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Impacts of Plant-Microbe Interactions on Seedling Performance in a Riparian Forest Invaded by Lonicera maackii

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Impacts of Plant-Microbe Interactions on Seedling

Preformance in a Riparian Forest Invaided by Lonicera maackii

(TITLE)

BY

Taylor E. K. Strehl

THESIS

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Abstract

Soil microbes have profound impacts on plant growth and survival and can either promote or inhibit plant dominance. Exotic plants are often strongly invasive because they have escaped their natural enemies, potentially including antagonistic soil microbes. I examined how the invasive shrub *Lonicera maackii* and a common native tree, *Acer negundo*, responded to soil microbial communities to determine the role of soil microbes in regulating invasion success. This was done by growing both species with microbes from invaded (*L. maackii*) and uninvaded (*A. negundo*) soils collected from three locations within a riparian forest. Seedlings were grown both in isolation (Experiment 1) and in combination (Experiment 2) with both live and sterilized soil inocula from these locations. Despite the expectation of minimal microbial inhibition due to a lack of natural enemies, *L. maackii* was strongly inhibited by 1/3 *A. negundo* and 3/3 *L. maackii* soil microbiome collections when grown in isolation. The native *Acer negundo* was strongly inhibited by 2/3 *A. negundo* and 3/3 *L. maackii* microbiome collections. Conversely, when grown together the soil microbiome largely mitigated negative interspecific interactions (i.e. plant-plant, plant-microbe) leading to a net advantage for *L. maackii* in many cases. This dynamic is likely key to *L. maackii* seedling success when it occurs with seedlings of other species, allowing *L. maackii* a competitive advantage through biotic interactions.

Key words: soil microbes, soil communities, soil inocula, invasion

1

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Table of Contents

Introduction

With modern global commerce, anthropogenically-facilitated biological invasions and their negative consequences are ubiquitous aspects of plant communities worldwide. One of the biggest concerns about biological invasions is loss of biodiversity through competitive exclusion (Shochat et. al 2010; French and Watts 2015; Wardle and Peltzer 2017). This impact is especially evident in plant communities where resource partitioning is a stabilizing force. Niche specialization allows plants that are usually weak competitors to be locally more dominant given the right environmental conditions (Shriver 2017; De Deurwaerder et al. 2018). The addition of an invasive plant to a community often displaces competitively inferior plants from their niche. Niche specialists can easily be excluded through new competitive interactions (Strickland et al. 2010) or the alteration of the environment by an invader (Hilton et al. 2006), leading to a dramatic loss in species richness within invaded patches.

Typically, direct competitive effects of an invasion are studied because they are more immediately evident than indirect or secondary invasion impacts. In many cases, especially for plant invasion, secondary effects may have much further reaching effects than direct competitive interactions. In particular, exotic plants have been shown to alter carbon, nitrogen and water cycling in soils (Ehrenfeld 2003; Van Der Heijden et al. 2008; Hickman et al. 2013; Delgado-Baquerizo et al. 2017), resource availability (Krishna and Dart 1984; Chen et al. 2003; Van Der Heijden et al. 2008), and ultimately competitive interactions (Pendergast et al. 2013; Mariotte et al. 2018). This alteration of soil conditions and competition can strongly impact community composition of both soil microbial (Kourtev et al. 2002) and plant communities (Ehrenfeld 2003; Bever et al. 2010).

5

An often-overlooked indirect effect of plant invasion is their impact on soil microbial communities. Most plant species culture the adjacent rhizosphere with a unique suite of root exudates, accumulating a species-specific soil microbiome of mutualist, parasites, pathogens, and commensal organisms (Broeckling et al. 2008; Liang et al. 2012, 2016). Obligate mutualism between plants and microbes are not uncommon as many species are dependent on mycorrhizae to improve nutrient and water uptake (Krishna and Dart 1984; Hart et al. 2003; Basu et al. 2018). Species invasions may alter soil microbial communities in ways that may indirectly alter the performance of other plant species. For example, *Alliaria petiolata*, a forest understory invader, releases root exudates with anti-fungal properties that suppress mycorrhizal populations (Hale and Kalisz 2012) leading to poor seedling recruitment of mycorrhizae-dependent plant species (Prati and Bossdorf 2004; Stinson et al. 2006). In another example, *Solidago canadensis* has been shown to produce allelopathic chemicals that suppress soil pathogens. This not only improves conditions for itself but also lowers pathogen loads for neighboring plants (Zhang et al. 2009).

