MICROBIAL EXO-ENZYME ACTIVITIES IN SOIL AS INFUENCED BY POLYPHENOL CHEMISTRIES

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MICROBIAL EXO-ENZYME ACTIVITIES IN SOIL AS INFLUENCED BY POLYPHENOL CHEMISTRIES

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Plant and Environmental Sciences

by
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August 2013

Accepted by:
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ABSTRACT

Soil organic matter decomposition is directly driven by the soil extracellular enzymes excreted by soil microorganisms. Therefore, factors which impede extracellular enzyme activity would result in retarding the rate of soil organic matter decomposition. Several factors in the soil may influence soil extracellular enzymes including the plant secondary metabolites, polyphenols- tannins and lignins. However, thus far few investigations have considered the direct and indirect impacts of polyphenols on soil enzyme activities. Hence, three investigations were conducted to understand the ecological impacts of tannins and lignins on soil extracellular enzyme activities.

Tannins, further classified as condensed and hydrolysable tannins, can hamper decomposition through the formation of the tannin-protein complex. However, investigations had not compared the reactivities of the two groups. Hence, in the first investigation the enzyme inhibition efficiency of hydrolysable and condensed tannins was compared against almond β-glucosidase and soil enzymes collected from two soils with opposing tannin exposures. It was discerned that against almond β-glucosidase hydrolysable tannins exhibited a higher enzyme inhibition capacity than condensed tannins. Meanwhile, in the soil enzyme inhibition was dependent upon the enzyme class and the litter chemistry history. Moreover, tannins could prevent oxidoreductase activity through their antioxidant potential, which is a novel mechanism elucidated in the decomposition setting. In the second investigation, it was discerned that the inhibiton capacity of mixed tannins is positively related to the hydrolysable content.
In the final study, the relationship of soil lignin chemistry to the degradation efficiency of soil peroxidase was investigated. Until recently, lignins were considered to be chemical resistant to enzyme mediated degradation, which suggests that the enzymatic mechanisms mediated the degradation of lignin are not fully understood. One factor that could be affecting the rate of lignin turnover in the soil is the degradation efficiency of soil peroxidase, an oxidoreductase enzyme that mediates the transformation of soil polyphenols. The degradation efficiency of soil enzymes are described by the Michaelis-Menten kinetics and Activation energy. In this study it was discerned that the degradation efficiency of peroxidase was positively associated to the vanillyl abundance of lignin. This suggests that lignin chemistry may be one of many factors governing peroxidase kinetics in the soil.

Combined, the three studies conducted discerned the direct and indirect effects of soil polyphenols on soil extracellular enzymes. By associating soil organic matter chemistry with soil extracellular activities, these basic studies have advanced our understanding of soil decomposition and bring forth new questions.
DEDICATION

I dedicate this work to my parents, Harold and Josefina Triebwasser, who always encouraged studying, pursuing research, and whose only requisite was that I do my best. I hope to continue to make my parents proud.

“Learn from yesterday, live for today, hope for tomorrow. The important thing is to not stop questioning.” – Albert Einstein
ACKNOWLEDGMENTS

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A special thanks to Dr. Tharayil for serving as a mentor through my undergraduate and graduate career and always pushing me to do better. My aptitude to pursue research, mentor, and my success would not be what it is today without his support.

Lastly, and most importantly, I would like to acknowledge my husband, Nowlan Freese, who is always supportive of my exhaustive research habits, the love for the sciences, and for always smiling when it was difficult.

Collectively, I would not be in the position I am today without my advisors, mentor, and husband. Thank you everyone.
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CHAPTER I
LITERATURE REVIEW

1.1 Significance

The global carbon cycle is a process that ties the atmospheric CO$_2$ to the terrestrial vegetation and the soil (Johnson et al., 2004; Melilo et al., 2002). Through the process of photosynthesis, atmospheric CO$_2$ is assimilated into organic carbon. Upon leaf senescence the leaf litter falls to the soil floor where it is incorporated as soil organic matter (SOM). During the process of soil decomposition, soil microorganisms degrade the organic matter, releasing it back to the atmosphere as CO$_2$, thus completing the cycle (Fig 1-1; Bond-Lamberty et al., 2004; Yuste et al., 2011). The rate of decomposition is strongly affected by climate, including temperature and precipitation (Davidson and Jonhsson, 2006; Kirschbaum, 2010). In the advent of rising global temperatures, the decomposition process is proposed to exhibit positive and/or negative feedback mechanisms to global climate change where SOM could display accelerated rates of decomposition or enhanced carbon sequestration respectively (Davidson and Johnsson, 2006). Interestingly, because the soil holds more carbon than the atmosphere and terrestrial vegetation combined, alterations to the rate of SOM decomposition can strongly influence the trajectory of anthropogenic induced climate change (Melilo et al., 2002; Davidson and Johnsson, 2006; Kirschbaum, 2009). Accelerated rates of decomposition could cause an increase in SOM released into the atmosphere as CO$_2$, whereas enhanced carbon sequestration, to our benefit, could result in loss of atmospheric CO$_2$ (Davidson and Johnsson, 2006). In order to gauge the response of decomposition to
climate change, it is important to have a basic understanding of the factors that moderate the rate of SOM decay.

Figure 1-1. The Global Carbon cycle. The cycle is initiated with the conversion of Atmospheric CO$_2$ to plant assimilates. During senescence in the fall, the leaf litter is incorporated into the soil floor as soil organic matter (SOM). Through the process of decomposition, microbes actively degrade SOM resulting in the release of CO$_2$ and/or the re-synthesis of SOM with microbe derived compounds (i.e. microbe synthates).

