RESTORATION ECOLOGY
The Journal of the Society for Ecological Restoration

SER SOCIETY FOR ECOLOGICAL RESTORATION

RESEARCH ARTICLE

High-throughput sequencing provides insight into manipulated soil fungal community structure and diversity during temperate forest restoration

Andrew C. Lance[1,2], David J. Burke[1,3], Constance E. Hausman[4], Jean H. Burns[1]

The process of community assembly in fungal communities is poorly understood and may have important implications for restoration. However, there is a shortage of data describing fungal community composition at various stages of restoration. This study describes how microbial inoculation with field-collected soils or a commercial inoculum influenced fungal communities during temperate tree restoration. We utilized Illumina Mi-Seq sequencing technology to examine fungal community structure in the rhizosphere soils of trees at the conclusion of one growing season. Inoculation treatment was found to be a significant determinant of fungal community structure in one of our three experimental tree species (*Liriodendron tulipifera*). We also found a marginally significant influence of inoculation method on fungal community structure in the rhizosphere soils of *Quercus rubra*, an ectomycorrhizal tree species. Importantly, within these taxa, the use of commercial inocula, while failing to lead to detectable abundances of the inoculated taxa, strongly influenced the resulting fungal community structure after 4 months in the field, relative to control trees that received no such inoculation. We observed lower abundances of Hebeloma, a potentially important ectomycorrhizal genera, in Quercus trees receiving the commercial inoculum compared with control trees; thus, the commercial inoculum might have unexpected consequences for fungal community assembly. Such unintended legacy effects of soil inoculation should be considered in ecological restoration. Furthermore, by taking a time series approach to sampling, high-throughput sequencing approaches could be used to test the principles of ecological assembly theory, including legacy effects of taxa no longer detectable in the community.

Key words: fungi, high-throughput sequencing, inoculation, temperate trees

Implications for Practice

- Inoculation with commercial inocula or forest-collected soils can influence soil fungal community structure in temperate trees, relative to control, uninoculated trees.
- Taxa contained within commercial inocula may not be detectable after extended periods but still influence soil fungal community structure.
- A diversity of saprotrophic, mutualistic, and parasitic fungal taxa can be recovered in association with temperate trees at sites with extensive disturbance histories.

Introduction

Soil microbial communities have been proposed to be important determinants of the success of ecological restoration projects (Harris 2009). Many sites targeted for restoration have undergone extensive disturbance and may therefore have altered or degraded soil microbial communities (Johnson et al. 2003; Kohl et al. 2014). As such, the manipulation of soil microbial community structure has become a commonly employed practice which intends to improve the performance of plants by providing access to a functionally diverse soil microbial community. Common methods of soil microbial community manipulation include inoculation of plants with soil collected from a reference plant community ("whole soil transfers") or the application of commercially produced inocula. Meta-analyses have indicated that whole soil transfers are more effective at increasing mycorrhizal colonization and plant growth than commercially produced inocula (Maltz & Tresder 2015); however, the mechanisms underlying these results are poorly understood. Furthermore, community assembly processes in fungal communities are still poorly understood (Bruns 2019), but critical to designing successful inoculations for restoration.

Modern genomic methods allow for the characterization of a soil fungal or bacterial community from small amounts of

Author contributions: CEH, JHB conceived and designed the experiments; ACL, JHB analyzed the data; ACL, JHB wrote the manuscript; all authors performed the experiments and provided editorial advice.

[1]Department of Biology, Case Western Reserve University, Cleveland, OH 44106, U.S.A.
[2]Address correspondence to A. C. Lance, email acl91@case.edu
[3]Holden Arboretum, 9500 Sperry Road, Kirtland, OH 44094, U.S.A.
[4]Cleveland Metroparks, 2277 W Ridgewood Drive, Parma, OH 44134, USA

© 2020 The Authors. Restoration Ecology *published by Wiley Periodicals, Inc. on behalf of Society for Ecological Restoration.*
This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
doi: 10.1111/rec.13120
Supporting information at:
http://onlinelibrary.wiley.com/doi/10.1111/rec.13120/suppinfo

environmental DNA (Schmidt et al. 2013). High-throughput sequencing (HTS) efforts can potentially play an important role in restoration efforts, allowing for rapid and detailed assessment and monitoring of soil microbial communities (Williams et al. 2014). HTS efforts often recover thousands of operational taxonomic units (OTUs) compared to hundreds often recovered from older methodologies such as terminal restriction fragment length polymorphism (TRFLP). This increased resolution provides a more complete characterization of the soil microbial community and may allow for insight into the functional diversity of a given soil profile. However, the cost of implementing HTS and the associated difficulty in analyzing soil fungi data have precluded the inclusion of HTS data in many papers that examine soil microbial inocula and subsequent soil microbial community development.