Furthermore, some of the indirect effects are likely mediated by interactions between the soil microbial communities of adjacent plant species. Plants are usually influenced by a cocktail of unique microbiomes from surrounding plants as well as their own (Zhang et al. 2009; Wardle and Peltzer 2017). The prevalence and potential strength of such plant-microbe interactions elevates soil microbes to be one of the primary controllers of plant community structure and dynamics (Bezemer et al. 2018) and species invasions (Andonian et al. 2012)

Lonicera maackii (Amur Honeysuckle) is one of the most widespread invasive plants in Eastern North America, widely colonizing riparian and mesic forests. Originally introduced as an

6

ornamental from Asia as an ornamental and for erosion control, the shrub quickly escaped

cultivation via bird dispersal into the surrounding landscape (Hutchinson and Vankat 1997,

1998; Bartuszevige and Gorchov 2006). *Lonicera maackii* is a fast-growing shrub that often

forms large monocultural stands in disturbed forests (Hutchinson and Vankat 1998). The leaf

phenology of *L. maackii* provides an extended growing season relative to native plant species. It

is likely that the species' aggressive growth and extended phenology are responsible for the

decline in understory plants (Collier and Vankat 2002; Gorchov and Trisel 2003) and canopy tree

growth (Hartman and McCarthy 2009) in areas invaded by *L. maackii*. *Lonicera maackii* has

been shown to reduce decomposition rates, leading to a buildup of organic matter, ultimately

improving nutrient availability to the shrub (Arthur et al. 2012). Therefore, the indirect effects

of *L. maackii* via changes to the soil microbiome are also likely to be an important factor in this

species' success relative to native species (Arthur et al. 2012) and warrant further attention.

   As *L. maackii* commonly invades riparian forests, I focused on microbial interactions

with the dominant native tree (*Acer negundo*) to determine whether microbially mediated

effects are contributing to the success of *L. maackii* in this system. To do this I conducted two

experiments using the soil microbiomes of both the invading shrub and the native tree. The first

experiment studied the reciprocal effects of each soil microbiome on seedlings of both species

grown in isolation. The second experiment repeated the design of the first, but with both

species grown together in co-culture. This allowed for comparison between microbiome

culturing effects. Specifically, I asked: 1) Does the soil microbiome of *L. maackii* differ from that

of native *Acer negundo* in its effects on seedling growth when gown in isolation? 2) Do the net

impacts of the soil microbiome change when seedlings are grown together in co-culture? 3) How do the soil microbiome communities differ between *A. negundo* and *L. maackii*.

Materials and Methods

Soil was collected from the study site (The Ainsley Farm) located in Monroe Center, Ogle Co, IL (42.116307,-88.987253) for use as inoculum in the experiments. The study site was split into three sub-sites for sampling: west, middle and east sites. All subsites were within a riparian forest that stretched intermittently along a stream for approximately 8 km from its source to its confluence with the south branch of the Kishwaukee River. From each sub-site, two samples were taken from the top 5-10 cm of the soil, avoiding large root material. One sample was taken from beneath a well-established *Lonicera maackii* patch and the other from beneath the uninvaded canopy of *Acer negundo* trees. The samples were transferred to the lab and mechanically homogenized with a 4 mm sieve to remove intact plant parts. Processed soils were then stored at 4°C until use. Soils were used as inocula for two greenhouse experiments and for microbiome analysis. After 90 days in each experiment the plant material from both experiments was harvested and dried at 60°C for 5 days. For plants grown in isolation, both above and below ground biomass was harvested. As initial analyses indicated very similar responses for both roots and shoots, I present only total biomass for the isolation experiment. Only above ground biomass was harvested for the plants grown in co-culture because of difficulty in differentiation.

Experiment 1 - Impacts of microbial communities on seedling growth in isolation

For each sub-site, I generated three unique soil inocula types (invaded alive, uninvaded alive, and dead - Fig. 1). To control for abiotic heterogeneity in the inocula, each treatment consisted of a 1:1 mixture of both invaded and uninvaded soil types with either both dead (autoclaved at 121 °C for 90 minutes) or one alive, depending on the treatment. This isolated the direct biotic effects of each unique microbial community from any abiotic differences in soil properties. Therefore, any differences in biomass production across treatments can be attributed solely to the biotic effects of the microbe community and not abiotic differences across locations (Pendergast et. al 2013).

Seedlings of each species were started on soilless greenhouse medium (Pro-Mix, Premier Tech Ltd, Quebec, Canada). 164 ml cone-tainers (Ray Leach Inc) were filled 2/3 with the same sterile potting mix. 10 mL of mixed inoculum was added and integrated into the top 2 cm of this layer. The small size of the inoculum relative to soil volume further minimizes the potential for abiotically generated variation across treatments. The inoculum layer was then covered with an additional 2.5 cm of sterile potting mix to minimize microbial spread across treatments. Seedlings were transplanted into the sterile layer of each tube, and any that died within the first 10 days were replaced.

