Effects of Foliar Microorganisms in Native and Exotic Plant Species in Old-Field Communities

by

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Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Biological Sciences

Program

YOUNGSTOWN STATE UNIVERSITY

August, 2017

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Abstract: Much is known about the effects of antagonistic and mutualistic soil microorganisms on plant growth, but scant information exists on the effects of *foliar* microorganisms (i.e., bacteria and fungi that reside on plant surfaces, the phyllosphere). Nearly all studies on this issue investigate foliar microbes or fungi separately, but not both simultaneously, despite the potential for them to interact. Moreover, no one has studied this aspect of plant ecology in temperate zones and, as such, this study provides a significant first step in understanding the potentially complex interactions between plants and the diverse foliar microorganisms that likely inhabit them. By removing fungal and microbial communities on the leaf surfaces of native and exotic old-field plants through antibiotic and fungicide applications, we elucidate the separate and interactive effects of various foliar microorganisms on plant growth under different nitrogen regimes. These factors have the potential to alter competitive hierarchies, and thus, plant community composition as well as invasibility. Specifically, the success of many exotic plants may in part be attributed to their escape of foliar pathogens present in their native range. This "enemy release" hypothesis was explicitly tested by comparing and contrasting the effects of removing some or all of the phyllosphere community on native and exotic plant species, and assessing their subsequent biomass, a measure of their fitness response.

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Introduction

The worldwide cost for managing invasive species is estimated at about $1.4 trillion while the United States alone is about $120 billion, 30% of which accounts for plants (Pimentel et al. 2005). Some species become invasive when they are introduced outside of their native range, where they increase in abundance and range. Not only do these organisms cause ecological change, often resulting in lower local and regional biodiversity (Clay and Holah 1999, Pimentel et al. 2001), but also result in economic loss (Pimentel et al. 2005). While research has focused on how invasions are affected by recipient native plant community composition (Meiners et al. 2002, Dickie et al. 2014, Kazenel et al. 2015), soil nutrients (Renne et al. 2006, Parepa et al. 2013, Inderjit 2015), disturbance regimes (Brouwer et al. 2015, Kulmatiski et al. 2006, Tognetti and Chaneton 2012), and soil microorganisms (van der Heijden et al. 2007, van der Putten et al. 2013, Zarraonaindia et al. 2015), less is known about the role of resident microorganisms on leaf surfaces, and importantly, how they affect plant fitness components, of growth and reproduction. The potential for foliar interactions between bacteria, fungi, and host plants is great, since bacteria and fungi on leaf surfaces can affect plant performance (Griffin et al. 2016). Current information about these foliar microorganisms is based primarily on research in tropical forests and agricultural systems (Clay 1990, Rodriguez et al. 2009, Vorholt 2012, Griffin and Carson 2015), and research has yet to combine treatments that manipulate soil nutrients, fungal, and bacterial variables on native and exotic plant species performance into one comprehensive study.

Invasion Processes

Studies have shown that complex underground interactions are involved in mechanisms that facilitate invasive species success in a new region (van der Putten et al. 2013, Dickie et al. 2014, Inderjit 2015, Inderjit and Cahill 2015). Invasions can be facilitated by soil microorganisms (Inderjit and Cahill 2015), as well as by plant soil-feedbacks (Larios and Suding 2015). In these cases, chemicals in soil and those released by plants and microorganisms affect not only mutualistic relationships between fungi, bacteria, and plants (Han and Micallef 2016) but also soil and plant community structures. This may facilitate further invasions, a circumstance known as invasional meltdown (Simberloff and Von Holle 1999). Alternatively, it may prevent other species from invading an area (Blank et al. 2015). Some plants are able to recruit microorganisms that could be pathogenic to other plants, or that release toxic chemicals, thus preventing the establishment and/or spread of other plant species within the area (see Calloway and Aschehoug 2000, Ibanez et al. 2012). Not only do these belowground interactions affect invasions, the interactions between roots of plants and soil communities can affect aboveground mutualisms and enhance invader success (Rodríguez-Echeverría and Traveset 2015).

The Phyllosphere

The phyllosphere, or the outer plant surface, is the largest surface on earth with a surface area of over 1 billion km² that is estimated to host more than 10^{26} bacteria (Lindow and Brandl 2003). Not only do microorganisms inhabit the phyllosphere as epiphytes, they can be present within plant tissue as endophytes. Some microorganisms on plants are decidedly pathogenic, but others can promote growth, whereby both species

receive resources from each other (Beattie and Lindow 1995, Hirano and Upper 2000, Rodriguez et al. 2009, Vorholt 2012, Gonçalves et al. 2014). The fungal and microbial community compositions on plants depend on geography, season, and plant species (Whipps et al. 2008, Redford and Fierer 2009, Redford et al. 2010). Differences in tropical and temperate systems, like warmer temperatures and increased soil aluminum levels in the former and cooler temperatures with higher calcium levels in the latter, affect the types of foliar microorganisms found in those environments and how they affect their plant host (Kembel et al. 2014, Griffin and Carson 2015). Some bacteria (*Proteobacteria, Bacteroidetes*, and *Actinobacteria*) and fungi (*Ascomycota* and *Basidiomycota*) can be commonly found on plant surfaces despite differences in plant geography, season , and phylogeny (Kembel et al. 2014, Fu et al. 2016), suggesting some microorganisms share traits that promote their survival on plant surfaces and as such, we are likely to encounter similar organisms in our study.

