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Legacy effects post removal of a range-expanding shrub influence soil fungal communities and create negative plant-soil feedbacks for conspecific seedlings

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Nuttapon Pombubpa · **Jeffrey M. Diez**

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, we know less about the soil legacy effects post removal of native range expanding species.

Methods We used a controlled greenhouse experiment with a range-expanding sagebrush species (*Artemisia rothrockii* (Asteraceae)) to determine how multiple metrics of sagebrush seedling performance (plant-soil feedback (PSF) ratio, height, leaf functional traits, and root:shoot biomass) were influenced by soil legacy effects in both the native and expansion range and over time since removal. We inoculated seedlings with field-collected soils from under sagebrush canopies and in herbaceous interspace, as well as in areas where sagebrush had been removed for 1 or 5 years. We then used ITS2 sequencing and extracellular enzyme assays to characterize the structure and function of soil microbial communities and to determine what microbial mechanisms drove seedling responses.

Results Conspecific sagebrush seedlings responded negatively to soil legacy effects of shrub removal, with a more negative PSF ratio, reduced height, and

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higher root:shoot ratios in shrub removal inoculum than in shrub and herbaceous soil inoculum. Seedlings in shrub removal inoculum also had enriched foliar isotope ratios, reflecting higher resource use efficiency. Soil communities of 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. Despite changes in functional group richness, fungal diversity and community composition proved the strongest drivers of seedling performance overall.

Conclusions This work provides novel insight into how soil legacy effects post removal of a native range expanding species may *limit* rather than promote the performance of conspecifics over short and long time periods, with important implications for management as global change continues to shift the geographic ranges of woody plants.

Keywords Soil legacy · Plant-soil feedbacks · Woody removal · Woody encroachment · Range expansion

Introduction

Global climate and land use change are increasing the establishment of plant species beyond their current ranges, both through range shifts and non-native invasions (Walther et al. 2009; Morrien et al. 2010). These 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 (Tomiolo and Ward 2018). These changes in the soil environment may then feedback to influence the growth of conspecific and neighboring plant species, a process known as plant-soil feedbacks (PSFs) (Bever et al. 1997; Kulmatiski et al. 2008). Furthermore, these changes to the soil ecosystem may persist long after the novel species is removed, dies or naturally declines (i.e., extirpated), at which point they are considered 'soil legacy effects' (Van de Voorde et al. 2011; Kostenko and Bezemer 2020; Li et al. 2022). 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 much less about the soil legacies of native range expanding species and how they may feedback to influence plant performance. 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, 2020; 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 species' (i.e. conspecific) growth and recruitment, known as positive PSFs, while negatively influencing currently established plant species (Van der Putten et al. 2013; Duell et al. 2019). This can include increases in generalist soil pathogens (i.e. pathogen spillover), decreases in beneficial soil mutualists, and changes to the soil saprotroph community and microbial enzyme activity (Eppinga et al. 2006; Coats and Rumpho 2014; Caravaca et al. 2020; Semchenko et al. 2022). Alternatively, novel species may cause changes to soil microbial communities that are beneficial for heterospecifics and limit the growth of conspecifics, known as negative PSFs (Kulmatiski et al. 2008). This can include an accumulation of species-specific soil pathogens (and/or dilution of generalist pathogens), but also higher microbial enzyme activity and larger mycorrhizal networks, especially if the novel species are nurse plants such as shrubs, nitrogen fixers or cushion plants (Rodríguez-Echeverría et al. 2016; Semchenko et al. 2022). 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; Austin et al. 2014; Cantarel et al. 2015). Overall, the net effects of novel plant species on the soil environment, and whether these changes persist over time, will ultimately feedback to influence intra and inter-specific plant performance and plant species coexistence (Bever 2003; Brandt et al. 2013; Revilla et al. 2013; Chung and Rudgers 2016).

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). For example, 6 years after 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, in grassland plant species, soil fungal legacies lasted much longer than bacterial legacies, however both fungal and bacterial legacies were conserved inside newly establishing plant roots and continued to shape future plant growth even after the soil legacy receded (Hannula et al. 2021). For range expanding species, soil legacies may also differ in the historic versus expansion range and may differentially feedback to influence subsequent plant growth and population dynamics. For example, in a range expanding subalpine shrub, soil legacy effects on bacterial and fungal diversity were much stronger in the expansion range (alpine zone) versus the historic range (subalpine zone) (Collins et al. 2016, 2018). In addition, range-expanding species across Europe grew better in soil legacies from congeneric natives than conspecifics, but these effects differed by soil origin (expansion range vs historic range) across species (Li et al. 2022). 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.

While soil microbial legacies can directly affect plant growth (i.e. biomass), growing evidence suggests that they also can affect plant functional traits (Friesen et al. 2011; Lau and Lennon 2011; Xi et al. 2018), which can in turn influence plant performance and ecosystem processes (Van Nuland et al. 2016). Incorporating plant functional traits into a multi-dimensional framework of PSFs is now widely accepted (Gundale and Kardol 2021); however, most work to date has considered the relationship of PSFs and plant functional traits at the species or community level (Baxendale et al. 2014; Kardol et al. 2015; Ke et al. 2015; Kuťáková et al. 2018; Xi et al. 2021). Yet evidence suggests that soil microbial communities can also alter intraspecific trait variation and local adaptation in plants (Petipas et al. 2021), and that more diverse microbial communities may promote reproductive traits associated with increased fitness (Lau and Lennon 2011). Despite recognizing its importance, few studies have considered the role of intraspecific trait variation in PSFs, both in response to, and in cultivating of novel soil microbial communities (Westerband et al. 2021). Functional traits which promote rapid resource acquisition or growth may enhance the performance of range expanding plants in novel ecosystems (Angert et al. 2011; MacLean and Beissinger 2017); thus, an improved understanding of how intraspecific traits are influenced by soil microbial communities during range expansion is vital.

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). In many cases, woody encroachment is considered land degradation and as a result, there is an increasing use of woody plant removal practices in land management (Archer et al. 2017). We lack a broad understanding of the soil legacy effects of woody species post-removal, however recent work has shown that abiotic soil legacies may be predicted by woody plant traits (Eldridge and Ding 2021) and can last decades after removal 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. 2016, 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)). 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. 2016, 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. 2016, 2018). We focus on legacy effects in soil fungal communities, because they form important mutualistic connections in the plant rhizosphere as well as contain numerous plant pathogens (Lee Taylor and Sinsabaugh 2015; Nguyen et al. 2016).

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 influence conspecific seedling performance across the species' range. We expected that historic and short-term (1 year) removal legacies will have a more negative effect on conspecific performance as they will be most similar to intact sagebrush soil communities. We further aimed 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, with the expectation that mutualist:pathogen ratios will have the strongest direct effects on seedling performance (above and belowground biomass ratios, height, functional traits).

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) and *A. rothrockii* individuals in this range have moderate to high AMF root colonization (~60–80% average, Collins, C.G.- unpublished data).