Biomass data were converted into response ratio where each data point is scaled relative to its sterile control. By doing so, variance created by the differences in growth between the species, or abiotic differences across soils were removed from the analyses. Transformed data were then analyzed with ANOVA. All analyses were conducted in R (R Core Team 2017).

Experiment 2 - Impacts of soil microbial communities on seedlings grown in co-culture

To contrast with the single species experiment, the influences of soil microbial communities on seedlings grown in co-culture were examined by measuring biomass production of plants grown together in a pot. I used an identical experimental design and inocula as the first experiment, except each pot (a 20 cm diameter, 3.05 L azalea pot) contained six seedlings, three of each species planted in an alternating pattern (Figure 2). This design was replicated 6 times per inoculum type. As in the first experiment, seedlings of each species were started on soilless greenhouse medium (Pro-Mix, Premier Tech Ltd, Quebec, Canada). Each pot was filled 80% with sterile potting mix, and 250 mL of inoculum was added and integrated into the top 2 cm of the potting mix. The inoculum layer was then covered with an additional 2.5 cm of sterile potting mix to minimize microbial spread across treatments. Seedlings were transplanted into each pot, and any that died within the first 10 days were replaced. Seedlings were harvested after 60 days and the data analyzed as above. However, as seedlings within pots are not independent, I averaged biomass data for each species in each pot to generate a single independent response value.

Microbiome Community make-up and variation

The inocula used in the two greenhouse experiments were analyzed to determine the makeup of the bacterial and eukaryotic components of the microbial community. The rRNA/eDNA from each soil sample was extracted and cleaned with the DNeasy Power Soil Kit (Qiagen, Hilden, Germany). Each soil sample yielded a unique community genome. PCR reactions for each

10

sample were run to amplify the V4 region of the 16S and 18S (prokaryote- and eukaryote-specific, respectively) rRNA using previously described primers (Kozich et at. 2013). The amplicons were then barcoded for multiplex sequencing analysis using the Illumina Nextera primer and index system (Illumina, Madison, WI) and KAPPA HiFi Hot Start Polymerase (Roche, Indianapolis, Indiana). Each sample was amplified using a unique set of indices that allow pooling of the amplicons for sequencing. PCR products were run through a 2% agarose gel at 100 V for 30 minutes. Amplified bands were identified by comparison to a lane run with O'GeneRuler 1 kb DNA standard ladder (Thermo Scientific, Waltham, MA). The correct bands were extracted, and the gel was removed with an MPBIO gel clean kit (MP Biomedicals, Santa Ana, Ca). The resulting amplicons were diluted to a standard concentration and pooled by amplified region. Two pooled samples (16S and 18S) were sent to the UIUC DNA Services Lab (Champaign, IL) for Illumina sequencing. The resulting sequence data was then compared against SILVA version 132 SSU reference database (Quast et al. 2013) using MOTHUR SOP (Schloss et al. 2013) to determine the community make-up and structure for each sample. Differences between the samples was analyzed with principal coordinates analysis (PCoA) using amplicon counts as a measure of relative abundance for prokaryotic and eukaryotic data separately.

Results

Impacts of microbial communities on seedling growth in isolation

Overall, microbial impacts on growth were inhibitory across locations and microbial communities for both species (Fig. 3, Table 1). *Lonicera maackii* was inhibited by both the *A.*

11

negundo and *L. maackii* microbiomes across all sampling locations. However, *A. negundo* was only inhibited by its own microbe community, with minimal reduction in growth when grown with *L. maackii* microbes. *Acer negundo's* response to the *L. maackii* microbiome was statistically indistinguishable from the sterile control. This difference in microbial effects demonstrates that established *L. maackii* individuals likely alter the soil microbiome. *Acer negundo's* lack of inhibition by *L. maackii* soil microbes was unexpected, however.

Impacts of soil microbial communities on plants in co-culture.

In marked contrast to seedlings grown in isolation, there was a much different relationship between seedling growth and microbial community identity when grown in co-culture (Fig. 4). In co-culture, site and seedling species was significant, but not which soil microbial community was used. While most (9/12) plants grown individually were inhibited by live microbes, all but one group of plants grown in co-culture had a neutral (7/12) or positive (4/12) relationship with live microbial communities. Half of the *L. maackii* microbe treatments (3/6) had a positive effect and the remaining were neutral relative to the biomass of sterile controls. Only in *A. negundo* soil from the eastern sub-site was the inhibition of *A. negundo* growth seen in plants grown alone maintained. In that same soil, *L. maackii* seedlings increased in growth, leading to a significant soil community × species interaction.