**1.2 The Soil Decomposition Process**

Carbon cycling and nitrogen cycling are two interlocked fundamental processes that occur during soil decomposition (Cotrufo et al., 2013). Broadly, the SOM is composed of organic biopolymers of plant and microbial origin (Kelleher and Simpson, 2006). The degradation of SOM proceeds with the excretion of soil extracellular enzymes.
The exoenzymes directly mediate the depolymerization of SOM polymers into their monomeric constituents (Burns, 1978; Marx et al., 2005). In the monomeric form, the organic carbon and nitrogen compounds can be assimilated for catabolic (resulting in CO$_2$ respiration) or anabolic purposes (cellular development and maintenance; Burns, 1978). The successful completion of SOM decomposition requires sufficient oxygen, moisture, temperature, enzyme diffusion from the microbe and substrate diffusion to the microbe are all essential (Prescott, 2010). Due to the number of factors, the decomposition of SOM can become difficult to achieve, resulting in the slow turnover of soil organic matter.

Apart from the climate affecting soil organic matter decomposition, two additional factors that are reported to affect the decomposition process are i) the leaf litter chemistry (Aerts, 1997; Canadell et al., 2007; Cornwell et al., 2008) and ii) the soil microbial community composition (Strickland et al., 2009a; Strickland et al., 2009b). The plant litter is the primary source of carbon and nitrogen components, stored in the form of biopolymers (Preston et al., 2009). The active degradation of plant detritus by microbes in conjunction with the transformation to microbial matter is the process that forms soil organic matter (Berg and McClaugherty, 2003; Preston et al., 2009). In the absence of aberrant climate conditions, the decomposition process is inter-regulated between plant community and below-ground microbial community (Cou^teaux et al., 1995). The leaf litter chemistry dictates the SOM chemistry and these two factors determine the carbon and nitrogen availability. Soil microorganisms are inherently C and N limited (Schimel
and Bennett, 2004). Hence, the liberation of C and N resources from the SOM serves to fulfill their metabolic demands (Schimel and Weintraub, 2003).

The rate of decomposition could be accelerated if the leaf litter comprised of compounds that can be easily degraded by microbes and/or if microbial productivity can be increased (Prescott, 2010). Alternatively, the process could be hampered if the leaf litter consisted of compounds relatively resistant to degradation, or if microbial activity is hampered (Prescott, 2010). Under ideal climatic conditions, the SOM composition and concentration can strongly influence the C/N microbial resource demands. In turn this can impact the allocation of exoenzymes for SOM degradation, and hence the rate of degradation (Allison, 2012). Due to the direct role of exoenzymes in the decomposition process the overall objective of this research was to expand the understanding of the linkage between SOM/litter dynamics and exoenzyme activities in the soil.

### 1.3 Soil Organic Matter

The soil is a heterogenous matrix of minerals, SOM, and microbes which results in a complex environment to study (Glexiner et al., 2001). This also amplifies the difficulty in elucidating SOM chemical profile and processes governing decomposition (Glexiner et al., 2001; Simpson and Simpson, 2012). The persistence of OM in the soil can range from weeks to centuries (Schmidt et al., 2011). This unusual stability in the soil is proposed to be a consequence of the SOM chemical composition (Grandy and Neff, 2008a). The molecular composition of SOM can range from easily degradable compounds such as
polysaccharides and proteins to ones relatively resistant to decay including plant derived waxes and aromatics (Kögel-Knabner, 2002).

Two mechanisms are proposed to stabilize and protect SOM from degradation-selective preservation and physical protection (aggregate formation and mineral-OM interactions; von Lützow et al., 2006; Kogel-Knabner et al., 2008). The mechanism of selective preservation is linked to the plant derived compounds lignins, tannins, cutins and suberins, whose structures are relatively resistant to decay (Kögel-Knabner, 2002). These compounds are hypothesized to display resistance to decomposition due to their aromaticity (lignins and tannins) and insolubility (cutins and suberins). However, the persistence of aromatics in the soil is currently controversial (Kiem and Kögel-Knaber, 2003; Rasse et al., 2006; Marschner et al., 2008). While in the past it has been suggested that aromatic compounds display greater lifespan in the soil (Waksman, 1938), using $^{13}$C-NMR recent investigations have reported an accumulation of alky-C and alkyl-N. These functional groups are associated to polysaccharides, aliphatics and proteins, of microbially-derived compounds rather than the expected aromatics of plant derived origin (Knicker, 2004; Rumpel et al., 2004). Collectively, this would suggest that the process of selective preservation may not contribute to the refractory pool of SOM.

The alternative mechanism for SOM stabilization is through the mechanism of physical protection (Balesdent, 1996; Rumpel et al 2002; Eusterheus et al., 2003; Helfrich et al., 2006; Kleber et al., 2007). This mechanism is associated with the easily degradable compounds of microbial origin rather than the plant derived metabolites
(Grandy et al., 2008a). Stabilization of SOM by physical protection can be divided into two main mechanisms. Firstly, the occlusion of SOM in aggregates, in conjunction with mineral-OM interactions, resulting in the formation of macro- and micro-aggregates (Six et al., 2000; Christensen, 2001). The aggregates < 50 uM and the fine particle fractions, carry SOM of older origin than the coarse fraction and macro-aggregates. A second mechanism, similar to the mineral-OM interaction, is the ligand-OM interaction. OM forms a ligand-OM complex in soils rich with oxides consisting of Fe$^{3+}$ and Al$^{3+}$ (Christensen, 2001; Mikutta et al., 2006; Eusterhues et al., 2003). Both mechanisms are thought to stabilize organic matter for over 100 years. From a microbial standpoint, the stabilization of OM by physical protection is hypothesized to limit the accessibility of substrates to the microorganisms (von Lützow et al., 2006) which actively engage in the degradation of SOM.

