BIOAVAILABILITY OF PHOSPHORUS TO

LOBLOLLY PINE (*PINUS TAEDA* L.) AND RED MAPLE (*ACER RUBRUM* L.)

IN CLAY AND SAPROLITE FROM THE SOUTHEASTERN PIEDMONT, USA

by

ZHINE WANG

(Under the direction of Daniel Markewitz)

ABSTRACT

Identifying the bioavailability of soil P pools and quantifying the amount of potential P uptake is important for defining long-term ecosystem productivity. My research investigates how loblolly pine (*Pinus teada* L.) and red maple (*Acer rubum* L.) take up P from soil clay (60-100 cm) and saprolite (450-500 cm) of the Calhoun Critical Zone Observatory in the Piedmont of South Carolina USA. Locally sourced seeds were germinated and grown in pairs of maple, pine, or maple and pine. I measured total P in plants relative to changes in soil P pools defined by Mehlich P, Hedley P, or Total P fractions, and indexed the extent of Fe reduction and phosphatase activity as potential mechanisms of P release. I found resin Pi and NaOH Pi supplied most to plant growth, and stable P pools (concentrated HCl P and residual P) buffered losses from these P pools.

INDEX WORDS: phosphorus, Hedley fractionation, soil total P, plant total P, soil iron reduction, mycorrhizal fungi

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CHAPTER I

INTRODUCTION

Forests are an important natural resource in the southeastern USA. Around 900,000 km^2 of the southeastern USA land area is covered by forest (Huang et al., 2011). These lands support the largest forest industry in the USA, primarily through pine plantation management, and supply 55% of national timber demand (Cubbage & Albert, 1998). Phosphorus (P), as one of the most limited and critical plant nutrients, impacts forest health and the yield of pine plantations. Soils in the growing region of the southeastern Piedmont are predominantly acidic Ultisols that in the native condition are low in P (<400 $\mu g\ g^{-1}$ total P). This region, however, has undergone a series of land use changes from forest clearing and agriculture (Boggs et al., 2013), which altered the pools of soil P through both erosion and fertilization. As such, understanding P supply and soil distribution of P in the Piedmont area requires continued investigation.

To assess P availability in Piedmont soils a number of studies have applied the Hedley fractionation approach (Hedley et al. 1982) on a decadal timescale. For example, Richter et al. (2006) quantified changes in Hedley P fractions over 28 years (1962 to 1990) as pines grew from age from 5 to 33 in an old agricultural field. They found that despite ~8 $g\ m^{-2}$ of plant P uptake, the most labile P fractions (resin Pi and $NaHCO_3$ Pi) barely decreased (0.08 $\mu g\ g^{-1}$) while the NaOH and 1M HCl pools declined by 4.5 $\mu g\ g^{-1}$. They concluded that these moderately labile P pools were resupplying the labile pools and sustaining plant P uptake. In a similar approach, Schmidt et al. (1996) studied P dynamics during crop cultivation in the Piedmont from 1975 to 1992. However,

they found that during an unfertilized period (1986 – 1992), resin Pi and $NaHCO_3$ P both decreased by around 4 μg g^{-1} and NaOH P decreased by 5 μg g^{-1} suggesting that these three pools were plant available.

In contrast to these decadal studies, the more recent work of Niederberger et al. (2017) applied the Hedley fraction to a short-term greenhouse study (6 months) to assess P availability to a hybrid poplar clone (*Populus nigra* L. x *Populus maximoviczii* Henry). This study used glaciated soils in Germany that were Entisols and Inceptisols with sand, silty loam, and loamy silt textures and had 2 – 5% C. In this study, resin Pi and $NaHCO_3$ Pi and Po significantly decreased after six months.

Given these previous studies, my goal was to apply the Hedley fraction in a short-term (one year) greenhouse study following Niederberger et al. (2017), but working in Piedmont soils similar to Richter et al. (2006). As such, this study focuses on identifying changes in soil P fractions and understanding plant P extraction mechanisms. I used loblolly pine and red maple, which are both widely distributed species throughout the Southeast region and grow on a diversity of soil types. These species differ, however, in their mycorrhizal symbionts with pine being ectomycorrhizal and maple being arbuscular. Ectomycorrhizal plants (like pine) are dominant in subtropical and temperate forests, while arbuscular plants (like maple) dominate in tropical forests (Plassard & Dell, 2010). Studies of ectomycorrhizae and arbuscular mycorrhizae demonstrate that both can facilitate P uptake either through increasing root surface area or through release of the enzyme phosphatase (van der Heijden, 2001; Wallander, 2000).

I also investigated two portions of the soil profile: clay and saprolite. Ultisols of this region often have a thick (~1m) argillic (Bt) horizon comprised of 15 – 60% clay. Clay mineralogy can vary, but is dominated by kaolinite. Saprolite, which is isovolumetrically weathered bedrock, is

below the Bt, has low clay content (<5%), and although still dominated by kaolinite can have some primary minerals in the larger size fractions of biotite or orthoclase. Neither clay nor saprolite in this region has the P bearing mineral apatite (Austin et al., 2018).

To assess plant P availability in these substrates I, too, use the Hedley fractionation to analyze the concentration of different soil P pools prior to and at the end of a greenhouse study. The Hedley fractionation has been widely applied in short-term and long-term studies (Niederberger et al., 2017; Richter et al., 2006) and has been applied in many soil types (Cross and Schlesinger 1995; Figure 1.1).

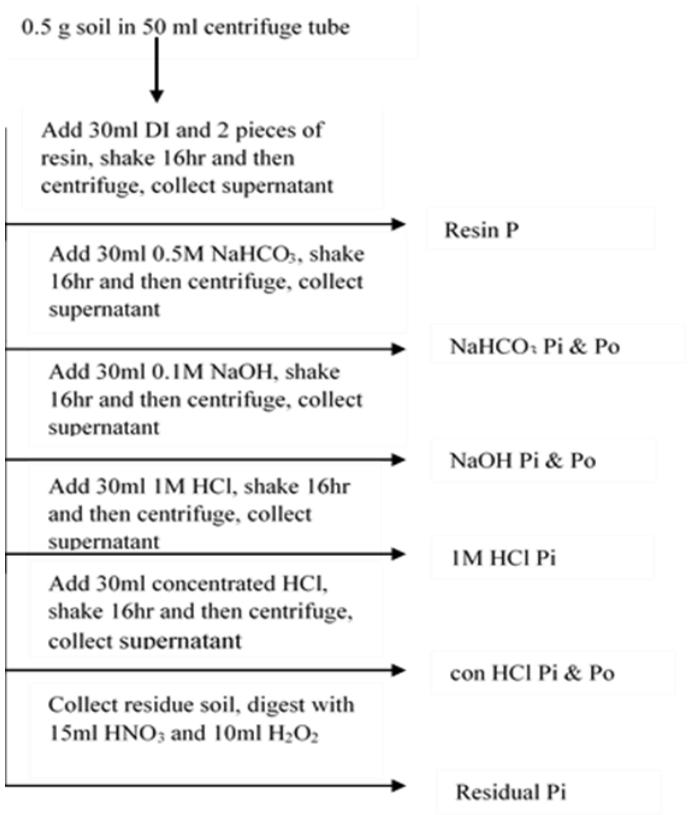

Figure 1.1. Hedley P fraction extraction sequence (after Tiessen & Moir, 1993).

Resin P and $NaHCO_3$ Pi (inorganic P) are ionically bound forms of P, which are readily available to plants, exist in equilibrium with the soil solution P, and are usually highly limited (<0.1 – 3 mg L^{-1}) (Frossard et al., 2000). I use labile Pi to represent the sum of resin and $NaHCO_3$ Pi and

identify these as the most likely plant available pools. With the addition of $NaHCO_3$ Po these three fractions make up Labile P. NaOH Pi is associated with soil Fe and Al oxide mineral surfaces, which can be abundant (3 – 10 mg g^{-1}) in clay rich Ultisols (Zhang et al., 2017). NaOH Po typically represents P associated with soil organic matter, which is elevated in A horizon (0.7-0.8%) but is low in Bt horizons and saprolite (~0.3%) (Richter et al., 1999). NaOH Po is also called extractable Po. The 1M HCl Pi represents Ca-bounded P, which is low in undisturbed Ultisols of the Piedmont but can be elevated in forest soils that were in agriculture previously and received inputs of lime and P. Finally, concentrated (con) HCl P and residual Pi (that portion recovered through a total digestion after removal of all other fractions) are considered as stable P and not plant available. Con HCl Pi likely represents P that is strongly bound to the interlayers of secondary minerals and residual Pi is large molecular weight complexes that correspond to tightly bound P in organic matter (Hedley et al., 1982) . Especially in regards to the Hedley fractions, I hypothesize that resin Pi and $NaHCO_3$ Pi will decline in response to plant P demand but will be lower than total plant P uptake as other pools (NaOH Po) will buffer the labile P pools.

In addition to the Hedley fractionation, I also used the Mehlich III (MIII) extractant to test changes in readily available soil P. MIII is closely related to other conventional methods of P extraction such as Bray and Kurtz P-1 extractions, but MIII is less aggressive towards calcium phosphates (CSSS, 2006). Here I wanted to assess MIII P relative to the labile P pools (Resin Pi, $NaHCO_3$ Pi and $NaHCO_3$ Po) and relative to plant P uptake.

Along with these budgets of plant P uptake relative to soil P decline, I wanted to assess specific plant mechanisms of P acquisition. Soil Fe oxide surfaces absorb 100 to 500 μmol g^{-1} phosphate under laboratory conditions (Parfitt et al., 1975) and this mechanism can cause P immobilization in the field. A potential mechanism for plant to acquire some of this P is by either

facilitating or taking advantage of ancillary Fe reduction. As Fe converts from insoluble trivalent to highly soluble bivalent forms, Fe bounded P can be released (Lin et al., 2018). My research aims to study the correlation between potential soil iron reduction and the decline of NaOH Pi, which is closely correlated to Fe bound P. I used rusted iron bars in soil as indicators of the potential for Fe reduction. I hypothesize that Fe reduction is more intense in the clay layers than in saprolite given that clay has greater iron oxide abundance and poorer drainage compared with the saprolite and that higher potential for Fe reduction will be correlated with higher total plant P.

Another potential mechanism of interest is plant release of the enzyme phosphatase through mycorrhizal fungi to acquire less available forms of P. Mycorrhizae are important symbionts on plant roots (Frioni et al., 1998), breaking down Po through release of enzymes and providing a P transporter to carry Pi into plant roots. Mycorrhizae are classified as ectomycorrhiza (EM) and arbuscular (AM). Previous research has shown a discrepancy between the two mycorrhizal fungi species regarding the effect on plant growth. In a study of *Salix repens* L., a dual mycorrhizal plant, AM fungi tended to contribute to plant growth mostly in the short-term (7 weeks and 12 weeks after planting), whereas EM fungi was found to be more beneficial in the long-term (30 weeks after planting) (van der Heijden, 2001). As such, I planted only AM (maple), only EM (pines), and a mix of AM and EM to evaluate effects over time. I expect that ectomycorrhizae (i.e., pines) alone will release more phosphatase into the soil. To test this, I extract soil samples during the greenhouse study to analyze for phosphatase activity as an indirect indicator of phosphatase concentration. I hypothesize that soil growing with pines would present higher enzyme activity than soil growing with maples.

CHAPTER II

LITERATURE REVIEW

Phosphorus limitation

Phosphorus (P) is one of the most critical but also the least abundant plant macro-nutrients in soil. Organic phosphorus (Po) takes part in major plant biochemical activities such as photosynthesis and respiration (Raghothama & Karthikeyan, 2005). Soil P supply is thus an essential driver of agricultural production and forest health. Unfortunately, more than 80% of the total soil P can become immobile and unavailable for plant uptake as the result of adsorption, precipitation, or conversion to recalcitrant organic forms (Holford 1997). Data from 135 soil samples in the U.S. showed that PO_4^{3-} concentration in soil solution is never higher than 8 μM, and median concentration is 1.5 μM (Bieleski, 1973). Therefore, defining different P pools and understanding the bioavailability in the soil is critical to managing both natural and plantation forests.

Phosphorus bioavailability

Apatite, the primary mineral source of P, contains 0.4 to 0.5 mg g^{-1} exchangeable P (Chadwick et al., 1999). Phosphate is initially released from apatite through carbonation weathering (Equation 1) in a congruent reaction (Schlesinger & Bernhardt, 2013),

$$Ca_5(PO_4)_3OH + 4H_2CO_3 \rightarrow 5Ca^{2+} + 3HPO_4^{2-} + 4HCO_3^- + H_2O \quad (1)$$

Mobilized phosphate from this reaction can be available to plants although most phosphate will quickly be sorbed to secondary Fe and Al minerals or leached from the soil in hydrological movement. P that is sorbed on mineral surfaces may become occluded over time and become unavailable to plants. These occluded forms of P likely result as absorbed P is coated by stable Fe and Al oxides and hydrous oxides. As apatite weathers, mobile forms of P tend to decline and these occluded forms of P tend to increase with continuing soil development (Figure 2.1). P can also become unavailable when tightly bounded with recalcitrant organic matter (Evans & Syers, 1971).