Research took place at the White Mountain Research Center (37.3609° N, 118.3269° W), 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 391 mm (Hall 1991)). This area is characterized by a short growing season (June 1st- Oct 31st) and moderate average growing season temperature

(5.23 °C) and precipitation (513 mm) which have increased and decreased respectively since 1961 (Kopp and Cleland 2014). 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). Furthermore, soil moisture, soil organic C and N increase from the low to high elevation sites (Collins et al. 2016).

Field sampling

In October 2015, approximately 1000 seeds were collected from 10 mature sagebrush individuals (100 seeds per individual) 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 and Karrfalt 2008). In September 2016, we sampled soils (soil cores 1.3 cm diameter × 10 cm deep) for use as greenhouse inoculum from under five sagebrush individuals (< 100 m apart), along with paired shrub interspaces, at the low (3100 m- historic range) and high (3700 m- expansion range) elevation sites. 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 × 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. 2016, 2018). Root systems were left intact, as is customary in shrub
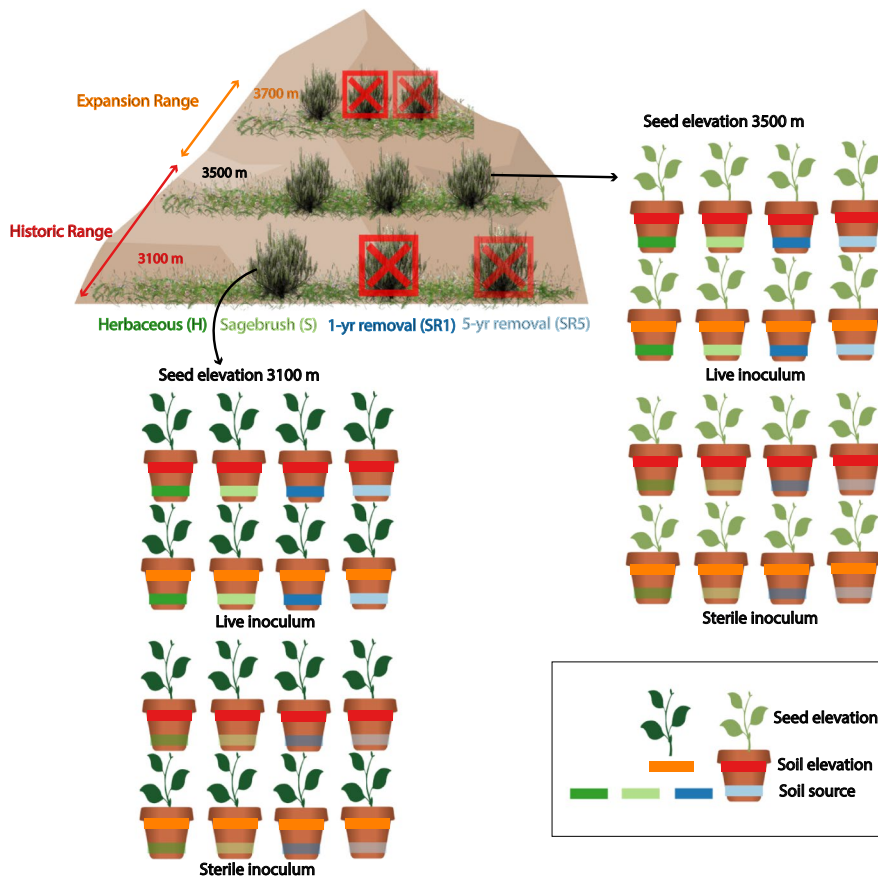
Fig. 1 Greenhouse experimental design. Seeds were collected from two intact *A. rothrockii* populations at 3500 m elevation (dark green seedlings) and 3100 m elevation(light green seedlings), both within the historic range of *A. rothrockii*. Soil inoculum was collected from the low elevation site (historic range, 3100 m, red) and the high elevation site (expansion range, 3700 m, orange) from each of four soil sources: intact herbaceous (H, green), intact sagebrush (S, light green) or areas where intact Sagebrush had been manually removed for 1 (SR1, blue) or 5 (SR5, light blue) years. For each pot icon, the color of the seedling reflects the elevation of the seed source population, the top bar color pot reflects the elevation of the soil inoculum and the lower bar color reflects the source (i.e. vegetation type) of soil inoculum. Full colored bars reflect live inoculum and faded color bars reflect paired sterile inoculum. Each pot represents 5 replicate seedlings for a total of N = 160 seedlings

removal experiments to avoid significant disturbance to soil communities (Berlow et al. 2003; Yin et al. 2017; Kopp and Cleland 2018), however decaying roots likely influenced soil legacies.

We use two time points post shrub removal to ensure that soil legacies were adequately accounted for after the large initial pulse of organic (root) material. 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 (Fig. 1). 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.

Microbial activity (extracellular enzyme assays)

Extracellular enzyme activities (nmol hr^{-1} g^{-1}) 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.

Greenhouse experiment

Seeds were surface sterilized with 10% bleach solution and germinated in trays of sterilized soil (autoclaved at 120˚C for 90 min). After one month, seedlings were transplanted to larger pots (1600 mL) of sterile background soil at the University of California Riverside (33.9737° N, 117.3281° W, elevation 252 m). 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 soil inocula 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$_4$P$_2$O$_8$), 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, we dug a small hole in the background soil in the center of each pot and placed 25 g (~50 mL, 3% total pot volume) of either sterile or live soil inoculum and then directly above, the roots of each seedling being transplanted, to allow for microbial inoculum to infect the plant rhizosphere. We grew two seedlings (one per seed elevation) for each soil sample (paired live and sterile) for a total of 160 seedlings (one seedling per pot) (Fig. 1) (ISS-MSS design type, Gundale et al. 2019). We include two seed elevations (3100 m and 3500 m) in our greenhouse study to assess whether plant responses were consistent across multiple sagebrush populations

within the historic range that may serve as source populations for the expansion range. Seedlings were grown for 4 months (between 126–130 days, ~ one alpine growing season) from October 2016-February 2017. 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. 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 h 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) ratio 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. During harvest, we also collected rhizosphere soil from the roots of seedlings from the low elevation seed source growing in live inoculum (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.

Leaf traits

We measured the following leaf functional traits for each seedling in live inoculum (N = 80): leaf dry matter content LDMC (g/g), specific leaf area SLA (cm^2/g), leaf N (%), leaf C (%), δ^{13}C, and δ^{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 h 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 h) 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 m, N = 40, Fig. 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′-GCA TCGATGAAGAACGCAGC-3′) and ITS4 (5′-TCC TCCGCTTATTGATATGC-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 2 × 250 paired end run on the Illumina HiSeq 2500 sequencing platform (San Diego, CA).

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 mixed effects models (see below).

