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Legacy effects of a range-expanding shrub influence soil fungal communities and reduce conspecific seedling performance

Courtney Grace Collins (courtneygrace125@gmail.com)
University of California Riverside https://orcid.org/0000-0001-5455-172X
Jeffrey Diez
Marko Spasojevic
Nuttapon Pombubpa

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Abstract

Aims

Soil legacy effects can have long-term impacts on soil microbial communities with implications for plant growth and community structure. These effects are well studied for invasive plants, particularly after removal of invasive species, however very little work has considered the soil legacy effects of native range expanding species.

Methods

We used a controlled greenhouse experiment with a range-expanding sagebrush species (*Artemisia rothrockii* (Asteraceae)) to determine how sagebrush seedling performance was influenced by soil legacy effects of sagebrush in both the native and expansion range and over time since removal. We then used ITS2 sequencing to determine what soil microbial mechanisms drove these feedbacks.

Results

Conspecific sagebrush seedlings responded negatively to sagebrush soil legacy effects overall, with more negative plant-soil feedbacks, reduced height, and higher root:shoot ratios in shrub removal inoculum than in shrub and herbaceous soil inoculum. Seedlings with shrub removal inoculum had increased fungal diversity, pathogen, and saprotroph richness, and altered fungal community composition. Legacy effects on soil fungal diversity and functional group richness were present in seedlings with 1-year shrub removal inoculum, while effects on fungal community composition were found in 1 and 5-year shrub removal inoculated seedlings. Similarly, negative plant-soil feedbacks occurred for seedlings growing in both 1-year and in 5-year shrub removal inoculum.

Conclusions

This work provides novel insight into how soil legacy effects of native range expanding species may *limit* rather than promote the recruitment of conspecifics, with important implications for management as global change continues to shift the geographic ranges of woody plants.

Introduction

Global climate and land use change are increasing the establishment of novel plant species, both through range shifts and non-native invasions (Walther et al. 2009; Tomiolo and Ward 2018). Novel plant species can alter both biotic and abiotic components of the soil ecosystem such as soil microbial community structure, nutrient availability and soil C storage, and feedback to influence the growth of conspecific and neighboring plant species (Van de Voorde et al. 2011; Kostenko and Bezemer 2020). These changes to

the soil ecosystem may persist long after the novel species is removed or naturally declines (i.e., extirpated), at which point they are considered 'soil legacy effects.' While soil legacies of non-native 'invasive' plant species are well documented (Elgersma et al. 2011; Suding et al. 2013; Lankau et al. 2014), we know little about the soil legacies of native range expanding species and how they may influence the trajectory of range expansions. This is particularly true for biotic soil legacies, including changes in soil microbial community structure and function, as the impacts and feedbacks of native range expansions on soil microbial communities is still an active area of study (Collins et al. 2019; Manrubia et al. 2019; Ramirez et al. 2019; Koorem et al. 2020).

Novel plant species may alter soil microbial communities in ways that benefit their own growth and recruitment, while negatively influencing currently established plant species. This often includes increases in generalist soil pathogens, decreases in beneficial soil mutualists, and changes to the soil saprotroph community and microbial enzyme activity (Coats and Rumpho 2014; Caravaca et al. 2020). Some of these changes are directly induced, for example through the exudation of secondary compounds (Stinson et al. 2006; Callaway et al. 2008; Lankau et al. 2014), while others occur indirectly, through changes in litter chemistry, root and leaf traits, and nutrient acquisition strategies (Williams et al. 2013; Cantarel et al. 2015; Collins et al. 2020). Alternatively, novel species may influence changes to soil microbial communities that are beneficial for other plants, including higher microbial biomass and activity and larger mycorrhizal networks, especially if they are nurse plants such as shrubs, nitrogen fixers or cushion plants (Rodríguez-Echeverría et al. 2016). The net effects of these changes to the soil environment, and whether they persist over time, will ultimately determine how soil legacies feedback to influence intra and inter-specific plant performance.

Soil legacy effects can change over time and after the removal or extirpation of the novel plant species, but we still have a limited understanding of the temporal dynamics of soil legacies post-removal (Grove et al. 2015; Esch and Kobe 2021; Hannula et al. 2021). Many factors can allow soil legacies to persist for long periods of time, even after removal of the novel species and/or subsequent recovery of historic plant communities (Elgersma et al. 2011; Kulmatiski et al. 2011; Lankau et al. 2014). For example, after 6 years of removal, soil legacy effects of invasive *Alliaria petiolata* (Brassicaceae) were still detectable in arbuscular mycorrhizal communities, and continued to slow the re-establishment of other plant species (Lankau et al. 2014). Furthermore, biotic soil legacies are generally thought to persist longer in soil fungi than bacterial communities (Hannula et al. 2021), however few studies have directly tested this claim.

For range expanding species, soil legacies may differ in the historic versus expansion range and may differentially respond to and feedback to influence subsequent plant growth – a process known as 'plant-soil feedbacks'. In a range expanding subalpine shrub species, soil legacy effects on bacterial and fungal community diversity were much stronger in the expansion range (alpine zone) versus the historic range (subalpine zone), as there was still detectable differences in soil microbial communities after 4 years of shrub removal in alpine but not subalpine zones (Collins et al. 2016a, 2018). This pattern may be caused by more limited microbial inoculum and dispersal potential from neighboring host plants at higher elevation sites and/or by slower microbial growth and turnover in alpine soils. Overall, the long-term

trajectory of biotic soil legacies will depend not only on the magnitude of change generated by the novel plant species, but also the dispersal ability of nearby plants and soil microbes and the environmental differences in the historic versus expansion range. However, the critical next question – how temporal and spatial variation in soil legacy effects will feedback to affect plant seedling establishment and performance during range expansions – has yet to be determined.

Woody plant encroachment is a range expansion type occurring in numerous ecosystems worldwide (Archer et al. 2017; García Criado et al. 2020) that alters many components of belowground ecosystems including soil microbial community structure (Collins et al. 2020) as well as nutrient availability and soil C pools (Eldridge et al. 2015; Throop et al. 2020). Very little research has examined soil legacy effects post-removal of range expanding woody species despite the increasing use of this practice in land management (Eldridge and Ding 2021). Recent work in drylands shows that after management via herbicide removal, abiotic soil legacies of woody encroachment can last decades and far outweigh the influence of grazing on soil organic matter pools (Throop et al. 2020). Furthermore, in alpine zones, soil legacy effects of woody shrubs on soil fungal and bacterial communities can last several (2–4) years after shrub removal (Collins et al. 2016a, 2018; Broadbent et al. 2022) and can interact with other abiotic factors such as snowmelt timing when influencing soil biota (Broadbent et al. 2022).

Here, we used a controlled plant-soil feedback greenhouse experiment and soil DNA sequencing from a native range-expanding sagebrush species (*Artemisia rothrockii* (Asteraceae)). We sought to determine: 1) How soil legacies of this species differ both between the historic and expansion range and over time since removal, and 2) how these different soil legacies feedback influence conspecific seedling performance and leaf traits across the species' range. We focus on legacy effects in soil fungal communities, because they form important mutualistic connections in the plant rhizosphere as well as contain numerous species-specific soil pathogens (Lee Taylor and Sinsabaugh 2015; Nguyen et al. 2016). We further aim to 3) identify which components of sagebrush soil legacies (changes in soil fungal diversity, community composition, and/or functional group richness) most strongly influence conspecific seedling performance.