We propose that HTS methodologies can effectively answer several important questions for restoration ecologists and practitioners who intend to utilize microbial inoculants. First, HTS is capable of providing a more complete profile of soil microbial communities than many alternatives (e.g. TRFLP), enabling practitioners to more accurately deduce whether inoculation elicits the formation of distinct communities (e.g. whether fungal communities associated with inoculated plants different from those in noninoculated plants). Second, HTS data could accurately assess the influence of inoculation on microbial diversity in field site soils. This is particularly important because increased taxonomic diversity may correlate with increased functional diversity (Van der Heijden et al. 2008). Third, HTS is capable of detecting pathogenic members of the soil microbial community, which may inhibit plant performance (Packer & Clay 2000). Knowledge of the pathogen community present at a given restoration site can lead to the implementation of best management practices (e.g. species selection). Finally, HTS data could be essential to detecting the presence of introduced microbial taxa in a soil community. The fate of many introduced taxa used for inoculation is often unknown and could have important consequences (Schwartz et al. 2006).

The objective of this work was to utilize HTS technologies to analyze and describe soil fungal communities in a temperate tree restoration project in eastern North America. We focused on three experimental tree species: *Liriodendron tulipifera* L., *Prunus serotina* Ehrh., and *Quercus rubra* L. Each of the species was sourced from three geographical "provenances": Indiana, Missouri, and West Virginia. Trees were inoculated with either a commercially produced microbial inoculum or reference forest soil collections (whole soil transfers); an equal number were left uninoculated to serve as controls. We sampled soils at the conclusion of one growing season (4 months following inoculation) and utilized Illumina Mi-Seq methods to describe soil fungal community structure and diversity. Our hypotheses were that the inoculation method would lead to distinct fungal community structure and that soil samples from trees inoculated with whole soil transfers would display greater OTU richness and Shannon's diversity than commercial or control trees. Previous work in this system demonstrated that inoculation with whole soil transfers resulted in an increase in the number of terminal restriction fragments recovered in rhizosphere soils (Lance et al. 2019). Our current approach implements HTS technology to further examine the influence of inoculation on soil fungal community structure and diversity.

Methods

Experimental Design Overview

Our experiment consisted of three tree species (*L. tulipifera*, *P. serotina*, and *Q. rubra*) sourced from three geographical provenances (Indiana, Missouri, and West Virginia). We examined the influence of two soil inoculation treatments and control on tree growth and establishment. Fifteen trees per species and provenance were randomly selected to be inoculated with a commercially produced microbial inoculant or soil collected from one of the three replicate locations in a nearby reference forest (see protocol below); an equal number were left unmanipulated and did not receive any form of microbial inoculation. The total initial number of trees in this experiment was thus 405 trees (3 species × 3 provenances × 3 soil treatments × 15 replicate trees).

Plant Material

We sourced second-year saplings from three nursery suppliers in spring 2015. Suppliers included Forrest Keeling Forestry Suppliers (Elsberry, MO), Indiana State Tree Nursery (Vallonia, IN), and West Virginia State Tree Nursery (Clements, WV). While the *precise* location of the seed source from which these trees were grown could not be guaranteed, each of these nurseries guaranteed that the material was grown from sources located within the geographical boundaries of each state. We therefore use the term provenance to describe the different source locations (Kranabetter et al. 2015). Trees were shipped bare root from each nursery.

We controlled for differences in root-associated fungal communities between nursery suppliers by dipping the roots of each tree in a soluble fungicide (ZeroTol; BioSafe Systems, Ltd., Indianapolis, IN) before planting bare root trees into 7.5 L pots filled with a sterilized potting medium. Trees were then placed in a lath-house at Holden Arboretum (Kirtland, OH) for one growing season to facilitate the development of secondary root structure. We sampled rhizosphere soils from 10 randomly selected pots of each species in spring of 2016 to assess the effectiveness of our fungicide treatment using molecular methods described below.

Inoculation Methodology

Trees were inoculated 2 weeks prior to outplanting in May 2016. We selected a commercial inoculum which consisted of a diversity of general fungi, arbuscular mycorrhizal fungi (AMF), and bacteria (MycoGrow Soluble; Fungi Perfecti, Olympia, WA, U.S.A.; Table S1). This diversity is representative of a natural soil microbiome, while other commercially produced products often contain a single microbial guild. The commercial inoculum was prepared by mixing 28 g of the inoculum with 4,000 mL of water. This concentration exceeded the

manufacturer's recommended concentration by 10×; however, evidence for herbaceous plants suggests that plant response to inoculation is limited with commercial products prepared at the manufacturer-recommended concentration (Rowe et al. 2007; Paluch et al. 2013). Therefore, increasing concentration may be a more accurate indicator of potential plant response to commercial inocula. Each tree receiving the commercial inoculum received 400 mL of the prepared solution (trees were inoculated in batches of 10).

