ABSTRACT

HACKMAN, JACOB J. Phosphorus Carryover in Southern Pine Plantations: Abiotic and Biotic Indicators of Bioavailability. (Under the direction of Dr. Rachel Cook as Chair).

Phosphorus (P) fertilization is common in intensively managed loblolly pine (*Pinus taeda* L.) plantations on P-deficient sites. A single application of 28 kg ha P at planting has been observed to remain in a single rotation for 20-plus years after fertilization in highly-weathered soils. P that remains in the stand after harvest and into the subsequent rotation is referred to as residual P or carryover P. Much of the P contributing to carryover is thought to be stored and released in the mineralization of organic matter in the forest floor in subsequent rotations. However, the contribution of P built up in the mineral soil, the rate at which the forest floor is mineralized, and how long these effects can supply P to a following rotation remain unknown for most soils. This research uses multiple methods to track carryover P to assess carryover P in two highly weathered soil types: (1) a somewhat poorly drained Spodosol and (2) a poorly drained Alfisol, both sites on the Atlantic Coastal Plain. O-horizon mass and P content were collected in the first rotation, and Mehlich III (M3P) and anion exchange resin probes were used to track carryover P on different pools of P in the following rotation. Microdialysis (MD), a novel technique for assessing diffusive soil P, was deployed in both bulk soils collected from the field and within actively growing *Pinus taeda* trees within each stand to capture differences in translocated P. Ectomycorrhizal (ECM) biomass, tree height, and foliar P concentrations were used to detect P carryover effects through biological growth and ECM community changes. ECM DNA was extracted from the rhizosphere and ingrowth mesh bags to compare differences in the ECM community between carryover P treatments and sites. O-horizon P content from samples collected in the first rotation had a positive relationship to tree height in the second rotation, indicating that the magnitude of previously applied P fertilization is closely linked to the amount of P used in the first rotation for both soils. M3P, resin probes, MD, and ECM biomass were all found to be sensitive to first rotation carryover P rates, with notable differences between sites. ECM biomass in the Spodosol responded to carryover P but was not responsive to carryover rates on the Alfisol. Fungal community diversity was higher in Alfisols than in Spodosols. Still, it was only weakly influenced by carryover P rates for ingrowth mesh bags and rhizosphere. Overall, the above methods, when compared to growth rates of *Pinus taeda,* were highly

dependent upon site-specific characteristics and sampling timing for proper assessment of carryover P in the Atlantic Coastal Plain.

P Carryover in Southern Pine Plantations: Abiotic and Biotic Indicators of Bioavailability

by
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APPROVED BY:

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DEDICATION

I dedicate this work to every moment lost, not exploring the woods or having drag spun off my reels, and to every cherished moment between playing fetch with my dogs and video games with my brothers. I dedicate it to the homies who always picked up calls when I was on the road for 6 hours to talk it up. I dedicate it to my family and friends, who supported me fully but constantly asked me when I was going to finish school. I dedicate this paper to all my colleagues and friends I have made along the way. I dedicate this paper to my mentor Dr. Cook and my committee members who supported my research. I dedicate it to Alexis for your constant support and love. Finally, I dedicate this paper to me for every moment I stayed late, for every time I re-ran some analysis, for every day I walked through the rain and crossed the absolute nightmare that is Western, and every time I said no to fun and decided to put in the work. But, without fail, I dedicate this paper to the most important one. Coffee. Without your friendship and company, I would never have had the courage, gumption, or ability to maintain a waking life where I was not in the woods or on the water. Thank you...delicious, sweet, bitter coffee.

BIOGRAPHY

A long time ago, in a galaxy far, far away, there lived a small boy from a small town in Illinois who had dreams of becoming the greatest soil scientist of all time. He wanted to be the absolute best like no one ever was. To discover all the soils was his real test, and to study them was his cause. He traveled across the lands, searching far and wide to study Spodosols and Alfisols to understand the P that was inside. In all seriousness, I grew up with a mixed bag of experiences ranging from the Midwest of Illinois to the Florida Keys fishing, hunting, and exploring the woods with my father and brothers. Deep into the swamps, rivers, and woods, I occasionally went purposefully, trying to get lost so that I could find my way back. Cataloging all the species I had seen or caught and printing them out into a flipbook of snakes, insects, fish, birds, and animals that I printed using my mom's printer, with color…oh, the ink costs I must have incurred.

My love and appreciation for the natural world and all things inside it manifested itself into a career in agriculture early on, learning how to manipulate and change how we grow crops to sustain an ever-growing human population. After deciding I had enough of that, I shifted my talents toward micro-communities, forests, and the fungi that thrive beneath our feet. There I found opportunities to pursue a master's degree and eventually a Ph.D. the rest, you can read about below.

ACKNOWLEDGMENTS

I want to thank all the staff of the Forest Productivity Cooperative, the USDA, the Bruce and Barbara Zobel Foundation, Sarah Slover for handling all the minutiae and scheduling of all the events, and Rayonier for their continued assistance and support. Funding for this research was provided by the Forest Productivity Cooperative, USDA Project 2022-67013-36864, Long-term P Carryover in Loblolly Pine Plantations, and NSF IUCRC Award 1916552, Center for Advanced Forestry Systems INTERN program.

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P Carryover in Southern Pine Plantations: Abiotic and Biotic Indicators of Bioavailability.

Introduction

Phosphorus (P) fertilization in loblolly pine (*Pinus taeda* L.) plantations have been shown to benefit overall tree productivity and growth in P-deficient soils (Pritchett et al*.,* 1961; Gent et al*.,* 1986). *Pinus taeda* has been fertilized with P on the Atlantic Coastal Plain since the late 1960s. Peak applications of P took place in 1999, with approximately 781,000 acres fertilized (Gent et al., 1986; Pritchett and W.R. Llewellyn, 1966;). By 2016, it was estimated that P fertilizer alone was applied to only 115,000 acres at site establishment (Albaugh et al., 2019). The decrease in P application can be partly attributed to high fertilizer costs and strong evidence that a single P fertilization 28 kg ha^{-1} P can supply crop trees for up to 20 years or more during a single rotation (Pritchett et al., 1982; Turner & Lambert, 2002). The longevity of applied P in forest plantations led many to wonder if that response would carry over into subsequent rotations, potentially saving growers on up-front fertilizer costs. The carryover P effect also called the legacy P effect (Doydora et al., 2020), is studied commonly in agricultural systems but not in forestry, where significantly lower rates and frequencies of P applications are typical, and rotations are much longer. To study P carryover effects in production forest plantations (Ballard, 1978; Comerford et al., 2002; Crous et al., 2007), only one major study has been conducted on *Pinus taeda* plantations in the Atlantic Coastal Plain (Everett and Palm-Leis, 2009). Everett and Palm-Leis' (2009) study was transformative in how we view P fertilization in plantations on the Atlantic Coastal Plain; however, the study was limited to a single poorly drained Albaqualt that was not fully representative of all soils in the region. Considering *Pinus taeda* plantations cover many different soils across the Atlantic Coastal Plain, a more comprehensive study of the P carryover effect is needed to predict carryover responses.

Atlantic Coastal Plain Soils

The Atlantic Coastal Plain contains highly weathered, sandy, acidic soils dominated by 1:1 clay layers with high P adsorption capacities citation. These soils are inherently P deficient due to the depletion of P in the parent material over time (Vitousek et al., 2010; Comerford et al., 2002). Any native P in these soils is found in fixed, non-labile Fe and Al oxides or absorbed onto soil particles (Vitousek et al., 2010; Harris et al., 2010). Due to these limitations, these soils are

hypothesized to be in a "terminal steady state" of P depletion as the parent material is slowly depleted of P (Walker & Syers, 1976). The primary source of available P for these soils originates from organic matter decomposition and mineralization of the forest floor (Comerford et al., 2002). Because most of the P in these soils are present in fixed organic forms or inorganic forms absorbed onto mineral complexes, tree growth is highly responsive to P fertilization on these sites when applied (Gent et al., 1986; Amateis et al., 2001).

Fate of Applied P

P fertilizer applied is taken up or sequestered into the following abiotic or biotic pools of P: (1) adsorbed to mineral surfaces and precipitated into secondary compounds; (2) solution phase inorganic P or plant available P; (3) immobilized by microorganisms or taken up by mycorrhizal fungi; A subset of P assessment methods can be used to quantify these pools: (1) Mehlich III, a standard soil test to assess acid-extractable P from the soil structure (Mehlich, 1984). (2) Anion exchange resins (AER) to assess diffusive P in the soil as a metric of plant available P in the soil solution (Qian & Schoenau, 1995). (3) Microdialysis (MD) is a novel method to test plant-available soil P by mimicking a root (Demand et al., 2017). (4) Ectomycorrhizal (ECM) fungal biomass and fungal community composition on rhizosphere and soil (Smith and Read, 2008). The following three chapters will examine the relationships between P availability and tree uptake to improve soil P management decisions that drive productivity in *Pinus taeda* plantations in the southeastern U.S.

Chapter 1: Carryover Phosphorus Effects on Soils and Loblolly Pine Growth in the Atlantic Coastal Plain

Highlights

- High P Fertilization rates carry over into the next rotation on Alfisols.
- Spodosols cycle P fertilization more rapidly than Alfisols
- O horizon and mineral P soil pools are strongly related to long-term forest productivity or maintenance.
- Resin-P was responsive to mineral soil P but not tree productivity.

Abstract

In intensively managed loblolly pine (Pinus taeda L.) plantations on P-deficient sites, phosphorus (P) fertilization is commonly practiced. Fertilizer P that remains in the soil after harvest and into the subsequent rotation is referred to as carryover P. The storage and release of P in the mineralization of organic matter in the O horizon are believed to contribute significantly to carryover P. However, the specific contribution of P in the mineral soil, the rate of forest floor mineralization, and the duration of these effects in supplying P to the subsequent rotation are not well understood for most soils. To address this knowledge gap, we conducted a study on two highly weathered sites located on the Atlantic Coastal Plain: a somewhat poorly drained Spodosol and a poorly drained Alfisol. We employed multiple methods to quantify the dynamics of carryover P. In the first rotation, we collected O horizon samples to determine mass P concentration and P content of the forest floor. We used Mehlich III (M3P) soil testing and resin probes to track carryover P in different P pools during the following rotation. The application of carryover fertilizer treatments of 121 kg P ha^{-1} and 81 kg P ha^{-1}, as well as fertilized treatments for the Alfisol, resulted in notable increases of 13% to 17% in height responses compared to control plots during the first two years of growth. The presence of carryover P was confirmed with resin probes. However, the relationship between carryover P and tree height and growth was limited to specific burial periods after planting and was only observed in the Alfisol. M3P soil testing showed varying relationships with tree height, depending on sampling timing, depth, and site. Importantly, we found that O horizon mass and P content from the first rotation, approximately seven years prior to harvest, exhibited a positive linear relationship with one-year-old heights in the Spodosol and both one and two-year-old heights in the Alfisol. These findings shed light on the importance of the O horizon characteristics and its potential as an indicator for tree growth in subsequent rotations.

Introduction

Phosphorus (P) fertilization in loblolly pine (*Pinus taeda* L.) plantations benefits overall tree productivity in P-deficient soils (Pritchett et al*.,* 1961; Gent et al*.,* 1986). *P.taeda* has been fertilized with P on the Atlantic Coastal Plain since the late 1960s. Peak applications of P took place in 1999, with approximately 316,059 hectares fertilized (Gent et al., 1986; Pritchett and W.R. Llewellyn, 1966;). By 2016, P fertilizer alone was applied to only 115,000 acres at site establishment (Albaugh et al., 2019). The decrease in P application can be partly attributed to high fertilizer costs and evidence that a single P fertilization of 28 kg ha^{-1} P can supply crop trees for up to 20 years or more during a single rotation (Pritchett et al., 1982; Turner & Lambert, 2002).

The P carryover effect, also called the legacy P effect (Doydora et al., 2020), is studied commonly in agricultural systems but not in forestry. In forestry applications, P is applied one to three times in 25 years on the same stand and at significantly lower rates than in row crop agriculture which typically ranges from 18 to 40 kg ha^{-1} P per application. Only a few studies have observed P carryover effects on in forest plantations (Ballard, 1978; Comerford et al., 2002; CrousPlain (2007; Turner et al., 1986), and only one study has been conducted on *P. taeda* plantations in the Atlantic Coastal Plain (Everett and Palm-Leis, 2009). Evertt and Palm-Leis (2009) demonstrated that rates of previously applied P influenced the productivity of the subsequent rotation in *P. taeda* plantations; however, the study was limited to a single poorly drained Albaquult that is not fully representative of all soils in the region. However, *P. taeda* plantations cover many soils across the Atlantic Coastal Plain. A more comprehensive study of the P carryover effect is needed to predict carryover responses under varying soil and environmental conditions. In addition to the P carryover effect, the assart effect (Kimmins, 1987; Kiser & Fox, 2012) is a temporary increase in available nutrients caused by significant disturbance effects such as tillage and harvesting. These disturbances cycle additional organic material into surface and subsurface horizons where microbes can quickly mineralize the material. The extent and magnitude of this assart effect for P can vary considerably depending on site and environment and has been demonstrated to change based on previously applied P rates (Fox et al., 2011).

The Atlantic Coastal Plain contains highly weathered, sandy, acidic soils dominated by 1:1 clay layers with high P adsorption capacities (Everett and Palm-leis, 2009). These soils are inherently P deficient due to the depletion of P in the parent material over time (Vitousek et al., 2010; Comerford et al., 2002). Any native P in these soils is found in fixed, non-labile Fe and Al

oxides or absorbed onto soil particles (Harris et al., 2010). Due to these limitations, these soils are hypothesized to be in a "terminal steady state" of P depletion as the parent material is slowly depleted of P (Walker & Syers, 1976). The primary source of available P for these soils originates from organic matter decomposition and mineralization of the forest floor (Comerford et al., 2002). Because most of the P in these soils are present in fixed organic forms or inorganic forms absorbed onto mineral complexes, tree growth is highly responsive to P fertilization on these sites when applied (Gent et al., 1986; Amateis et al., 2001).

Applied P fertilizer is taken up or sequestered into the following abiotic or biotic pools of P: (1) adsorbed to mineral surfaces and precipitated into secondary compounds; (2) solution phase inorganic P or plant available P; (3) immobilized by microorganisms. Soil P testing and foliar P testing are the main methods used to test for P deficiencies in forest plantations (Everett and Palm-leis, 2009). However, in highly P-limited soils, soil testing and foliar testing results are difficult to correlate with tree growth, and tree responsiveness to soil P varies by site (Wells et al., 1986; Everett & Palm-Leis, 2009). The optimal soil test for P varies depending on which P pool is being targeted and the characteristics of the soil itself. Mehlich 3 (M3P) soil testing is widely used in agriculture and forestry on acidic soils in the US to recommend fertilizer additions (Mehlich, 1984; Wells et al., 1986). M3P extracts inorganic and organic forms of P from the soil (Cade-Menun et al., 2018) and is quantified colorimetrically or by inductively coupled plasma spectrometry (ICP). Both methods quantify orthophosphate in solution. However, ICP also quantifies inorganic and organic P forms that may not have been plant-available (Cade-Menun et al., 2018; Fox et al., 2011), potentially providing inaccurate estimates for plant P availability. M3P soil samples are also limited temporally and require multiple samplings to track them over time.

Anion exchange resin probes are an alternative to traditional soil testing methods used to measure available soil P (Van Raij et al., 1986). Anion resins passively adsorb solution phase phosphorus (resin-P) providing an estimation of plant-available inorganic P (Qian & Schoenau, 1995; Schoenau et al., 1993). These probes have been used in modified Hedley sequential fractionalization procedures as the first step to extract the available soluble P fraction in the soil as PO_4^{3-}, $H_2PO_4^{-}$, and HPO_4^{2-} (Tiessen et al., 1993). Resin probes have proven applications in agriculture, forestry, and ecology to measure resin-P from various ecosystems (Tejowulan et al., 1994; Meason et al., 2009; Karamanos et al., 2013; Meason et al., 2009). The resin creates a

diffusive sink for P, which is saturated with a counter ion. Most resins are saturated with HCO_3^- which desorbs readily into the soil and biologically mimics rhizospheric respiration (Qian & Schoenau, 2002).

The primary objective of this study is to provide more accurate predictions regarding P build-up and carryover effects in intensively managed *P. taeda* plantations. To achieve this goal, a comprehensive understanding of the determining factors that influence the availability of P in subsequent rotations across multiple soils and parent materials is crucial. This understanding will ensure the optimization of early P fertilization regimes across the southeastern US. The experiment has two primary objectives: 1) determining the extent of height responses in *P. taeda* resulting from the P carryover effect, and 2) evaluating P availability indicators (M3P, foliar P, resin-P, and O horizon P content) in two distinct sites within the Atlantic Coastal Plain and examining their relationship with height growth

Materials and Methods

Site Description and Study Design

This experiment utilizes plots established from a previous regional study on nitrogen (N) and phosphorus (P) fertilization rates conducted by the Forest Productivity Cooperative between 1998 and 2001 (Tacilla, 2015). The original regional experiment aimed to investigate the growth response of juvenile Pinus taeda plantations to N and P applications across various site conditions in the southeastern United States. This paper focuses on two specific sites selected from the original study, each characterized by distinct soil characteristics (Figure 1.1).

The first site is located in northeast Florida and features a poorly drained soil with a fine, mixed, active, thermic Typic Albaqualf (Meggett series) profile. The parent material consists of marine sediment, and the soil includes an argillic horizon (CRIFF group A) (Fisher & Garbett, 1980). The second site, situated in southeast Georgia, is somewhat poorly drained and exhibits a sandy over loamy, siliceous, active, thermic Typic Haplohumods (Leon series) soil profile. The parent material is also marine sediment, and the soil contains multiple spodic horizons, without the presence of an argillic or kandic horizon within the top 100 cm of soil depth (CRIFF group D). Relevant information about the soil properties, including drainage class, texture, and depth to

subsurface layers, is presented in Table 1.1: Selected site characteristics and corresponding soils. CRIFF soil classification. CRIFF group A soils are very poorly to somewhat poorly drained, with a shallow argillic horizon less than 50 cm into the subsoil, and group D soils are sandy soils with no argillic horizon in the top 152 cm of depth and one or more spodic horizons. Site physical and chemical properties were collected in 2013, as well as during the first rotation by Tacilla (2015), as documented in Table 1.2 and Table 1.3.

Table 1.1: Selected site characteristics and corresponding soils. CRIFF soil classification. CRIFF group A soils are very poorly to somewhat poorly drained, with a shallow argillic horizon less than 50 cm into the subsoil, and group D soils are sandy soils with no argillic horizon in the top 152 cm of depth and one or more spodic horizons

Site	Physiographical province	Parent material	Series name	Soil Drainage	Taxonomy	CRIFF Group
Alfisol	Flatwoods	Marine sediments	Meggett	Poorly	Fine, mixed, active, thermic, Typic albaqualf	A
Spodosol	Flatwoods	Marine sediments	Leon	Somewhat Poorly	Sandy, siliceous, thermic Grossarenic Alaquods	D

Source: Official Soil Series Description – USDA NCRS Soil Survey Division and the University of Florida IFAS Extension

Table 1.2: First rotation *of* physical and chemical properties for the Spodosol and the Alfisol sites collected at pre-harvest in 2013. Percentages of sand, silt and clay, texture, bulk density (BD), pH (1:1 soil/water by volume), CEC (Cation Exchange Capacity), C (Carbon), N (Nitrogen), P (Phosphorus), K (Potassium), Ca (Calcium), Mg (Magnesium), Fe (Iron), Al (Aluminum), and Cu (Copper) collected from the first rotation (Tacilla, 2015).

Soil Properties	Alfisol	Spodosol
Sand (%)	81.5	92.6
Silt (%)	11.3	4.4
Clay (%)	7.2	3
BD (g cm^{-3})	1.32	1.27
pH	4.5	4.5
CEC (cmolc kg^{-1})	4.9	3.5
C (mg kg^{-1})	12337	8688
N (mg kg^{-1})	608.5	303.5
P (mg kg^{-1})	9.5	7.8
C:N	20.3	28.6
K (mg kg^{-1})	13.6	4.6
Ca (mg kg^{-1})	121.9	21.9
Mg (mg kg^{-1})	13.9	4.8
Fe (mg kg^{-1})	143.2	48.1
Al (mg kg^{-1})	626.3	305.9
Cu (mg kg^{-1})	0.1	0.1

Table 1.3: Both sites' location, site, and stand properties from the first rotation. The Alfisol study was established at a stand age of three, and the Spodosol was established at stand age of 5.

Site	County and State	Latitude	Longitude	Species	Study Establishment	"Base" Site Index *	Years since P Fertilization in 2019	Stand Age at Harvest (2019)
First rotation 1998 – 2019								
Alfisol	Nassau, Florida	30.6661	-81.8361	*Pinus taeda*	1999	45	21	26
Spodosol	Brantley, Georgia	31.3353	-81.8217	*Pinus taeda*	1998	67	22	25
***Base Site Index at 25 years old**								

The previous experiment, referred to as the "first rotation," was harvested in 2019. Height measurements for the first rotation were collected manually, starting from age 3 for the Alfisol site and age 5 for the Spodosol site. The experimental treatments in the first rotation were arranged in a completely randomized block design with four replicates for each treatment. These treatments

consisted of different combinations of N and P fertilizer applications, varying in dose and frequency, from 1999 to 2011. The application frequencies were 2, 4, or 6 years over a period of 12 years (Tacilla, 2015). Cumulative N rates ranged from 404 kg N ha^{-1} to 1210 kg N ha^{-1}, applied as urea (Table 1.4). Fertilizer P was applied at the establishment of the study, at a rate of 10% of the total cumulative amount of N applied, using triple superphosphate. For example, the treatment receiving 404 kg N ha^{-1} at the establishment also received 40 kg P ha^{-1}, and the treatment receiving 1210 kg N ha^{-1} at the establishment received 121 kg P ha^{-1}.

The current rotation, known as the "second rotation," was established on the same plots as the first rotation. In the second rotation, the four replicates of each treatment from the first rotation were divided into two treatments. The first treatment, referred to as carryover treatments, did not receive any additional P fertilization at establishment. The second treatment, called re-fertilized treatments, received an additional rate of 45 kg P ha^{-1} broadcasted as triple superphosphate (see Table 4). The cumulative P rate for the two rotations is expressed as X + Y, where X represents the rate of P in the first rotation and Y represents the rate of P in the second rotation. Additionally, all treatments in the second rotation received an additional 52 kg N ha^{-1} as urea with a urease inhibitor, 29 kg K ha^{-1} as KCl, and a micronutrient mix to address nutrient limitations other than P. As part of the site preparation for weed control, all treatments in the second rotation received 6 oz of Arsenal© herbicide after bedding in the spring (Table 1.4).

Figure 1.1: Regional map of intensive (sites currently harvested and planted for the second rotation) and extensive sites that will be harvested in the next 3-5 years. The red circle indicates both areas that encompass the scope of this experiment.

Table 1.4: Treatments and cumulative rates of applied fertilizer for the first and second rotations. The first number under the "P fertilization treatments" column represents P applied from the first rotation in kg ha^{-1}, and the second number represents the amount of P in kg ha^{-1} applied in the second rotation. All treatments received NPK in the first rotation and N and K in the second rotation, but only select treatments received P. X + Y, where X = the first rotation rate and Y = the second rotation rate.