Functional guilds were assigned to fungal sequences using FUNGuild v1.0 (Nguyen et al. 2016) with functional guilds assignments (mutualist, plant pathogens and saprotrophs). We filtered all unique FUNGuild 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, resulting in a total of 295 OTUs (Table S3). All mutualists had the confidence ranking of 'highly probable' and all plant pathogens had the confidence ranking of 'highly probable' or 'probable.' Any taxa with the with the confidence ranking of 'possible' (i.e. undefined saprotrophs), we further examined the literature citation to confirm their role (Table S3). We then calculated the richness of saprotrophs, pathogens, and mutualists in each soil sample as determined by these FUNGuild assignments. However, it is important to note that members of all fungal guilds (pathogenic, saprotrophic, mutualistic) may switch modes or play multiple roles depending on environmental

context (Johnson et al. 1997; Olson et al. 2012; Zanne et al. 2020) and thus all assignments are broad generalizations.

Statistical analyses

We tested soil microbial (fungal) community structure (alpha diversity, functional group richness) and function (extracellular enzyme activity) using two-way analysis of variance in the function 'aov' in R (R Core Team 2020) to determine how soils differ between vegetation types in the historic and expansion range and over time since removal. We used the following categorical predictors with an interaction 1) soil source (i.e., vegetation type- shrub, herbaceous, shrub removal 1 yr, shrub removal 5 yr) 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

For microbial community composition (beta diversity) 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).

We analyzed seedling responses (PSF, height, root:shoot, leaf traits) 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 type- shrub, herbaceous, shrub removal 1 yr, shrub removal 5 yr), 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 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 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

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 metrics where we found evidence of an effect of shrub removal (determined in the above analyses), as we aimed to understand how soil legacy effects post removal influenced seedling responses through soil microbial mechanisms. Because we measured fungal community composition on rhizosphere soils from low elevation (3100 m) 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 type) 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

(Figs. 2 and 3, Table 1, S1). 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 S1). In terms of elevation, seedlings inoculated with high elevation soils (3700 m) had lower alpha diversity, mutualist and pathogen richness and altered community composition when compared to seedlings inoculated with low elevation (3100 m) soils, regardless of soil source (Table 1, S1).

Legacy effects of shrub removal were consistent between soils from the expansion (high elevation) and historic (low elevation) range, as we found no interactions between soil source and soil elevation (Table S1). We also found no evidence that shrub removal inoculum influenced mutualist richness in seedling rhizosphere soils (Fig. 2, Table 1, S1). 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 and NAG
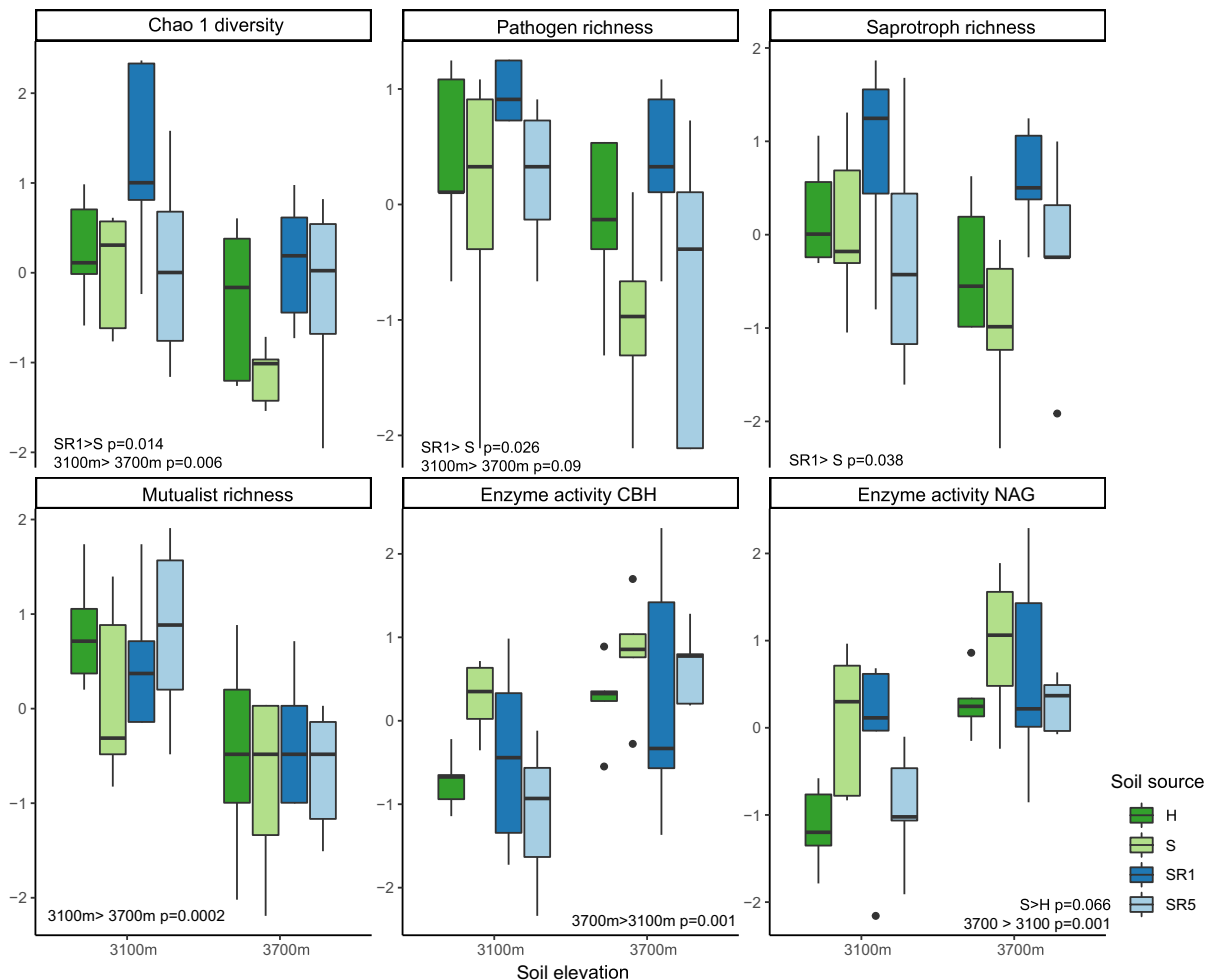

