**Soil Legacy Effects and Arbuscular Mycorrhizal Fungi Throughout an Invasive Plant’s Range**

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**Introduction**

Invasive species reduce species diversity worldwide because of their ability to dominate natural areas (Mack et al 2000). It has been theorized that invasive species’ pervasiveness comes from their lack of predators and pathogens, their ability to colonize disturbed sites, or their novel interactions outside of their native environment, among other mechanisms (Keane & Crawley 2002; Simberloff 2010). However, invasion success can be variable, with only a fraction of species becoming invasive and only in certain areas outside of their endemic range (Mack et al 2000). Environmental factors, such as climate and nutrient availability, as well as the interactions between invasive and native species can all affect the range and severity of a species’ invasion (Hayes & Barry 2007; Yang et al 2013).

Interactions between native soil microbes and non-native plants are important determinants of whether a species becomes a successful invader. Plant-soil interactions range from mutualistic to parasitic, often depending on whether plants gain or lose nutrients from the interaction (Johnson et al 1997). Interactions with mutualistic soil microbes could be responsible for a plant species’ success in a nutrient-limited environment (Richardson et al 2009; Yoshida et al 2004). Whereas in nutrient-rich environments, microbes can become parasitic, which could inhibit a plant species’ dominance in the community (Bever 2002). Invasive plants often interact with soil microbes in their novel range and these interactions have proven important in enhancing or inhibiting invasion in several species (Reinhart & Callaway 2006; Yang et al 2013).

While plants can have large ranges spanning continents, soil microbial communities are highly variable depending on their plant hosts and under different environmental conditions (Afkhami et al 2014; Van der Heidjen 2002). When an invasive plant expands beyond its historic range, it may escape coevolved parasitic and pathogenic soil microbes or novel interactions could enhance or suppress its growth, influencing whether the species becomes invasive (Klironomos 2002; Maron et al 2013; Shah et al 2009). Several studies demonstrate invasive plants with negative soil feedbacks in their native range and positive feedbacks in their introduced range, suggesting that pathogen escape and/or new associations with mutualistic microbes may explain plants success in certain areas over others (Callaway et al 2004; Dostálek et al 2016; Reinhart et al 2003).

Some invasive plants have adapted to drive shifts in the microbial community around them, which could expand their range by increasing suitable habitat or give them an edge over native species (Davis et al 2021). By exuding chemical compounds through their roots or leaf litter, these plants change the soil community to increase their own mutualists or to suppress their competitors’ (Shah et al, 2009; Zubek et al 2016). Some of the most aggressive invasive plants in North America exert changes to the soil microbial community around them, indicating that this strategy is key to their success (Gibbons et al 2017). Plant-induced changes to soil microbial communities often have a lasting effect, which can influence the plant community for many years (Lankau 2010). These lasting effects, hereby referred to as legacy effects, can drive the future of a plant community by influencing different plant species to become more or less abundant.

Legacy effects of invasion often include changes to both the microbial community and nutrient dynamics important to plant growth. Plant-induced changes to the microbial community can have indirect effects on nutrient dynamics if plants influence the abundance of microbes important to nutrient uptake. Invasive plants can also directly alter nutrient availability by absorbing more or less than native species or by changing the soil’s texture, which could influence the rate of leeching from the soil (Evans et al 2001; Corbin & D’Antonio 2004). For instance, if an invasive plant changes either the N availability in the soil or the mycorrhizal community, which is often important in facilitating plant N uptake, its invasion could have downstream effects on native plant growth.

*Linaria vulgaris* (Plantaginaceae), commonly referred to as ‘Yellow Toadflax’ or ‘Butter and Eggs’, is a forb native to Southeastern Europe that is invasive throughout North America (Sing et al 2016). It reproduces both sexually from seed and asexually from rhizomes, making it extremely difficult to eradicate (Sing et al 2016). *Linaria vulgaris* is widespread throughout the U.S. but is particularly successful in Western states throughout the Rocky Mountain region (USDA, NRCS 2021). Outside of its clonal reproduction, little is known about why *L. vulgaris* is such a tenacious invader in the Western U.S. Ambient soil N is historically lower in the Rocky Mountain region, where *Linaria* aggressively proliferates, relative to soil N in the Midwestern U.S., where *Linaria* is non-aggressive (Bowman et al 2012; Jelinski & Kucharik 2009). This suggests that *L. vulgaris* alters the microbial community or outcompetes native plants for N in the Rocky Mountain region to invade more aggressively. Alternatively, *L. vulgaris* could be suppressed by soil microbes in the Midwestern U.S., making it an ineffective invader in that region.

This study addresses the biogeography of *Linaria vulgaris* invasion by asking why this speciesdominates Western subalpine meadows and not Midwestern prairies. Specifically, it aims to uncover whether 1) *Linaria vulgaris* invasion leaves legacy effects that influence native plants and its own success, 2) if soil from Colorado and Illinois influence *L. vulgaris* success, and 3) if N-availability explains the range of aggressive *L. vulgaris* invasion. We examined plant biomass accumulation and N uptake in *L. vulgaris* and two native plants grown in previously invaded and uninvaded soil collected from IL and CO, and in the presence or absence of N-fertilization. Using soil samples collected from *L. vulgaris* populations at the Rocky Mountain Biological Lab (RMBL) in Gothic, CO (aggressive) and Cook County, IL (non-aggressive), We grew plants in all possible treatment combinations in growth-chambers for 120 days. We then supplied 15N-labeled tracer (NH4NO3) and analyzed plant tissue for isotope abundance to detect plant N uptake capacity. We also used Next Generation Sequencing to characterize arbuscular mycorrhizal fungal (AMF) communities from previously invaded and uninvaded soils from CO and IL. The findings of this study will support ongoing research at RMBL of *L. vulgaris* impacts and may present insight into invasion biology more broadly, particularly in how invasive plants use legacy effects to regulate competition with native plants, and how regional soil biota and N availability influence invasion biogeography.