	First rotation (X)		Second rotation (Y)		
P Fertilization Treatments	**Cumulative P**	**Cumulative N**	**Cumulative N**	**Cumulative K**	**Cumulative P**
	kg ha^{-1}				
Carryover					
0 P + 0 P	0	0	52	29	0
40 P + 0 P	40	400	452	29	40
60 P + 0 P	60	600	652	29	60
81 P + 0 P	81	807	859	29	81
121 P + 0 P	121	1210	1262	29	121
Re-fertilized					
40 P + 45 P	40	400	452	29	85
60 P + 45 P	60	600	652	29	105
81 P + 45 P	81	807	859	29	126

Each treatment plot contained six rows with 12 trees per row for 72 trees per treatment. All 70 trees in the treatment plots were identified with aluminum tags to track individual tree growth and mortality over time. During the first rotation, tree height and volume data were collected each year, starting at year five after planting for the Alfisol and three years after planting for the Spodosol until the end of the first experiment in 2015. For the second rotation (established in 2019), individual tree heights, root collar diameter, and mortality were collected for each measurement plot in the first two years of growth for the Alfisol and the Spodosol (Table 1.5). Trees were measured using a height pole in January 2021 and January 2022, approximately 1 and 2 years after establishment. At this early stage in growth, tree height and root collar diameter were highly correlated; therefore, tree height was used as our indicator for P responsiveness.

Table 1.5: Sampling timeline for stand assessment considering: soil P testing, soil resin-P, and treatment years. M3P testing was conducted in year 0, year 1, year 2, and year 3. Foliar P testing was undertaken in years 2 and year 3. Resin-P was evaluated 90 days before pre-harvest and every 90 days after planting. Tree heights were collected at years one and two.

Sampling	Mature Stand	Planting	One-year-old Heights	Two-year-old Heights
O Horizon (2013)	2013	N/A	N/A	N/A
Foliar P (Yearly) **M3P (Yearly)**	Year 0	Year 1	Year 2	Year 3
Resin Probes Burial Periods (90 days)	Burial Period 0 (-90 to 0)	Burial Periods 1-4 (0-360)	Burial Periods 5-6 (360-540)	N/A
Time				

Foliar P

Foliar P was determined by collecting 100 first flush fascicles from a primary branch each winter from five dominant trees with the greatest height after one year of growth. The samples were combined into a single sample and dried before analysis.

Anion Extractable Resin Probes

Resin probes were deployed in a subset of all established field treatments to measure resin-P due to the cost and logistics of installing and removing the probes. The subgroup used to measure resin-P contained the following treatments: 0 + 0 P, 40 + 0 P, 60 + 0 P, 40 + 45 P, and 121 + 0 P.

All probes were installed after planting in the winter of 2019 to assess nutrient supply rates and dynamics. Seven 90-day burial periods were analyzed from the sites, with the last extraction in the fall of 2021 (Table 1.5). A 90-day burial period was chosen based on the known nutrient limitations for the site due to the low acid-extractable P concentrations found in each soil. Probes were isolated from plant roots using 25 cm diameter by 30 cm deep PVC collars (open on top and bottom) to prevent roots from penetrating the resins and nutrient uptake. Four pairs of probes were installed in each plot (Figure 1.2). Two pairs were placed between the planting rows (Interbed) and two within tree rows (Bed). After each 90-day burial period, probes were extracted from PVC collars, washed, and placed into labeled Ziploc® bags. The probes were then returned to Western Ag Innovations Inc., Saskatoon, SK, for further analysis Probes: all standard nutrient extraction protocols considered that all probes in a sample were transferred to Ziploc® bags, and 17.5 mL of 0.5 mol/L HCl was added per probe and eluted for one hour. Inorganic N (ammonium and nitrate) in the eluant is then determined colorimetrically using automated flow injection analysis using a Skalar San + Analyzer (Skalar Inc., Netherlands). Remaining nutrients (P, K, S, Ca, Mg, Al, Fe, Mn, Cu, Zn, and B) were measured using inductively coupled plasma (ICP) spectrometry (Optima ICP-OES 8300, PerkinElmer Inc., USA). All standards and controls were prepared in a 0.5 mol L^{-1} HCl matrix equivalent to that of the samples.

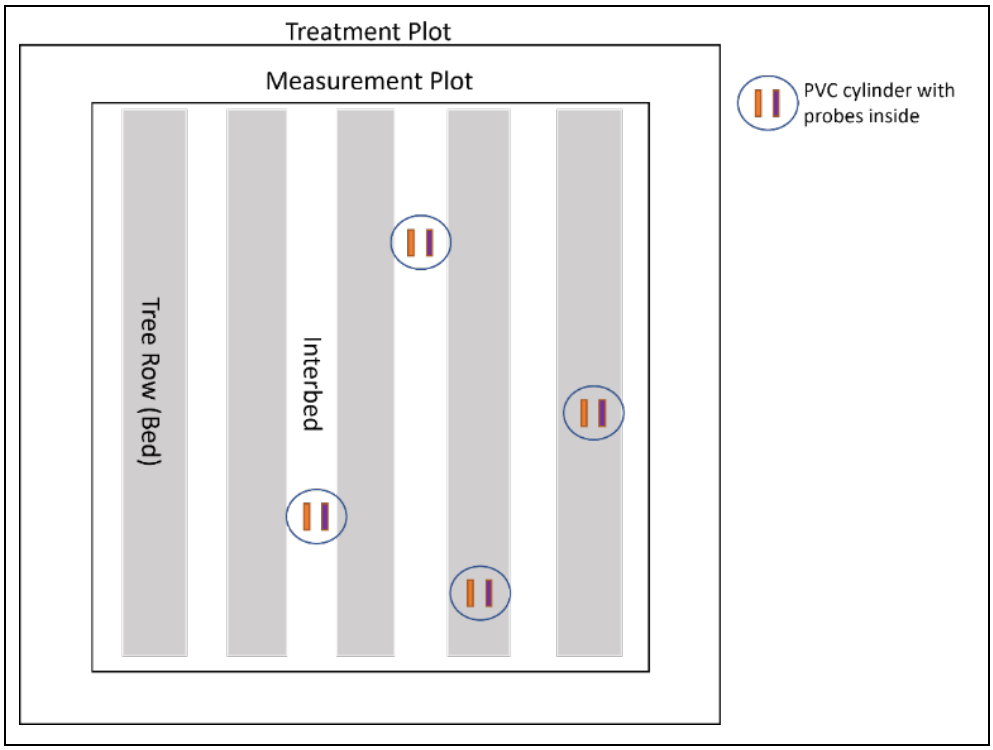

Figure 1.2: Resin probe sampling placement for each treatment plot. One pair, consisting of a cation and an anion probe, were each placed within a single PVC cylinder, isolated from roots, for seven 90-day burial periods

Mineral Soil and O Horizon Sampling

Soil samples were collected at three different time points over a three-year timespan. Year 1 data were collected in January 2020 at planting before P fertilization. Samples were collected from the Bed and Interbed to capture microsite variability and track the nutrient cycling between the disturbed and undisturbed soil. Year 2 data were collected in February 2021, one year after planting and fertilization, and year 3 data were collected in May 2022, approximately two years post-fertilization and planting (Table 1.5). Two composite samples were collected from each treatment replication from each site combining eight soil cores from a depth of 0 to 15 cm with a 4 cm diameter soil auger: one from the Bed and one from the Interbed. Soil samples were air-dried and sieved using a 2 mm mesh. These soil samples were then analyzed using the M3P soil extraction method and analyzed using ICP.

O horizon samples were collected and analyzed for P content between May and June 2013, approximately 13 years after the P fertilization treatment in the first rotation before year 0 (Table 1.5). The O horizon, including twigs and small branches less than 2.5 cm in diameter, was sampled at four random locations chosen via random quadrat sampling in each plot with a 0.25 m^2 frame.

The four subsamples were composited for each plot. The O horizon's three sub-horizons (Oi, Oe, and Oa) were composited and analyzed. Total P concentration in the O horizon was determined by dry-ashing 0.5 grams at 500°C and dissolving the ash in 10 mL of 6 N HCl solution and 40 mL of deionized water. Solution P was analyzed as described by Hansen et al. (2013) on a Varian Vista MPX Inductively Coupled Plasma atomic emission Spectrophotometer (ICP-AES, Varian, Palo Alto, CA, USA). Total P content was determined by multiplying P concentration and O-horizon mass.

Statistical analysis

Resin-P was averaged across treatment replicates for each site for each burial period. Repeated-measures two-way analysis of variance (ANOVA) was used to test changes in P adsorption over time on resin probes between treatments and burial periods and M3P samples over time (Maxwell et al., 2017). This design treats time as an independent variable using an unconstructed variance-covariance matrix simultaneously with each treatment. A mixed model ANOVA was used to test for differences between P levels for each site, treating P fertilization treatment and site as fixed effects and placement (Bed vs. interbed) as random effects. Means were separated from the control plots using Dunnett's post hoc multiple comparison tests using a 0.10 alpha value. Each treatment was compared to the control (0 + 0 P) within each site using Dunnett's multiple comparisons. Error bars represent treatment standard error for all figures. Asterisks in parentheses for all statistical comparisons were (*) Weak p-value < 0.10 (**) Moderate p-value < 0.05 (***) Strong p-value < 0.01 (Muff et al., 2021. Simple linear regressions using 95% confidence intervals were performed on tree heights and years of M3P, resin probes, foliar P, and O horizon samples taken to determine optimal timing and location of sampling for best overall predictors of plant growth. All analyses were conducted using GraphPad Prism 9.5.0.

Plot and Treatment Issues

Most of our plots were established correctly; however, as with many large-scale field experiments, operational errors affected a few of our plots. Overall, one replication of the 60 + 0 P for the Spodosol, one replication of the 81 + 0 P for the Alfisol, and half of one of the 121 + 0 P treatment plots for the Spodosol were planted on a harvest landing. To maintain continuity, the 121 + 0 P samples were left in the dataset to determine how they continue to develop in the future.

Results

Tree Height Responses to Carryover and Fertilized P Treatments

In the first rotation, before any fertilization was applied, there were no height or size differences for the Spodosol or the Alfisol at years three and, respectively, five compared to the control plots (p-values = 0.79, 0.49) (data not shown). In the second rotation, by the end of the first year of growth, apparent differences between applied rates of N and P in the first rotation were observed, implying that carryover fertilization effects were influencing tree growth and height (Figure 3). For the second rotation in the Alfisol, the highest carryover treatment, 121 + 0 P, had no evidence of a tree height response in the first year of growth compared to the control (p-value = 0.19). However, at age 2, trees at the 81 + 0 P, the 121 + 0 P, and the 40 + 45 P treatments were significantly larger than the control and ranged from 13% to 17% increases in total height and growth increments from the control plots. For the Spodosol, the two treatments with the tallest trees were the 81 + 45 P (p-value = 0.02) and the carryover 60 + 0 P treatment (p-value = 0.02) on the Spodosol, a 16% and a 14% change, respectively, in mean height compared to the control (Figure 1.3).

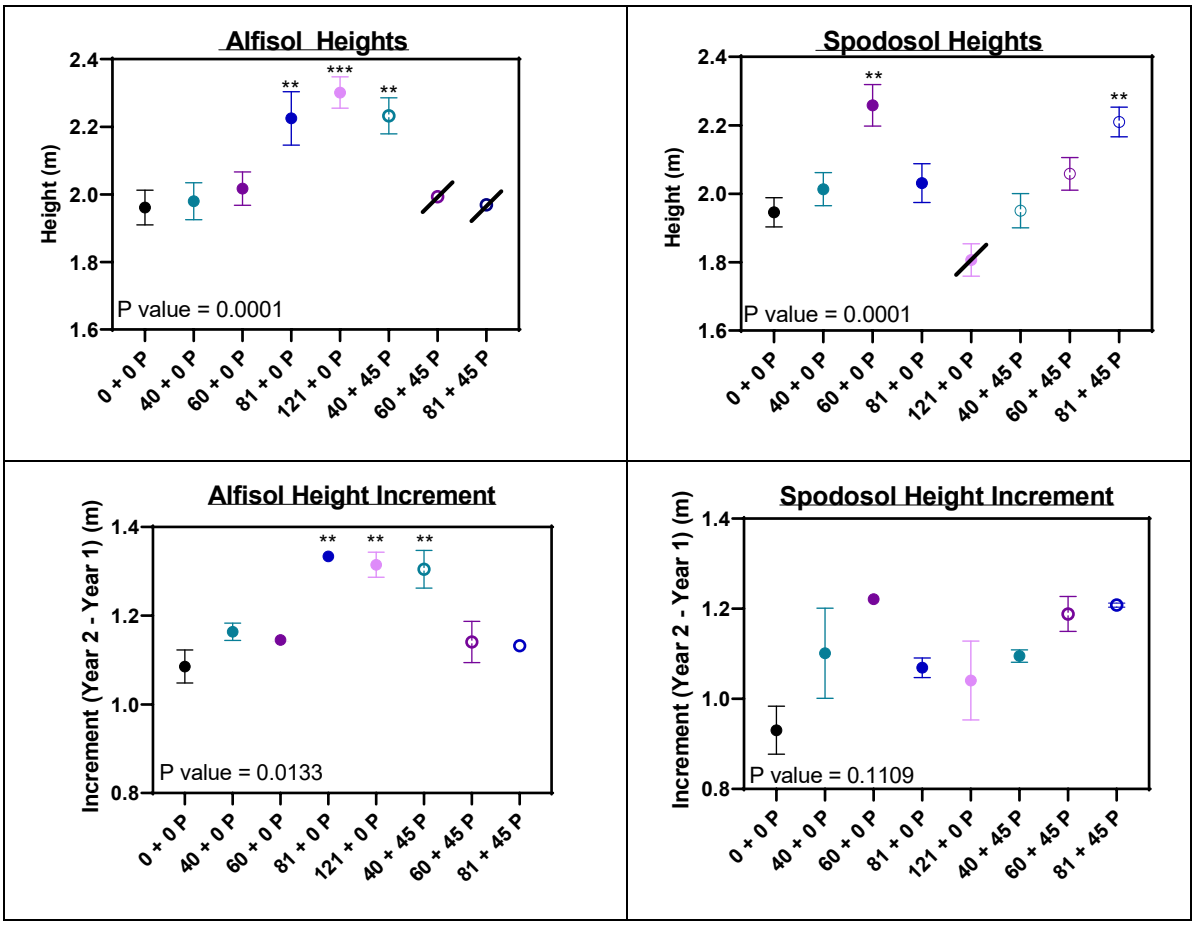

Figure 1.3: (Top) Second rotation, 2-year-old tree heights by P fertilization treatment show effects for the Alfisol and Spodosol. Re-fertilized plots (hollow circles) have an additional 45 kg P ha^{-1} applied at the planting establishment to the base fertilization rates from the first rotation. Carryover treatments (solid circles) only had fertilizer applied in the first rotation. Slashed circles had issues with weed control or harvesting error. (Bottom) Height increment increases from one-year-old trees to two-year-old trees. Each treatment was compared to the control (0 + 0 P) within each site using Dunnett's multiple comparisons (*). Error bars represent treatment standard error. Asterisk's statistically significant differences from the control are (*) weak p-value < 0.10, (**) moderate p-value < 0.05 (***), and strong p-value < 0.01 (Muff et al., 2021).

Foliar P

The Alfisol and the Spodosol had 70 % to 150 % significant decreases in needle concentration P from year 1 to year 2 (p-value < 0.01). At the Alfisol, re-fertilized carryover treatments were higher in foliar P than the control treatment for year 2. The re-fertilized at-planting treatments had the lowest decrease in needle concentration P from year 1 to year 2 for the Alfisol (*Figure 1.4*).

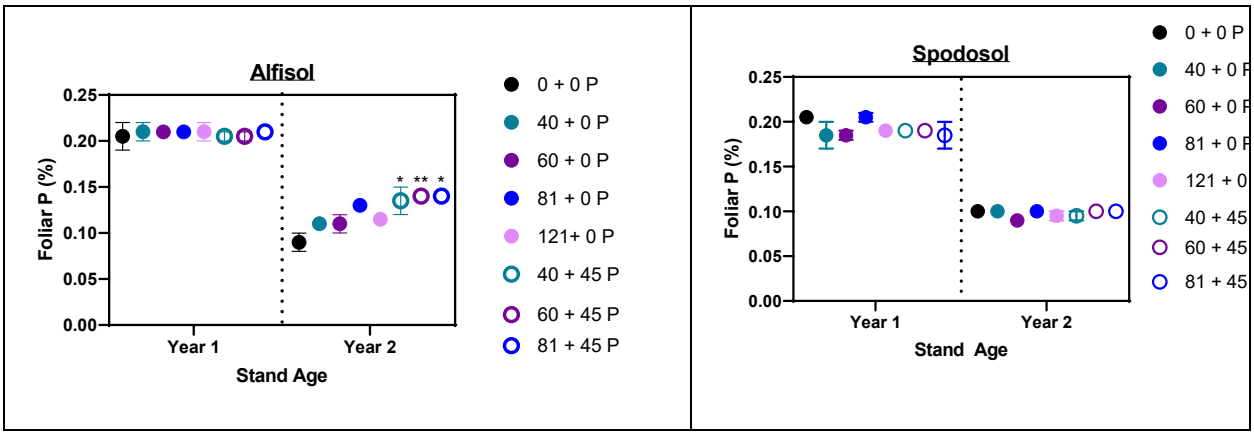

Figure 1.4: Foliar P concentration change from year 1 to year 2 for the Alfisol (left) and the Spodosol (right).Right) soil sites. Asterisks (*) indicate differences between year 2 two foliar P concentrations of each treatment from the control. Error bars represent the standard error of the treatment mean. (*) Weak p-value < 0.10 (**) Moderate p-value < 0.05 (***) Strong p-value < 0.01. (Muff et al., 2021).

O horizon P

In the first rotation, the Spodosol accumulated more O horizon mass and P content than the Alfisol for almost all treatments (p-value = 0.04). This relationship was strongly related to the amount of P applied during the first 12 years of growth for both sites (p-value ≤ 0.01); Tacilla, 2015; Figure 1.5).

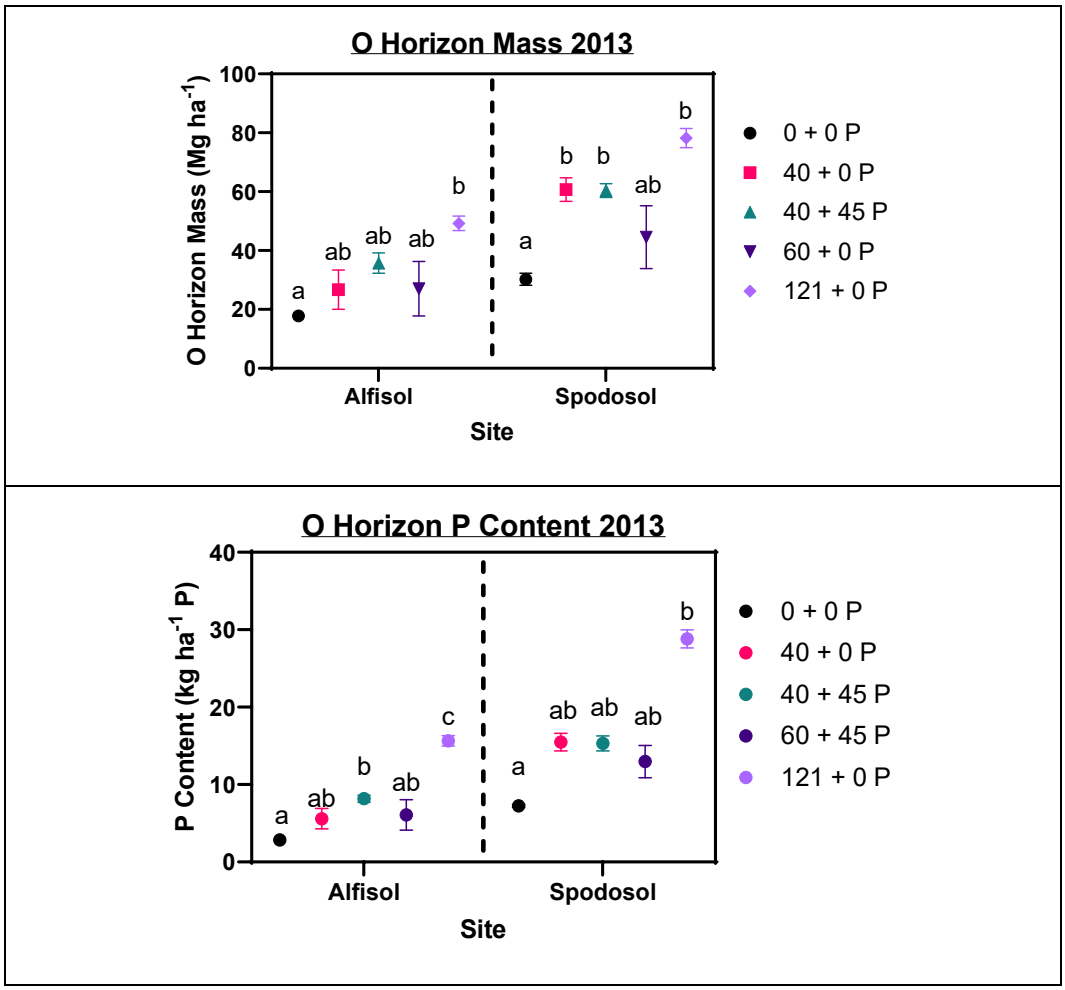

Figure 1.5: O horizon mass (Top) and P concentration (Bottom) was collected 13 years after the establishment of the first rotation. Each site was modeled separately and separated by the dotted line. Error bars represent standard error for each treatment mean. Non-connecting letters significantly differ using a 0.10 alpha value generated via Tukey's HSD.

In the Alfisol, resin-P was highest in the re-fertilized 40 + 45 P treatment, and the highest carryover treatment, 121 + 0 P. These increases occurred during the second and third burial periods, 0–90 and 90–180 days post-planting and fertilization (p-value = 0.03; Figure 1.6). The average increase in resin P from pre to post harvest on the 40 + 45 P and the 121 + 0 P in the 0-90 day burial period was 598 % and 191 %, respectively. Carryover effects were only observed for the 121 + 0 P plots compared to the control (p-value ≤ 0.01. The control plots had no measurable carryover resin-P in any year. Mean carryover resin-P increased from 0 to 270 days post-planting in control and 40 + 0 P treatments and up to 400 days in the post 60 + 0 P treatments (Figure 1.6). Comparing sites, the Spodosol had almost one order of magnitude

greater resin-P than the Alfisol (p-value ≤ 0.01). Resin-P increased in all carryover treatments except the 121 + 0 P treatment until the 4th or 5th burial period. The 40 + 0 P and the 60 + 0 carryover treatments had significantly higher resin-P across burial periods than the control (p-value = 0.04). The re-fertilized treatment, 40 + 45 P, had an immediate response, resulting in a 945 % average increase in resin-P to fertilization in the 0 to 90 days post-planting, post-fertilization burial period. This fertilized treatment maintained higher levels of resin-P for the subsequent five burial periods.