Forest soils were collected in May 2016 from a mature beech-maple forest located approximately 5 km from our study site (Squire Valleevue Farm, Hunting Valley, OH). A Mahoning silt loam soil characterizes the forest (USDA 2018). We collected soil from three replicate locations located >400 m apart in order to accurately assess the variability associated with forest soil inoculation (Reinhart & Rinella 2016); collection implements were sterilized with 95% ethyl alcohol between each of the three collection sites. We collected soil to a depth of 10 cm in areas void of herbaceous vegetation. The dominant trees surrounding all three collection sites were American Beech (*Fagus grandifolia* Ehrh.) and Sugar Maple (*Acer saccharum* Marshall). We chose donor sites for our forest soil to avoid the specific focal trees in our experiment, in order to avoid tree species-specific pathogens. However, we sampled near trees that were both AMF and ectomycorrhizal to include a broad suite of potential mutualists. Following collection, soils from each of the three replicate sites were separately dried for 1 week at room temperature, sieved with a 0.5-cm sieve, and homogenized within each replicate collection. Trees selected to receive the forest soil inoculum received 50 g of dried forest soil (from one of the three replicate collections) applied to the top of the pot soil followed by 400 mL of water.

Unmanipulated control trees received 400 mL water on the day of inoculation.

Site Description and Planting

Our study site was located at Acacia Reservation (Cleveland Metroparks) in Lyndhurst, OH. A humid continental climate characterizes the area. A Mahoning silt loam soil type dominates our study site (USDA 2018), which is the same soil classification as our donor site. Our site was situated on a former golf course that is actively being restored. Exotic turf grasses (*Poa pratensis* L. and *Agrostis gigantea* Roth) dominated our study site before the area was mechanically tilled twice (once in fall 2015 and again in spring 2016) prior to the establishment of our experiment. The site was seeded with a diverse grass-dominated seed mix following the second mechanical preparation.

Our study site was divided into 15 blocks measuring approximately 15 m^2. Trees were planted in June 2016 into randomized positions located within these 15 blocks. A 4-m buffer zone separated each block. Trees were planted in rows with approximately 2.5 m spacing between each position. We utilized treatment-specific planting implements to prevent cross-contamination between soil treatments. Mixed conifer-hardwood wood chips were placed in an approximately 1-m-diameter circle around the stem of each tree and irrigation was provided during the summer of 2016. We weeded manually throughout the course of this study.

Soil Sampling

We collected rhizosphere soil samples from 30 randomly selected trees per species (10 per soil treatment) in October 2016. We defined rhizosphere soil as soil which adhered to tree roots upon initial collection. Two 3-cm-wide cores targeting the top 10 cm of soil 0.25 m from the tree stem were collected and homogenized in the field. Samples included small root fragments and the associated soil. Samples were immediately placed on ice following collection and then frozen at −70°C for molecular analysis (see protocol below).

HTS with Illumina Mi-Seq

Eighty-one soil samples were subjected to HTS of soil fungal communities. Samples equally represented all three soil treatments for each tree species (3 tree species × 3 soil treatments × 9 replicates). DNA was extracted from samples using a phenol–chloroform protocol (Burke 2008). We made amplicons of the fungal ITS-2 gene region using the primers 58A2F and NLB4 (Martin & Rygiewicz 2005) (Eurofins, Louisville, KY, U.S.A.) with Illumina overhang adapters (Burke et al. 2019). The 58A2F-metagenome primer was TCGTCGGCAG CGTCAGAT GTGTATAAGAGACAGATCGATGAAGAAC GCAG and the NLB4-metagenome primer was TCTCGTGGG CTCGGAGATGTGTATAAGAGACAGGG ATTCTCACCCT CTATGAC, where the underlined portions of the primers are the overhang adapters. Primers 58A2F and NLB4 were selected as they have been shown to discriminate against plant DNA making them useful in plant–fungi studies. Each reaction included 2 U of FastStart Taq DNA polymerase (Sigma-Aldrich, Inc.), 2mM $MgCl_2$, 0.2 μM of both primers, 0.5 μg/μL bovine serum albumin, and 0.8mM dNTP mix. Our thermocycling conditions were an initial denaturation at 95°C for 5 minutes, followed by 25 cycles at 95°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 5 minutes. Amplicons were then purified, indexed, and sequenced as 2 × 250 bp reads on one lane of the Illumina Mi-Seq V3 sequencer (Illumina Inc., San Diego, CA)at the Case Western Reserve University Genomics Core facility.

Blaxter's sample processing pipeline (version 1.0.1; 2016) was used as a guide for our sample processing. We joined forward and reverse reads with the fastq_join command in USEARCH (Edgar 2010). We removed control phiX prior to joining reads using the filter_phiX command. Forward and reverse reads were joined (instead of merged) due to the variable length of the ITS2 region. This enabled us to retain longer ITS2 sequences. Reads were mapped to OTUs using the UPARSE pipeline (Edgar 2013); primers were removed with Cut Adapt (v1.10) (Martin 2011). We implemented the UCLUST algorithm (Edgar 2010) for OTU clustering at 97% similarity and removal of chimeras using the UCHIME algorithm (Edgar et al. 2011). Dataset-wide singletons and OTUs that occurred in only one sample were removed prior to analysis. We made

taxonomic assignments for each OTU by utilizing the SINTAX algorithm (Edgar 2016) and comparing against UNITE databases (Aberenkov et al. 2010; Koljalg et al. 2013). The search local command in USEARCH was used to make local alignments with 97% identity and 80% query coverage included. Matches to this site database confirmed the SINTAX matches to the UNITE database (v.7.1). We truncated the forward MiSeq reads to 230 base pairs for taxonomic assignment to the UNITE database (as we joined reads for OTU processing) (Burke et al. 2019).