Plant litter is the primary source of organic polymers into the soil environment (Kögel-Knabner et al., 2002). As such, from a spatial perspective, the top soil exhibits the greatest concentrations of SOM, which declines with soil depth (Kögel-Knabner et al., 2008). Loosely, the rate of decomposition is positively linked to the SOM abundance. Therefore, in general, the topsoils exhibit the highest rates of decomposition. Over time, leaching of compounds from the leaf litter and lysed microbial material results in the transfer of OM from the topsoil to the lower soil layers (Grandy et al., 2008b). This causes the quantity and chemistry of OM to transition from plant derived compounds, to microbe derived compounds with depth (Grandy et al., 2008b). Consequently, the stability of OM also transitions moving down the soil profile, increasing with soil depth.
(Rumpel et al., 2002). While the stability and accessibility of SOM for degradation by microbes is governed by the SOM chemistry, the plant litter chemistry is the primary factor that dictates the SOM chemistry (Grandy and Neff, 2008b).

1.4 Litter Decomposition Trends and Litter Chemistry

Broadly, the rate of SOM decomposition is linked to the species composition of the plant community (Berg and Meentemeyer, 2002). In general, gymnosperm related soils exhibit lower rates of decomposition than deciduous tree soils (Cornwell et al, 2008). Similarly, graminoid soils including agricultural fields, non-cultivated fields, are characterized with faster rates of decomposition than the latter two (Preston and Trofymow, 2000; Hobbie, 1996). Understanding the chemistry of plant detritus is essential because the leaf litter is the primary regulator of the SOM chemistry (Aerts, 1997). Generally, the three classes of ecosystems are addressed in these studies: the southern pine forests (gymnosperm dominant), deciduous forests (Oak-Maple; angiosperm dominant), and agricultural fields.

The leaf litter contains an array of carbon metabolites that range in their susceptibility to decomposition. Due to the variation in their susceptibility to decay, the relative abundance of each polymer class can strongly influence the chemical nature of SOM (Aber et al., 1990; Berg and Meentemeyer, 2002; Melilo et al 1982; Schwietzer et al., 2005). Polymer resistance to degradation is driven by the monomeric composition and the chemical linkages uniting monomers (von Lützow et al., 2006). The polymers that are relatively non-resistant to degradation include proteins, and the polysaccharides - cellulose, hemicellulose and pectin. Cellulose is the most abundant polymer of the plant
litter and is composed of D-glucose units coupled by β-1-4-glycosidic bonds (Kögel-Knabner, 2002). In contrast to cellulose, hemicellulose can contain several sugar monomers that are bonded by β-1-4 glycosidic bonds. The primary classes of sugar monomers incorporated into the hemicellulose structure are pentoses and hexoses (Kögel-Knabner, 2002; Santhanam et al., 2012). Generally, deciduous trees contain hexoses whereas gymnosperms accumulate more pentoses (Fengel and Wegener, 1984). Although the enzymatic degradation of these polymers is complex, the polysaccharides are the primary source of organic carbon from the plant litter for soil microorganisms (Berg, 2000; Fioretto et al., 2005; Gillon and David, 2001). As such, the presence of polysaccharides in the plant litter is proposed to accelerate decomposition by enhancing microbial productivity (Grandy and Neff, 2008). From a species level perspective, the grasses and deciduous trees accumulate more polysaccharides and proteins in the leaf litter than gymnosperms (Berg and Meentemeyer, 2002). This factor has been shown to contribute to accelerated rates of litter decay (Cornwell et al., 2008). However, at the same time, these plant litters may comprise of compounds resistant to decay that can counteract this effect (Kögel-Knabner, 2002).

The compounds less susceptible to degradation in the plant litter are associated with hindering decomposition and are structurally diverse in the plant kingdom. The extensive collection of metabolites, in turn, diversifies the potential consequences that these compounds can inflict on the decomposition process and which largely remains to be investigated. A primary class of plant metabolites that interferes with decomposition is the plant phenolics (Appel, 1993; Schimel et al., 1996; Qu and Wang, 2008). The
phenolics are further subdivided into simple phenolic acids (Tharayil et al., 2006), polyphenolics (Tharayil et al., 2011) and lignins (Vanholm et al., 2010). Simple phenolic acids are of low molecular weight, ranging between 200-300 g/mol, and are derivatives of benzoic acid, cinnamic acid, vanillic acid, catechin or gallic acid (Fig 1-2a; Chapuis-Lardy et al., 2002; Tharayil et al., 2006). Due to their water-soluble properties, phenolic acids readily leach from the plant material into the soil matrix, temporarily impeding SOM processes (Tharyail et al., 2006). Alternatively, polyphenolics (i.e. tannins) and lignins, which combined can comprise at least 30 % of the leaf dry weight, are significantly greater in molecular size (Fig 1-2 b1, b2, c) and are reported to strongly hamper SOM decomposition (Berg, 2000; Kraus et al., 2003b; Kraus et al., 2004). While both lignins and tannins could hinder the decomposition process, the causative mechanism is different for each polymer class.
Figure 1-2. Structures of the phenolic compounds in plant litter. (a) the simple phenolic acid, gallic acid, (b1) condensed tannin (b2) hydrolysable tannin (c) lignin.