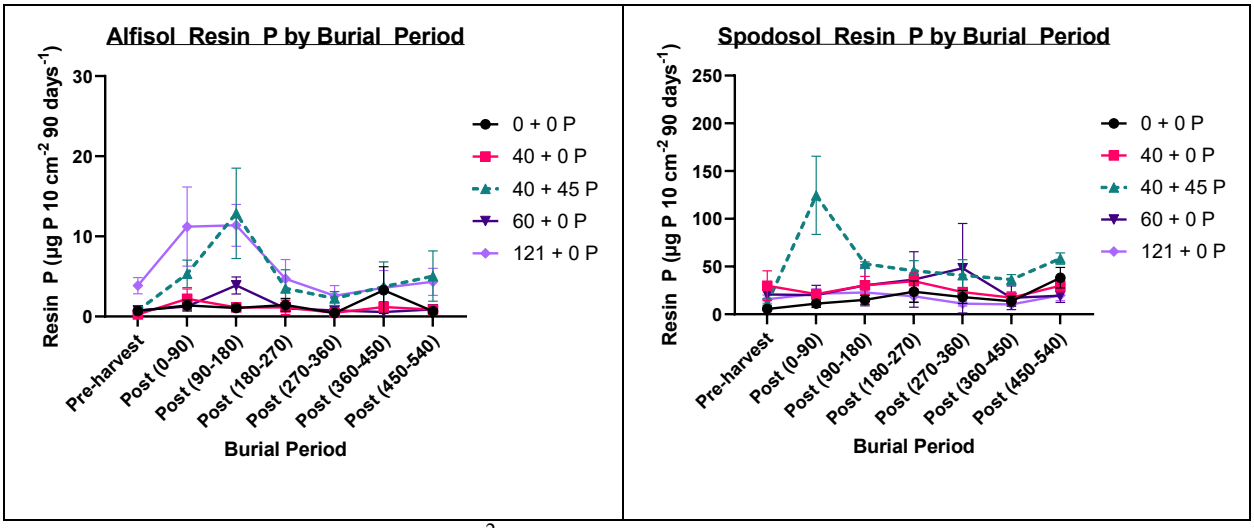

Figure 1.6: Resin-P (µg P per 10 cm^2 of resin over 90 days) changes over seven burial periods (total of 600 days) for Alfisol (Left) and Spodosol (Right). Each line and color represent a different treatment through time. The dashed line represents the re-fertilized treatment at planting in the second rotation. Error bars represent one standard error of the mean for each burial period and P fertilization treatment.

Mehlich 3 Phosphorus

Mehlich results changed significantly from the main effects of year and P fertilization treatment (p-value ≤ 0.01). M3P was above the five ppm P threshold for *P. taeda* growth (Wells et al., 1986) in all treatments and years collected, including the control for the Alfisol. ANOVA results indicated significant differences between P treatment means (p-value = 0.03) for year 1, showing that 121 + 0 P carryover treatment had a 360 % increase from the control treatment. Year 2, one-year post-fertilization, indicated that the re-fertilized 40 + 45 P and the 121 + 0 P treatments were significantly higher than the control by 393 % and 437 % (p-value ≤ 0.01 and 0.014, respectively). By year 3, the 40 + 45 P and the 121 + 0 P treatments returned to year 1 levels in

the top 15 cm of soil. No significant carryover effect was detected for the Spodosol treatment at year 1 (pre-fertilization). In year 2, one year after fertilization, all treatments were significantly higher in M3P than the previous year (p-value = 0.02) but were not proportional to the amount of carryover or fertilized P applied. By year 3, three years post-planting and fertilization, all treatments were significantly higher in M3P than year 1 pre-fertilization levels (p-value ≤ 0.01). Only the 121 + 0 P (p-value = 0.05) treatment was higher in year 3 than in the previous year (Figure 1.7).

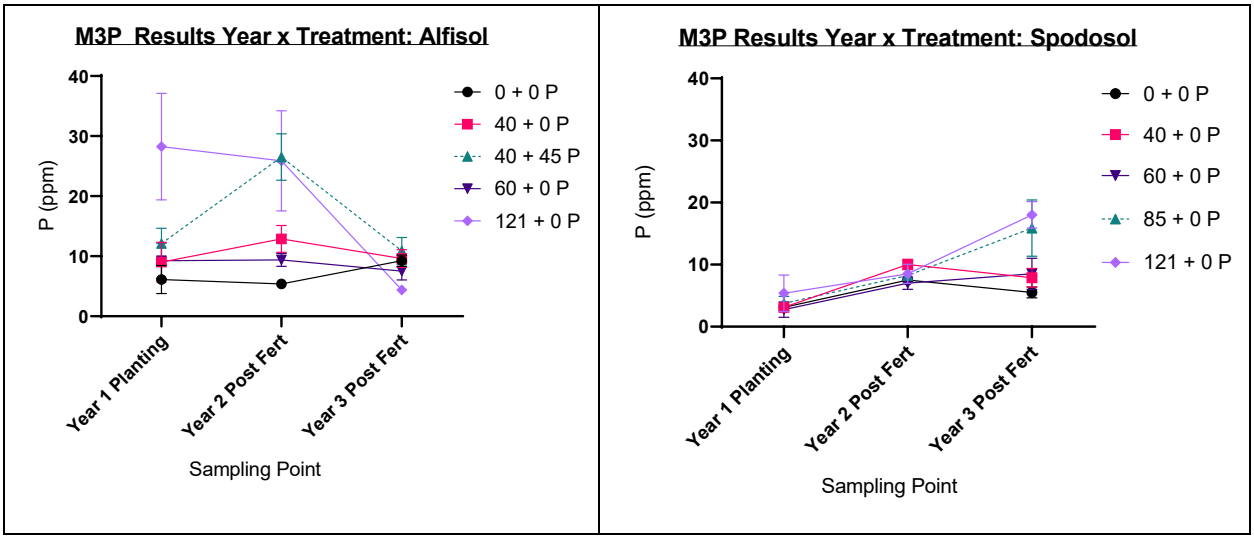

Figure 1.7: Mehlich 3 Phosphorus results in the top 15cm of soil between Alfisol and Spodosol over the first three years from the establishment of the stand. Carryover treatments (solid lines) show decreases in M3P in the highest P fertilization treatment (121 + 0) by Sampling Point. In contrast, the highest treatments for Spodosol are significantly higher after three years post-fertilization. In the Spodosol results indicate 0 + 0 P, 40 + 0 P, and 60 + 0 P are dropping off in M3P after year 2 while the highest (121 + 0 P) and the re-fertilized treatments (40 + 45 P) are continuing to increase in year 3 Post Fertilization. Error bars represent one standard error of the mean for each sampling point and P fertilization treatment.

Resin-P and Tree Height

For the Alfisol, it appeared that pre-harvest samples (p-value = 0.02; R^2: 0.49) and the post-planting (180–270 days) (p-value ≤ 0.01; R^2: 0.58) burial periods had the best overall relationship to tree height growth by year 2. Only the pre-harvest resin-P samples collected for the Spodosol had a weak correlation to plant height at year 2 (p-value = 0.08; R^2: 0.37). Samples collected after harvest had little relationship to tree height responses (Table 1.6).

Mehlich 3 and Tree Height

The relationships between tree growth and production varied depending on the location of the sample within the plot, the year of measurement, and the site. In the case of the Alfisol, tree heights exhibited a stronger relationship with samples collected from the Bed (p-value = 0.03; R^2: 0.46), while there was no significant relationship with samples taken from the interbed (p-value = 0.84; R^2: = 0.016). Specifically, the height of two-year-old trees in the Alfisol positively correlated with the quantity of M3P obtained from each soil sample collected in the second year. On the other hand, for the Spodosol, the relationships between tree growth and soil samples varied significantly depending on the year of measurement and the timing of sampling (Table 1.6, Figure 1.8).

Table 1.6: Regression analyses between two-year-old plot height mean against foliar P, Mehlich 3 Phosphorus, and resin-P assessments considering all treatments (detail of treatments) at each sampling time for the Alfisol and Spodosol sites.

Method	Sample Collected	R^2	P-value	Eq.
		Alfisol		
Foliar P	Year 1	0.01481	0.67	Y = 1.026*X + 0.6702
	Year 2	0.09325	0.28	Y = 0.03741*X + 0.04141
M3P 0-15 cm	Year 1	0.4943	**0.02	Y = 1.180*X – 7.061
	Year 2	0.4658	**0.02	Y = 16.45*X – 94.29
Resin-P	Post (0-90)	0.3575	*0.06	Y = 7.405*X – 45.70
	Post (90-180)	0.3806	*0.05	Y = 10.01*X – 59.93
	Post (180-270)	0.5773	**0.01	Y = 0.9192*X – 5.221
	Post (270-400)	0.1245	0.31	Y = 0.8718*X – 4.740
	Post (400-450)	0.1167	0.33	Y = -2.983*X + 23.85
	Post (450-540)	< 0.018734	0.79	Y = 0.6961*X – 2.139
		Spodosol		
Foliar P	Year 1	0.5303	***<0.01	Y = 0.07511*X + 0.1233
	Year 2	0.06583	0.35	Y = -<0.016397*X + 0.1102
M3P 0-15 cm	Year 1	0.3714	*0.081	Y = 7.621*X – 37.23
	Year 2	0.69	***<0.01	Y = -0.5821*X + 7.803
Resin-P	Post (0-90)	< 0.01234	0.90	Y = -5.597*X + 89.93
	Post (90-180)	0.03814	0.61	Y = -4.562*X + 53.10
	Post (180-270)	< 0.012781	0.89	Y = 0.5348*X + 16.40
	Post (270-400)	0.01876	0.81	Y = -1.757*X + 26.38
	Post (400-450)	0.01494	0.76	Y = -2.651*X + 34.97
	Post (450-540)	0.2813	0.17	Y = -19.55*X + 160.1

(*) Weak p-value < 0.10 (**) Moderate p-value < 0.05 (***) Strong p-value < 0.01

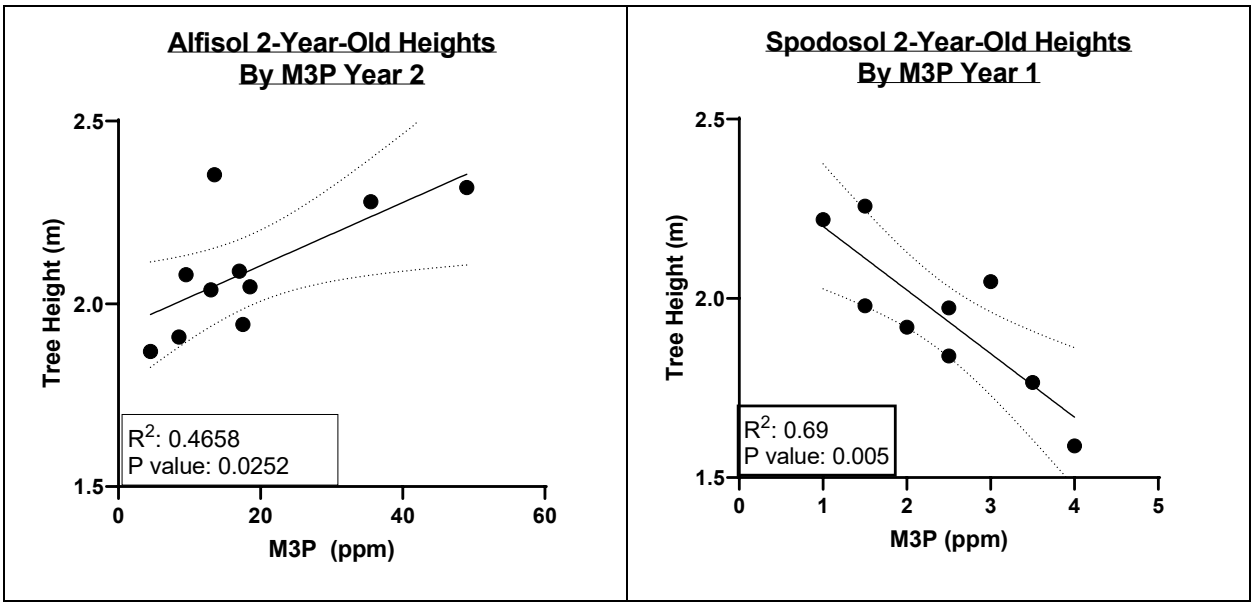

Figure 1.8: Linear regression with 95 % confidence intervals (dotted lines) between Mehlich 3 Phosphorus (ppm) and tree height (m). Note scale on the x-axis for the Spodosol shows deficient levels of M3P, well below critical levels, *so* regression results may be suspect.

O Horizon and Tree Height

The relationship between the O horizon P content from the previous rotation and the growth of the second rotation was examined for both year 1 and year 2 at both sites (Table 1.7). The results showed that the P content, P concentration, and forest floor mass from the first rotation were moderately to strongly correlated with the height outcomes of the Spodosol in the first year of growth. However, these variables did not show a significant relationship with the height results in the second year of growth. In the case of the Alfisol, the O-horizon P content exhibited a moderate association with the growth outcomes in the first year and a strong correlation with the growth results in the second year (Figure 1.9).

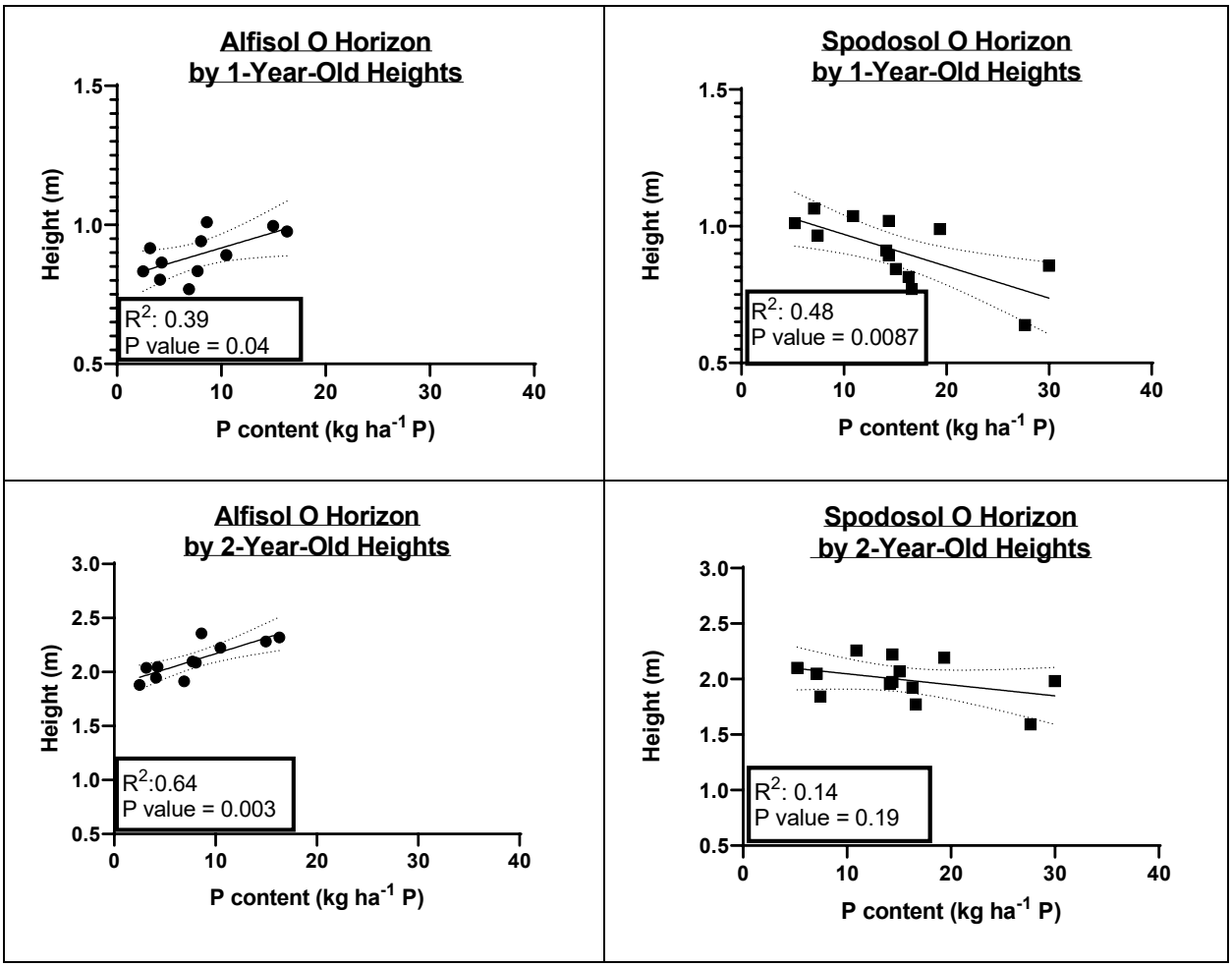

Figure 1.9: First rotation O horizon P content relationships to average plot-level tree height for the second rotation's first and second year of growth. a: Alfisol year 1, b: Alfisol year 2, c: Spodosol year 1, d: Spodosol year 2.

Table 1.7: First rotation O horizon P content, concentration, and mass collected in 2013 as it relates to second rotation one-year-old and two-year-old tree heights.

Alfisol				
Method	**Rotation Age**	**R^2**	**p-value**	**Eq.**
O Horizon P Content (kg ha^{-1} P)	Year 1	0.3912	**0.039	Y = 35.23*X – 23.58
	Year 2	0.6431	***<0.01	Y = 22.03*X – 38.60
O Horizon P Conc. (ppm)	Year 1	0.3828	**0.04	Y = 437.0*X – 157.5
	Year 2	0.6748	***<0.01	Y = 283.0*X – 403.6
O Horizon Mass (Mg ha^{-1})	Year 1	0.2678	0.10	Y = 77.78*X – 37.53
	Year 2	0.5042	**0.01	Y = 52.06*X – 77.76
Spodosol				
O Horizon P Content (kg ha^{-1} P)	Year 1	0.4799	***<0.01	Y = -41.15*X + 52.66
	Year 2	0.1496	0.19	Y = -15.07*X + 45.34
O Horizon P Conc. (ppm)	Year 1	0.2412	*0.08	Y = -186.5*X + 452.8
	Year 2	0.07491	0.40	Y = -68.16*X + 419.3
O Horizon Mass (Mg ha^{-1})	Year 1	0.4488	**0.01	Y = -103.2*X + 146.2
	Year 2	0.1098	0.26	Y = -33.46*X + 119.3

(*) Weak p-value < 0.10 (**) Moderate p-value < 0.05 (***) Strong p-value < 0.01

Discussion

Our experiments aimed to distinguish the two factors influencing the need for fertilization at planting: the growth response of *P. taeda* based on soil P build-up in the first rotation and the identification of soil tests and conditions that best represent this build-up for each site (Ballard, 1978; Comerford et al., 2002; Crous et al., 2007; Everett and Palm-Leis, 2009). Initial tree height data collected in 1999, before any fertilization treatments, showed no significant differences among plots for either site (data not shown). However, data from year 2 of the second rotation indicated that carryover P positively affects productivity (Figure 1.3). Our findings suggest that applied P at both sites directly influences tree height in the subsequent rotation, compared to control plots that received no P in either the first or second rotation. However, the relationship between increasing P rates and height growth responses for two-year-old *P. taeda* was not consistently proportional. In the case of the Alfisol, our data indicates that application of P exceeding 81 kg P ha^{-1} from either the first or second rotation results in a 13% to 17% increase in total height responses. Treatments with less than 81 kg P ha^{-1} did not show growth responses compared to the controls. On the other hand, results for the Spodosol were highly variable, and we

cannot provide reliable suggestions for the minimum P amounts required to enhance overall growth. We attribute some of this variability to weed control issues and harvesting errors in the first rotation (Albaugh et al., 2015). Carryover treatments from the first rotation had moderate to strong effects on the overall heights of both the Spodosol and the Alfisol. In the case of the Alfisol, responses appeared to increase when more than 81 kg P ha^{-1} was applied from the first rotation, resulting in a 13% increase in total height.

Foliar P

By the second year, all plots and treatments dropped in foliar P concentration significantly, with many treatments at or just above the 0.10 % critical threshold. We attribute this flush of P uptake by the trees during year 1 to the increased rates of mineralization of organic material due to the disturbance of harvest (Kiser & Fox, 2012). The duration of nutrient release from the initial flush from decomposition, as shown by foliar P, appears to be highly dependent on site and soil characteristics (Everett and Palm-Leis, 2009). The Spodosol significantly dropped across all treatments in foliar P, even compared to the Alfisol. This shift in foliar P was inconsistently related to the O horizon content collected in 2013. We found that the P content of O horizon in the first rotation was negatively associated with increases in foliar P during year 1 of the second rotation for the Spodosol but not the Alfisol. The results from the Spodosol agree with Comerford et al. (2002), who concluded that the breakdown of organic matter in the O Horizon would only supply enough P for the first year of growth. This result from year 1 did not carry into year 2 for the Spodosol, nor did it have any connection to each other on the Alfisol. Compared to Everett and Palm-Leis (2009), which took place on an Albaquult in the Lower Coastal Plain of South Carolina, our results appear accelerated by comparison. Peak foliar P concentrations on the Albaquult took place in year 2 and fell off by year 3, indicating the possibility that P cycling and mineralization from the Assart effect was occurring slower on their soil than our two soils.

Foliar P results highlight an apparent response to fertilization on the Alfisol, with all the treatments fertilized with P having significantly higher foliar P than the control plots. Interestingly, the response to fertilization was not reflected in Spodosol foliar P for any of the carryover or re-fertilized treatments. The Spodosol results suggest that many treatments are at or below the critical threshold for foliar P in year 2 (*Figure 1.4*). Yet, the same treatments are highly variable in height,

implying that foliar P might not be the best metric to use when testing for P deficiencies in these sites at this stage. of development.

P in Mineral Soil

Resin-P results were variable between sites, with the Spodosol adsorbing 100 % more resin-P over each burial period than the Alfisol for some treatments. These resins appeared to capture the magnitude and extent of the initial pulse of nutrients from harvest and site preparation for each treatment as the excess organic material was mineralized into the inorganic P pool in the top 15 cm of soil (Comerford & De Barros, 2005a). The most significant differences occurred in the fertilized treatment for the Alfisol and the Spodosol shortly after fertilization. Both soils had resin-P peaks, but the Spodosol peaked one burial period sooner than the Alfisol by 90 days. This could prove that fertilizer P is cycling into the soil much faster in the Spodosol than the Alfisol. Both 40 + 45 P treatments, for each site dropped off in the following burial periods but were significantly higher than any other treatment in the subsequent burial periods. The magnitude of this response was site dependent, with the fertilization causing the Spodosol to increase by 990 % while only 142 % for the Alfisol. In the following burial period, the Spodosol decreased by 52 %, while the Alfisol decreased by almost 267 % from the peak, even though the same amount of fertilizer was applied at both sites. These results are similar to previous results showing how the initial flush of nutrients is quickly sequestered away into plants (Condron et al., 2005) or mineral soil horizons (Kiser & Fox, 2012). Only the 121 + 0 P carryover treatment for the Alfisol differed from the control treatments during the first 180 days. In the Spodosol, all P fertilization treatment rates were higher than the control for most burial periods, indicating that carryover P from the previous rotation, regardless of rate, had some influence on how much P was in the top 15 cm of soil.

This 121 + 0 P treatment on the Alfisol also had the highest overall P content in the O horizon, indicating that the probes were capturing the increased mineralization of the O horizon in the resin probes (Polglase et al., 1992; Comerford & De Barros, 2005a). The lack of a response to the lower P carryover treatment rates might lead one to assume that the P is being sequestered into deeper horizons in the Alfisol; however, Tacilla (2015) found, on the same site, that the majority of extractable P was found in the top 20 cm of soil. M3P results from the Alfisol show P levels are above critical (Wells et al., 1986) for all treatments. The first rotation's O horizon mass and P

content data provided exciting results. In agreement with several previous studies (Everett and Palm-Leis, 2009; Comerford et al., 2002), O horizon P cycling is integral in providing Psystems. The rates at which these systems cycle can change depending on the site and soil characteristics. The Spodosol had significantly higher amounts of O horizon mass and P content across most treatments than the Alfisol. P content, concentration, and O horizon mass were related considerably to one-year-old heights but not two-year-old heights in the Spodosol.