Materials And Methods

Study species and site

Artemisia rothrockii (Timberline Sagebrush; A. Gray; Asteraceae) is a California endemic and dominant (sub)shrub species in the White Mountains in subalpine and alpine zones (Rundel et al., 2008, Mooney et al., 1962-described as *A. arbuscula*). *A. rothrockii*'s distribution has been moving upwards in elevation at an average rate of 30 m/decade over the last 60 years (Kopp and Cleland 2014). Sagebrush is an obligate arbuscular mycorrhizal (AMF) host (Weber et al. 2015)d *rothrockii* individuals in this range have moderate to high AMF root colonization (~ 60–80% average, Collins, C.G.- unpublished data). Previous work in this system has shown that soil microbial communities in the expansion zone of *A. rothrockii* differ in both structure and function from those in the historic range (Collins et al. 2016b, 2018).

Furthermore, sagebrush removal plots were established in 2011 and 2015 to assess soil legacy effects and previous work showed that after 4 years, microbial communities in sagebrush removal plots possessed an intermediate community composition and diversity compared to shrub and non-shrub (herbaceous) soils (Collins et al. 2016b, 2018).

Research took place at the White Mountain Research Center, located in the White Mountains in eastern California and western Nevada, at the western edge of the Great Basin (mean annual temperature − 0.4°C; mean annual precipitation 391mm (Hall 1991). Experimental plots were established at 3100 m, 3500 m and 3700 m elevations, representing the historic (low and middle elevation) and range expansion (high elevation) zones of this species (Kopp and Cleland, 2014). In 1961, *A. rothrockii* was found in moderate to high densities at the 3100 and 3500 m sites and not present at the 3700 m site (Mooney et al., 1962). This shift between subalpine and alpine communities encompasses the transition from sagebrush steppe to true alpine plant communities dominated by prostrate cushion plants and perennial bunchgrasses (Rundel et al. 2005, 2008).

Field sampling

In October 2015, approximately 1000 seeds were collected from 10 mature sagebrush individuals at low (3100 m) and middle (3500 m) elevation populations (both within historic range, Fig. 1) and stored in a desiccator at 4 ˚C for an 11-month cold stratification treatment (Bonner 2008). In September 2016, we sampled soils for use as greenhouse inoculum from under five sagebrush individuals (< 100 m apart) at the low (historic range) and high (expansion range) elevations (soil cores 1.3 cm diameter x 10 cm deep), along with paired shrub interspaces. Shrub interspace cores were taken between 1 and 5 m from the edge of the canopy (based on sagebrush density of the site) in non-shrub, herbaceous plant cover. The corer was sterilized between each sample with a 10% bleach solution to prevent cross-contamination, and two replicate cores were combined into one sample. Soils were sampled in the same location as seeds at the low elevation site, and soils at the high elevation site were collected in 2 areas of recent sagebrush establishment (~ 200 m apart) as determined by Kopp and Cleland (2014). We also took soil samples using the same coring method from five separate (1 x 1 m) plots where sagebrush has been manually removed (cut at the base of the stem and trimmed back yearly) for 1 year (SR1) and for 5 years (SR5) at both high and low elevation sites (Collins et al. 2016b, 2018).

All soil samples were placed on ice in the field and then kept cool in a refrigerator (4 ˚C) for one month prior to use. During this time, soils were sieved through a 2 mm mesh to remove stones and large plant material. All sampling locations had granitic soils (Colluvium derived from granite) and east-/south-east-facing slopes to control for edaphic and aspect variation. As described in Collins et al. (2016), soil VWC, SON and SOC increase from the low to high elevation sites, however we diluted these abiotic differences in our greenhouse experiment (see below) by adding a small volume of inoculum to sterile background soils (Pernilla Brinkman et al. 2010). All soil samples were kept separate (N = 40) to retain the variation in soil microbial communities within and across elevations and vegetation types (Gundale et al., 2019) and each sample was divided in half, with one half sterilized, and the other half live for paired inoculation (Table 1).

Table 1
Greenhouse experimental design

Soil source	Non-shrub (herbaceous community)	Intact shrub (*A. rothrockii* community)	1-yr post shrub removal (SR1)	5-yr post shrub removal (SR5)
Soil elevation	3100m (low) + 3700 m (high)	3100m (low) + 3700 m (high)	3100m (low) + 3700 m (high)	3100m (low) + 3700 m (high)
Inoculum	Live + sterile	Live + sterile	Live + sterile	Live + sterile
Seed elevation	3100m (low) + 3500m (middle)	3100m (low) + 3500m (middle)	3100m (low) + 3500m (middle)	3100m (low) + 3500m (middle)
# Seedlings	5 seedlings x 2 soil elevation x 2 inoculum x 2 seed elevation = 40	5 seedlings x 2 soil elevation x 2 inoculum x 2 seed elevation = 40	5 seedlings x 2 soil elevation x 2 inoculum x 2 seed elevation = 40	5 seedlings x 2 soil elevation x 2 inoculum x 2 seed elevation = 40

Microbial activity (Extracellular Enzyme Assays)

Extracellular enzyme activities were measured on all live soil inoculum (N = 40 samples) following a modified protocol Saiya-Cork et al. (2002) as described in German et al. (2011). We measured two common microbial hydrolytic enzymes: Cellobiohydrolase (CBH) and β-N-acetylglucosaminidase (NAG) involved in C and N cycling respectively (Treseder and Lennon 2015). Fluorescence readings were run on a Promega GloMax Multiplus Plate Reader at 365/450 nm excitation/ emission at the UCR Genomics Core to calculate enzyme activity (nmol hr^{-1} g^{-1}).

Greenhouse experiment

Seeds were surface sterilized with 10% bleach solution and germinated in trays of sterilized soil (autoclaved at 120˚C for 90 minutes). After one month, seedlings were transplanted to larger pots (1600 mL) of sterile background soil and initial seedling height at time of transplanting was used to estimate initial biomass (g) for each seedling via an allometric equation of dry biomass to height, generated from 10 additional seedlings. Background soil in all pots was identical to control for abiotic differences across the soil inoculum types and consisted of a custom mix of equal parts #30 silica sand and peat moss and a 15:10:1 ratio of Dolomite lime ($CaMg(CO_3)_2$), Triple Superphosphate ($CaH_4P_2O_8$), and Potassium nitrate (KNO_3) respectively. This closely resembles the granitic soil type where sagebrush grows in the White Mountains, characterized by high percent sand, coarse texture, low organic matter and low water retention (Smithers 2017). During transplanting, pots were inoculated with 25g (~ 50 mL, 3% total pot volume) of either sterile or live soil inoculum in the center of the pot of each seedling being transplanted. We grew two seedlings (one per seed elevation) for each soil sample (paired live and sterile) for a total of 160 seedlings (Table 1) (ISS-MSS design type, Gundale et al., 2019). Seedlings were grown for 4 months

(between 126–130 days) from October 2016-February 2017 (one alpine growing season). Greenhouse temperatures ranged from 10°C (low) to 22°C (high) which closely mirror average temperatures during the growing season at these elevations in the White Mountains and supplemental lighting was used in the evenings to extend day length to match the growing season (http://www.wmrc.edu/weather/). Seedlings were watered twice weekly with DI water.

