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Invasive Species Shift Fungal Driven Decomposition in Midwestern Forests

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INVASIVE SPECIES SHIFT FUNGAL DRIVEN DECOMPOSITION IN MIDWESTERN FORESTS

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requirements for the degree of
Master of Science

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Adam M. Reed ENTITLED Invasive species shift fungal driven decomposition in Midwestern forests BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Reed, Adam M. M.S., Department of Biological Sciences, Wright State University, 2020. Invasive species shift fungal driven decomposition in Midwestern forests

Midwestern forests are currently impacted by two prominent invaders, *Agrilus planipennis* and *Lonicera maackii*. The *Ag. planipennis* induced loss of *Fraxinus* spp. trees can facilitate *Lo. maackii* invasion, which is likely altering microbial driven forest nutrient cycling. To assess these changes in microbial processes, I conducted litter bag and culture-based decomposition experiments using leaf litter from *Acer* spp., *Quercus* spp., *F. nigra*, *F. pennsylvanica*, *Lindera benzoin*, and *Lo. maackii*. For the culture-based decomposition experiment, I inoculated six species of fungi separately onto both single species and multispecies (half *Lo. maackii* and half native spp.) leaf litter and measured decomposition rate, fungal growth and enzymatic activity. Both *Lo. maackii* and multispecies leaf litter had faster decomposition, increased fungal growth, and higher carbon degrading enzyme activities than native species leaf litter. Thus, forests affected by this dual invasion will have faster decomposition, potentially resulting in an influx of nutrients for nutrient cycling.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

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INTRODUCTION

Forests throughout the world have undergone several major changes that affect entire ecosystems and will have lasting impacts on their overall health. These changes include dominant species loss, habitat destruction and exotic species introductions and invasions, each of which greatly alter ecosystem functioning (Tilman et al., 1994; Pyšek and Richardson, 2010). In the Midwestern and Eastern United States, forests are greatly impacted by the introduction of the insect, *Agrilus planipennis* (emerald ash borer), and subsequent loss of *Fraxinus* spp. (ash) trees as well as the introduction of the invasive shrub *Lonicera maackii* (Amur honeysuckle). While the independent roles these two events play in structuring plant communities are widely studied (i.e., Collier et al., 2002; Gould, 2000; Hartman and McCarthy, 2008; Schradin and Cipollini, 2012), the interactive effects of these events on nutrient cycling and microbial processes are largely unexplored. By losing a prominent native tree species and gaining an invasive shrub species, the nutrient cycling within these forests is likely altered due to the influx of different plant material.

Background

Originally from Southeast Asia, *Ag. planipennis* is an invasive pest with no natural predators in North America (Muirhead et al., 2006; Herms and McCullough, 2014). Since its discovery in 2002 in southeast Michigan, *Ag. planipennis* has spread to 24 states within the United States as well as two neighboring Canadian provinces (Nisbet et al., 2015). With mortality rates near 100% in the most infected areas, damages from

Ag. planipennis infestation are estimated to cost over $26 billion (Klooster et al., 2014; Sydnor et al., 2011). Consequently, *Ag. planipennis* induced *Fraxinus* spp. tree mortality has the potential to change ecosystem function and species dynamics in many Midwestern forests (Lovett et al., 2006).

Extreme losses of prominent plant species like *Fraxinus* trees from forests is likely to impact key functions in forest ecosystems like nutrient cycling, which encompasses the movement of inorganic matter into organic matter to facilitate new growth. *Fraxinus* spp. leaf litter has higher amounts of nitrogen (N), magnesium and calcium leading to faster decomposition and a higher turnover rate in the litter than both *F. excelsior* (European ash) and *Tilia* spp. (lime; Langenbruch et al., 2012). Thus, decomposition of *Fraxinus* spp. leaf litter leads to a lower carbon to nitrogen (C:N) in the soil than other native species including *Quercus rubra* (red oak), *Acer saccharum* (sugar maple), *Ac. rubrum* (red maple), *Fagus grandifolia* (American beech) and *Tsuga canadensis* (eastern hemlock; Finzi et al., 1998). This change in soil nutrients reflects interspecific differences in the quality of leaf litter which drive differences in the decay rates of leaves. Therefore, losing *Fraxinus* spp. in forest ecosystems can potentially lead to a large loss of nutrients from forest ecosystems.

The loss of *Fraxinus* spp. from forest ecosystems is also likely to cause increased turnover in plant species composition due to gap formation in the overstory and understory (Klooster et al., 2018). Gaps in the canopy resulting from increased *Fraxinus* spp. mortality will lead to higher light availability in the understory, which could lead to an increase in shrubs and saplings with high light tolerance (Dolan and Kilgore, 2018). While replacement of *Fraxinus* spp. by other native species can potentially happen

(Smith et al., 2015), replacement by an invasive species such as *Lo. maackii* or *Elaegnus umbellata* (autumn olive) is more likely since such species are better adapted to higher light availability than native species (Hoven et al., 2017; McNeish and McEwan, 2016). Indeed, where *Fraxinus* spp. were removed from the overstory of an infected forest, 18% of cover from where the trees were removed were invasive (Hoven et al., 2017). Further, the control strategies for *Ag. planipennis* must now consider the removal of *Lo. maackii* from the understory to ensure this invasive shrub does not become the dominant understory species and further alter forest functioning (Woods et al., 2019).

Lo. maackii is an invasive shrub present throughout the understory of Midwestern forests, causing significant changes to ecosystems. Since its introduction in 1896 in southwest Ohio, *Lo. maackii* has spread to over half of North America (Hutchinson and Vankat, 1997). This invasion disrupts ecosystems in a variety of ways. When *Lo. maackii* is present in terrestrial ecosystems, overall plant species richness declined by 53% (Collier et al., 2002). *Lo. maackii* invasion is an ongoing problem and cause for major concern as it takes multiple treatments to remove it fully from an affected area (Hartman and McCarthy, 2004). Costs due to damages and attempts to control invasive species are estimated at $120 billion per year in the US (Pimentel et al., 2005).

The preponderance of *Lo. maackii* in forest ecosystems is also altering nutrient cycling in these systems. Invasive plant species differ in multiple ways from native species - including their biomass, productivity, tissue chemistry, plant morphology and phenology - all of which alter the availability of soil nutrients (Ehrenfeld, 2003; Vitousek, 1987; Cox, 1999). *Lo. maackii* has a higher N content and decomposes faster than native species, thus, potentially driving an increase of N in invaded forests (Arthur et

al., 2012). There is also evidence that *Lo. maackii* may increase decay rates of native species and alter nutrient cycling in forest ecosystems, possibly due to excess amounts of N present (Blair and Stowaser, 2009). Invasive plant species can deplete native plant species growth by limiting space and resources, such as light, water and nutrient availability (Arthur et al., 2012; Lieurance and Cipollini, 2013). The combination of increased decomposition rates, reduction in native vegetative growth and introduction of new chemical components from *Lo. maackii* could have a major impact on nutrient cycling in affected forests.

Across ecosystems, nutrient cycling is a major process that begins with decomposition. Decomposition is the process of breaking down complex organic carbon compounds (primarily lignin during plant decay) into its simpler organic or inorganic material. This process makes nutrients readily available for surrounding organisms and drives carbon cycling in many ecosystems (Aerts, 1997; Pan et al., 2011). In forests, both dead woody debris and leaf litter represent important substrates for decomposition, however, litter decomposition is a faster process. Litter from individual plant species has unique chemical properties that render it either labile or recalcitrant during decay, which drives plant species decay rates. Labile leaves are "higher quality" leaves due to higher N content and decompose quicker compared to recalcitrant leaves, which are "lower quality" leaves due to higher C content (Cotrufo et al., 2012). One way to assess recalcitrant vs. labile leaf material is through the concentration of lignin, a polymer for cellular wall structure and cellulose, which is a primary, recalcitrant component of plant cell walls (Kirk, 1987). Leaves are more labile when they have a lower lignin:N, which leads to a faster decomposition rate (Melillo et al., 1992; Bhatnagar et al., 2018). In

general, leaf litter from invasive plant species has a quicker decay rate compared to native species due to a combination of having both higher N content and a lower amount of lignin (Table 1; Arthur et al., 2014; Ehrenfeld, 2003; Ashton et al., 2005). As a result, the loss of a native plant species and the gain of an invasive plant species can lead to drastic changes in nutrient cycling due to changes in litter composition.

Table 1. Known leaf properties and decay rates of focal plant species

Species	**Common Name**	**Labile or Recalcitrant**	**Decay Rate *k* (g/year)**	**Location**	**Citation**
Acer spp.	Maple	Labile	1.77	Field	Stoler et al., 2017 Jo et al., 2016
Quercus spp.	Oak	Recalcitrant	0.06	Field	Kominoski et al., 2007 Blair and Stowasser, 2009
Fraxinus nigra	Black Ash	Labile	5.475	Field	Nisbet et al., 2015 Swan et al., 2009
Fraxinus pennsylvanica	Green Ash	Labile	3.796	Laboratory	Nisbet et al., 2015 Peterson and Cummins, 1974
Lindera benzoin	Spicebush	Unknown*	0.7	Field	Jo et al., 2016
Lonicera maackii	Honeysuckle	Labile	6.61	Field	Arthur et al., 2012

*Likely recalcitrant based off the decomposition rate

Decomposition of Native and Invasive Species

Leaf litter decomposition rates are dependent on leaf chemistry and invasive species typically have faster decomposition than natives (Table 1). *Lo. maackii* should decompose the quickest due to having a low C:N compared to native trees, and *Quercus* spp. should have a slower decomposition rate than *Acer* spp. or *Fraxinus* spp. (Howard and Howard, 1974; Madritch and Cardinale, 2007). Since leaf litter often consists of mixtures of several plant species, decomposition dynamics of different leaf species combinations are not easily predictable based off single species decomposition patterns (Gartner and Cardon, 2004). When native species are combined with invasive species, decomposition rates of native species are usually accelerated (Ashton et al., 2005; Arthur et al., 2012), but labile litter decomposition can be slower when mixed with native species litter (Grossman et al., 2020). Since *Lo. maackii* litter is highly labile, mixed species litter combining native and invasive leaves should have a faster decomposition rate than single species litter.

Fungal Roles in Decomposition

To fully understand changes to decomposition driven by changes in forest species composition, the function of fungi in nutrient cycling must be examined. Saprobes and mycorrhizal fungi are key players for nutrient cycling in forest ecosystems. Saprotrophic fungi, including soft rot, white rot and brown rot fungi, are free-living fungi that solely decompose dead plant matter such as branches, fallen trees and leaves (Riley et al., 2014). On the other hand, ectomycorrhizal (ECM) fungi form nutritional symbioses with plant hosts, exchanging immobile soil nutrients for carbon from the plant host, but can act as saprobes and colonize decaying plant material (Bödeker et al., 2009). These guilds

exhibit niche partitioning with specialized roles in decomposition: white rot fungi degrade lignin and hemicellulose (Worrall et al., 1997; Baldrian, 2009); brown rot fungi primarily degrade cellulose, but also degrade some lignin and labile litter faster than white rot (Worrall et al., 1997; Baldrian, 2009); and ECM fungi degrade lignin (Bödeker et al., 2016). Since decomposition is the main function of saprobes, they are better suited for decomposing organic material and will likely decompose organic material faster than ECM fungi.

Decomposition is mainly driven by microorganisms whose community composition and function can be altered by invasive plant species (Vitousek et al., 1997). Early work suggests that *Lo. maackii* has a unique community of microbes associated with its leaves that are not present on native *Fraxinus* spp. or *Carya* spp. (hickory) leaves in Midwestern forests (Arthur et al., 2012). This change in the microbial community may alter microbially driven nutrient cycling by changing the decomposers of plant litter, potentially explaining why invasive species decompose faster and lead to an abundance of soil nutrients (Van der Putten et al., 2007). The primary way microorganisms function is through the secretion of extracellular enzymes. Enzyme activities scale with target nutrient availability, leading to greater rates of decomposition with excess nutrients (Sinsabaugh and Moorhead, 1994). This trend occurs when microbial decomposers allocate more enzymatic activity towards C breakdown (Allison and Vitousek, 2004). Since *Lo. maackii* has higher amounts of N compared to native species in Midwestern forests, microorganisms that target N may increase decomposition rates in the presence of *Lo. maackii*. Consequently, changes in plant composition will drive changes in available

leaf litter, potentially altering fungal community composition, function, and changing nutrient availability in invaded ecosystems.

Enzymatic Decomposition

Decomposing fungi release extracellular enzymes to chemically break down organic matter into usable inorganic matter. Each class of fungi has a specialized set of enzymes that allow it to break down different components of organic matter (Hankin and Anagnostakis, 1975). For example, saprobes have higher activity for enzymes associated with C and phosphorus (P) acquisition (Talbot et al., 2015), while mycorrhizal fungi typically have higher activity for enzymes associated with N acquisition due to obtaining their C from the host plant (Burke et al., 2011). A few of the enzymes that target C breakdown include β-glucosidase (which hydrolyzes cellobiose into glucose), cellobiohydrolase (which hydrolyzes cellobiose), polyphenol oxidase (which oxidizes phenols), and peroxidase (which degrades lignin with oxidases; Talbot et al., 2015; Yin and Koide, 2019). Some of the enzymes that target N and P breakdown include leucine aminopeptidase (which breaks down polypeptides), and acid phosphatase (which releases inorganic phosphate from organic matter; Talbot et al., 2015). ECM fungi can act as saprobes and have similar, if not greater peroxidase activity than saprotrophic fungi (Bödeker et al., 2009). Therefore, ECM fungi should have higher peroxidase and leucine aminopeptidase activity and break down labile litter faster while saprobes may have higher activities of acid phosphatase, β-glucosidase, cellobiohydrolase and polyphenol oxidase to break down both labile and recalcitrant litter. White rot fungi break down more recalcitrant litter faster than brown rot fungi, so they have a higher polyphenol oxidase activity than brown rot fungi (Kirk and Farrell, 1987). Finally, brown rot fungi

have higher β-glucosidase activity than white rot fungi since brown rot fungi breaks down labile litter faster than white rot fungi.

In addition to fungal characteristics, plant invasion can also alter enzyme activity levels related to decomposition. Plant species invasion can lead to increases in enzyme activity, most noticeably for N and P decomposing enzymes (Zhou and Staver, 2019) but can also contribute to an increase in C related enzymes such as peroxidase and polyphenol oxidase (Woods et al., 2019). This could be due to invasive species causing shifts in fungal communities (Yang et al., 2019; Lui et al., 2019). Understanding how fungi drive changes in available leaf litter enzymatic activities could further clarify how the changes in leaf litter affect nutrient cycling.