In contrast, although the Alfisol had lower overall P content, concentration, and O horizon masses, the relationship to height response increased from year 1 to year 2 for the Alfisol, indicating that the Alfisol was still cycling through the O horizon P from the first rotation. These results also agree with our resin probe data which shows that the cycling of fertilized phosphorus took place at a slower rate in the Alfisols compared to the Spodosols. This indicates that the Spodosol is either leaching P into the subsoil faster or the P in these soils is being sequestered away into unavailable forms more quickly in the Spodosol than the Alfisol. The lack of a relationship with O horizon P content, concentration, or mass to two-year-old heights for the Spodosol also agrees with the foliar P results, which had significant drops in foliar P concentrations for two-year-old heights. This also implies that most P taken up by the tree comes from the decomposition of the O horizon in Spodosols and Alfisols and that the decomposition or sequestration of available P forms into unavailable forms is happening faster than in Alfisols.

Management recommendations

Our study aimed to determine optimal fertilization strategies for Alfisols and Spodosols in terms of tree productivity and development. For Alfisols, our results indicate that re-fertilization with 45 kg P ha^{-1} at planting is recommended when soil M3P levels fall below 20 ppm (as measured at planting from the Bed) or when resin probe data indicates less than 10 µg P 10 cm-2 over a 90-day burial period within the first 180 days post-planting. Low carryover rates of P up to 60 + 0 kg P ha^{-1} from the first rotation were found to be ineffective in maintaining optimum tree productivity, compared to higher rates that may not be economically feasible.

Regarding Spodosols, the carryover rates of P exhibited high inconsistency in relation to overall height responses of two-year-old trees. From our data analysis, only the 60 + 0 kg P ha^{-1} carryover treatment and the re-fertilized 81 + 45 kg P ha^{-1} treatment showed a significant difference from the control plots. Interestingly, resin-P measurements showed no significant relationship with tree

heights in the first two years of measurements. These findings strongly suggest that sampling from the O horizon before the harvest of a mature stand may provide better fertilization recommendations compared to sampling from the mineral soil. Overall, our study highlights the importance of appropriate fertilization strategies for different soil types. For Alfisols, re-fertilization with 45 kg P ha^{-1} at planting is recommended when specific soil P thresholds are met, while for Spodosols, carryover rates of P may not consistently influence tree growth. These findings contribute to the understanding of optimal fertilization practices and emphasize the significance of considering soil sampling techniques in making effective fertilization recommendations.

Conclusion

With the rising costs of P fertilization, improved placement and management of P fertilization is a requirement for landowners and growers. Determining if and for how long fertilized P remains in the forest system and if that soil P can be carried over into subsequent rotations would save growers on up-front fertilization costs and P testing. Our results demonstrated that the carryover effect occurs on both soils in our experiment. The rate, duration, and magnitude of these responses varied between sampling method, type, and timing of sample taken regarding tree height and plant available P. With our dataset, making confident fertilization recommendations is not currently viable, and the carryover effect will need to be tracked for more time before a clearer picture of P-deficiencies develops on these sites.

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Chapter 2: Using Microdialysis to Assess Soil Diffusive P and Translocated Sap Flow P Concentrations in Southern *Pinus taeda* Plantations.

Highlights

- Microdialysis detected changes in carryover rates for both sites using diffusive P.
- The Spodosol had nearly ten-fold higher rates of diffusive P than the Alfisol across all P fertilization treatments.
- There was a 138 % increase in sap flow P from the control plots to the plots that received 121 kg ha^{-1} P.

Abstract

To investigate the impact of phosphorus (P) fertilization from a previous rotation on the growth of newly established loblolly pine (*Pinus taeda* L.) in a subsequent rotation, a phosphorus carryover fertilization experiment was conducted in the southeastern United States. In this study, a novel technique called microdialysis was employed to assess diffusive soil P. The technique was applied to bulk soils under laboratory conditions and within actively growing *P. taeda* trees in situ in the field. Significant variations in diffusive P were observed between the two sites (Alfisol and Spodosol) concerning the timing of sampling and P fertilization treatments. The Spodosol soils exhibited diffusive P levels 15 to 50 times higher than those collected from the Alfisol. On average, diffusive P increased by 137% in the Spodosol and 166% in the Alfisol from bulk soil samples collected at planting to one year after planting. The effects of P fertilization treatments on diffusive P varied depending on the site and the rate of P fertilization. In the Alfisol site, diffusive P showed a strong relationship with tree height, whereas no significant association was observed in the Spodosol. The Mehlich III soil extraction method did not demonstrate a strong correlation with diffusive P for either site. Additionally, in a unique assessment to determine if P fertilization treatments affected translocated P concentrations within the trees, 16 in-situ microdialysis samples were collected from a single tree within each treatment plot and extracted over four hours for 16 consecutive days. The site did not influence the results measuring sap flow P but was responsive to changes in P fertilization rates compared to the control. It was observed that atmospheric conditions substantially impacted sap flow P, with samples collected in full sunlight exhibiting an average increase of 100% compared to samples collected during overcast conditions. Overall, these findings highlight the potential of microdialysis as a valuable tool for soil P testing and its potential for expanding applications to address more complex questions related to P translocation and tree physiology in silvicultural settings.

Introduction

Phosphorus (P) availability has a complex assortment of chemical and physical environmental controls, determining its availability to plants. P is critical for plant growth but is the least mobile and available macronutrient, making it the most common limiting nutrient after nitrogen (N) (Raghothama & Karthikeyan, 2005). P deficiencies are common on the Atlantic Coastal Plain, characterized by highly weathered acidic soils with little native exchangeable P remaining in the underlying parent material (Polglase et al., 1992). Plants have developed strategies to accumulate P, such as increasing total root surface through macroscopic and microscopic root growth (Jungk et al., 2001), releasing extracellular enzymes called phosphatases and phytases to mine organic sources or develop associations with mycorrhizal fungi, which enhances the first two strategies (George & Jakobsen, 1995). These mining strategies create P depletion zones that cause a passive diffusion gradient around the root (Bhat & Nye, 1973). The strength of this diffusional gradient and its effects on the diffusion of P from soil surfaces are dictated by the native soil P present in the system and the overall buffering capacity (Holford, 1997). Therefore, to properly understand P uptake from the surrounding environment, methods need to be developed to model this diffusional gradient with higher resolutions (Santner et al., 2012).

Natural Sources of P and Fertilization

In P-limited sites where soil supply cannot meet plant demand, fertilization is used to substitute for the deficit. P fertilization in *Pinus taeda* L. stands on the Atlantic Coastal Plain has been shown to benefit growth in P-deficient soils (Pritchett & Swinford, 1961; Gent et al., 1986) and has become common practice in plantation management (Fox et al., 2011). It has been estimated that almost 80 % of applied P as fertilization is quickly sequestered away into insoluble forms, leaving the remaining P either in solution or taken up by the trees for immediate use (Holford, 1997). It has been found that a single application of P on deficient sites can maintain optimized productivity for *P. taeda* for the entire length of a stand rotation and even into the subsequent rotation as residual P (Pritchett & Comerford, 1982, Comerford & De Barros, 2005; Everett & Palm-Leis, 2009). This residual P from the fertilization remaining in the stand from the forest floor and subsoil and its availability to the subsequent rotation is referred to as the P carryover effect or legacy effect (Everett & Palm-Leis, 2009). Build-up from fertilization has been

studied extensively in agricultural soils but is poorly understood in forest systems (Zhang et al., 2020; Zhu et al., 2018; Blackburn et al., 2018). Thus, understanding the relationships between soil chemical properties and long-term availability is essential to predict response to fertilization. Everett and Palm-Leis (2009) found on highly weathered Ultisols that tissue concentrations of P for young *P. taeda* plantations began to fall below peak values two years after the harvest, implying that much of the P from these sites came from the mineralization of organic material and the previous forest floor and not from the mineral soil. However, Everett and Palm-Leis (2009) studied only one soil on the Atlantic Coastal Plain. A more comprehensive understanding of how residual P fertilizers carry over into subsequent rotations across multiple soils and parent materials is critical to optimize early P fertilization regimes across the southeastern U.S.

Soil Extraction Methods

Plants absorb P as soluble inorganic orthophosphate ions in solution (PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$) via diffusion by active membrane transporters on the root's surface, as well as through the of mycorrhizal fungi (Schachtman et al., 1998; Plassard et al., 2019). The soluble phase of P without plant root interference can be viewed as the soil's equilibrium of P's dissolution, desorption, and mineralization in that soil at any given time (Stevenson & Cole, 1999). The soluble phase P pool is the smallest, often containing less than 0.2 mg L^{-1} P even on highly fertile agricultural soils, implying that most plant-available P originates from the solid phases of soil P (Grant et al., 2005). Solution P can be immobilized via soil microbes or plants, absorbed onto mineral surfaces, or precipitated with secondary Fe and Al hydrous oxide compounds found in the soil (Schachtman et al., 1998). Depending on environmental and soil conditions, the remaining unavailable pools of P can be multiple orders of magnitude higher than solution P.

Methods such as Mehlich III (Mehlich, 1984), resin-P (Sibbesen, 1978), and fractionation (Hedley et al., 1982) separate or incorporate one or more P pools into a final reported value. These approaches, however, are often difficult to relate to soluble phase soil P because they also mobilize one or more of the unavailable forms of P that may not be available to plants. Mehlich III soil testing is widely used in agriculture and forestry on acidic soils to provide fertilizer recommendations (Mehlich, 1984; Wells et al., 1986). Mehlich III strips both inorganic and organic forms of P from the soil using a combination of acetic acid (CH_3COOH), ammonium nitrate (NH_4NO_3), ammonium fluoride (NH_4F), nitric acid (HNO_3), and

ethylenediaminetetraacetic acid (EDTA) at pH 2.5 to determine both readily and slowly available P. The resulting solution is quantified colorimetrically or by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and contains both inorganic and organic forms of P that were stripped from the soil and dissolved into solution. Mehlich III is a valuable tool to test for acid-extractable forms of P; however, in highly P deficient soils, with < 6 mg kg^{-1} of extractable P, Mehlich III soil tests are difficult to correlate to increases in *P. taeda* productivity (Wells et al., 1986). This difficulty in establishing a relationship between Mehlich III and *P. taeda* on these soils highlights the need for an alternative soil test to predict responses to growth and available P better.

Microdialysis

Microdialysis bears many comparisons to a plant root (Demand et al., 2017; McKay et al., 2021) and potentially offers researchers a relatively simple method to assess P uptake from diffusive gradients under various circumstances. Microdialysis emerged from neurobiology and pharmacokinetics to study the concentrations of neurotransmitters and compounds in both *in vivo* and *in vitro* solutions via passive diffusion (Kalant, 1958; Plock & Kloft, 2005). The process of microdialysis requires an infusion pump, a probe, a perfusate solution, and a collection tube or vial. The probe consists of a thin inner tube surrounded by an outer semi-permeable membrane, which pushes the perfusate through the membrane allowing solutes to passively exchange across the surface into the perfusate solution depending on the concentration of gradients of both the probe and exterior solution. The microdialysis pump pushes the perfusate solution through the microdialysis probe at a constant push-pull rate ranging from 0.5 – 5 µl/min. The dialysate (perfusate that has passed through the probe) is collected in a small collection receptacle (Figure 2.1). The result is a dialysate solution mimics the concentrations of metabolites, compounds, and overall composition of the tissue or matrix being tested.

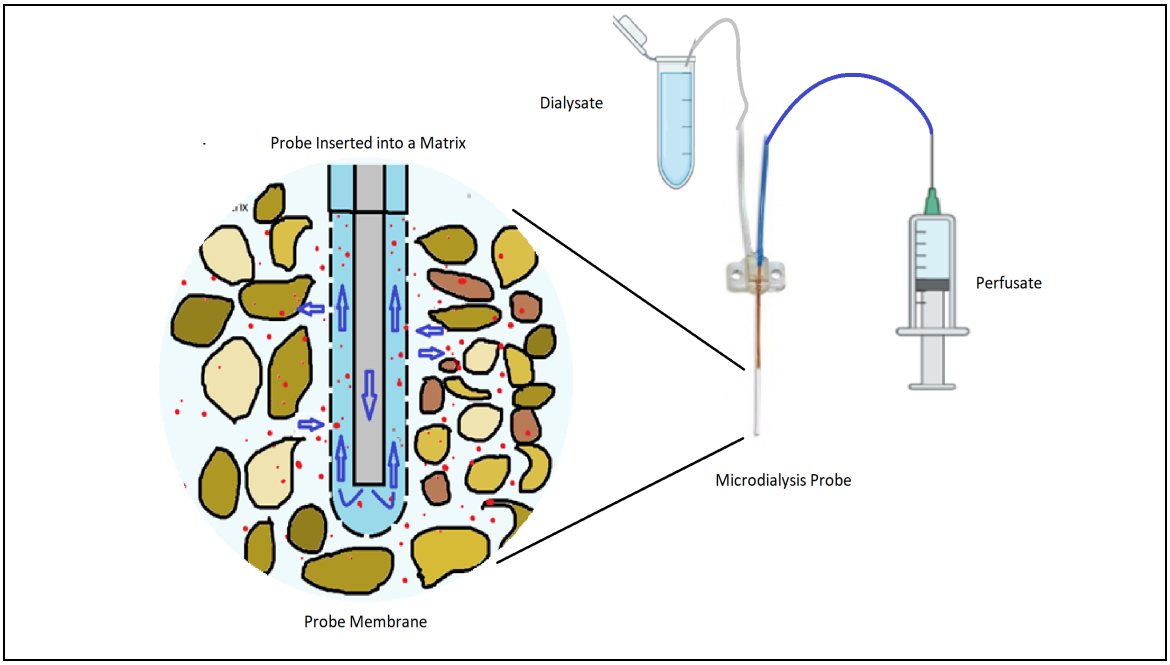

Figure 2.1: Experimental setup of a microdialysis probe is inserted into a matrix; input into the probe is called the perfusate; the output collected is the dialysate. A syringe pump controls the pump rate. Image of microdialysis probe adapted from Harvard apparatus. Holliston, MA.

Microdialysis has made significant breakthroughs in studying environmental equilibrium samples by offering a non-destructive method for assessing ion concentrations in solution. Microdialysis testing of soils has led to a greater understanding of diffusive N (Inselsbacher & Näsholm, 2012), heavy metal concentrations in soil solutions (Mosetlha & Wibetoe, 2007), and P availability in soil solutions (Demand et al., 2017). Equilibrium methods are commonly used to measure soils' adsorption and desorption of P ions. These equilibrium methods can be performed on inorganic or organic soil phases by adding a known amount of P into the soil and waiting for the soil to reach equilibrium before the solution is remeasured (Barrow, 1978; Oburger et al., 2011). These experiments provide valuable information regarding P dynamics in bulk soil; however, they fail to account for the processes on and near the plant roots as simulated by the probe (Figure 2.1). Microdialysis takes the root interactions into account by creating a pseudo-rhizosphere mimicking both the exudation of P mobilizing compounds, such as citrate (Ryan et al., 2014), and the creation of the diffusional sink that occurs near the plant roots (Demand et al., 2017; Oburger et al., 2011; McKay et al., 2021). The P that is mobilized and sampled by microdialysis should be analogous to a plant root and provide an additional method for analyzing

plant available quantities of nutrients (Shaw et al., 2014; Demand et al., 2017; Inselsbacher & Näsholm, 2012).

P deficiencies in soil for plants manifest in the xylem sap with increases or decreases in the production of various amino acids and inorganic PO_4^{3-} (Sung et al., 2015; Lima et al., 2000; Mason and Larsen, 1965). Current approaches to observe these changes in xylem sap composition require destructive sampling methods to extract these compounds from plant tissue. Microdialysis allows researchers to sample metabolite concentrations within living plant tissue in a non-destructive sampling method. To our knowledge, only two experiments have attempted to study *in vivo* plant metabolites: Pretti et al. (2014) used microdialysis to study ascorbic acids and antioxidants in water-stressed *Opuntia fixus indica*, and Jeřábek et al. (2020) was the first report to show high-frequency *in situ* measurements of PO_4^{3-} in the xylem sap of living beech trees using microdialysis probes. Diurnal fluctuations detected in these probes over 24 hours were consistent with destructive sampling methods (Clark et al., 1986; Siebrecht et al., 2003), albeit at significantly lower overall concentrations in some cases, demonstrating this method is a viable non-destructive option to track xylem PO_4^{3-} within the sap with the probes. Because we know that P deficiencies manifest in xylem sap in *P. taeda* (Mason and Larsen, 1965) and that microdialysis probes are an effective method to track these changes in trees (Jeřábek et al., 2020), we propose that *in vivo* measurements using microdialysis probes of xylem sap in P-deficient *P. taeda* plantations offer an additional approach to tracking nutrient deficiencies in actively growing *P. taeda*.

Goal and Objectives

Our main goal for this research was to explore the viability of microdialysis probes as an effective tool to assess P deficiencies and P carryover effects in southern pine plantations. Our objectives to achieve this goal were determining if (1) soil microdialysis PO_4^{3-} (diffusive P) obtained from the soil is affected by P fertilization carryover treatment; (2) diffusive P collected via microdialysis has relationships to M3P soil testing; (3) diffusive P obtained from the soil is related to tree growth; (4) *in vivo* sap flow PO_4^{3-} (sap flow P) is affected by P fertilization carryover; and (5) *in vivo* translocated sap flow PO_4^{3-} is related to tree growth or diffusive P. To evaluate these objectives, we collected data on soil diffusive P using the microdialysis technique from two sites previously used as N and P fertilization rate experiment (Tacilla, 2015) that were harvested in December 2018. The sites were re-established with *P. taeda* in the spring of 2020 to

test the P carryover effect, where paired treatments were left with only carryover P or re-fertilized with P at planting to track the impact of residual P in the soil from the previous rotation. Sap flow P data were collected using microdialysis in *P. taeda* trees, *in vivo*, under field conditions under specific carryover treatments during the summer of 2022.

Materials and Methods

Site Design and Treatments

This study is established on long-term plots, previously used in an N and P fertilization rate experiment (the "Regionwide 18") established in 1999 by the Forest Productivity Cooperative (formerly Forest Nutrition Cooperative, Albaugh et al., 2015). Of the original sites, three sites have been harvested and replanted, and the remaining sites will be converted in the next 1 to 6 years (Figure 2.2)

Figure 2.2: Regional map of intensive (sites currently harvested and planted for the Second Rotation) and extensive sites that will be harvested in the next 3-5 years. The red circle indicates both sites that encompass the scope of this experiment.

For this project's scope, we focus on the first two sites replanted in winter 2020 (Figure 2.2). Both sites are located on the Atlantic Coastal Plain and represent two different soils.

Cooperative Research in Forest Fertilization (CRIFF) groups A through F are often used in plantation silviculture as a classification system primarily based on drainage class, texture, and depth to subsurface layers (Fisher and Garbett, 1980). The first site, referred to as the Alfisol, in Northeast (NE) Florida is a poorly drained, fine, mixed, active, thermic, Typic Albaqualf (Meggett series) with marine sediment parent material and an argillic horizon (CRIFF group A). The second site, referred to as the Spodosol, located in Southeast (SE) Georgia, is a somewhat poorly drained, sandy over loamy, siliceous, active, thermic Typic Haplohumods (Leon series), with marine sediment parent material, CRIFF group D, with multiple spodic horizons and no argillic or kandic horizon present within the first 100 cm of soil depth.

The previous Regionwide 18 experiment was harvested in 2019 and will be referred to as the "First Rotation," though the site was planted with pine before the First Rotation of this study (Table 2.1). The current rotation (now named the "Regionwide 28") is called the "Second Rotation." Experimental treatments are arranged at each site in a completely randomized block design with two replicates (Table 2.2).

Table 2.1: Both sites' location, site, and stand properties from the first rotation. The Alfisol study was established at a stand age of three, and the Spodosol was established at stand age of 5.

Site	County and State	Latitude	Longitude	Species	Study Establishment	"Base" Site Index *	Years since P Fertilization in 2019	Stand Age at Harvest (2019)
First rotation 1998 – 2019								
Alfisol	Nassau, Florida	30.6661	-81.8361	*Pinus taeda*	1999	45	21	26
Spodosol	Brantley, Georgia	31.3353	-81.8217	*Pinus taeda*	1998	67	22	25
***Base Site Index at 25 years old**								

Table 2.2: Treatments and cumulative rates of applied fertilizer for the First and Second Rotation. All treatments received NPK in the First Rotation. All treatments received N and K in the Second Rotation—only one treatment for sap flow P received fertilization with P.

	First Rotation Carryover Rates		Second Rotation Re-Fertilization Rates		
Soil or sap flow P Microdialysis	**Cumulative P Fertilization (kg ha^{-1})**	**Cumulative N Fertilization (kg ha^{-1})**	**N at planting (kg ha^{-1})**	**K at planting (kg ha^{-1})**	**P at planting (kg ha^{-1})**
Treatment	Diffusive P				
0 + 0 P	0	0	52	29	0
40 + 0 P	40	400	52	29	0
60 + 0 P	40	600	52	29	0
121 + 0 P	121	1210	52	29	0
Treatment	Translocated Sap flow P				
0 + 0 P	0	0	52	29	0
40 + 0 P	40	400	52	29	0
40 + 45 P	40	600	52	29	45
121 + 0 P	121	1210	52	29	0

The First Rotation plots received a range of cumulative fertilizer applications. The First Rotation treatments sampled for this study included four replications of the following treatments (1) 0 kg P ha^{-1}, (2) 40 kg P ha^{-1}, (3) 60 kg P ha^{-1}, (4) 121 kg P ha^{-1}. The Second Rotation split the four replications of each treatment from the First Rotation into two replications for two different P fertilization treatment groups, 1) "Carryover," no fertilizer P at the establishment for the Second Rotation, 2) "Re-fertilized" with 45 kg P ha^{-1} at the establishment for Second Rotation broadcasted as triple super phosphate (X + Y; X = Carryover rate from First Rotation, Y = Re-fertilization rate from Second Rotation; (Table 2.2). In the Second Rotation, all plots receive N as urea plus a urease inhibitor, potassium (K) as KCl, and a micronutrient mix to remove nutrient limitations besides P. All treatments received 30 ml of Arsenal® herbicide after bedding in the spring for herbaceous weed control.

Soil and Sap Sampling

Soil samples for diffusive P and Mehlich III (M3P) extractions were collected from the same soil samples from two time points. The first time point was collected at "Year 0" in the fall of 2018 pre-harvest. The second time point, "Year 1", was collected in March 2020, after planting but before fertilization (Table 2.3). Two composite samples were collected from each treatment replication: one from the bed (tree row) and one from the interbed (interrow of trees). Each composite sample combined eight soil cores to a depth of 0 to 15 cm. Soil samples were dried for two days at 50 °C, sieved using a 2 mm mesh, and stored at -20 °C for future analysis. Soil samples collected were tested for acid-extractable P using the M3P method and analyzed via ICP-MS (Waters Agriculture Lab. Camilla, GA). Sap flow P was measured from one tree per treatment replication in the summer of 2022, two years after planting in the following treatments (in kg ha^{-1} P): 0 + 0 P, 40 + 0 P, 40 + 45 P, and 121 + 0 P. Diffusive P was collected at Year 0 and Year 1 post-planting with the following treatments used: 0 + 0 P, 40 + 0 P, 60 + 0 P, and 121 + 0 P. Two treatment replications were sampled on each site. Mineral soil pH (1:1, soil/water by volume) was determined using a combination of glass electrode pH, and CEC was determined by the Virginia Tech Soil Testing Lab.

Table 2.3: Sampling points collected for Mehlich 3 (M3P), soil diffusive P and Sap flow P.