After 4 months, all seedlings were harvested and soils in pots were sieved thoroughly to remove all belowground biomass. During harvest, we collected rhizosphere soil from the roots of low elevation seedlings (N = 40) by gently shaking all excess soil from the roots of each seedling into a Whirlpak bag, which were then immediately frozen (-20 C) for molecular analyses. Roots were washed in soapy water to remove any remaining soil and all plant material was placed in the drying oven at 60 ˚C for 72 hours and then weighed. Height of each seedling was calculated by subtracting initial height from final height and total biomass was calculated by subtracting initial biomass from final biomass. We calculated a Plant-Soil Feedback (PSF) value for all seedlings in live inoculum using the equation PSF= {(total biomass (g) live soil – total biomass (g) sterile soil)/total biomass (g) sterile soil}. Biomass in sterile soil was the average biomass of all seedlings from the same soil elevation and soil source (i.e. vegetation type) as described in (Pernilla Brinkman et al., 2010, FB1 2nd equation). A negative PSF signifies lower growth in live soil versus sterilized soil, indicating an overall negative effect of the soil microbial community. Finally, we calculated seedling root: shoot ratio by dividing the total belowground biomass (g) by the total aboveground biomass (g) of each seedling.

Leaf traits

We also measured the following leaf functional traits for each seedling: leaf dry matter content LDMC (g/g), specific leaf area SLA (cm^2/g), leaf N (%), leaf C (%), $\delta^{13}C$, and $\delta^{15}N$ following standard protocols (Pérez-Harguindeguy et al. 2016). During harvest, one average-sized leaf was collected from each plant and placed into a coin envelope, weighed within 24 hours on a microbalance for fresh weight (g), and scanned on a flatbed scanner to calculate leaf area (cm^2) using ImageJ software (https://imagej.nih.gov/ij/). Leaves were then placed in the drying oven (60 ˚C for 72 hours) and then weighed for dry weight (g). LDMC was calculated as the ratio of fresh weight (g) to dry weight (g) and SLA was calculated as leaf area (cm^2) to dry weight (g). Leaf chemical and isotope analyses were analyzed on dried leaf material at the University of Wyoming Stable Isotope Facility (Laramie, WY).

Molecular analyses and Bioinformatics

DNA sequencing analyses were conducted on rhizosphere soils from each low elevation greenhouse seedling with live inoculum (i.e., seed elevation 3100, N = 40, Table 1). We extracted microbial DNA from 0.25 g of soil using a Qiagen DNeasy PowerSoil Kit (Germantown MD, USA) and all DNA extracts were sent on dry ice to Novogene Corporation (Sacramento, CA) for sequencing of the ITS2 region for fungi. Forward and reverse primers ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990) respectively, were used to amplify the ITS2 region. Sample libraries were created using the Illumina TruSeq DNA PCR-Free Library Preparation Kit and

sequenced in a multiplexed 2x250 paired end run on the Illumina HiSeq 2500 sequencing platform (San Diego, CA).

Bioinformatics pipeline

Demultiplexed paired-end sequences data were pre-processed by trimming forward and reverse reads to 240 bp (reads length less than 100 bp were dropped), trimming primer sequences, and merging paired-end reads using USEARCH v9.1.13 (Edgar 2010). After pre-processing steps, valid output contained 4,156,070 reads. Quality filtering was proceeded with an expected error less than 0.9 in which 3,656,832 reads passed quality filtering. After pre-processing and quality filtering steps, UPARSE (Edgar 2013) clustering was performed at 97% percent identity to create an Operational Taxonomic Unit (OTU) table which generated 2,797 OTUs. Next, we ran chimera filtering using VSEARCH (v 2.3.2) (Rognes et al. 2016) which removed 181 reference chimeras. Lastly, taxonomy assignment was run using AMPtk hybrid approach (Palmer et al. 2018) which resulted in 2,470 assigned fungal OTUs and 3,218,660 reads. This output was then rarefied to 21,000 reads per sample with all samples were retained and run through the 'core_diversity_analyses.py' command in QIIME version 1.9.1 (Caporaso et al. 2010).

Fungal Community Structure

We estimated alpha diversity using the Chao1 diversity outputs from the core diversity analyses in QIIME. Beta diversity (community composition) was estimated using Non-Metric Multidimensional Scaling (NMDS) of the Bray-Curtis dissimilarity outputs from the core diversity analyses in QIIME with the functions 'MetaMDS' in the package *vegan* in R (Oksanen et al. 2016). We also ran a principal coordinate analysis (PCoA) using the function 'cmdscale' in the *stats* package in R (R Core Team 2020). PCoA axis scores were used as predictors in subsequent analyses (see below).

Functional guilds were assigned to fungal sequences using FUNGuild v1.0 (Nguyen et al. 2016) resulting in 749 OTUs with functional guilds assignments. We first filtered FUNGuild assignments, removing all taxa that were at the confidence level of 'possible' and retaining only assignments that were at the confidence level of 'highly probable' and 'probable. We then calculated the relative richness of saprotrophs, pathogens, and mutualists in each soil sample as determined by FUNGuild assignments. All unique OTUs assigned to trophic modes beginning with "Saprotroph" and notes mentioning presence in soil were counted for saprotroph richness. These taxa were confirmed as soil saprotrophs through further literature review (Jančič et al. 2015; Tedersoo et al. 2018; Purahong et al. 2019). All unique OTUs assigned to the functional guild "Plant Pathogen" were counted for pathogen richness and all unique OTUs assigned to the functional guild "Arbuscular Mycorrhizal" were counted for mutualist richness, as *A. rothrockii* associates with AMF fungi.

Statistical analyses

To determine how sagebrush soil legacies differ between the historic and expansion range and over time since removal, we tested whether soil microbial (fungal) community structure (alpha diversity, functional group richness) and function (extracellular enzyme activity) differed across soil inoculum types using

two-way analysis of variance in the function ‘aov’ in R (R Core Team 2020). We used the following categorical predictors with an interaction 1) soil source (i.e., vegetation community- shrub, herbaceous, shrub removal 1 year, shrub removal 5 year) and 2) soil elevation (3100 m-historic range, 3700 m-expansion range). We then calculated pairwise contrasts using a Tukey test for models with evidence of a relationship (see Muff et al. (2022)). Prior to modeling, we logged or square root transformed microbial data for normality and removed outliers greater than 3 standard deviations from the mean.

Model structure: Microbial response ~ soil source x soil elevation

To determine if community composition (beta diversity) differed across soil inoculum types we used a Permutational multivariate analysis of variance (perMANOVA) in the function ‘adonis’ the package *vegan* in R (999 permutations; Oksanen et al. 2016) with the same model structure as above. We then calculated pairwise contrasts between soil sources, elevations with a strata (blocking) variable of soil elevation, soil source respectively using the function ‘pairwise.adonis2’ in the package *pairwiseAdonis* in R (Martinez Arbizu 2020). We also tested for within group heterogeneity using the *vegan* functions ‘betadisper’ and ‘permutest’ (Oksanen et al. 2016).

We used indicator species analysis to further elucidate which fungal taxa characterized soils from each soil elevation x soil source combination using the function ‘mulitpatt’ in the ‘indicspecies’ package in R (Cáceres and Legendre 2009). We calculated Indicator Values ($Indval_g$) based on species (OTU) abundance and report indicator taxa with moderate to strong evidence based on permutation tests (N = 999; Dufrêne and Legendre 1997).

To determine how sagebrush soil legacies feedback to influence conspecific seedling performance and leaf traits across the species’ range, we analyzed seedling responses (PSF, height, root:shoot, leaf traits) to the different soil inoculum types using three-way analysis of variance in the function ‘aov’ in R (R Core Team 2020). We used the following categorical predictors with an interaction 1) soil source (i.e vegetation community- shrub, herbaceous, shrub removal 1 year, shrub removal 5 year), 2) soil elevation (3100 m-historic range, 3700 m-expansion range) and 3) seed elevation (3100 m-historic population low, 3500 m-historic population mid). We then calculated pairwise constrats using a Tukey test for models with evidence of a relationship (see Muff et al. (2022)). Prior to modeling, we logged or square root transformed plant responses for normality and removed outliers greater than 3 standard deviations from the mean.