Microorganisms and Plant Fitness

Several studies have looked at microbial addition or removal on the fitness of plant hosts, and showed that both beneficial and/or pathogenic microorganisms may be able to proliferate on plant surfaces, and either promote or deter plant growth based on nutrient availability (Heil et al. 2000, Traw et al. 2007, Borer et al. 2015, Griffin et al. 2016). Specifically, Nitrogen, as a macronutrient is an essential amino acid component and a limiting factor in plant systems. Zhang et al. (2013) showed that nitrogen is used in chlorophyll production and is important for plant growth and fitness. Several studies concluded that increased levels of nitrogen absorption allow plants to become more

competitive and can promote changes in plant community hierarchies (Fynn et al. 2005, Gao et al. 2005, Leskovšek et al. 2012).

Despite knowledge that bacteria and fungi in the rhizosphere can modulate the effects of plant fitness, these putative interactions in the phyllosphere are unknown. Although Griffin et al. (2016) did not investigate phyllospheric fungal communities, they found that the effects of bacteria could be augmented, or even reversed, across a nutrient gradient in conjunction to antibiotic application; in some plant species antibiotic by itself promoted plant growth, however addition of soil nutrients, like potassium, resulted in no change in biomass or decreased biomass. Possible mechanisms suggested by Griffin et al. (2016) are host plant allocation of resources for growth instead of defense when pathogens are removed from plant surfaces (Heil et al. 2000, Traw et al. 2007), and the benefits of soil nutrient addition. Plants that contain foliar pathogens must defend themselves instead of grow to ensure survival of offspring in the future, however when pathogens are not present, a plant can rapidly grow and reproduce. Soil nutrients are important in plant fitness; specifically nitrogen is used in plant chlorophyll production that is important for light absorption in photosynthesis (Leskovšek et al. 2012, Zhang et al. 2013). In their study testing fungal and bacterial ratios based on nitrogen managements, de Vries et al. (2006) noted that while bacterial numbers increased with more nitrogen, the actual fungal-to-bacterial ratios decreased due to decreased fungal biomass. One likely mechanism for the decrease in fungal biomass in the de Vries et al. (2006) study is the niche overlap of bacteria and fungi on their host plant with increased bacterial numbers promoting competition between these microorganisms, ultimately resulting in decreased fungal organisms (Gu et al. 2010, Redford et al. 2009, Kazenel et

al. 2015). Evidence thus suggests these factors may interact to affect plant performance, but to our knowledge, no study has simultaneously integrated the effects of nitrogen, foliar fungi, and foliar bacteria on plant growth.

As such, this study will be a significant first step in understanding the potentially complex interactions between plants and the diverse foliar microorganisms that may inhabit them. Moreover, these phyllosphere communities have strong potential to alter plant competitive hierarchies, and thus may influence the floristic diversity of numerous temperate systems. Many foliar organisms are also involved in nitrogen and carbon fixing (Woods et al. 2012), and with so many organisms present on leaf surfaces, they have a potential to significantly contribute to global nitrogen and carbon cycles. We add that the invasion success of many introduced species may in part be attributed to the "enemy release hypothesis", which proposes that invasion success is due to the exotics' escape from enemies within their native ranges (Vilà et al. 2005, Cincotta et al. 2009, and Castells et al. 2013). Of particular relevance to the present study, successful exotic plants may have escaped their native fungal and/or bacterial pathogens, and may further possess a novel chemistry to which pathogenic microorganisms in their recipient ecosystems are not adapted to (Callaway and Aschehoug 2000, Keane and Crawley 2002).

Objectives

Here, we test the separate and combined effects of bacterial and fungal phyllosphere communities on performance of native and exotic plants across a nitrogen regime. Our study was conducted in a temperate old-field community which is a system that underwent disturbances due to its previous agricultural use before its abandonment (Dölle et al. 2008). As ecosystems that may experience numerous disturbances, old-

fields can facilitate exotic plant colonization which has the potential to affect plant community composition (Meiners et al. 2002, Tognetti and Chaneton 2012, and Kuebbing et al. 2014).

The main hypothesis of our study is that microbial and fungal leaf communities differentially affect the growth of native and exotic plant species, which is tested by comparing average host plant biomass across different antibiotic, fungicide, and nitrogen treatments. We hypothesized that removal of some or all microorganisms may increase or decrease plant growth, depending on whether the microorganisms were pathogenic or mutualistic, and that nitrogen addition may amplify these results. We also tested the "enemy release hypothesis" by measuring the differences in biomass between native and exotic species after different treatment regimens were applied. We hypothesized that our exotic focal plants have an advantage over the native plants and that by removing foliar microorganisms from the native plant species, they will be able to grow as well as the exotic species do with intact foliar microorganism communities. By integrating the potentially complex interactions affecting plant performance, and even competitive hierarchies, an understanding of how these factors may affect plant fitness may be elucidated, with potential application to managing exotic, invasive species.