Fig. 2 Soil fungal community structure (Chao 1 diversity, pathogen, saprotroph and mutualist richness) from sagebrush seedling rhizosphere soils and extracellular enzyme activity (Cellobiohydrolase (CBH) and β-N-acetylglucosaminidase (NAG)) from live field soil inoculum. Box- plots include the median (black line), first and third quartiles (bottom and top of the box, respectively), 1.5 times the interquartile range (whisk-ers), 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 or soil elevations. Full ANOVA results and pairwise contrasts can be found in Tables 1 and S1
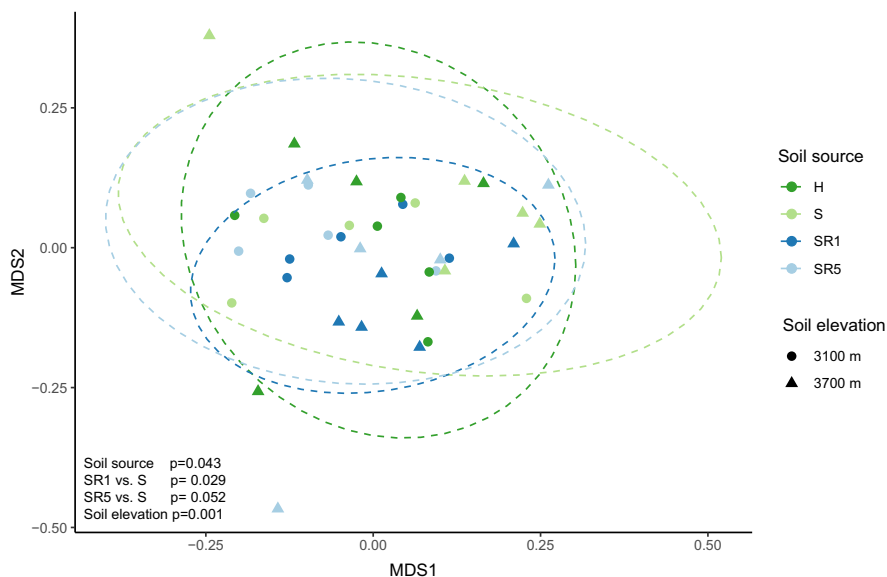
Fig. 3 Non-metric multidimensional scaling (NMDS) of the Bray–Curtis dissimilarity of soil fungal communities from sagebrush seedling soils. Soil source indicates that inoculum was sourced from intact herbaceous (H), intact sagebrush (S) or areas where intact sagebrush had been manually removed for 1 (SR1) or 5 (SR5) years. Soil elevation indicates that inoculum was sourced from either low elevation (3100 m- sage-brush 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 or soil elevations. Full perMANOVA results and pairwise contrasts can be found in Tables 1 and S1

Microbial response	Predictor	DF	Sum Sq	F val	P val
Enzyme activity (NAG)	**Soil source**	**3**	**5.978**	**3.034**	**0.043**
	Soil elevation	**1**	**11.349**	**17.279**	**0.000**
	Soil source: soil elevation	3	0.654	0.332	0.802
Enzyme activity (CBH)	Soil source	3	4.129	1.947	0.142
	Soil elevation	**1**	**10.074**	**14.250**	**0.001**
	Soil source: soil elevation	3	2.173	1.025	0.395
Alpha diversity (Chao1)	**Soil source**	**3**	**7.913**	**3.593**	**0.024**
	Soil elevation	**1**	**6.292**	**8.570**	**0.006**
	Soil source: soil elevation	3	1.300	0.590	0.626
Saprotroph richness	**Soil source**	**3**	**7.764**	**3.027**	**0.044**
	Soil elevation	1	2.279	2.665	0.112
	Soil source: soil elevation	3	1.595	0.622	0.606
Pathogen richness	**Soil source**	**3**	**7.815**	**3.360**	**0.031**
	Soil elevation	**1**	**5.965**	**7.693**	**0.009**
	Soil source: soil elevation	3	0.408	0.175	0.912
Mutualist richness	Soil source	3	1.491	0.664	0.580
	Soil elevation	**1**	**12.911**	**17.258**	**0.000**
	Soil source: soil elevation	3	0.656	0.292	0.831
Beta diversity (Bray–Curtis)	**Soil source**	**3**	**0.806**	**1.426**	**0.043**
	Soil elevation	**1**	**0.814**	**4.322**	**0.001**
	Soil source: soil elevation	3	0.536	0.949	0.572

Table 1 Results of two-way ANOVA (alpha diversity, functional group richness, extracellular enzyme activity) and PERMANOVA (beta diversity) testing whether soil microbial (fungal) community structure (3100 m seed elevation only) differed across seedling rhizosphere soils or across live field soils for function (extracellular enzyme activity). Results are bolded where we found evidence of an effect of model predictors Models include the following categorical predictors with an interaction 1) soil source (i.e.vegetation type-sagebrush, herbaceous, shrub removal 1 yr, shrub removal 5 yr) and 2) soil elevation (3100 m-historic range, 3700 m-expansion range)

and CBH enzyme activity were higher in high than low elevation soils (Fig. 2, Table 1, S1). 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 S2). 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 S2). 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 S2). 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 S2).
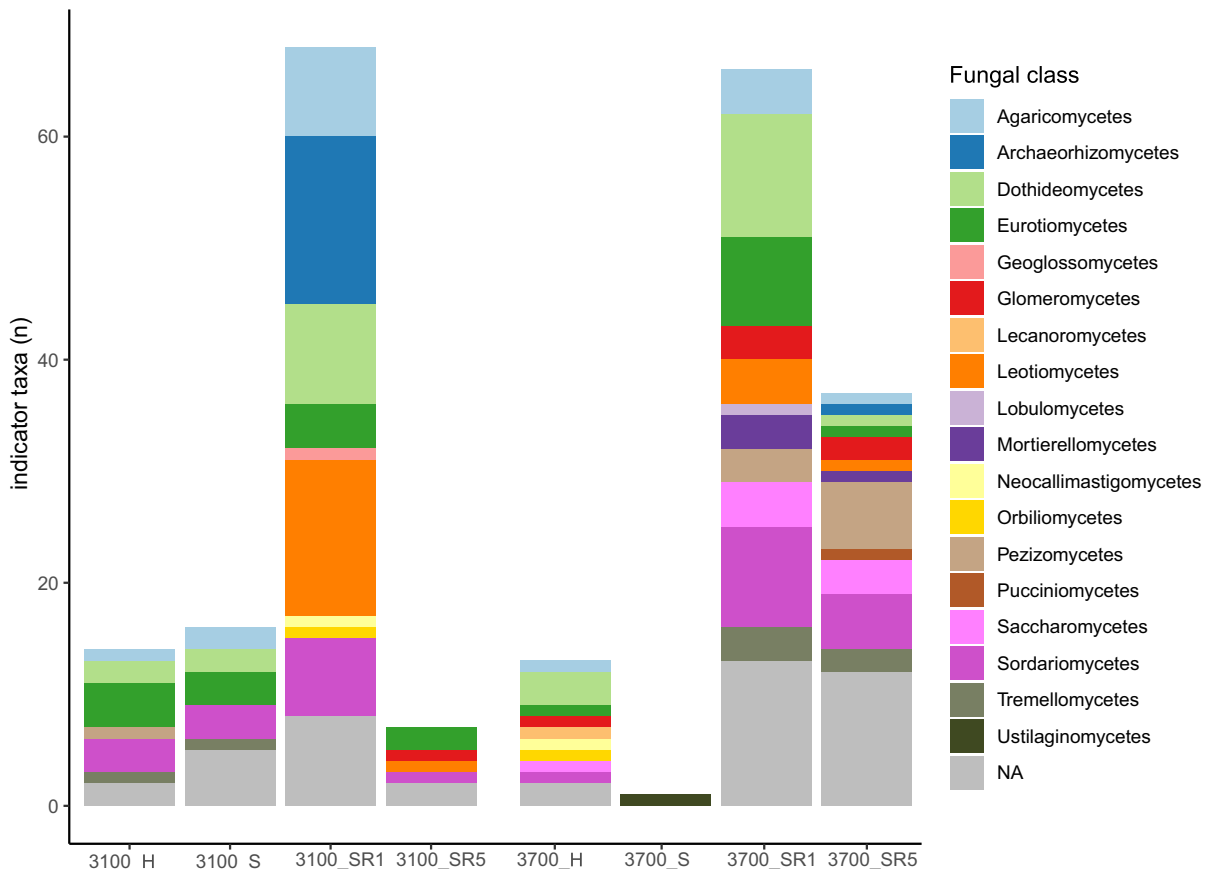