**Methods**

*Study Sites*

Fieldwork was conducted from July-August 2020 at the Rocky Mountain Biological Lab in Gothic, CO and in the Forest Preserves of Cook County in Cook County, IL. The Rocky Mountain Biological Lab (RMBL) is a research station located in Gothic Colorado, within Gunnison National Forest. Soil and rhizomes were collected from subalpine meadows (~2895m a.s.l) characteristically made up of diverse grasses, forbs, and shrubs (Price and Waser 1998). *Linaria vulgaris* is an aggressive invader at the Rocky Mountain Biological Lab and many populations are sprayed with herbicide (Glyphosate, RoundUp) to control the spread. Soil and rhizomes were collected from populations that had no history of herbicide use. The Forest Preserves of Cook County (~178m a.s.l.) are semi-urban natural areas in the Chicagoland area made up of prairies, woodlands, and wetlands (FPDCC 2020). Soil and rhizomes were collected from *L. vulgaris* populations without visible herbicide use, but herbicide history was not available at the time of collection.

*Study Species*

*Linaria vulgaris* (Plantaginaceae) is a forb native to Eurasia that has spread throughout North America since the 1600s (Sing et al 2016; Parker and Gassmann 2021). *L. vulgaris* is invasive in the Western U.S. and Alaska but it can be found throughout North America (McCartney et al. 2019). *Linaria vulgaris* can reproduce both from seed and clonally from rhizomes, but the latter is believed to be much more prevalent as its seed viability is ≤25% (Parker and Gassmann 2021). Reproducing clonally allows *L. vulgaris* to grow in dense patches and subsequently exclude native species. *Linaria vulgaris* is an undesirable species for cattle forage and it invades both rangeland and agriculture lands (Parker and Gassmann 2021). While the stem-mining weevil has shown some success as a biological control, management typically consists of herbicide application (Toševski et al. 2018; Parker and Gassmann 2021).

*Field Soil and Rhizome Collection*

We identified populations of *L. vulgaris* from research records in Gothic and using iNaturalist in Cook County, IL. In the summer of 2020, soil samples were collected from ten paired plots of *L. vulgaris* invaded and adjacent uninvaded plots in each site. Paired plots were at least 75m apart and were demarcated using N-S and E-W tape measures to calculate plot area. In plots greater than 100m2, we sampled from the centermost 5m2 area to capture the area with the longest invasion history. For plots smaller than 100m2, we sampled throughout the plot. To locate uninvaded plots, we selected a random intercardinal direction from the paired invaded plot and established an uninvaded plot 5-15m from the invaded plot and of the same size. To ensure random sampling throughout plots, we used a random number generator to select points on the tape measures to collect each sample. In each site (IL and CO), we collected 160 soil cores (152.4 mm depth, 101.6 mm width; *n* = 320): eight samples from each of 10 invaded plots and 10 adjacent uninvaded plots. We also collected 32 2.4cm rhizomes of *L vulgaris* from each invaded plot, for a total of 640 rhizomes. The soil and rhizomes were used in growth-chamber experiments, soil nutrient analyses, and Next Generation sequencing at the Chicago Botanic Garden.

*Growth-Chamber Experimental design*

This experiment consisted of a complete factorial design (2 x 2 x 2) in which we measured the growth and N uptake in *Linaria* and two native plant species (*Rudbeckia hirta,* *Leptoloma cognata*) in response to combinations of three factors: (i) soil site, either CO or IL ; (ii) invasion history, uninvaded (control) or *L. vulgaris*-invaded soil, and (iii) two levels of N, ambient or N-fertilized (N kg ha-1 as NH4NO3). Pots contained either *L. vulgaris* or both native species grown together. This combination resulted in 8 treatments across two plant combinations with 10 pots per treatment (*n* = 160). To prepare the soil for the experiment, fresh soil from each location and invasion type was gently mixed 1:1 (v/v) with coarse sterile sand (autoclaved). Pots (10.1 cm3) were filled with prepared soil and planted with either *L. vulgaris* alone (two rhizomes); *L. vulgaris* with native plants (two rhizomes and three seeds per native species)*,* or native plants only (three seeds per native species).

The two native species used in the growth-chamber experiment, *Rudbeckia hirta* (Asteraceae)and *Digitaria cognata* (Poaceae), are commonly found in disturbed sites, making them more likely to coexist with *L. vulgaris*. However, *R. hirta* and *D. cognata* are both native to IL, as obtaining and cold-stratifying seed native to CO would have delayed planting. *Rudbeckia hirta* is also native to CO but does not commonly occur alongside *L. vulgaris* at RMBL (USDA PLANTS 2021). Additionally, *R. hirta* is a forb, whereas *D. cognata* is a grass, which can respond differently to soil legacy effects; by growing both we hoped to capture broader impacts of *L. vulgaris* invasion on native species success. Seeds were donated by the Dixon National Tallgrass Prairie Seed Bank at the Chicago Botanic Garden. Seeds were dry stratified for 30 days at 4°C and then seeds were surface sterilized in a bleach solution (10%) before planting. *L. vulgaris* rhizomes were washed in DI water to remove excess soil. Rhizomes collected from CO and IL were planted in their soil of origin across all treatments.

Plants were grown in growth chambers using 12:12 hr photoperiod (day: night), 25/15°C (day/ night) and 75% relative humidity for four months. Plants were N-fertilized every 30 days at rates of 8.9 kg N ha yr-1 (6.1 kg N ha-1 yr as NO3, 2.8 kg N ha yr-1 as NH4) or were unfertilized (control). The level of N fertilization represents double the average N input from anthropogenic N deposition in Gothic, CO (4.05 kg N ha-1 year, 3:1 NO3:NH4, 20 yr-average; NADP 2022; Yoshida & Allen 2004).