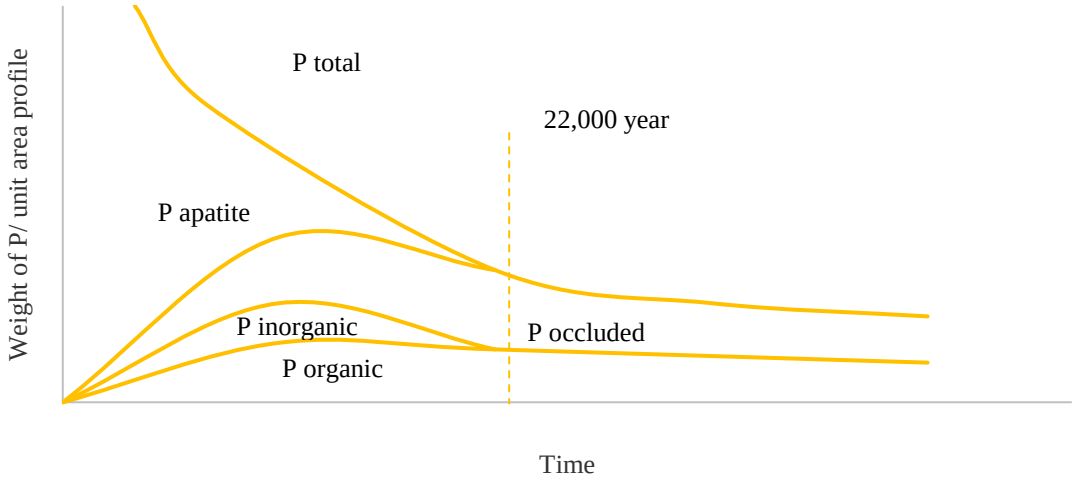

Figure 2.1. Change in the forms of phosphorus found during soil development on sand dunes in New Zealand (modified from Walker and Syers, 1976)

The increasing immobility of soil inorganic phosphate is the result of the reactivity of phosphate (P) ions relative to different soil components and to the consequent strong retention of most soil P onto those components. Fe, Ca, and Al form strong P complexes that have been identified to hold as much as 30 – 60% of inorganic P (Pi) (Herbert & Fownes, 1995). Organic P (Po) can also comprise a major portion of total soil P ranging from 10 – 40% in native soils (Cross and Schlesinger 1995) or in agricultural soils up to 30 – 80% of total P. The largest fraction of organic P, approximately 50%, appears to be in the form of phytin and its derivatives (Tarafdar &

Claassen, 1988). Po is present in living soil organisms and dead soil organic matter (Achat et al., 2009).

Phosphorus in forests

Studies of forest P cycling indicate an annual P requirement ranging from 0.1 – 0.6 g m^{-2} and aboveground biomass contents from 1 – 5 g m^{-2} (Johnson et al., 2003, Markewitz et al., 2004). P storage in soil O horizons ranges from 0.5 – 7 g m^{-2} and the first 10 cm of soil may contain 0.1 – 1.0 g m^{-2} of labile P depending on soil type. In southern US loblolly pine stands, in particularly, Mehlich III extractable P ranged from 4 – 6 μg g^{-1} or ~0.4 – 0.6 g m^{-2} in the upper 10 cm (Bünemann et al., 2011). One pine plantation in the southern Andean region of Ecuador had A horizon soil (0 – 20 cm) with only 0.05 g m^{-2} of P (Quichimbo et al., 2019). In addition, annual precipitation generally provides <0.01 g m^{-2} P (Markewitz et al., 2004). In soil solutions, labile P is also limited. A five-year study on a Piedmont loblolly pine plantation indicated below 20 cm depth, soil solution only contains 1.1 – 5.4 mg L^{-1} P (Wells et al., 1986).

Plant mechanisms for acquiring phosphorus

Different methods have been identified through which plants acquire P to survive in a low P environment. First, plants can directly uptake PO_4^{3-} (readily available Pi) from soil solution, although as noted above solutions typically have low concentrations. In temperate agricultural systems concentrations may range from 0.01 – 3 mg L^{-1} of P in soil solution (Frossard et al., 2000), while in temperate forests P concentrations below the forest floor may range from 0.01 – 0.03 mg L^{-1} (Yanai 1991). This P is directly available to plants but the mass of P in soil solution at any time only meets a small portion of annual demand (Frossard et al., 2000).

Plants are able to increase specific root length and density to reach more soil per root area and potentially capture more soil solution P (Hill et al., 2010). In addition, associated changes to the physical, chemical and biological properties of rhizosphere soil can influence P dissolution from the solid phase to solution with a subsequent benefit to the growth and health of plants (Richardson et al., 2009).

Plants also increase P mobility by modifying soil pH, and most cases of microbial P solubilization are completed via acidification (Bünemann et al., 2011). A major process that contributes pH changes in the rhizosphere is the release of ionic charges carried by H^+ or OH^- to compensate for an unbalanced cation-anion uptake at the soil–root interface (Hinsinger et al., 2003). Field study in Australian and New Zealand sites indicated plant roots could release H^+ to increase the dissolution of phosphate rocks and hence the mobility of inorganic P (Bolan et al., 1990). Organic acid is also a possible source for acidifying the rhizosphere in response to P deficiency. This mechanism has been detected for species such as chickpea (*Cicer arietinum* L.) (Ohwaki and Sugahara, 1997), maize (*Zea mays* L.*)* (Peterson & Bottger, 1991) and tomato (*Lycopersicon esculentum* L.) (Imas et al., 1997). Root respiration in the rhizosphere can exude great amounts of CO_2, which later forms carbonic acid in soil solution and decreases soil pH. Carbohydrate flux used for plant respiration ranged from 29 – 67% of total CO_2 translocated to roots in *Daucus carota* and *Helianthus annuus* (Lambers et al., 2000).

Given the insufficient supply of readily available solution and solid phase Pi, plants also attempt to access less available P, such as that adsorbed on iron (Fe) and aluminum (Al) oxides. It is well understood that phosphate has a relatively strong affinity for mineral surfaces and it is tightly adsorbed to the surface of metal (hydro)oxides. Laboratory experiment showed natural ferrihydrite can adsorb around 190 μmol g^{-1} of phosphate after shaking with KH_2PO_4 while

goethite can adsorb up to 120 μmol g^{-1} (Parfitt, 1989), and the adsorbed amount was positively correlated with specific mineral surface area. Liptzin and Silver (2009) provided a model of Fe reduction and P dynamics (Figure 2.2). During this process, trivalent Fe is reduced to divalent Fe by organic acid such as citric acid or oxalic acid and hence the Fe-P bound is broken. Mobilized P can be taken up by plants or combine with soil organic matter. Plant exudates released through plant roots and mycorrhizal fungi serve as an energy source for soil bacteria that reduce Fe^{3+} (equation 2).

$$24Fe^{3+} + C_6H_{12}O_6 + 6H_2O \rightarrow 24Fe^{2+} + 6CO_2 + 24H^+ \quad (2)$$

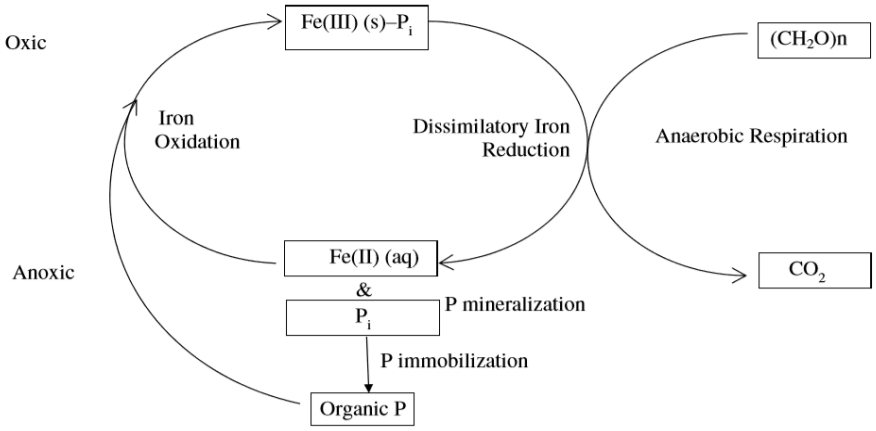

Figure 2.2. Conceptual model of Fe reduction linked to P availability and C oxidation (Liptzin & Silver, 2009)

Organic P is another less available form for plants. Most plants cooperate with mycorrhizal fungi to capture P. Mycorrhizae are important symbionts on plant roots (Frioni et al., 1998), breaking down Po through release of enzymes and providing a P transporter to carry Pi into plant roots. Mycorrhizae are classified as ectomycorrhiza (EM) and arbuscular (AM). Ectomycorrhizal hyphae cover the roots but do not penetrate the cell, while arbuscular fungal hyphae directly penetrate the root cell's membrane. Compared with non-mycorrhizal plants, mycorrhizal plants are

more effective at P acquisition belowground (Bünemann et al., 2011).

Studies have tested the growth and P uptake differences between mycorrhizal colonized plants and non-mycorrhizal colonized plants. Wallander (2000) cultivated *Pinus sylvestris* L. in a pot system with the existence of apatite. This study demonstrated that seedlings colonized by ectomycorrhizal (EM) fungi on average gained 80% more biomass than in seedlings without EM fungi. With the persistence of EM fungi, seedlings have significantly higher P concentration (1.1 – 1.5 mg g^{-1}) than that (0.6 – 1 mg g^{-1}) of control seedlings. Griffiths (1994) suggested that EM fungi improve the uptake of soil P by exuding low molecular-weight organic acids, especially oxalic acid. The two mycorrhizal fungi species (ecto vs arbuscular) also show discrepancy regarding the effect on plant growth. In a long-term study of *Salix repens L.,* a dual mycorrhizal plant, AM fungi tended to contribute to plant growth mostly in the short-term (7 weeks and 12 weeks after planting), whereas EM fungi was found to be more beneficial in the long-term (30 weeks after planting) (van der Heijden, 2001).

Phosphatases are the key enzymes that break down Po. These group of enzymes exuded by root systems use water to cleave a phosphoric acid monoester into a phosphate ion and an alcohol. Induction of phosphatases during Pi deficiency is a universal response in higher plants (Goldstein, 1992). According to the pH condition, phosphatase can be classified as acid phosphatase, alkaline phosphatase, and purple acid phosphatase. These enzymes are present in intracellular compartments or extracellular spaces (Kraè & Green, 2000). Plant roots are major producers of acid phosphatase, while soil bacteria and fungi generate most of the alkaline phosphatase (Vincent et al., 1992). Acid phosphatases are involved in pH-dependent hydrolysis of monoester soil organic P in the rhizosphere. Acid phosphatases hydrolyze phosphate esters in a mechanism as reported by Vincent et al. (1992):

$$R\text{-}O\text{-}PO_3^{2-} + H_2O = R\text{-}OH + H\text{-}O\text{-}PO_3^{2-} \quad (3)$$

where R represents a monoester group.

Alkaline phosphatases are typically dimers of 94 kDa subunits that hydrolyze a wide variety of phosphate monoesters, with a pH optimum of 8. The distinguishing feature of alkaline phosphatase is the existence of two Zn and one Mg ions per subunit (Raghothama & Karthikeyan, 2005). The zinc ions facilitate the cleavage of the substrate-P bonds. Coordination of H_2O to Zn can produce an OH-nucleophile to displace the phosphoryl group from the phosphoserine intermediate.

Purple acid phosphatases are among the commonly observed phosphatases secreted into the rhizosphere during P deficiency. They are distinguished from acid phosphatase by the existence of binuclear transition metal centers (i.e., Fe(III)-Fe(II), Fe(III)-Mn(II) or Fe(III)-Zn(II)) (Vincent et al., 1992) and their resistance to inhibition by tartrate, a potent inhibitor of most other acid phosphatases. The pink or purple color arises from a low energy charge transfer transition from tyrosine phenoxide to ferric iron.

Since both the purple acid phosphatases and some of the acid phosphatases that lack metal cofactors appear to employ similar overall mechanisms that utilize a phosphohistidine intermediate, the role of the binuclear iron center in the former needs to be clarified. There is no evidence that the iron center produces a superior catalyst. It is true that the Fe(II) in purple acid phosphatases provide plausible sources of positive charge to bind and anchor the anionic phosphate ester substrate, since ferric ion is a strong Lewis acid and hydrolyses-readily in aqueous solution (Vincent et al., 1992). A mechanism for the purple acid phosphatases is shown below (Figure 2.3). This figure also illustrates how binding of both the phosphate ester substrate and water to the di-iron center might facilitate phosphate ester hydrolysis.