1.4.1. Tannins

Tannins are polymeric water-soluble phenolics that readily interact with proteins forming soluble or insoluble tannin-protein complexes (Hagerman et al., 1998; Hagerman and Robbins, 1987). Structurally, tannins are divided into condensed (CTs) and hydrolysable tannins (HTs) (Triebwasser et al., 2012). Catechin is the monomeric unit for condensed tannins forming carbon-carbon linkages whereas hydrolysable tannins are derivatives of gallic acid residues conjugated to a glucose moiety (Hagerman, 1992). CTs and HTs production segregates by plant species: gymnosperms have the biochemical pathways to produce condensed tannins whereas angiosperms commonly generate
condensed and hydrolysable tannins simultaneously (mixed tannins; Mueller-Harvey, 2001). Tannin production is molded by the environment as well as the species (Kienanen et al., 1999; Tharayil et al., 2011). Abiotic and biotic stresses generally increase tannin production and alter the chemistry to enhance their biological reactivity with proteins (Lees et al., 1994; Peltonen et al., 2005; Tharayil et al., 2011). Due to the malleability of tannin chemistry to the environment, it has been difficult to characterize the natural variation in the tannin chemistries and their associated biological reactivities.

Currently, it is proposed that tannins interfere with decomposition through the formation of tannin-protein complexes. This could result in the direct interference with decomposition by physically protecting proteins from decomposition (Northup et al., 1995; Northup et al., 1998; Bradley et al., 2000) or indirect hinderance by deactivating soil enzymes (Joanisse et al., 2007). Physical protection of proteins can limit the accessibility of the protein for enzyme mediated degradation (Adamczyk et al., 2009) and is associated with increases in the soil organic nitrogen concentrations (Northup et al., 1995; Northup et al., 1998). Due to the necessary utilization of nitrogen in proteins and enzymes for metabolic activity, microbes are sensitive to nitrogen limitations (Schimel and Weintraub, 2003). Hence, the sequestration of protein in the tannin-protein could hamper decomposition by limiting nitrogen availability. Studies have further reported that the biological reactivity of tannins is linked to the tannin chemistry (Kraus et al., 2003a; Nierop et al., 2006, Norris et al., 2011). However, when a spectrum of CTs chemistries is compared against each other in their efficacy to halt soil nitrogen fluxes, it is difficult to link a structural feature to soil responses (Norris et al., 2011). Hence, it
appears that much remains to be understood of the mechanisms by which tannins directly interfere with decomposition.

Interestingly, Johanseen et al. (2007) demonstrated that enzyme deactivation capacity of tannins was linked to the tannin chemistry and concentration. Because enzymes are directly responsible for the depolymerization of SOM (Sinsabaugh et al., 2008), this pathway poses an interesting direction to pursue. However, despite our understanding of the roles enzymes play in SOM decomposition, few investigations have conducted in-depth characterization of tannin-enzyme interactions in the soil environment. Moreover, few studies have considered the long-term impacts of tannins in affecting exoenzyme activities.

From a structural standpoint, CTs have been the dominant focus of tannin-decomposition studies while little work has considered the ecological significance of HTs. This has been attributed to the greater protein precipitation capacity of CTs in comparison to HTs (Zucker, 1983). However, this is in spite of the fact that HTs can be a significant proportion of tannin in the leaf litter per weight basis in certain classes of angiosperms (Tharyil et al., 2011) and have overwhelming affinity for proteins that in some instances are more competitive for protein binding than CTs (see chapter II). Collectively, these studies reaffirm that much remains to be explored in our understanding of tannin-decomposition interaction. Questions that need to be fully addressed include:

a) Natural variation in the tannin chemistry as mediated by environmental stress
b) the ecological significance of mixed tannins (HTs+CTs) in soil decomposition

c) turnover rate of the tannin-protein complex in the soil environment

d) the ecological significance of tannin-enzyme interactions

1.4.2. Lignin

In contrast to the reactivity displayed by tannins, the hindering capacity of lignins is attributed to their inert behavior (Berg, 2000). In plant cells, lignin is deposited into the cell walls to provide structural support and a physical barrier against pathogens (Vogel, 2008). Its resistance to degradation in the plant cell is also conferred during litter decomposition. Lignin is frequently conjugated with polysaccharides forming a lignocellulotic matrix in the leaf litter. In this complexed form, lignin is shown to physically protect the sugars from degradation (Grabber, 2005). Thus, the microbial mediated degradation of lignin is thought to be driven by the sugars and proteins encased by the lignin (i.e resource mining; Craine et al., 2007; Sinsabaugh, 2010) or co-metabolism. Like tannins, the susceptibility to degradation is tied to the lignin chemistry and the biochemical production of lignin is governed by the species (Thevenot et al., 2010) and environment (Dixon and Paiva, 1995; Moura et al., 2010).

Lignins comprise of the three phenol derivatives: vanillin (V), syringyl (S), cinnamyl (C) and (Otto and Simpson, 2006; Talbot et al., 2010; Barriere et al., 2008). Generally, gymnosperms contain approximately 80 % V units, whereas angiosperms are comprised of S and V monomers, and grasses contain equal contributions of V, S and C (Thevenot et al., 2010). The relative abundance of each phenol class in turn affects lignin’s predisposition to decay (Talbot et al., 2010). Lignins abundant in V residues are
less prone to degradation than those that accumulate S-V mixtures and C units respectively (Bahri et al., 2006). Hence, the predisposition of lignin to decay can be linked back to the species. Like tannins, environmental stresses can modify the chemical composition and increase the total abundance of lignin leading to lignin chemistries less susceptible to degradation (Moura et al., 2010).