Sampling	Mature Stand (Year 0)	Planting (Year 1)	One-year-old Heights (Year 2)	Two-year-old Heights (Year 3)
M3P (Yearly)		X	X	X
Soil Diffusive P	X	X		
Sap Flow P				X

Tree Height Sampling

Each treatment replicate contains six rows with 12 trees per row for ~70 trees per treatment. All 70 trees in the treatment plots were tagged with aluminum tags to track individual tree growth and mortality over time. For the Second Rotation (2019-present), individual tree heights, root collar diameter, and mortality were collected for each measurement plot in the first two years of growth for the Alfisol and the Spodosol. Trees were measured by hand and collected during the winter of January 2021 and January 2022, approximately one and two years after establishment ().

At this early stage in growth, tree height and root collar diameter were highly correlated; therefore, tree height was used as our indicator for P responsiveness.

Microdialysis in Soil

The microdialysis system used for soil diffusive P under laboratory conditions consisted of a single CMA 4004 Syringe Pump with four 5 mL micro syringes loaded with perfusate solution composed of 15 mmol L^{-1} of potassium nitrate (KNO_3) and 1 mmol L^{-1} of citrate, pH was held at 5.8 ± 0.3 using sodium citrate to buffer. Concentrations of KNO_3 and citrate used in the perfusate were modeled after Demand et al. (2017), which had the highest overall recovery rates of P in stirred solutions. Paired microdialysis probes 10 mm long, 500 µm outer and 400 µm inner diameter with a 20 kDa molecular weight cut-off permeability of the membrane (CMA 20; CMA Microdialysis, Solna, Sweden) were inserted into 50 g of fully saturated soil (Figure 2.3). A total of 2.5 mL of perfusate solution was pumped through each soil probe at a consistent rate of 2 µL per minute for a final dialysate volume of 5 mL, which lasted 20 hours and 41 minutes. Increasing the pump rate decreases sample time but reduces the recovery rate of P. Dialysates were collected in two 5 mL falcon tubes with the tops sealed with parafilm to limit evaporation. Dialysates were stored at -20 °C until further downstream analysis. Diffusive P dialysates were analyzed for PO_4^{3-} on a Seal-Analytical AA3 segmented flow auto-flow analyzer. Dialysate P concentrations were measured using PO_4^{3-} concentration of dialysate P in µg L^{-1}. Eighty dialysates were collected from our P fertilization treatment plots, 40 from Year 0, and 40 from Year 1 soil samples collected from the same plots. Nine samples were below the 2.5 µg L^{-1} threshold outside the standard curve and could not be accurately quantified even after repeated attempts. To account for this in these data, these samples were halved below the 2.5 µg L^{-1} detection limit to 1.25 µg L^{-1} because we could not classify them as missing values. All nine halved samples were collected from the Alfisol, not the Spodosol, and seven were collected from Year 0 from the mature *P. taeda* stand (before harvest and replanting).

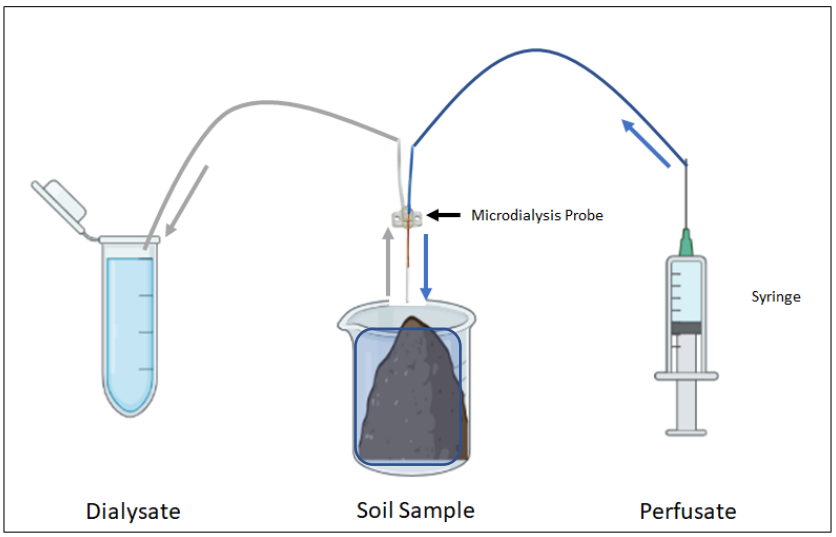

Figure 2.3: Soil microdialysis. Simplified representation of microdialysis system to assess $PO4^{3-}$ in soil. The microdialysis probe is fully inserted into a homogenized soil sample slurry of soil and H_2O, and perfusate is pumped into the probe and expelled as dialysate.

Because tree size can influence sap flow, only healthy trees with diameters between 9.5 and 10 cm were chosen. Four microdialysis probes were inserted into the sapwood of sixteen 3-year-old *P. taeda* trees (1 tree per plot, two plots per treatment, four treatments per site, two sites) (Figure 2.4). Eight samples were collected from the Alfisol and eight from the Spodosol. Probes were drilled into the base of each tree using a 1 mm drill-bit and flushed using 5 mL of deionized (DI) H_2O. Perfusate solution passed through the probes was DI H_2O treated with 15 mmol of KNO_3 to offset the potential osmotic imbalance (Demand et al., 2017). Microdialysis probes were inserted into the base of each probe and sealed around the hole using a silicone sealant. Probes were set to run for four hours at a constant flow rate of 10 µL min^{-1} until reaching a final volume of approximately 5 mL (Figure 2.5). The same microdialysis pump for diffusive P in the soil was used for sap flow P.

Figure 2.4: Gas-powered generator and microdialysis pump was set up next to each tree after removing the flammable substrate from around the generator. (Middle) The microdialysis system consisted of four syringes and four microdialysis probes drilled into the tree after carefully removing the outer layer of bark. (Right) Each hole was flushed with DI water and sealed using silicone paste to prevent leaking.

Sap flow rates in *P. taeda* can vary drastically on both daily and seasonal scales (Ford et al., 2004). To control this, samples collected from each site were collected over twelve days intermittently from June through July 2022. Samples were only attempted on sunny days with minimal cloud cover from 9 a.m. to 1 p.m. and from 1 p.m. to 5 p.m. if the weather stayed constant throughout the day. Weather data was collected visually daily as probes were monitored over the 4-hour run time. Consistent sunny weather was important because solar radiation influences the sap flow translocation and transpiration rates of the trees (Xia et al., 2008). Eleven of the sixteen samples were set up during sunny periods; however, intermittent or coastal storms caused five samples to be collected during complete cloud cover, which was noted in the field. Samples were classified as sunny if greater than 50 % of the total run time was in full sunlight. Samples were classified as cloudy if less than 50 % of the total run time was in full sunlight. All days classified as cloudy were 100 % overcast of the total run time. We evaluated tree heights relative to sap flow P concentrations using a standard linear regression to determine if sap flow p-values were related to increased height responses. Unfortunately, only visual assessments were made based on the weather. To improve the accuracy of further experimentation, photosynthetically active radiation sensors will be deployed. These sensors will allow us to more accurately correct for fluctuations in ambient weather.

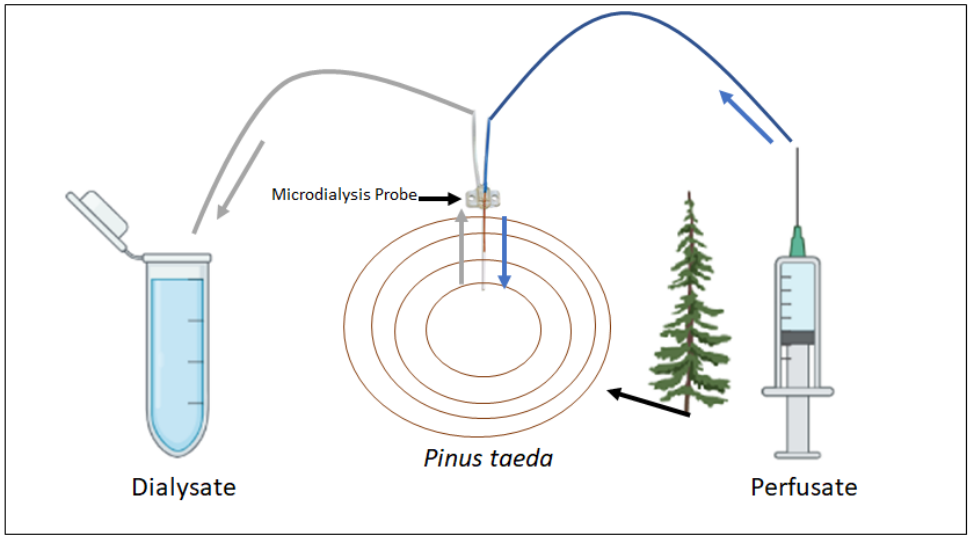

Figure 2.5: In vivo microdialysis in a tree. Perfusate solution is passed through a microdialysis probe sealed within the tissue of a *P. taeda* tree. The resulting dialysate is collected from the sap concentration of PO_4^{3-}. Probes were inserted the full 20 mm length into the tree, with the top 10 mm holding the semi-permeable membrane.

Statistics

Diffusive P and M3P were analyzed using mixed model designs. This design treated bed and interbed as random effects and P fertilization treatment as a fixed effect. Treatments were blocked by site and year. Sap flow P was initially analyzed with atmospheric conditions as a random effect, but the variance was too high between sunny and cloudy conditions. Translocated sap flow P collected during cloudy conditions were adjusted by increasing their values by 100 %, which is the relative increase observed from similar studies during overcast conditions for translocated P (Xia et al., 2008; Jian et al., 2019). Differences between carryover treatments from year 0 to year 1 were analyzed via a two-way ANOVA with P fertilization treatment and treatment year as fixed effects. Carryover treatments were analyzed separately within each treatment year and site by one-way ANOVA. Šidák's multiple comparison tests were performed for each P fertilization treatment and Year by site. Šidák's multiple comparison tests were chosen because it is more conservative than Tukey's HSD when comparing unequal sample sizes. Dunnett's multiple comparison tests were used to analyze carryover treatments against the control treatment with an alpha value of 0.10 to determine how P fertilization treatment affected diffusive P. Sap flow P did not have a site-dependent effect. Therefore, carryover and re-fertilized treatments were analyzed by combining both sites using a one-way ANOVA. Dunnett's multiple comparison tests were

performed using an alpha value of 0.10 against the control P fertilization treatment (0 + 0 P). Standard linear regressions were performed for M3P, sap flow P, and diffusive P against tree heights collected at year two using GraphPad Prism v9.0 software. All other analyses were completed in JMP Pro v16.0, SAS Institute Inc., Cary, NC, USA.

Results

Carryover and Site Effects on Diffusive Phosphorus Over Time

Mean Year 0 bulk soil pH values were similar for both sites and years, the Spodosol had a mean pH in the top 15 cm of 4.08 ± 0.06, and for the Alfisol, pH was 4.48 ± 0.05. The total amount of P recovered from the probes varied greatly by site, P fertilization treatment, and Year. In most treatments, concentrations of PO_4^{3-} were over 100 times greater in the Spodosol than the Alfisol for both Year 0 and Year 1 samples (Figure 2.6). Diffusive P samples collected in Year 0, at the end of the First Rotation, averaged across all P fertilization rates, had significantly lower concentrations of PO_4^{3-} than samples taken at Year 1 for both the Spodosol and Alfisol with a 137 % increase for the Spodosol and a 166 % increase for the Alfisol from Year 0 to Year 1 (p-value < 0.01). Year 0 diffusive P was highly responsive to P treatments for the Spodosol (p-value < 0.01), but diffusive P was unresponsive in the Alfisol at Year 0 (p-value $= 0.20$). The highest carryover treatment (121 + 0 P) had the lowest overall concentration of PO_4^{3-} at Year 0 samples for the Spodosol. Comparing individual carryover treatments within the site from Year 0 to Year 1 highlights the changes and magnitude of response with increasing rates of P from the First Rotation (Figure 2.6).

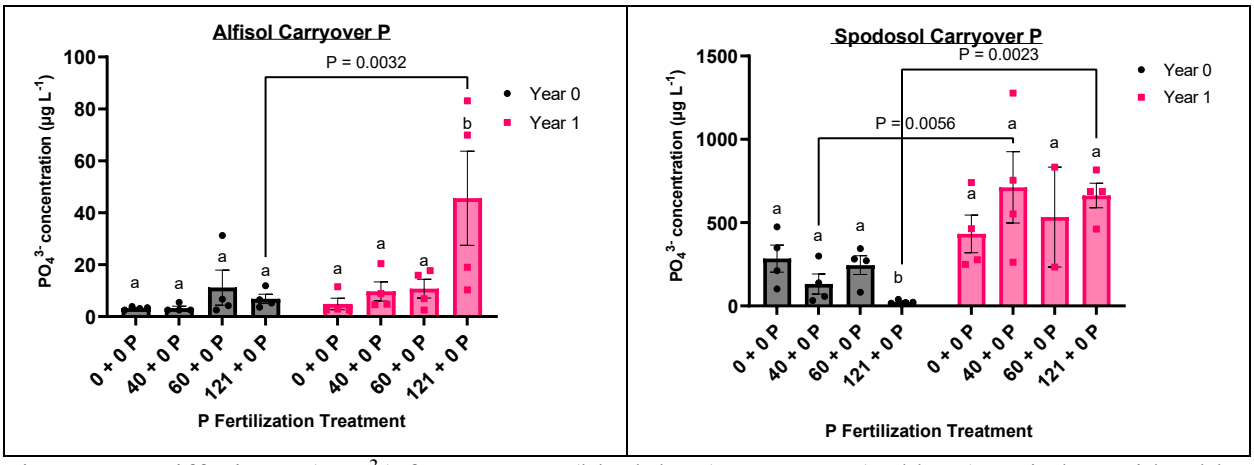

Figure 2.6: Diffusive P (PO_4^{3-}) from Year 0 (black bars) to Year 1 (red bars) varied considerably between sites with significantly less PO_4^{3-} in the Alfisol (left) vs. the Spodosol (right). Responses from Year 0 to Year 1 varied between carryover treatments. Šidák's multiple comparisons tests showed significant responses within carryover treatment from Year 0 to Year 1. Within site and Year, differences showed responses to carryover treatment separated by connecting letters. Error bars represent the standard error of the mean for each treatment within the site and year. Note that Alfisol and Spodosol values are not shown at the same scale.

Soil Diffusive P Relationships to Tree Height

Diffusive P samples collected in Year 1 showed moderate evidence of a relationship to tree heights collected in Year 2 for the Alfisol (p-value ≤ 0.01). No association was found between Year 1 diffusive P collected and Year 1 heights. The Spodosol, although having accumulated significantly more PO_4^{3-} than the Alfisol, showed no evidence of a relationship to tree height for either Year 1 or Year 2 (Figure 2.7).

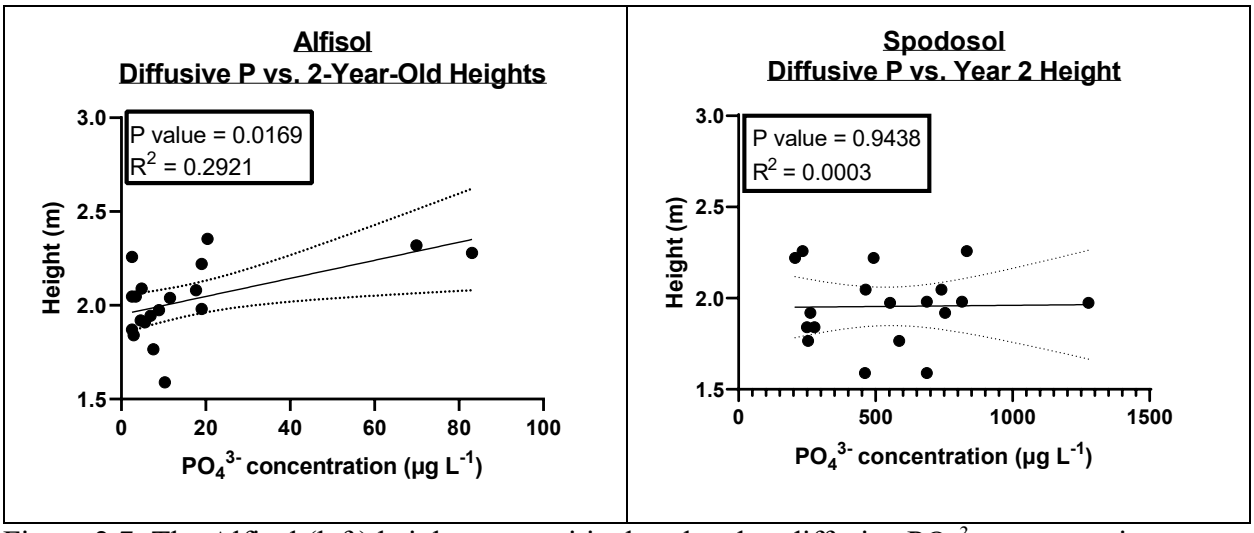

Figure 2.7: The Alfisol (left) height was positively related to diffusive PO_4^{3-} concentrations. Spodosol (right) height was not associated with diffusive PO_4^{3-} concentrations.

Detecting Translocated Sap Flow P using Microdialysis Probes

Initially, a very strong relationship was found between PO_4^{3-} concentration and solar radiation, with significantly higher PO_4^{3-} concentrations on sunny days vs. cloudy days (p-value < 0.001) (Figure 2.8).. Solar radiation is known to influence sap flow by up to 100 % (Xia et al., 2008; Jian et al., 2019); because of this, we also suspect that the reduction in sap flow led to a proportional decrease in PO_4^{3-} concentrations in the probes on cloudy days vs. sunny days. To correct for the effect of solar radiation on sap, cloudy day values were increased by 100 % before additional analysis was performed based on results from Xia et al. (2008) which found roughly a 100 % decrease in sap flow from cloudy vs. sunny days on *Caragana korshinskii* a shrub native to northern China. Corrected PO_4^{3-} concentrations accumulated by the microdialysis probes had a weak interaction with carryover treatments (p-value = 0.06). The highest overall concentrations of PO_4^{3-} occurred on the 121 + 0 carryover treatments and the lowest concentrations for control (0 +

0 P) carryover treatment (Figure 2.8), resulting in a 138% increase in PO_4^{3-}. The re-fertilized treatment (40 + 45 P) was not different from the carryover treatment (40 + 0 P) for either site.

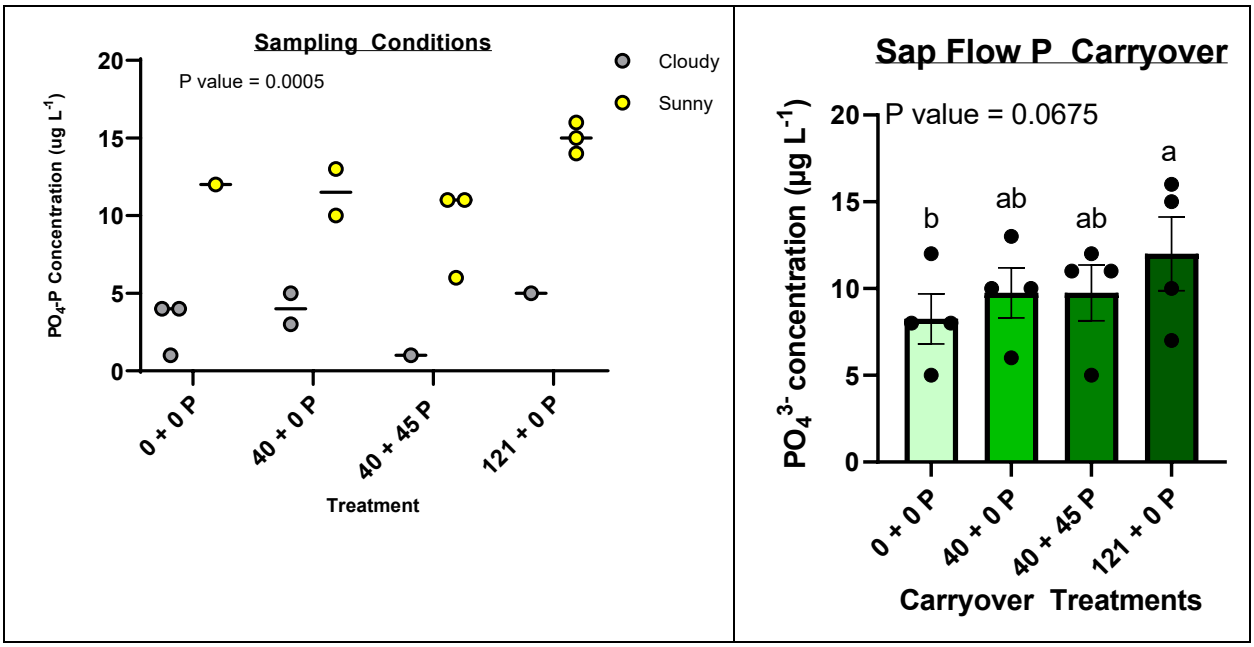

Figure 2.8: In vivo sap flow P results show (Left) samples collected during sunny weather (yellow circles) accumulated significantly more PO_4^{3-} in the sap than during cloudy days (gray circles). (Right) PO_4^{3-} was weakly influenced by carryover treatment (0 + 0 P, 40 + 0 P, 121 + 0 P) and re-fertilized (40 + 45 P) treatments after a 100 % increase correcting for cloudy day values. Error bars represent the standard error of the mean for individual P fertilization treatments. Unconnected letters are significantly different (alpha value = 0.10) using Dunnett's test.

Translocated Sap Flow P Relationship to Tree Heights

Sap flow P concentrations of PO_4^{3-} showed moderate evidence of a relationship to tree heights collected from Year 2 for the Alfisol but not the Spodosol (Figure 2.9). No significant association between Year 1 heights and sap flow P was found. We did not find any relationships between sap flow P and soil diffusive P collected in either Year 0 or Year 1.

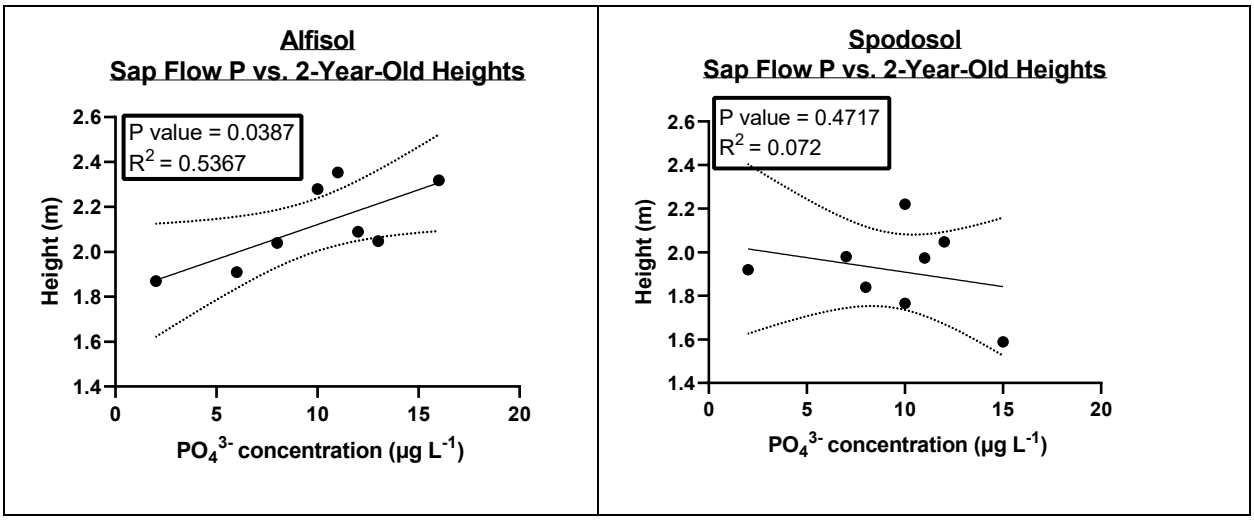

Figure 2.9: Sap flow P predicted tree heights with strong confidence in the Alfisol (a) but had poor prediction in the Spodosol (b).