Soil community composition

Although community composition at the phylum and order levels appear to be quite consistent across all samples, resolution at the family level revealed clear variation across microbial

12

communities. The prevalence of many families fluctuated between samples, as two families were not detected and some accounted for a significant portion of other soil communities. The least diverse community was from the WN (West-ACNE) inocula which contained only 29 of the 35 most common families representing the most common 50% of microbe families found overall.

Microbial composition varied greatly across sampling locations and species (Fig 5 & 6) for both prokaryotic and eukaryotic components. The differences between the communities were mapped using a PCoA (principal coordinates analysis). Using R the PCoA plots were overlaid with a two-dimensional heat map representing relative Shannon-Wiener diversity. This analysis reveals that both soil history (species origin) and site are likely important determinants of microbiome make up. For both the prokaryotic and eukaryotic community, variation is more strongly associated with site.

Shannon diversity was correlated with both PCoA axes in each ordination. In both cases points lower and to the right have the lowest Shannon diversity while those towards the top left have the highest. For example, WN is the furthest to the right and low because its composition was most different from the others and had the lowest Shannon diversity. *L. maackii* soils always had a higher Shannon diversity than *A. negundo* soils and soils from the western site had consistently lower Shannon diversity than other sites.

Discussion

13

The Enemy Release hypothesis suggests that exotic species may benefit from a loss of natural enemies. This loss of enemies often leads to increased vigor in the invaded range, allowing species to become invasive (Colautti et al. 2004; Agrawal et al. 2008). Enemy release is thought to be a factor behind *Lonicera maackii* invasion (Lieurance and Cipollini 2012, 2013). It is likely that *L. maackii* has few natural enemies in newly invaded areas. Expectation of enemy release would be that non-native species should experience relatively fewer antagonistic interactions with soil microbes than associated native species, which retain their full complement of antagonistic soil organisms (Andonian et al. 2012; Dawson and Schrama 2016). However, the data from the greenhouse experiments do not support enemy release as an explanation for continued *L. maackii* success. Exotic plants tend to acquire new enemies over time (Diez et al. 2010) potentially explaining why L. maackii was inhibited by its own microbiome more than by the *A. negundo* microbiome.

Data from the isolated seeding response experiment indicates that the soil microbiomes of *L. maackii* and *A. negundo* vary significantly in their influence on seedling growth. *Lonicera maackii* was inhibited significantly by both the *A. negundo* and *L. maackii* microbe communities but *Acer negundo* was only inhibited by its own microbes. *Acer negundo* is the dominant native tree at the study site, therefore its natural enemies would would be expected to be abundant throughout the site. This is the likely mechanism for Acer negundo self-inhibition at the study site. Conversely in the Rhone valley of France where Acer negundo is highly invasive, adult acer trees were found to promote the growth of seedlings (Girel et al. 2010). This stark difference between native and invaded ranges is evidence that enemy release is likely a factor for *A. negundo* invasiveness (Reinhart and Callaway 2009).

14

Lonicera maackii on the other hand is a relatively new invader. Interestingly, *Acer negundo* was not inhibited by the *L. maackii* microbiome but by its own microbiome. This suggest that *L. maackii* cultures a unique soil community where *A. negundo* enemies are less abundant. It is likely that soils directly adjacent to *L. maackii* plants have accumulated this community over time displacing disused microbes antagonistic to *A. negundo*. This would in-turn generate microbiome conditions advantageous to *A. negundo*.

When grown in co-culture, seedling growth of both species almost always showed a neutral or positive association with soil microbes regardless of their source. This result is in marked contrast to the predominately antagonistic effects observed in the first experiment. The only difference between the experiments was that in the second experiment multiple species share a single pot. This results in a unique suite of biotic interactions determined by the initial inocula and selective pressures exerted by both plants (Bever et al. 1997). The reversal of soil microbiome effects could be due to reduction of plant-plant competition brought about by a limited plant-microbiome interaction. In other words, the direct negative impacts of the microbe community on each plant is outweighed by the indirect effect of reduced competition caused by harm that the microbe community causes to its plant competitor. However, as plants were overall larger in the second experiment, any competition appears minimal.

Another possible mechanism is the microbe-culturing effect of individual plant species. When grown in isolation, a soil microbial community unique to that plant species only was generated via interactions between the seedling and the inoculum. When grown together with overlapping root systems, the single inoculum will interact with both plant species: preventing dominance of the soil microbial community by specialist species of either plant species.

15

Furthermore, soil microbial communities may directly interact, further altering composition and therefore biotic impacts. (Czaran et al. 2002; Hibbing et al. 2010; Kinkel et al. 2010). As seedlings often occur in mixed assemblages the effects in mixed culture appear more relevant to the natural systems being invaded.