*Stable Isotope and Biomass Analysis*

At the end of the growing period, 5 ml of 14.37 mM 15NH4-15NO3 solution (> 98.9 at %15N; Cambridge Stable Isotopes, Tewksbury, MA, USA) was injected into the root zone of each pot using 100mm 11-gauge biopsy needles (Fisher Scientific, Waltham, MA, USA; Yoshida & Allen 2004; Dresboll & Thorup-Kristensen 2012), leading to a total of 2.012 mg N added per pot. After 24 hours, plants were destructively harvested with each plant separated into root and shoot fractions, dried in biomass ovens (72 hours, 80˚C), and weighed. After weighing, leaf samples (*n* = 68) were collected from all treatments in which there was sufficient leaf material for analysis. Each leaf sample was ground to a fine powder using ceramic beads in Fastprep-24 MG with sub-samples (1-1.5mg), packed into aluminum capsules, and analyzed for 15N atom% and N concentrations in a Costech 4010 ECS (Costech Analytical Technologies, Inc., Valencia, CA, USA) coupled to an ion ratio mass spectrometer (IRMS; Delta V Plus, Thermo Scientific) at the Northwestern University Stable Isotope Laboratory.

We used the 15N data to calculate the rate of N uptake per day (as NO3 + NH4). The 15N atom percent excess (APE) was calculated as the difference in atom% 15N between labelled and non-labeled plants from literature (typically atom% 15N < 0.1; Torres-Poché et al 2020). The net uptake rate (NUR, µg N g-1 shoot per day-1) of N was calculated following McKane et al. (2002) as:

**NUR = [Plant N content (µg.g-1) x (APE/100)]/ ([time (days) x (atom%15N/ 100) x shoot mass (g)].**

The percentage of 15N recovered in plants (15N recovery %) was calculated as:

**15N recovery (%) = (15N uptake/ 15N supplied) \*100.**

The 15N uptake (µg N plant-1) was defined as the amount of 15N recovered from N pool (shoots) and calculated by multiplying APE (%) with the moles of N in the plant and 15N supplied refers to the 15N mass added to the soil per pot.

For our calculations of N uptake, we assumed that the shoot 15N levels in each plant species were proportional to the amount of 15N label taken up from the soil. While this approach simplifies differences in plant N allocation, our experiment was conducted when plant growth (native plants) and reproductive demands (*Linaria*, in flower) were high, so that N was more likely allocated to above- rather than below-ground structures. We also determined bulk %N from the same leaf samples used for IRMS to compare the effect of treatments on plant total N content for *L. vulgaris* and native plants.

*Soil Edaphic Factors*

Soil samples from field sites were analyzed for NO3, NH4, P, and pH. Soil extractions were created for 320 soil samples collected from CO and IL (160 each; 80 invaded and 80 uninvaded). To create extractions, five grams of soil was mixed in 45 mL of deionized water by shaking for 30 mins and centrifuging. Extractions were tested for pH using a pH probe (Vivosun, Ontario, CA). Extractions and reagents were added to 96-well plates and analyzed for NO3, NH4, and P using colorimetry on an Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, VT, USA). Absorbance was reported in Gen5 software (BioTek Instruments, Winooski, VT, USA) and μ/g was calculated across all samples. Soil texture was analyzed using the Bouyoucos method (Bouyoucos 1962). Soil samples were pooled per sampling plot and by invasion, (IL invaded, IL uninvaded, CO invaded, CO uninvaded) with 10 samples in each combination (*n* = 40) and analyzed for the percentage of clay, sand, and silt.

*AMF Community Sampling*

Genomic DNA was extracted from 0.25g of soil for 80 samples using the DNeasy PowerSoil Kit (QIAGEN-MO BIO, Carlsbad, California, USA). Extractions were cleaned using Cytiva Sera-Mag Select beads prior to amplification (Global Life Sciences Solutions, Marlborough, MA, USA). A 550 bp section of small-subunit ribosomal DNA (18S rRNA) was amplified using the forward primer NS31 and the reverse primer AML2 (Lee et al. 2008). PCR reactions entailed 2 minutes at 94°C, 30 cycles of 30 seconds each at 94°C, 1 minute at 59°C, 2 minutes at 72°C, and 10 minutes at 72°C. DNA amplification was verified by gel electrophoresis on a 1.5% agarose gel, which was then visualized on a Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290, Eastman Kodak Company, Molecular Imaging Systems, Rochester, NY, USA). Amplified DNA was cleaned again using Cytiva XP beads. Sample concentrations were then quantified in Qubit using a dsDNA HS assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples were indexed using a Nextera XT Index Kit (Illumina, San Diego, CA). Equimolar aliquots of each sample were pooled and sequenced at Northwestern on Illumina MiSeq version 3, 300-bp MiSeq Reagent Kit for paired-end reads (Illumina, San Diego, CA, USA). The first run yielded low-complexity sequences and as a result, samples were re-sequenced using Illumina MiSeq version 3, 150 bp MiSeq Reagent Kit for paired-end reads (Illumina, San Diego, CA, USA). Fragment size was measured using an Agilent 2100 Bioanalyzer System (Agilent Technologies, Waldbronn, Germany).

*Sequence Processing and Analysis*

Samples were demultiplexed in Illumina BaseSpace and processed in QIIME2 (Bolyen et al, 2019). DADA2 was used to denoise samples and reads were truncated at 120bp where quality dropped below a q value of 20 (Callahan et al 2016). Only forward sequences were included in further analyses, as previous studies have shown that forward sequences are sufficient to capture AMF diversity (Davison et al 2012), totaling 7,092,099 reads. Sequences were included if they were reported at least 10 times and in 3 or more samples. OTUs were clustered at 97% (96 OTUs) using vsearch and chimeras and borderline chimeras were removed. Taxonomy was assigned using the q2-feature-classifier (Bokulich et al 2018) naïve bayes classifier with MaarjAm reference sequences (Öpik et al 2010). OTUs with less than 80% alignment to MaarjAm reference sequences were removed. OTUs were blasted against the MaarjAm Database (Öpik et al 2010) and GenBank (Benson et al. 2012) to determine AMF diversity.