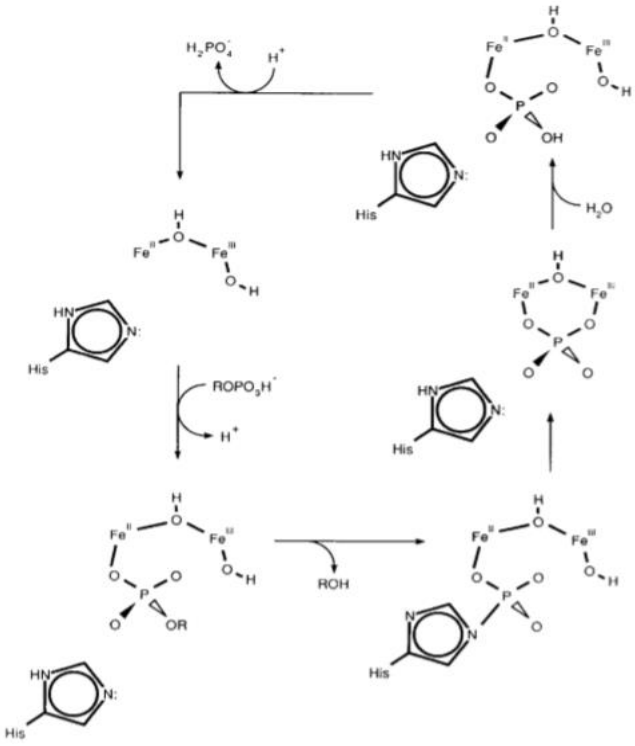

Figure 2.3. Possible role of the iron centers in phosphate ester hydrolysis by purple acid phosphatases (Karandashov & Bucher, 2005)

In addition to lysing organic P, enzymes may also facilitate P uptake by impacting uptake kinetics (Casper & Jackson, 1997). Nutrient uptake in most ecosystems is governed by Michaelis-Menten kinetics:

$$V = V_{max}C_l / (C_l + K_m)$$

where V is the flux of ion into roots per unit time, V_{max} is the maximum of V, C_l is soil solution concentration at the root surface, and K_m is soil solution concentration where $V = 0.5V_{max}$. Mycorrhizal plants can exude more enzyme per root area, which leads to a greater V_{max} and a higher ion affinity of enzymes (lower K_m).

Plant species used in this study

Loblolly pine (*Pinus taeda L.*) is a native tree of North America and is a common host for

ectomycorrhizal fungi. It also is the most dominant and commercially important evergreen tree in the southern United States used for pulp and wood products. Humid, warm-temperate with long, hot summers and mild winters are the most preferred climate for loblolly pine growth. Loblolly pine grows on a wide variety of soils, ranging from the flat, poorly drained soil of the Atlantic Coastal Plain to the relatively dry inland soils of the Piedmont (USDA, 1975). This species has been shown to be responsive to P fertilization in plantations (Wells et al., 1986).

Red maple (*Acer rubrum L.*) is a native tree of North America and an arbuscular mycorrhizal fungal host. It is also widespread throughout eastern and central North America. Red maple is adapted to a broad range of soil types. Although it develops best on moderately well-drained, moist sites at low to intermediate elevations, red maple is common in the mountainous country on the drier ridges and on south and west exposures of upper slopes. Red maple is also common, however, in swampy areas, on slow-draining flats and depressions, and along small sluggish streams (Hutnick & Yawney, 1961). Red maple (and its varieties) are a commonly planted horticultural tree. It is also desirable as a wood species but is rarely grown in plantation in the Southeast.

CHAPTER III

METHODS

Study substrates

I used two substrates, clay and saprolite, both of which were collected in the Calhoun Experimental Forest of the Sumter National Forest in Union County, South Carolina in January 2018. Annual precipitation is around 1170mm (1950 – 1987) and average temperature is 16°C (NOAA, 1994).

Ultisols are most common in the Calhoun, which typically formed from igneous rock weathering in humid and warm temperate regions (Richter et al., 1994). Ultisols have an argillic or kandic horizon with low base saturation, and clay content increases with depth and often reaches the highest concentrations at the top of the Bt horizon (Schmidt et al., 1996). Appling and Cataula series soils (Fine, kaolinitic, thermic Typic Kanhapludults or Fine, kaolinitic, thermic, Oxyaquic Kanhapludults) cover most slopes (<3%) (USDA, 2014). Dominated by cotton and corn cultivation for nearly 150 years, most soils in the Calhoun area were degraded due to this intensive agricultural land-use. From the middle of the twentieth century, the US forest service started to regenerate the Calhoun area and it is now mainly covered by pine forest (Richter et al., 2000). Clay substrate was collected from the upper Bt horizon, 60 – 100 cm below the surface, while saprolite substrate was collected from within the BC horizon, 450 – 500 cm belowground.

Experiment Design

I used a randomized block design, which has two factors (soil substrate and tree species). Tree

species has four levels: 2 maple (MM), 2 pine (PP), 1 maple + 1 pine (MP), and no seedling blank (B). Soil substrate has two levels: clay and saprolite. Treatments within each substrate and block had a sample size of 3 PP tubes, 3 MP tubes, 3 MM tubes, and 3 no seedling blank tubes. Thus, there were 24 growth tubes per block. In total there were 24 tubes x 7 blocks for 168 tubes. I moved blocks around in a clockwise fashion every week to make sure every block received the same greenhouse conditions that might be present at a specific position on the growing bench.

Soil Preparation

At the time of substrate collection, clay and saprolite moisture content (by mass) were similar with 4.7 and 4.6%, respectively. Before planting, both soils were dried in an oven (Kysor Corporation, Michigan) at 35°C for 48 h to facilitate homogenization and planting. During this time, all trays and tubes were cleaned with disinfectant and bleach. After substrates were dry, each was separately homogenized in a 12101 mixer (Bouldin & Lawson Inc, Tennessee). Samples were then sieved through a 2mm sieve and left air dried.

Seed germination & Planting

Loblolly pine (*Pinus taeda* L.) seeds were provided by the Georgia Forestry Commission. Red maple (*Acer rubrum* L.) seeds were collected from Whitehall forest in Athens GA. I collected red maple seeds from nine different maple trees by encircling immature samaras with organza bags until they fell from the stem and I collected the bags on April 2nd, 2018. Pine seeds were hydrated on wet paper towels in a half-sealed zip-lock bag for 48h and afterwards I saturated the pine seeds in water for 24h to ensure full saturation. Thereafter, seeds were chilled in a refrigerator for 40 d, under a temperature of 4.0°C. Maple seeds needed no treatment after collection. Pine and maple

seeds were planted in 60 x 30 cm trays in the greenhouse on April 2nd, 2018. Maple seeds were planted 1 cm under the soil and pine seeds were placed on the soil surface. During April and May, I watered seeds once a day with deionized water from the bottom of the trays, which avoided seed disturbance. During the first month of seed germination, fertilizer was not applied. In May, after germination, plants were transplanted into clay and saprolite substrates in growth tubes that have a volume of 135 cm^3. On average this tube volume required 175 g of clay or 180 g of saprolite.

Plant Growth

Plants were grown in tubes from May 2018 to May 2019. Plants were watered daily with deionized water and were fertilized with P-free Hoagland fertilizer (Hoagland & Arnon, 1950) solution twice a week. Growth lights were applied to the plants 16 hours per day. In December 2018, due to poor growth and survival, I applied 10 μg of P per growth tube in three randomly-selected blocks (2, 4, and 5) in hopes of priming the plants and facilitating greater growth. In 2019, surviving plants were harvested and each individual plant separated into root, stem, and leaf. I gently washed off any soil before oven drying plant components and grinding in an 8000-D mixer mill® grinder (Spex CertiPrep, New Jersey) for 5 min.

Soil and Plant Analysis

In April 2018, prior to planting, soil available P was analyzed using Mehlich III (CSSS, 1993) in a 1:10 soil:solution ratio using air-dried clay (n=5) and saprolite (n=5). Samples were shaken at 125 rpm on a G-33 shaker (New Brunswick Scientific, New Jersey) for 5 min. After shaking, samples were filtered through pre-rinsed Whatman 42 filters. Filtrates were analyzed using the Murphy-Riley chemistry on a Genesys™2 spectrophotometer (Thermo Electron,

Massachusetts). In December 2019, after plant growth and harvest, all 168 soil samples were dried, sieved through a 2 mm screen and run for Mehlich III P along with re-analysis of stored clay and saprolite substrate.

In a similar approach, the Hedley P fractionation (Hedley et al., 1982) was performed both before and after seedling growth to quantify soil P pools. The first fractionation analysis was performed in July 2018 on clay (n=10) and saprolite (n=10). I used 1 X 6 cm strips of BDH No. 55164 resins (CSSS, 2006) for the resin extraction step. After pH adjustment, all extracts (resin Pi, $NaHCO_3$ Pi, NaOH Pi,1M HCl Pi, Con HCl Pi, and Residual P) were analyzed as the Mehlich III above. For HCO_3, NaOH, and Con HCl, a subsample of the extract was digested using the persulfate digest and reanalyzed for Pi with the difference between digested and undigested used to estimate Po. Following plant growth and harvest, another Hedley fractionation analysis was conducted in January 2020. In the second extraction, I analyzed resin Pi, $NaHCO_3$ Pi, NaOH Pi and 1M HCl Pi following the analytical methods above. However, I measured total P on an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) instead of using the persulfate digest. I also ran a subset of samples by persulfate digest as a quality control check. Paired t-test for results from the two methods indicated an insignificant difference (p value= 0.15) and Pearson correlation coefficient between the two methods was 0.97 (supplemental Figure 1).

Total P for initial substrates and for substrates after the growing year were analyzed using 0.1 g of sample following EPA method 3050b (1996). Standard reference material (SRM) 2711 Montana soil was used for quality control. Total P for plant tissues were digested following the same method using SRM 1547 Peach leaves as quality control samples. Approximately 0.1 gram of ground plant sample was used for analysis. If a sample's total recovered weight was < 0.1 gram the total available mass was utilized. This was true for ~33% of samples with the lowest mass

weight of 0.001 g. For complete digestion, up to 10 ml H_2O_2 was added to samples. P concentration was analyzed via ICP-OES as described above.

Index of iron reduction

I conducted two assays of the potential for iron reduction as developed by Hodges et al. (2018). The first assay was in May 2018, the second in Aug 2018. I used 2x10 cm iron bars that were ~2 mm thick. Prior to placing rods in the soil, iron bars were rusted thoroughly by spraying a mist of 0.1M HCl on the surface. Thereafter, one bar was inserted into each plant tube and extracted after four weeks. I gently rinsed off attached soil on the iron bars and photographed both sides. Pictures of the bar were analyzed using Adobe Photoshop 6®. In brief, reduced pixels are selected and then all pixels in the appropriate color range are counted. After pixel analysis, the ratio of reduced pixels to total pixels is reported as a percentage with values representing an index of iron reduction potential in the soil.

Phosphatase activity

In November 2018 and March 2019, a plastic straw (8 mm diameter) was used to subsample the upper 3 cm of soil from the three P-primed blocks. I also sampled rhizosphere soil of three red maple trees from which I earlier collected seeds and three local loblolly pine trees. Soil samples were stored at -4º C until analysis. Phosphatase activity was analyzed following Bell et al. (2013), although instead of 2.75 g of soil only 1 g was used. Soil was blended in 125 ml of 50 mM Sodium Acetate for 30 seconds by Rival IB954W handheld blender (New Brand, California). The soil slurry was then pipetted into 96-well plates and then stored overnight at 24°C. After incubation, the plates were read on a Synergy H1 microplate reader (Biotek, Vermont). A calibration curve

following Bell et al. (2013) was used to estimate activity. Enzyme activity was expressed in the unit of nmol 4-Methylumbelliferone (4-MUB) g^{-1} h^{-1} and calculated as followed:

$$Activity = \frac{4\ MUB\ released(\mu mol) * 125ml * 1000}{0.2ml * Time(h) * Soil\ (g)}$$

Where, 4-MUB released is calculated by calibration curve, 125 ml is the soil slurry volume, and 0.2 ml is the volume of soil slurry used in the test.

Statistical Analyses

I used tubes from block 1,3,6, and 7, which were not primed with 10 μg P during planting for MIII, Hedley, total soil and plant P per tube analysis. Blocks 2, 4, and 5 were only used for phosphatase analysis pre-priming and post-priming. Sample sizes under the soil x plant treatment combinations were unbalanced due to the plant mortality during the study. As such, for the individual soil substrates, means in MIII P concentration, Hedley P fractions, and soil total P were tested after growth with an unbalanced one-way ANOVA with plant treatments as the main factor. Pairwise differences were tested for significance at $p<0.05$ using Tukey's Honestly Significant Difference (HSD). Means in P were tested with the same unbalanced ANOVA with plant treatments as a main factor, and differences in tissue P were tested with an unbalanced two-way ANOVA with tissue and soil as two main factors. Tukey HSD was also applied to test pairwise significant differences at $p<0.05$. Multiple linear regression modeling was generated for both soil total P (P~Plant.treatment*Soil, random=~1|Block) and plant total P per tube (Plant.P~Plant.combo*Soil, random=~1|Block). In the soil total P model, regression was applied to the soil types and four plant treatments; in plant total P per tube, regression was applied to soil types and three plant treatment combinations without the no seedlings "Blank". Regressions were conducted by package "nlme" (Pinheiro, et al., 2019) using R version 3.6.1 (R Core Team, 2019).

CHAPTER IV

RESULTS

Plant mortality

After germination, seedlings struggled to grow presumably due to P deficiency, which was suggested by poor foliar greenness. In December 2018, I applied 10 μg P to every tube in three randomly-selected blocks (2, 4, and 5) in hopes of priming plant growth to avoid high mortality and augment future plant P demand. However, this action did not meet my expectation as growth remained limited and rates of mortality in these blocks did not decrease. For phosphatase analysis, however, I decided to use the tubes from these blocks with surviving plants both before and after priming to determine the potential effect of priming on phosphatase. Otherwise, I used only the unamended four blocks (1,3,6, and 7) to budget P relative to plant uptake and soil pool changes. In all blocks, if seedlings died, I harvested them to keep track of P uptake but for further analysis I excluded tubes that only had one remaining plant alive at final harvest. Mortality in this group of blocks was 23%, and I had 55 tubes that had both plants survive.