In this study, I focused on six woody species, which are representative of a typical Midwestern forest: *Acer* spp. (maple), *Quercus* spp. (oak), *F. nigra* (black ash), *F. pennsylvanica* (green ash), *Lindera benzoin* (spicebush) and *Lo. maackii*. These species vary in decomposition rate and leaf litter chemistry (Table 1; Jo et al., 2016; Blair and Stowasser, 2009; Peterson and Cummins, 1974; Swan et al., 2009; Poulette and Arthur, 2012; Arthur et al., 2012; Stoler et al., 2017; Kominoski et al., 2007; Nisbet et al., 2015). Each of these studies measured leaf litter decomposition rates in both single species and or in mixtures of multiple species, but no previous work has examined the microbial responses driving changes in decomposition rates. My thesis will fill this knowledge gap by investigating how *Lo. maackii* leaf litter addition changes fungal facilitated decomposition in forests that also have *Ag. planipennis* induced *Fraxinus* spp. loss.

Specific Research Aims:

I investigated the importance of nutrient cycling in a Midwestern forested ecosystem with dual invasions of *Ag. planipennis* and *Lo. maackii* through four specific aims:

1. Evaluate litter decomposition rates of *Acer* spp., *Quercus* spp., *F. nigra, F. pennsylvanica, Li. benzoin,* and *Lo. maackii*.
2. Determine differences in decomposition rates between field and lab-based approaches.
3. Identify differences in decomposition rates as a function of three fungal guilds: brown rot, white rot and ECM.
4. Delineate difference in fungal activity measuring six enzymatic activities: acid phosphatase, β-glucosidase, cellobiohydrolase, leucine aminopeptidase, polyphenol oxidase and peroxidase.

MATERIALS AND METHODS

Study Site

This study utilized material from an undisturbed section of the Wright State University (WSU) woods (primary woods, which are approximately 127 years old; DeMars and Runkle, 1992) in Dayton, Ohio (39.785253 °N, 84.05424 °W). The overstory of the woods consists primarily of *Quercus* spp. and *Acer* spp., but also contains other species such as *Carya* spp., and *Ulmus americana* (American elm; DeMars and Runkle, 1992). *F. americana* (white ash), *F. pennsylvanica* and *F. quadrangulata* (blue ash) used to be present in the woods but have been reduced due to *Ag. planipennis* and now there is primarily only *F. quadrangulata* with a few *F. americana* and *F. pennsylvanica* trees (Cipollini and Runkle, *personal communication*). Like other Midwestern forests, the understory has been invaded by and is now primarily composed of *Lo. maackii*, but *Li. benzoin* is also present in the shrub layer (Dorning and Cipollini, 2006).

Leaf Litter Collection

Leaves from *Acer* spp., *Quercus* spp., *Li. benzoin* and *Lo. maackii* were hand collected in the WSU woods from September-December 2017 and September-December 2018. During the same period, leaves from *F. nigra*, originating from Baileys Nursery in St. Paul, Minnesota, and *F. pennsylvanica*, originating from the WSU woods, were collected from greenhouse grown trees. These trees were kept in pots with commercial soil outside the WSU greenhouse to prevent *Ag. planipennis* infection. All leaves were

collected after abscission from September-November 2017 and September-November 2018. Following collection, leaf litter was dried in a drying oven at 80 °C for two days to obtain dry weight. To kill any microbes present, leaf litter was autoclaved for 20 minutes at 121 °C on a standard dry (wrapped) cycle twice within 24 hours. Litter was stored at room temperature until the start of the experiments, approximately seven days.

Litter Bag Decomposition

Experimental Set-up

To evaluate natural rates of litter decomposition, I performed a standard litter bag decomposition experiment. I placed 10 g of leaf litter from each of five focal species (*Acer* spp., *Quercus* spp., *F. nigra*, *Li. benzoin* and *Lo. maackii*) in individual litter bags constructed by folding 300 µm nylon mesh into 8 x 6 inches and stapling all four edges. Decomposition bags with mixed species were created by combining 5 g of native plant leaf litter from *Acer* spp.,

Quercus spp. or *Li. benzoin* and 5 g of *Lo. maackii* leaf litter. A total of 56 litter bags, seven replicates of each experimental unit, were haphazardly placed in primary forested areas of the WSU woods for 100 days (17 November 2018 - 25 February 2019). After 100 days, leaf litter was removed from litter bags and dried at 80 °C for two days to obtain dry weight to determine final leaf mass. The decomposition rate (k) was calculated in g/year by taking the natural log of the final weight of the leaf litter (W_f) divided by the initial weight of the leaf litter (W_o) (Equation 1; Olson, 1963).

$$k = -\frac{\ln\left(\frac{W_f}{W_o}\right)}{t}$$

Equation 1: Exponential decay model (Olson, 1963)

Culture Decomposition

To better understand the role fungi play in structuring changes in decomposition rate based on changes in leaf litter composition, I created a culture-based experiment with six species of fungi collected from the WSU woods (*Mycena galericulata, Amanita parcivolvata, Schizophyllum commune, Laetiporus sulphureus, Inocybe rimosa,* and *Marasmius rotula*; Table 2). Fungi were isolated and maintained on Modified Melkin-Norkans (MMN) agar for approximately 21 days prior to amplification. Streptomycin sulfate was added to the plates to prevent bacterial contamination. To obtain enough fungal biomass for the experiment, I first cultured 1 cm^3 plugs from isolated fungal cultures for each species on to twelve plates and incubated for four weeks at 22 °C. I then inoculated 1 cm^3 plugs from these plates onto the leaves for the culture decomposition experiment.

Experimental Set-up

To isolate the effect of fungal species and guild on leaf litter decomposition, I performed a culture-based decomposition experiment using different species of leaf litter and fungi. Cultures consisted of deep plate Petri-dishes (100 mm x 20 mm) filled with 30 ml MMN agar. Each plate received 1 g of a single leaf species or a 1 g combination of 0.5 g of *Lo. maackii* and 0.5 g of the native leaf species (*Acer* spp., *Quercus* spp. or *Li. benzoin*), which were hand crushed and homogenized before being placed on each plate. Each fungal species by leaf litter type was replicated 7 times for a total of 378 plates; however, some plates were discarded due to contamination resulting in a total of 369 plates (Table 2).

Table 2. Fungal guilds and associated fungal species. Dates included are for the initial collection of fungi in woods, date the fungal species was inoculated on leaf litter and the date the cultures were collected. Replicates represent total number of plates per fungal species.

Round (1 or 2)	**Fungal Guild**	**Fungal Species**	**Date Collected (Fungi)**	**Replicates (Single Species)**	**Replicates (Mixed Species)**	**Date Inoculated**	**Date Collected (Cultures)**
1	Brown rot	*Mycena galericulata*	14 August 2017	42	21	21 June 2018	30 September 2018
1	Ectomycorrhizal fungi	*Amanita parcivolvata*	5 July 2017	42	21	20 June 2018	29 September 2018
1	White rot	*Schizophyllum commune*	6 September 2017	42	21	21 June 2018	30 September 2018
2	Brown rot	*Laetiporus sulphureus*	15 June 2017	38	17	2 April 2019	11 July 2019
2	Ectomycorrhizal fungi	*Inocybe rimosa*	20 September 2017	42	21	15 March 2019	23 June 2019
2	White rot	*Marasmius rotula*	13 September 2017	41	21	18 March 2019	26 June 2019

Each plate was inoculated with a single species of fungi. Four 1 cm^3 plugs of fungi were removed from amplified four-week-old cultures by a sterilized scalpel blade and placed on top of leaves. After the 100-day incubation, the fungal material was removed from the leaves and placed back into the agar to measure fungal biomass as described below. The remaining leaf litter was collected and weighed to determine rate of decomposition (Equation 1). Approximately 0.25 g of remaining litter was stored at -20 °C until ready for enzyme assays. The experiment was performed twice with three different species of fungi in each round (Table 2).

Fungal Hyphae

To determine the growth rate of fungi in culture, fungal hyphal length was measured with a caliper twice a week from the edge of the initial fungal plug to the end of the hyphae until the hyphae reached the edge of the plate. Hyphal growth rate was calculated in mm/day by taking the natural log of the difference between initial hyphal length (H_i) and final hyphal length (H_f) over the time maximum hyphal length was reached (t; Equation 2).

$$\text{Hyphal growth rate} = \frac{\ln(Hf - Hi)}{t}$$

Equation 2: Hyphal Growth Rate

Fungal Biomass

To assess total fungal biomass, both the agar and the fungal material that was removed from the leaf litter were melted in a beaker in an autoclave following a procedure outlined by Maynard et al. (2017). Cultures were autoclaved for 20 minutes at 121 °C to separate the fungal material from the agar, then poured through a 45 µm sieve

to isolate the fungal material from the agar. Fungal material was further separated from agar by rinsing with ~100 mL of 90 °C DI water. The remaining fungi was placed in a drying oven at 65 °C for 12-24 hours until dry and weighed to determine fungal biomass in mg.

Enzyme Activities

To determine differences in fungal function between leaf species and translate these differences into potential impacts on nutrient cycling, I tested six enzymatic activities commonly in decomposition. I measured the enzyme activities of acid phosphatase, β-glucosidase, cellobiohydrolase, leucine aminopeptidase, peroxidase and polyphenol oxidase following the procedures outlined in Woods et al. (2019). I selected incubation times by testing for maximum potential of each enzymatic activity for leaf litter. Each enzyme assay was conducted using homogenous leaf slurries made with 13 mg of leaf and 4 ml of 50 mM sodium acetate buffer at a pH of 5.6 and incubated in the dark at 4 °C. For the fluorometric enzymes acid phosphatase, β-glucosidase, cellobiohydrolase, and leucine amino peptidase, I measured assay absorbance and fluorescence values using a BioTex Synergy HT microplate reader (BioTek, Winookski, Vt, USA). For the colorimetric enzymes, polyphenol oxidase and peroxidase, I measured assay absorbance using a Molecular Devices Corporation SpectraMax 190 microplate reader (Molecular Devices Corporation, Sunnyvale, Ca, USA).

Fluorometric Enzyme Analysis

I measured the enzyme reactions of acid phosphatase, β-glucosidase, cellobiohydrolase, and leucine aminopeptidase fluorometrically, or by the intensity of fluorescence. Each assay included three replicates of a substrate blank, buffer blank, leaf

homogenate blank, and leaf homogenate reaction as well as a standard curve of methylumbelliferyl (MUB) for acid phosphatase, β-glucosidase and cellobiohydrolase. Acid phosphatase was assayed using 50 µl of 250 µM MUB-phosphate and incubated in the dark at 22 ℃ for 30 min. β-glucosidase was assayed using 50 µl 100 µM MUB β-D-glycopyranoside and incubated in the dark at 22 ℃ for 1 hour. Cellobiohydrolase was assayed using 50 µl 100 µM MUB β-D-Cellobioside and was incubated in the dark at 22 ℃ for 4 hours. Leucine aminopeptidase was assayed using 50 µl 100 µM L-Leucine-7-amido-4-methylcoumarin hydrochloride with a standard curve of methylcoumarin and was incubated in the dark at 22 ℃ for at 4 days. Following incubation, 10 µl of 1M NaOH was added to each well and plates were read at 360 nm excitation and 450 nm emission 10 minutes after NaOH addition using a BioTex Synergy HT microplate reader. Using the standard curve, excitation coefficients and fluorescent measurements, enzyme activities were calculated in µmol h^{-1} g^{-1} by taking net fluorescence units (NFU = assay fluorescence – homogenate blank – substrate blank) divided by the product of extinction coefficient (Ɛ-the slope of the linear regression of MUB concentration by MUB fluorescence from the standard curve) divided by 0.25 ml (assay volume), then divided by the product of incubation time (*t*) * (1/ dilution factor (*DF*= volume of buffer (mL) / mass of dry leaf litter) * 0.2 mL (homogenate volume); Equation 3).

$$\text{Fluorescent Activity} = \frac{\left(\frac{\text{NFU}}{\frac{\text{Ɛ}}{0.25}\,\text{mL}}\right)}{\left(t * \left(\frac{1}{DF}\right) * 0.2\ \ mL\right)}$$

Equation 3: Fluorometric Enzyme Activity

Colorimetric Enzyme Analysis

I measured polyphenol oxidase and peroxidase activities colorimetrically, or by the intensity of color. Optimal incubation time varied between 4-10 days for polyphenol oxidase, at 22 ℃ in the dark, and was measured once every 24 hours for peak activity. Optimal incubation time varied between 3-8 days for peroxidase, at 22 ℃ in the dark, and was measured every 4 hours for peak activity. Polyphenol oxidase was conducted using 50 µl of a 25 µM 3,4-Dihydroxyphenylalanine (L-DOPA), and peroxidase assays had both 50 µl of a 25 µM L-DOPA and 10 µl of 3% hydrogen peroxide. Both assays included three replicates of each of the following for each sample: substrate blank, buffer blank, leaf homogenate blank, and leaf homogenate with substrate. All assays were measured at 450 nm using a Molecular Devices Corporation SpectraMax 190 microplate reader (Molecular Devices Corporation, Sunnyvale, Ca, USA) and enzyme activities were calculated in µmol $h^{-1}g^{-1}$ by taking the product of net absorbance units (NAU=assay absorbance – homogenate blank – substrate blank) * buffer volume (*bv* = buffer added to dried leaf litter) divided by the product of extinction coefficient (Ɛ) (2.4942 for polyphenol oxidase, 1.8446 for peroxidase) * homogenate volume (*hv* = 0.2 mL * incubation time (*t)* * litter dry weight (*w;* Equation 4).

$$\text{Fluorescent Activity} = \frac{\text{NAU (abs)} * bv}{(\text{Ɛ}\left(\frac{\text{abs}}{\mu\text{mol}}\right) * hv * t * w}$$

Equation 4: Colorimetric Enzyme Activities

Data Analysis

All statistical analyses were performed in the statistical programming environment R version 3.6.2 (R Core Team, 2020). I tested all models for both single

species litter and multispecies litter combinations. For the culture experiment, I repeated all models using fungal species instead of fungal guild to understand how decay rate differs between fungal species within the same guild. No models included fungal species and guild in the same model. In cases where fungal guild was included as a predictor variable, I used linear mixed effects models with the function *lme* from the package *nlme* (Pinheiro et al., 2019) with a random effect to account for experimental round. Models with fungal species as a predictor variable were performed using the function *lm* from the *stats* package because there were different species between rounds 1 and 2 (R Core Team, 2020). All results were visualized using *ggplot2* (Wickham, 2016) unless otherwise noted. Significant interactions for all models were tested with ANOVA and post hoc analysis using the *emmeans* package with adjustment for Tukey HSD (Lenth, 2019).