Sequences that could not be identified to AMF family level were aligned in CLUSTAL Omega using default parameters, and the percent identities among sequences determined. Pairwise sequences or multiple-sequence alignments with >97% similarity were classified as the same OTU. This resulted in 47 unique sequences for phylogenetic analyses. Unique sequences were then concatenated with a reference set of SSU sequences (Krüger et al. 2012) for major AMF genera (*Scutellospora, Gigaspora, Acaulospora*) as well as *Glomus* species and allied taxa (*Archaeospora, Claroideoglomus, Ambispora, Funneliformis, Paraglomus, Rhizophagus*). Alignments were trimmed and a maximum likelihood phylogenetic tree was inferred for the concatenated data set using RAxML with default options. Eighteen sequences could be placed within the *Glomus* clade, while the remaining 29 sequences were consistent with Glomeraceae but showed no specific phylogenetic placement. Clustal Omega and RAxML analyses were undertaken on the CIPRES gateway (v3.3) at the San Diego Supercomputer Center ([www.phylo.org](http://www.phylo.org/)).

*Statistical Analysis*

Statistical analyses were performed in R version 3.6.2 (R Core Team, 2019). We used linear models and linear mixed-effects models to determine the effects of state, invasion history, and fertilization as well as their interactions on biomass, plant presence, plant NUR, and plant %N (Appendix 1). Model fit was verified using histograms and the qqPlot function in the ‘car’ package (Fox & Weisberg 2019). Statistical significance was designated by a p-value ≤0.05 (Fox & Weisberg 2019). We also performed Tukey post-hoc tests to determine which treatments were significantly different. Results were then visualized using the ggplot function in the ‘ggplot2’ package (Wickham 2016).

Presence and absence of plants was designated with a 1 or 0, respectively, across all pots by treating all pots with shoot biomass greater than 0 as a 1 and all other pots as a 0. Mean plant presence was used as the response variable in models for each treatment. To compare presence/absence among treatments, we used generalized linear models (GLM) with binomial distributions across all possible combinations of variables (fertilization and invasion history) within all datasets (site and plants grown).

To compare plant biomass across treatments, we divided root and shoot biomass into datasets. Biomass was analyzed separately among plants grown (*L. vulgaris* or native species), and for each combination of states and invasion histories due to varying sample sizes. We also removed pots without biomass as these were evaluated in presence/absence analyses. We used linear mixed-effect models to compare biomass between site and invasion histories and plot

as a random effect using the lmer function in the ‘lme4’ package (Bates et al 2015). Biomass data for each combination of state and invasion histories was transformed by taking either the log or square to account for non-normally distributed data. The package ‘emmeans’ was used to generate Tukey pairwise-comparisons (Lenth 2021).

We log-transformed plant NUR and used linear models to compare NUR across invasion histories and fertilization treatments. We created linear models to compare bulk %N across treatments. We also generated linear mixed-effects models to test differences in soil pH, PO4, NO3, NH4 (log-transformed) and percent clay, silt, and sand (untransformed), across sites and invasion histories with plot as a random effect.

We performed permutational multivariate ANOVAs (PERMANOVAs) using the adonis2 function in the ‘vegan’ package to test whether AMF communities were influenced by invasion history, state, and individual edaphic factors (Oksanen et al. 2022). We performed an indicator species analyses using the multipatt function in the indicspecies package (Appendix 2,3; Cáceres et al 2010). We also compared individual OTU and family mean relative abundances across states and invasion histories using Kruskal-Wallis tests. Relative abundances of AMF OTUs and edaphic factors were visualized on Nonmetric Multidimensional Scaling (NMDS) plots using the metaMDS function in the ‘vegan’ package (Oksanen et al. 2020). We used the diversity function in the ‘vegan’ package to calculate Shannon’s diversity, which we used to calculate Pielou’s evenness for all OTUs (Oksanen et al 2022). We used linear mixed-effects models to compare OTU and family richness between states and invasion histories with plot as a random effect.

**Results**

*Plant Presence and Biomass*

In CO soil, overall plant presence varied significantly by the interaction of invasion history and species (Fig. 1, *X2* = 12.1, *P* < 0.001). In CO soil, *L. vulgaris* presence was significantly greater in invaded soil than uninvaded soil, irrespective of N fertilization treatment (*X2* = 7.72, *P* = 0.005). In contrast, native plant presence in CO soil was not affected by invasion history or N fertilization. Overall, in IL soil, plant presence was not significantly different between species, invasion histories, or their interaction. In IL soil, *L. vulgaris* was only present in uninvaded control pots, so we could not compare presence across treatments. Native plant presence in IL soil was not affected by invasion history or N fertilization.

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**Figure 1.** Percent of pots with plant growth representingplant presence/absence (1, 0) in pots containing CO soil (*n* = 80). Error bars represent mean plus one standard error. Asterisks represent a P value ≤ 0.01.

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**Figure 2.** Percent of pots with plant growth representingplant presence/absence (1, 0) in pots containing IL soil (*n* = 80). Missing bars signify treatments without any plant presence. Error bars represent mean plus one standard error.

In CO soil, *L. vulgaris* shoot biomass was significantly greater in invaded than uninvaded soil (Fig. 2, *X2* = 3.98, *P* = 0.046), but not different between fertilized and control treatments. However, in IL soil, there was not enough data to compare *L. vulgaris* shoot biomass between invasion histories or fertilization treatments. In both CO and IL soils, native plant shoot biomass was not influenced by invasion history or fertilizer.

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**Figure 3.** Shoot biomass for *L. vulgaris* and native plants grown in soil from CO (n = 47). Error bars represent mean plus standard error. Asterisks represent a P value ≤ 0.05.

In CO soil, both *L.* *vulgaris* and native plant root biomass was significantly different between invaded and uninvaded soil but was not different between fertilizer treatments or the interaction between invasion history and fertilizer (Fig. 3). *Linaria vulgaris* root biomass was greater in invaded than uninvaded soil (*X2* = 4.38, *P* = 0.036), whereas native plant root biomass was greater in uninvaded than invaded soil (*X2* = 4.31, *P* = 0.037). In IL soil, native plant root biomass was not different between invasion histories, fertilizer treatments, or their interaction. However, not enough *L. vulgaris* plants sprouted in IL soil to make comparisons across treatments.