Plant tissue P

Biomass growth into leaf, stem, and root differed by substrate and species (Table 4.1). In clay, red maple leaves (0.06±0.01 g) and roots (0.05±0.01 g) gained significantly more biomass than the stems (0.02±0.00 g). In saprolite, however, maple leaf biomass (0.22±0.04 g) was significantly lower than root (0.41±0.06 g) and stem (0.48±0.05 g) biomass (Table 4.1). Loblolly

pine had similar biomass distributions in both clay and saprolite. In clay, loblolly pine leaves (0.24±0.04 g) and roots (0.17±0.01 g) gained significantly more biomass than the stems (0.07±0.01 g) and in saprolite, stem biomass (0.25±0.06 g) was significantly lower than leaf (0.49±0.09 g) and root (0.50±0.07 g) biomass (Table 4.1). Soil substrate had a significant effect on biomass accumulation for both red maple and loblolly pine, with growth in saprolite being significantly greater than in clay. Tissue P concentrations were similar between leaf, root, and stem except for maple trees in clay, in which leaf P concentration (1.47±0.20 mg g^{-1}) was significantly higher than root (0.81±0.10 mg g^{-1}) and stem (0.86±0.12 mg g^{-1}) P concentration (Table 4.1). Soil substrate had a significant effect on loblolly pine leaf and root P concentration, with tissue in saprolite having significantly higher P concentration than that in clay.

Based on the above biomass growth and P concentration, total plant P in clay tubes under 1 maple + 1 maple (MM), 1 maple + 1 pine (MP), and 1 pine + 1 pine (PP) treatments averaged 222±33, 360±40 and 736±156 μg (mean ± SE), with MM being significantly lower than PP (Figure 4.1). In saprolite, total plant P in tubes under MM, MP, and PP treatments averaged 1860±368, 1997±326 and 2352±346 μg. None of the treatments, however, were significantly different while saprolite was a significant main effect having greater plant P than clay.

In the two-factor models for plant tissue P, substrate had a significant main effect while plant treatment effect as well as block was not significant and there was no significant interaction (Table 4.2). In the model of plant tissue P the best and only significant predictor was saprolite. This model explained 43% of the variability.

Soil analysis

In a similar two-factor model but used for soil total P, substrate again had a significant main

effect while the plant treatment and the block effect were not significant, and there was no significant interaction (Table 4.3). The best predictor of soil total P was again saprolite, and overall, this model explained ~87% of the variability. Given the large differences observed in substrate, the following analyses for soil P fractions evaluate treatment differences individually by substrate.

Before planting, clay and saprolite MIII P concentrations were 0.5±0.1 and 10.9±0.7 μg g^{-1} (mean ± SE), respectively (Table 4.4). These concentration differences result in saprolite MIII P content (1966 μg) being 20 times greater than that of clay (96 μg) (Figure 4.2). After one-year of plant growth, the Blank clay treatment did not show a significant change relative to the Initial clay, while MM, MP, and PP treatments showed significant decreases in soil MIII P relative to the Initial clay. These three treatment decreases were 44, 37, and 37% on average, respectively. MIII P in saprolite under the Blank treatment did not significantly decrease from Initial MIII P concentration after one-year. MIII P in the three plant treatments (MM, MP, PP), however, declined significantly by 89, 87, and 85 % on average, respectively, relative to the Blank saprolite MIII P (Table 4.4 and Figure 4.2)

Responses in P pools also differed between clay and saprolite (Table 4.5). In clay, Resin Pi, NaOH Pi, con HCl Po, and residual Pi concentrations decreased relative to initial P by 62, 20, 90, and 32%, respectively, although plant treatments (MM, MP and PP) did not differ from the blank treatment with regards to these P pools. $NaHCO_3$ Po, NaOH Po, and 1M HCl Pi pools in clay did not change for any treatment. The remaining two pools, $NaHCO_3$ Pi and con HCl Pi significantly increased in all treatments relative to the initial but did not differ from each other. In saprolite, Resin Pi, $NaHCO_3$ Po, NaOH Pi, and residual Pi decreased by 65, 70, 27, and 32% relative to the Initial P but, again, treatments with plants did not differ from the no-plant blank treatment. NaOH Po, 1M HCl Pi, con HCl Pi, and con HCl Po in saprolite did not change for any treatment. Finally,

the $NaHCO_3$ Pi pool in saprolite significantly increased in all treatments relative to the initial $NaHCO_3$ Pi (Table 4.5).

Summing across the various P fractions, Labile P comprised only a small portion (4% in clay and 10% in saprolite) of the total Hedley P (Table 4.6). Labile P in clay under MM and MP significantly increased relative to the initial P while the blank and PP had an insignificant increase relative to the initial P, and only MP significantly increased relative to the blank. Labile P in saprolite under MM, MP and PP, however, decreased significantly relative to initial and blank (Table 4.6). Extractable Po (i.e., NaOH Po) is also a minor proportion (8% in clay and 3% in saprolite) of Hedley total P. In clay, extractable Po under the treatments did not differ significantly; in saprolite, only the MM treatment differed from the Initial but did not differ from the other treatments (Table 4.6). Finally, summing across all the Hedley P fractions (i.e., Total Hedley P), before planting Total Hedley P concentration in clay and saprolite were 168.0±4.1μg g^{-1} and 290.1±21.3μg g^{-1} (mean ± SE). After one-year, Total Hedley P concentration only slightly decreased from the initial level and did not differ between any treatment.

The independent soil total P analyzed post-planting was moderately correlated with the post-planting Hedley total P (Pearson correlation coefficient =0.78, supplemental figure S2). In clay, the Initial, MM, MP, and PP treatments were all greater than the Blank, but only the MM and MP were significantly lower than the Initial (Table 4.7). In saprolite, Blank, MM, MP, and PP were all lower than the Initial but only PP was significantly greater than the other treatments.

Index of potential iron reduction

In the first round of measurement for potential iron reduction index, when seedlings were quite small (week 11 – 15), loss of Fe coating in the Blank treatment in clay (25%) was

significantly higher than loss of Fe coating in saprolite (16%), but the iron reduction index under the treatments showed no significant differences within clay or saprolite (Figure 4.3). In the second round of measurement (week 22 – 24) the iron reduction index of the Blank in clay (16%) reversed and was significantly lower than that in saprolite (22%); again, iron reduction index under the treatments did not differ. Pooled over both rounds of measurement, the MM treatment had significantly higher iron reduction index than the PP treatment in both clay and saprolite (Figure 4.3).

Phosphatase activity

Before priming, phosphatase activity in clay averaged ~251 nmol MUB g^{-1} h^{-1} across plant treatments and there was no significant difference of enzyme activity between treatments. The same pattern was evident in saprolite: enzyme activity averaged ~272 nmol MUB g^{-1} h^{-1} across plant treatments and no significant difference was found between treatments. There was also no significant difference between phosphatase activity of MM tubes and surface soils collected around parent maple trees, or between PP tubes and soils collected around local loblolly pines (Supplemental Table 2). After priming, phosphatase activity in clay averaged ~130 nmol MUB g^{-1} h^{-1} across plant treatments and averaged ~372 nmol MUB g^{-1} h^{-1} across plant treatments in saprolite, no significant difference was found between treatments (Figure 4.4). Enzyme activity of the three plant treatments in clay significantly decreased after priming. In saprolite, except for enzyme activity of MP tubes that remained unchanged, activity of MM and PP both significantly increased.

Table 4.1. Plant tissue biomass and P concentration (mean ± SE) of loblolly pine and red maple. An asterisk indicates a significant (α=0.05) difference between substrate within a species and different letters indicate significant (α=0.05) differences between tissues within a substrate and tree species.

Species	Tissue	----------Soil Substrate----------			
		Clay		Saprolite	
		Biomass (g)	Concentration (mg g^{-1})	Biomass (g)	Concentration (mg g^{-1})
Loblolly pine	Leaf	0.24 a	0.71±0.09 a	0.49 a*	1.37±0.27 a*
	Stem	0.07 b	0.51±0.06 a	0.25 b*	0.68±0.07 a
	Root	0.19 a	0.56±0.05 a	0.50 a*	1.52±0.36 a*
Red maple	Leaf	0.06 a	1.47±0.20 a	0.21 b*	0.98±0.20 a
	Stem	0.02 b	0.86±0.12 b	0.48 a*	0.86±0.10 a
	Root	0.05 a	0.81±0.10 b	0.41 a*	0.78±0.11 a

Table 4.2. Model parameters for predicting total plant P (Plant.P~Plant.combo*Soil, random=~1|Block) per tube one-year after planting, n=60. MM = Maple + Maple; MP = Maple + Pine; PP = Pine + Pine

Parameter	Value	Std.error	T value	*p*
Intercept (MM)	222.150	281.769	0.788	0.434
MP	136.912	358.123	0.382	0.704
PP	513.832	370.318	1.388	0.171
Saprolite (MM)	1636.877	398.482	4.108	0.000
MP: Saprolite	1.362	527.464	0.002	0.998
PP: Saprolite	171.150	523.708	0.327	0.745

Model p= 0.43
Model R^2= 0.56
Model R^2 adjusted = 0.56

Table 4.3. Model parameters for predicting soil total P concentration (Soil Total P~Plant.treatment*Soil, random=~1|Block) one-year after planting, n=62. MM = Maple + Maple; MP = Maple + Pine; PP = Pine + Pine

Parameter	Estimate	Std.error	T value	*p*
Intercept (Clay)	133.250	9.762	13.443	$2e^{-16}$
MM	12.000	11.956	0.990	0.327
MP	8.058	11.163	0.669	0.474
PP	23.295	11.400	2.014	0.045
Saprolite	68.750	14.912	4.907	$8.91e^{-6}$
MM: Saprolite	12.400	18.608	0.698	0.508
MP: Saprolite	24.692	17.301	1.475	0.159
PP: Saprolite	23.405	17.180	1.421	0.179

Model p= $2.2e^{-16}$
Model R^2= 0.87
Model R^2 adjusted = 0.86

Table 4.4. MIII P concentration (mean ± SE) in tubes after one-year of growth in the greenhouse. Different letters indicate significant difference between tree species. MM = Maple + Maple; MP = Maple + Pine; PP = Pine + Pine. Initial clay n=1 (with five laboratory replicates), blank clay n=13, MM clay n=11, MP clay n=13, PP clay n=11; Initial saprolite n=1 (with five laboratory replicates), blank saprolite n=12, MM saprolite n=10, MP saprolite n=12, PP saprolite n=12. Letters indicate significant differences among treatments within a substrate.

Soil	Tree species				
	Initial	Blank	MM	MP	PP
	---------- $\mu g\ g^{-1}$ ----------				
Clay	0.57±0.06 a	0.54±0.06 a	0.34±0.01 b	0.35±0.01 b	0.35±0.01 b
Saprolite	10.91±0.66 a	9.33±0.47 b	1.06±0.14 c	1.19±0.34 c	1.44±0.16 c

Table 4.5. P fractions in clay (a) and saprolite (b) before and after one-year of growth as determined using the Hedley P fractionation. Different letters indicate significant difference between treatment within a fraction. Initial clay n=1 (with five laboratory replicates), Blank clay n=4, MM clay n=8, MP clay n=13, PP clay n=11; Initial saprolite n=1 (with five laboratory replicates), blank saprolite n=3, MM saprolite n=5, MP saprolite n=8, PP saprolite n=10. MM = Maple + Maple; MP = Maple + Pine; PP = Pine + Pine

Substrate	Fractions	Tree species				
		Initial	Blank	MM	MP	PP
		----------µg/g----------				
Clay	Resin Pi	1.9 a	0.5 b	0.7 b	0.7 b	0.8 b
	$NaHCO_3$ Pi	3.4 c	6.9 bc	10.7 a	8.3 ab	8.6 ab
	$NaHCO_3$ Po	2.2 a	1.3 a	2.1 a	2.0 a	0.7 a
	NaOH Pi	44.6 a	39.2 b	36.6 b	37.2 b	37.8 b
	NaOH Po	9.8 a	7.5 a	8.1 a	10.5 a	10.7 a
	1M HCl Pi	0.8 b	2.9 a	1.1 b	1.3 b	1.2 b
	con HCl Pi	48.1 b	63 a	59.2 a	58.5 a	58.2 a
	con HCl Po	1.1 a	0.1 b	0 b	0 b	0.1 b
	Residual Pi	55.8 a	30.9 b	37.2 b	37.6 b	38.5 b
Saprolite	Resin Pi	14.5 a	2.6 b	3.3 b	3.3 b	2.4 b
	$NaHCO_3$ Pi	13.0 b	24 a	22.3 a	21.3 a	20.7 a
	$NaHCO_3$ Po	5.3 a	0.7 b	0.3 b	0.7 b	1.2 b
	NaOH Pi	60.0 a	21.2 b	30.9 b	24.3 b	25.5 b
	NaOH Po	4.6 a	2.7 a	2.1 a	3.4 a	4.1 a
	1M HCl Pi	82.7 a	90.9 a	80.1 a	80.9 a	81.8 a
	con HCl Pi	71.7 a	49.9 a	65.4 a	51.5 a	57.2 a
	con HCl Po	0.6 a	0.8 a	3.3 a	2.5 a	2.4 a
	Residual Pi	40.3 a	24.7 b	26.8 b	27.7 ab	27.3 b

Table 4.6. Total Hedley P, labile P (resin Pi, $NaHCO_3$ Pi, $NaHCO_3$ Po), and extractable Po (mean ± SE) in tubes before and after one-year of growth. Different letters indicate significant difference among treatments within a substrate. MM = Maple + Maple; MP = Maple + Pine; PP = Pine + Pine.