Methods and Materials

The six annuals or short-lived perennials plant species were grown in a 40m by 40m tilled plot located at the Pymatuning Laboratory of Ecology Donald S. Wood site, part of the University of Pittsburgh in northwestern Pennsylvania, for nine weeks. Three native species (*A. artemisiifolia* L., *A. virginica* L., *P. capillare* L.) and three exotic species (*B. vulgaris* R. Br., *S. glauca* L., *P. lanceolata* L.) were treated every five to

seven days with EPA approved antibiotics (Agri-mycin 17 and FireLine) and fungicides (Captan 50% and Mancozeb). For each species, we had 80 focal individuals, and for each treatment we had 10 replicates, resulting in a total of 480 focal plants. Absorbent towels were used to cover the soil during antibiotic and fungicide applications to prevent runoff into the soil. The antibiotics and fungicides were alternated and applied to focal plants weekly to reduce the risk of resistance by bacteria and fungi to any single treatment.

In week 1, nitrogen was applied (40 kg N/ha) along with the first treatment rotation of antibiotic (Agrimycin-17) and fungicide (Captan 50%). In week 2, the rotation of plant treatments included FireLine antibiotic and Flowable Mancozeb fungicide. In week 7 and 9, 250 randomly selected leaves (5 from each treatment of each species) were removed by forceps cleaned with 90% ethanol and placed in autoclaved amber vials. In week 10, plants were harvested and placed in brown paper bags to air-dry for two weeks after which dry weight biomass of plants was measured.

The leaves collected were pressed onto individual nutrient agar plates (front and back) that were then incubated at 37˚C for 24 hours (Dubey and Maheshwari 2002). Colonies formed were counted manually and plates were categorized in one of four colony counts: less than 100 colonies, greater than 100 but less than or equal to 200 colonies, greater than 200 colonies, and confluent growth (indiscrete growth). Between 28 and 30 plates were inoculated per each treatment combination of all six species resulting in a total of 236 plates.

The average mass for each treatment per species was calculated and the data were inputted into SPSS statistical software as a three-way ANOVA with two levels of

nitrogen, antibiotic, and fungicide. Levene's Test was used to ascertain variance homogeneity and no post-hoc tests were performed. Log-transformation was done for *P. capillare* and *B. vulgaris* biomasses. One-way ANOVA with two levels was done for the two-way interactions seen in *P. capillare* and *B. vulgaris.*

Results

Across host plant species the control, nitrogen, antibiotic, and nitrogen-by-antibiotic treatments had the largest percentages of plates with confluent growth (53.6%, 55.2%, 60%, and 63.3% respectively; see Table 1). In contrast the fungicide, nitrogen-by-fungicide, antibiotic-by-fungicide, and nitrogen-by-antibiotic-by-fungicide treatments had the largest percentages of plates that had minimal growth (72.4%, 83.3%, 83.3%, and 86.7% respectively; Table 1).

Tables 2-7 are the ANOVA tables for each plant species. The average biomass of all plant species except the exotic *P. lanceolata* demonstrated significant treatment effects. Figures 1-5 show the average biomasses from the significant interactions in the ANOVA table of the remaining 5 plant species. Figure 1 shows a significant 78% biomass increase in the exotic *S. glauca* with fungicide application (98.915g) compared to the control group (56.925g) ($F_{1, 70} = 13.574$, $p < 0.0001$). Figure 2 shows a marginally significant 28.2% biomass increase in the native *A. artemisiifolia* with the fungicide application (216.946g) compared to the control group (169.198g) ($F_{1, 74} = 3.661$, $p < 0.06$). Figure 3 shows the average biomass of the native *A. virginica* with a significant 60.2% biomass increase with fungicide application (58.066g) compared to the control group (36.258g) ($F_{1, 71} = 20.089$, $p < 0.0001$). Figure 4 shows the average biomass in the native *P. capillare* comparing nitrogen and fungicide treatments. *P. capillare* had an

insignificant 14.3% decrease in average biomass in the nitrogen-by-fungicide treatment (69.487g) compared to the nitrogen-only treatment (81.089.g), while the fungicide-only treatment (91.697g) compared to the control group (59.917g) had a significant 53% increase in average biomass ($F_{1, 77} = 7.390$, $p < 0.008$). Figure 5 shows the average biomass of the exotic *B. vulgaris* comparing antibiotic and fungicide treatments. *B. vulgaris* had an insignificant 12.8% biomass decrease in the antibiotic-by-fungicide treatment (6.402g) compared to the antibiotic-only treatment (7.345g), while the fungicide-only treatment (12.498g) compared to the control group (2.410g) had a significant 418.6% increase in average biomass ($F_{1, 60} = 4.512$, $p < 0.04$).

There were no differences in biomass between native and exotic species after treatment. Figures 1, 2, and 3 show biomass increases with fungicide treatment compared to control in *S. glauca* (exotic), and *A. artemisiifolia* (native), and *A. virginica* (native) respectively. Similarly, *P. capillare* (native) and *B. vulgaris* (exotic) had increased biomass with fungicide treatment compared to control in Figures 4 and 5 respectively.