Regardless of the mechanism (plant-plant, plant-microbe, microbe-microbe, etc.), the net effects are altered when *L. maackii* and *A. negundo* were grown together. Plant-microbe interactions can mitigate negative environmental effects on plant growth. This mitigating effect of microbial co-culture may be important for plant community diversity and long-term persistence of less competitive plants (Kinkel et al. 2010; Bezemer et al. 2018).

In the case of *Lonicera maackii* invasion, plant-microbe interactions are likely integral to success, but indirectly. While enemy release was not shown to occur, plant-microbe interactions in co-culture proved to be advantageous for L. maackii in 5/6 microbiomes tested, despite strong inhibition when grown in isolation. This complex soil-mitigated interaction helps explain how a plant that with seemingly vulnerable seedling can be such an aggressive invader in riparian systems.

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L. maackli

A. negundo

**Loma: Live**   **Acne: Live**   **Both: Dead**
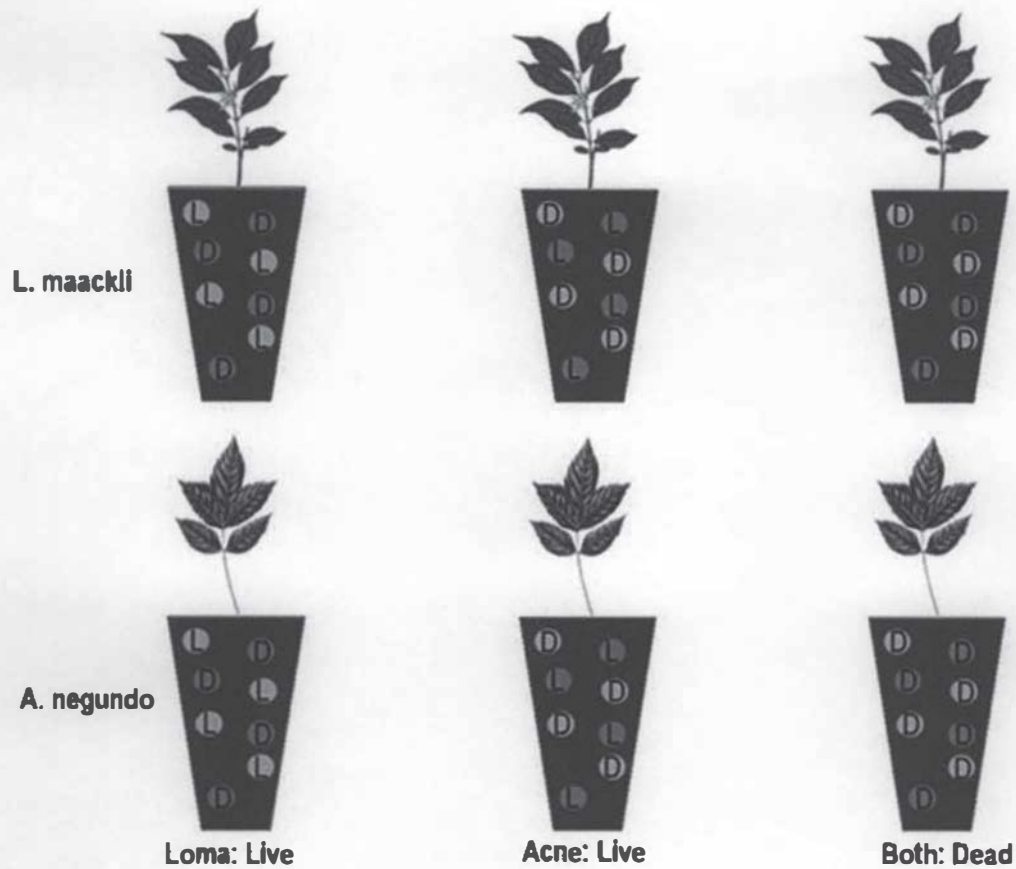