Fractions	Soil	Tree species				
		Initial	Blank	MM	MP	PP
		----------μg g^{-1}----------				
Total Hedley	Clay	168.0±4.0 a	151.9±6.1 a	156.3±3.0 a	157.2±2.8 a	157.0±5.1 a
	Saprolite	290.1±21.3 a	216.5±18.6 a	232.8±16.9 a	214.0±15.2 a	220.5±15.5 a
Labile P	Clay	7.5±0.3 c	8.7±0.9 bc	13.5±1.2 a	11.0±0.6 ab	10.0±0.6 abc
	Saprolite	31.2±0.8 a	27.3±1.3ab	25.9±1.4 b	25.3±0.9 b	24.3±1.4 b
Extractable Po	Clay	12.0±0.4 a	8.4±0.9 a	9.8±2.1 a	12.8±1.5 a	10.4±2.6 a
	Saprolite	8.3±1.0 a	2.4±1.3ab	1.3±0.8 b	2.6±1.2 ab	4.1±1.3 ab

Table 4.7. Soil total P concentration (mean ± SE) before and after one year of growth. Different letters indicate significant differences among treatments within a substrate. Initial clay n=1 (with five laboratory replicates), blank clay n=4, MM clay n=8, MP clay n=13, PP clay n=11; Initial saprolite n=1 (with five laboratory replicates), blank saprolite n=3, MM saprolite n=5, MP saprolite n=8, PP saprolite n=10. MM = Maple + Maple; MP = Maple + Pine; PP = Pine + Pine.

Soil	Tree species				
	Initial	Blank	MM	MP	PP
	----------μg g^{-1}----------				
Clay	157.3 a	133.3±9.7 b	145.3±2.5 ab	141.2±3.2 ab	156.6±6.3 a
Saprolite	277.5 a	201.8±18.4 c	226.3±7.6 bc	234.7±5.4 bc	248.6±9.5 b

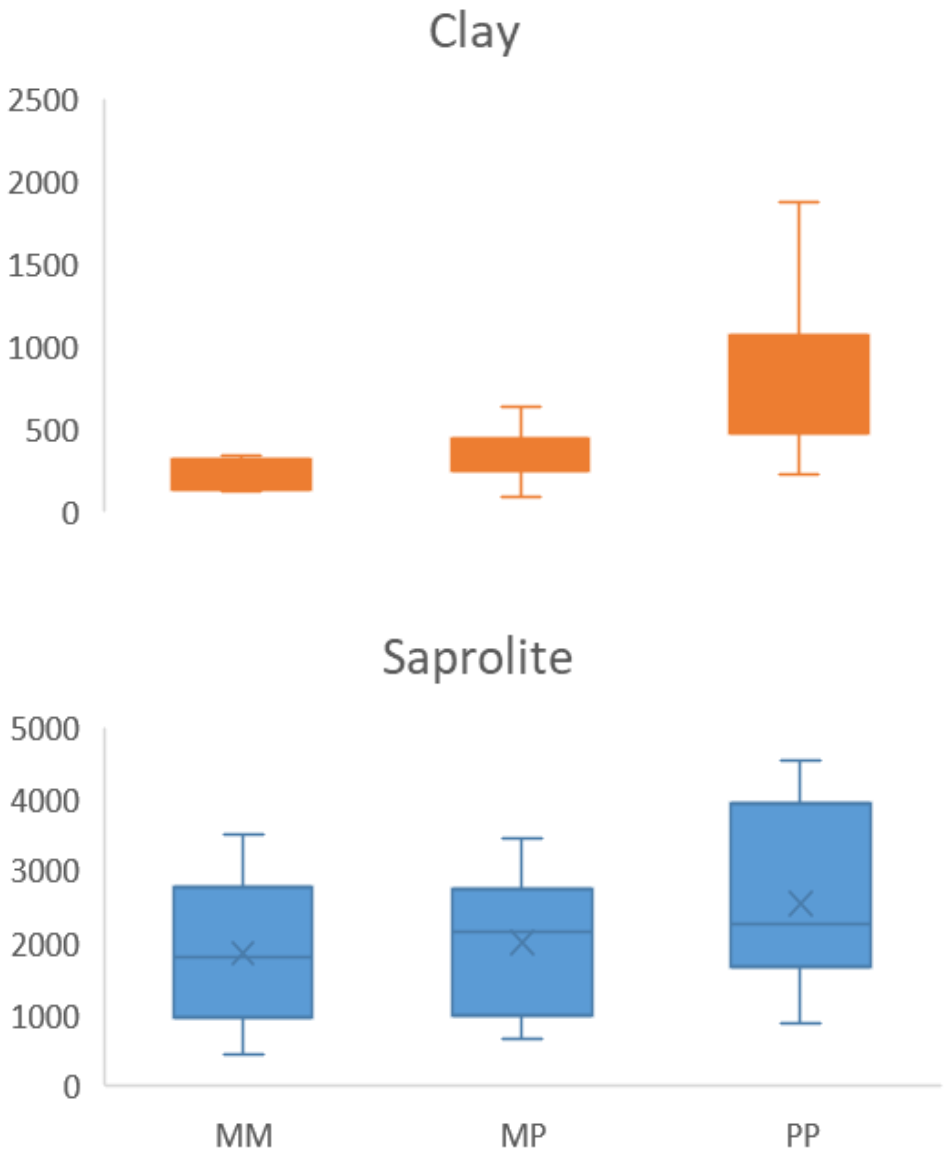

Figure 4.1. Plant total P content in clay and saprolite per tube. MM = Maple + Maple; MP = Maple + Pine; PP = Pine + Pine. Bars above and below the box represent the maximum and minimum value, the box is the interquartile range, line and cross in the box represent median and mean, respectively.

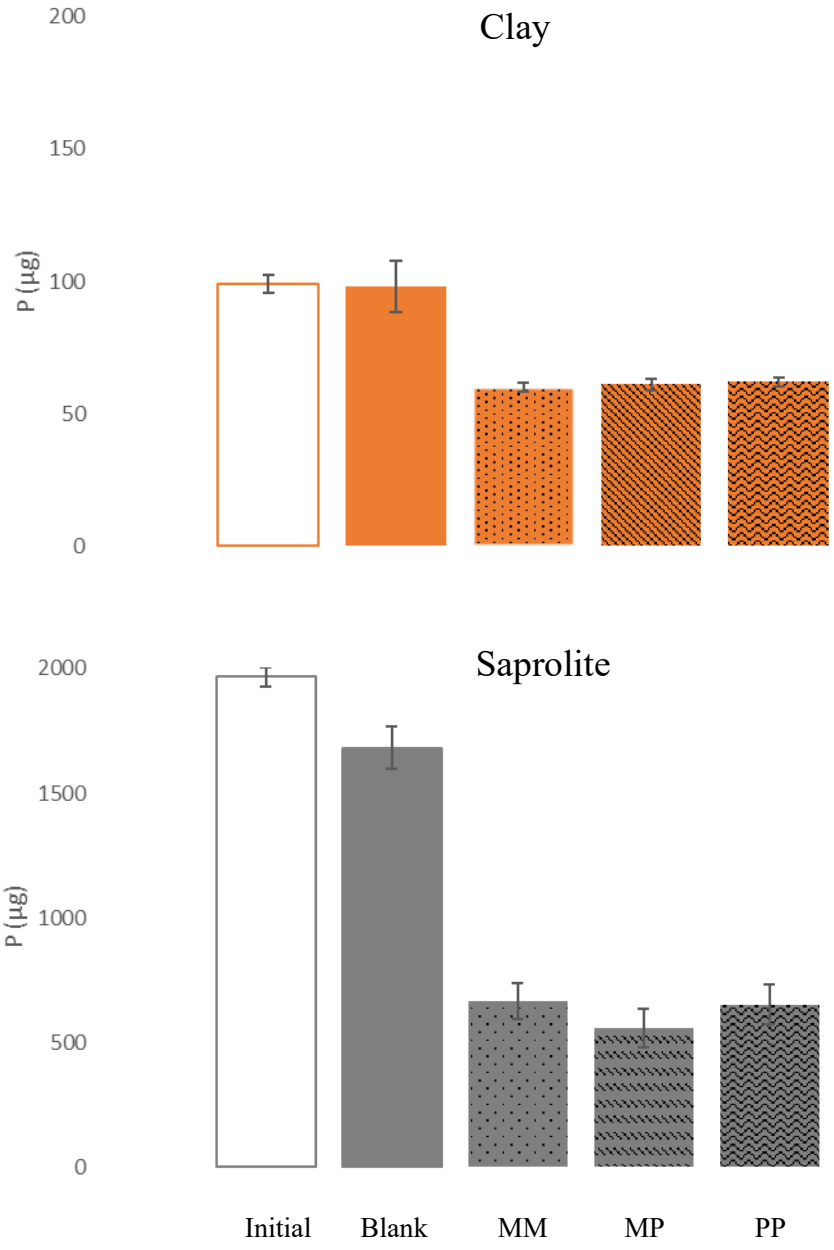

Figure 4.2. Mehlich III P content in clay and saprolite per tube after one-year of seedling growth. MM = Maple + Maple; MP = Maple + Pine; PP = Pine + Pine. The scale of MIII P in clay is 200 and of MIII P in saprolite is 2000. Bars represent mean ± SE.

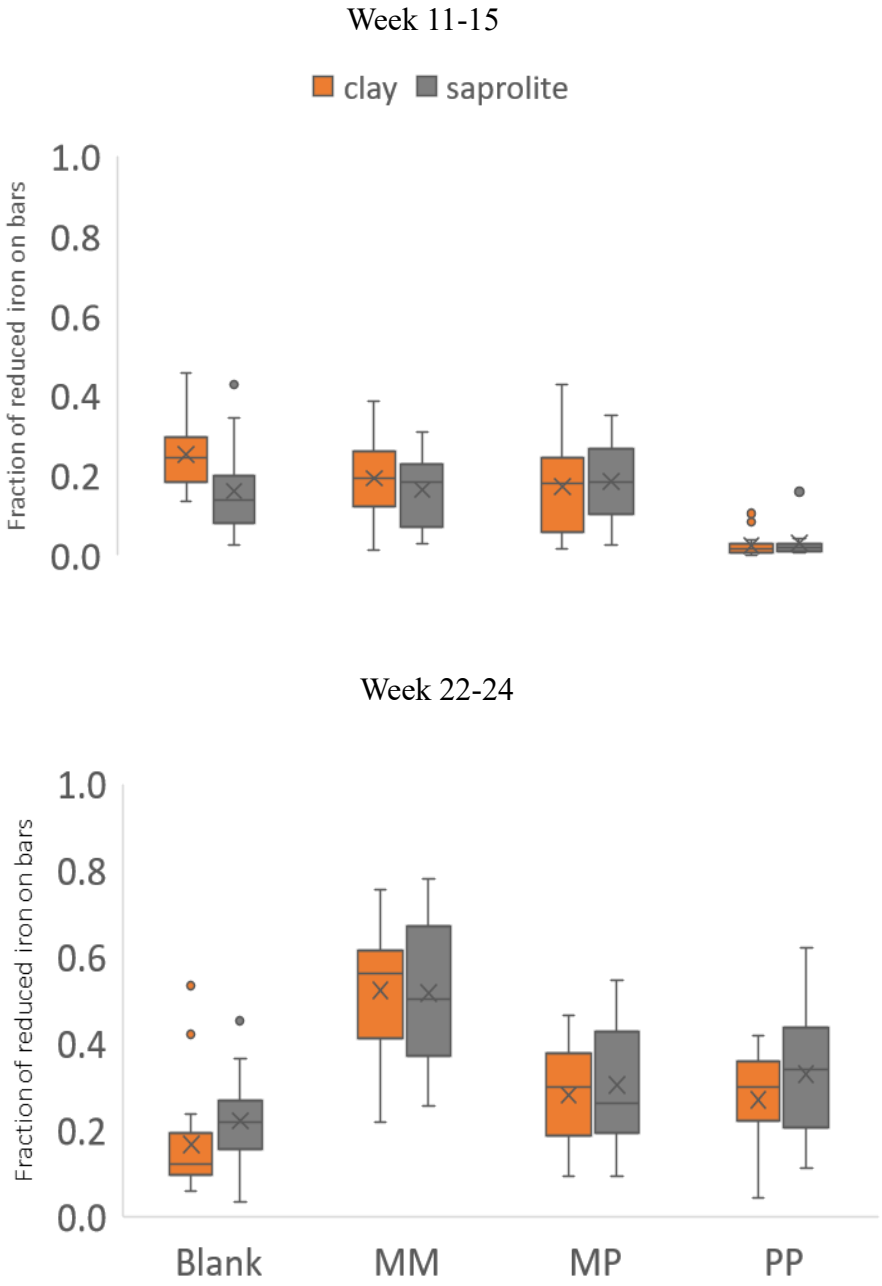

Figure 4.3. Fraction of reduced iron on iron bars during week 11-15 and week 22-24. MM = Maple + Maple; MP = Maple + Pine; PP = Pine + Pine. Bars above and below the box represent the 95% confidence interval, line and cross in the box represent median and mean, respectively, dots represent outliers.