Discussion

To our knowledge, this study is the first to deploy microdialysis to measure and assess its applicability for detecting and quantifying soil diffusive P in *P. taeda* silviculture systems. Our first objective was to determine if the diffusive P detected via microdialysis could be used to predict growth response due to the carryover effect. Comparing diffusive P results from Year 0 to Year 1 (pre-harvest to post-harvest) by carryover treatment rate, the disturbance of harvesting and site preparation liberated a significant portion of P (Everett and Palm-Leis, 2009) for multiple carryover treatments when compared to the control treatments, demonstrating that the diffusive P is responsive to the magnitude of applied fertilizers one-year post-harvest. From these data points, it is clear using microdialysis to detect soil P build-up and carryover treatments is highly soil dependent. Diffusive P was poorly related to carryover treatment rates in all but the highest carryover rate of 121 + 0 P on the Alfisol, making its viability in determining critical p-values in Alfisols and Spodosols uncertain and may require additional optimization and sampling replications.

For our second objective, M3P results from the top 15 cm of soil in the Alfisol showed sufficient P above the commonly used 5 ppm critical level for fertilization (Wells et al., 1986). The results revealed relatively similar amounts of M3P from the Alfisol and the Spodosol in the first year of growth, except for the carryover 121 + 0 P treatments. Counter-intuitively to this observation, soil PO_4^{3-} concentrations detected via microdialysis were 100 times higher in the

Spodosol than the Alfisol across all time points and treatments. Still, no relationships between diffusive P and M3P were found (data not shown). Our hypothesis for this discrepancy is that the concentrations of citrate and KNO_3 used in the perfusate dramatically affected the mobilization of P in the Spodosol but not the Alfisol (Mckay et al., 2021). This hypothesis is also supported by Demand et al. (2017), who found that the same concentrations of solutes within the perfusate had variable accumulations of P depending on the sampling environment. Additional research and optimization will need to be performed on various soils and concentrations of solutes to confirm this hypothesis. Potential drivers of these differences between sites are (1) the Spodosol naturally had higher amounts of sorbed inorganic P to soil surfaces that could be liberated thanks to the perfusate composition (Fletcher, 2021); (2) there was higher microbial mineralization of organic matter occurring in the Spodosol than the Alfisol (Achat et al., 2010); or (3) the P in the Alfisol was sequestered into more difficult-to-access forms of P that the perfusate composition could not liberate. These PO_4^{3-} concentration disparities between sites could also result from combining these three factors. It is uncertain which soil surfaces, or factors determine the rates and amounts of soil diffusive P accumulated by microdialysis probes. Additional research and consideration must be given to soil characteristics, such as texture, and perfusate compositions to optimize this method for forestry and agricultural applications.

Our third objective to determine if the diffusive P pool had relationships to *P. taeda* tree growth and productivity. As with the previous results, we found relationships highly dependent on sampling timing and site. It appears from these data that the diffusive P pools sampled in Year 0 have little to no connection to the growth rates of trees in the subsequent rotations, probably because the litter layer and organic material have not yet had time to be incorporated into the subsurface horizons (Comerford et al., 2002). However, diffusive P data points collected in Year 1 showed moderate evidence of a positive relationship between increases in diffusive P and height responses for the Alfisol two years after planting. Previous studies demonstrated that microdialysis is a powerful tool for evaluating nitrogen pools in Norway Spruce (Shaw et al., 2014; Inselsbacher & Näsholm, 2011), P availability in soil solutions (Demand et al., 2017), and its spatial and temporal similarities to plant roots (McKay et al., 2021). These studies have demonstrated that microdialysis could have far-reaching implications in studying mass flow and diffusive nutrients in agriculture, forestry, and environmental samples. Microdialysis probes provide researchers with a pseudo-rhizosphere to simultaneously efflux-controlled amounts of solutes outward from the

probe (in our case, citrate) and to passively collect solutes by generating an infinite diffusive sink for specific nutrients in the soil (McKay et al., 2021).

To our knowledge, this is the first study to evaluate the use of *in situ* microdialysis probes to study sap flow P concentrations in *P. taeda* and the second in any tree species. While attempts were made to specifically target only days with high forecasted solar radiation, intermittent clouds, and rainfall throughout the day during multiple sampling events, influencing the total PO_4^{3-} accumulated by the probes (Xia et al., 2008; Jian et al., 2019). The diurnal changes to sap flow in *P. taeda* have been verified and correlate well with temperature, solar radiation, and vapor pressure deficit (Ford et al., 2004). Although these data were not directly recorded in this experiment, a general assessment of cloud cover was our best metric to correct for differences in P recovery within the sap.

Though our sample size was limited, the absence of any relationship between diffusive P and sap flow P for either site implies that the diffusive P pools from the soil do not represent the total P distributed in the sap. Alfisol heights collected at Year 2 show a positive relationship to sap flow P but not for the Spodosol. We hypothesize that this is because the Spodosols were not as P limited as the Alfisols based on our diffusive P data. Supporting these results, weak associations between carryover rates and sap flow P were also detected. Indicating that carryover rates from the previous rotation had some relationship to the amounts of sap flow P in the trees. This relationship shows that there could be a strong temporal component that we currently do not have enough data to uncover. Future height measurements and soil sampling with these and additional sites will reveal whether some of these data points are artifacts or actual responses that need further exploration.

Limitations and Future Research

A total of 12 probes were used throughout the sap flow P microdialysis experiments. Almost all probes were broken by the end of the experiment due to the fragile construction of the probes themselves. Extraction from the trees after each run had the highest probability of probe failure. Other shortfalls of the sap flow experiment included fluctuations in weather which dramatically affected the sap flow P rates of the trees. Many probes used in soil were also broken over the duration of the experiment. To improve upon these results, implementation of a photosynthetically active radiation sensor will dramatically corrections in atmospheric conditions.

A total of 18 probes were broken for the diffusive P soil experiments over the testing of 120 soil samples. More robust probes and testing procedures must be developed for this method to see any widespread adoption. The microdialysis recovery rate of solute is affected primarily by pump flow rates and perfusate composition (Demand et al., 2017). Further optimization areas of study will be important in identifying perfusate compositions and flow rates that provide optimal recovery of targeted compounds in environmental settings. Deciding which depths and soil horizons to analyze with the probes could also be crucial when assessing soil deficiencies.

Conclusion

These results highlight the applicability of these probes to assess diffusive P supply in the soil. Considering no relationships were found between any treatments, years, or sites when running simple linear regressions between diffusive P and M3P, we can conclude that these soil tests were sampling very different subsets of P pools in the system. Although sap flow P was only weakly related to carryover treatments, our results highlight that microdialysis probes could have broader applications in plant physiology than once thought. Additional work on how these diffusive P rates relate to overall plant growth later in the stand's rotation could help elucidate long-term relationships between available P and stand growth.

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Chapter 3: Fungal Biomass and Ectomycorrhizal Community Assessment of P-Deficient *Pinus taeda* Plantations

Highlights

- Mesh bags treated with P had a 25% increase in biomass over untreated bags.
- Biomass accumulation in the bags were only influenced by P fertilization treatments one year after planting.
- Twelve ectomycorrhizal species overlap between P-Treated mesh bags and samples collected from the rhizosphere.
- A 212% increase in the effective number of taxa was observed in Alfisols that received P fertilization in both the first and second rotation compared to the control.

Introduction

Forest soils in the southeastern US are often deficient in bioavailable nutrients, particularly P, due to the inherent P deficiency of the highly weathered and acidic soil groups that dominate the region (Comerford et al., 2002;). These soils are hypothesized to be in a "terminal steady state" of P depletion as the parent material slowly loses its P content (Walker and Syers, 1976). As a result of this deficiency, loblolly pine (*Pinus taeda* L.) stands in some sites and responds well to P fertilization, which can increase their overall productivity (Albaugh et al., 2019). Although P fertilization has been used on these sites since the late 1960s, it is now reserved for sites that will benefit most in terms of crop tree growth response due, in part, to the rising cost of fertilizer.

With and without P additions, trees rely on symbiotic ectomycorrhizal fungi (ECM) to enhance their ability to take up P from the soil. These fungi invade the cortex of pine roots and create a network of hyphae that trade soil minerals for carbohydrates from the plant (Doidy et al., 2012). The tree provides the ECM with carbohydrates in the form of sugars, and the ECM transfers water and nutrients from the soil to colonized roots via specific transport proteins (Becquer et al., 2019; Garcia et al., 2016). ECM fungi can increase the nutrient assimilation capacity of P by up to two orders of magnitude by increasing the total root surface area (Smith and Read, 2010) and assimilating non-available P by releasing acid phosphatases by external hyphae (Ho, 1989).

P availability of the soil directly affects the growth of ECM fungi, their nutrient exchange, and their interactions with host plants. Research has consistently shown that P levels in plants are much higher when they form associations with ECM fungi than those without (Smith and Read, 2008). However, under extreme P-limiting conditions, more carbon is allocated to ECM hyphal exploration into the soil to scavenge for nutrients at the expense of aboveground host biomass allocation (Bahr et al., 2015). P fertilizer additions in nutrient-limited environments have been linked to significant effects on the biomass and community composition of the ECM fungal communities. P additions in P-limited environments are associated with reduced underground biomass for roots and ECM fungi, favoring communities of low-biomass taxa specialized for short-distance soil exploration (Treseder et al., 2004; Bae, 2015; Bahr et al., 2015). In contrast, high-biomass ECM species specialized for long-distance soil exploration thrive in P-limited environments (Wallander et al., 1992). In summary, hyphae production increases under deficient P levels, and when P is sufficiently available, fungal biomass and hyphal exploration are suppressed (Jones, 1990; Wallander et al., 1992; Callahan et al., 2016).

We hypothesize that ECM fungi have the potential to be used as a biological indicator for P availability in loblolly pine plantations in the southeastern U.S. Fine mesh bags, which exclude all plant roots but allow fungal hyphae to enter, can be "baited" with a P source, to evaluate both the abundance and community of ECM fungi (Callahan et al., 2016; Hagerberg et al., 2003; Hedh 2008). Preliminary analyses conducted by Tacilla (2015) on our two sites indicate increasing rates of P fertilization led to increased productivity responses of *Pinus taeda* in both sites. Because of this increase in productivity associated with increased rates of P fertilization, we assume that the ECM community will shift to communities adapted to higher rates of P in the soil. Connecting productivity responses with the characterization of fungal communities and biomass, it may be possible to predict response to fertilization better as ECM fungi are crucial to nutrient uptake for *Pinus taeda* and are sensitive to nutrient inputs.

The primary goal of this experiment was to evaluate how varying levels of P fertilization in the previous rotation affect fungal biomass and fungal communities at two sites with contrasting soils. We developed five primary objectives for these experiments regarding fungal biomass and ECM communities to reach this goal. (1) Evaluate biomass accumulation from pre-harvest to 18 months after planting. (2) Determine if P fertilization treatments affect fungal biomass accumulation (3) Determine if fungal biomass is related to the richness and abundance of taxa

extracted from soil or mesh bags. (4) Determine if P fertilization treatment changes the community composition of ECM and Non-ECM taxa extracted from soil DNA. (5) Determine if the ECM or Non-ECM communities in the mesh bags are associated with the rhizospheres of *Pinus taeda*

Methods

Site Description and Design

This study builds on a long-term experiment established by the Forest Productivity Cooperative (formerly Forest Nutrition Cooperative, Albaugh et al., 2015) to investigate the effects of nitrogen and P fertilization rates (Figure 3.1). The experiment comprises eight sites across the southeastern US, of which three have been harvested and replanted, and the remaining five will be converted in the next 1-3 years. We focus on the first two sites that have already undergone harvesting and replanting. Both sites are located on the Atlantic Coastal Plain and have contrasting soil characteristics. The first site in northeastern Florida has a poorly drained, fine, mixed, active, thermic, Typic Albaqualf (Meggett series, CRIFF group A) with marine sediment parent material. The second site, located in Southeast (SE) Georgia, has a somewhat poorly drained, sandy over loamy, siliceous, active, thermic Typic Haplohumods (Leon series, CRIFF group D), with marine sediment parent material, multiple spodic horizons, and no argillic or kandic horizon present within the first 100 cm of soil depth (Table 3.1).

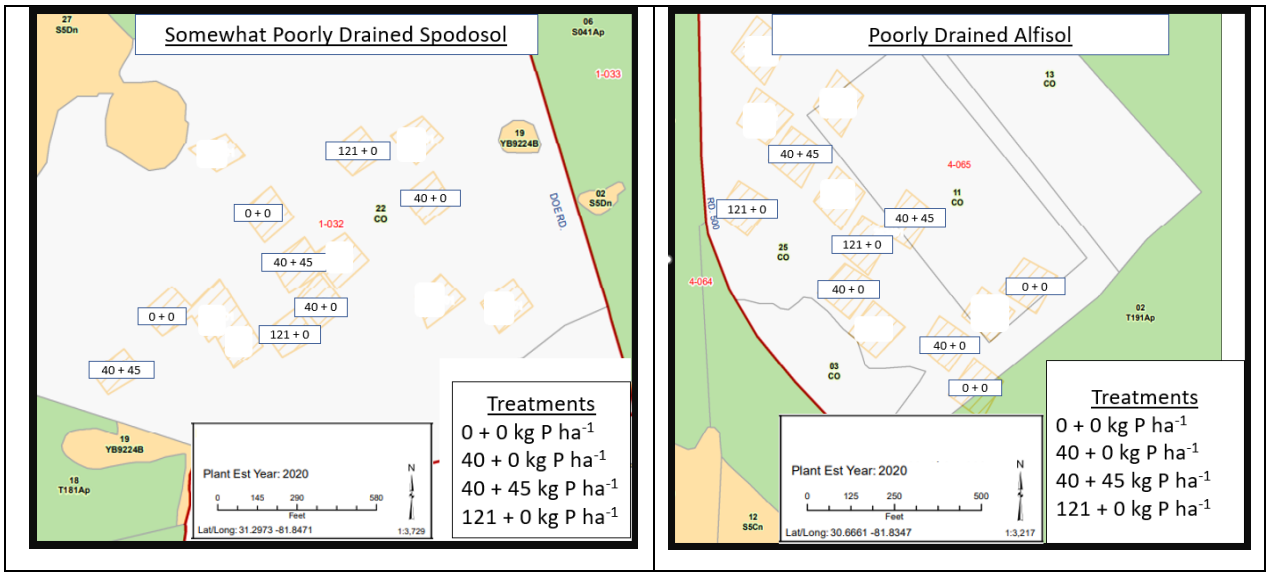


Figure 3.1: Plot layout and replications for the poorly drained Alfisol and the somewhat poorly drained Spodosol. Plots whited out are part of the more extensive study but were not used as the subset of treatments in this study.

Table 3.1: First rotation and second rotation site designs and descriptions.

Site	County and State	Latitude	Longitude	Species	Study Establishment	"Base" Site Index*	Years Since P Fertilization	Harvest Date
Alfisol	Nassau, Florida	30.6661 N	81.8361 W	Loblolly	1999	45	2004	Jan-19
Spodosol	Brantley, Georgia	31.3353 N	81.8217 W	Loblolly	1998	67	2005	Jan-19
***Base Site Index at 25 years old**								

The first rotation of the experiment was harvested in 2019 and will be referred to as the "first rotation." The current rotation is referred to as the "second rotation," even though the sites were planted with pine before the first rotation of this study. Experimental treatments are arranged at each site in a completely randomized block design with two replicates. A two-row buffer of trees surrounds each measurement plot, and all receive fertilization. Each treatment plot contains six rows with 12 trees per row for approximately 70 trees per treatment, all labeled with aluminum tags to track individual tree growth and mortality over time. The first rotation plots received various cumulative fertilizer applications. Treatments for the second rotation were overlaid on previous rates and either received an additional re-fertilization of P (40 + 45 kg P ha^{-1}) applied at tree establishment as broadcast triple super phosphate or no fertilizer P (X + Y, where X = the Carryover Rate and Y = Re-Fertilization Rates) at the establishment (Table 3.2). second rotation P treatments were (in kg ha^{-1}): 0 + 0 P, 40 + 0 P, 40 + 45 P, and 121 + 0 P. All plots, except the control, received N as urea plus a urease inhibitor, K as KCl, and a micronutrient mix to remove nutrient limitations besides P. All treatments received 6 oz of Arsenal herbicide after bedding in the spring for herbaceous weed control.

Table 3.2: P fertilization treatments for carryover and re-fertilization rates. Cumulative N and P rates from the previous rotation established in 1999 and current rates from the second rotation in 2020.

	First rotation (Carryover Rates)		Second rotation (Re-Fertilization Rates)		
P Fertilization Treatments	**Cumulative P**	**Cumulative N**	**N**	**K**	**P**
	kg ha^{-1}				
0 + 0 P	0	0	52	29	0
40 + 0 P	40	400	52	29	0
40 + 45 P	40	600	52	29	45
121 + 0 P	121	1210	52	29	0

Mesh Bag Construction and Deployment

To accomplish our first objective to (1) Determine if levels of P fertilization treatment affect fungal biomass accumulation or fungal community composition in P-Treated or Untreated bags, biomass samples were collected from the surrounding bulk soil using mesh bags Treated or Untreated with P. The bags were constructed using a 50 µm nylon mesh (9 x 5 cm), fine enough to exclude plant fine roots but not fungal hyphae from entering the bag (Wallander et al., 2001). Previous sequencing analyses using similar mesh bags found that ECM fungi dominate the community compositions within the bags (Parrent & Vilgalys, 2007; Wallander et al., 2013). Each bag was heat sealed and filled with 30 grams of acid-washed fine quartz sand sieved to 2 mm. The sand is devoid of nutrients and carbon, which only ECM fungi receiving carbon from above-ground biomass can pass through to produce hyphae. To incentivize the colonization of the bags with ECM that harvest P, half of the bags are baited with finely ground apatite as a readily available source of P that the ECM can use to exchange with the trees. One hundred twenty-eight bags were constructed for each field location; 64 of those bags in each site received a treatment of 1 gram of finely ground apatite added to each bag and homogenized (P-Treated). The remaining 64 were left as control bags filled only with 30 grams of acid-washed quartz sand (Untreated). Each treatment plot received 16 bags (8 P-Treated and 8 Untreated). These bags were randomly placed evenly between the row and Interbed throughout the plot. Each bag was buried 15 cm below the soil

surface using a soil auger. Bags burials were marked using flagging and colored nylon string tied to the tops of each bag affixed to the flagging (Figure 3.2).

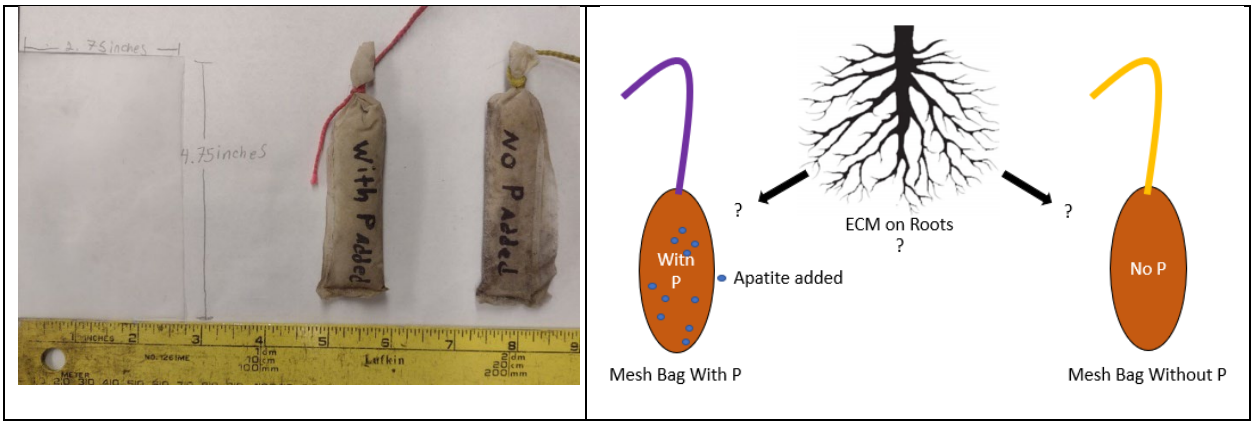


Figure 3.2: Single sheets of 50 um nylon mesh were cut out and folded to create a bag, then sealed via melting (Left). Bags were individually labeled and equipped with two colors of nylon twine to assist in relocating and differentiating between treated and non-treated bags. (Right) ECM on the roots scavenging for P will accumulate in bags baited with P and not in bags without P.

A total of four burial periods were included in this experiment (Table 3.3). The mesh bags were left in the ground for six-month burial periods except for the first burial period, three months from August-November 2018. After harvest, the next set of bags was installed when the plots were replanted in the spring of 2020, ending in the summer of 2022. A total of 4 burial periods were included in this experiment, one three-month burial period Pre-harvest and three six-month burial periods post-harvest. After each six-month burial, bags were harvested from the soil and separated by Bed vs. Interbed and P-treated vs. Untreated. Mesh bags were stored on ice and then frozen at –20º C.

Table 3.3: The sampling timeline for fungal biomass shows that burial period 1 was only three months and burial periods 2-4 were six months long. Initial installation for burial period 1 was in the Fall of 2018 on a fully developed *Pinus taeda* plantation. The 2nd burial period was installed in January 2020, and burial periods 3 and 4 were installed 6 and 12 months after.

Sampling	Burial Period 1	Burial Period 2 (six months)	Burial Period 3 (six months)	Burial Period 4 (six months)
Year	2018	2020	2021	2022
Fungal Biomass	X	X	X	X
DNA		X		

Biomass Method for Processing

To homogenize samples based on site and carryover treatment, mesh bags were opened and subjected to grinding using a mortar and pestle for 30 seconds. This grinding procedure ensured an even distribution of biological material in the sample. Subsequently, samples were split in half; one half was stored in the freezer for DNA extraction, while the other half was subjected to loss-on-ignition treatment. Although the loss-on-ignition method is initially intended for soil organic carbon quantification (Tiessen et al., 1981), we assume that only fungal hyphae will enter the mesh bags. While the method cannot distinguish how much biomass is non-ectomycorrhizal or mycorrhizal fungi (Martin et al., 1990), we propose it as a viable alternative to ergosterol sampling when dealing with mycorrhizal colonization in sand mesh bags, as it is generally assumed mycorrhizae are the predominant colonizer of the bags considering there is no organic material present in the bags (Wallander et al., 2010, 2013). After covering samples with foil, they were dried in an oven for 12 hours at 40°C, subsampled using 20 ml ceramic crucibles, and labeled with a wax pen. Ceramic crucibles were pre-heated to 100°C to eliminate residual moisture. Samples were weighed using a high-accuracy scale to the nearest milligram and inserted into a blast furnace heated to about 650°C for 12 hours to eliminate any hyphae or organic material present. The difference in weight between the initial and final measurements was used to estimate the amount of organic material and hyphae in the bags.

Rhizosphere Sampling and DNA Extraction

We harvested fine roots from five dominant *Pinus taeda* trees in each treatment plot to collect root sections and stored them at -20 ºC for downstream use. The root clippings were placed in a 50 ml centrifuge tube with ten large glass beads and vortexed for 2 minutes with DI H_2O. We then extracted the root clippings from the centrifuge tube and vortexed the remaining fluid for 3 minutes at 10,000 x g. We subsampled 0.25 grams for DNA extraction from this substrate using DNeasy Powersoil Pro kits from QIAGEN.