Fig. 4 Results of Indicator species analysis for each unique inoculum (i.e. soil elevation (3100 m, 3700 m) by soil source (herbaceous-H, shrub-S, 1-year shrub removal-SR1, 5-year shrub removal-SR5)). Bars reflect the total number of indicator species (colored by fungal class) sampled from rhizosphere soils of (n=5) sagebrush seedlings per soil inoculum. Full results can be found in Table S2

Seedling performance

Across all soil sources, seedlings had lower total biomass in live versus sterile soil inoculum, confirming the sterilization treatment was effective (t = 2.4764, p = 0.014). Sagebrush seedlings responded negatively to soil legacy effects of sagebrush removal, with more negative PSFs, reduced height, and higher root: shoot ratios in shrub removal inoculum when compared to those growing in shrub and herbaceous soil inoculum (Fig. 5, Table 2, S4). Seedlings had more negative PSF ratios in 1-year and 5-year shrub removal inoculum than when growing in shrub inoculum (Fig. 5, Table 2, S4). 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 S4). In addition, seedling height was lower in 5-year shrub removal than in herbaceous soil inoculum for the mid elevation seed source (Fig. 5, Table S4). 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 S4).

Soil legacy effects of shrub removal also influenced leaf isotope ratios where seedlings growing in 5-year shrub removal inoculum had higher (less negative) leaf $\delta^{13}C$ values, indicating a higher water use efficiency (WUE) or more water stress (May and Oberbauer 2021; Spasojevic and Weber 2021), than seedlings growing in shrub or herbaceous inoculum overall (Fig. 5, Table S4). Leaf $\delta^{13}C$ values were also higher in 5-year shrub removal than 1-year shrub removal inoculum overall (Fig. 5, Table S4). We found a similar pattern for leaf N isotope ratios, but only within certain soil, seed elevations. Specifically, seedlings had higher leaf $\delta^{15}N$ values in low elevation 5-year shrub removal inoculum than low elevation shrub inoculum. We found the same pattern in low elevation 5-year shrub removal inoculum versus herbaceous inoculum for the mid elevation seed source (Fig. 5, Table S4). Foliar $\delta^{15}N$ values are more complex to interpret than $\delta^{13}C$ values and can reflect differences in nitrogen form, mycorrhizal symbioses and depth of N acquisition among other factors (Spasojevic and Weber 2021).

Seed elevation influenced overall seedling growth and leaf traits, as seedlings from low elevation (3100 m) seed were taller, with more positive PSF ratios, higher root: shoot ratios, higher leaf C, LDMC, and $\delta15N$ and lower $\delta^{13}C$ (Fig. 5, Table 2, S4). However, seedling responses to shrub removal inoculum did not differ consistently by seed elevation, as differences in seedling height and leaf $\delta^{15}N$ were stronger for mid elevation seeds (3500 m), while differences in root:shoot ratio were stronger for low elevation seeds (3100 m) (Fig. 5, Table 2, S4). Soil elevation also influenced seedling leaf traits as seedlings growing in low elevation inoculum 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 PSF ratio, height and leaf $\delta^{15}N$ (Fig. 5, Table 2, S4).

Microbial mechanisms of seedling performance

Mixed-effects modeling revealed that fungal alpha diversity, saprotroph richness and beta diversity (community composition) best predicted seedling responses (low seed elevation only) (Table 3). Fungal alpha diversity (Chao1) had a negative relationship with seedling PSF ratios and a positive relationship with seedling root:shoot ratios and leaf $\delta^{15}N$. Fungal community composition (PCoA2) influenced had a positive relationship with seedling height and leaf $\delta^{13}C$ values and saprotroph richness had a negative relationship with leaf $\delta^{15}N$ values (Fig. 6, Table 3).

Principal coordinates analysis showed that shrub removal soils loaded most heavily on PCoA2 (Fig. S2) with a positive PCoA2 values reflecting soil communities of seedlings with shrub removal inoculum and negative PCoA2 values reflecting soil communities of seedlings with shrub and herbaceous inoculum. 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), so this axis was used as a predictor in mixed-effects models (above).

Discussion

Removal of a range expanding shrub species left distinct signatures on soil microbial community
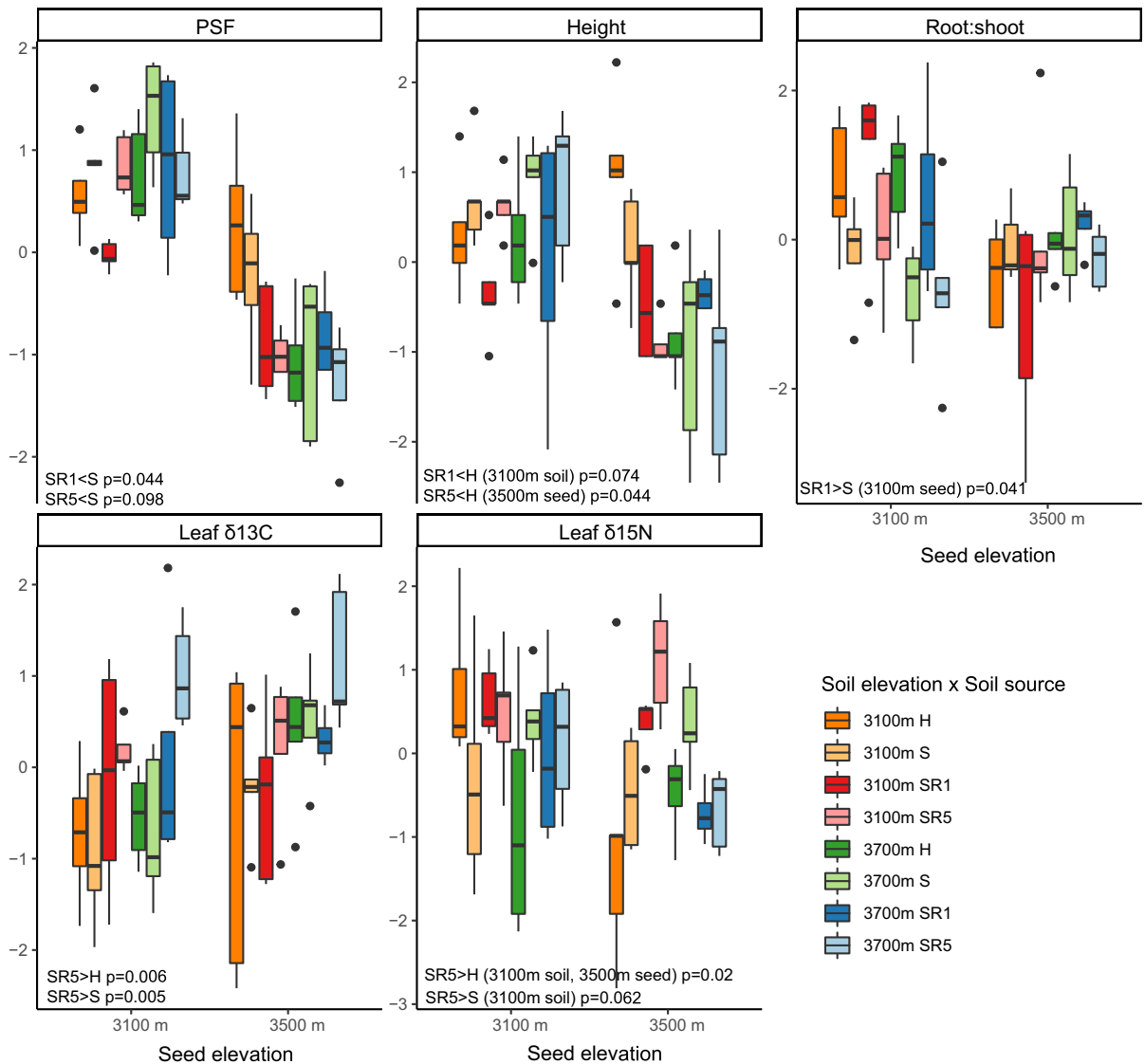
Fig. 5 Sagebrush seedling growth and leaf functional trait responses in each soil inoculum (i.e. soil elevation (3100 m, 3700 m) by soil source (herbaceous (H), sagebrush (S), 1-year shrub removal (SR1), and 5-year shrub removal (SR5)) for seedlings from low (3100 m) and mid (3500 m) elevation seed sources. Seedling responses that differed in shrub removal inoculum (PSF ratio, height, root:shoot ratio, leaf δ^{13}C and leaf δ^{15}N) are shown, and all measured plant responses can be found in Figure S1. 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 2 and S4. 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