Figure 1. Experimental design to determine microbiome effect on seedling growth in isolation. Each species (ACNE & LOMA) were grown singly in a 164 ml cone-tainer inoculated with either live LOMA, live ACNE or sterilized field soil. To isolate abiotic effects all inoculum consisted of a 50:50 mixture of LOMA & ACNE soils where either one or both portions sterilized. This experiment was further replicated with inoculum from three sites (East, Central, and West).
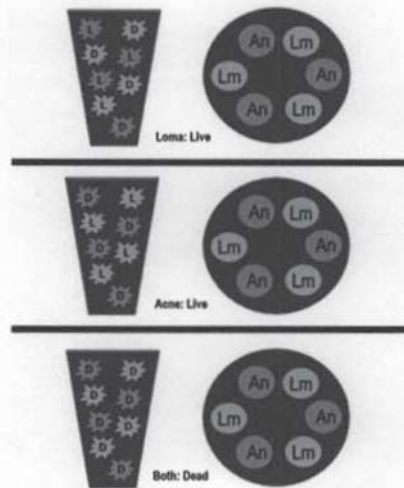
Figure 2. Experimental design to determine microbiome effects on seedlings grown in co-culture.
Three *A. negundo* (An) & three *L. maackii* (Lm) seedlings were grown together with either live *L. maackii*,
live *A. negundo* or sterilized field soil. To isolate abiotic effects all inoculum consisted of a 50:50 mixture
of both soil inocula where either one or both portions were sterilized. This experiment was replicated six
times per treatment and further replicated with inoculum from three sites (East, Central, and West).
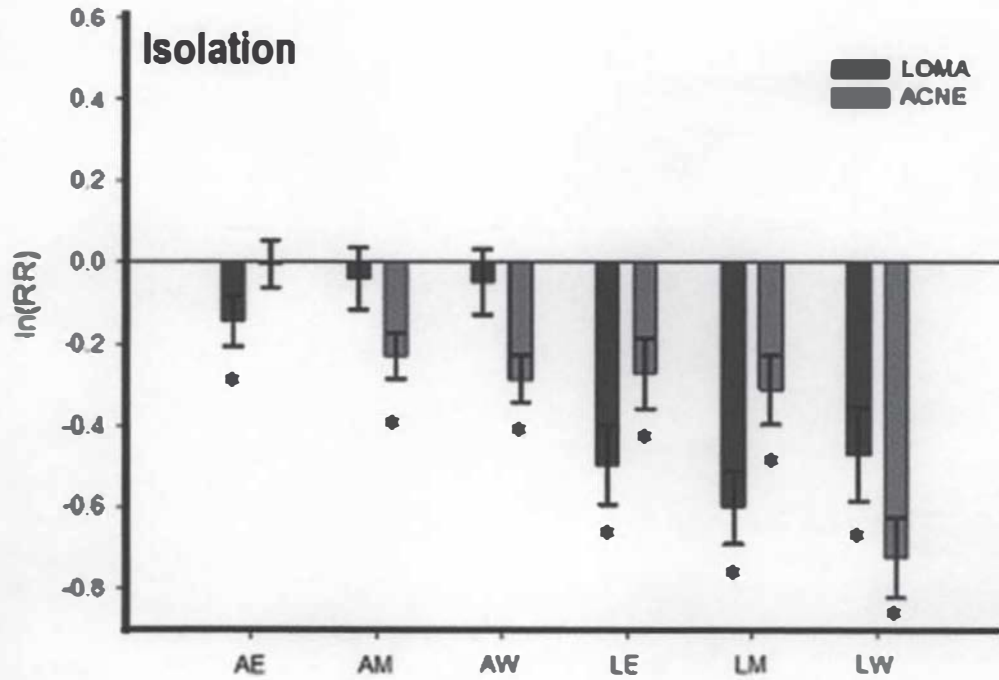
Figure 3. Plant response to microbiome grown individually. Bars represent the mean ln (response ratio)