To understand how decay rates differ between plant species for the litter bag experiment, decay rate was tested as a function of plant species using linear models. This test was repeated to examine differences in decay rates for laboratory and field methods using a linear model for decay rate as a function of plant species with an interaction for experimental condition (lab vs. field). I tested for significant interactions between lab and field decay rates for each plant species using the ANOVA.

In the culture experiment, decay rate was assessed for differences using various response variables. I tested decay rate as a function of either hyphal growth rate, fungal biomass or enzyme activity with interactions for both fungal guild and plant species using a linear mixed effects model from the package *nlme* (Pinheiro et al., 2019) with a random effect for experimental round. To examine differences in fungal traits, fungal biomass

and hyphal growth rate were separately tested as a function of fungal species and interactively with plant species using linear models.

To determine if the six enzyme activities responded similarly based on fungal guild, single species plant litter, or multispecies plant litter, I performed a principal component analysis (PCA) using the *prcomp* function from the *stats* package using enzyme activity values standardized to have a mean of 0 and a variance of 1 (R Core Team, 2020). I ran seperate PCAs for each round to detect differences in round. I broke enzyme activities into principal components and visualized them using the function *fviz_pca_biplot* from the *factoextra* package (Kassambara and Mundt 2019). To evaluate if any grouping in space was due to predictor variables, I performed an analysis of similarity with the *ANOSIM* function with 999 permutations from the *vegan* package (Oksanen et al., 2019).

RESULTS

Litter Bag Decay Rates

Plant species identity predicted litter decay rate ($F_{4,30} = 83.51$, $P < 0.0001$, Figure 1) and each multispecies mixture had decay rates between *Lo. maackii* and its associated native species ($P < 0.0001$, Figure 2). *Lo. maackii* leaf litter decayed ~100% faster than *Li. benzoin* and *F. nigra*, ~200% faster than *Acer* spp. and ~250% faster than *Quercus* spp (Figure 1). Additionally, *Lo. maackii + Li. benzoin* decayed ~43% faster than *Li. benzoin* ($F_{2,18} = 19.82$, $P < 0.0001$, Figure 2A), *Lo. maackii + Acer* spp. decayed ~90% faster than *Acer* spp. ($F_{2,18} = 62.57$, $P < 0.0001$, Figure 2B) and *Lo. maackii + Quercus* spp. decayed ~90% faster than *Quercus* spp. ($F_{2,18} = 63.41$, $P < 0.0001$, Figure 2C).

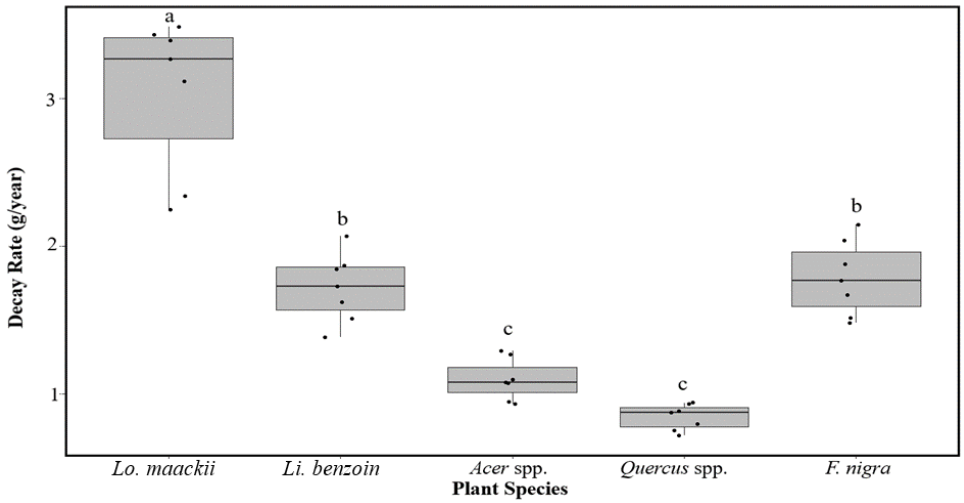

Figure 1. Plant species identity drives decay rates in single species litter bags ($P < 0.0001$). *Lo. maackii* litter decayed faster than each native species litter. Letters indicate significant differences based on the ANOVA.

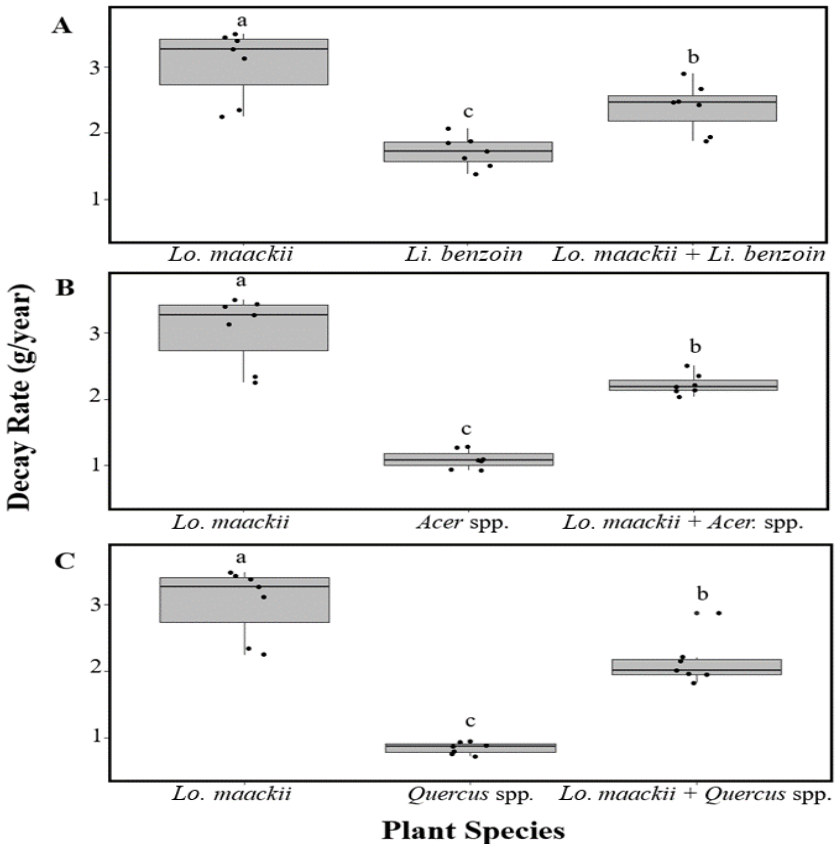

Figure 2. Plant species identity drives decay rates in multispecies litter bag mixtures for A) *Li. benzoin* ($P < 0.0001$), B) *Acer* spp. ($P < 0.0001$) and C) *Quercus* spp. ($P < 0.0001$). Decay rate was highest for *Lo. maackii*, lowest for the three native species (*Li. benzoin* *Acer* spp, and *Quercus* spp.) and intermediate for the multispecies litter bags. Letters indicate significant differences based on the ANOVA.

Lab vs. Field Decay Rates

Litter decay rates were different between the lab and field for most plant species combinations ($F_{7,367} = 4.936$, $P < 0.0001$, Figure 3). Decay rates on average were higher for the field than in the lab across all single species litter: *Lo. maackii* litter decayed ~70% faster; *Li. benzoin* litter decayed ~85% faster; and *F. nigra* litter decayed ~ 95% faster (Figure 3). Decay rates for *Acer spp.* and *Quercus* spp. did not differ across lab and field experiments. Multispecies litter also decayed quicker in the field than in the lab: *Lo. maackii* + *Li. benzoin* litter decayed ~100% quicker; *Lo. maackii* + *Acer* spp. litter

decayed ~90% faster; and *Lo. maackii* + *Quercus* spp. litter decayed ~ 65% faster (Figure 3).

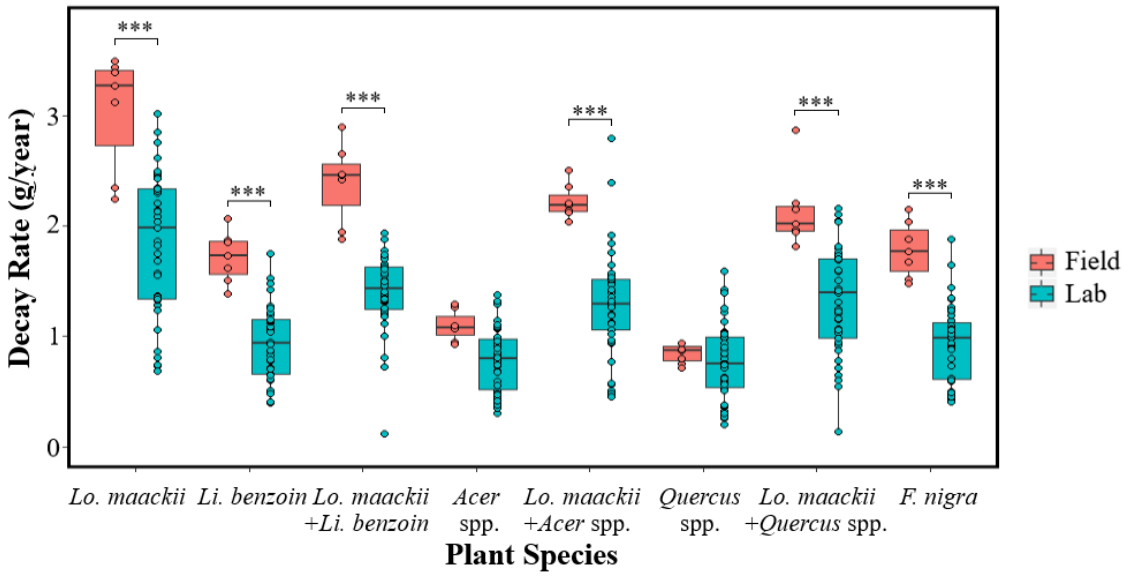

Figure 3. Experiment type (field vs lab) drives decay rates. Decay rate was higher in the field than the lab for all litter species ($P < 0.0001$) except for *Acer* spp. and *Quercus* spp. litter. Colored boxes represent experiment type: field (pink), lab (blue). *** denotes $P < 0.001$ ** denotes $P < 0.01$, * denotes $P < 0.05$ based on the ANOVA.

Culture Litter Decay Rates

Decay Rate by Hyphal Growth Rate

Hyphal growth rate, fungal guild and plant species identity explained leaf litter decay rates in single species litter ($F_{10,209} = 7.744$, $P < 0.0001$, Figure 4). Litter decayed slower with increased hyphal growth rate for *Lo. maackii*, *F. pennsylvanica*, *Quercus* spp. and *F. nigra*, but decay rates increased with an increase in hyphal growth rate for *Li. benzoin* and *Acer* spp. litter. For both brown rot and white rot fungi, decay rates increased with increased hyphal growth rate, but decay rates for ECM fungi remained constant regardless of hyphal growth rate (Figure 4).

Each multispecies culture followed similar trends to *Lo. maackii* litter where decay rates decreased with increasing hyphal growth rate: *Lo. maackii* + *Li. benzoin*

($F_{4,103}$ = 11.55, P <0.0001, Figure 5A), *Lo. maackii* + *Acer* spp. ($F_{4,105}$ = 8.536, P < 0.0001, Figure 5B) and *Lo. maackii* + *Quercus* spp. ($F_{4,105}$ = 4.224, P = 0.0033, Figure 5C). For multispecies cultures with brown rot fungi, decay rates increased with increasing hyphal growth rates for *Lo. maackii* + *Li. benzoin* but decreased with *Lo. maackii* + *Acer* spp. and *Lo. maackii* + *Quercus* spp. compared to the respective single species litter (Figure 5). For all multispecies cultures with white rot fungi, decay rates increased with increased hyphal growth rates. For multispecies cultures with ECM fungi, decay rates decreased with increasing hyphal growth rates for *Lo. maackii* + *Li. benzoin* and *Lo. maackii* + *Acer* spp. but increased with increasing hyphal growth rates for *Lo. maackii* + *Quercus* spp. compared to native single species (Figure 5).

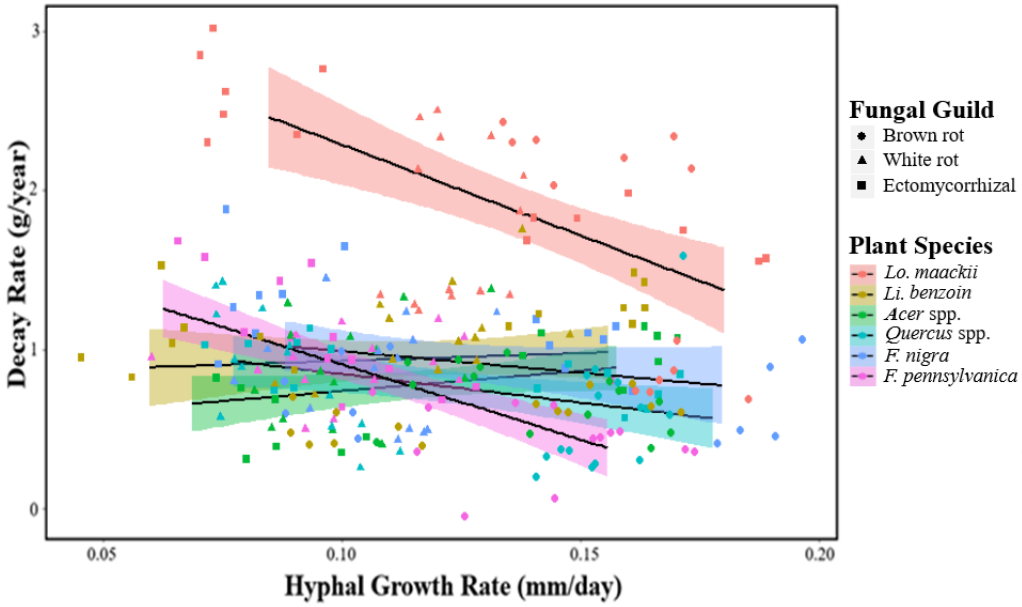

Figure 4. Hyphal growth rate, plant species identity and fungal guild drive litter decay rate in single species cultures (P < 0.0001). All plant species had decreased decay with increasing hyphal growth rate except for *Li. benzoin* and *Acer* spp. Shaded regions represent 95% confidence intervals for *Lo. maackii* (pink), *Li. benzoin* (brown), *Acer* spp. (green), *Quercus* spp. (light blue), *F. nigra* (dark blue), and *F. pennsylvanica* (purple) while point shapes represents brown rot fungi (circles), white rot fungi (triangles) and ECM fungi (squares).