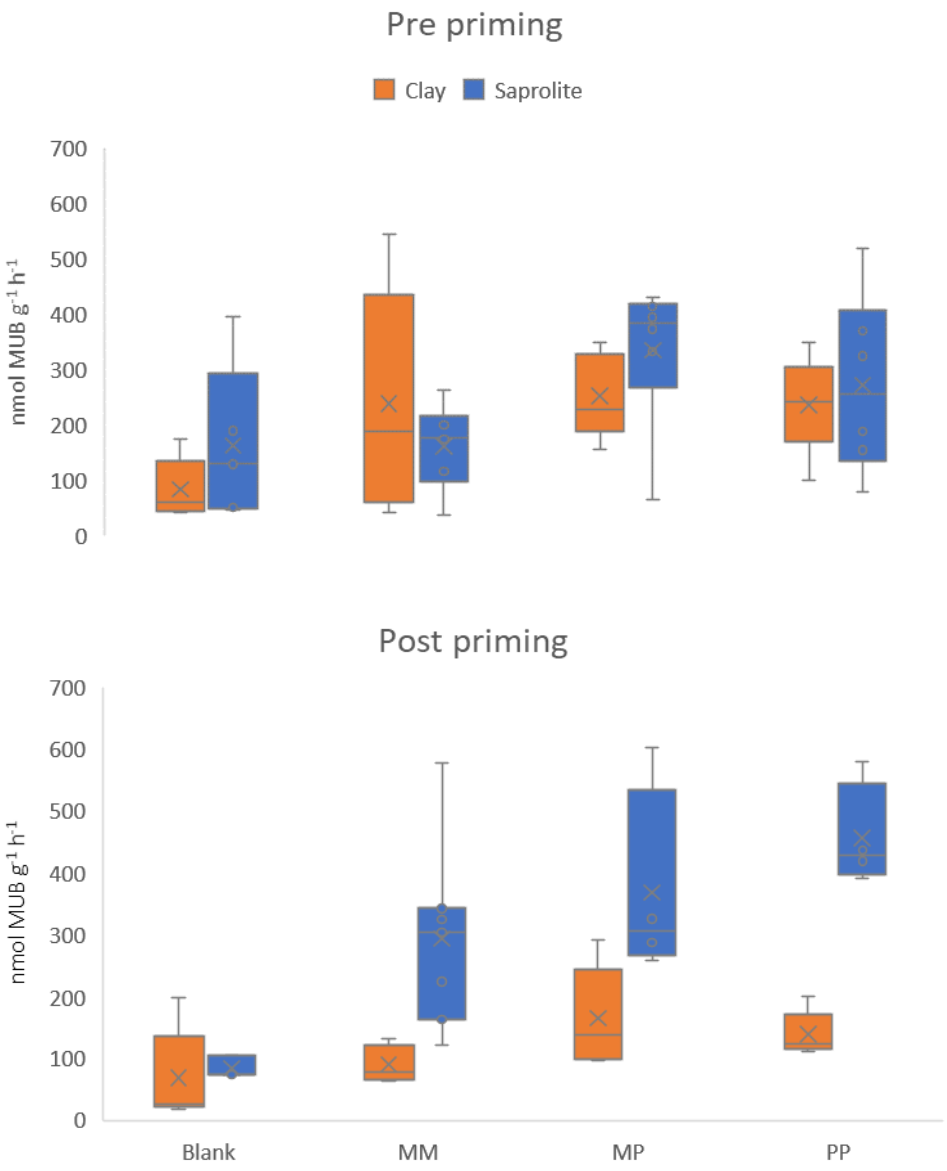

Figure 4.4. Phosphatase activity by treatment in clay and saprolite in May 2018 before priming and Dec 2018 after priming. MM = Maple + Maple; MP = Maple + Pine; PP = Pine + Pine. Bars above and below the box represent the 95% confidence interval, line and cross in the box represent median and mean, respectively.

CHAPTER V

DISCUSSION

Soil P availability has been studied due to its critical role as a plant macronutrient. In the current study I used an ectomycorrhizal and arbuscular mycorrhizal tree species to create a plant demand for soil P. Furthermore, I used the clay rich portions of a southeastern Piedmont Ultisol that has high iron and aluminum oxide contents as well as a portion of the saprolite at the base of the profile with limited hydroxide and carbon content. I expected that plant demand would exceed P availability in the labile P fractions (assessed using a Hedley fractionation), but that Hedley labile P fractions would be resupplied and buffered against changes by P in more recalcitrant Hedley P fractions.

Plant P uptake

Plant P uptake ranged from 300 – 800 μg per tube within clay and was four to eight times greater (500 – 4500 μg) in saprolite (Figure 4.1). This difference in uptake is consistent with the lower MIII P in clay relative to saprolite. In general, I observed significantly less biomass in stems relative to root and leaf in the seedlings. A similar biomass distribution of root and leaf was also found in the greenhouse study of Niederberger et al. (2017), where root and leaf had similar biomass in four out of five study soils. The contents of accumulated plant P in this study were substantially below those in Niederberger et al. (2017), where they found up to 9000 μg of P uptake per tree after growing poplar tree cuttings for 6 months. This discrepancy may be reasonable, however, since I grew seedlings from seeds while Niederberger et al. (2017) used cuttings from

two-year-old poplar trees ranging from 24.5 – 26 cm tall and from 19.4 – 60.5 g. One limitation in my experimental method was the loss of dead leaves. Although dead leaves were collected and a portion of the dead leaf biomass was allocated to all remaining maple or pine in the tube, it was not always possible to assign the leaf to its original plant. Dead leaves averaged 0.01 g per tube, considering the small biomass growth for each plant within a tube, and the higher P concentration of plant leaves, missing those leaves clearly created an under estimate of P content in plants.

Soil phosphorus

The MIII P content in clay tubes (~200 µg) was tenfold below that in saprolite (Figure 4.2). MIII P concentrations in clay tubes is consistent with Richter et al. (2006), which indicated MIII P- concentration in 0.35 – 0.60 m clay varied from 0.5 – 1.5 µg g^{-1} from 1962 to 2005. Relative to plant P uptake in clay tubes (~500 µg), MIII P in clay tubes was not sufficient to meet demand. I found a significant decline in MIII P within clay tubes accounting for ~38 µg of P, and thus requiring P provisioning from other fractions of soil P. For saprolite, MIII P content (~2000 µg per tube) would be sufficient to meet plant P uptake in 43% of cases (i.e., <2000 µg of plant P per tube). In saprolite, however, MIII P significantly declined but, on average, only by ~1700 µg per tube, again suggesting buffering and/or utilization of other less available soil P fractions.

The sum of the labile P fractions contains about 1890 µg per tube in clay and 4626 µg per tube in saprolite (Table 4.6). Labile P pools measured by Hedley fractionation are often three to six times greater than MIII P in saprolite or clay. Similar differences were observed in a previous comparison where labile P was measured to be three to five times greater than Mehlich or Bray P in both slightly and heavily weathered forest soils (Johnson et al., 2003). There is evidence that acidic extractants result in lower extractable P than neutral and diluted alkaline extractants in

highly weathered soil (Sharpley et al., 1987). Our result again addressed concern about the validity of plant available P extractants in highly weathered soil. The observed increase in labile P in clay is also consistent with Richter et al. (2006), where Resin Pi and $NaHCO_3$ Pi significantly increased in the 0.35-0.6 m layer. In contrast, I found labile P in saprolite treatments (MM, MP, and PP) significantly decreased relative to Initial and Blank. This finding is paralleled in the sandy soils of Niederberger et al. (2017), in which Labile P significantly decreased in soils used from two poplar plantation sites. The current results suggest that tree seedlings can make use of Labile P but that there is a buffer effect on Labile P by other P fractions since in some cases Labile P increased while in others declines in Labile P were insufficient to account for total plant P uptake.

Within individual components of the Hedley fractionation, I found a significant reduction in Resin Pi of ~210 µg-P averaged across all plant treatments within clay and ~2100 µg-P in all plant treatments within saprolite (Figure 5.1), which supported my hypothesis. The Resin Pi decrease relative to Initial satisfied ~18% of plant demand for P in clay and ~46% of plant demand in saprolite. There was not a significant difference between Blank and other plant treatments, however, suggesting some Resin P may be lost to leaching and thus contributing less to plant uptake. Either way, it is clear that Resin Pi is not sufficient to supply plant P demand. Niederberger et al. (2017) also found a significant decrease of Resin Pi in three of four soils.

$NaHCO_3$ Pi had a contrary change to Resin Pi. I found a significant augmentation of ~1015 µg-P across the three plant treatments in clay and a 1440 µg-P augmentation across the three plant treatments in saprolite (Figure 5.1). In both clay and saprolite, plant treatment was not significantly different from Blank in the $NaHCO_3$ Pi pool. Richter et al. (2006) supports these results in that they identified significant increase of $NaHCO_3$ Pi in 0.075 – 0.15, 0.15 – 0.35 and 0.35 – 0.6 m soil layers in these same Ultisols of the Calhoun CZO.

The NaOH Pi pool significantly decreased in all treatments of both clay and saprolite, with declines ranging from 1207 to 6214 μg per tube (Figure 5.1). Relative to plant P uptake these declines are sufficient to meet demand. Niederberger et al. (2017) found significant decreases in NaOH Pi (3.9 and 8.3 $\mu g\ g^{-1}$) in two fertilized forest soils, but their other two soils barely changed. In contrast, Richter et al (2006) found no change of the NaOH Pi pool in the 0.35-0.6 m layer. In a study on Inceptisols in New Zealand (Chirino-Valle et al (2016), during ten years of grassland afforestation, the NaOH-I Pi pool did not decline, while the NaOH-II Pi pool significantly increased. NaOH-II Pi, also called sonicate Pi, represents P that is held tighter at internal surfaces of soil aggregates (Hedley et al., 1982). These studies suggest the NaOH Pi pool is dynamic and may be an important moderately labile P pool.

Within this study I hypothesized that NaOH Pi decrease might be greatest where potential for soil iron reduction was greatest. My iron bar reduction measurements found a greater reduction rate in plant treatments in two soils compared to Blank, although this distinction was not significant (Figure 4.3). However, there was almost no correlation between fraction of reduced iron and changes in NaOH Pi from the two iron reduction measurements (Supplemental Figure 3). NaOH Pi was suggested to slowly buffer labile Pi in the 28-year study of Richter et al (2006), in which a significant decrease of NaOH Pi was found in the 0 – 0.075 m layer of these Ultisols. The fraction of reduced iron on the bars was not significantly different between clay and saprolite, which was not my expectation. Fe concentration measured in digests for total soil P was also inconsistent with my experiment result, which indicated initial clay has significantly higher Fe concentration than saprolite (Supplemental Figure 4). Hodges et al. (2018), working in basaltic soils from Maui, Hawaii found that Fe reduction increased with rainfall. As such, I hypothesized that the potential moisture retention difference and greater iron oxides in clay relative to saprolite would result in

higher potential for iron reduction, but results indicated that substrate did not have a significant impact on the fraction of reduced iron on the bars. One possible explanation is that daily watering within the greenhouse minimized any potential moisture difference.

One finding, when pooling the data from both measurements of iron reduction potential, indicated that fraction of reduced iron in MM tubes was significantly higher than that in PP, both within clay and saprolite. PP generally had greater plant biomass than MM, which may have impacted soil moisture. However, this difference in the fraction of reduced iron was not consistent with my Hedley data, in which MM and PP showed no significant effect on the NaOH Pi decrease.

The next most likely fraction to support plant P uptake is NaOH Po. This pool is less labile but still considered available to plants through long-term biogeochemical processes. I only found minor decreases (~105 μg in clay tubes and 288 μg in saprolite tubes) in the NaOH Po pool. Again, this amount of P decrease is insufficient to fully satisfy plant growth. Minor changes of this pool were also found in Richter et al. (2006), in which NaOH Po significantly decreased in the two top layers (0 – 0.075 m and 0.075 – 0.15 m). Niederberger et al. (2017) found insignificant decreases of NaOH Po in the loamy Cambisol and sandy Anthrosol soils used in their greenhouse study. In contrast, Townsend et al. (2002), in a study of Oxisol in Brazil and Costa Rica, found NaOH Po increased by nearly 25 μg g^{-1} after five years under primary forest.