Model structure: Seedling response ~ soil source x soil elevation x seed elevation

To determine which components of sagebrush soil legacies most strongly affect conspecific seedling performance, we tested the influence of soil microbial communities on measured seedling responses using mixed effects models in the package *lme4* in R (Bates et al. 2014). For these models, we only included microbial and plant parameters where we found evidence of an effect of shrub removal (determined in the above analyses), as we aimed to understand how soil legacy effects influenced seedling responses through soil microbial mechanisms. Because we measured fungal community

composition on rhizosphere soils from low elevation (3100m) seedlings only, we predict low elevation seedling responses with fungal diversity, functional group richness, and community composition metrics, with a random effect of soil source (i.e., vegetation community) nested within soil elevation.

Model structure: Seedling response ~ microbial community+ (1| soil source: soil elevation)

Results

Soil microbial legacies

After 4 months of growth, rhizosphere soils of seedlings inoculated with shrub removal soils had higher alpha diversity, saprotroph and pathogen richness and altered community composition when compared to rhizosphere soils of shrub soil inoculated seedlings (Fig. 2, 3, Table S1, S2). These patterns were primarily in seedlings with 1-yr shrub removal inoculum, with the exception of beta diversity, which was also distinct in seedlings with 5-yr shrub removal inoculum (Fig. 3, Table S2). Legacy effects of shrub removal were consistent between soils from the expansion (high elevation) and historic (low elevation) range, except for alpha diversity, which was higher in seedlings with historic (low elevation) shrub removal inoculum only (Fig. 2). We found no evidence that shrub removal inoculum influenced mutualist richness in seedling rhizosphere soils (Fig. 2, Table S1, S2). Furthermore, we found no evidence that shrub removal influenced soil microbial community function (extracellular enzymes), however NAG enzyme activity was higher in intact shrub than herbaceous soils (Fig. 2, Table S1, S2). It is important to note that extracellular enzymes were measured on live field soil inoculum rather than post seedling growth (i.e. greenhouse soils), so they are not directly comparable to the DNA sequencing results.

Indicator species analysis resulted in 220 fungal indicator taxa comprising 7 phyla, 18 classes and 37 unique orders across all soil elevation and soil sources. It revealed a disproportionate number of fungal indicator taxa in seedling soils with shrub removal and in particular, 1-year shrub removal inoculum comprising about 80% and 60% respectively of the total indicator species identified (Fig. 4, Table S3). Seedlings with shrub removal (especially 1-year shrub removal) inoculum had the highest number of indicator taxa in the classes *Leotiomycetes* (primarily *Helotiales*) and *Archeaorhizomycetes*, and *Dothideomycetes* (primarily *Pleosporales* and *Capnodiales*) (Fig. 4, Table S3). There was a very high richness of indicator taxa from these clades in seedlings inoculated with shrub removal soils (20- *Leotiomycetes*, 16- *Archeaorhizomycetes* and 21- *Dothideomycetes* taxa respectively), and there were no indicator taxa from *Leotiomycetes* and *Archeorhizomycetes* in either shrub or herbaceous soil inoculated seedlings (Fig. 4, Table S3). Seedlings with shrub removal inoculum from the expansion range (high elevation) also had indicator taxa from the class *Mortierellomycetes* not present in seedling soils from any other inoculum type (Fig. 4, Table S3).

Seedling performance

Sagebrush seedlings responded negatively to sagebrush soil legacy effects overall, with more negative PSFs, reduced height, and higher root: shoot ratios in shrub removal soils when compared to those

growing in shrub and herbaceous soils (Fig. 5, Table S4, S5). Seedlings had more negative PSFs in 1-year and 5-year shrub removal soils than when growing in shrub soils overall (Fig. 5, Table S4, S5). We found similar patterns with seedling height but only within certain seed and soil elevations. Specifically, seedling height was lower in 1-year shrub removal than herbaceous soil inoculum for low elevation soils (Fig. 5, Table S5). In addition, seedling height was lower in 5-year shrub removal soils than in herbaceous soil inoculum for the mid elevation seed source. Finally, seedlings from the low elevation seed source had higher root:shoot ratios in 1-year shrub removal than in shrub soil inoculum overall (Fig. 5, Table S5)..

Shrub soil legacy effects also influenced leaf isotope ratios where seedlings growing in 5-year shrub removal soils had higher (less negative) leaf $\delta^{13}C$ values, indicating a higher water use efficiency (WUE) or more water stress, than seedlings growing in shrub or herbaceous soils overall (Fig. 5, Table S5). Leaf $\delta^{13}C$ values were also higher in 5-year shrub removal than 1-year shrub removal soils overall (Fig. 5, Table S5). We found a similar pattern for leaf N ratios, but only within certain soil, seed elevations. Specifically, seedlings had higher leaf $\delta^{15}N$ values, indicating a higher N-use efficiency or more N-stress, in low elevation 5-year shrub removal soils than low elevation shrub soils. We found the same pattern in in low elevation 5-year shrub removal soils than herbaceous soils for the mid elevation seed source (Fig. 5, Table S5).

Seed source influenced overall seedling growth and leaf traits, as seedlings from low elevation (3100m) seed were taller, with more positive PSFs, higher root:shoot ratios, higher leaf C, LDMC, and Δ15N and lower $\delta^{13}C$ (Fig. 5, Table S4, S5). However, seedling responses to shrub removal inoculum did not differ consistently by seed source, as differences in seedling height and leaf $\delta^{15}N$ were stronger for the mid elevation seed source (3500m), while differences in root:shoot ratio were stronger for the low elevation seed source (3100m) (Fig. 5, Table S4, S5). Soil elevation influenced seedling leaf traits as seedlings growing in low elevation soils had higher leaf $\delta^{15}N$ and lower $\delta^{13}C$ and LDMC. Furthermore, seedling responses to shrub removal inoculum were stronger in low elevation (historic range) soils for height and leaf $\delta^{15}N$ (Fig. 5, Table S4, S5).

Microbial mechanisms of seedling performance

Mixed-effects modeling revealed that fungal alpha diversity, saprotroph richness and beta diversity (community composition) best predicted seedling responses (Table S6). Fungal alpha diversity (Chao1) had a negative influence on seedling PSF ratios and a positive influence on seedling root:shoot ratios and leaf $\delta^{15}N$. Fungal composition (PCoA2) influenced seedling height and leaf $\delta^{13}C$ values and saprotroph richness had a negative effect on leaf $\delta^{15}N$ values (Fig. 6, Table S6).

Principal coordinates analysis showed that shrub removal soils loaded most heavily on PCoA2 (Fig S2) so this was used as a predictor in mixed-effects models (above). Overall, the first Principal coordinates axis (PCoA1) explained 20.23% of the variation in fungal community composition and herbaceous and shrub soil communities primarily loaded on this axis (Fig S2). The second Principal coordinates axis

(PCoA2) explained 17.2% of the variation in fungal community composition and shrub removal communities (1 and 5 year) primarily loaded on this axis (Fig S2).

Discussion

Removal of a range expanding shrub species left distinct signatures on soil microbial community structure including fungal alpha diversity, functional group richness and community composition. These legacy effects were detectable in rhizosphere soils of conspecific seedlings after 1–5 years of shrub removal and 4 months of seedling growth. Overall, legacy effects of shrub removal created negative plant-soil feedbacks on conspecific seedlings that were not present for seedlings growing in intact shrub or herbaceous soil inoculum, highlighting how shrub removal may have add-on benefits of limiting further range expansion of woody species through biotically mediated plant-soil feedbacks.