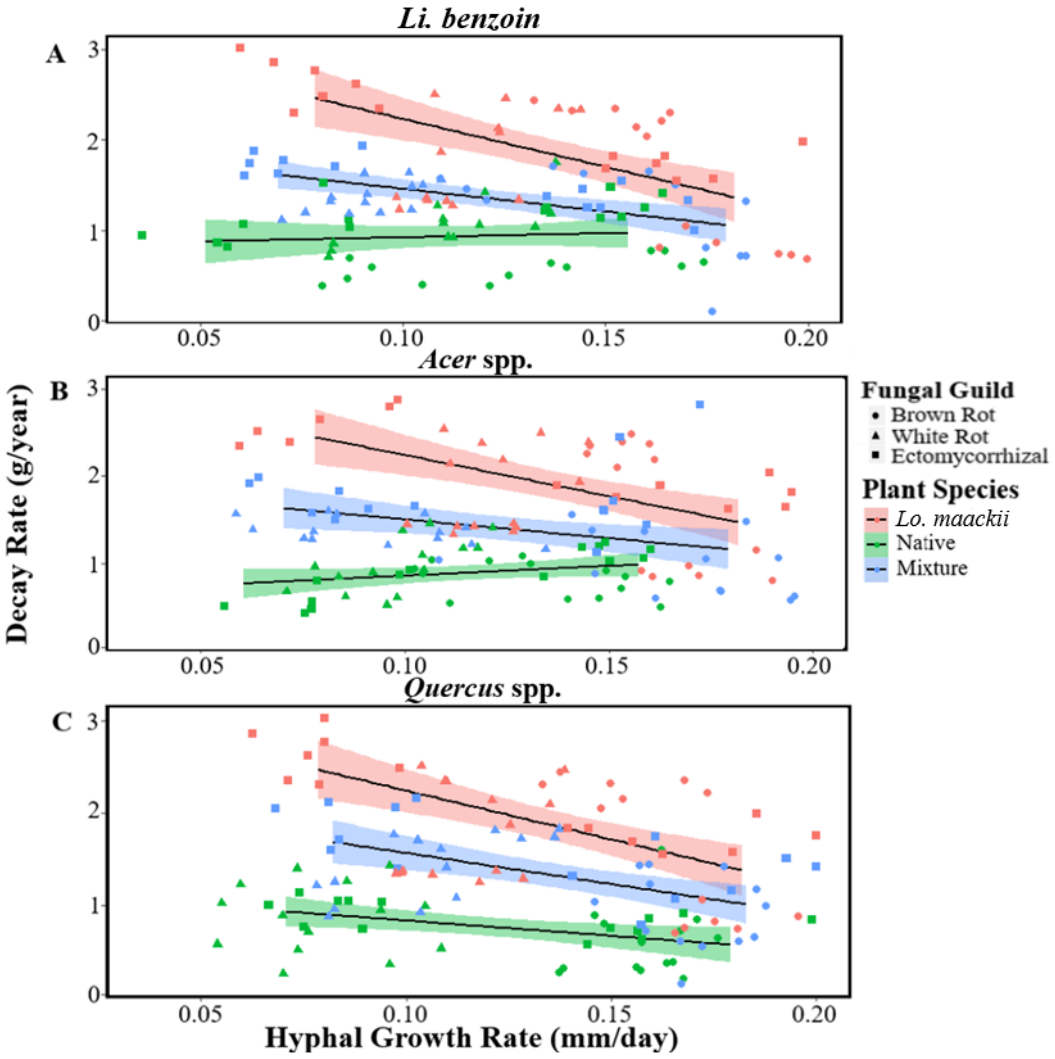

Figure 5. Hyphal growth rate, plant species identity and fungal guild drive litter decay rate in multispecies cultures for A) *Li. benzoin* ($P < 0.0001$), B) *Acer* spp. ($P < 0.0001$), and C) *Quercus* spp. ($P = 0.0033$). Decay rates for multispecies cultures followed similar patterns to *Lo. maackii* with decreasing decay rates with increased hyphal growth rate. Colored regions represent 95% confidence intervals for *Lo. maackii* (pink), native species (green) and mixtures (blue) while point shapes represents brown rot fungi (circles), white rot fungi (triangles) and ECM fungi (squares).

Decay Rate by Fungal Biomass

Fungal biomass, fungal guild and plant species identity predicted litter decay rate for all single species cultures ($F_{10,209} = 9.017$, $P < 0.0001$, Figure 6). *Lo. maackii* litter decay rate decreased with increasing fungal biomass while decay rate increased for all native species litter with increasing fungal biomass: *Li. benzoin*, *Acer* spp., *Quercus* spp.,

F. nigra, *F. pennsylvanica* (Figure 6). For both white rot and ECM fungi, decay rate increased with higher fungal biomass, but decay rates for brown rot fungi decreased with higher fungal biomass (Figure 6).

Multispecies cultures decay rates decreased with increasing fungal biomass for *Lo. maackii* + *Li. benzoin* ($F_{4,103} = 15.19$, $P < 0.0001$, Figure 7A) and *Lo. maackii* +*Acer* spp. ($F_{4,105} = 10.39$, $P < 0.0001$, Figure 7B), but decay rate remained constant for *Lo. maackii* + *Quercus* spp. ($F_{4,105} = 6.386$, $P = 0.0033$, Figure 7C). For multispecies cultures with brown rot fungi, decay rates decreased with greater fungal biomass for *Lo. maackii* + *Li. benzoin*, increased for *Lo. maackii* + *Acer* spp. and remained constant for *Lo. maackii* + *Quercus* spp. compared to the associated native species (Figure 7). For multispecies cultures with white rot fungi, decay rates increased with increasing fungal biomass for *Lo. maackii* + *Li. benzoin* and *Lo. maackii* + *Quercus* spp., but decreased for *Lo. maackii* + *Acer* spp. compared to the associated native species (Figure 7). For cultures with ECM fungi, decay rates increased for each multispecies culture with increased fungal biomass compared to the associated native species (Figure 7).

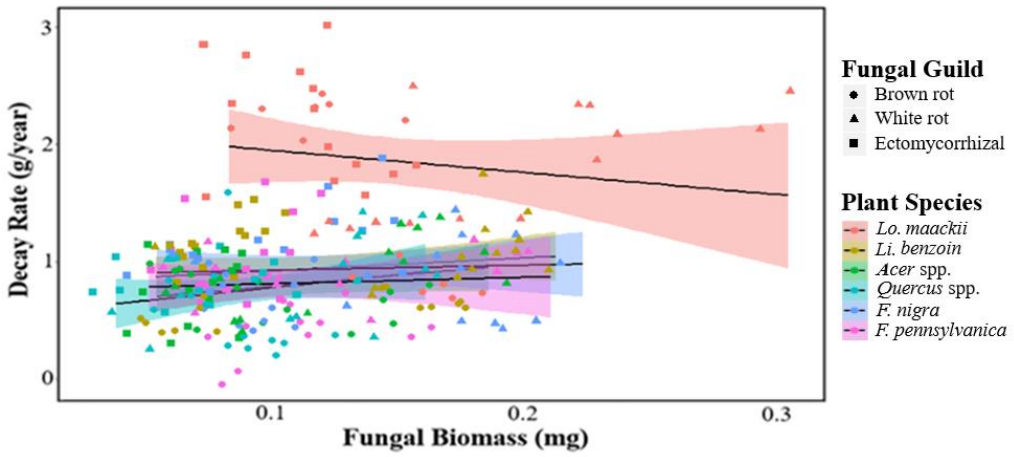

Figure 6. Fungal biomass, plant species identity and fungal guild drive litter decay rate in single species cultures ($P < 0.0001$). *Lo. maackii* litter decay rate decreased with increased fungal biomass while all native species decay rates slightly increased with increasing fungal biomass. Shaded regions represent 95% confidence intervals for *Lo. maackii* (pink), *Li. benzoin* (brown), *Acer* spp. (green), *Quercus* spp. (light blue), *F. nigra* (dark blue), and *F. pennsylvanica* (purple) while point shapes represents brown rot fungi (circles), white rot fungi (triangles) and ECM fungi (squares).

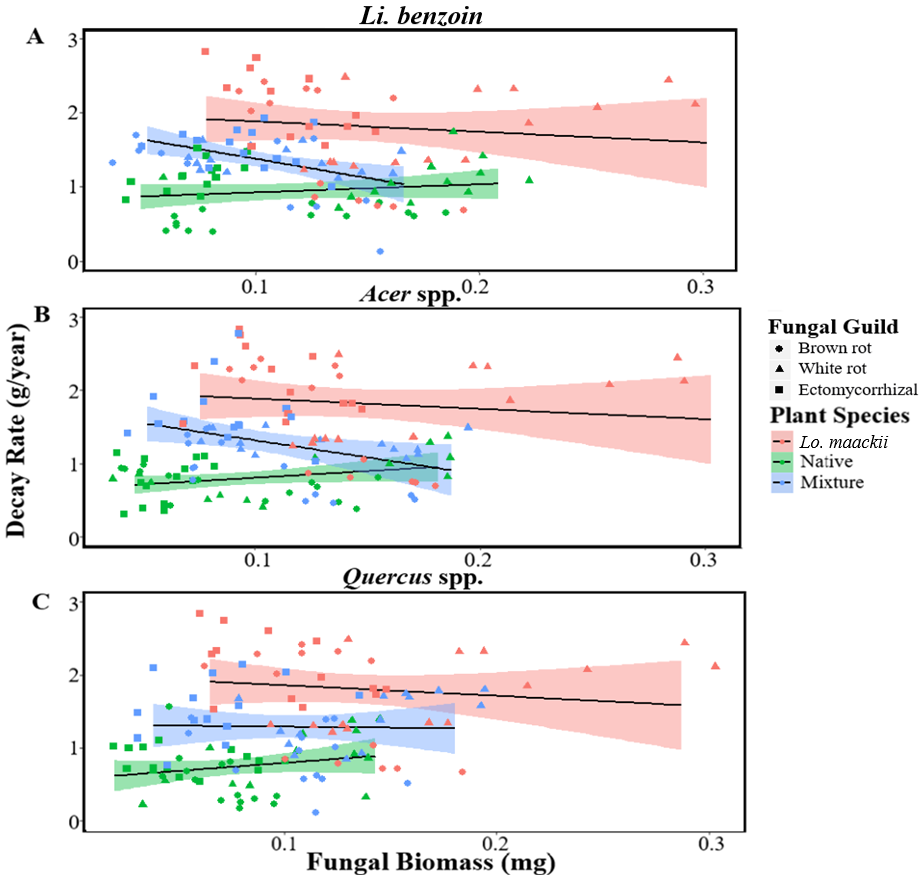

Figure 7. Fungal biomass, plant species identity and fungal guild drive litter decay rate in multispecies cultures for A) *Li. benzoin* ($P < 0.0001$), B) *Acer* spp. ($P < 0.0001$), and C) *Quercus* spp. ($P = 0.0033$). Multispecies litter decay rates decreased with increasing fungal biomass compared to native single species litter. Shaded regions represent 95% confidence intervals for *Lo. maackii* (pink), native species (green) and mixtures (blue) while point shapes represents brown rot fungi (circles), white rot fungi (triangles) and ECM fungi (squares).

Hyphal Growth Rate

Fungal species and plant species interacted to predict hyphal growth rates for each fungal guild in multispecies cultures: brown rot fungi ($F_{6,79} = 21.57$, $P < 0.0001$, Figure 8A), white rot fungi ($F_{6,83} = 17.18$, $P < 0.0001$, Figure 8B) and ECM fungi ($F_{6,84} = 3.780$, $P = 0.0022$, Figure 8C). Both brown rot multispecies cultures had a faster hyphal growth rate than the associated native species (Figure 8A). For *My. galericulata*, hyphae grew ~45% faster on *Lo. maackii* + *Li. benzoin*, ~10% faster on *Lo. maackii* + *Acer* spp., and

~5% faster for *Lo. maackii* + *Quercus* then with *La. sulphuerus* (Figure 8A). Both white rot fungi *S. commune* and *Ma. rotula* grew hyphae faster on *Lo. maackii* + *Quercus* spp. than *Quercus* spp. and slower on *Lo. maackii* + *Li. benzoin* than *Li. benzoin* (Figure 8B). *S. commune* hyphae grew ~12% faster on *Lo. maackii* + *Li. benzoin*, ~55% faster for *Lo. maackii* + *Acer* spp., and ~ 32% faster on *Lo. maackii* + *Quercus* spp. (Figure 8B) than for *Ma. rotula*. Both ECM fungi *Am. parcivolvata* and *I. rimosa* hyphae grew faster on *Lo. maackii* + *Quercus* spp. than *Quercus* spp. (Figure 8C). *I. rimosa* hyphae grew 100% faster on *Lo. maackii* + *Li. benzoin*, ~ 87.5% faster on *Lo. maackii* + *Acer* spp., and ~ 94% faster on *Lo. maackii* + *Quercus* spp. than *Am. parcivolvata* (Figure 8C).