Table 1. Colony numbers of all eight treatments groups in all host plant species. Plates were separated into four categories: 100 colonies or less, greater than 100 but less than or equal to 200 colonies, greater than 200 colonies, and confluent growth (CG). Numbers of plates (per treatment combination) are shown out of 30. Treatment combinations were abbreviated based on the applications; N= nitrogen, A= antibiotic, F= fungicide, and control.

Colony Count	Treatment Group							
	Control*	N**	A	N A	F**	N F	A F	N A F
X ≤ 100	10	4	9	6	21	25	25	26
100 < X ≤ 200	0	6	1	2	1	0	0	0
X >200	3	3	2	3	0	0	0	0
CG	15	16	18	19	7	5	5	4

**28 plates total*

***29 plates total*

Table 2. ANOVA test of between-subject effects of the average biomass of *P. lanceolata*. No significant interactions are seen in this table.

Dependent Variable: Biomass

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3107.243[a]	7	443.892	1.109	.368
Intercept	34527.927	1	34527.927	86.226	.000
Nitrogen	93.590	1	93.590	.234	.630
Antibiotic	203.396	1	203.396	.508	.478
Fungicide	825.722	1	825.722	2.062	.155
Nitrogen * Antibiotic	759.406	1	759.406	1.896	.173
Nitrogen * Fungicide	556.550	1	556.550	1.390	.242
Antibiotic * Fungicide	180.666	1	180.666	.451	.504
Nitrogen * Antibiotic * Fungicide	420.219	1	420.219	1.049	.309
Error	28030.626	70	400.438		
Total	65596.375	78			
Corrected Total	31137.869	77			

a. R Squared = .100 (Adjusted R Squared = .010)

Table 3. ANOVA test of between-subject effects of the average biomass of *S. glauca.* A one-way interaction is seen in the fungicide treatment ($F_{1,\ 70} = 13.574$, $p < 0.0001$).

Dependent Variable: Biomass

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	36268.844[a]	7	5181.3	2.281	.039
Intercept	424642.2	1	424642.2	186.979	.000
Nitrogen	1081.8	1	1081.8	.476	.493
Antibiotic	1255.6	1	1255.6	.553	.460
Fungicide	30828.3	1	30828.3	13.574	.000
Nitrogen * Antibiotic	908.5	1	908.5	.400	.529
Nitrogen * Fungicide	1224.4	1	1224.4	.539	.466
Antibiotic * Fungicide	913.4	1	913.4	.402	.528
Nitrogen * Antibiotic * Fungicide	561.5	1	561.5	.247	.621
Error	143077.2	63	2271.1		
Total	627950.3	71			
Corrected Total	179346.1	70			

a. R Squared = .202 (Adjusted R Squared = .114)

Table 4. ANOVA test of between-subject effects of the average biomass of *A. artemisiifolia.* A one-way interaction is seen in the fungicide treatment ($F_{1,\ 74} = 3.661$, $p < 0.06$).

Dependent Variable: Biomass

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	89494.829[a]	7	12784.976	1.105	.370
Intercept	2770505.573	1	2770505.573	239.440	.000
Nitrogen	16787.412	1	16787.412	1.451	.233
Antibiotic	218.046	1	218.046	.019	.891
Fungicide	42360.646	1	42360.646	3.661	.060
Nitrogen * Antibiotic	2275.975	1	2275.975	.197	.659
Nitrogen * Fungicide	18867.007	1	18867.007	1.631	.206
Antibiotic * Fungicide	4462.256	1	4462.256	.386	.537
Nitrogen * Antibiotic * Fungicide	391.464	1	391.464	.034	.855
Error	775243.040	67	11570.792		
Total	3701034.202	75			
Corrected Total	864737.869	74			

a. R Squared = .103 (Adjusted R Squared = .010)

Table 5. ANOVA test of between-subject effects of the log-transformed average biomass of *A. virginica.* A one-way interaction is seen in the fungicide treatment ($F_{1, 71} = 20.089$, $p < 0.0001$).

Dependent Variable: logbiomass

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.835[a]	7	.119	3.288	.005
Intercept	190.922	1	190.922	5264.875	.000
Nitrogen	.015	1	.015	.420	.519
Antibiotic	.021	1	.021	.580	.449
Fungicide	.728	1	.728	20.089	.000
Nitrogen * Antibiotic	.012	1	.012	.337	.564
Nitrogen * Fungicide	.024	1	.024	.669	.417
Antibiotic * Fungicide	.031	1	.031	.857	.358
Nitrogen * Antibiotic * Fungicide	9.582E-05	1	9.582E-05	.003	.959
Error	2.321	64	.036		
Total	197.709	72			
Corrected Total	3.155	71			

a. R Squared = .264 (Adjusted R Squared = .184)

Table 6. ANOVA tests of between-subject effects of the average biomass of *P. capillare.* A two-way interaction is seen in the nitrogen-by-fungicide treatment ($F_{1, 77} = 7.390$, $p < 0.008$).