Incubation studies have shown that as the microbial mediated degradation of lignin advances the total concentration of lignin (V+S+C=total) declines and the acid-to-aldehyde ratio (Ac:Ad) of V and S units increase (Hedges et al., 1988; Otto and Simpson, 2006). Hence, in the soil these parameters are used to reflect the degradation state of lignin in comparison to the native, un-decomposed state (Otto and Simpson, 2006). Soil studies have shown that the yield and degradation state of lignin in the soil is linked to the soil depth and particle size. Moving down the soil profile, the total concentration declines (Triebwasser-Freese et al., in review) and Ac:Ad ratio of V and S rises (Thevenot et al., 2010). Similarly, the concentration and degradation state of lignin in the fine particle fractions, comprising of clays, are lower in comparison against coarse particles (Thevenot et al., 2010). Moreover, some investigations have reported that the relative yields of V-S-C isolated from the soil can reflect the composition of the above-ground plant community, which in turn can be used as a biomarker of the plant composition.

Overall, the combination of lignins and tannins in the plant litter results in the formation of unique aromatic signatures in plant detritus linked back to the genotype of
the plant and its environment. Generally, as the phenolic concentration increases in the plant litter, the polysaccharide and nitrogen content declines (Murphy et al., 1998). Because polysaccharides contribute to the carbon content, the ratio aromatics: N of plant litter, rather than the C:N, has been used as an preliminary indicator of its susceptibility to microbially mediated decomposition (Constantinides and Fownes, 1994; Aerts, 1997). As such, as the aromatic: N ratio increases, the capacity for degradation declines in the leaf litter (Hobbie, 2000). In turn, the aromatic: N ratio associates back to the genetic identity of the plant (Preston et al., 2000). Gymnosperms exhibit the highest aromatic: N ratio, followed by the deciduous trees and grasses (Preston et al., 2000).

For several decades it was hypothesized that by-products of lignin degradation would undergo condensation and polymerization reactions in the soil resulting in an amorphous, unrecognizable SOM that was resistant to decay (Hammel, 1997: fungal degradation of lignin). However, $^{14}$C and $^{13}$C labeling studies and $^{13}$C NMR investigations suggest that the turnover of lignin in the soil could occur as quickly as 5 years (Hobbie, 2000; Rasse et al., 2006). Specifically, Rasse et al. (2006) concluded that a majority of the lignin in plant detritus exhibited a turnover rate of 1 year. Collectively, these authors concluded that lignins appear to be less impervious to degradation than previously anticipated.

Oxidoreductase enzymes are the primary class of exoenzymes that depolymerize lignins and other phenolics in the soil (Sinsabaugh, 2010). However, accurate assessment of oxidoreductase activity in the soil remains a challenge due to limitations in the
availability of appropriate artificial substrates (Sinsabaugh, 2010). However, if lignin is in fact degraded at such an accelerated pace, this would suggest that the efficacy of soil microbes to degrade lignin, directly mediated by oxidoreductases, is a poorly understood pathway. Due to this paradigm shift in thought, the mechanisms controlling the degradation of lignin in the soil need to be further assessed to understand their contribution to lignin degradation.

1.5 Soil Microbial Community

The microorganisms responsible for SOM decomposition are heterotrophic organisms that scavenge SOM for carbon and nitrogenous resources by extracellular enzyme activity (Sinsabaugh et al., 2002) to sustain metabolic activity (Kieblinger et al., 2010). Although the rate decomposition is associated to the leaf litter chemistry, what drives decomposition are the microbial demands for C and N resources (Kieblinger et al., 2010). High resource demands could stimulate fast decomposition, whereas low demands could hamper it. Microbial resource demands are predominately tied to the genetic identity of the microbe. However, a secondary factor that can impact the apparent microbial resources demands is the resource availability. The resource availability in turn is defined by the SOM chemistry and concentrations.

From a metabolic standpoint, microbial demands for carbon and nitrogen resources are driven by the carbon- (CUE) and nitrogen-use efficiency (NUE) of individual microbes. These parameters dictate the C and N requirements as well as nutrient allocation (anabolism versus catabolism) in the microbial body (Manzoni et al.,
Importantly, this factor influences how microbes pursue the degradation of SOM (Vance and Chapin III, 2001; Schimel and Weintraub, 2003). Does a microbe expend energy on carbon acquisition, or nitrogen acquisition? For example, a microbe with a low nitrogen use efficiency may dedicate more exoenzymes towards nitrogen liberation from SOM rather than carbon. In turn this could alter the total N pool in the SOM and impact neighboring microbes. Alternatively, low nitrogen availability has been reported to decrease microbial CUE, resulting in higher rates of overflow catabolism (i.e. CO₂ respiration; Manzoni et al., 2008; Manzoni et al., 2012). Collectively, CUE and NUE demands would govern how a microbe allocates extracellular enzymes towards C and N liberation from SOM (Allison and Vitousek, 2005; Allison et al., 2011; Sinsabaugh and Follstad Shah, 2011). At the same time, SOM stability creates a highly competitive environment for the microbial community. Hence, the microbe which successfully out-competes for C and N resources, while withstanding its CUE and NUE metabolic pressures, exhibits the greatest fitness and becomes the dominant decomposer of the soil community.