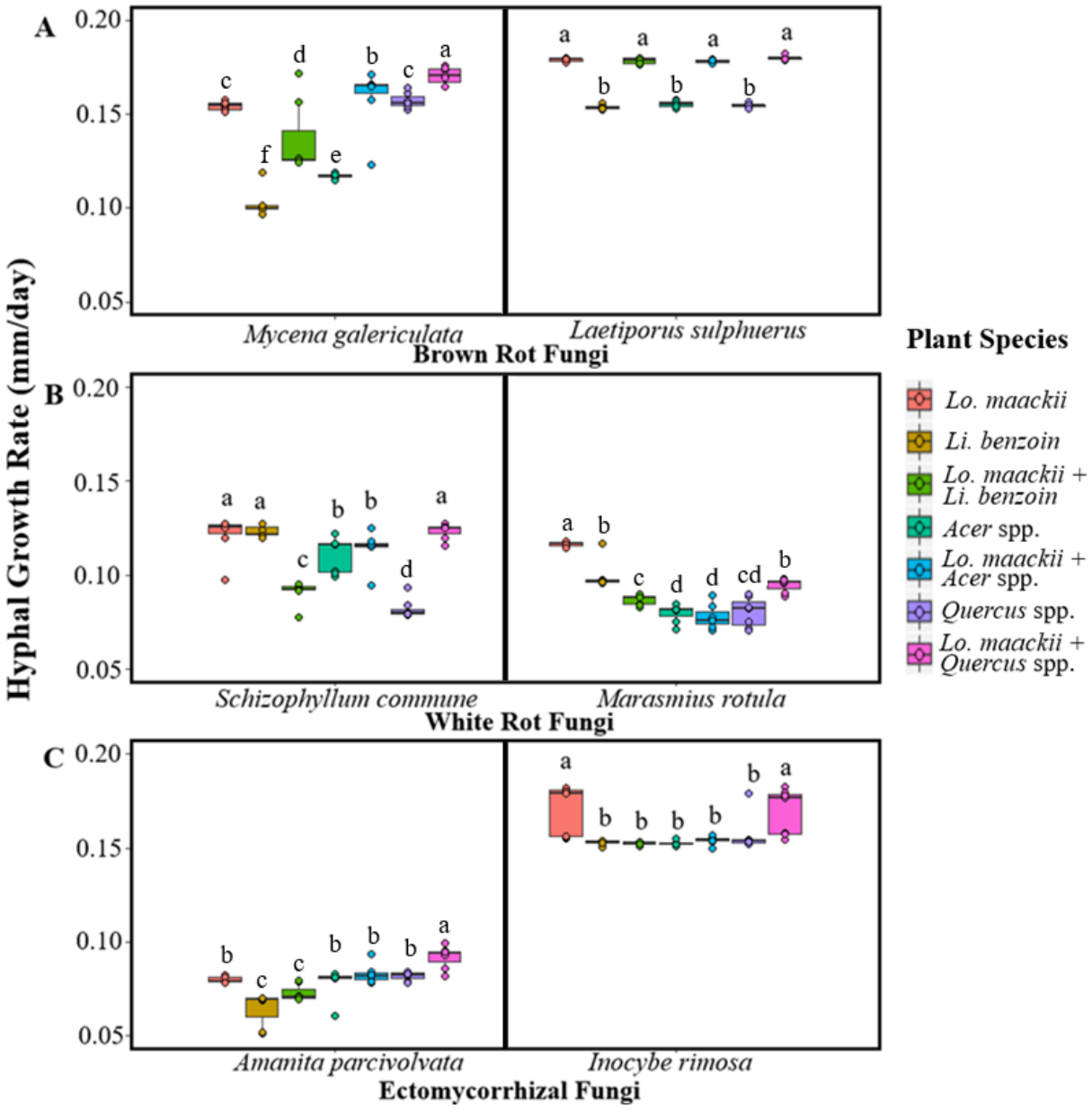

Figure 8. Plant species identity and fungal species drive hyphal growth rate for A) brown rot fungi ($P < 0.0001$), B) white rot fungi ($P < 0.0001$) and C) ECM fungi ($P = 0.0022$). Colored bars represent leaf litter species: *Lo. maackii* (pink), *Li. benzoin* (brown), *Lo. maackii + Li. benzoin* (green), *Acer* spp (teal), *Lo. maackii + Acer* spp. (light blue), *Quercus* spp. (purple), and *Lo. maackii + Quercus* spp. (pink). Letters indicate significant differences based on Tukey's post-hoc analyses within a species.

Fungal Biomass

Fungal species and plant species interacted to predict fungal biomass for both brown rot fungi ($F_{6,79} = 8.438$, $P < 0.0001$, Figure 9A) and white rot fungi ($F_{6,83} = 2.465$, $P = 0.0304$, Figure 9B), but not for ECM fungi ($P = 0.0729$, Figure 9C). For brown rot cultures, fungal biomass increased for *Lo. maackii* + *Quercus* spp. leaf litter compared to *Quercus* spp. leaf litter for both fungal species, and *La. sulphuerus* fungal biomass increased for *Lo. maackii* + *Acer* spp. leaf litter compared to *Acer* spp. leaf litter (Figure 9A). *My. galericulata* fungal biomass increased by ~150% for *Lo. maackii* + *Li. benzoin* leaf litter, ~100% for *Lo. maackii* + *Acer* spp. leaf litter and ~10% for *Lo. maackii* + *Quercus* spp. leaf litter compared to *La. sulphureus* (Figure 9A). For both species of white rot cultures, fungal biomass increased for *Lo. maackii* + *Acer* spp. and *Lo. maackii* + *Quercus* spp. leaf litter and decreased fungal biomass for *Lo. maackii* + *Li. benzoin* leaf litter compared to the associated native species (Figure 9B). Between white rot species, *S. commune* fungal biomass increased by ~40% on *Lo. maackii* + *Li. benzoin* leaf litter, ~70% on *Lo. maackii* + *Acer* spp. leaf litter and ~40% on *Lo. maackii* + *Quercus* spp. litter compared to *Ma. rotula* (Figure 9B).

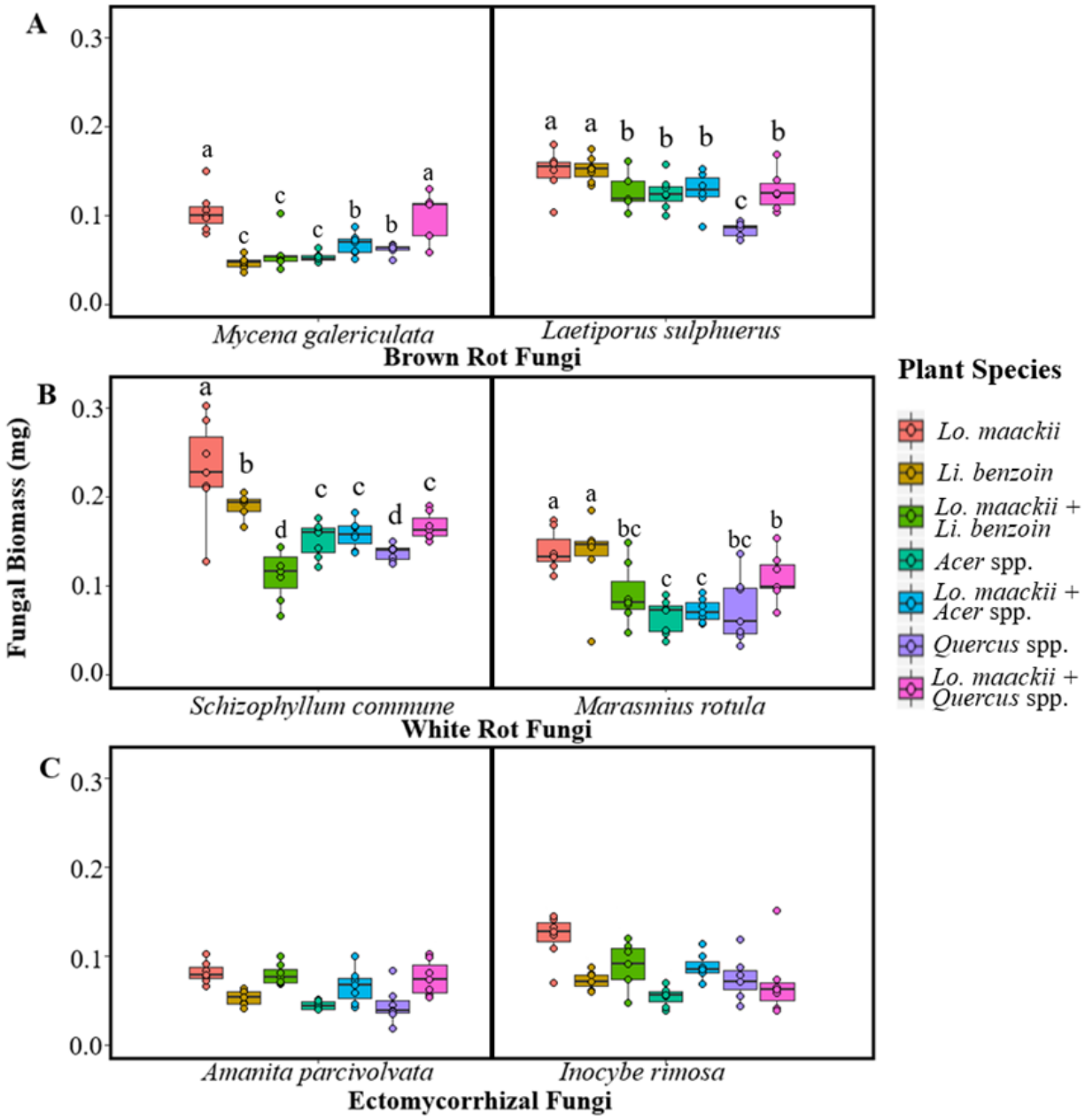

Figure 9. Plant species identity and fungal species drive fungal biomass in A) brown rot fungi ($P < 0.0001$) and B) white rot fungi ($P = 0.0304$), but not for C) ECM fungi ($P = 0.0729$). Colored bars represent litter species: *Lo. maackii* (pink), *Li. benzoin* (brown), *Lo. maackii* + *Li. benzoin* (green), *Acer* spp (teal), *Lo. maackii* + *Acer* spp. (light blue), *Quercus* spp. (purple), and *Lo. maackii* + *Quercus* spp. (pink). Letters indicate significant differences based on Tukey's post-hoc analyses within a species.

Univariate Enzyme Activities

Single Species Cultures

Enzyme activity, fungal guild and plant species identity predicted litter decay rate for single species cultures ($F_{10,200} = 2.394$, $P = 0.0105$, Figure 10A for acid phosphatase; $F_{10,198} = 6.007$, $P < 0.0001$, Figure 10B for cellobiohydrolase; and $F_{10,208} = 4.624$, $P < 0.0001$, Figure 10C for leucine aminopeptidase). Acid phosphatase activity was highest for *Quercus* spp., but increases in acid phosphatase activity increased decay rate across all littler species and fungal guilds (Figure 10A). Increased cellobiohydrolase activity also increased decay rates across all plant species and fungal guilds except for *Li. benzoin* litter (Figure 10B). Finally, increased leucine aminopeptidase activity increased decay rates for *Lo. maackii, Quercus* spp., and across all fungal guilds, but decreased for *Li. benzoin, Acer* spp., *F. nigra* and *F. pennsylvanica* (Figure 10C).

Polyphenol oxidase activity and plant species identity interacted to predict litter decay rate for single species cultures ($F_{5,233} = 5.908$, $P < 0.0001$, Figure 11). Decay rates were increased for *Lo. maackii* leaf litter and decreased for *Acer* spp., *Quercus* spp., and *F. pennsylvanica* (Figure 11).

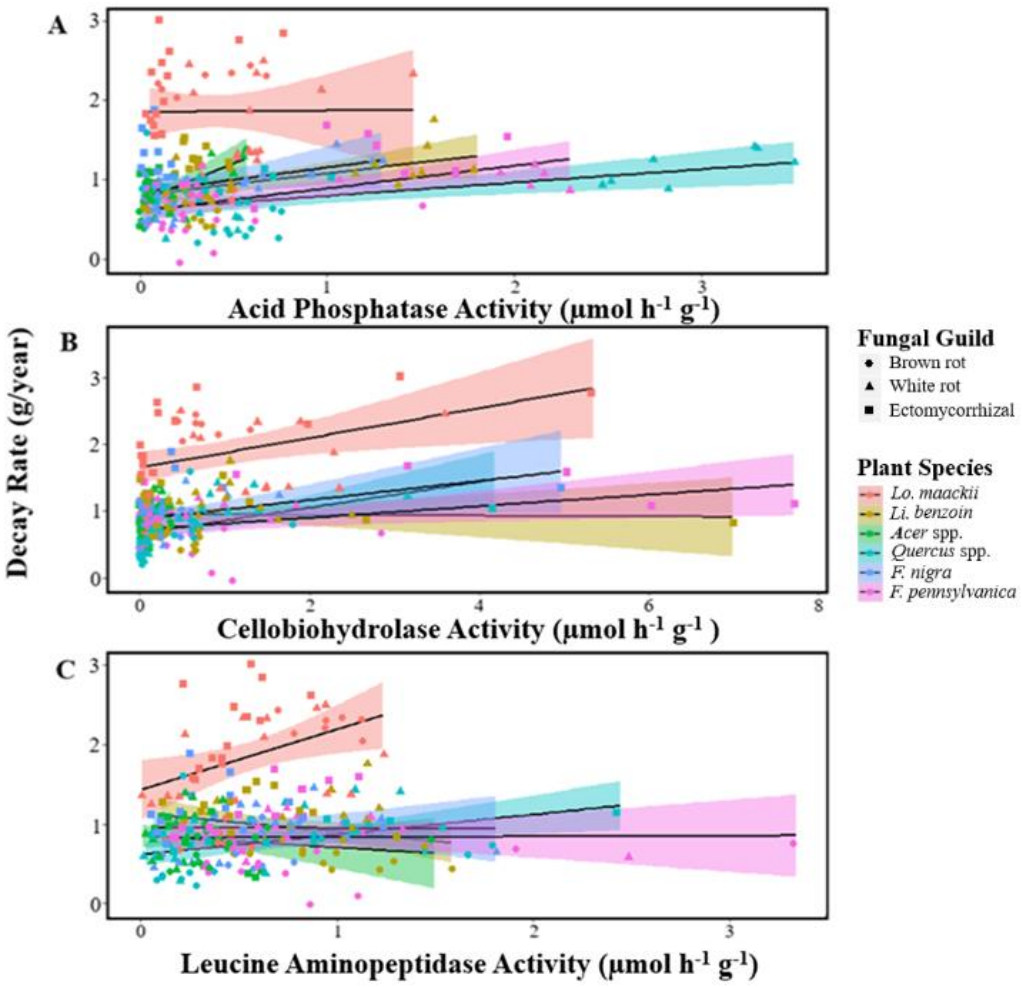

Figure 10. Enzyme activity, plant species identity and fungal guild drive decay rate in single species cultures for A) acid phosphatase activity (P = 0.0105), B) cellobiohydrolase activity (P < 0.0001) and C) leucine aminopeptidase activity (P < 0.0001). Increased enzyme activity increased decay rates across most plant species for each enzyme with exception for *Li. benzoin* for cellobiohydrolase activity and *Acer* spp. for leucine aminopeptidase activity. Shaded regions represent 95% confidence intervals for *Lo. maackii* (pink), *Li. benzoin* (brown), *Acer* spp. (green), *Quercus* spp. (light blue), *F. nigra* (dark blue), and *F. pennsylvanica* (purple) while point shapes represents brown rot fungi (circles), white rot fungi (triangles) and ECM fungi (squares).