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

Table 2 Results of three-way ANOVA testing whether seedling responses (PSF, height, root:shoot, leaf traits) differed across soil inoculum. Results are bolded where we found evidence of an effect of model predictors

Seedling response	Predictor	DF	Sum Sq	F val	P val
PSF	**Soil source**	**3**	**3.190**	**3.382**	**0.024**
	Soil elev	1	0.280	0.906	0.345
	Seed elev	**1**	**42.990**	**136.940**	**0.000**
	Soil source: soil elev	**3**	**2.740**	**2.904**	**0.042**
	Soil source: seed elev	**3**	**2.670**	**2.833**	**0.045**
	Soil elev: seed elev	**1**	**4.460**	**14.212**	**0.000**
	Soil source:soil elev:seed elev	3	1.200	1.278	0.290
Height	Soil source	3	3.440	1.870	0.144
	Soil elev	1	1.700	2.781	0.100
	Seed elev	**1**	**15.080**	**24.631**	**0.000**
	Soil source: soil elev	3	3.770	2.050	0.116
	Soil source: seed elev	**3**	**8.690**	**4.727**	**0.005**
	Soil elev: seed elev	**1**	**4.590**	**7.502**	**0.008**
	Soil source:soil elev:seed elev	3	1.760	0.960	0.417
Root:shoot ratio	Soil source	3	3.640	1.497	0.224
	Soil elev	1	0.050	0.063	0.803
	Seed elev	**1**	**3.600**	**4.446**	**0.039**
	Soil source: soil elev	3	2.330	0.958	0.418
	Soil source: seed elev	**3**	**12.390**	**5.099**	**0.003**
	Soil elev: seed elev	**1**	**2.920**	**3.601**	**0.062**
	Soil source:soil elev:seed elev	3	1.870	0.772	0.514
SLA	Soil source	3	3.170	1.039	0.382
	Soil elev	1	1.220	1.203	0.277
	Seed elev	1	1.050	1.037	0.312
	Soil source: soil elev	3	2.640	0.865	0.464
	Soil source: seed elev	3	1.340	0.440	0.725
	Soil elev: seed elev	1	0.200	0.200	0.656
	Soil source:soil elev:seed elev	3	4.370	1.433	0.242
LDMC	Soil source	3	1.640	0.610	0.611
	Soil elev	**1**	**4.280**	**4.790**	**0.032**
	Seed elev	**1**	**2.910**	**3.250**	**0.076**
	Soil source: soil elev	3	1.790	0.667	0.576
	Soil source: seed elev	3	4.030	1.501	0.223
	Soil elev: seed elev	1	0.030	0.030	0.863
	Soil source:soil elev:seed elev	**3**	**6.880**	**2.566**	**0.063**
Leaf C:N	Soil source	3	3.040	0.927	0.433
	Soil elev	1	0.100	0.092	0.762
	Seed elev	1	0.500	0.461	0.500
	Soil source: soil elev	3	1.380	0.420	0.740
	Soil source: seed elev	3	1.890	0.576	0.633
	Soil elev: seed elev	1	0.090	0.078	0.781
	Soil source:soil elev:seed elev	3	2.230	0.679	0.568

Table 2 (continued)

Seedling response	Predictor	DF	Sum Sq	F val	P val
Leaf C	Soil source	3	1.220	0.479	0.698
	Soil elev	1	2.260	2.655	0.108
	Seed elev	**1**	**16.750**	**19.697**	**0.000**
	Soil source: soil elev	3	0.760	0.298	0.827
	Soil source: seed elev	3	0.270	0.105	0.957
	Soil elev: seed elev	1	0.210	0.248	0.621
	Soil source:soil elev:seed elev	3	2.820	1.104	0.354
Leaf N	Soil source	3	2.940	0.877	0.458
	Soil elev	1	0.000	0.001	0.979
	Seed elev	1	0.040	0.032	0.860
	Soil source: soil elev	3	1.320	0.394	0.758
	Soil source: seed elev	3	1.910	0.570	0.637
	Soil elev: seed elev	1	0.140	0.128	0.722
	Soil source:soil elev:seed elev	3	1.230	0.368	0.777
Leaf δ^{15}N	Soil source	3	4.450	1.944	0.132
	Soil elev	**1**	**2.960**	**3.873**	**0.054**
	Seed elev	**1**	**2.340**	**3.071**	**0.085**
	Soil source: soil elev	**3**	**10.090**	**4.407**	**0.007**
	Soil source: seed elev	3	1.590	0.692	0.560
	Soil elev: seed elev	1	0.040	0.050	0.823
	Soil source:soil elev:seed elev	**3**	**8.220**	**3.592**	**0.018**
Leaf δ^{13}C	**Soil source**	**3**	**12.560**	**5.235**	**0.003**
	Soil elev	**1**	**6.520**	**8.157**	**0.006**
	Seed elev	**1**	**3.660**	**4.583**	**0.036**
	Soil source: soil elev	3	0.610	0.256	0.857
	Soil source: seed elev	3	2.890	1.206	0.315
	Soil elev: seed elev	1	0.930	1.164	0.285
	Soil source:soil elev:seed elev	3	0.250	0.102	0.958

We used the following categorical predictors with an interaction 1) soil source (i.e.vegetation type- sagebrush, herbaceous, shrub removal 1 yr, shrub removal 5 yr), 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)

in intact shrub or herbaceous soil inoculum, highlighting how shrub removal may limit further range expansion of conspecific woody species through biotically mediated plant-soil feedbacks.