The soil microbial community may be comprised of bacteria (DeForest et al., 2005), saprophic fungi (basidomycetes and ascomycetes; Osono, 2007) and the arbuscular mycorrhizal fungi (ectomycorrhizae and ericoid mycorrhizae) (Talbot et al., 2008). Arbuscular mycorrhizae are soil symbionts of plant organisms (Talbot et al., 2008) and their contribution to soil decomposition is warrants future assessment (Talbot et al., 2008). The relative abundance of each microbial taxa in a given ecosystem is driven by selection pressures of the litter/SOM chemistry (as discussed above) and the climate,
namely temperature and moisture levels (Cou^teaux et al., 1995). Bacteria are relatively fast growing microorganisms that can rapidly take advantage of pulses in low molecular compounds (Ohotonen et al., 1999). While fungi do respond to changes in SOM resource availability, the rate of response is more limited than bacteria due to the slower growing nature of fungi (Ohotonen et al., 1999). Generally, bacteria are dominant in soils that are more abundant in nitrogen and polysaccharides whereas fungi are most prevalent in soils that are relatively more abundant in aromatics and low in nitrogen (Bossyut et al., 2001, Wardle, 2002). Although the C/N microbial demand plays a major role in developing the soil microbial community composition (McMahon et al., 2005), the secondary factor that affects the competitive ability of a microbe is its genetic potential to release the appropriate exoenzymes that liberate resources from the SOM.

### 1.5.1 Exoenzymes

Soil enzymes are functionally divided into hydrolases and oxidoreductases (Caldwell, 2005). Hydrolases are substrate specific and utilize the mechanism of hydrolysis to cleave residues (Caldwell, 2005). Hydrolases facilitate the depolymerization of proteins, polysaccharides, and chitin whereas oxidoreductases primarily serve to degrade the phenolic rich polymers- lignins and polyphenolics (Sinsabaugh et al., 1991; Theurel and Buscot, 2010). The substrate specificity exhibited by hydrolases is defined by the type of bond the enzyme can cleave. For instance, β-glucosidase specifically cleaves the β-1-4 glycosyl bond. Similarly, β-N-
acetylglucosaminidase strictly hydrolyzes the β-N acetylglucosamine linkage (Caldwell, 2005).

Enzyme mediated degradation of polysaccharides, protein, and chitin occurs in several sequential steps to obtain the monomeric residues (Sinsabaugh et al., 1991; Baldrian and Valášková, 2008). Consequently, these enzyme pathways contain a suite of enzymes that work in sequential order to generate the final product. For instance, the decomposition of cellulose can be initiated by the cellulbiohydrolase or endoglucanase hydrolytic enzymes, converting cellulose into cellullobiose (Baldrian and Valášková, 2008). At this stage, the β-glucosidase is the terminal enzyme and cleaves the β-1-4 bond in cellobiose to release D-glucose (Caldwell, 2005; Baldrian and Valášková, 2008). Chitin, a biopolymer of microbial origin, undergoes similar biochemical transformations to its residues. To compensate for the complexity in polymer degradation, soil decomposition studies generally monitor the enzyme activity of the terminal enzymes (German et al., 2011). The most commonly monitored hydrolase enzymes and their substrates in the soil include: lucine aminopeptidase- proteins, N-acetyl-glucosaminidase-chitin, β-glucosidase-cellulose, α-glucosidase-starch (German et al., 2011).

In contrast to hydrolases, oxidoreductases do not exhibit substrate specificity and utilize redox reactions to oxidize phenolic substrates. Moreover, substrates are selected for degradation based on the substrate redox potential. Oxidoreductases are subdivided into phenoloxidase and peroxidases (Wang, 2009; Sinsabaugh, 2010). To initiate catalytic activity oxidoreductases are require to donate 2e- to an initial electron acceptor. For
phenoloxidase the electron acceptor is H$_2$O whereas peroxidases are dependent upon H$_2$O$_2$ (Wang, 2009). Upon catalytic activation, the enzyme transitions from the parent state to an oxidized condition. In this oxidized condition, the enzyme can withdraw 2e- from an electron-donating substrate and return to the parent state. Generally, peroxidases have lower pH optima (ca. pH 3) than phenoloxidases (ca. pH 5-6; Wang, 2009). Consequentially, this enables peroxidases to utilize phenolic substrate with high redox potentials, such as lignins and tannins, whereas phenoloxidases are more limited in this regard (Wang, 2009). Hence, from an ecological perspective, understanding peroxidase activity could be more relevant than phenol oxidase. In general, phenoloxidase is the more commonly assessed enzyme in the soil. Due to the difference in the redox potential of the substrates, is likely that peroxidase has a greater ecological significance than previously anticipated. However, few investigations have conducted in-depth function of peroxidase in respect to soil decomposition studies.

**1.6 Conclusion**

Collectively, the hydrolases and oxidoreductases directly facilitate the degradation of SOM. Their activity is controlled by the microbes which pursue the degradation of SOM based on their C/N metabolic needs. In turn, the SOM chemistry, which is dictated by the plant detritus chemistry, determines the abundance and the availability of C and N resources for microbial uptake. Hence, the SOM can influence the metabolic demands of soil microbes and determine the components preferentially degraded by the soil microbial community. Overall, the decomposition process is a highly
complex system that requires an understanding of the plant litter chemistry, SOM
dynamics, and the soil microbial community activity.