Dependent Variable: Biomass

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	12100.923[a]	7	1728.703	1.395	.221
Intercept	444250.989	1	444250.989	358.578	.000
Nitrogen	5.251	1	5.251	.004	.948
Antibiotic	240.213	1	240.213	.194	.661
Fungicide	1980.760	1	1980.760	1.599	.210
Nitrogen * Antibiotic	28.937	1	28.937	.023	.879
Nitrogen * Fungicide	9155.619	1	9155.619	7.390	.008
Antibiotic * Fungicide	705.700	1	705.700	.570	.453
Nitrogen * Antibiotic * Fungicide	148.014	1	148.014	.119	.731
Error	86724.814	70	1238.926		
Total	548237.230	78			
Corrected Total	98825.737	77			

a. R Squared = .122 (Adjusted R Squared = .035)

Table 7. ANOVA tests of between-subject effects of the log-transformed average biomass of *B. vulgaris.* A two-way interaction is seen in the antibiotic-by-fungicide treatment ($F_{1, 60} = 4.512$, $p < 0.04$).

Dependent Variable: logbiomass

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.186[a]	7	.312	1.986	.074
Intercept	26.896	1	26.896	171.064	.000
Nitrogen	.006	1	.006	.041	.840
Antibiotic	.015	1	.015	.098	.755
Fungicide	1.096	1	1.096	6.973	.011
Nitrogen * Antibiotic	.175	1	.175	1.112	.297
Nitrogen * Fungicide	.036	1	.036	.232	.632
Antibiotic * Fungicide	.709	1	.709	4.512	.038
Nitrogen * Antibiotic * Fungicide	.010	1	.010	.061	.806
Error	8.333	53	.157		
Total	39.058	61			
Corrected Total	10.519	60			

a. R Squared = .208 (Adjusted R Squared = .103)

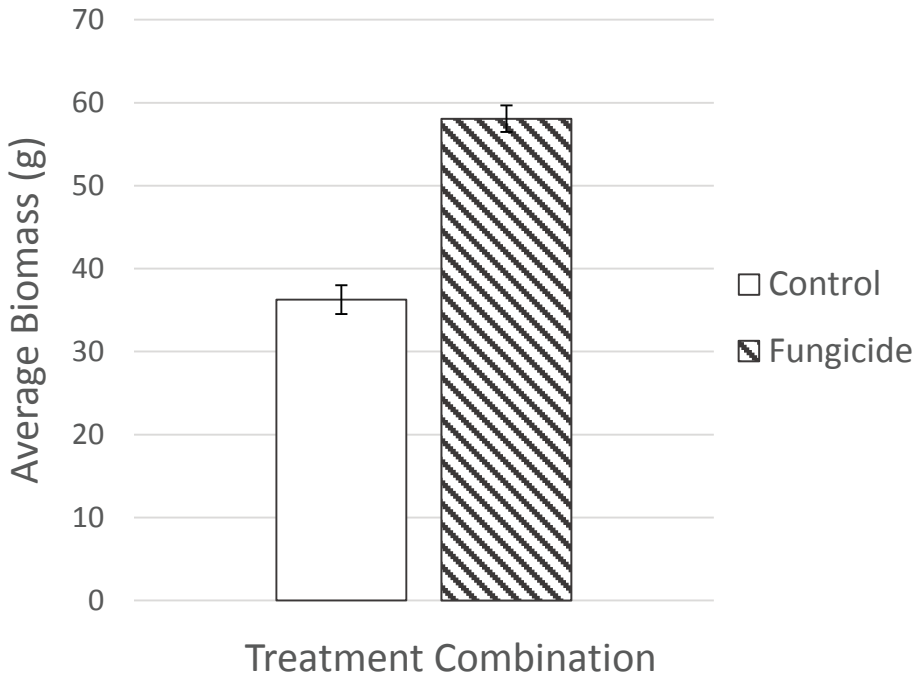

Figure 1. Average biomass of *S. glauca* comparing control and fungicide treatment groups. Control plants had an average biomass of 56.925g while fungicide treatment plants had an average biomass of 98.915g ($p<0.0001$). Standard error bars are shown.