Data Analysis

We analyzed fungal community structure with nonmetric multidimensional scaling (NMDS) procedures in the "vegan" package of R (Oksanen et al. 2017). NMDS utilized the Sorensen (Bray–Curtis) distance metric, three dimensions, and a randomized starting configuration with 100 random starts using the metaMDS function. Three dimensions were selected as this resulted in the lowest stress for our permutation analysis (in comparison to a two-dimensional analysis). We examined differences in fungal community composition between different soil treatments and tree source provenances by implementing nonparametric permutation procedures [e.g. permutational multivariate analysis of variance (PERMANOVA)] in the "vegan" package of R using 4,999 iterations. Our initial universal PERMANOVA model included species; treatment; provenance; an interaction of species, treatment, and provenance; and block. Our within-species models included treatment, provenance, an interaction of treatment and provenance, and block.

Fungal community richness and diversity were examined with natural log-transformed response ratios (Brinkman et al. 2010; Larios & Suding 2015). We calculated richness and Shannon's diversity of fungal OTU communities with the "vegan" package of R. Response ratios were calculated as $\ln(X/Y)$, where X was either richness or Shannon's diversity in rhizosphere soils of trees receiving commercial or forest inoculants and Y was the same measure in rhizosphere soils of control trees. Thus, each tree species had four comparisons calculated (2 treatments – commercial or forest × 2 measures – fungal richness or Shannon's diversity). We maximized physical proximity when calculating response ratios. For example, we paired richness or Shannon's diversity measurements by row (position in the experimental design) when possible; data from adjacent rows were used when no measurements from the same row were available. This resulted in 6–8 log response ratios per species paired by spatial location in the experimental design, analogous to including a block effect in a mixed model. We then calculated 95% confidence intervals for all richness and Shannon's diversity measures.

Results

We were able to retrieve useable reads for 77 of our 81 samples. One commercial inoculated Liriodendron, two commercial inoculated Quercus, and one forest soil inoculated Quercus did not provide useable reads (see Table S3 for number of reads per sample).

Our HTS effort generated over 8.9 million reads which were mapped to 1,095 OTUs. Seven fungal phyla were represented: Ascomycota, Basidomycota, Chrytridiomycota, Entorrhizomycota, Mortierellomycota, Mucoromycota, and Rozellomycota. A total of 545 OTUs were mapped to Ascomycota and 295 OTUs to Basidomycota. We were able to achieve genus- or species-level resolution for 344 OTUs (approximately 31%). Of the genera that had multiple OTUs mapped to them, the most abundant was Mortierella (21 OTUs), followed by Entoloma (14 OTUs), Exophiala (12 OTUs), and Penicillium (10 OTUs). Sixty-three OTUs (approximately 5%) were unable to be mapped to any domain with 80% confidence. One hundred and five OTUs (approximately 9%) could only be mapped to "fungi."

A preliminary analysis indicated that tree species differed significantly in their response to microbial inoculation ($F_{[2,73]} = 2.48$, $p = 0.0002$); therefore, we conducted separate PERMANOVA within each tree species. Our metaMDS ordination comparison found that for Liriodendron, the two-dimensional stress was 0.17 while the three-dimensional stress was 0.11; for Prunus, the two-dimensional stress was 0.19 while the three-dimensional stress was 0.14; and for Quercus, two-dimensional stress was 0.17 and three-dimensional stress was 0.09. We found that soil inoculation treatment had a significant influence on fungal community structure in rhizosphere soils of Liriodendron ($F_{[2,21]} = 1.77$, $p = 0.02$; Table S2; Fig. 1). Treatment did not significantly influence general fungi community structure in Prunus ($F_{[2,24]} = 0.99$, $p = 0.48$; Table S2; Fig. 1) and had a marginally significant influence on fungal communities in Quercus ($F_{[2,18]} = 1.32$, $p = 0.09$; Table S2;

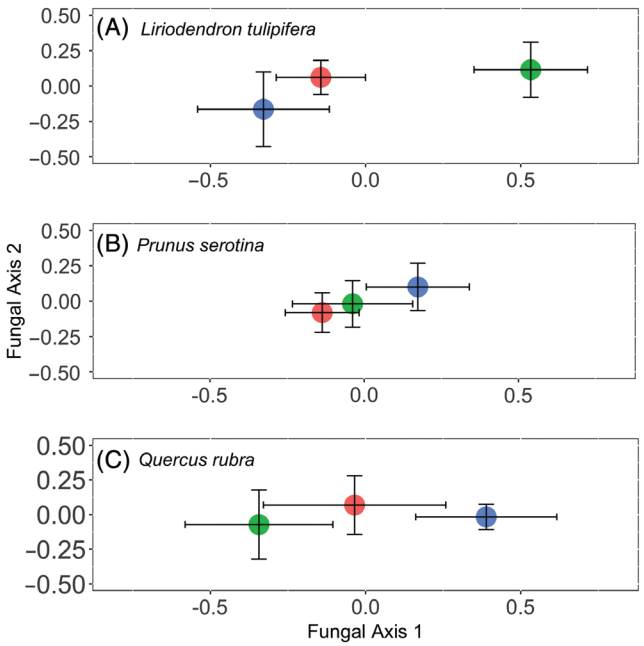

Figure 1. NMDS plots for general fungi communities in the rhizosphere soils of Liriodendron (A), Prunus (B), and Quercus (C). Means ± 1 SE. Control (red), reference forest soil inoculation (blue), and commercial inoculum (green).