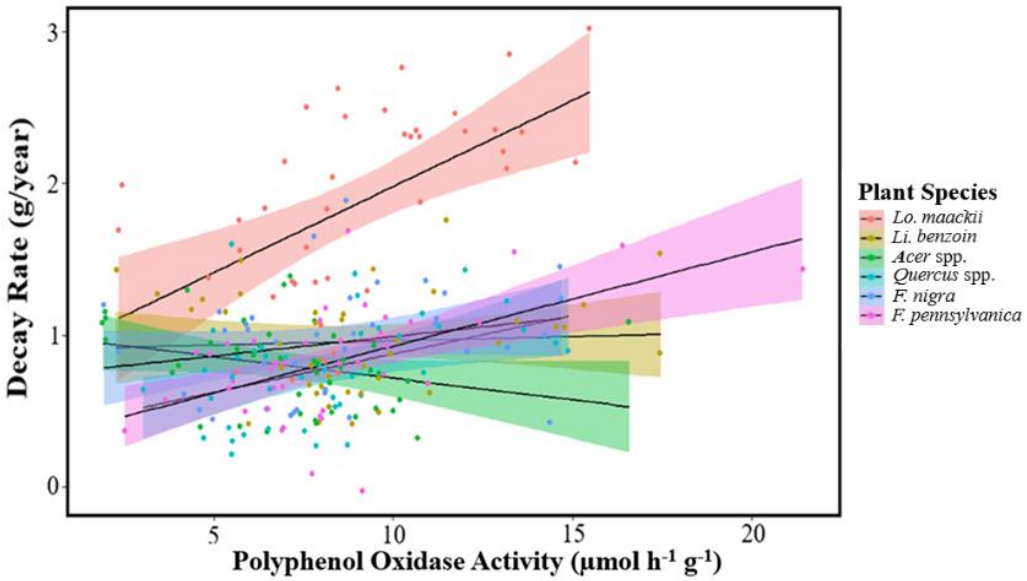

Figure 11. Polyphenol oxidase activity and plant species drive decay rate in single species cultures ($P < 0.0001$). Increased polyphenol oxidase activity increased decay rates for all litter species except *Li. benzoin* and *Acer* spp. Shaded regions represent 95% confidence intervals for *Lo. maackii* (pink), *Li. benzoin* (brown), *Acer* spp. (green), *Quercus* spp. (light blue), *F. nigra* (dark blue), and *F. pennsylvanica* (purple).

Multispecies Cultures

Enzyme activity, fungal guild and plant species identity interacted to predict litter decay rates for *Lo. maackii* + *Li. benzoin* cultures ($F_{4,103} = 4.398$, $P = 0.0025$, Figure 12A for acid phosphatase; $F_{4,103} = 2.863$, $P = 0.027$, Figure 12B for leucine aminopeptidase; $F_{4,102} = 9.221$, $P < 0.0001$, Figure 12C for cellobiohydrolase; $F_{4,103} = 3.219$, $P = 0.016$, Figure 12D for β-glucosidase). Decay rates decreased with increasing acid phosphatase activity for white rot fungi but increased with brown rot and ECM fungi for *Lo. maackii* + *Li. benzoin* compared to *Li. benzoin* leaf litter (Figure 12A). Decay rates increased with higher leucine aminopeptidase activity for both brown and white rot fungi but decreased for ECM fungi compared to *Li. benzoin* leaf litter (Figure 12B). Finally, with increasing cellobiohydrolase and β-glucosidase activity, decay rates decreased for both brown and

white rot fungi but increased for ECM fungi for *Lo. maackii* + *Li. benzoin* litter compared *Li. benzoin* leaf litter (Figure 12C and 12D).

Polyphenol oxidase activity and plant species identity interacted to predict litter decay rates for *Li. benzoin* multispecies cultures ($F_{2,115}$ = 5.582, P = 0.0049, Figure 13). Decay rates decreased with increasing polyphenol oxidase for *Li. benzoin* + *Lo. maackii* in relation to *Li. benzoin* litter (Figure 13).

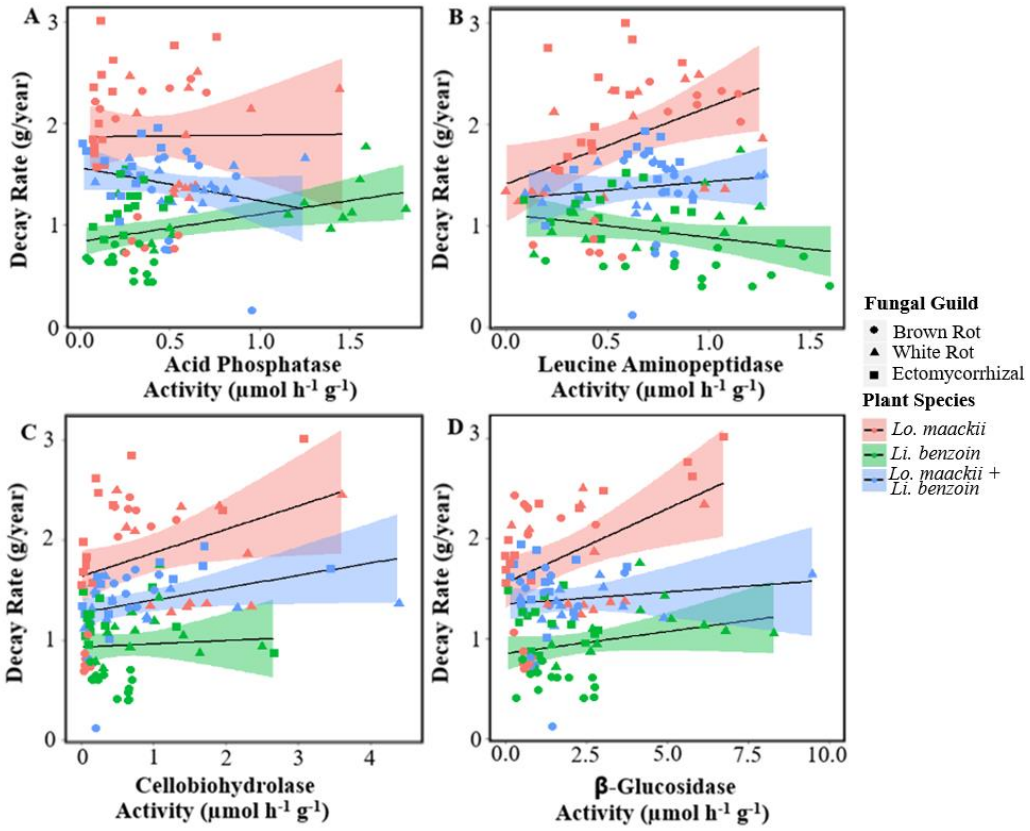

Figure 12. Enzyme activity, plant species identity and fungal guild drive decay rate in *Lo. maackii* + *Li. benzoin* litter for A) acid phosphatase activity (P = 0.0025), B) leucine aminopeptidase activity (P = 0.027), C) cellobiohydrolase activity (P < 0.0001) and D) β-glucosidase activity (P = 0.016). *Lo. maackii* + *Li. benzoin* litter decay rates decreased with increasing acid phosphatase but increased with the other enzymes compared to *Li. benzoin* litter. Shaded regions represent 95% confidence intervals for *Lo. maackii* (pink), *Li. benzoin* (green) and *Lo. maackii* + *Li. benzoin* (blue) while point shapes represents brown rot fungi (circles), white rot fungi (triangles) and ECM fungi (squares).

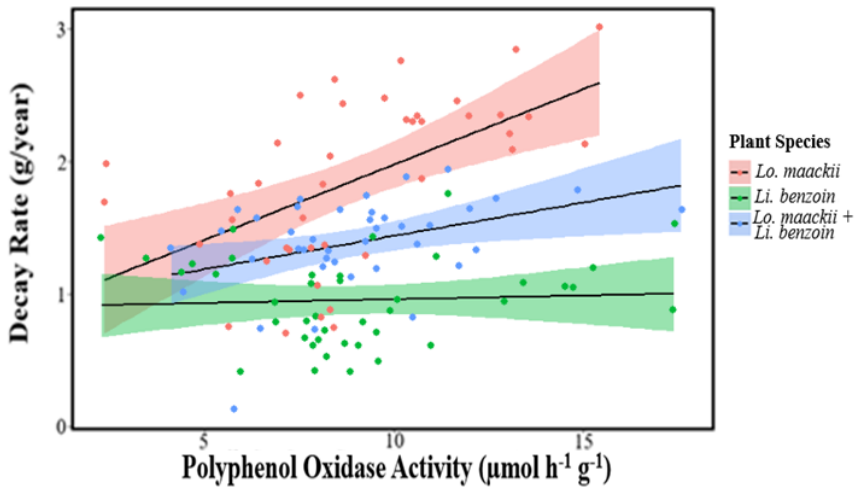

Figure 13. Polyphenol oxidase activity and plant species identity drive decay rate for *Lo. maackii* + *Li. benzoin* litter (P = 0.0049). *Lo. maackii* + *Li. benzoin* litter decay rates increased with increased polyphenol oxidase activity compared to *Li. benzoin* litter. Shaded regions represent 95% confidence intervals for *Lo. maackii* (pink), *Li. benzoin* (green) and *Lo. maackii* + *Li. benzoin* (blue).

Leucine aminopeptidase activity, plant species identity and fungal guild interacted to predict litter decay rates ($F_{4,104}$ = 2.571, P = 0.042, Figure 14A), while polyphenol oxidase activity and plant species identity interacted to predict litter decay rates for *Lo. maackii* + *Acer* spp. litter ($F_{2,117}$ = 7.527, P < 0.0001, Figure 14B). Decay rates increased with increased leucine aminopeptidase activity for all fungal guilds for *Lo. maackii* + *Acer* spp. compared to *Acer* spp. litter (Figure 14A). Decay rates increased with increased polyphenol oxidase activity for *Lo. maackii* + *Acer* spp. compared to *Acer* spp (Figure 14B).

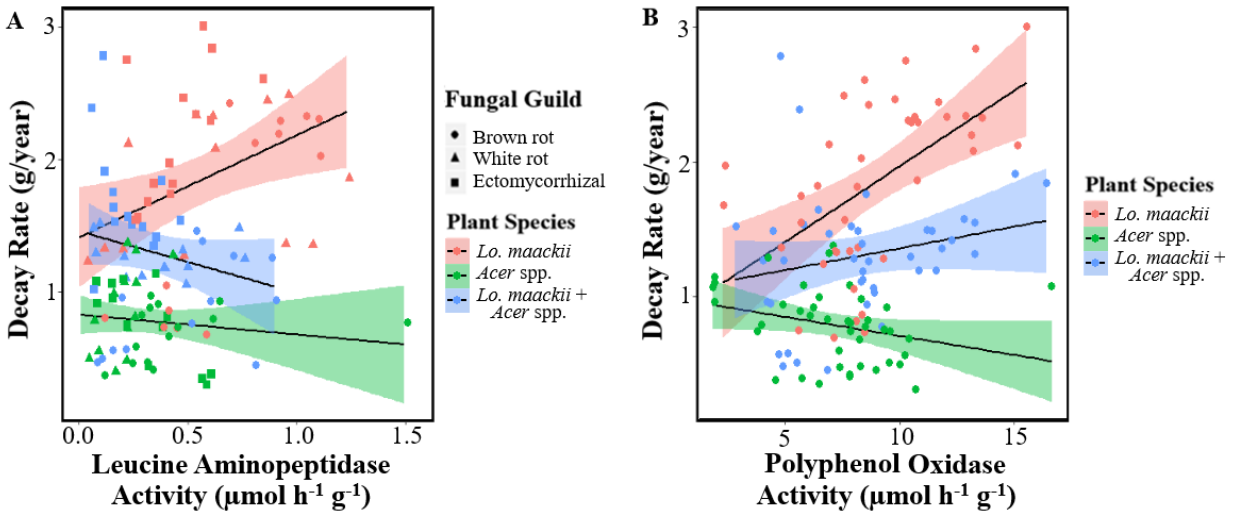

Figure 14. Enzyme activity, plant species identity and fungal guild drive decay rate for A) leucine aminopeptidase (P = 0.042), while only enzyme activity and plant species identity drive decay rate for B) polyphenol oxidase (P < 0.0001) for *Acer* spp. multispecies cultures. *Lo. maackii* + *Acer* spp. litter decay rates decreased with increasing leucine aminopeptidase and increased with increased polyphenol oxidase activity. Shaded regions represent 95% confidence intervals for *Lo. maackii* (pink), *Acer* spp. (green) and *Lo. maackii* + *Acer* spp. (blue) while point shapes represents brown rot fungi (circles), white rot fungi (triangles) and ECM fungi (squares).

Enzyme activity, fungal guild and plant species identity interacted to predict litter decay rates for *Quercus* spp. multispecies cultures ($F_{4,104}$ = 4.441, P = 0.0024, Figure 15A for cellobiohydrolase; $F_{4,105}$ = 3.125, P = 0.018, Figure 15B for polyphenol oxidase; $F_{4,105}$ = 2.519, P = 0.0456, Figure 15C for leucine aminopeptidase; $F_{4,100}$ = 2.468, P = 0.0496, Figure 15D for peroxidase). Higher cellobiohydrolase, polyphenol oxidase and leucine aminopeptidase activities increased decay rates across all fungal guilds for *Lo. maackii* + *Quercus* spp. compared to *Quercus* spp. (Figure 15A, Figure 15B, Figure 15C). Higher peroxidase activity decreased decay rates across all fungal guilds for *Lo. maackii* + *Quercus* spp. compared to *Quercus* spp. (Figure 15D).

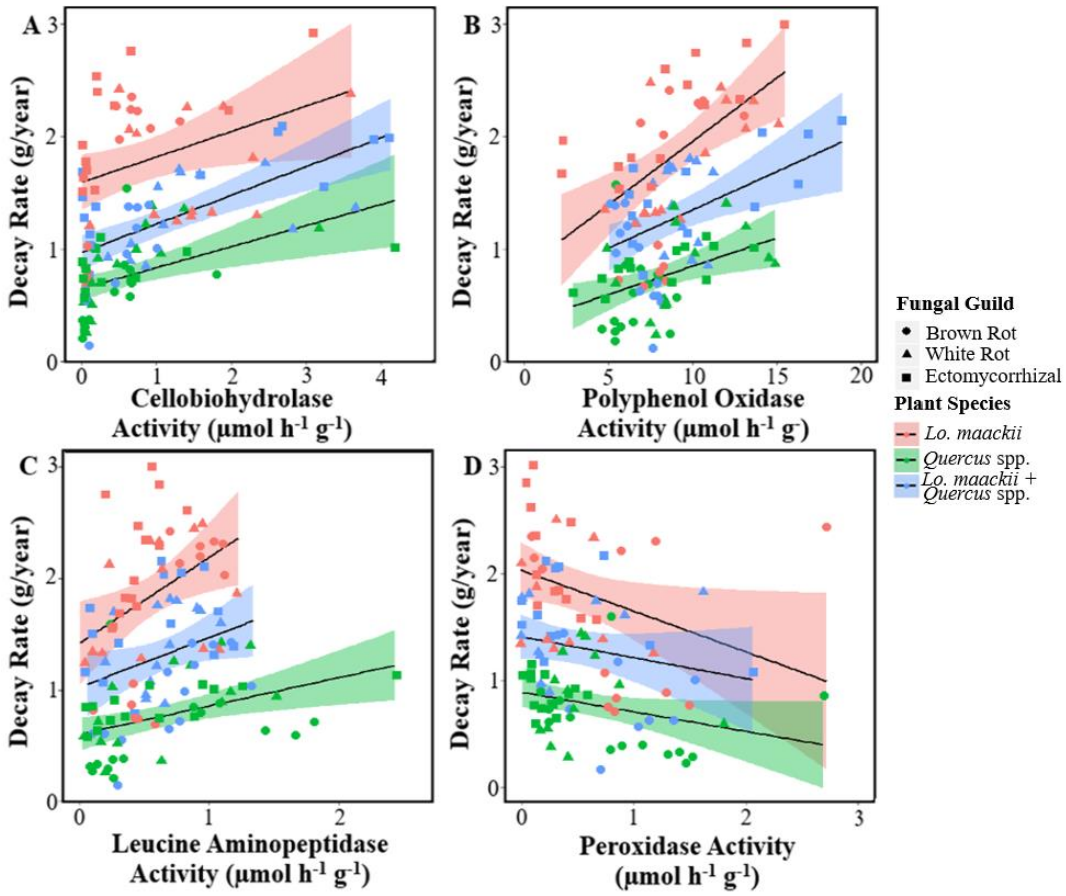

Figure 15. Enzyme activity, plant species identity and fungal guild drive decay rate for *Quercus* spp. multispecies cultures for A) cellobiohydrolase activity (P = 0.0024), B) polyphenol oxidase activity (P 0.018), C) leucine aminopeptidase activity (P 0.0456) and D) peroxidase activity (P 0.0496). *Lo. maackii* + *Quercus* spp. litter decay rates increased with higher cellobiohydrolase, polyphenol oxidase and leucine aminopeptidase activity, but decreased with increased peroxidase activity compared to *Quercus* spp. litter. Shaded regions represent 95% confidence intervals for *Lo. maackii* (pink), *Quercus* spp. (green) and *Lo. maackii* + *Quercus* spp. (blue) while point shapes represents brown rot fungi (circles), white rot fungi (triangles) and ECM fungi (squares).