We assessed whether soil legacy effects of sagebrush would change over time after shrub removal and between the historic and range expansion zone of the woody species. The duration of soil legacy effects differed across distinct microbial metrics. For example, legacy effects on soil fungal diversity, pathogen richness and saprotroph richness were strongest in seedlings with 1-year shrub removal inoculum, while effects on fungal community composition (beta diversity) were present in both seedlings with 1 and 5-year shrub removal inoculum (Fig. 2, 3). This suggests that initial legacy effects of woody shrubs on microbial diversity and functional group richness may start to decline in less than 5 years; however, changes in microbial community composition may last for a longer period.

On the other hand, soil legacy effects on fungal community structure were relatively consistent between seedlings in historic versus expansion range inoculum, except for fungal diversity, where effects were much stronger in seedlings with shrub removal inoculum from the historic range (low elevation soils) (Fig. 2). This is the opposite pattern of what we observed previously, where shrub removal effects on soil microbial diversity were stronger in the expansion versus historic range (Collins et al 2016a, 2018), however the previous studies measured soil microbial diversity on live field soils rather than the rhizosphere soil of (field) inoculated seedlings. These findings suggest that despite large differences in the time since establishment of woody species in historic and range expansion zones, the soil legacy effects that occur after woody plant removal in each range may be very similar.

Sagebrush soil legacies contained distinct fungal taxa in addition to changes in diversity and community composition. Increases in fungal diversity were concomitant with increased pathogen and saprotroph richness in seedlings inoculated with shrub removal soils, suggesting that the majority of changes in diversity were in plant pathogens and saprotrophic taxa. Indicator species analysis showed that sagebrush seedlings with 1-year shrub removal inoculum had rhizosphere soils with the highest number of indicator taxa, mostly from the classes *Leotiomycetes* (primarily *Helotiales*) and *Archeaorhizomycetes* (Fig. 4). The former are primarily root inhabiting dark septate endophytes with widespread distribution in arctic and alpine environments (Newsham 2011). The latter is an ancient clade of saprotrophic rhizosphere inhabiting soil fungi also shown to have high abundance in alpine tundra soils (Schadt et al

2003). In terms of pathogens, seedlings with shrub removal inoculum had increased richness of *Dothideomycetes*, in particular *Pleosporales* and *Capnodiales*, known clades of soil pathogens (Ohm et al. 2012). Contrary to expectations, legacy effects of shrub removal did not alter the richness of mutualist fungi in the rhizosphere of sagebrush seedlings (Fig. 3). This may be due to methodological limitations of the ITS region primers including low resolution for arbuscular mycorrhizal fungi (Glomeromycota) (Schoch et al. 2012). However, we observed a slight increase in *Glomeromycete* indicator taxa in shrub removal soils as compared to herbaceous and shrub soils (Fig. 4).

Plant-soil feedbacks on conspecific seedlings growing in shrub removal soils were mostly negative, including reduced height and biomass compared to sterile soils (i.e., PSF ratio) and leaf traits indicating water and nitrogen stress (increased leaf $\delta^{13}C$, $\delta^{15}N$) as compared to seedlings growing in intact shrub and herbaceous soils (Fig. 5). Seedlings in shrub removal soils also had higher root:shoot ratios, which in combination with leaf isotope ratios, suggests that they were more resource stressed, and thus invested more in belowground root growth. This is likely a result of the increased pathogen and saprotroph richness in shrub removal soils. This is similar to patterns observed in temperate grasslands, where the diversity of pathogenic and saprotrophic fungi were key drivers of biotic soil feedbacks, and resource acquisitive seedlings had a higher diversity of pathogens and specialist saprotrophs in their rhizosphere, resulting in strong plant growth suppression in conspecific soils (Lozano et al. 2018). Furthermore, negative plant-soil feedbacks occurred in seedlings growing in both 1-year and 5-year shrub removal soils, indicating that both short term changes in fungal diversity and functional group richness and longer-term changes fungal community composition had important influences on seedling growth (Fig. 5).

Finally, fungal alpha diversity, saprotroph richness and beta diversity influenced seedling responses across all soil sources suggesting that the negative growth responses of seedlings in shrub removal soils are likely due to these underlying changes in fungal community structure. Of these parameters, alpha diversity proved the best indicator of the PSF ratio, as seedlings had higher PSFs in low diversity soils (Fig. 6). This is somewhat counter-intuitive, but a potential explanation is that low diversity soils also had lower pathogen richness (Fig. 2). However, surprisingly, pathogen richness itself was not a predictor of seedling performance, but this may be due to many unassigned taxa in the FunGuild database, including key indicator species in shrub removal soils within the *Leotiomycetes, Dothideomycetes* and *Archeaorhizomycetes.* This further emphasizes the need for determining the ecological role of myriad of soil microbial species whose ecosystem functions are still unknown (Nannipieri 2020). It also suggests that changes in fungal diversity may encapsulate underlying shifts in functional guilds important for plant-soil feedbacks.

Our results have implications for the management of woody species and range expanding species worldwide. First, we found that negative plant-soil feedbacks on conspecific seedlings were driven by changes in soil biota that occurred *after* woody plant removal, but not from soils of intact shrubs. This is an important caveat whereby the legacy effects, rather than current soil conditioning of conspecifics most strongly limited further conspecific growth. Next, we found that biotic soil legacy effects from both

short term (1 year), and long term (5 years) woody plant removal persisted after several months of seedling establishment and generated negative feedbacks on seedling performance. These negative feedbacks were driven by distinct changes in soil fungal communities that occurred in short vs. long-term removal (i.e. fungal diversity and functional group richness vs. community composition), suggesting that woody plant removal on multiple time scales may leave soil legacy effects that can meaningfully alter subsequent plant growth. While the spread of many woody species to track changing climate is a beneficial process, from a conservation perspective, their spread into restricted herbaceous systems, such as alpine communities or montane meadows, may require management (via removal) to protect resident species with nowhere to move. Indeed, as woody plants become increasingly prevalent with global change, woody plant removal is becoming an important management strategy with complex biotic and abiotic ecosystem consequences (Eldridge and Ding 2021). In this context, the occurrence of negative legacy soil effects is encouraging, as it would serve to slow down reinvasion by the removed species. Our work is among the first to highlight the biotic soil consequences of woody plant removal and their implications for the growth of range expanding woody plant species.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Field sampling, experimental design, data collection and analysis were performed by Courtney G. Collins. Nuttapon Pombubpa completed the bioinformatics for the DNA sequencing data. The first draft of the manuscript was written by Courtney G. Collins and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

Fungal sequencing data will be archived in the NCBI SRA database and all other data will be archived in the Dryad database.

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Figures

Figure 1

Two intact *A. rothrockii* populations from which seeds were collected at 3500 m elevation (top) and 3100 m elevation (bottom), both within the historic range of *A. rothrockii.* Lower photo has Crooked Creek research station in the background.