In general, the overall objective of this research was to understand the impacts of
polyphenolics and lignins on exoenzyme activities. In the first investigation the
susceptibility of exoenzymes to inhibition by leaf litter tannins was considered (Chapter
2). In this study, the mixed tannin and CTs efficacy to inhibit soil enzymes was
compared. We hypothesized that mixed of tannin would have equal or greater enzyme
inhibition capacity than condensed tannins. In the second study an in-depth chemical
characterization of mixed tannins was conducted (Chapter 3). In particular, we focused
on identifying if HTs and CTs in the mixed tannins were covalently bonded to each other
and the enzyme/protein interacting implications. Lastly, the seasonal dynamics of the
Michaelis-Menten kinetics and Activation Energy behavior of soil peroxidase was
characterized (Chapter 4). Specifically we conducted spatiotemporal comparison across
ecosystems with distinct aromatic signatures in the litter chemistry. Our objective was to
observe how the Michaelis-Menten kinetics of the oxidative enzyme, peroxidase, differs
among ecosystems with different aromatic chemistries.
CHAPTER 2

THE SUSCEPTIBILITY OF SOIL ENZYMES TO INHIBITION BY LEAF LITTER TANNINS IS DEPENDENT ON THE TANNIN CHEMISTRY, ENZYME CLASS AND VEGETATION HISTORY

2.1 Introduction

Litter decomposition sustains ecosystem productivity through the recycling of nutrients stored in senesced plant tissues (Allison & Vitousek 2007; Prescott, 2010). The process of decomposition is primarily mediated by microbial extracellular enzymes that facilitate the depolymerization and mineralization of the various components of plant litter (Allison & Vitousek, 2007; Sinsabaugh et al., 2008). Hence, the properties of plant biopolymers that directly or indirectly affect the catalytic efficiency of extracellular enzymes partially regulate the rate of this decomposition process. Tannins, which are functionally characterized as water-soluble polyphenolic compounds that readily complex with proteins (Hagerman et al., 1998a), are one of the prominent classes of plant macromolecules that can control the rate of decomposition. Tannins interfere with decomposition biologically, through their toxic effects on microbial metabolism (Schimel et al., 1995), and chemically, by inactivating microbial enzymes (Joanisse et al., 2007). In addition, tannins leach from litter into the soil, where they undergo condensation and polymerization reactions with protein substrates, thereby protecting nitrogenous compounds from subsequent decomposition (Northup et al., 1995; Northup et al., 1998; Bradley et al., 2000; Hattenschwiler & Vitousek, 2000; Kraus et al., 2003a). The effect of
condensed tannins on protein precipitation is well understood (Norris et al., 2011). However, the enzyme inhibition capacity of different tannin groups, the differential susceptibility of various isoenzymes to tannin inhibition, and the possible contribution of the redox quenching capacity of tannins towards enzyme inhibition remains relatively unexplored. This lack of knowledge could limit the ability to predict the tannin-induced inhibition of soil processes in various ecosystems.

Based on their monomeric composition, tannins can be broadly divided into two classes: condensed tannins (CTs) and hydrolysable tannins (HTs). Condensed tannins are comprised of catechin and epicatechin units linked by C-C interflavon bonds at C4-C8 or C4-C6 positions, and the hydroxylation pattern of the B ring allows them to be further subdivided into procyandins (PCs; dihydroxylated B-ring) and prodelphinidins (PDs; trihydroxylated B-ring; Schofield et al., 2001; Kraus et al., 2003a). Hydrolysable tannins (HTs) are composed of gallic acid units linked via an ester linkage to a glucose moiety, and they can be further classified based on the absence or presence of C-C linked galloyl groups as gallotannins and ellagitannins, respectively (Mueller-Harvey, 2001). In general, gymnosperms produce CTs but lack the ability to produce HTs, whereas most angiosperms produce a mixture of HTs and CTs, collectively referred to as mixed tannins (MTs). The overall protein complexation capacity of tannins depends on the composition of condensed versus hydrolysable tannins, the hydroxylation pattern of the B-ring, the extent of polymerization, the types of cross-linkages between monomeric units, the substitution pattern of the A-ring, and the cis versus trans conformation at C2-C3 (Maie et al., 2003; Kraus et al., 2003a; Nierop et al., 2006). The chemical reactivity of tannins is
traditionally defined by their protein complexation affinities (Kraus et al., 2003b). Condensed tannins that interact with proteins through hydrogen-hydrogen bonding (between amine and hydroxyl groups) are thought to exhibit greater protein complexation efficiency on a per-weight basis than hydrolysable tannins that associate with proteins through hydrophobic interactions (Hagerman et al., 1998a). Thus, CTs are anticipated to play a greater ecological role in inhibiting microbial enzymes (Kraus et al., 2004). However the protein complexation is a poor predictor of enzyme inhibition (Juntheikki & Julkunen-Tiitto, 2000) and hence, the influence of tannins on soil C and N dynamics could be less associated with their protein precipitation characteristics (Norris et al., 2011). Considering the higher overall reactivity the tannins might attain due to the association of HT subunits, the MTs produced by angiosperms could exhibit efficient enzyme inhibition capacities that may be similar or more ecologically relevant. Although due to pro-oxidant activities, a similar greater inhibition of herbivore digestive enzymes has been reported by ellagitannins at higher pH (Barbehenn et al., 2006; Salminen & Karonen, 2011).