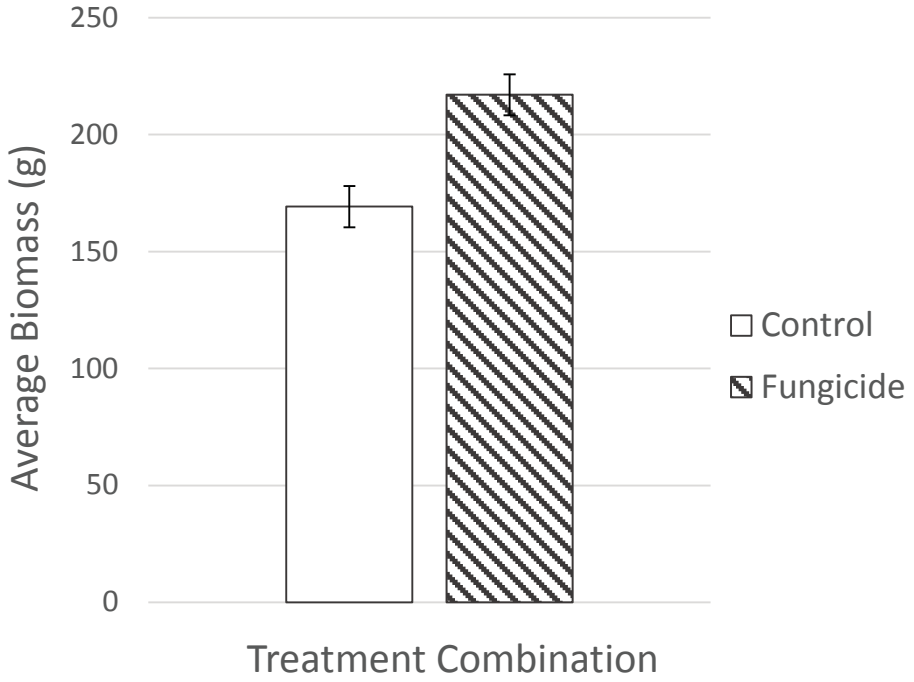

Figure 2. Average biomass of *A. artemisiifolia* comparing control and fungicide treatment groups. Control plants had an average biomass of 169.198g while fungicide treatment plants had an average biomass of 216.946g ($p<0.06$). Standard error bars are shown.

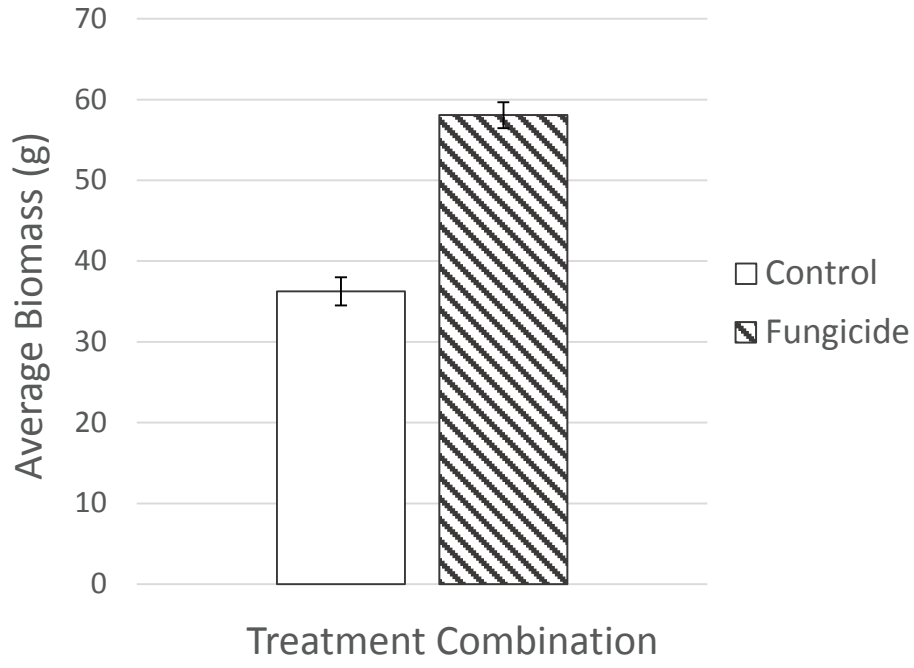

Figure 3. The average biomass of *A. virginica* comparing control and fungicide treatment groups. Control plants had an average biomass of 36.258g while fungicide treatment plants had an average biomass of 58.066g ($p<0.0001$). Standard error bars are shown.