In the current study, I did find a significant decrease (~540 μg) in $NaHCO_3$ Po in saprolite and some decline in the NaOH Po of both clay and saprolite. I had hypothesized that energy to use Po would be evident in high phosphatase activity. Before priming, phosphatase activity averaged ~251 nmol MUB g^{-1} h^{-1} in clay and 272 nmol MUB g^{-1} h^{-1} in saprolite (Supplemental Table 1). These values are similar to those reported by Bell et al. (2013) for 0-5 cm depth soil (280 nmol MUB g^{-1} h^{-1}), which is a bit surprising given my samples are at 60 cm or below. After priming,

activity declined in clay across plant treatments but increased in saprolite across plant treatments, which was not the expectation. Previous research has typically shown a decline in phosphatase activity with phosphate input (Spiers & McGill, 1979). I had also expected that activity under ectomycorrhizal pines would be higher. Phosphatase activity of PP was ~60% higher than that of MM in saprolite both before and after priming, which is consistent with my hypothesis. However, phosphatase activity of PP in clay is slightly lower than that of MM before priming. Overall, declines in Po and high phosphatase activity suggests use of extractable Po as a portion of plant demand, but given the magnitude of declines other moderately labile and even stable P pools may supply P to plant P uptake.

Beyond the labile P and extractable Po fractions, the remaining fractions are considered more recalcitrant. The 1 M HCl pool, which is expect to represent Ca bound P, is somewhat unique, however, as it is influenced by agricultural liming and P fertilization and would be expected to be more mobile as the result of Ca leaching. This was demonstrated in the study of soil chemical change from 1962 to 1990 at the Calhoun Critical Zone Observatory (CZO) where a significant decrease of Ca was observed in 0.35 – 0.60 m soil layer and was inferred to be a re-equilibration of soil after abandonment of agricultural fertilization. Richter et al. (2006) found Ca-P significantly decreased in 0 – 0.6 m soil during their study and indicated Ca-P as a major source of P for biological circulation. In the saprolite treatment of the present study, initial 1M HCl P concentration and contents were significantly greater than the concentration in clay, indicating some potential for Ca-P in saprolite. Ca measurements on digests for total soil showed initial saprolite contained 6-fold higher Ca than clay (Supplemental Figure 4). After one-year of growth, 1M HCl only had a slight change in clay (70 μg per tube) and in saprolite (306 μg per tube) (Figure 5.1). The Ca abundance in saprolite could be caused by the lime application during the past

agricultural activities. Across plant treatments, 1M HCl P was lower than that of Blank, although this decline was only significant in clay. Here it seems 1M HCl P supplied P to plant growth was resupplied and buffered by other less mobile P pools.

Stable P (con HCl P and residual Pi) is a major part of Hedley total P, representing 60% of total P in clay and 40% in saprolite. I expected stable P to have only a minor contribution to the P dynamics. However, con HCl Pi significantly increased by 1750 μg under all four treatments compared to Initial soil in clay. While in saprolite, con HCl Pi had an insignificant decrease (2745 μg) (Figure 5.1). Foroughi (2019), working in the same Calhoun CZO soils, also found an increasing con HCl Pi pool in the 0.35 – 0.60 m layer under pine growth from 2005 to 2017. In contrast to our result, however, Niederberger et al. (2017) found no significant change in con HCl P pool during one growing season. The mechanism for changing con HCl Pi is uncertain but might be related to Fe and Al oxide changes in the soil.

Residual Pi showed a significant decrease in both clay (3500 μg) and saprolite (2520 μg) (Figure 5.1). This is inconsistent with Richter et al. (2006), in which Residual Pi had no significant variation in any of the four soils layers during 28 years. In Niederberger et al. (2017), residual P also only showed a significant increase in one fertilized soil but did not change in the other soils. A similar trend to ours was found, however, in Townsend et al. (2002), in which residual Pi concentration decreased from 155 ($\mu g\ g^{-1}$) to 117 ($\mu g\ g^{-1}$) after five years in afforesting soil. In this previous research, the decrease of the residual fraction was paralleled with an increase of NaOH Po and $NaHCO_3$ Po. Our results supported this potentially more dynamic stable P pool in response to limited soil P availability. One reasonable explanation for changes in stable P was given in a grassland soil study where a consistent decline in organic C may indicate stable organic P was mineralized as a result of limited P availability (Tiessen et al., 1982). Our experiment showed

residual P might supply P to plant growth as a buffering mechanism related to the decrease of moderately labile P such as the NaOH Pi pool.

After one-year of growth, soil total P in clay and saprolite decreased by 2310 μg (8%) and 8937 μg (18%) per tube on average (Table 4.7). The declining trend in soil total P was correlated (Pearson correlation coefficient =0.79) with a decline in the Total Hedley P (2170 μg in clay and 12519 μg in saprolite) per tube (Figure 5.2), although the decrease of the later was not significant. In clay, all four treatments (Blank, MM, MP, and PP) showed an insignificant decrease relative to initial soil, while in saprolite, total P of all four treatments significantly declined. Plant treatments (MM, MP, and PP) did not differ relative to total P declines. In both clay and saprolite, MM and MP had no significant difference in total P from Blank, and total P in PP was significantly higher than the level in Blank. However, the decrease of total P (2000 – 9000 μg per tube) exceeded the amount of plant total P uptake in each tube (200 – 4500 μg), which raises the question as to what other factors could potentially cause P reduction during the experiment. Considering the limited amount of inorganic P in soil, daily watering seems unlikely to cause noticeable P loss. In forested ecosystems, streamwater loses of P are quite limited, which was recently measured in Calhoun soils (Foroughi 2019). Markewitz et al. (2006) measured P output in stream water from mature and secondary forests on clay-rich Oxisols as low as 0.01 kg $ha^{-1}yr^{-1}$. A few measurements from leachates in the current study also suggest low P (~16 μg L^{-1}) in leachate (Supplemental Table 2). The best index of soil P loss may be the Blank control. The greenhouse study of Niederberger et al. (2017) had no Blank controls, while here, controls declined by 4170 μg per tube in clay and 10196 μg per tube in saprolite. While I cannot identify the exact mechanism, future research could focus on colloidal P flux as there has been a suggestion that this is a missing flux in forest P cycling (Bol et al., 2016).

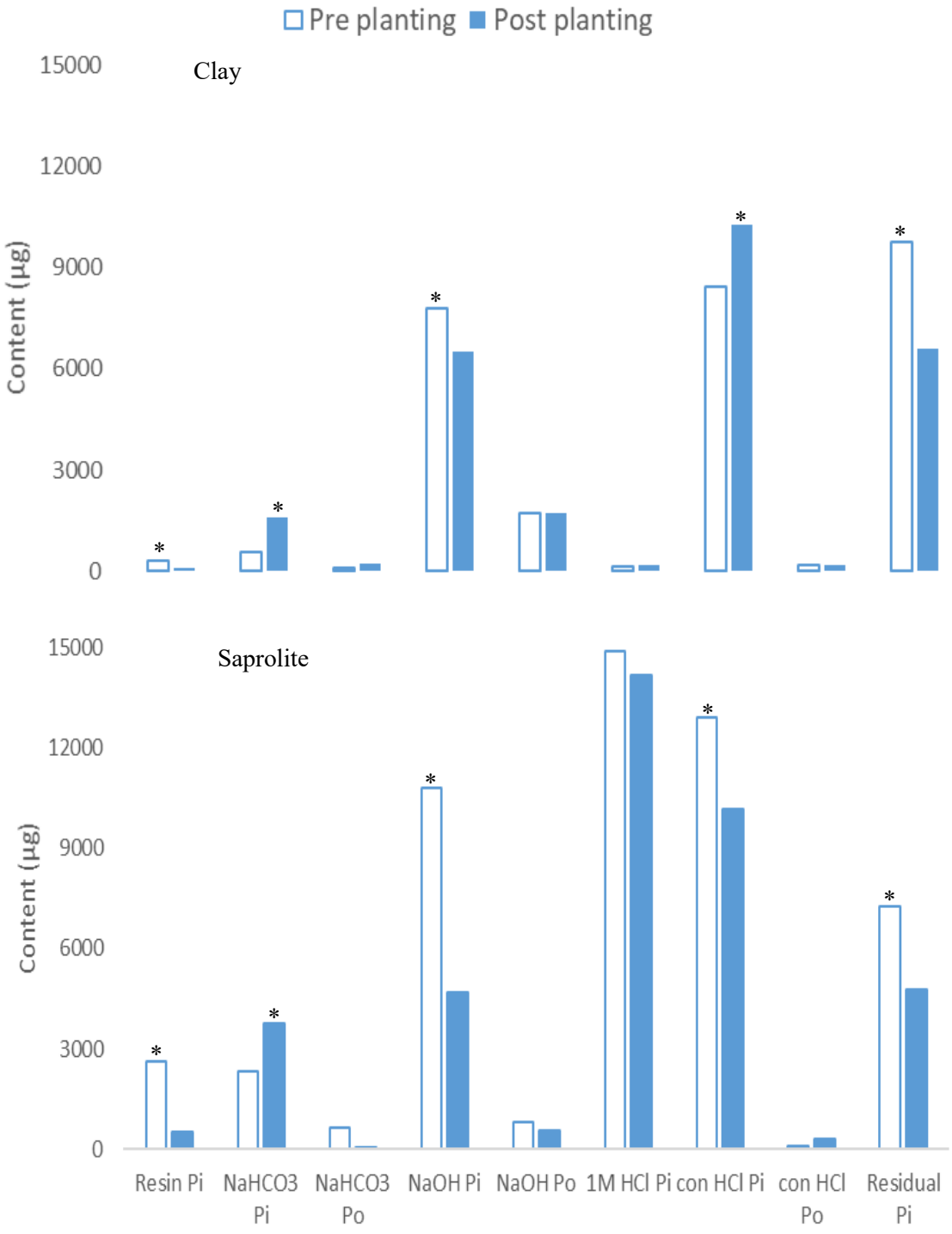

Figure 5.1. Content of pre and post planting Hedley P fractions per tube in clay and saprolite. Asterisks indicate significantly higher content between pre planting and post planting with in Hedley P fraction.

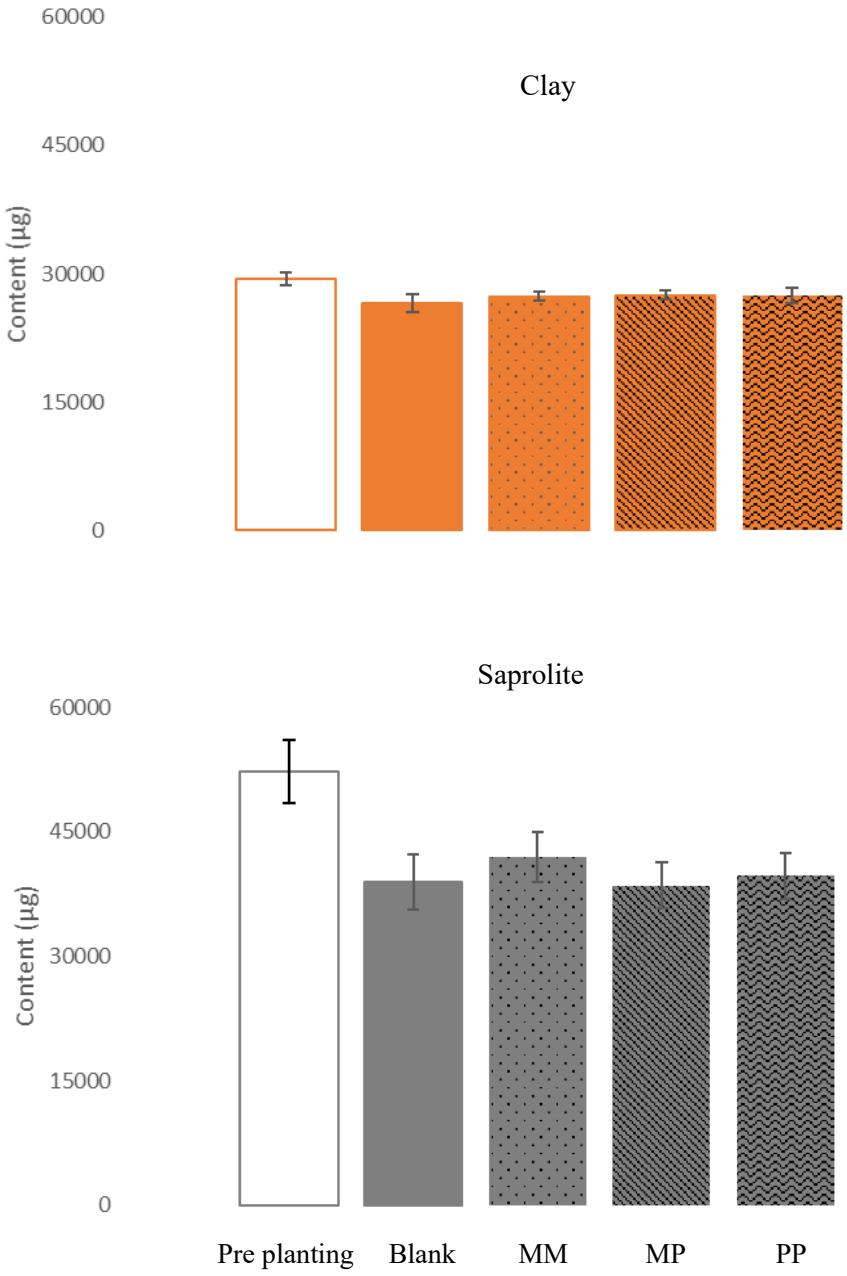

Figure 5.2. Content of pre and post planting Total Hedley P fractions per tube in clay and saprolite. MM = Maple + Maple; MP = Maple + Pine; PP = Pine + Pine. Bars represent mean ± SE.