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**Figure 4.** Root biomass for *L. vulgaris* and native plants grown in soil from CO (n = 47). Error bars represent mean plus standard error. Asterisks represent a P value ≤ 0.05.

*Stable Isotope Analyses*

In CO soil, *L. vulgaris* NUR and native plant NUR were not significantly different between invasion history or fertilizer treatments (Appendix 1, Fig. 3). In IL soil, native plant NUR was not significantly different between invasion history or fertilizer treatments (Appendix 1, Fig. 4). However, not enough *L. vulgaris* plants sprouted in IL soil to make a comparison of *L. vulgaris* NUR across treatments.

*Soil Chemistry and Texture*

All soil chemistry and texture variables varied significantly by state, except for NO3 and PO4 (Table 2, Fig. 4). Ammonium and clay were significantly higher in CO soil than IL soil. In contrast, pH, silt, and sand were significantly higher in IL soil than CO soil. None of the soil chemistry and texture variables were significantly different between invasion histories or the interaction between state and invasion history.

**Table 1.** Summary of soil chemistry and texture variables by state of collection and invasion history including means ± standard error and best-fit model results. Significant p-values are denoted in bold.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | CO Soil | | | IL Soil | | | Statistical Comparisons | | |
| Soil Factor | | Invaded | Uninvaded | | Invaded | Uninvaded | State | Invasion | State\* Invasion |
| pH | 6.65 ± 0.1 | | 6.66 ± 0.1 | | 7.89 ± 0.1 | 7.99 ± 0.1 | **<0.001**  X2 = 78  df = 68 | 0.230  X2 = 71  df = 68 | 0.588 |
| NO3 (μ/g) | 13.6 ± 1.0 | | 13.8 ± 0.9 | | 9.48 ± 0.9 | 13.1 ± 2.2 | **0.028**  X2 = 77  df = 73 | 0.367  X2 = 72  df = 73 | 0.967 |
| NH4 (μg/g) | 5.47 ± 0.1 | | 5.91 ± 0.1 | | 4.88 ± 0.2 | 4.82 ± 0.1 | **<0.001**  X2 = 72  df = 69 | 0.298  X2 = 70  df = 69 | 0.699 |
| PO4 (μ/g) | 17.5 ± 0.3 | | 18.3 ± 0.4 | | 22.1 ± 1.5 | 16.5 ± 0.7 | 0.545  X2 = 76  df =75 | 0.221  X2 = 78  df = 75 | 0.060 |
| Sand (%) | 60.9 ± 3.9 | | 59.6 ± 4.6 | | 70.0 ± 4.3 | 67.9 ± 4.0 | **0.004**  X2 = 78  df = 38 | 0.577  X2 = 78  df = 38 | 0.893 |
| Silt (%) | 28.4 ± 3.8 | | 29.0 ± 4.2 | | 17.9 ± 3.7 | 19.0 ± 3.4 | **<0.001**  X2 = 78  df = 38 | 0.747  X2 = 78  df = 38 | 0.941 |
| Clay (%) | 10.7 ± 0.5 | | 11.4 ± 0.7 | | 12.2 ± 0.9 | 13.1 ± 1.0 | **0.005**  X2 = 64  df = 32 | 0.154  X2 = 63  df = 32 | 0.915 |

**AMF Diversity**

Next Generation Sequencing resolved 61 OTUs. Of these 61 OTUs, 29 could not be identified to family level. The remaining 32 OTUs belonged to 7 genera and 6 families. Average family abundances per sample in order from highest to lowest are: Glomeraceae (23 OTUs, 50.0% of total reads), Clarideoglomeraceae (2 OTUs, 20.6% of total reads), Paraglomeraceae (3 OTUs, 12.2% of total reads), Diversisporaceae (1 OTU, 4.5% of total reads), Archaesporaceae (2 OTUs, 1.8% of total reads), and Ambisporaceae (1 OTU, <1% of total reads). The unidentified OTUs make up the remaining 10.7% of total reads. Our linear mixed-effects models demonstrated no significant difference in either OTU or family richness between states, invasion histories, or the interaction between state and invasion history.

**AMF Community Composition**

Our PERMANOVA revealed that there was a significant difference in AMF community composition between soil collected from CO and IL (Fig. 1, *df* = 1, *R2* = 0.12, *P* = 0.001). However, AMF OTU composition was not significantly different between invaded and uninvaded plots from both states (*df* = 1, *R2*=0.007, *P*=0.859). In CO soil, AMF OTU composition was not significantly different between invaded and uninvaded plots (*df* = 1, *R2* = 0.016, *P*=0.787). Similarly, in IL soil, AMF OTU composition was not significantly different between invaded and uninvaded plots (*df* = 1, *R2* = 0.021, *P*=0.638).

The indicator species analysis revealed several OTUs that differed by either state (Appendix 2) or invasion history (Appendix 3). Kruskal-Wallis tests on mean relative abundance of AMF families revealed that Diversisporaceae (*df* = 1, *X2* = 35.2, *P* <0.001) were more abundant in IL than CO soil and Archaeosporaceae (*df* = 1, *X2* = 10.8, *P* < 0.001) were more abundant in CO than IL soil. Additionally, Glomeraceae were more abundant in uninvaded than invaded soil across both sites (*df* = 1, *X2* = 4.64, *P* = 0.031).

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**Figure 5.** Non-metric multidimensional scaling plot of AMF OTUs, soil chemistry, and texture in CO and IL soil. Lines represent soil edaphic factors.

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**Figure 6.** Non-metric multidimensional scaling plot of AMF OTUs, soil chemistry, and texture in *L. vulgaris* invaded and uninvaded CO soil.

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**Figure 7.** Non-metric multidimensional scaling plot of AMF OTUs, soil chemistry, and texture in *L. vulgaris* invaded and uninvaded IL soil.