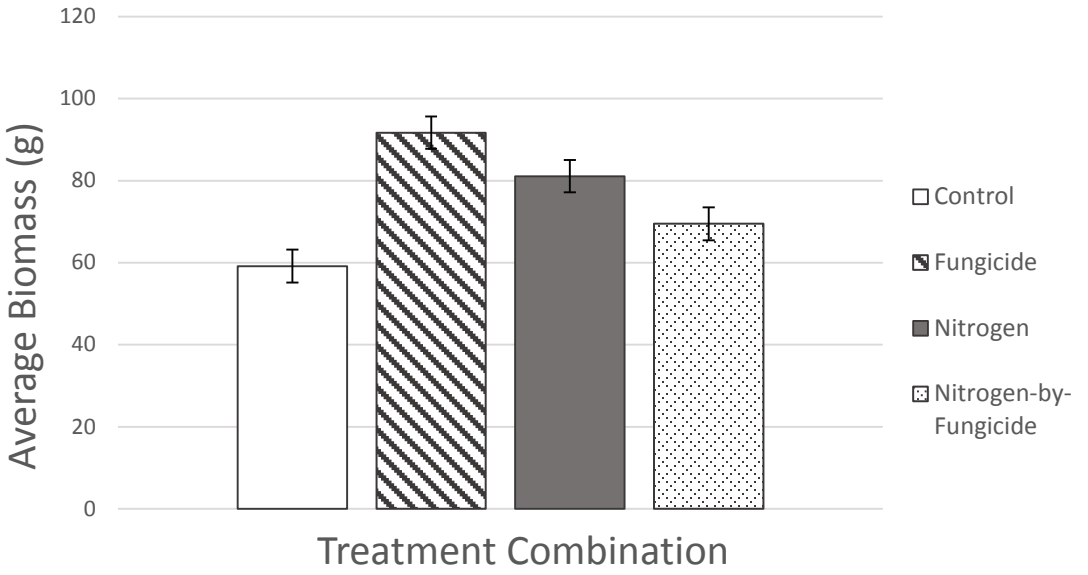

Figure 4. The average biomass of *P. capillare* comparing nitrogen-by-fungicide interactions ($p<0.008$). Control groups (no fungicide treatments) compared to fungicide treatments had a significant interaction. The average plant biomass for the control group was 59.917g while the fungicide treatment was 91.697g. Nitrogen-by-fungicide compared to nitrogen treatments did not have a significant interaction. The average plant biomass for the nitrogen-by-fungicide treatment was 69.487g while the nitrogen treatment was 81.089g. Standard error bars are shown.

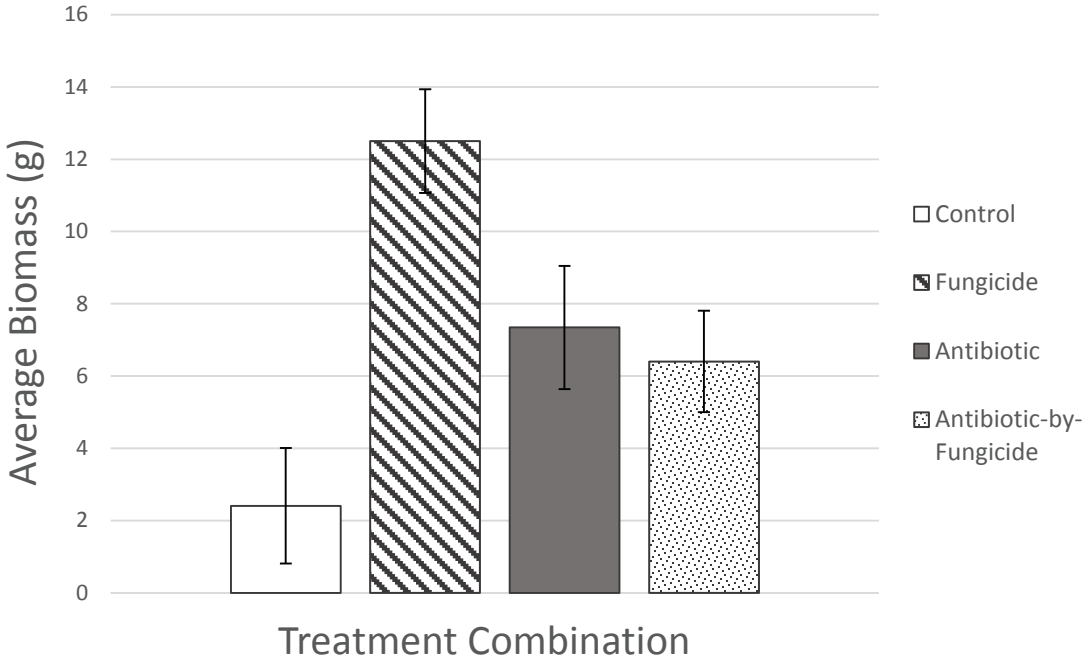

Figure 5. The average biomass of *B. vulgaris* comparing antibiotic-by-fungicide interactions ($p<0.038$). Control groups (no fungicide treatments)) compared to fungicide treatments had a significant interaction. The average plant biomass for the control group was 2.410g while the fungicide treatment was 12.498g. Antibiotic-by-fungicide compared to antibiotic treatments did not have a significant interaction. The average plant biomass for the antibiotic-by-fungicide treatment was 6.402g while the antibiotic treatment was 7.345g. Standard error bars are shown.

Discussion

It appears that fungicide application had a greater impact than antibiotic application on reducing microbe numbers on the plant leaves, regardless of whether nitrogen was added or not. The lowest colony numbers were seen in fungicide-only and fungicide-by-antibiotic treatments, with or without nitrogen. Surprisingly, antibiotic application only appeared to be effective in reducing microbial numbers in conjunction with the fungicide application, but not on its own or with added nitrogen. In fact, the nitrogen-by-antibiotic treatments actually had the greatest number of plates that produced confluent growth. It is possible that the antibiotic application could have caused a shift in the microbial community on plant surfaces due to foliar competition. The removal of some bacteria types that were competitively excluding other bacteria and fungi by the antibiotic could have allowed the non-affected organisms to proliferate in greater amounts due to greater resource availability (Redford and Fierer 2009, Remus-Emsermann et al. 2012).