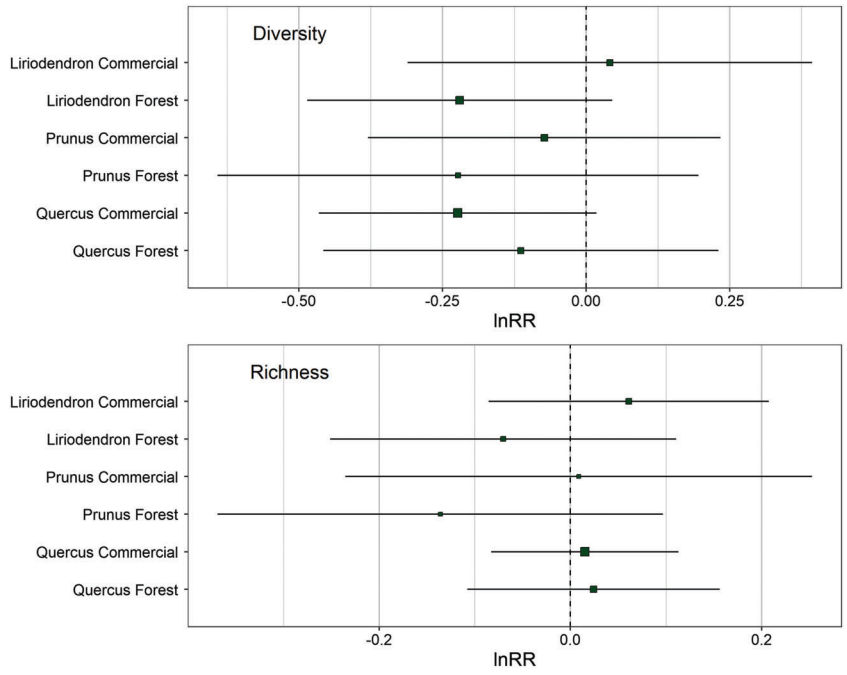

Figure 2. Forest plots representing fungal richness and Shannon's diversity for Liriodendron, Prunus, and Quercus. Natural log-transformed response ratios (lnRR) ± 95% confidence intervals are displayed. Response ratios are calculated in reference to the control uninoculated trees. "Commercial" refers to trees which received the commercial inoculum and "Forest" refers to trees inoculated with reference forest soils.