of each species to each control (sterilized) soil. Error bars represent the SEM of each sample (n=?).

Asterisks (*) denote samples that vary significantly from their respective control (i.e. 0).
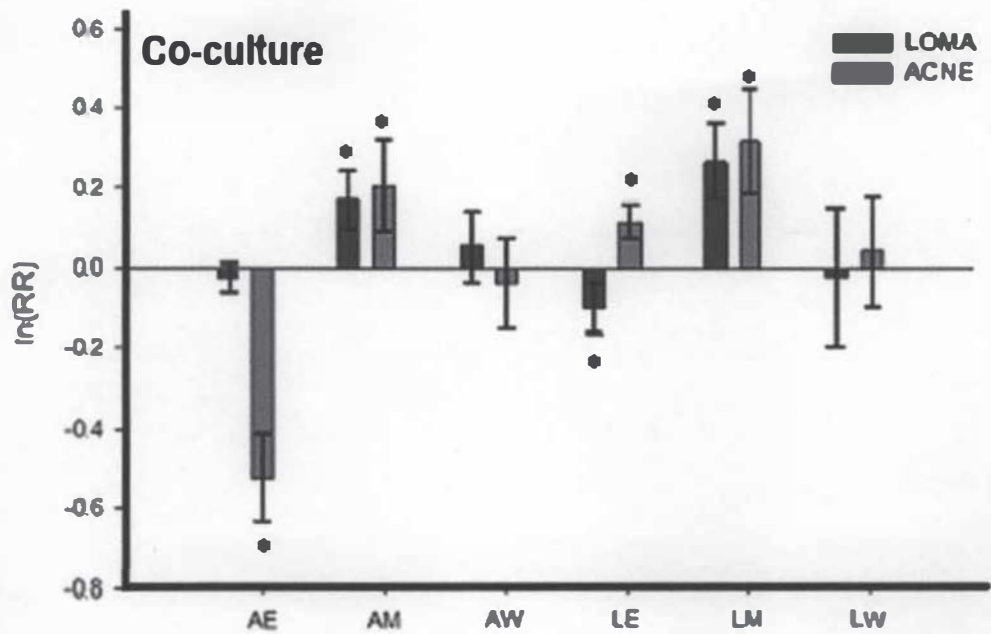
Figure 4. Plant response to microbiome grown in co-culture. Bars represent the mean ln (response ratio) of each species to each control (sterilized) soil. Error bars represent the SEM of each sample. Asterisks (*) denote samples that vary significantly from their respective control (i.e. 0)
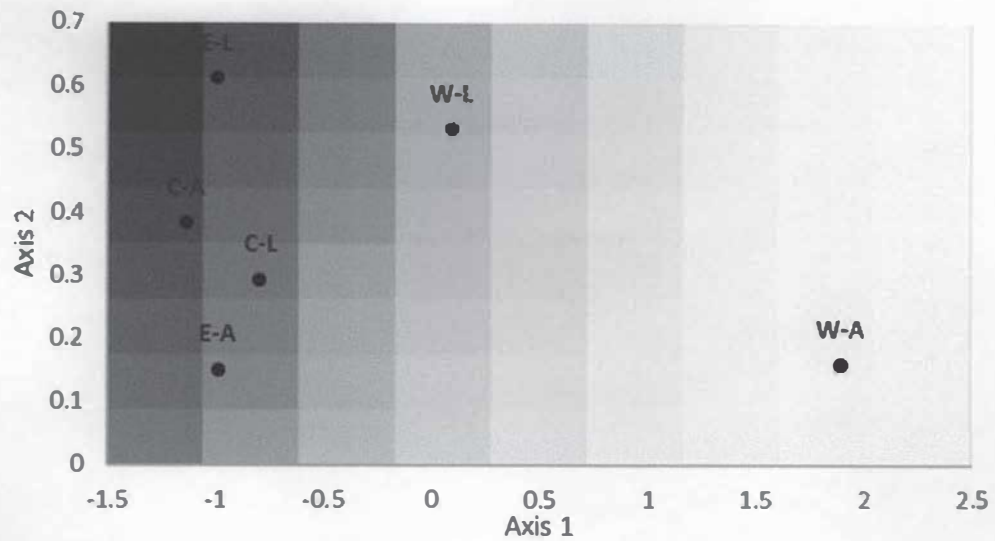
Figure 5. PCoA of ordination of prokaryotic community composition of *L. maackii* (L) and *A. negundo* (A) inocula from each collection site (E, C and W). Points representing each community are plotted based on a two-dimensional model. Each axis was correlated with Shannon diversity and represented in an overlaying heat map where darker regions represent communities of higher Shannon diversity.
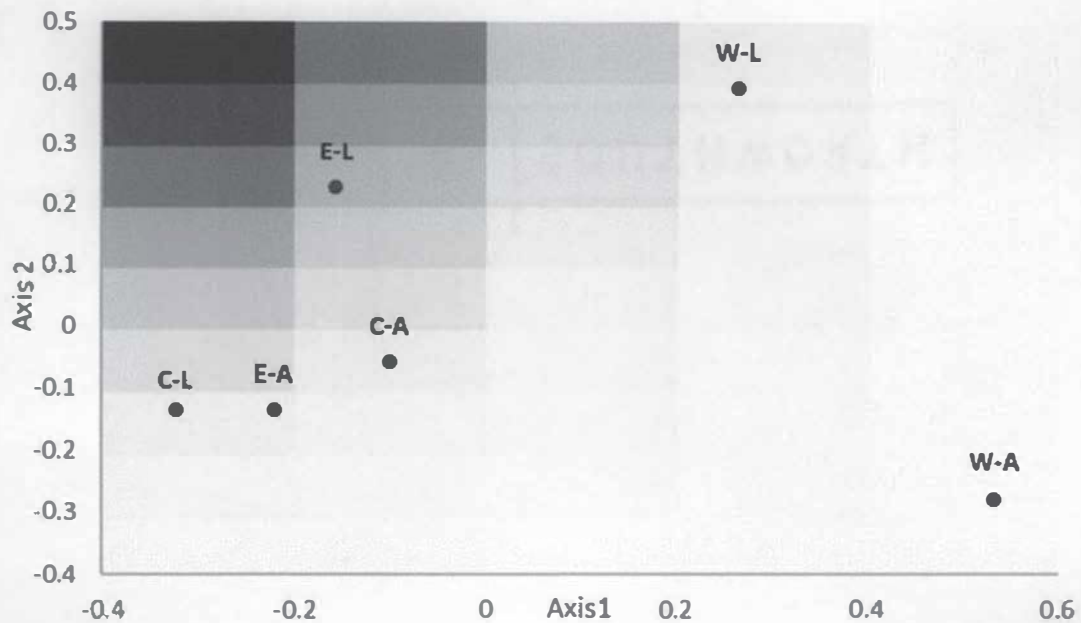
Figure 6. PCoA of ordination of eukaryotic community composition of *L. maackii* **(L) and** *A. negundo* **(A) inocula from each collection site (E, C and W).** Points representing each community are plotted based on a two-dimensional model. Each axis was correlated with Shannon diversity and represented in an overlaying heat map where darker regions represent communities of higher Shannon diversity.