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**Figure 8.** Mean relative abundance of families by site. OTUs that could not be identified to family are listed as NA. Significantly different mean relative abundances from Kruskal-Wallis tests are represented by asterisks (\*P < 0.05, \*\*P<0.01, \*\*\*P<0.001).

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**Figure 9.** Mean relative abundance of families by invasion history. OTUs that could not be identified to family are listed as NA. Significantly different mean relative abundances from Kruskal-Wallis tests are represented by asterisks (\*P < 0.05, \*\*P<0.01, \*\*\*P<0.001).

PERMANOVAs revealed that relative abundances of OTUs across sites were affected by NO3, sand, and silt (Table 4). Relative abundances of OTUs affected by NH4, sand, and clay depended on site. Relative abundances of OTUs were not affected by soil factors depending on invasion history or the interaction between state and invasion history.

**Table 2.** PERMANOVA of effects of edaphic factors and their interactions with state, invasion, and state and invasion together on AMF community composition. Significant p-values are denoted in bold.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Edaphic Factor | \*State | | \*Invasion | \*State\*Invasion |
| pH | 0.379, R2 = 0.01 | 0.772, R2 = 0.01 | 0.830, R2 = 0.01 | | 0.373, R2 = 0.01 |
| NO3 | **0.015,** R2 = 0.02 | 0.155, R2 = 0.02 | 0.571, R2 = 0.01 | | 0.164, R2 = 0.02 |
| NH4 | 0.101, R2 = 0.02 | **0.017**, R2 = 0.02 | 0.401, R2 = 0.01 | | 0.161, R2 = 0.02 |
| PO4 | 0.169, R2 = 0.02 | 0.148, R2 = 0.02 | 0.283, R2 = 0.01 | | 0.467, R2 = 0.01 |
| Sand | **0.006**, R2 = 0.03 | **0.050**, R2 = 0.02 | 0.923, R2 = 0.01 | | 0.630, R2 = 0.01 |
| Silt | **0.014**, R2 = 0.03 | 0.113, R2 = 0.02 | 0.913, R2 = 0.01 | | 0.677, R2 = 0.01 |
| Clay | 0.070, R2 = 0.02 | **0.040**, R2 = 0.02 | 0.756, R2 = 0.01 | | 0.471, R2 = 0.01 |

**Discussion**

This study investigated the impacts of *L. vulgaris* invasion legacy on plant success and associated arbuscular mycorrhizal fungi communities in two regions of its introduced range. When we grew *L. vulgaris* in soil collected from previously invaded and uninvaded plots, we found a difference in the root and shoot biomass and the number of plants growing depending on invasion history, indicating that *L. vulgaris* leaves legacy effects in the soil following invasion. However, this direction of legacy effects for *L. vulgaris* differed between CO and IL soil, with a positive effect on *L. vulgaris* in CO soil and a negative effect on *L. vulgaris* in IL soil. We also found a negative relationship between native plant root biomass and prior invasion in CO soil, signifying negative legacy effects on native species. These results indicate that soil characteristics, biological or chemical, unique to our two sites likely influence the direction and magnitude of *L. vulgaris*’ legacy effects. Arbuscular mycorrhizal fungi communities were different between our two sites, which could influence the extent of aggressive invasion and native plant susceptibility to legacy effects. However, AMF communities were not different between previously invaded and uninvaded soil, which suggests that legacy effects are not the result of changes to AMF community composition. Further, NUR was not affected by invasion history or fertilizer in soil from either site, demonstrating that legacy effects are not the result of changes to N availability. Taken together, these results suggest that *L. vulgaris* invasion changes soil microbial communities and/or soil chemistry, but that legacy effects vary across its introduced range.

*Legacy Effects*

Our results suggest that soil characteristics are at least partly responsible for the geographic distribution of aggressive *L. vulgaris* invasion in the U.S. Increased number of *L. vulgaris* plants growing in previously invaded CO soil supports our hypothesis that *L. vulgaris* shapes soil characteristics in CO, where it is aggressive, to become more successful. We attribute more *L. vulgaris* plants growing in invaded soil to increased sprouting of rhizomes, since we did not observe any plants produce seed during the study. Further evidence of positive legacy effects in CO soil is that both root and shoot biomass were greater among *L. vulgaris* plants grown in invaded than uninvaded soil. Increased biomass in previously invaded soil could indicate that *L. vulgaris* is better able to access resources such as water and nutrients due to microbial associations (Citation). Greater root mass creates more surface area for resource acquisition, resource storage, and could lead to increased clonal reproduction (Citation).

In contrast, *L. vulgaris* only grew in previously uninvaded soil from IL, indicating negative legacy effects following invasion. Negative legacy effects could be caused by suppressive microbial interactions, such as those in its native range (Klironomos 2002). Having some parasitic or pathogenic microbial associations does not rule out the possibility of mutualistic ones, since some invasive plants that benefit from mutualisms in their introduced range can only do so when their pathogens are absent (Klironomos 2002; Reinhart et al 2003). However, too few *L. vulgaris* plants grew in IL soil to compare biomass across treatments. The limited number of plants growing in IL soil could indicate suppressive microbial interactions, fewer mutualistic ones, or unsuitable soil chemistry. Still, the absence of the strong positive legacy effect we observed in CO soil shows that legacy effects across the species’ introduced range could influence the degree of its invasion.

Unfortunately, we cannot distinguish whether the amounts of plants or biomass is an indicator of legacy effects or genetic fitness of the different *L. vulgaris* populations we collected. Because we grew rhizomes in their soil of origin and did not study their population genetics, we cannot rule out genetically distinct populations as the source of *L. vulgaris’* increased growth in CO soil compared to IL soil. In some species, different genotypes of the same species can associate with different microbes, influencing plant fitness and nutrient dynamics (Brown et al 2020). We collected rhizomes from 10 paired plots within each site to maximize plants’ genetic diversity, but because we did not grow CO rhizomes in IL soil and vice versa, we cannot be certain that legacy effects are the result of population differences between the two sites. In the future, we hope that population genetics can be used to compare this species across both its native and introduced range.