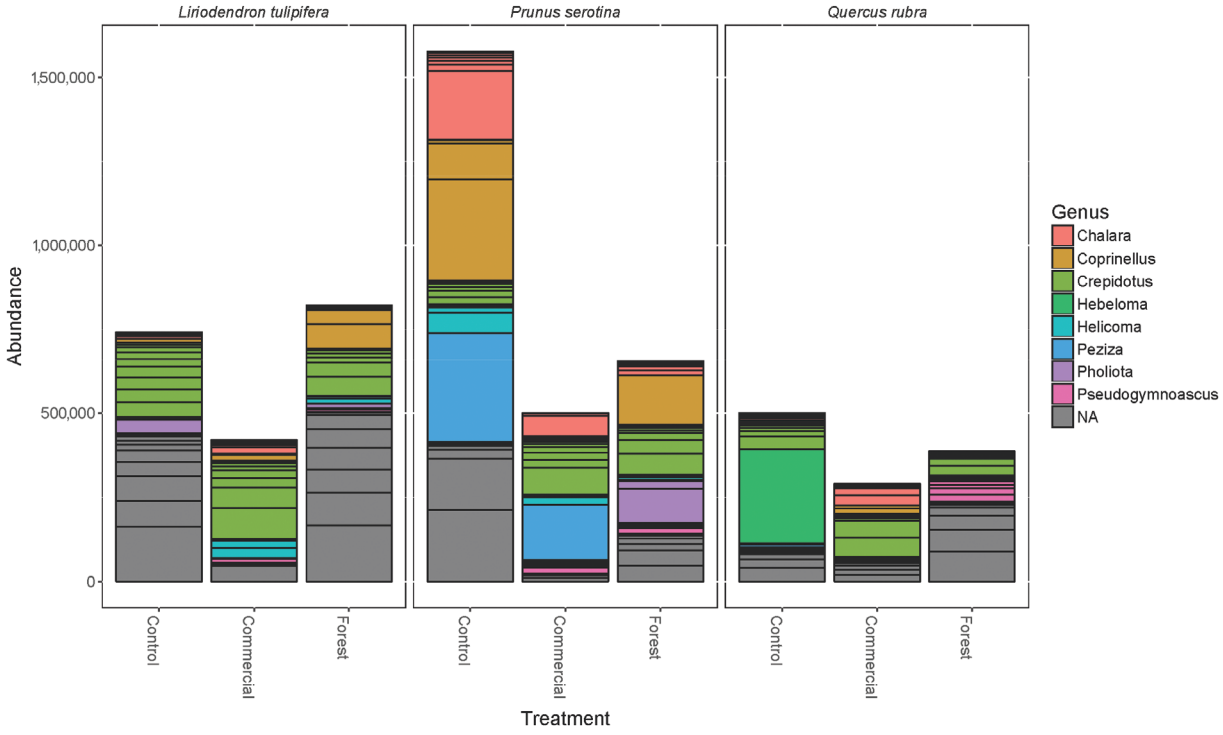

Figure 3. The abundance of fungal genera found in *Liriodendron tulipifera*, *Prunus serotina*, and *Quercus rubra* rhizosphere soils at the conclusion of one growing season. Trees were inoculated with a commercial inoculum ("Commercial"), inoculated with a whole soil transfer from a reference forest ("Forest"), or left unmanipulated as controls ("Control"). Black lines separate different taxa within a genus.

Fig. 1). We noted that in both Liriodendron and Quercus the forest soil inoculants shifted fungal communities in different directions than the commercial inoculum in relation to the controls (Fig. 1). Tree provenance did not significantly influence the structure of rhizosphere fungal communities in any of our three experimental species (Table S2).

Inoculation with either commercial inoculum or forest-collected soils did not significantly influence fungal richness or Shannon's diversity in rhizosphere soils of any of the three focal tree species (Fig. 2). However, trends hint that diversity may be lower in commercial than control trees for Quercus (Fig. 2).

In addition, we noted qualitative differences in the relative abundances of some fungal taxa between each experimental species and between soil inoculation treatments (Fig. 3). Among the 10 genera with the highest number of reads, the relative abundances of these taxa was lowest in the commercial inoculation treatment for all three tree species compared with control and forest treatments (Fig. 3). We noted that Chalara, a putative pathogen, was present in greater relative abundances in the rhizosphere soils of control Prunus trees, compared with commercial or forest treatments. Hebeloma, which can be ectomycorrhizal, was present in the control Quercus trees.

Discussion

Our study highlights several trends and patterns in fungal community composition of temperate tree rhizosphere soils following experimental inoculation with either commercial or whole forest soils. Fungal community responses were species specific for different planted tree species. We found a significant influence of inoculation of rhizosphere fungal community structure in Liriodendron and a weakly significant influence in Quercus; Prunus rhizosphere fungal community structure, however, did not differ between soil treatments. Species-specific responses to inoculation have been noted previously in temperate trees (St Denis et al. 2017; Lance et al. 2019) and our current study provides further evidence that broadly applicable inoculation protocols are unlikely to be easily developed. Species-specific inocula, which could be developed using HTS technologies, may result in the best tree growth and performance in restoration contexts.

Our findings add to a growing literature suggesting that commercial soil inocula, even diverse inocula such as this one, may lead to unexpected and undesirable consequences for community composition and abundance. Our HTS approach allowed us to examine the persistence of fungal species present within the commercial inoculum. We failed to find genetic evidence of the fungal species present in the commercial inoculum after 4 months of growth in the field. While our sequencing primers are not optimized for AMF, the commercial inocula contained general fungal taxa as well, and these were also not recovered with our approach. However, we still observed community-wide differences, indicating that the influence of the commercial inoculum could be lasting over 4 months in the field. Our understanding of the factors shaping fungal community composition in the soil is still developing (Bruns 2019), but community assembly theory suggests that "ghosts" of competition (Miller et al. 2009) can influence assembly processes, sometimes in unexpected ways. The composition of the fungal community in our commercial inoculation treatment does not resemble the composition of the inocula itself. Furthermore, it is distinct from control communities, suggesting that the ghosts from the inocula had an unintended legacy effect, influencing community composition even though they are not themselves detectable. This is consistent with theory, which suggests that unobserved taxa can play an important role in community assembly (Pimm 1991; Law & Morton 1996; Miller et al. 2009). Such unintended and unexpected effects of inoculation suggest that community assembly in fungal communities is likely influenced by unsampled taxa, and that characterizing the assembly process (e.g. by HTS) would benefit from a time series approach, to help identify how community composition in the soils changes over time following inoculation. The influence of inoculation can last for extended periods of time (Wubs et al. 2016) and our single sampling after 4 months provides limited insight into long-term fungal community assembly patterns following inoculation, but suggests that these effects might be hard to predict from inocula community composition alone.