PCAs of Enzyme Data

Fungal Guild

The enzyme activity PCA condensed acid phosphatase, β-glucosidase, cellobiohydrolase, leucine aminopeptidase, peroxidase and polyphenol oxidase activities into two principal components (PCs). Separate PCAs were run for each round to account

for differences in round. There were significant, but weak relationships between enzyme activity and fungal guild for round 1 (R = 0.22, P = 0.001, Figure 16A) and for round 2 (R = 0.18, P = 0.001; Figure 16B). For round 1, PC1 explained 34.8% of the variance and PC2 explained 18.5% of the variance totaling 53.3% of the variation within enzyme activities. Leucine aminopeptidase activity scaled negatively with PC2, peroxidase activity scaled positively with PC1 and acid phosphatase, β-glucosidase, cellobiohydrolase, and polyphenol oxidase activity scaled negatively with PC1. For round 2, PC1 explained 40% of the variance and PC2 explained 17.4% of the variance totaling 57.4% of the variation within enzyme activities. Leucine aminopeptidase activity negatively scaled with PC2, peroxidase activity positively scaled with PC2 and acid phosphatase, β-glucosidase, cellobiohydrolase, and polyphenol oxidase activities scaled negatively with PC1.

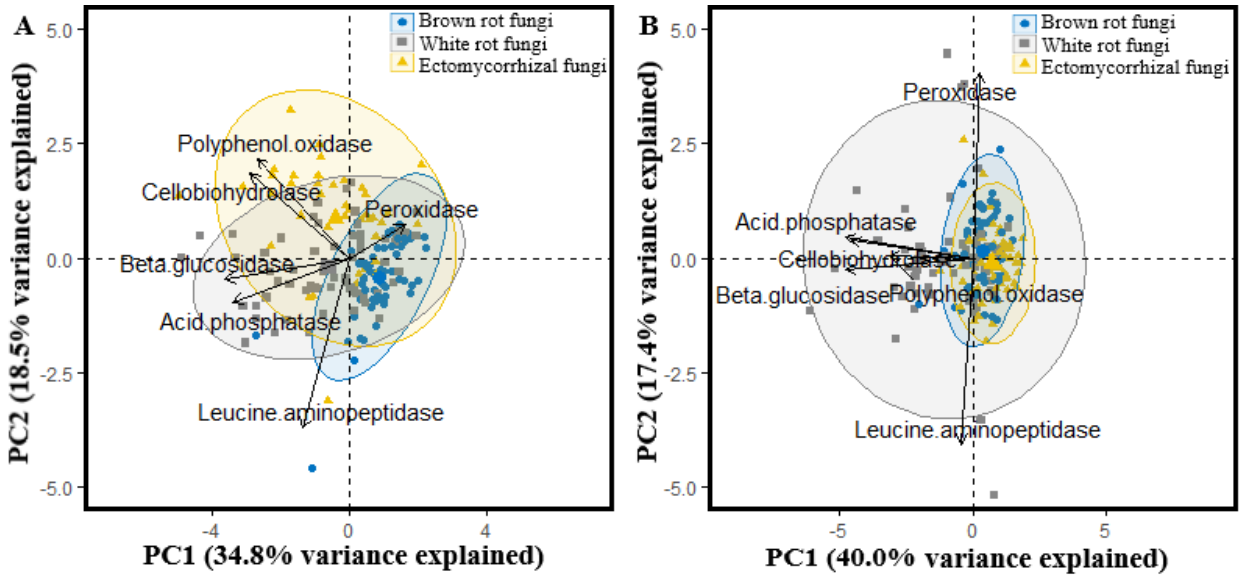

Figure 16. PCA of enzyme activity by fungal guild for A) round 1 and B) round 2. Fungal guild explains variance in enzyme activity (P = 0.001). Colored regions and shapes represent fungal guild: brown rot (blue), ectomycorrhizal (yellow), and white rot (grey).

Single Species Litter

There were also significant but very weak relationships between enzyme activity and single plant litter species for round 1 (R = 0.098, P = 0.001, Figure 17A) and round 2 (R = 0.094, P = 0.001, Figure 17B). For round 1, PC1 explained 33% of the variance and PC2 explained 17.4% of the variance totaling 50.4% of the variation within enzyme activities. For round 2, PC1 explained 36.8% of the variance and PC2 explained 17.9% of the variance totaling 54.7% of the variation within enzyme activities. For both rounds 1 and 2, leucine aminopeptidase activity scaled positively with PC2, peroxidase activity scaled negatively with PC1 and acid phosphatase, β-glucosidase, cellobiohydrolase, and polyphenol oxidase activities scaled negatively with PC1.

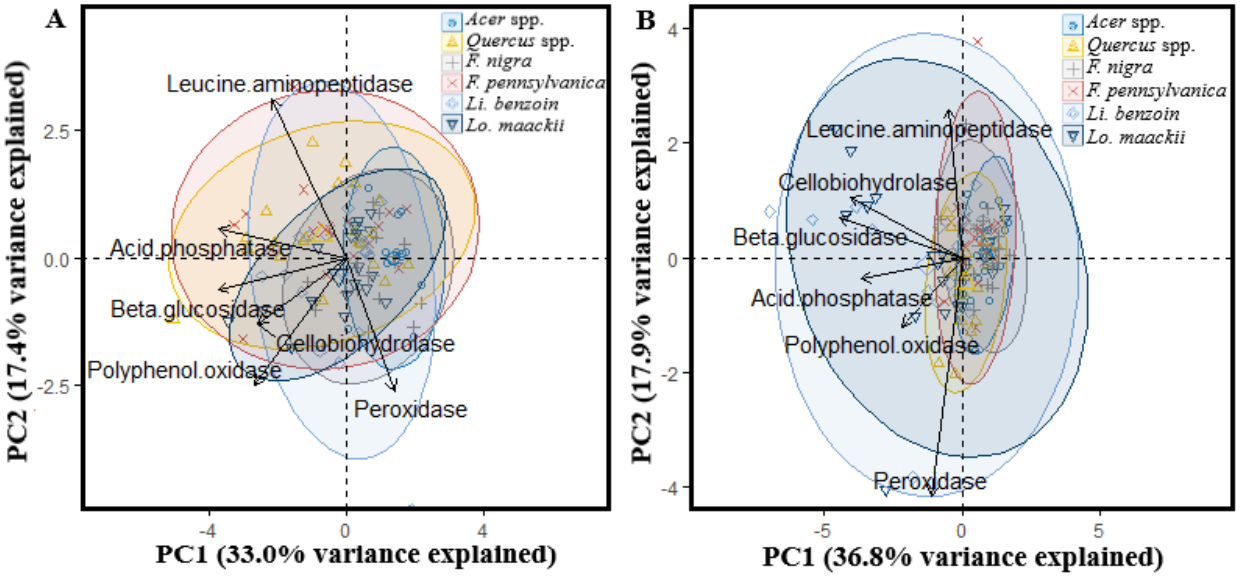

Figure 17. PCA of enzymes by plant species for A) round 1 and B) round 2. Plant species explained variance in enzyme activities between experimental rounds (P = 0.001). Colored regions and shapes represent plant species: *Acer* spp. (blue), *Quercus* spp. (yellow), *F. nigra* (grey), *F. pennsylvanica* (red), *Li. benzoin* (light blue), *Lo. maackii* (dark blue).

Multispecies Litter

There was no significance for the *Li. benzoin* multispecies litter for either round 1 (P = 0.105) or round 2 (P = 0.256), however there were significant, but weak relationships for the *Acer* spp. multispecies litter for round 1 (R = 0.19, P = 0.001, Figure 19A) and round 2 (R = 0.07, P = 0.008, Figure 19B). For round 1 *Acer* spp. multispecies, PC1 explained 34.1% of the variance and PC2 explained 18.6% of the variance totaling 52.7% of the variation within enzyme activities (Figure 19A). Leucine aminopeptidase activity scaled negatively with PC2, peroxidase activity scaled positively with PC1 and acid phosphatase, β-glucosidase, cellobiohydrolase, and polyphenol oxidase activities scaled negatively with PC1. For round 2, PC1 explained 39.2% of the variance and PC2 explained 17.9% of the variance totaling 57.1% of the variation within enzyme activities (Figure 19B). Leucine aminopeptidase activity scaled positively with PC2, peroxidase activity scaled negatively with PC2, and acid phosphatase, β-glucosidase, cellobiohydrolase, and polyphenol oxidase activities scaled negatively with PC1.

There were significant, but weak relationships for *Quercus* spp. multispecies litter for round 1 (R = 0.085, P = 0.007, Figure 20A) and round 2 (R = 0.087, P = 0.003, Figure 20B). For round 1 *Quercus* spp. PC1 explained 35% of the variance and PC2 explained 19.7% of the variance totaling 54.7% of the variation within enzyme activities. For round 1, both leucine aminopeptidase and peroxidase activities scaled positively with PC2, while acid phosphatase, β-glucosidase, cellobiohydrolase, and polyphenol oxidase activities scaled negatively with PC1. For round 2, PC1 explained 49.2% of the variance and PC2 explained 19.7% of the variance totaling 68.9% of the variation within enzyme activities. Leucine aminopeptidase activity scaled positively with PC2, peroxidase

activity scaled negatively with PC1 and acid phosphatase, β-glucosidase, cellobiohydrolase, and polyphenol oxidase activities scaled negatively with PC1.

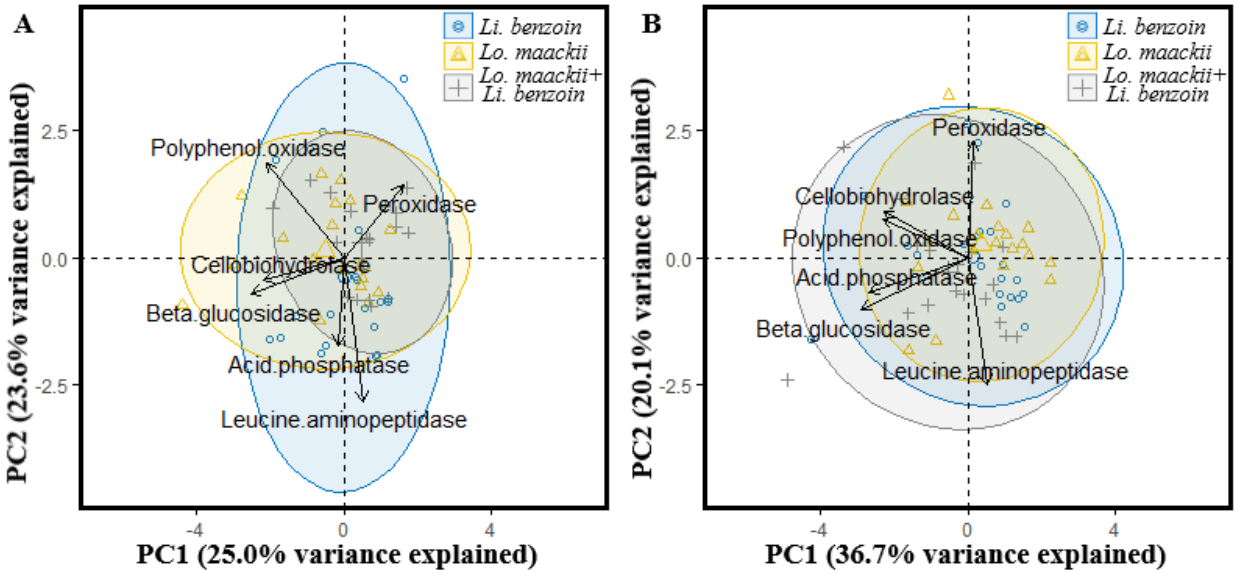

Figure 18. PCA of enzymes by *Li. benzoin* multispecies for A) round 1 and B) round 2. Plant species did not significantly explain variance in enzyme activities for round 1 (P = 0.105) or for round 2 (P = 0.256). Colored regions and shapes represent plant species: *Li. benzoin*. (blue), *Lo. maackii* (yellow), *Lo. maackii* + *Li. benzoin* (grey).