DNA quality and quantity for the rhizosphere and mesh bag samples were checked using a Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer and standardized to approximately ten ng mg-1. The mesh bags were pooled by treatment, site, and placement (Bed or Interbed). We used 30 grams of sand for each sample, which was hand-ground using a mortar and pestle and then subsampled. Samples were diluted using 30 ml of clean

deionized H_2O as an eluent and ten large glass beads. After vortexing for 1 minute to suspend any remaining biomass in the eluent, we extracted the mobile phase containing the organic material from the sample using a large pipette, which was then centrifuged at 10,000 x g for 3 minutes. We used 0.25 grams of the solid phase for further downstream DNA extraction using the MoBio DNA extraction kit for consistency. We performed PCR amplification using the fungal standard ITS1f and ITS4 primers to amplify the ITS1 region. We sequenced the samples on Illumina Miseq v3 (2 × 300 bp for fungi) at the North Carolina State University Genomic Sciences Laboratory. Mesh bag data collected in subsequent years, 2021 and 2022, is currently stored at 20 °C, awaiting further downstream analysis.

Bioinformatic Analysis of Mesh bag and Rhizosphere DNA

Illumina data was processed with dada2 v 1.10.6 (Callahan et al., 2016) in R v 4.2.2 (R Core Team, 2019). Default parameters in the dada2 pipeline were used to create amplicon sequence variants (ASVs). Sequences were trimmed, and low-quality ends were removed. After filtering and merging, 11,799 fungal ASVs remained in the dataset, comprising 7,145,573 reads. After chimera removal, 734 ASVs were removed for a dataset with 11065 fungal samples. Taxonomy for the dataset was assigned using the Unite vers. 9.0. fungal database for ITS reads. For improved estimates of biodiversity within the dataset, a post-clustering curation algorithm called LULU (Frøslev et al., 2017) was used to remove erroneous ASVs. After LULU curation, 3132 ASVs were removed, leaving 7933 ASVs. Functional parsing of ASVs into saprotrophic and mycorrhizal fungi guilds was performed using FunGuild functional database (Nguyen et al., 2016). Of the remaining 7933 ASVs, FunGuild assigned 1966 ASVs to a functional guild.

The guild assignments of the 1966 ASVs were as follows, 110 were assigned as ECM fungi, 73 as Arbuscular mycorrhizae, 17 as Ericoid mycorrhizae, and the remaining 1855 ASVs were classified as non-mycorrhizal. Non-mycorrhizal fungi consisted of various endophytes, pathogens, and saprotrophs. We have four datasets, two from the rhizosphere and two from mesh bags. Of the two datasets for each area, one has only ECM taxa, and the other has ECM and non-ECM taxa.

Site and P fertilizer treatment analyzed rhizosphere samples, while Mesh bags were analyzed by site, P fertilizer treatment, and mesh bag treatment. To determine which treatments influence the composition of fungi present in the samples, Bray-Curtis dissimilarity-based

permutation analysis of variance was performed for each treatment group. Indicator species analysis on the ectomycorrhiza dataset was performed to determine which taxa within those groups are unique to those individual groups (Figure 3.3). Alpha diversity was calculated using the effective number of species from each treatment group. This value is found by first finding the Shannon Diversity index (H) and taking the exp of that value (Jost, 2006)

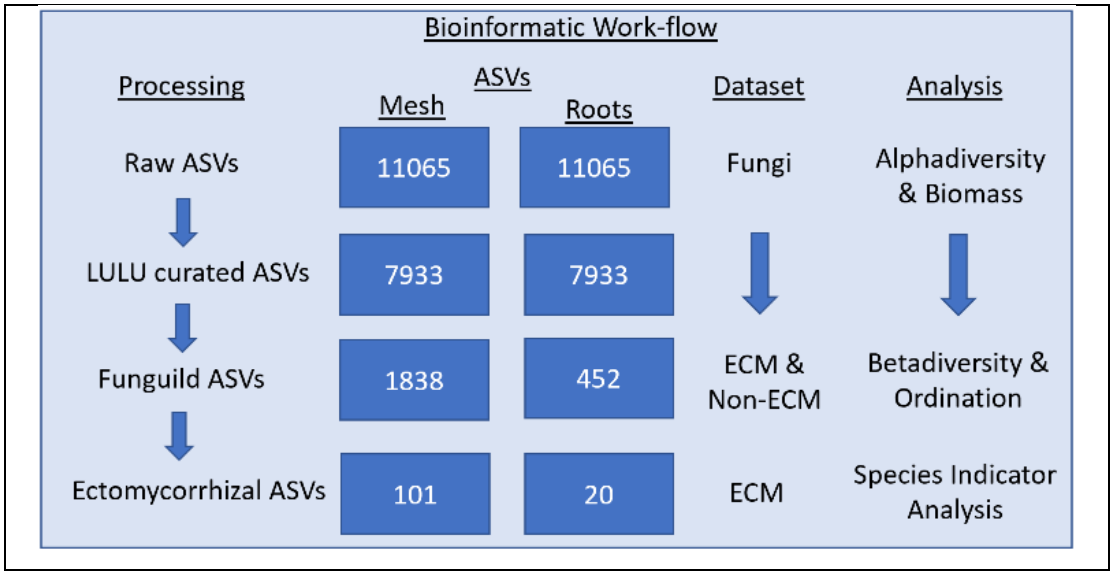


Figure 3.3: Functional Assignment of ASVs creates an ECM dataset with multiple processing steps to filter out non-mycorrhizal ASVs from our dataset. Because the mesh bags contain biomass of all organisms in our samples, diversity estimates were performed on the raw ASVs. Our final ECM dataset was too small to perform ordination plots; therefore, ordination plots and beta diversity measures were generated using FunGuild curated ASVs.

Statistics

Biomass samples were analyzed using a combination of mixed model designs and One-way ANOVAs in JMP v16.0 to explore interactions between the timing of sampling and P fertilization treatment effects. To compare how biomass changed over the burial period was affected by site, Biomass samples were blocked by site, and the fixed effect treatment was burial period. Multiple comparisons were performed on the mean total biomass accumulated from each burial period and separated using Tukey's HSD with an alpha level of 0.05. To examine biomass changes by P fertilization treatment, biomass was separated each year and blocked by a treatment consisting of either P-Treated vs. Untreated bags collected and run using a mixed model design with Placement (Bed or Interbed) treated as a random effect. Effects of P fertilization treatment on

Mesh bag Treatment collected in 2020 were run using a One-way ANOVA with multiple comparisons performed using Tukey's HSD. Alpha diversity between samples was blocked by Site and analyzed by P fertilization treatment using One-Way ANOVA on Shannon diversity indices generated using R v.4.0.0. Community differences by Site, Mesh bag treatment, and P fertilization effects separated by communities within the mesh bags or on the rhizosphere and was generated by PERMANOVA using the statistical software package PAST ver3.0 (Hammer et al., 2001).

Results

Biomass Changes by Treatments

For objective 1, biomass was very strongly influenced (p-value ≤ 0.0001) by the burial period for the mesh bags. On the Alfisol and Spodosol, samples collected in 2020 just after the harvest had the lowest overall accumulation of biomass across all burial periods, followed by the Pre-harvest samples collected in 2018, and finally, the highest overall accumulation of biomass coming from two years post-harvest in 2022 for the Alfisol. In contrast, Spodosol did not significantly increase biomass in 2021 vs. 2022 (Figure 3.4).

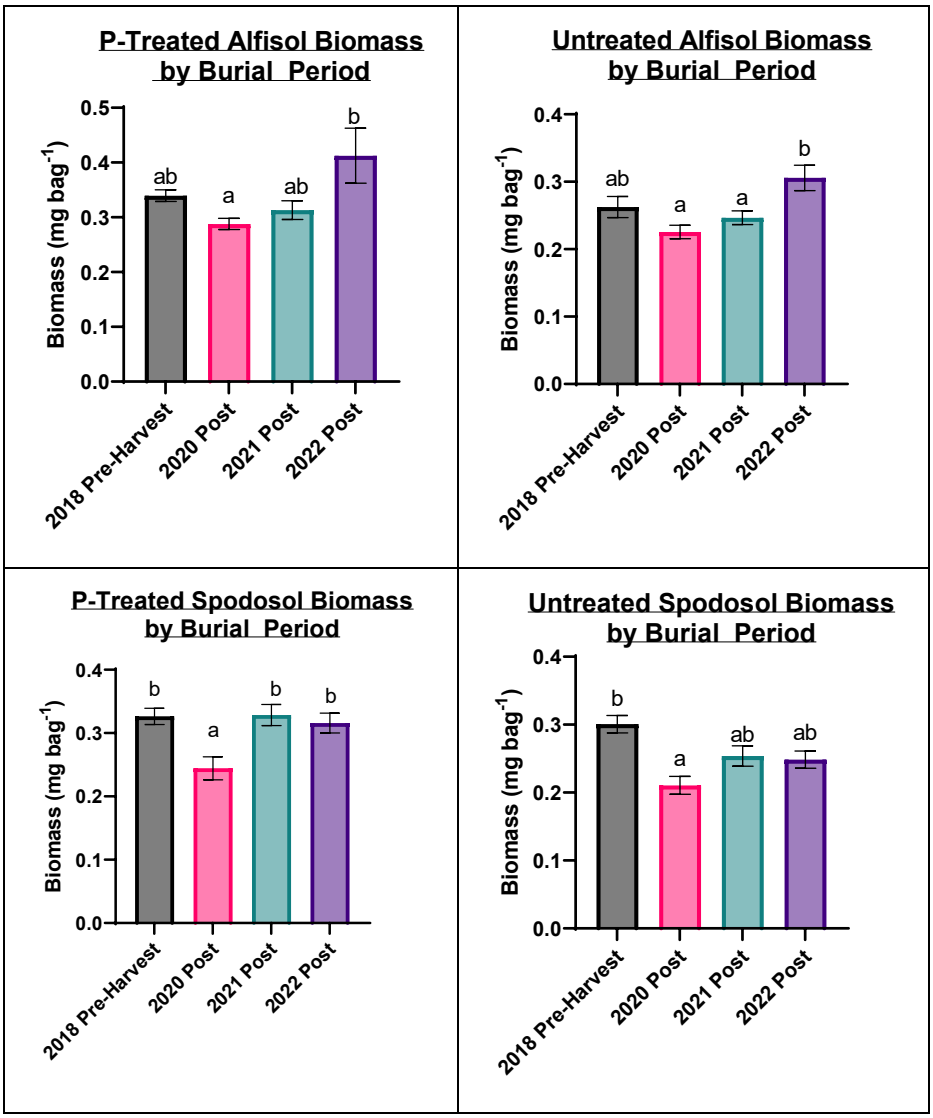


Figure 3.4: Results from biomass by burial period for Mesh bag treatment by Alfisol (top) and Spodosol (bottom). Biomass accumulations were higher in bags treated with P than in untreated bags. The lowest biomass accumulation occurred in the 2020 Post Planting set of bags across all treatments and sites.

For Objective 2, overall biomass was higher in P-Treated mesh bags than in Untreated mesh bags across all treatments (p-value ≤ 0.01), resulting in a 24.9% increase in total biomass in bags in P-treated bags vs. Untreated bags. Responses to P fertilization treatment P depended primarily on Mesh bag treatment and burial period. Biomass was only affected by P Fertilizer treatment in Pre-harvest 2018 and post-harvest 2020 in P-Treated bags (p-value = 0.0405, 0.0036, resulting in an increase in biomass from the control plots for 2020. No significant differences were found for 2021 or 2022. No differences due to P Fertilizer treatment were found in Untreated mesh

bags (data not shown). The Spodosol and the Alfisol had similar trends in response to P fertilization treatments, and their means were analyzed together to increase statistical power, separated by burial period. In the 2018 P-Treated bag samples, the control treatment (0 + 0 P) had the highest biomass (Figure 3.5). Conversely, in the 2020 samples, we saw the opposite trend, where P-Treated bags had greater biomass in higher P fertilizer application rates (40 + 45 P and 121 + 0 P).

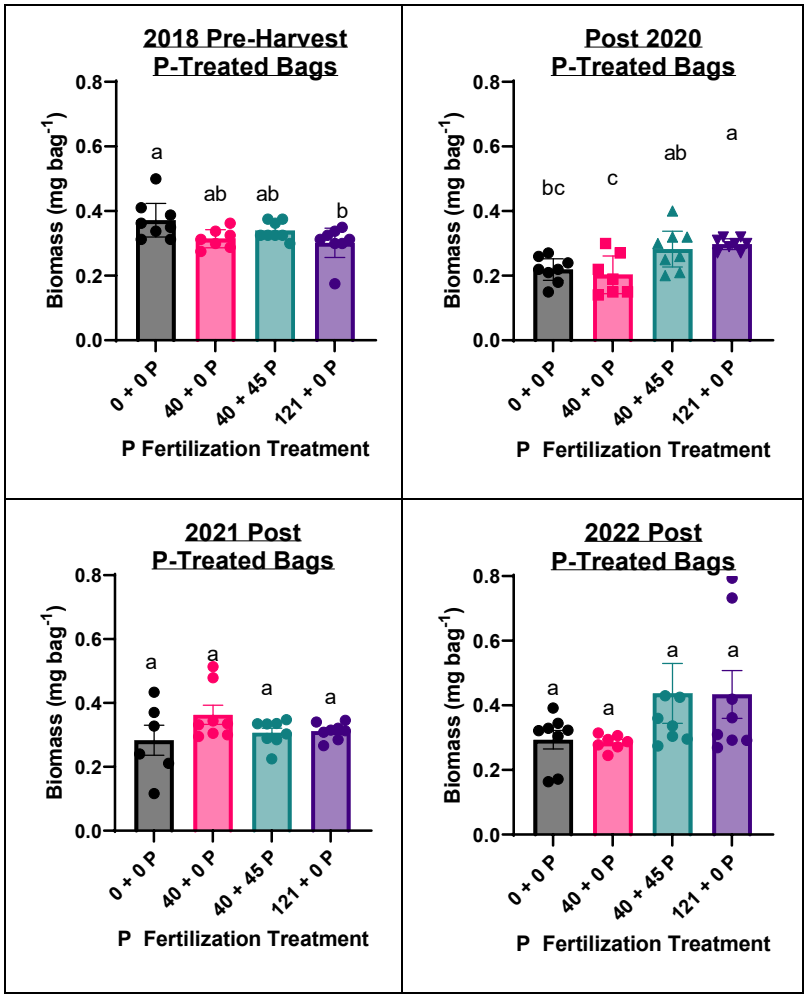


Figure 3.5: Fungal biomass by P fertilization treatment averaged together by site and separated by burial period 2018 and burial period 2020. Fungal biomass in the P-Treated bags responded positively to increased P-fertilization rates post-harvest in 2020 (p-value = 0.0294), while increasing P rates negatively affected biomass P-Treated mesh bags in 2018 (p-value ≤ 0.0130). P fertilization treatments did not influence fungal biomass in the 2021 and 2022 burial periods. Error bars represent the standard error of the mean.

Alpha Diversity

For objective 3, to determine if fungal community composition was related to P fertilization treatments, Shannon diversity indices were generated for each P fertilization treatment on the un-trimmed fungi dataset, including singletons. Only P-Treated bags located on the Alfisol had a strong interaction between P fertilization treatments for overall richness and abundance (p-value ≤ 0.0149). Results of this interaction revealed that any treatment on the Alfisol had, on average, a 212% increase in diversity to plots that received P fertilization from the first rotation were significantly higher in species diversity and richness than the control plots that did not receive any additional P fertilization (Figure 3.6).

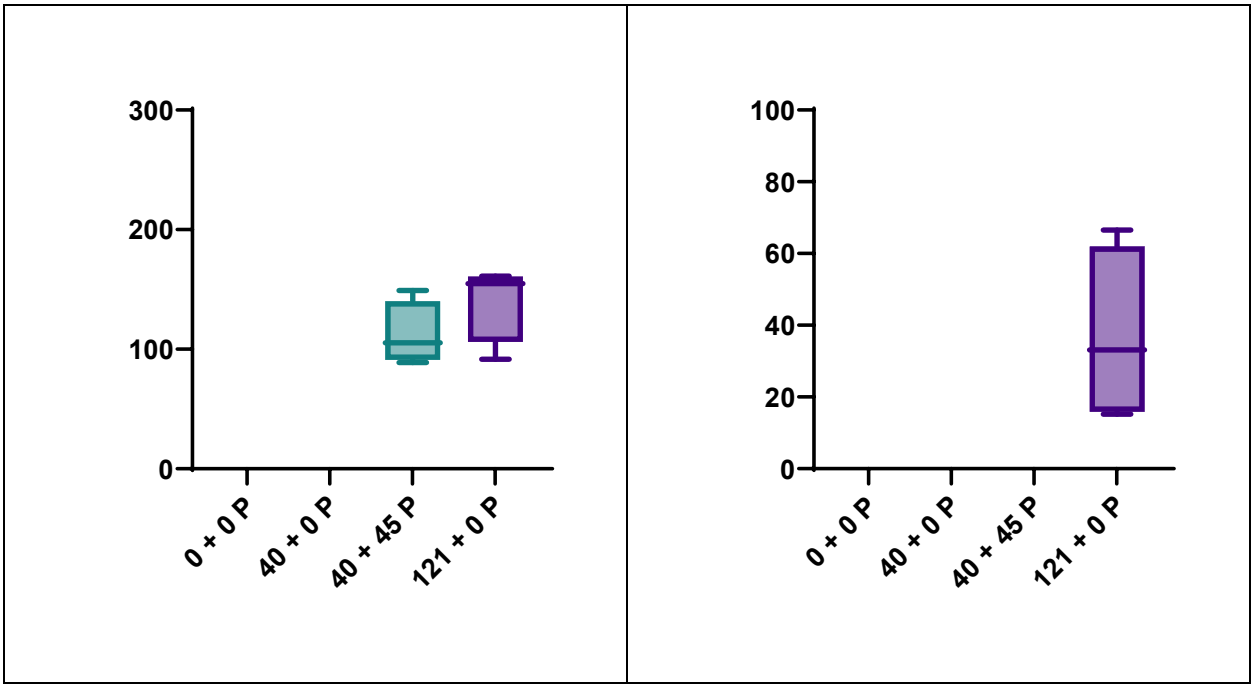


Figure 3.6: Fungi dataset true species diversities for P fertilization treatments by site. The Spodosol had less diversity and abundance than the Alfisol across P fertilization treatments except for the control (0 + 0 P). The Alfisol had significantly higher true species diversities in plots that had received fertilization than the control (0 + 0 P).

To determine if biomass in the bags had any relationship to abundance and species diversity within those bags, we compared the biomass results directly to the Shannon diversity index. We found that the control P fertilization treatments in the Alfisol (0 + 0 P) and the highest carryover P fertilization treatment in the Spodosol (121 + 0 P) had moderate relationships (R^2: 0.657, p-value≤

0.0147; R^2: 0.591, p-value = 0.0258), respectively, to total biomass accumulated in those treatments (Figure 3.7).

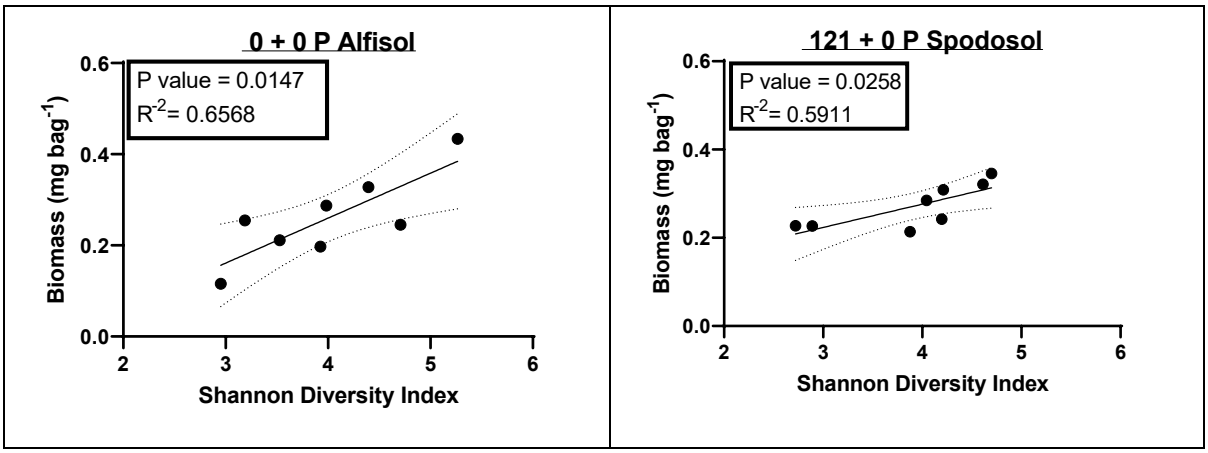


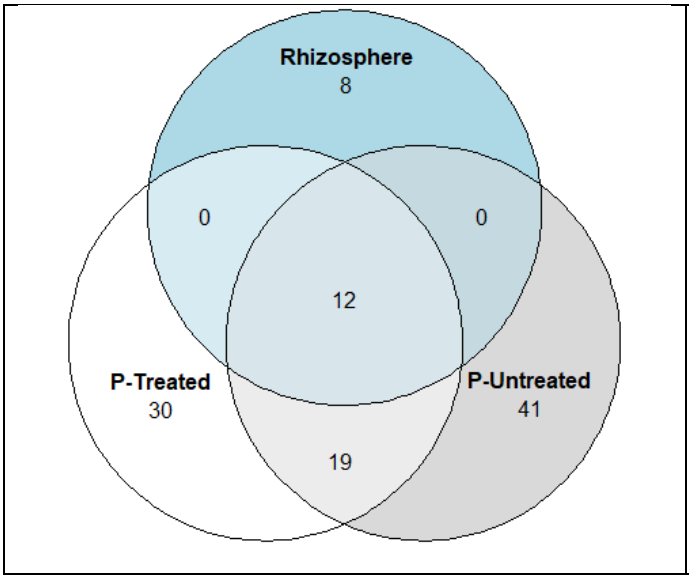
Figure 3.7: Regression of Biomass and Shannon diversity Index by P fertilization treatment and site. Standard linear regressions were performed on individual treatments to determine if biomass had a relationship to the observed diversity and abundance of taxa within the Mesh Bags. Two P fertilization treatments out of 8, the control (0+0 P) for the Alfisol and the high (121+0 P) for the Spodosol had relatively strong relationships between Mesh bag Shannon diversity Index and biomass.

For fungal bags collected in 2021, there was a weak interaction between site and Mesh bag treatments for richness (p-value = 0.0985) and a very strong interaction for overall species diversity estimated using the Shannon diversity index (a combination of species richness and their abundances) (p-value = 0.0049). Results of this interaction show that P-Treated bags on the Alfisol had a significantly higher number of species and their total abundances than P-Treated bags on the Spodosol.

Community Composition

Community composition analysis (objective 4), as determined by the FunGuild functional assignment, resulted in a total of 1966 taxa combined from the rhizosphere and mesh bags. Of this 1966, 110 were classified as ECM. Of the 110 ECM taxa detected, 101 taxa belonged to the ECM mesh bag dataset, and 20 taxa were in the rhizosphere ECM dataset. Of the 20 ECM found on the rhizosphere, twelve were unique to the Alfisol, six were unique to the Spodosol, and only two taxa were shared between the two sites. For mesh bag samples, 62 were unique to the Alfisol, 18 for the Spodosol, and 21 were shared between the two sites. The total ECM + non-ECM dataset had

452 taxa found on the rhizosphere and 1838 taxa in the mesh bags across both sites. Of the 1838 total fungal taxa found in the mesh bags, 832 were only on the Alfisols, 353 were on the Spodosol, and 653 were shared. For the 452 on the rhizosphere, 176 taxa were unique to the Alfisol, and 111 were unique to the Spodosol. Of the 110 ECM taxa, 12 were shared between mesh bag treatments and the rhizosphere (Figure 3.8). A total of 19 taxa were unique to the mesh bags regardless of treatment and 8 were only found within the rhizosphere.