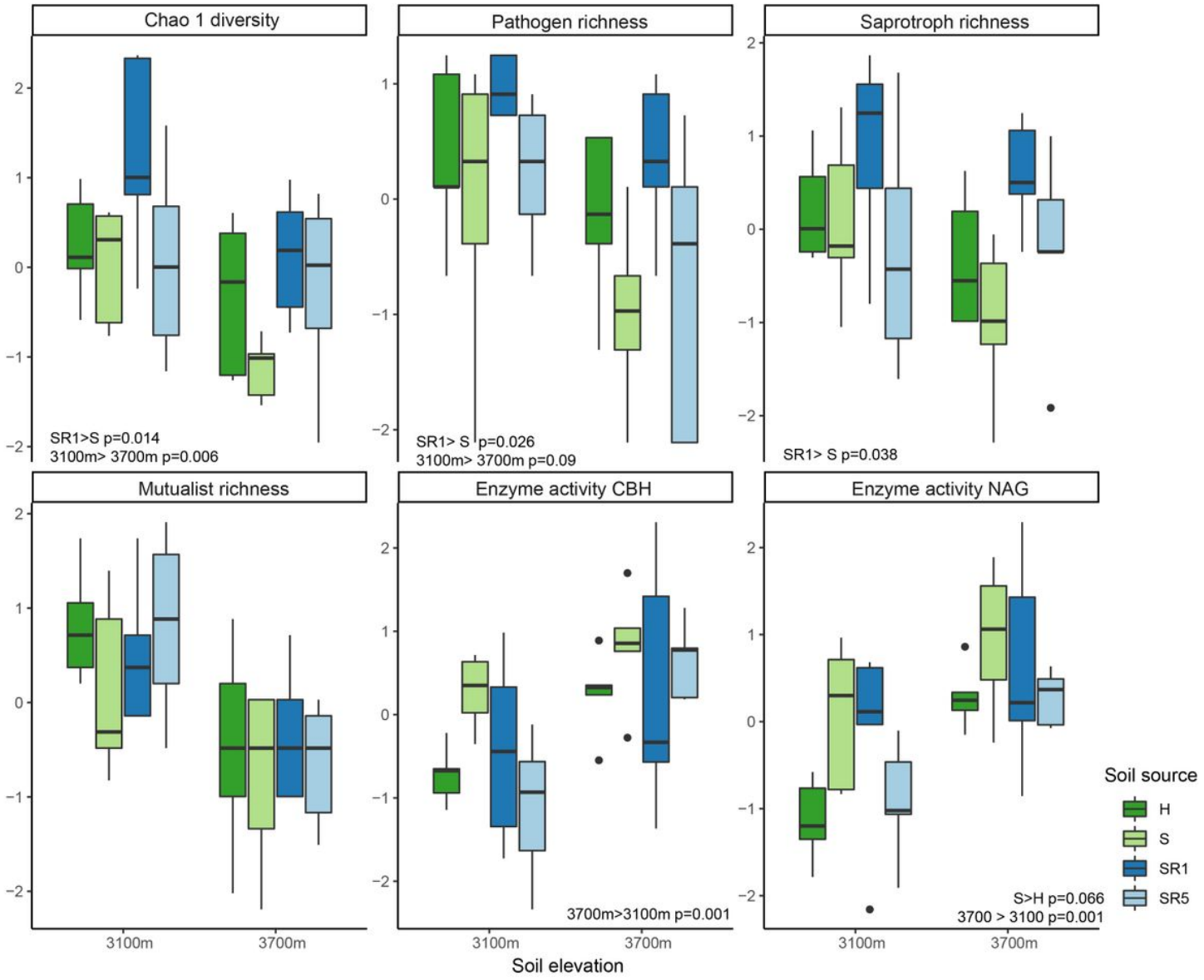

Figure 2

Soil fungal community structure (Chao 1 diversity, pathogen, saprotroph and mutualist richness) from sagebrush seedling rhizosphere soils and extracellular enzyme activity (Cellobiohydrolase (CBH) and β-N-acetylglucosaminidase (NAG)) from live field soil inoculum. Soil source indicates that inoculum was sourced from intact herbaceous (H), intact sagebrush (S) or areas where intact Sagebrush had been manually removed for 1 (SR1) or 5 (SR5) years. Soil elevation indicates that inoculum was sourced from either low elevation (3100 m-sagebrush historic range) or high elevation (3700 m- sagebrush expansion range) sites. Box- plots include the median (black line), first and third quartiles (bottom and top of the box, respectively), 1.5 times the interquartile range (whiskers), and outliers (black points) and each box includes (n=5) soil samples. All values are standardized with mean zero and unit variance for comparison. P-values are shown where we found evidence for differences between soil sources, elevations. Full ANOVA results and pairwise contrasts can be found in Tables S1 and S2.

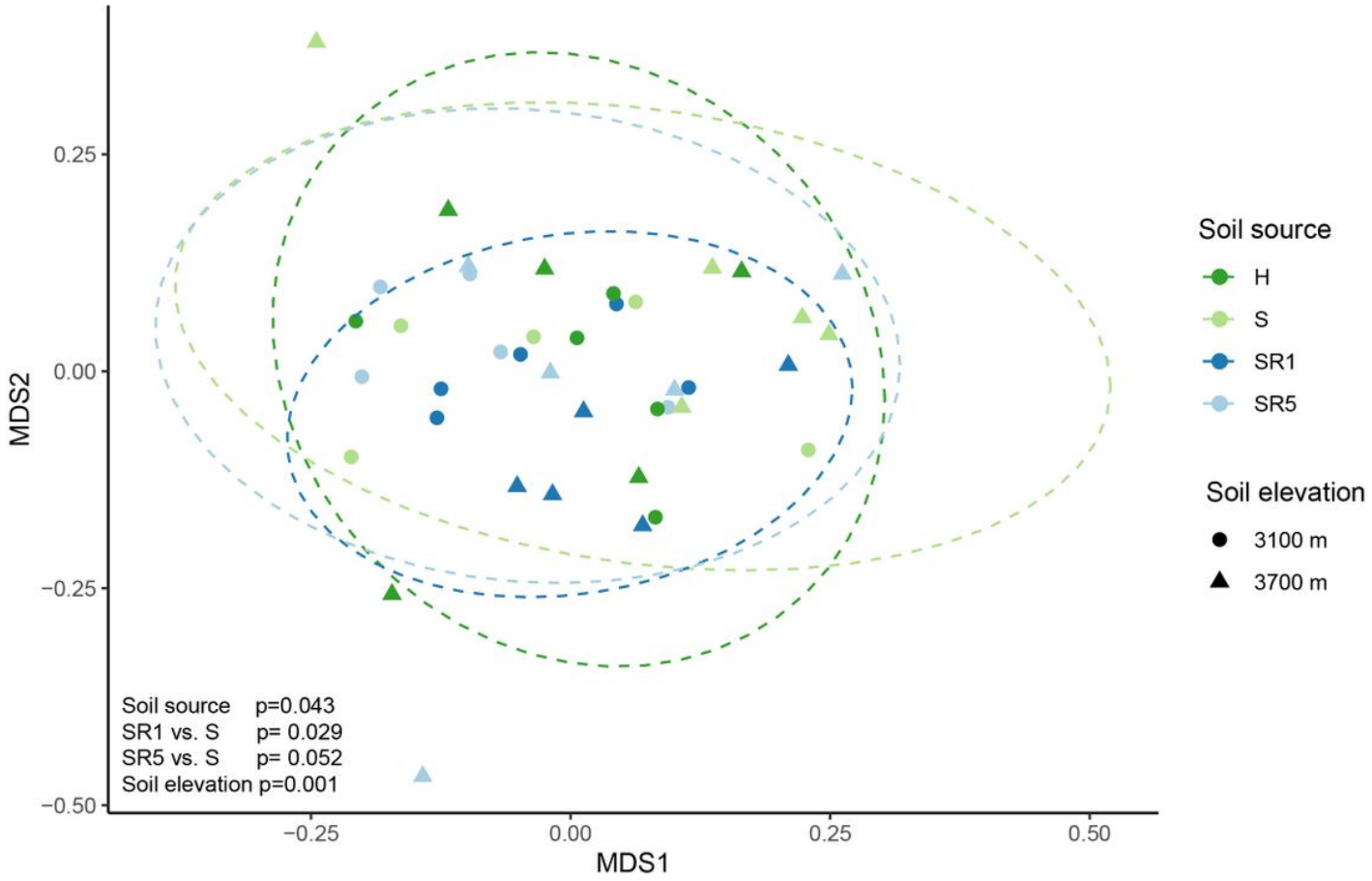

Figure 3

Non-metric multidimensional scaling (NMDS) of the Bray-Curtis dissimilarity of soil fungal communities from sagebrush seedling soils. Soil source indicates that inoculum was sourced from intact herbaceous (H), intact sagebrush (S) or areas where intact Sagebrush had been manually removed for 1 (SR1) or 5 (SR5) years. Soil elevation indicates that inoculum was sourced from either low elevation (3100 m- sagebrush historic range) or high elevation (3700 m- sagebrush expansion range) sites. Each point reflects one seedling from the low elevation seed source only (n=40). P-values are shown where we found evidence for differences between soil sources, elevations. Full perMANOVA results and pairwise contrasts can be found in Tables S1 and S2.