We noted several interesting trends related to relative abundances of dominant fungal genera recovered by our HTS approach. Total abundances of general fungi were lower in trees receiving the commercial inoculant than trees receiving forest soil or left unmanipulated. This pattern was present in all three of our study species. Trees receiving the commercial inoculant may have decreased performance, if decreased fungal abundance correlates with decreased function of the fungal community (e.g. reduced nutrient cycling) (reviewed in Van der Heijden et al. 2008). This would be consistent with some ecological studies, which have found greater plant biomass in communities with greater soil microbial diversity (Van der Heijden et al. 1998; Maherali & Klironomos 2007; Lau & Lennon 2011).

HTS approaches such as that conducted here can also identify particular soil taxa with functional implications, including saprotrophs, pathogens, and mycorrhizal fungi. Saprotrophic fungi including Coprinellus and Mortierella dominated the fungal communities present at our restoration site (Nguyen et al. 2016). Most saprotrophic fungi were present in the rhizosphere soils of all three species and distributed across soil treatments. We noted that Chalara, a potentially pathogenic genus, was present in greater relative abundances in the rhizosphere soils of control Prunus trees. This suggests that pathogens are likely host specific (Packer & Clay 2000; Bever et al. 2015) and that inoculation can influence the ability of pathogenic fungi to establish in restoration sites. Mutualistic members of the soil fungal community were most notable in Quercus, a genus which associates with ectomycorrhizal fungi (our primers amplified saprotrophic and ectomycorrhizal fungi). Within Quercus, the ectomycorrhizal fungal genus Hebeloma was a dominant member of control tree microbiomes but was found in little to no other species or treatments. Therefore, it is unlikely that Quercus trees were "mutualist limited" at this particular restoration site and ectomycorrhizal fungi may be able to tolerate and persist in sites subjected to severe physical soil disturbance.

Our HTS approach to fungal community description allowed for an analysis of community composition, richness, and diversity with important implications for restoration and community assembly. We noted effects of inoculation on general fungal community composition in Liriodendron lasting 4 months in

the field. Evidence for an effect of inoculation on fungal community structure was weak in Quercus and not existent in Prunus, highlighting the species-specific nature of tree response to inoculation. Several fungal guilds were detected by our HTS approach, including dominant saprotrophic fungi, ectomycorrhizal fungi, and pathogenic fungi. We suggest that HTS technologies could be utilized in longer-term monitoring of fungal community structure and used for more nontraditional methodologies such as constructing inocula best suited to a given restoration site's abiotic and biotic conditions. Furthermore, assessing the fate of introduced fungal taxa at a given restoration site may allow for the detection of persistent and potentially "invasive" fungal taxa (Schwartz et al. 2006). HTS technologies will play an important role in understanding the factors that influence restoration outcomes, allowing for the development of best practices and subsequently facilitating the return of ecosystem services to disturbed habitats. HTS data will also be critical to testing ecological theory of community assembly in fungal communities (Bruns 2019). This is especially critical when, as found here, commercial inocula unexpectedly suppress relative abundances and alter fungal community composition, relative to control uninoculated trees, suggesting unexpected legacy effects of inoculation for community composition and restoration.

Acknowledgments

We thank Erik Shaffer at Cleveland Metroparks for assisting with the implementation of our experiment at Acacia Reservation and Sarah Carrino-Kyker for assisting in the laboratory. This research was funded by support from the Charles L. Pack Charitable Trust (to C.E.H.), Holden Forest and Gardens (to D.J.B.), and Case Western Reserve University (to J.H.B.). A.C.L. was supported by a USDOE GAAAN fellowship.

LITERATURE CITED

Aberenkov K, Niellsen HR, Larsson KH, Aleksander RJ, Eberhart U, Erland S, et al. (2010) The UNITE database for molecular identification of fungi – recent updates and future perspectives. New Phytologist 186:281–285

Bever JD, Mangan SA, Alexander HM (2015) Maintenance of plant species diversity by pathogens. Annual Review of Ecology, Evolution, and Systematics 46:305–325

Brinkman EP, Van der Putten WH, Backer EJ, Verhoeven KJ (2010) Plant-soil feedbacks: experimental approaches, statistical analyses, and ecological interpretations. Journal of Ecology 98:1063–1073

Bruns TD (2019) The developing relationship between the study of fungal communities and community ecology theory. Fungal Ecology 39:393–402

Burke DJ (2008) Effects of *Alliaria petiolata* (garlic mustard: Brassicaceae) on mycorrhizal colonization and community structure in three herbaceous plants in a mixed deciduous forest. American Journal of Botany 95:1416–1425

Burke DJ, Carrino-Kyker SR, Burns JH (2019) Is it climate or chemistry? Soil fungal communities respond to soil nutrients in a multi-year high-resolution analysis. Ecosphere 10:1–20

Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460–2461

Edgar RC (2013) UPARSE: highly accurate OTU sequences form microbial amplicon reads. Nature Methods 10:996–998

Edgar RC (2016) SINTAX: a simple non-Bayesian taxonomy classifier for 16s and ITS sequences. bioRxiv 2016:074161

Edgar RC, Haas B, Clemente J, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194–2200

Harris J (2009) Soil microbial communities and restoration ecology: facilitators or followers? Science 325:573–574