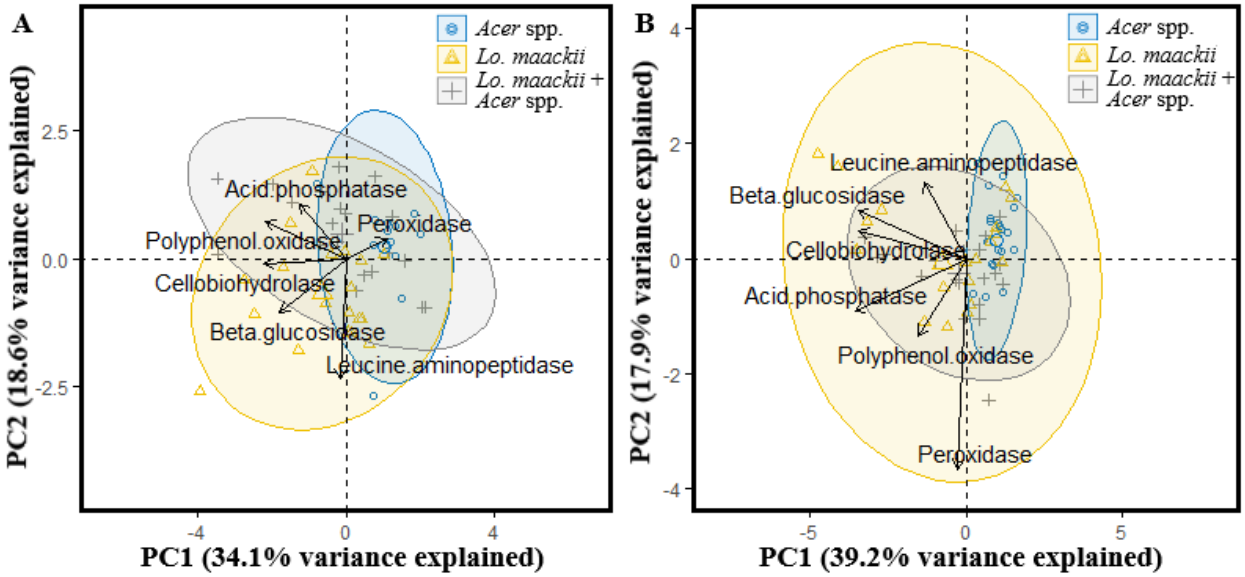

Figure 19. PCA of enzymes by *Acer* spp. multispecies for A) round 1 and B) round 2. Plant species explained variance in enzyme activities for round 1 (P = 0.001) and for round 2 (P = 0.008). Colored regions and shapes represent plant species: *Acer* spp. (blue), *Lo. maackii* (yellow), *Lo. maackii* + *Acer* spp. (grey).

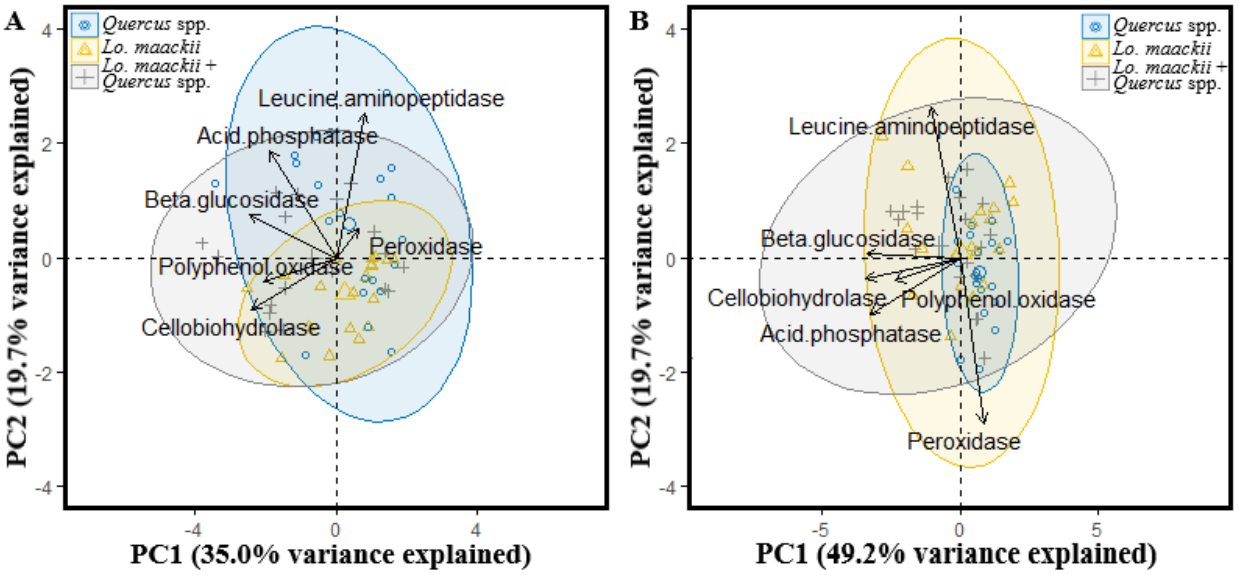

Figure 20. PCA of enzymes by *Quercus* spp. multispecies for A) round 1 and B) round 2. Plant species explained variance in enzyme activities for round 1 (P = 0.007) and for round 2 (P = 0.003). Colored regions and shapes represent plant species: *Quercus* spp. (blue), *Lo. maackii* (yellow), *Lo. maackii* + *Quercus* spp. (grey).

DISCUSSION

Invasive species are detrimental to forests by causing habitat destruction, altering plant community structure, and impacting ecosystem functions (Tilman et al., 1994; Pyšek and Richardson, 2010). The separate invasions of *Lo. maackii* and *Ag. planipennis* are significant disturbances to ecosystem function, but their interactive effect on many ecosystem functions are relatively unknown. One of the most obvious changes caused by these cooccurring invaders is declines in plant species richness (Hoven et al., 2017; Hartman and McCarthy, 2008). Ecosystem functions like nutrient cycling require further research to understand their long-term impacts of losing *Fraxinus* spp. and gaining *Lo. maackii* in invaded forests. Here, I found that the addition of *Lo. maackii* litter not only increased decomposition rates when combined with native species litter, but also altered fungal biomass, hyphal growth rates and enzyme activities, all of which contributed to increased decomposition rates. The addition of *Lo. maackii* further increased enzyme activity for several key enzymes associated with decomposition, particularly C associated enzymes. Across all native species, *Fraxinus* spp. litter decayed the quickest of the and had faster hyphal growth rates, increased fungal biomass and higher acid phosphatase, cellobiohydrolase, leucine aminopeptidase and polyphenol oxidase activities than the other native species. Due to the loss of *Fraxinus* spp., *Lo. maackii* invasion may further increase the rate that C is broken down by fungi, thus potentially increasing the C nutrient pools in invaded forests.

Invasive species leaf litter decomposes faster than native species leaf litter in both

field and lab studies (Jo et al., 2016; Nisbet et al., 2015; Arthur et al., 2012). In this study, *Lo. maackii* litter decomposed the quickest among the six litter species for both litter bag (Figure 1) and culture (Figure 3) experiments. The quick decomposition of *Lo. maackii* litter compared to native species in this study supports invasive species litter as more labile with faster decomposition than native species (Arthur et al., 2014; Ehrenfeld, 2003; Ashton et al., 2005). *Fraxinus* spp. litter decomposed slower than *Lo. maackii* litter but decomposed faster than other native species (Figure 3). This trend supports *Fraxinus* spp. litter being more labile than other native species, but less than *Lo. maackii* (Nisbet et al., 2015). Furthermore, mixing *Lo. maackii* and individual native species leaf litter increased decay rates of native species litter compared to native species decay alone in litter bags (Figure 2) and lab cultures (Figure 3). In previous studies, the addition of a more labile leaf litter such as *Lo. maackii* has led to mixed results for changes in decomposition rates of native leaf litter. Some studies have reported an overall increase in decomposition rate, which could be the result of increased N (Ashton et al., 2005; Arthur et al., 2012). Other studies have reported a decrease in decomposition rate, which may be driven by an increase in litter with diverse chemical traits (Grossman et al., 2020; Zhang et al., 2015). My results support an overall increase in litter decomposition due to the addition of the highly labile *Lo. maackii,* potentially reducing N limitation of fungi during decay.

Invasive species can also alter fungal growth, which is a primary driver of decomposition (Vitousek et al., 1997). In this study, fungi inoculated on *Lo. maackii* litter grew faster and had higher biomass than on native species litter, leading to a faster decay rate (Figure 4, 6). Fungi inoculated on *Fraxinus* spp. litter grew faster and had higher biomass than the other native species litter, but less than *Lo. maackii* (Figure 4, 6). This

increase in fungal growth drives faster decomposition since the fungi have increased ability for decay with increased size. The addition of *Lo. maackii* to native litter also increased both hyphal growth rates and fungal biomass compared to fungi inoculated on native species litter alone (Figure 5, 7). This trend for increased fungal growth leading to increased decomposition rates is typical with a mixture of labile and recalcitrant leaf litter (Cotrufo et al., 2012; Chapmen and Newman, 2009).

Changes in fungal traits can be further explained through differences observed in the various fungal guilds associated with decomposition. I used three fungal guilds commonly associated with decomposition: brown rot, white rot and ECM fungi. Both brown rot and white rot fungi are primary decomposers and typically decompose litter faster than ECM fungi (Riley et al., 2014; Bödeker et al., 2009). In this study, both white rot and ECM fungi decomposed litter quicker than brown rot fungi (Figure 4). ECM fungi may have had faster decomposition rates as it is specialized in N degradation, and there was likely higher N in the labile litter. Indeed there is evidence that ECM fungi can have similar importance in decomposition to other fungal guilds (Talbot et al., 2015; Burke et al., 2014). Examining changes in fungal traits can explain the increased decomposition for *Lo. maackii* litter. Hyphal growth rate was quickest for brown rot fungi with the most notable changes occurring on both *Lo. maackii* and the multispecies mixtures (Figure 8). Biomass for brown rot and white rot fungi can be higher than ECM fungi (Talbot et al., 2015), which is supported in this study with white rot fungi having the largest biomass (Figure 9). Overall, fungi that had faster hyphal growth rates had less fungal biomass (Figure 8, 9). Fungi inoculated on *Lo. maackii* and the multispecies litter had increased fungal biomass compared to native litter (Figure 9). This increase of fungal

biomass supports the trend of higher fungal biomass in both more labile species and in more labile mixtures of litter (Chapmen and Newman, 2009).

The primary way fungi facilitate decomposition is through the release of extracellular enzymes (Hankin and Anagnostakis, 1975) and *Lo. maackii* addition altered these activity levels. Enzyme activities were dependent upon plant species with more labile litter and had higher enzymatic activities related to C breakdown than either N or P breakdown (Figure 12, 13). Specifically, both leucine aminopeptidase and acid phosphatase enzymes had lower activity on *Lo. maackii* compared to the native species (Figure 10) whereas cellobiohydrolase and polyphenol oxidase enzymes had higher activities on *Lo. maackii* leaf litter compared to native species leaf litter (Figure 10, 11). *Lo. maackii* having lower enzyme activity for enzymes associated with N and P contradicts previous studies with invasive plants which suggests an increase in activity levels due to a larger amount of nutrient input (Zhou and Staver, 2019, Vila et al., 2011, Liao et al., 2008). However, my results with C associated enzymes reflect others results which have demonstrated increased C enzyme activity such as polyphenol oxidase (Woods et al., 2019, Liao, 2008), potentially due to C limitation during decay.

Fraxinus spp. litter had higher leucine aminopeptidase, acid phosphatase, cellobiohydrolase, and polyphenol oxidase activities compared to litter from the other species (Figure 10, 11). This trend was particularly prominent for *F. pennsylvanica*, which had the overall highest enzyme activities. This trend supports that *Fraxinus* spp. facilitate increased nutrient availability in forests and are more nutrient rich than other native tree species (Langenbruch et al. 2012). With the transition to *Lo. maackii* litter, which has decreased activities for leucine aminopeptidase and acid phosphatase, two

prominent N and P degrading enzymes, the loss of *Fraxinus* spp. may lead to a decrease in nutrient cycling for N and P.

Variation between individual enzyme responses can be further explained by evaluating the change in enzyme activities within multispecies litter (Figures 18, 19, 20). Multispecies litter followed similar trends to *Lo. maackii* litter with decreased leucine aminopeptidase and acid phosphatase enzyme activities, which are related to N and P degradation (Figure 12A, 12B, 14A, 15C) and increased enzyme activities for cellobiohydrolase and polyphenol oxidase, which are related to C degradation (Figure 12C, 12D, 13, 14B, 15B). With greater decomposition, there is increased nutrient availability in forest soils (Sinsabaugh and Moorhead, 1994). Increased nutrients lead microorganisms to allocate greater enzymatic activity towards C degradation instead of N and P degradation leading to increased C for nutrient cycling (Allison and Vitousek, 2004). For instance, by decomposing C at faster rates for multispecies litter, greater amounts of C would become available surrounding vegetation. However, since *Fraxinus* spp. were a large C source, there is an overall net loss of C within forested soils due to *Ag. planipennis* induced loss of *Fraxinus* spp. (Flower et al. 2015). Therefore, the dual invasion of *Ag. planipennis* and *Lo. maackii* would likely cause a loss of C in soils from the loss of *Fraxinus* spp. and increased C decomposition. In areas invaded and established with *Lo. maackii* following the loss of *Fraxinus spp*., soils would also have a net gain in N due to decreased N degradation when *Lo. maackii* is mixed with native species leaf litter, causing higher accumulation of N in forested soils. A shift towards an increase of C degrading enzymes and away from N degrading enzymes is important in understanding the transition from labile *Fraxinus* spp. to even more labile *Lo. maackii*.

In addition to the changes in enzyme activities between plant litter, there were also differences between fungal guilds (Figure 16). For single species litter, acid phosphatase activity was highest in litter inoculated with white rot fungi (Figure 10). Litter inoculated with brown rot fungi had higher associated enzyme activities for leucine aminopeptidase, while litter inoculated with ECM fungi had higher overall enzyme activities for cellobiohydrolase and polyphenol oxidase (Figure 10,11). Higher acid phosphatase activity is typical for white rot fungi (Talbot et al., 2015, Bödeker et al., 2009) since white rot fungi typically target C and P degradation more often than ECM fungi. The higher amounts of leucine aminopeptidase activity for brown rot fungi could be due to brown rot fungi degrading labile litter quicker than white rot fungi (Kirk and Farrell, 1987). ECM fungi having higher associated activities for cellobiohydrolase and polyphenol oxidase, two C degrading enzymes, conflicts with previous studies which show an increase in activity for ECM fungi compared to either brown rot or white rot fungi (Talbot et al., 2015; Bödeker et al., 2009). This increase in C degradation supports ECM fungi playing similar important roles in decomposition as both brown and white rot fungi, which are primary decomposers, and shows how the primary decomposers functions may shift in response to this dual invasion.

In summary, the addition of *Lo. maackii* litter is altering native species litter decomposition through several fungal traits: increased hyphal growth rates, increased fungal biomass, increases in activities of enzymes associated with C and decreases in activities of enzymes associated with both N and P. Forests that previously had abundant *Fraxinus* spp. populations are becoming overtaken by invasive shrubs where *Fraxinus* spp. have died due to *Ag. planipennis* (Hoven et al., 2017). Damages due to the dual

invasion of *Ag. planipennis* and *Lo. maackii* continue to rise as both species are spreading throughout the United States. Because forests are losing *Fraxinus* spp., which are a primary C source of forests, and it is likely replaced by N rich *Lo. maackii*, there will be less overall C and higher overall N available in invaded forests. This study represents an important first step in understanding how microorganism functions will change in response to the alteration in leaf litter from the transition of *Fraxinus* spp. to *Lo. maackii* in Midwestern US forests.

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