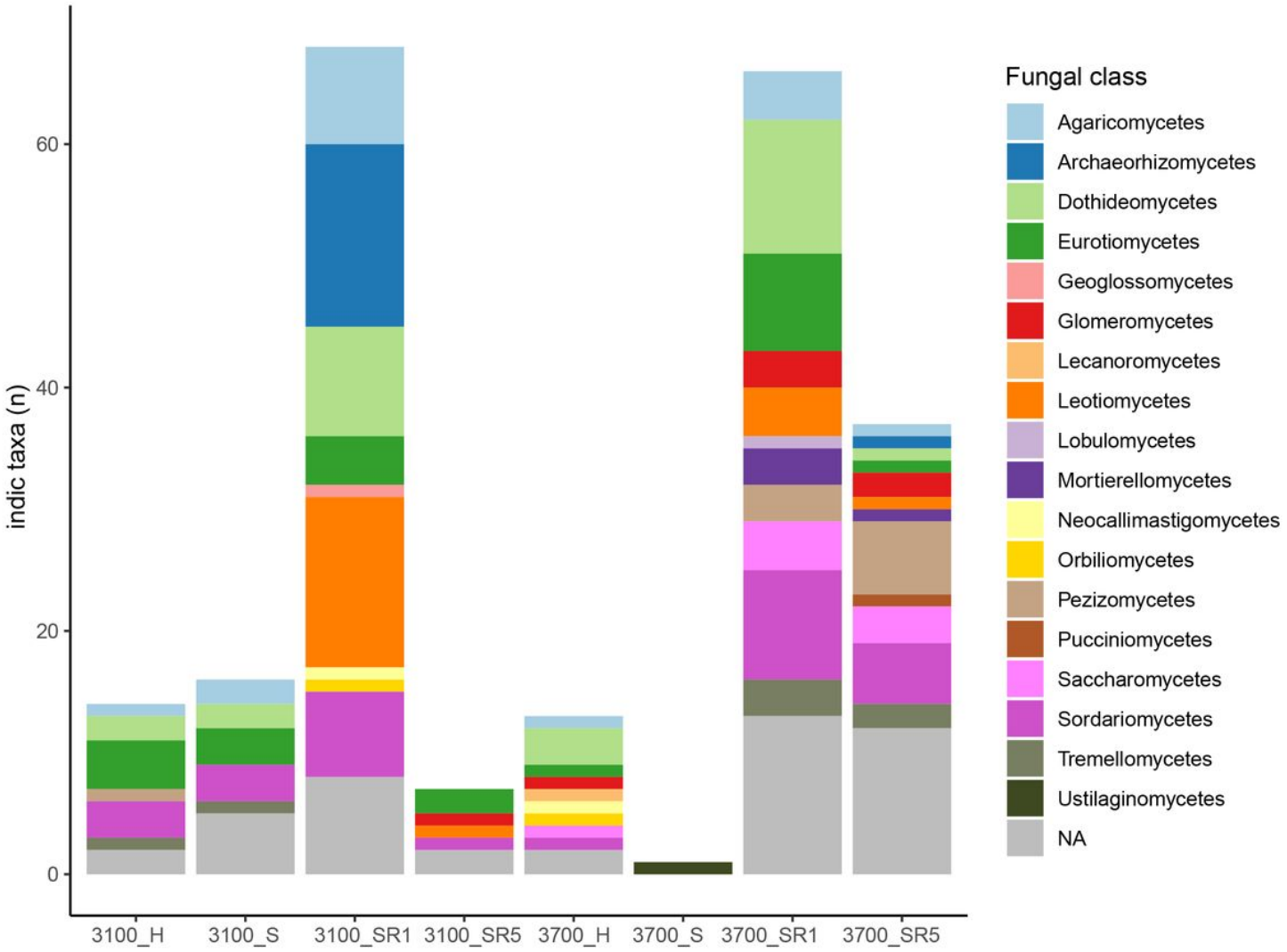

Figure 4

Results of Indicator species analysis for each unique inoculum type i.e. soil elevation (3100m, 3700m) by soil source (Intact herbaceous-H, Intact shrub-S, 1-year shrub removal-SR1, 5-year shrub removal-SR5). Bars reflect the total number of indicator species (colored by fungal class) sampled from rhizosphere soils of (n=5) sagebrush seedlings per soil inoculum type.