There was some evidence that legacy effects impacted native plants differently across the two sites. There was no relationship between invasion legacy and number of native plants growing in either CO or IL soil. However, native plants grown in CO soil had greater root biomass in uninvaded than invaded soil, but there was no effect of invasion on native plant biomass from IL soil. This signifies that *L. vulgaris* invasion negatively affects native plants grown in CO soil, but not IL soil. The negative effect of invasion on native plant root biomass and not abundance could be more indicative of impacts on native plant productivity and resource allocation. Still, decreased productivity, particularly in root mass of a rhizomatous species, could result in shrinking populations over time (Citation). For managers in CO, this could reflect challenges in native species reintroduction following invasion, but IL management can be centered around *L. vulgaris’* removal with the expectation that native plants will be able to recover.

Important to note is that the two native plants used in this study are likely not indicative of all native species’ responses to *L. vulgaris* invasion. *Rudbeckia hirta* and *Digitaria cognata* are much more likely to cooccur with *L. vulgaris* in IL, so the absence of negative effects on these species may not reflect consequences of *L. vulgaris* invasion on CO native plant communities. While some invasive species can have broad negative effects on native species, such as those that disrupt plant-mycorrhizal mutualisms, other invasive plants have a more targeted approach on their competitors (Zubek et al 2016). Additionally, legacy effects could impact plant diversity, but we could not evaluate diversity impacts using just two species (Citation). Certain invasive plants have limited effects on individual species but reduce species richness and evenness in the years following invasion (Citation). Future studies should evaluate invasion legacy in-situ to capture impacts on plant communities directly adjacent to invasion.

*Nitrogen Availability and Uptake Rates*

Interestingly, there was no effect of N fertilization on the number of plants growing or biomass in either *L. vulgaris* or native plants across our two sites. If plants were N-limited, we would expect fertilization to increase the number of plants growing and/or biomass. We hypothesized that *L. vulgaris* is a better competitor for N than native plants in CO soil where N is limited, which would allow it to spread rapidly. Because both *L. vulgaris* and native plants were unaffected by N addition, this suggests that the legacy effects we observed were not related to N limitation. Our soil chemical analysis confirmed that there was no significant difference in NO4 or NO3 availability between invaded and uninvaded soil, but showed significantly more NO3 and NO4 in soil from CO. This result contradicts statewide trends of NO3 and NH4, which could be indicative of sampling site quality (Citation). Due to the scarcity of *L. vulgaris* populations in IL, our sampling plots were located along trails, roadsides, and train tracks where soil may be contaminated with chemical runoff or physically disturbed. CO plots were located within a national forest with relatively low anthropogenic impact and where there is high animal activity, which could have resulted in higher N inputs. While N availability did not appear to influence soil feedbacks, other effects of site disturbance could have influenced soil microbial communities, chemical composition, and subsequent legacy effects.

Furthering our belief that N availability does not explain the range of *L. vulgaris’* invasion, N uptake rates for both *L. vulgaris* and native plants were not different across invasion or fertilizer treatments in either site. If N uptake was a strong determinant of plant success, we would expect to see higher N uptake rates in treatments where sprouting and growth was highest. Because invasion legacy did not impact N uptake rates, this suggests that legacy effects cannot be attributed to increased or decreased N absorption. Similar N uptake rates in plants from fertilized and unfertilized treatments illustrates that plants were not N-limited, implying that environmental N is not a driver for *L. vulgaris’* success.

*AMF Community Composition*

High-throughput sequencing revealed that AMF communities are significantly different between the two sites. This was expected, as previous research has shown a strong positive relationship between geographic distance and AMF community dissimilarity (Kivlin et al 2011). Different AMF communities associated with *L. vulgaris* in CO and IL is one possible explanation for its invasive distribution. However, our study did not compare *L. vulgaris* grown in live vs. sterile soil to establish whether soil biotic or abiotic characteristics influence *L. vulgaris* growth. At best, we generated one theory for *L. vulgaris* invasion success that requires further experimentation to be supported or disproven.

Unexpectedly, OTU and family richness was about the same between sites and each site had almost the same number of indicator OTUs, meaning that states had different communities, but neither had higher diversity. Because IL is lower elevation than CO, we expected to find greater OTU richness and taxonomic diversity in IL (Swaty et al 2016). Our sampling sites in IL were highly disturbed, which could have resulted in a loss of AMF diversity (Entry et al 2002; Sánchez et al 2012). Soil with high AMF diversity is often more resistant to disturbances like pathogens, moisture stress, and others (Van der Heijden et al 1998). If AMF community differences are responsible for *L. vulgaris’* invasion success in CO, OTU-level taxonomic differences could reflect more mutualistic and fewer parasitic AMF. Still, further study is required to attribute *L. vulgaris* invasion success to microbial diversity.

When we compared invaded and uninvaded soil from both sites, as well as each site individually, overall AMF community composition was not different, suggesting that invasion does not drive AMF community changes. Similarly, OTU and family richness were not different between invaded and uninvaded soil from both sites together and each site alone. Although, we did not sequence soil prior to and following invasion, so we cannot determine decisively whether invasion impacts AMF communities.

However, there were state-specific differences in indicator OTUs between invaded and uninvaded soil, with the same number of indicator OTUs between invaded and uninvaded CO soil and slightly more indicator OTUs in invaded than uninvaded IL soil. Differences in indicator OTUs reveal that sites had slightly different communities, but not enough to illustrate any effect of *L. vulgaris* invasion. Because OTU richness was unaffected by invasion, indicator OTUs in invaded and uninvaded sites are most likely the result of site differences unrelated to invasion.