Interestingly, soil microbial legacies post removal were highly distinct from the communities of intact sagebrush in diversity, functional group richness, and community composition (Figs. 2, 3 and 4). This challenges our initial hypothesis that post removal soils would create negative conspecific PSFs because they would be most similar to intact sagebrush soils. Rather, the distinct soil community cultivated in seedlings with shrub removal inoculum generated negative PSFs not present in seedlings with intact shrub soil inoculum. There may be several possible reasons for this discrepancy. First, soil pathogens in field sagebrush soils may be kept in check directly by allelochemicals or indirectly via recruitment of beneficial soil microbes through root exudates from live shrubs (Yuan et al. 2018; Scavo et al. 2019), both of which are no longer active post removal, thereby allowing the soil pathogen

Table 3 Results of linear mixed effects models testing the influence of rhizosphere soil microbial communities on measured seedling responses. Results are bolded where we found evidence of an effect of model predictors

For these models, we only included microbial and plant parameters that were distinct in shrub removal soils (see Tables 1 and 2). We predict seedling responses (3100 m seed only) with fixed effects of fungal diversity and functional group richness and a random effect of soil source nested within soil elevation. Conditional model R^2 values were calculated in the 'r.squaredGLMM' in the *MuMIn* package in R (Burnham et al. 2011)

Seedling response	Predictor	Est	SE	Df	t val	P val
PSF	**Chao1**	**−0.262**	**0.102**	**34.000**	**−2.578**	**0.014**
Model R^2c = 0.269	Saprotroph richness	0.093	0.106	34.000	0.870	0.390
	Pathogen richness	−0.032	0.081	34.000	−0.393	0.697
	PCoA2	0.551	0.473	34.000	1.164	0.252
Height	Chao1	−0.228	0.145	32.974	−1.576	0.125
Model R^2c = 0.371	Saprotroph richness	0.196	0.148	30.767	1.326	0.195
	Pathogen richness	0.040	0.112	30.646	0.362	0.720
	PCoA2	**1.544**	**0.680**	**32.953**	**2.271**	**0.030**
Root:shoot ratio	**Chao1**	**0.357**	**0.198**	**33.256**	**1.809**	**0.079**
Model R^2c = 0.319	Saprotroph richness	−0.269	0.202	31.416	−1.333	0.192
	Pathogen richness	0.054	0.152	31.285	0.354	0.726
	PCoA2	−1.184	0.927	33.268	−1.277	0.211
Leaf δ¹³C	Chao1	−0.002	0.176	33.840	−0.012	0.991
Model R^2c = 0.269	Saprotroph richness	0.105	0.181	32.504	0.582	0.564
	Pathogen richness	−0.058	0.136	32.212	−0.427	0.672
	PCoA2	**1.795**	**0.823**	**33.939**	**2.180**	**0.036**
Leaf δ¹⁵N	**Chao1**	**0.323**	**0.190**	**33.990**	**1.704**	**0.097**
Model R^2c = 0.184	**Saprotroph richness**	**−0.359**	**0.197**	**33.508**	**−1.821**	**0.078**
	Pathogen richness	0.138	0.149	33.165	0.930	0.359
	PCoA2	−0.380	0.888	33.792	−0.428	0.672

community to proliferate. Furthermore, a shift in organic matter substrate from predominantly leaf to (woody) root litter post removal is also likely to drive shifts in soil microbial community structure, including a higher diversity of saprotrophic taxa (Boddy and Watkinson 1995). We observe higher richness of both fungal pathogens and saprotrophs in seedlings grown in shrub removal compared to sagebrush soil inoculum (Fig. 2), however overall diversity and community composition were the strongest drivers of observed PSFs (Fig. 5).

Sagebrush soil legacies post removal 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 pathogenic 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 (DSE) with widespread distribution in arctic and alpine environments and commonly serving as mutualists in cold-stressed habitats (Newsham 2011), though the function of DSE is often unclear (Mayerhofer et al. 2013). 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; Rosling et al. 2011), however their ecological role in relation to plants is still mostly unknown (Pinto-Figueroa et al. 2019). 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. 2). 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).

We assessed whether soil legacies would change over time after shrub removal and between the
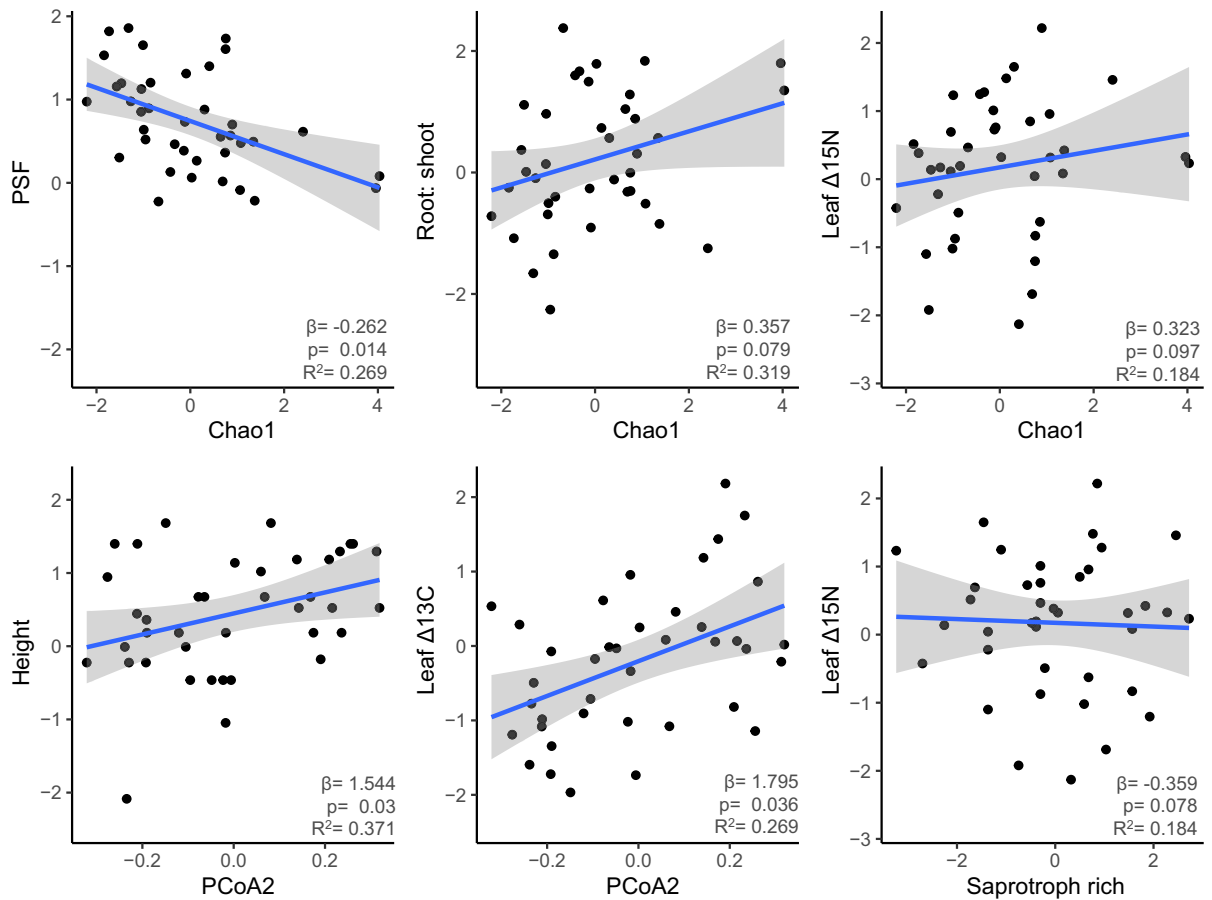
Fig. 6 Seedling responses (PSF ratio, seedling height, root:shoot ratios, leaf $\delta^{13}C$ and leaf $\delta^{15}N$) by soil fungal community metrics (Chao 1 diversity, saprotroph richness and Principal coordinates Axis 2 of the Bray–Curtis dissimilarity metric for Beta diversity (community composition) from sagebrush seedling rhizosphere soils. We plot seedling and soil (3100 m seed, soil elevation only) metrics where we found evidence of a relationship in linear mixed effects models (Table 3) 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