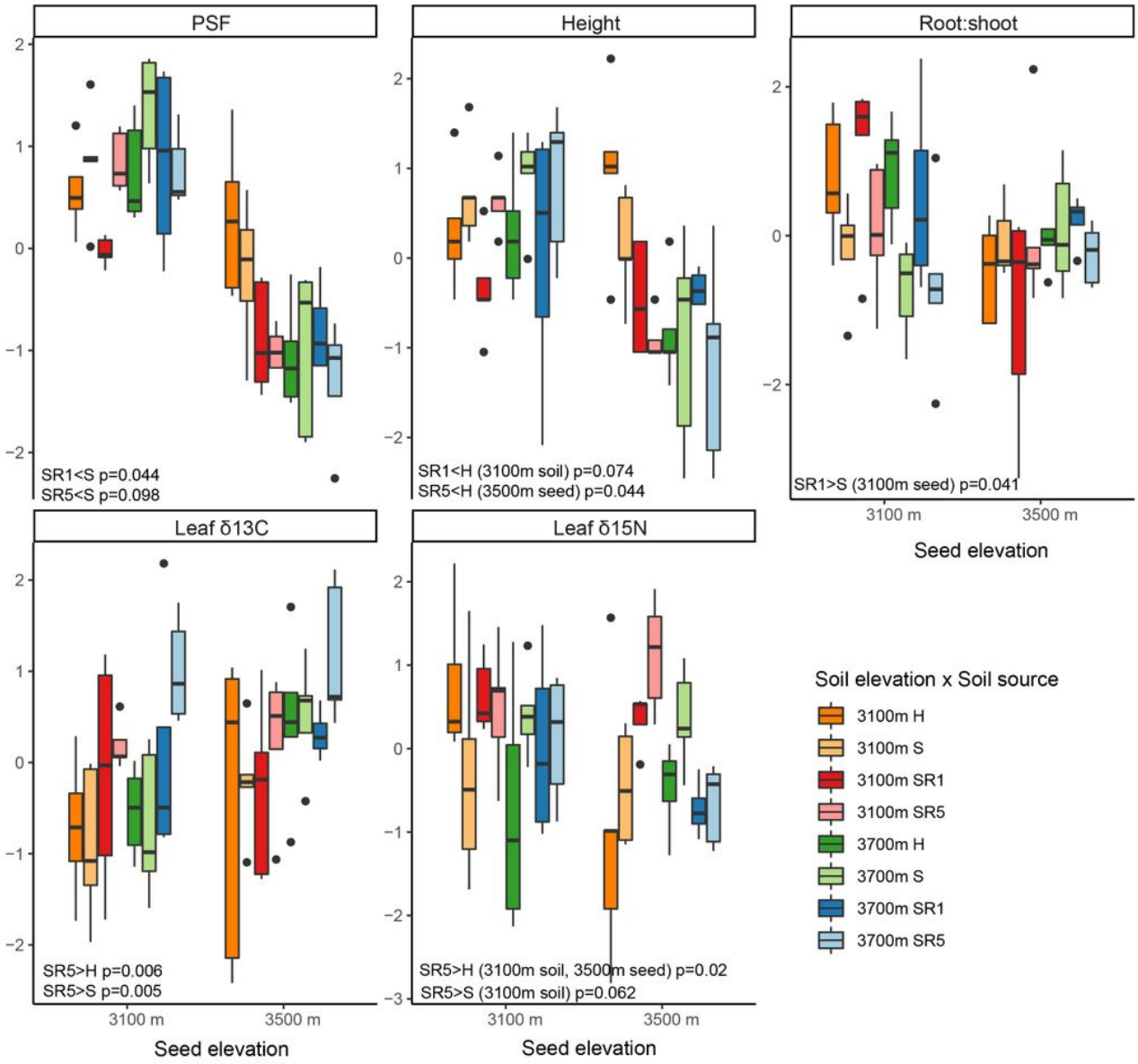

Figure 5

Sagebrush seedling growth and leaf functional trait responses in each soil inoculum type (i.e. soil elevation (3100m, 3700m) by soil source (herbaceous (H), sagebrush (S), 1-year shrub removal (SR1), and 5-year shrub removal (SR5) for seedlings from low (3100m) and mid (3500m) elevation seed sources. Seedling responses that differed in shrub removal inoculum (PSF ratio, height, root:shoot ratio, leaf $\delta^{13}C$ and leaf $\delta^{15}N$) are shown, and all measured plant responses can be found in Figure S1. Box-plots include the median (black line), first and third quartiles (bottom and top of the box, respectively), 1.5 times the interquartile range (whiskers), and outliers (black points) and each box includes (n=5) seedlings. All values are standardized with mean zero and unit variance for comparison. PSF= {(total biomass (g) live soil – total biomass (g) sterile soil)/total biomass (g) sterile soil}. Root: shoot ratio= total belowground biomass (g) / total aboveground biomass (g) of each seedling. P-values are shown where we found evidence for differences between shrub removal and other soil sources. Full ANOVA results and pairwise contrasts can be found in Tables S4 and S5.

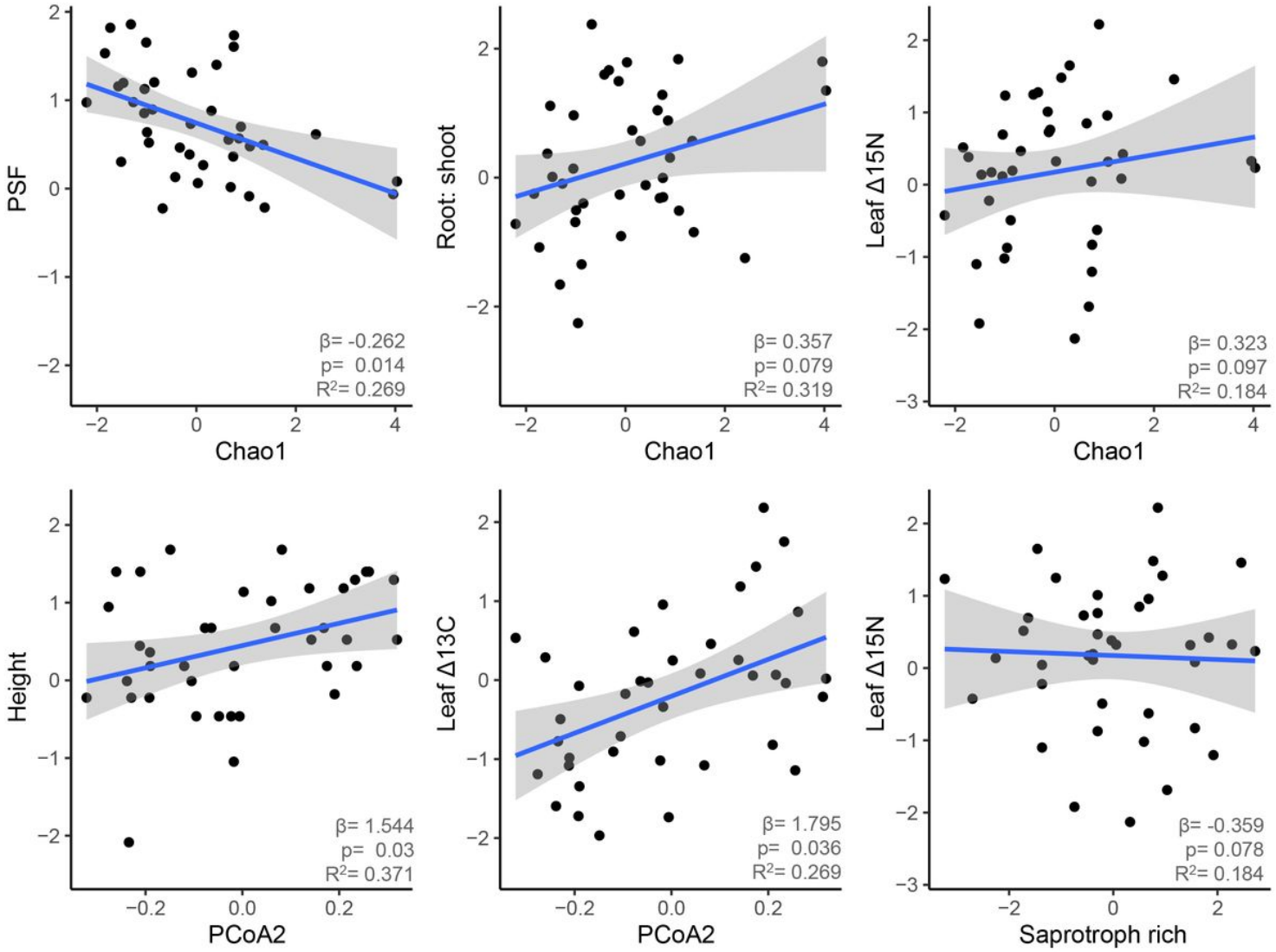

Figure 6

Seedling responses (PSF ratio, seedling height, root:shoot ratios, leaf $\delta^{13}C$ and leaf $\delta^{15}N$) by soil fungal community parameters (Chao 1 diversity, saprotroph richness and Principal coordinates Axis 2 of the Bray-Curtis dissimilarity metric for Beta diversity (community composition) from sagebrush seedling rhizosphere soils. We plot seedling and soil parameters where we found evidence of a relationship in linear mixed effects models (Table S6) and respective slopes (β), p-values and conditional R^2 values are reported. Each dot reflects (n=1) seedling and best fit lines are generated in the *geom_smooth* function in the R package 'ggplot2'(Wickham 2009) with the 'method= lm' argument. All values were standardized with mean zero and unit variance prior to modeling. PSF= {(total biomass (g) live soil – total biomass (g) sterile soil)/total biomass (g) sterile soil}. Root: shoot ratio= total belowground biomass (g) / total aboveground biomass (g) of each seedling.

Supplementary Files

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