While all the treatment combination containing fungicide efficiently reduced bacterial and fungal numbers on the nutrient agar plates, the biomass of the majority of the plant species were generally significantly affected by the fungicide treatment only. The average biomass of *S. glauca, A. artemisiifolia*, and *A. virginica* were significantly different when fungicide alone was added. The application of the fungicide could have removed many of the pathogenic fungi and allowed beneficial microorganisms that contain plant growth promoting factors or that fix nitrogen to reproduce in greater abundance, providing nutrients for the plants and enhancing their growth (Gu et al. 2010, Fu et al. 2016). Many fungi are known to cause plant diseases, and the susceptibility to

disease can be reduced by removing these organisms from plant surfaces (Rodriguez et al. 2009, Sylla et al. 2015), and *S. glauca, A. artemisiifolia*, and *A. virginica* benefited from likely pathogen removal. With removal of pathogenic microorganisms, the plant might be able to switch its resource use from defense to growth (Heil et al. 2000 and Traw et al. 2007).

Zhang et al. (2013) showed that nitrogen is important in increasing plant chlorophyll content, so it is likely that *P. capillare* benefited from the nitrogen applications. However, assessing the insignificant decrease in biomass of the nitrogen-by-fungicide treatment, it is possible that many beneficial fungi were eliminated, allowing other likely pathogenic organisms to quickly colonize and persist in the phyllosphere (Gu et al. 2010). These organisms could have taken advantage of greater nutrient availability and proliferated, causing detrimental effects on our focal plants, and thus resulting in decreased plant biomass that overshadowed any benefits of the nitrogen. However, nitrogen was added only once early in the season so the results in biomass might not depict the full potential of its effects on our focal plants and foliar microorganisms. Another possibility is that these plants were utilizing the available nitrogen for defense instead of extra growth (Heil et al. 2000 and Traw et al. 2007), preventing the plant from growing as much. The increase in biomass comparing the fungicide treatment to the control in *P. capillare* could be a result of proliferation of mutualistic bacteria and fungi on host plant surface that promoted plant growth once pathogenic fungi were removed (Hirano and Upper 2000, Fu et al. 2016). Similarly, *B. vulgaris* appeared to have greater biomass with only the fungicide applications compared to control treatments. The insignificant decrease in biomass of *B. vulgaris* in the

antibiotic-by-fungicide treatment compared to the antibiotic-only treatment could be caused by a detrimental chemical interaction of both antibiotic and fungicide on the plant, and/or by the persistence of resistant pathogenic fungi and bacteria preventing plant growth (Gu et al. 2010). The increase in *B. vulgaris* biomass with fungicide application compared to control treatment, similarly to the previous focal plants, can be a result of removing pathogenic fungi that prevented the proliferation of mutualistic bacteria (Hirano and Upper 2000, and Fu et al. 2016). It is also possible that once pathogenic organisms were removed, *B. vulgaris* could allocate its resources on growth rather than defense (Heil et al. 2000 and Traw et al. 2007).

There was no evidence supporting the "enemy release hypothesis" that proposes that non-native plant success is attributed to lack of enemies in their newly colonized ecosystem. The exotic plants did not have the assumed advantage over natives as predicted when it came to defense against pathogens; both native and exotic plants benefited from fungicide application in relation to plant growth, especially the exotic *B. vulgaris* with the greatest increase in biomass out of all the plant species. It is likely these exotic plants have already coevolved with our old-field system's pathogens and they no longer have advantage over native species in the same area (Mlynarek et al. 2015). Williamson and Fitter (1996) suggested that some invasive species hit a "bust" phase that results in a decrease in invasiveness over time while allowing the persistence of the organism in the new area possibly due to pathogen evolution having detrimental effects on plant fitness and growth. *S. glauca* has been in North America since the 1950s (Peters et al. 1963), *B. vulgaris* since the 1800s (MacDonald and Cavers 1990), and *P.*

lanceolata since the 1860's (Cavers et al. 1980), and 60 years is enough time for bacteria and fungi to evolve to prey on our focal plants (Inderjit 2015, Inderjit and Cahill 2015).

Conclusion

There was no evidence to support the success of exotic species via the enemy release hypothesis on the exotic species compared to the native ones. Neither the exotic species nor the native species in our study appeared to be hindered or profited from microorganism removal more than the other when it came to plant biomass. It is important, however, to note that the three exotic species have inhabited North America for over 60 years (some over 100 years). Decades can be enough time for plants and microorganisms to coevolve (into mutualistic or predator-prey relationships) and not affect each other as they would have if the exotic plants were recently introduced.

This study exemplifies that plant fitness and pesticide interactions appear to vary between species and can be very complex, involving host specificity and soil resource rates as seen in Griffin et al. (2016). If foliar microbes have the ability to affect plant fitness, they may be more involved in affecting plant community composition than previously believed. It is plausible that a combination of competitive exclusion between bacteria and fungi as well as the plants' ability to switch resources between defense mechanisms and growth could have caused the observable effects. We suggest quantification of fungal and bacterial species, via leaf microbial DNA extraction and qPCR or next-generation sequencing to provide information on the foliar inhabitants that are likely responsible for the changes in plant biomass. Nonetheless, we provide a mechanism by which phyllosphere communities affect temperate plant community structure, affecting their competitive abilities, and ultimately, their distributions.

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