Johnson NC, Rowland DL, Corkidi L, Egerton-Warburton LM, Allen EB (2003) Nitrogen enrichment alters mycorrhizal allocation at five mesic to semiarid grasslands. Ecology 84:1895–1908

Kohl L, Oehl F, Van der Heiden MG (2014) Agricultural practices indirectly influence plant productivity and ecosystem services through effects on soil microbiota. Ecological Applications 24:1842–1853

Koljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor A, Bahram M, et al. (2013) Towards a unified paradigm for sequence-based identification of fungi. Molecular Ecology 22:5271–5277

Kranabetter JM, Stoehr M, O'Neill GA (2015) Ectomycorrhizal fungal maladaptation and growth reductions associated with assisted migration of Douglas fir. New Phytologist 206:1135–1144

Lance AC, Burke DJ, Hausman CE, Burns JH (2019) Microbial inoculation influences arbuscular mycorrhizal fungi community structure and nutrient dynamics in temperate tree restoration. Restoration Ecology 27: 1084–1093

Larios L, Suding KM (2015) Competition and soil resource environment alter plant–soil feedbacks for native and exotic grasses. AoB Plants 7:1–9

Lau JA, Lennon JT (2011) Evolutionary ecology of plant–microbe interactions: soil microbial structure alters selection on plant traits. New Phytologist 192:215–224

Law R, Morton RD (1996) Permanence and the assembly of ecological communities. Ecology 77:762–775

Maherali H, Klironomos JN (2007) Influence of phylogeny on fungal community assembly and ecosystem functioning. Science 316:1746–1748

Maltz MR, Tresder KK (2015) Sources of inocula influence mycorrhizal colonization of plants in restoration projects: a meta-analysis. Restoration Ecology 23:625–634

Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet Journal 17:10–12

Martin KJ, Rygiewicz PT (2005) Fungal specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. BMC Microbiology 5:28

Miller TE, terHorst CP, Burns JH (2009) The ghost of competition present. American Naturalist 173:347–353

Nguyen NH, Song Z, Bates ST, Branco S, Tedersoo L, Menke J, Schilling JS, Kennedy PG (2016) FUNGuild: an open annotation tool for parsing fungal community datasets by ecological guild. Fungal Ecology 20: 241–248

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, et al. (2017) Vegan: community ecology package. R package version 2.4-3. http://CRAN.R-project.org/package=vegan

Packer A, Clay C (2000) Soil pathogens and spatial patterns of seedling mortality in a temperate tree. Nature 404:278–281

Paluch EC, Thomsen MA, Volk TJ (2013) Effects of resident soil fungi and land use history outweigh those of commercial mycorrhizal inocula: testing a restoration strategy in unsterilized soil. Restoration Ecology 21:380–389

Pimm SL (1991) The balance of nature? Ecological issues in the conservation of species and communities. University of Chicago Press, Chicago, Illinois

Reinhart KO, Rinella MJ (2016) A common soil handling technique can generate incorrect estimates of soil biota on plants. New Phytologist 210: 786–789

Rowe HI, Brown CS, Claassen VP (2007) Comparisons of mycorrhizal responsiveness with field soil and commercial inoculum for six native montane species and *Bromus tectorum*. Restoration Ecology 15:44–52

Schmidt PA, Balint M, Greshake B, Bandow C, Rombke J, Schmitt I (2013) Illumina metabarcoding of a soil fungal community. Soil Biology and Biochemistry 65:128–132

Schwartz MW, Hoeksema JD, Gehring CA, Johnson NC, Klironomos JN, Abbott LK, Pringle A (2006) The promise and the potential consequences on the global transport of mycorrhizal fungal inoculum. Ecology Letters 9:501–515

St Denis A, Kneeshaw D, Belanger N, Simard S, LaForest-LaPointe I, Messier C (2017) Species-specific responses to forest soil inoculum in planted saplings in an abandoned agricultural field. Applied Soil Ecology 112:1–10

USDA (United States Department of Agriculture) (2018) Web soil survey. Natural Resources Conservation Service, United States Department of Agriculture. https://websoilsurvey.sc.egov.usda.gov/ (accessed 9 Jun 2018)

Van der Heijden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders I (1998) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396:69–72

Van der Heijden MGA, Bardgett RD, Van Straalen NM (2008) The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecology Letters 11:296–310

Williams AV, Nevill PG, Krauss SL (2014) Next generation restoration genetics: application and opportunities. Trends in Plant Science 19:529–537

Wubs J, van der Putten WH, Bosch M, Bezemer TM (2016) Soil inoculation steers restoration of terrestrial ecosystems. Nature Plants 2:16107

Supporting Information

The following information may be found in the online version of this article:

Table S1. Contents of MycoGrow Soluble microbial inoculant.
Table S2. Results of PERMANOVA of rhizosphere soil fungal communities.
Table S3. Initial number of reads per sample, final number of reads following data process, and filtered number of reads with less than one expected error.

Guest Coordinating Editor: Martin Breed

Received: 29 August, 2019; First decision: 9 October, 2019; Revised: 18 December, 2019; Accepted: 12 January, 2020