historic and range expansion zone of the woody species. The duration of soil legacy effects differed across microbial metrics, as soil fungal diversity, pathogen richness and saprotroph richness were highest in seedlings with 1-year shrub removal inoculum, while seedlings with 1 and 5-year shrub removal inoculum had distinct fungal community composition (beta diversity) (Figs. 2 and 3). This suggests that initial legacy effects post removal 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. Soil legacy effects post removal were relatively consistent between seedlings grown in historic versus expansion range inoculum, except for fungal diversity, where effects were much stronger in seedlings with 1-year shrub removal inoculum from the historic range (low elevation soils) (Fig. 2). 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. Likewise, soil origin (expansion vs. historic range) effects were weaker than plant

conditioning effects (native vs. range-expander) on congeneric seedling performance in riparian plant communities across Europe (Li et al. 2022). Although not part of the main questions of this study, seedling rhizosphere communities also had lower fungal diversity, lower pathogen and mutualist richness, and distinct community composition when inoculated with high elevation soils (3700 m- expansion range) regardless of soil source (i.e. vegetation type). In a previous study, we found similarly that fungal diversity declined and community composition shifted with increasing elevation in this range (Collins et al. 2018) and these results suggest that strong environmental filtering of alpine (3700 m) versus subalpine (3100 m) soil communities persist post inoculation and growth of conspecific seedlings.

Plant-soil feedbacks (PSFs) on conspecific seedlings in shrub removal inoculum were mostly negative, including reduced height and biomass in live versus sterile soil inoculum (i.e., PSF ratio). These negative PSFs occurred in seedlings growing in both 1-year and 5-year shrub removal inoculum, indicating that both short term changes in fungal diversity and functional group richness and longer-term changes fungal community composition had important influences on conspecific seedling growth (Figs. 4 and 5). Aspen seedlings inoculated with soils from dead adult aspen stands similarly had reduced growth and survival, highlighting that soil legacies can last for years after the death of the conditioning plant and continue to exert a negative influence on conspecific PSFs (Bennett et al. 2022). Furthermore, seedlings growing in shrub removal inoculum had leaf traits indicating higher water and nitrogen use efficiency (increased leaf δ^{13}C, δ^{15}N, Lloret et al. 1999; Spasojevic and Weber 2021) and higher root:shoot ratios as compared to seedlings growing in intact shrub and herbaceous inoculum (Fig. 5). High root:shoot ratios in combination with enriched leaf isotope ratios, suggests that these seedlings employed a more resource acquisitive trait strategy (Lloret et al. 1999). This may reflect coordinated responses with soil microbial communities to limiting resources (Lozano et al. 2021) and may have enhanced pathogenic and saprotrophic fungi already present in shrub removal soils. A similar pattern was observed in temperate grasslands, where plants with resource acquisitive traits such as thin roots cultivated a higher diversity of pathogens and specialist saprotrophs in their rhizosphere, resulting

in strong plant growth suppression in conspecific soils (Semchenko et al. 2018). By incorporating two seed elevations (i.e. populations) from the within historic range of sagebrush (Fig. 1), we aimed to broadly account for whether phenotypic plasticity influenced seedling responses across soil sources. Distinct PSF ratios and leaf δ^{13}C values in shrub removal inoculum were observed in seedlings from both seed elevations, while changes in height, root:shoot ratio and leaf δ^{15}N only occurred in one of the two seed elevations, suggesting that some PSF responses may reflect locally adapted phenotypes or be more plastic while others are more fixed. The biological underpinning for why certain seedling responses were shared across seed elevations, or not, requires further study, as there was no consistent pattern between the low vs. mid elevation seed responses despite their clear differences in size and traits overall (Table 2, S4).

Finally, fungal alpha diversity, beta diversity (PCoA2) and saprotroph richness influenced seedling PSF responses across all soil sources suggesting that the responses of seedlings in shrub removal soils are likely due to these underlying changes in fungal community structure. Of these, 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, we did not find evidence that pathogen richness itself was 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*, many of which are saprotrophic taxa that can also behave pathogenically (Olson et al. 2012; Ohm et al. 2012). Nonetheless, we can infer a role of plant pathogens in PSF responses, as seedlings had lower biomass in live than sterile soil inoculum overall (Bennett et al. 2022). Seedlings also had higher root:shoot and leaf δ^{15}N ratios in high diversity soils, which reflects resource acquisitive strategies for water and N belowground. For beta diversity, the PCoA2 axis reflects differences in fungal community composition among seedlings with shrub removal versus intact shrub and herbaceous soil inoculum (Fig. S2) which influenced seedling height and leaf δ^{13}C ratios, both reflective of light and nutrient acquisition strategies (May and Oberbauer 2021).

Our results have clear implications for the management of woody species and range expanding species. First, we found that negative PSFs 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 growth and generated negative feedbacks on seedling performance. These negative feedbacks were driven by distinct changes in soil fungal communities that occurred after short and 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 herbaceous systems, such as alpine communities or montane meadows, may require management (via removal) to protect resident species. 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 promising, as it would serve to slow down reinvasion by the removed species. Our work fills an important gap in understanding 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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