CHAPTER VI

CONCLUSION

This one-year assay of soil P availability in clay and saprolite from Ultisols within the Calhoun Critical Zone Observatory in the Piedmont of South Carolina, USA demonstrated that P is limited in both substrates as demonstrated by high mortality and low seedling P uptake. Further, however, saprolite was demonstrated to higher than clay in MIII P, total P, and plant available P. After one-year of growth in the greenhouse, resin Pi significantly decreased, while $NaHCO_3$ Pi increased as the potential result of buffering from other P pools. NaOH Pi was a dynamic pool that supplied P to plant growth, although there is no clear evidence showing plants have an effect on the soil iron reduction as a mechanism of P release. Saprolite is high in HCl Pi, which is presumed to be Ca bound P. Residual P decline suggested stable P pools may replenish the loss of labile and moderate labile P pools. Phosphatase activity of the ectomycorrhizal plant (loblolly pine) did not significantly differ from that of the endomycorrhizal plant (red maple) during one-year of growth. Plant P uptake was not the only driver of soil P decline as plant P uptake alone could not account for soil total P reduction. Overall, my experiment indicates labile P and total extractable Po are buffered by moderately labile P or stable P during plant growth in a P limited environment. Furthermore, considering the significantly greater plant biomass growth in saprolite, I concluded that saprolite may be an unrecognized source of soil P sustaining forest growth.

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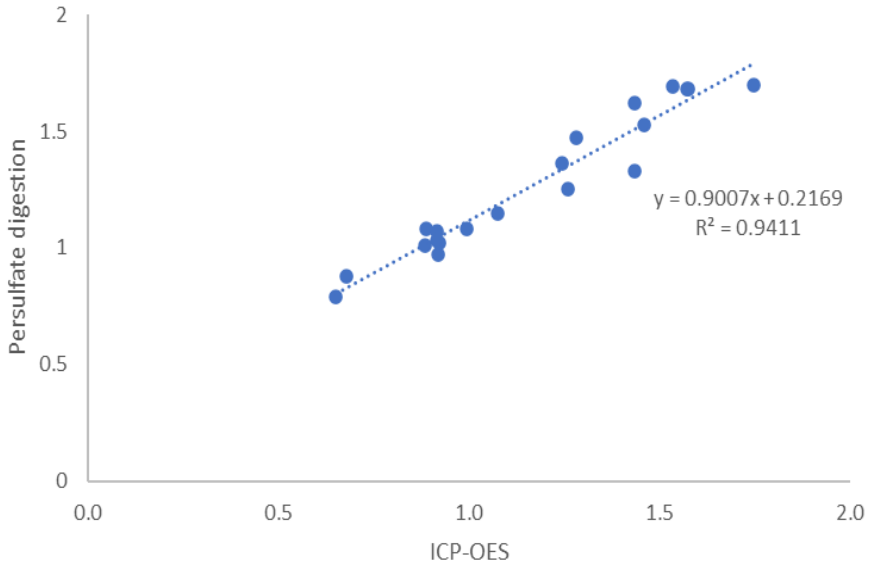
SUPPLEMENTAL MATERIAL

Supplemental Table 1. Phosphatase activity in tubes before P application and in 0-10 cm depth rhizosphere soil of parent red maple (Parent M) and parent loblolly pine (Parent P) collected in Whitehall Forest, Athens, GA in June 2020. Different letters indicated significant differences

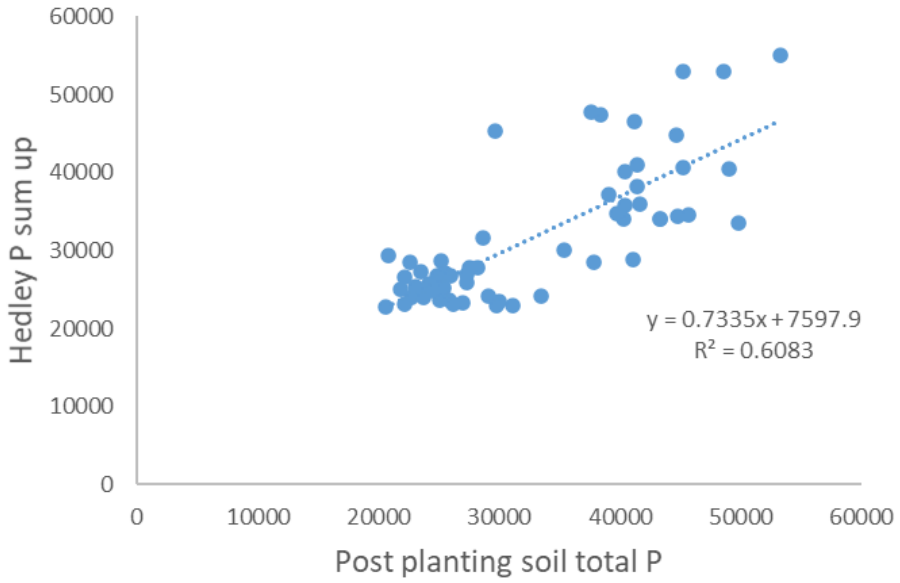
Soil	Tree species			
	Blank	MM	MP	PP
	Phosphatase activity (nmol MUB g^{-1} h^{-1})			
Clay	83.1±24.7 a	237.1±89.8 a	251.3±34.1 a	235.6±42.3 a
Saprolite	162.0±64.0 a	161.2±31.5	334.4±55.6	272.0±66.2 a

Supplemental Table 2. Leachate collected in the greenhouse from the bottom of tubes in September 2018, clay and saprolite n=8.

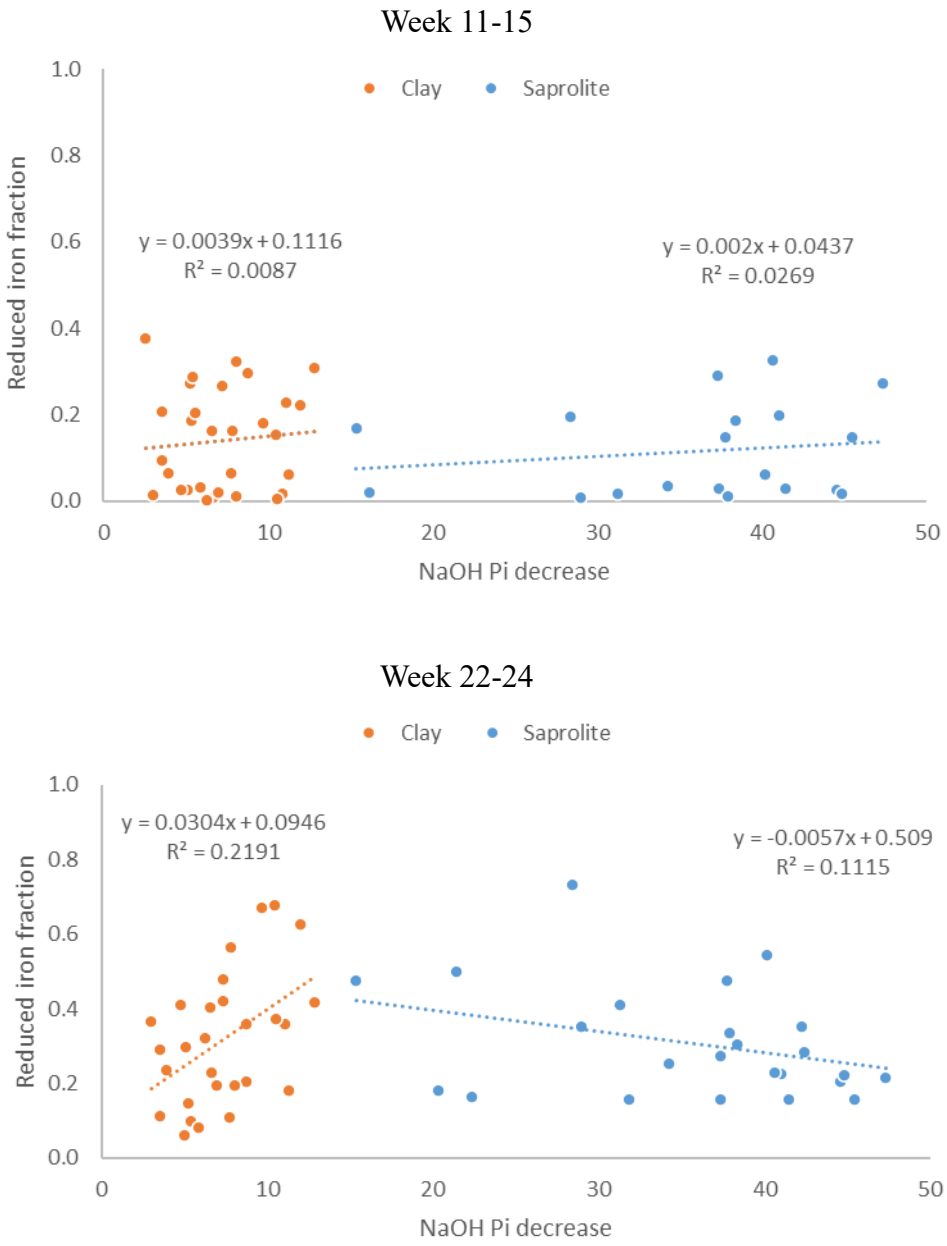
Soil	P concentration ($\mu g\ L^{-1}$)
Clay	14.9±2.9
Saprolite	17.0±2.8



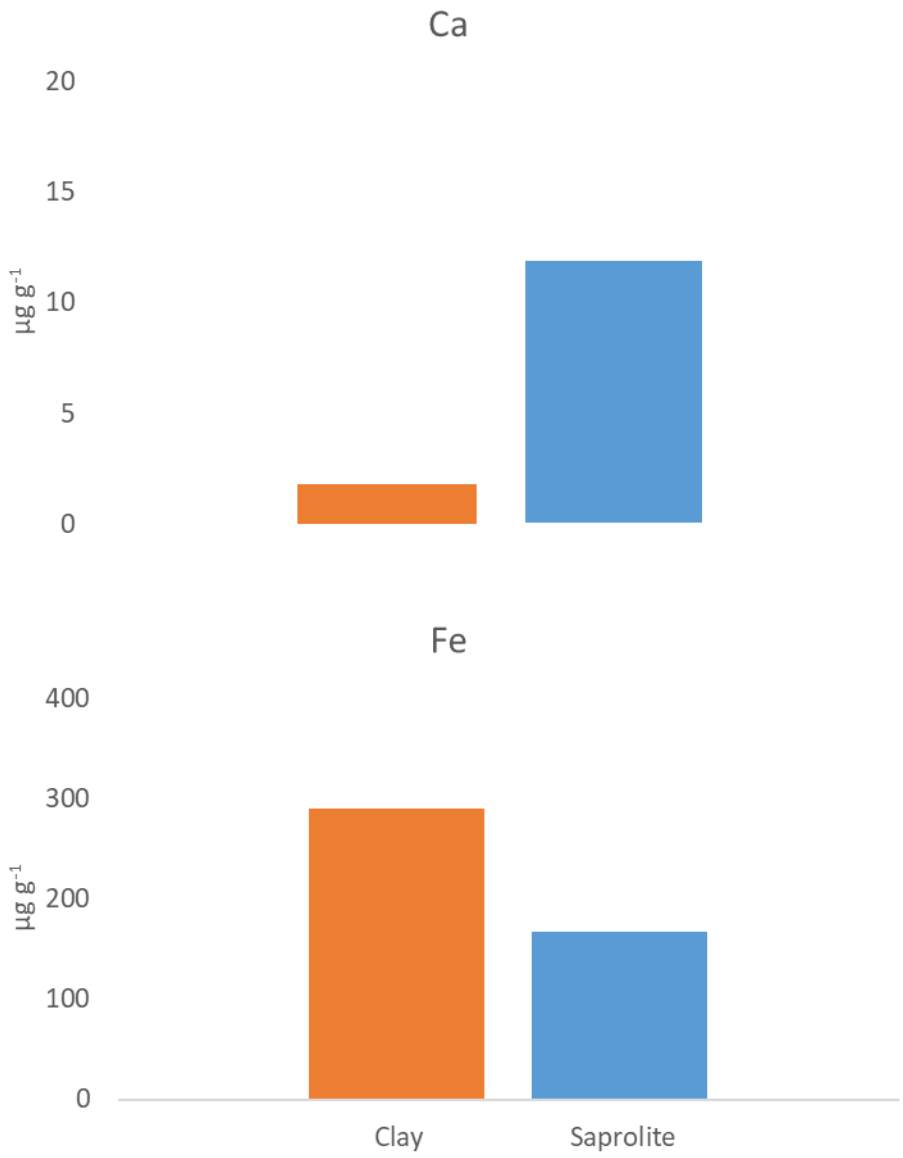
Supplemental Figure 1. Regression line between soil total P result from ICP-OES and result from persulfate digestion.



Supplemental Figure 2. Regression line between Hedley total P and post planting soil total P.



Supplemental Figure 3. Regression line between NaOH Po decrease and reduced iron fraction on iron bars.



Supplemental Figure 4. Ca and Fe concentration in initial clay and saprolite from total P digestion by ICP-OES.