Genus	Species	Guild
Laccaria	*Spp.*	ECM
Tomentella	*Spp.*	ECM
Pisolithus	*arhizus*	ECM
Pisolithus	*arhizus*	ECM
Rhizopogon	*Spp.*	ECM
Rhizopogon	*Spp.*	ECM
Sistotrema	*Spp.*	ECM
Suillus	*cothurnatus*	ECM
Wilcoxina	*mikolae*	ECM
Xerocomus	*Spp.*	ECM
Chloridium	*Spp.*	ECM
Chloridium	*Spp.*	ECM

Figure 3.8: (Left) A Venn diagram of ECM species shows the 110 taxa classified as ectomycorrhiza. Only 12 taxa were found in the rhizosphere and mesh bags. (Right) Rhizosphere and Mesh Bag Shared Taxa. Twelve taxa were found to be shared between the rhizosphere and the mesh bags treated with P. Only three of these ASVs were classified to the species level.

Of the twelve taxa shared between the rhizosphere and P-Treated mesh bags (Figure 3.8), all genera found except *Sistotrema, Chloridium, and Xerocomus* are known early successional colonizers of *Pinus taeda* in North Carolina (Hackman et al., 2022). These genera are artifacts generated by the FunGuild database and many species in these genera do not classify as ECM. Many of the ECM taxa found in the Untreated bags were also known colonizers of *Pinus taeda*; however, these were not found to be associated with the roots.

To determine if community abundances and distributions of taxa were unique to any individual treatment to continue answering objective 4, two-way PERMANOVA analysis using P fertilization treatments and site as fixed effects found no strong evidence of interaction for the ECM community within the mesh bags (p-value = 0.218), however, individually both fixed effects

had a strong relationship with ECM community composition in the mesh bags for both P-Treated and Untreated bags (p-value ≤ 0.01, 0.01). ECM community changes within mesh bags indicate a strong community response to P fertilization rates (p-value ≤ 0.01). Comparisons among P fertilization treatments suggest that the high treatment (121 + 0 P) and the re-fertilized treatment (40 + 45 P) had significantly different communities vs. the control (0 + 0 P). Overall, the Spodosol had significantly less abundance and diversity of taxa; however, much of the higher abundance for the Alfisol can be attributed to *Russula, Rhizopogon,* and *Pisolithus*. The Spodosol had higher abundances of *Tomentella* across all treatments. Site was more critical than P fertilization treatment in our species indicator analyses for ECM within mesh bags. Among sites, four taxa were found for the Alfisol, *Protubera, Rhizopogon, Wilcoxina,* and *Enteloma,* and *Tomentella* was found for the Spodosol (Table 3.4).

Table 3.4: Species indicator analysis table of ECM taxa significantly associated with one or more mesh bag ECM sample treatment groups.

Site				
Site	Genus	Dataset	p-value	Reference
Alfisol	*Protubera*	ECM	0.01	Chen et al., 2000
	Rhizopogon	ECM	0.018	Molina & Trappe, 1994
	Wilcoxina	ECM	0.049	
	Entoloma	ECM	0.048	Khalid et al., 2022
Spodosol	Tomentella	ECM	0.002	Kuyper and Suz, 2023
P Fertilizer Treatments				
Labels	Genus	Dataset	P-value	Reference
0+0 P	none			
40+0 P	*Laccaria*	ECM	0.049	Desai et al., 2014
40+45 P	none			
121+0 P	Entoloma	ECM	0.029	Khalid et al., 2022

**p-value likelihood that a species is unique to a particular group based on its Indicator value (Dufrêne and Legendre, 1997)

For objective 5, to determine whether mesh bags and rhizosphere share commonalities between taxa, a total of 20 ECM taxa were detected from the rhizosphere of *Pinus taeda* trees. Of those 20 ECM taxa, only 13 were detected using mesh bags. One-way PERMANOVA tests with P Fertilizer treatment and site as fixed effect treatments showed no significant differences between

either P fertilization treatment or site. Abundance plots show a site and P fertilization treatments had drastically different abundances of the ECM genera in these data. Plots re-fertilized in the second rotation (40 + 45 P) had the least overall diversity among genera for both sites. Indicator species analysis did not find any of the 20 ECM taxa detected to be favored by any treatment group. The top three most abundant ECM genera were *Rhizopogon, Laccaria,* and *Chloridium* across sites and P fertilization treatments. A significant contribution to the *Rhizopogon and Laccaria* abundances came from the 121 + 0 carryover P treatment on the Alfisol (Figure 3.9).

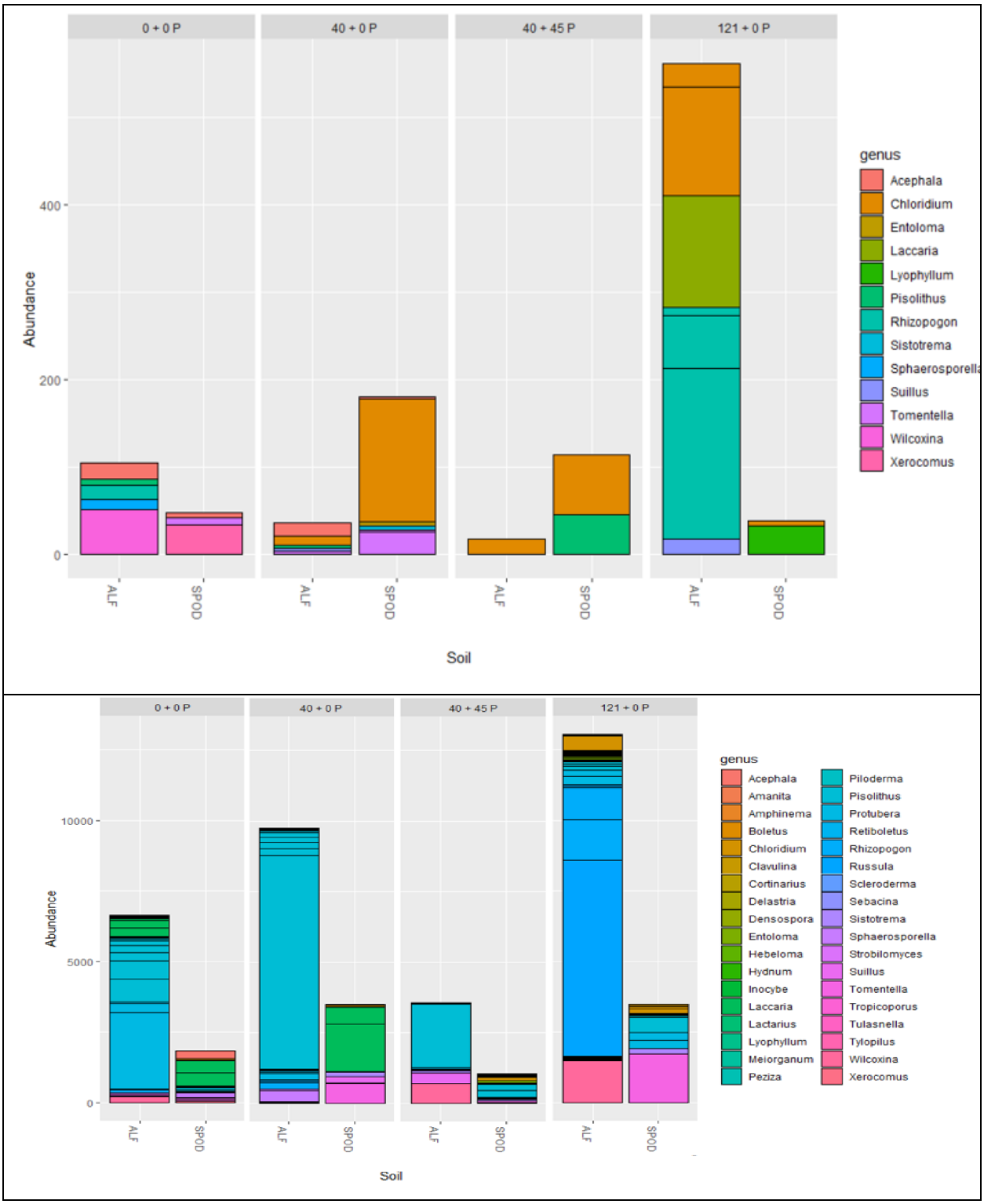


Figure 3.9: Rhizosphere Richness of ECM taxa between site (Alfisol = Alf, Spodosol = Spod) by P fertilization treatment (Top). Mesh bags Richness of ECM taxa between site and P fertilization treatments (Bottom). Total abundances for the mesh bag samples were nearly an order of magnitude higher than the abundances of ECM collected from the rhizosphere.

The rhizosphere ECM + non-ECM community was influenced by both site and P fertilization treatments. PERMANOVA tests on the rhizosphere using the ECM and non-ECM dataset consisting of 452 total taxa showed dramatic shifts between both site (p-value ≤ 0.001) and P fertilization treatments (p-value ≤ 0.001) (Figure 3.10). Species indicator analysis of site found 24 species for the Alfisol and 18 for the Spodosol out of the 452 detected in the original dataset. For P Fertilization effects, two were found for the 121 + 0 P treatment and three for the 40 + 0 carryover P treatment (Table 3.5). None were found for the re-fertilized, 40 + 45 P, or the control treatments (0 + 0 P).

Table 3.5: Species indicator analysis of rhizosphere ECM + non-ECM dataset fungi by site and significant interactions with P fertilization treatment from both sites.

Alfisol			**Spodosol**		
Genus	Species	P-value	Genus	Species	P-value
Umbelopsis		0.001	*Umbelopsis*	*autotrophica*	0.001
Umbelopsis	*angularis*	0.001	*Penicillium*		0.001
Talaromyces		0.001	*Umbelopsis*	*isabellina*	0.001
Penicillium		0.027	*Penicillium*	*zonatum*	0.001
Penicillium	*rolfsii*	0.001	*Trichoderma*		0.005
Talaromyces		0.001	*Umbelopsis*	*longicollis*	0.002
Umbelopsis	*dimorpha*	0.001	*Penicillium*	*canariense*	0.011
Coniochaeta	*velutina*	0.039	*Umbelopsis*	*isabellina*	0.003
Penicillium		0.022	*Aspergillus*	*japonicus*	0.015
Didymella	*bellidis*	0.007	*Rhodotorula*	*dairenensis*	0.035
Metapochonia	*bulbillosa*	0.006	*Talaromyces*		0.027
Occultifur		0.015	*Umbelopsis*	*isabellina*	0.005
Cryptotrichosporon	*argis*	0.007	*Sporothrix*		0.005
Rhodotorula	*toruloides*	0.014	*Paraphaeosphaeria*	*michotii*	0.034
Tremella	*phaeophysciae*	0.007	*Myrmecridium*	*schulzeri*	0.032
Selenophoma	*mahoniae*	0.028	*Sporothrix*	*rossii*	0.032
Rhodotorula	*toruloides*	0.031	*Talaromyces*		0.029
Occultifur		0.033	*Exophiala*	*abietophila*	0.028
Talaromyces	*yunnanensis*	0.024	**P fertilization treatment 121 + 0**		
Chaetosphaeria		0.026	Coniochaeta	deborreae	0.005
Melanospora	*kurssanoviana*	0.019	Umbelopsis	isabellina	0.014
Didymella	*bellidis*	0.031	**P fertilization treatment 40 + 0**		
Penicillium		0.03	Umbelopsis	isabellina	0.009
Penicillium	*dodgei*	0.024	Umbelopsis	longicollis	0.41
			Aureobasidium	pullulans	0.45

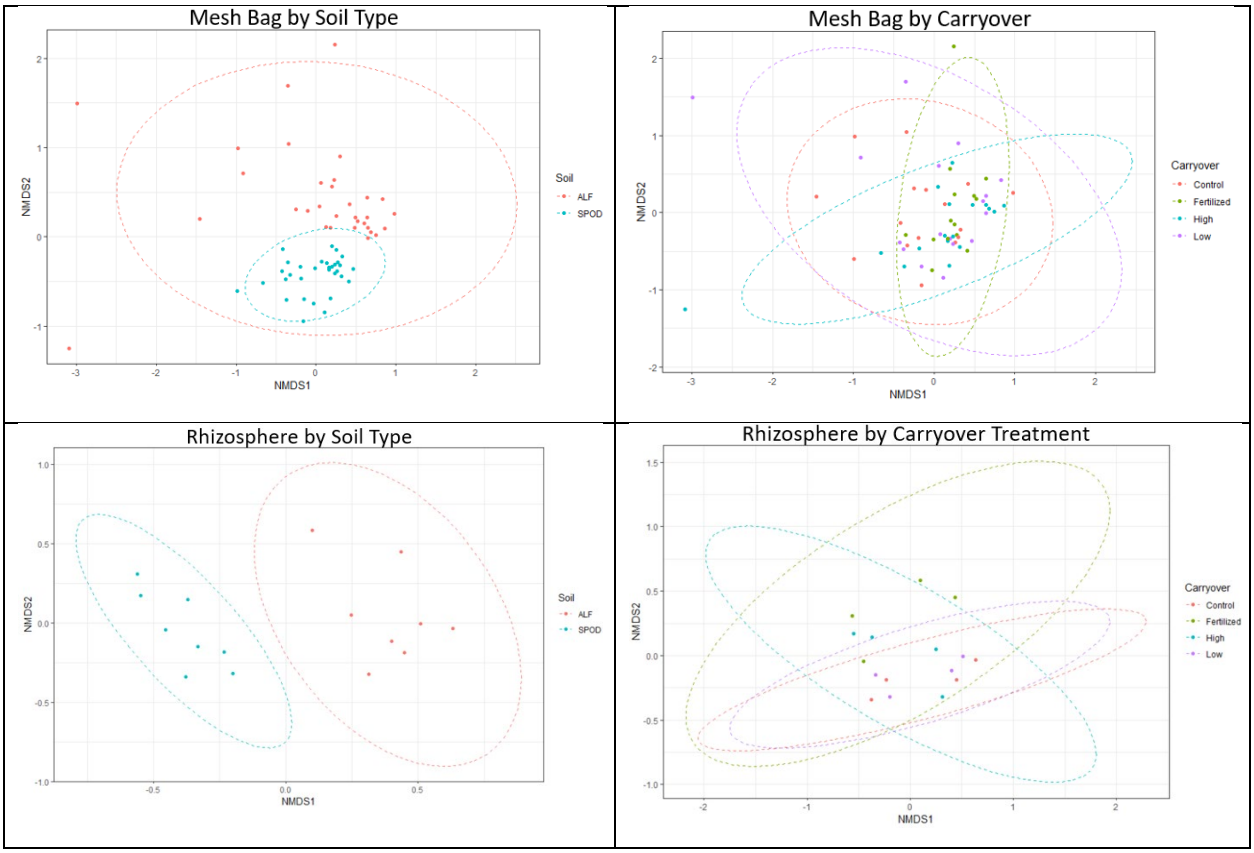


Figure 3.10: Root and Mesh Bag by site and Treatment. Root and mesh bag sample NMDS plots were transformed using Bray-Curtis dissimilarities plotted for mesh bags (top row) and rhizosphere (bottom row). Permutation ANOVA tests indicate significant shifts between sites for mesh bags (p-value = 0.02) and P fertilization treatment mesh bags (p-value ≤ 0.001). Rhizosphere plots using ECM + non-ECM species had significant shifts between site (p-value ≤ 0.0001) and treatment (p-value = 0.03) with 95% ellipses.

Discussion

The responses to site and P fertilization treatments indicate that the ECM community is highly responsive to changes in environment and nutrient availabilities, supported by Smith and Read (2008). For our first objective to determine how biomass changes after harvest, we tracked changes to biomass across four burial periods for both sites. Results show that burial period dramatically influenced biomass accumulation within the mesh bags. Samples collected in 2018 had the additional benefit of having large and established organic and litter layers where most ECM biomass is typically located in mature forest systems (Harvey et al., 1976). Samples collected in 2020, six months after site preparation and planting, decreased in total accumulated biomass from Pre-harvest samples in 2018. We attribute this decrease mainly to the effects of our site

preparation techniques which included bedding and herbicide treatments. Both methods eliminate any competing vegetation the ECM could colonize without *Pinus taeda* saplings (Lazaruk et al., 2008; Byrd et al., 2000).

Wallander et al. (2010) also noted that mycelium production increases later in the stand's rotation after canopy closure in Norway spruce (*Picea abies*) forests. Interestingly, similar amounts of biomass were recovered from Pre-harvest 2018 samples and post-harvest 2022 samples, even though 2018 samples only had a 3-month burial period as opposed to post-harvest samples which had a six-month burial period. This discrepancy between burial periods makes comparisons between the two difficult because we do not know if an additional three months in the ground would increase the amount of biomass accumulated. Hagenbo et al. (2018) reported that increases in burial period changed the fungal composition of mesh bags with growing proportions of mycorrhizal fungi increasing with increasing burial periods up to one year, highlighting the importance of "stable" burial periods when performing such experiments. When examining only post-harvest burial periods 2 through 4, site influenced fungal biomass accumulation over time. The current data shows biomass accumulation in the Alfisol could still increase over time, especially in P-Treated bags and in P fertilization treatments that received additional P in the second rotation. In contrast, biomass collected from the Spodosol may have plateaued only one year post-planting in 2021, implying that disturbance effects such as site preparation and planting are soil dependent. These site-specific patterns may influence P allocation and indicate a need for assessments across many soils to understand mycorrhizal communities (Averill et al., 2016).

For our second objective of finding if there are relationships between biomass and P Fertilization treatments, mycorrhizal communities are known to respond to P in multiple ways depending on species and P availability in the soil (Smith and Read, 2008). Due to these interactions, we hypothesized that P inputs from the previous rotation, known to remain in the soil for up to 30 years in southern pine plantations (Everett and Palm-leis, 2009), would still influence the biomass into the subsequent rotation. Interestingly, only P-Treated mesh bags from burial periods 1 and 2 had any biomass response to P fertilization treatments. Biomass was lower in plots fertilized with P during burial period 1, but higher in plots that received fertilization and higher rates of P Fertilization in burial period 2. From these results, we hypothesize a shift in community

composition from less specialized fungi for P acquisition from Pre-harvest burial period 1 to more specialized exploration and exploitation types of fungi post-harvest.

Our third objective was to determine if there are relationships between biomass collected in the mesh bags and DNA extracted from those mesh bags. Additional evidence that the loss-on-ignition method was suitable for detecting fungal growth and colonization of the mesh bags was found when biomass samples were compared to Shannon diversity indices by sample, a popular ecological metric used to estimate species richness relative abundance. Although relationships between the two metrics appear to be site and treatment specific, the positive relationship between the two metrics highlights loss-on-ignition as a promising, cheaper, and significantly less labor-intensive alternative to ergosterol sampling. A follow-up experiment comparing the two methods under more controlled conditions will be extremely valuable in determining the method's efficacy (Gessner, 2020; Martin et al., 1990).

Fungal Community Composition

For our fourth objective, looking only at the ECM community in the mesh bags, which consisted of 110 taxa functionally assigned by FunGuild, permutation ANOVA detected strong relationships between site and P fertilization treatments to ECM community changes within fungal mesh bags. The responses to site and P fertilization treatments indicate that the ECM community is highly responsive to changes in environment and nutrient availabilities, supported by Smith and Read (2008). These changes to the ECM's abundances and community compositions within the bags indicate that the ECM community could indicate P deficiencies or surpluses on these two soils. Species indicator analysis highlighted responses to individual community abundances varied dramatically by site and P Fertilization, with the Alfisol having significantly higher abundances of *Rhizopogon, Pisolithus, Wilcoxina,* and *Entoloma*, and the Spodosol having higher abundances of *Tomentella*, all known genera that associate with early plantations of *Pinus taeda* (Hackman et al., 2022).

PERMANOVA analysis of the rhizosphere ECM community did not reveal any significant interactions with either site or P fertilization treatment. This shows that these two factors did not significantly affect the ECM community, but some individual taxa were shown to have interactions with certain treatment effects using species indicator analysis. The rhizosphere had approximately 20 known ECM taxa that were detected using sequencing. We are confident that these are true

interactions because many of the species occurring on the rhizosphere are known as early plantation colonizers of *Pinus taeda* (Hackman et al., 2022). *Rhizopogon* and *Laccaria spp*. are known to improve the growth and uptake of P in *Pinus taeda*; however, it is surprising that it is in such high abundance in the treatments with the highest rates of P fertilization (Torbert & Burger, 1985).

Species Indicator Analysis on the ECM + non-ECM dataset revealed that *Umbelopsis isabellina* was found on the rhizosphere in 121 + 0 P and 40 + 0 P, P fertilization treatments. *Umbelopsis isabelline* belongs to the phylum *Mucoromycota* and is used in the production of biomaterials, specifically chitosan, a deacetylated homopolymer of chitin with various applications in medicine, agriculture, and wastewater industries (Kumar, 2000). Due to these applications, this species has been studied extensively and is highly sensitive to changes in inorganic P (Ye et al., 2015; Dzurendova et al., 2022). Although this species is not known to be mycorrhizal, its response to P as a possible biological indicator of P deficiency or surplus on the rhizosphere is an interaction that needs further exploration.

When comparing the presence and absence of taxa between the rhizosphere and the Mesh bag treatments, we found approximately 12 ECM taxa associated with both the rhizosphere of the trees and P-Treated mesh bags. Of those 12 taxa, all are known to have ECM associations with *Pinus taeda* (Hackman et al., 2022). Surprisingly, we found zero overlap in ECM taxa between untreated bags and the rhizosphere or between untreated bags and treated bags. This result implies that the P-treated bags not only had a unique ECM community as opposed to the Untreated bags but also that the ECM on the rhizosphere was not exploring the Untreated bags. These observations raise additional questions about how sensitive some of these ECM taxa are to changes in available P in the soil. Many taxa found in the Untreated bags are also known genera that associate with *Pinus taeda,* so the fact there were zero overlaps was surprising. These associations and overlaps may change over time, and only further examination will show the long-term effects of fertilization and site on fungal communities and their impact on nutrient uptake.

Conclusion & Management Implications

To our knowledge, this is the first study to compare the rhizosphere ECM communities with *Pinus taeda* under variable P fertilization rates using the mesh bag capture method. Biomass in mesh bags was responsive to P fertilization treatments, supporting the method's feasibility to

indicate P deficiencies/amendment. With additional sequencing data from that biomass, we could ascertain that at least some of the biomass in the bag was ECM fungi; however, additional sequencing or culturing is needed to identify ECM species in the bags accurately. For our ECM dataset, we did not find significant community changes in the rhizosphere of *Pinus taeda* due to site; however, when using the combined ECM and Non-ECM data, there were highly significant differences between rhizosphere communities by site and P fertilization treatments. Improving the ECM dataset with additional replications and sampling depth would help support our results. In optimizing P-use efficiency using ECM, we have only scratched the surface of what could be possible. Identifying genera that both colonize *Pinus taeda* and are optimized for gathering P in specific sites and environments will be vital in creating future experimental designs targeting direct applications of ECM with the appropriate conditions to maximize plant growth. Here we provide improved data on a range of ECM taxa that associate with *Pinus taeda* and are optimized for gathering P in P-limited conditions. This data further improves upon the ECM-Host knowledge base, which can lead to innovative inoculation strategies for *Pinus taeda* in the future for P uptake optimization.

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