Table 1. Effects of site and soil microbiome identity on *A. negundo* and L. maackii biomass when grown in isolation. Data analyzed are ln(response ratios) for each sample relative to its control. Significant P values indicated in bold. Model R^2= 0.098 for A. negundo and 0.198 for *L. maackii*.

Response	df	MS	F	P
Acer negundo				
Site	2	0.1024	0.842	0.4325
Microbiome	2	2.0182	16.603	**<0.0001**
Site*Microbiome	4	0.2189	1.802	0.1307
Residuals	171	0.1215		
Lonicera maackii				
Site	2	0.0019	0.024	0.9763
Microbiome	2	0.5309	6.476	**0.0019**
Site*Microbiome	4	0.1123	1.370	0.2464
Residuals	171	0.0819		

Table 2. Effects of site soil microbiome identity and species on A. negundo and L. maackii biomass when grown in co-culture. $R^2 = 0.870$

Response	df	MS	F	P
Site	2	20232	5.458	**0.0053**
Microbiome	2	5275	1.446	0.241
Sp.	1	2513058	689.147	**2.2e-16**
Site:Microbiome	4	11179	3.066	**0.020**
Site:Sp.	2	8373	2.296	0.107
Microbiome:Sp.	2	7251	1.989	0.143
Site:Microbiome:Sp.	2	10071	2.762	**0.0032**
Residuals	90	3647		

Table 3. Soil Microbiome make-up by OUT Phylum

Phylum	Fraction					
	EI	EN	CI	CN	WI	WN
Prokaryote						
Actinobacteria	0.2722	0.2497	0.2560	0.2513	0.2495	0.2383
Proteobacteria	0.2052	0.2614	0.2461	0.2420	0.2452	0.1989
Unknown *Bacteria*	0.2549	0.2234	0.2209	0.2160	0.2199	0.1321
Acidobacteria	0.1311	0.1546	0.1290	0.1541	0.1118	0.1530
Verrucomicrobia	0.0343	0.0304	0.0351	0.0383	0.0649	0.1370
Firmicutes	0.0328	0.0190	0.0168	0.0242	0.0454	0.0856
Planctomycetes	0.0203	0.0209	0.0282	0.0287	0.0219	0.0428
Crenarchaeota	0.0243	0.0133	0.0229	0.0159	0.0278	0.0052
Unknown *Archaea*	0.0133	0.0065	0.0248	0.0093	0.0000	0.0000
Bacteroidetes	0.0044	0.0065	0.0095	0.0111	0.0053	0.0009
Gemmatimonadetes	0.0041	0.0079	0.0072	0.0051	0.0056	0.0037
Nitrospirae	0.0011	0.0027	0.0015	0.0018	0.0019	0.0012
Chloroflexi	0.0022	0.0027	0.0011	0.0018	0.0003	0.0009
Armatimonadetes	0.0000	0.0008	0.0008	0.0006	0.0000	0.0003
Chlamydiae	0.0000	0.0000	0.0000	0.0000	0.0006	0.0000
Eukaryote						
Ascomycota	0.6656	0.5778	0.6250	0.6501	0.6836	0.5476
Basidiomycota	0.1297	0.1912	0.1536	0.1584	0.0786	0.1675
Unknown	0.1081	0.1131	0.1141	0.1109	0.1193	0.1582
Mucoromycota	0.0710	0.0860	0.0866	0.0605	0.0863	0.0875
Chytridiomycota	0.0210	0.0255	0.0165	0.0170	0.0281	0.0303
Amoebozoa	0.0023	0.0037	0.0031	0.0022	0.0026	0.0029
Blastocladiomycota	0.0021	0.0023	0.0010	0.0006	0.0012	0.0057
Ciliophora	0.0001	0.0002	0.0001	0.0001	0.0000	0.0002
Aphelida	0.0001	0.0001	0.0000	0.0000	0.0004	0.0000