States also had strong differences in soil factors, with higher pH and sand in IL soil and higher silt, NO3, and NH4 in CO soil. I was surprised that N availability was higher in CO soil than IL soil, contrary to previously documented state-wide trends, but I attribute this difference to site disturbance. Sites in IL where I was able to locate *L. vulgaris* were adjacent to roads, railroads, or in other highly disturbed areas, making them highly susceptible to leaching and less likely to harbor N-fixing bacteria (Perakis et al 2015). Whereas in CO, deer, moose, and other wildlife were often found inhabiting sites and most likely contributed N inputs to the soil. Interestingly, PO4 was affected by both state and invasion, with higher PO4 in invaded than uninvaded IL soil, but no effect of invasion in CO. It seems likely that PO4 availability is driven by microbial community differences, as microbes are essential to plant P uptake and disturbance has less of an effect on soil P (Richardson et al 2011; Redel et al 2013). While between-state AMF differences could be partly responsible for PO4 changes, AMF communities were not very different between invaded and uninvaded sites in CO and IL. Bacteria, saprotrophic fungi, and other microorganisms could influence PO4 differences between invaded and uninvaded sites, but I only addressed AMF community differences in this study.

When I tested the effects of soil factors on AMF community composition, not all soil factors different by state resulted in community differences. Nitrate, sand, silt, (and clay marginally) influenced AMF communities across both states. Sand, clay, and ammonium influenced AMF community differences between states. I hypothesize that because NO3 and NH4 were abnormally low in IL sites and abnormally high in CO sites, N availability had stronger effects on AMF communities than expected. Sand, clay, and silt ratios can affect soil nutrient and moisture retention and because CO has a dry climate, I hypothesize that moisture is at least partly responsible for AMF composition there (Kivlin et al 2011). While pH was higher in IL than CO soil, pH did not drive AMF community composition in both or either state. For all soil factors, the R2 values were very low (0.01-0.03), meaning that the AMF communities I observed may be correlated with soil factors, but the variation I saw was not explained by the soil factors I tested.

Soil factors didn’t drive AMF community differences between invaded and uninvaded soil in both states together and separately. Despite the differences in soil PO4 by state and invasion history, PO4 did not affect AMF community composition across state and invasion treatments, which could be because other microorganisms are more important for plant P acquisition. If AMF were responsible for plant PO4 uptake, I would expect to see strong effects on AMF communities, since PO4 availability would drive the direction of the plant-mycorrhizal symbioses (Johnson et al 1997). While invaded and uninvaded soil AMF communities were not different overall, unique indicator OTUs in invaded and uninvaded soil may be unimportant to plant nutrient acquisition or otherwise not functionally diverse (Lee et al 2013). The NMDS revealed that AMF communities in invaded IL soil were a subset of uninvaded IL soil, despite having more indicator OTUs. This could illustrate that within-family taxonomic diversity was greater in uninvaded than invaded IL soil, but this does not appear to correspond to greater functional diversity.

Unfortunately, the strength of my data was limited, as I had to remove many low-quality base pairs that are important in species-level identification. My library preparation did not yield the quantity or quality of reads I would have liked for strong AMF community analysis. While I am confident in my identification of OTUs to either family or genus, I likely did not completely capture AMF diversity in my samples. Furthermore, I only sequenced soil that had either been invaded by *L. vulgaris* or not but did not do any experimental manipulation to determine whether growing *L. vulgaris* changes the AMF community. While I used paired plots to reduce site variation, I can’t be sure that invasion and not site differences contributed to my observed AMF community differences. In future studies, I suggest that researchers grow *L. vulgaris* in previously uninvaded soil prior to sequencing in addition to performing more broad-scale sequencing of AMF, bacteria, and saprotrophic fungi to better capture microbial diversity.

My sequencing revealed that AMF communities are distinct between CO and IL soil, but not between *L. vulgaris* invaded and uninvaded soil in both locations. This leads us to believe that state differences may contribute to *L. vulgaris’* invasion, but that *L. vulgaris* does not directly cause changes to the AMF community. I compared AMF diversity and found that both OTU and family richness was not affected by either state or invasion, furthering my belief that *L. vulgaris* invasion does not impact AMF community composition. I also established that soil factors including NO3, sand, silt, and clay contributed to AMF compositional differences between states. Whereas PO4 was different between invaded and uninvaded sites in IL soil, PO4 had no effect on AMF community differences between invaded and uninvaded soil. There is no strong linkage between *L. vulgaris* invasion, soil nutrients, and AMF community composition, but I hypothesize that other microbial differences between sites are responsible for nutrient dynamics and subsequently impact *L. vulgaris’* invasion.

While AMF do not appear to be directly impacted by *L. vulgaris* invasion, other soil characteristics seem to be important in shaping *L. vulgaris’* aggressive distribution. Specifically, soil PO4 and microbial communities apart from AMF could either influence or be influenced by *L. vulgaris* invasion. Understanding the relationships between soil and invasive plant success could help managers better restore previously invaded sites and prevent future invasions. I hope that this study draws more attention to studying the relationships between invasive plants, nutrient availability, and soil microbial communities to uncover additional restoration solutions.

*Conclusion*

Ultimately, our study shows that soil is an important variable shaping *L. vulgaris’* aggressive distribution and its impacts on native plant communities. Differences in genotypes, climate, competition, and other variables may also play a role in *L. vulgaris*’ aggressive range. However, reducing soil legacy effects could be a feasible way to manage this species once we understand what microbial and chemical changes result from invasion. In CO, fully eradicating *L. vulgaris* will necessitate repeated removals, as legacy effects will promote re-invasion. Whereas in IL, legacy effects may help control *L. vulgaris* invasion, so removal appears unnecessary. Native plant presence appears unaffected by invasion legacy in both states, but plants appear to be allocating resources differently and other characteristics could be affected by invasion. Furthermore, N availability does not appear to influence *L. vulgaris’* success, but future studies should evaluate how other soil nutrients, including PO4, and soil microbes influence and are influenced by invasion. My results support previous research in invasive plant soil legacy effects, namely that soil-feedbacks are strong drivers of invasion distributions. I hope that this study will encourage more research in invasive plant impacts on soil communities throughout their range.

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