In addition to the structural features of tannins, the formation of tannin-protein complexes could also be governed by the structural characteristics of the proteins involved. Proteins with a higher structural flexibility, such as gelatin, have been shown to interact better with tannins compared to compact protein molecules, including cytochrome, lysozyme and myoglobin (Hagerman & Butler, 1981). Additionally, proteins that are abundant in hydrophobic amino acids are more susceptible to complexation with tannins than those containing charged amino acids (Hagerman & Butler, 1981; Bacon & Rhodes, 2002). From a decomposition perspective, similar to tannins, soil enzymes are
functionally defined based on the characteristics of the chemical reaction they catalyze (Caldwell, 2005). Hence, enzyme classes that perform similar catalytic functions could differ structurally, resulting in different isoforms of the enzymes (isoenzymes; Di Nardo et al., 2004; Khalil et al., 2011; Stone et al., 2012, German et al., 2012). The structural differences that accompany the existence of different isoforms include variations in protein size, the number of domains, amino acid qualities, the percent of glycosylation, the isoelectric point and pH optimum (Caldwell, 2005; Baldrin & Valášková, 2008). For example, bacterial β-glucosidase (which catalyzes the cleavage of β-1,4 linkages in polysaccharides) ranges from 50 to 210 kDa in size, while fungal β-glucosidase, which performs the same function, exhibits an average size of approximately 100 kDa (Barrera-Islas et al., 2007; Baldrin & Valášková, 2008; Canizares et al., 2011). The production of these enzyme isoforms could be determined by the identity of the microbes involved, which in turn, is a function of the abiotic and biotic conditions, including the vegetation history (Strickland et al., 2009b; Stone et al., 2012), to which the microbes are acclimated. Enzyme isoforms that perform the same catalytic function could exhibit different tannin-binding affinities due to differences in their protein structures which in part is governed by the litter quality to which the microbial community is acclimatized. Our current understanding of how vegetation history affects the susceptibility of isoenzymes to tannin complexation is limited, and this lack of knowledge could hinder our ability to predict soil processes during plant invasions. Additionally, this differential inhibition of iso-enzymes could cause shifts in the microbial community in ecosystems
where the resident isoenzymes are more susceptible to novel tannins and, thus fail to sustain the native microbial communities.

In addition to binding directly to enzymes, tannins can reduce the catalytic efficiency of enzymes by altering the chemical environment in which they operate. Functionally, soil enzymes are broadly categorized as hydrolases and oxidoreductases (Allison et al., 2007). The hydrolytic enzymes are substrate specific because their identity is strictly based on the chemical bonds they cleave in their substrates (Caldwell, 2005). In contrast, oxidoreductases, including lignin peroxidases, manganese peroxidases and laccases, are less substrate specific and undertake oxidation based on the redox potential of the substrate (Wang, 2009). Lignin peroxidases (LiP) undergo two-electron oxidation in the presence of H₂O₂ as the electron acceptor, forming the oxidized intermediaries LiP-I and LiP-II (Wang, 2009). In turn, these intermediates oxidize aromatic substrates with a lower redox potential (<1.4 V at pH 3) to form unstable phenoxy radicals that propagate ring cleavage in lignins. Because of the inherent antioxidant properties of tannins (Wei et al., 2010; Hagerman et al., 1998b), they could form resonance-stabilized phenoxy-radicals upon donation of an electron (Bors & Michael, 2002), and could readily prevent the formation of a reactive enzyme intermediary or quench an oxidized enzyme (Hemeda & Klein, 2006), resulting in the preferential protection of other substrates from oxidation by peroxidase.

The objectives of this study were to compare the inhibition efficiencies of tannins produced by angiosperms (mixed tannins, MTs) and gymnosperms (pure CTs) i) to different soil enzyme classes and ii) to different isoforms within a same enzyme
functional class that were acclimated to different vegetation histories. We hypothesized that i) contrary to the traditional understanding, MTs produced by angiosperms will exhibit a similar or greater enzyme inhibition capacity than CTs produced by gymnosperms, ii) the enzymes in soils acclimatized to gymnosperms will be more inhibited by MTs, and the inhibitory effect of CTs in soils acclimatized to angiosperms will be on par with that of MTs; and iii) because of their dual mode of interaction (protein complexation and redox buffering), tannins will exhibit greater inhibition of oxidoreductases than hydrolases.

2.2 Materials and Methods

Sites and soil description

Soils were collected from two forest ecosystems common to the southeastern US: an *Acer rubrum-Quercus alba* forest (AQ soil) and a *Pinus taeda-Pinus echinata* forest (Pinus soil). The stands were located in the Clemson University Experimental Forest, approximately 16 km apart, and had been established for more than 100 years. The two sites had similar soil type- Typic Kanhapludult, described as a pacolet fine sandy loam soil, climatic conditions and soil pH (5.0). These two stands were chosen because they represented distinct differences in the tannin chemistries of the litter, with *Acer/Quercus* producing an HT + CT mixture (mixed tannins, MTs) and *Pinus* solely producing CTs. At both sites, the understory vegetation was sparse, minimizing the impacts of other vegetation on soil enzyme dynamics. During April 2011, soil samples were collected from each stand at three randomly chosen sampling points that were at least 15 m apart.
At each sampling point, the surface litter and organic matter layer were carefully removed, and the mineral soil was sampled to a depth of 5 cm. To increase the homogeneity of the sampling, at each sampling point, three cores were collected along the three corners of an equilateral triangle (20 cm). The cores were combined, mixed, placed on ice, and then immediately transferred to the lab. There, the samples were further homogenized, sieved through a 2 mm mesh and stored at -20°C for analysis.

**Tannin Purification**

Mixed tannins (MTs) were purified from *Acer rubrum*, *Quercus alba*, and *Betula papyrifera*, and condensed tannins (CTs) were isolated from *Pinus banksiana*, *Abies balsamea*, *Picea mariana*, *Vaccinium boreale*, and *Thuja plicata*. The CTs are those previously described by Norris et al. (2011); the *Betula* tannin, from foliage collected in Newfoundland, has not been previously reported and was prepared similarly to the other CTs. The *Acer* and *Quercus* tannins were extracted as described by Tharayil et al. (2011). Briefly, ground dry plant material was extracted with 70% acetone three times and once with 100% methanol. The extracts were pooled and evaporated under nitrogen. The aqueous phase was extracted three times with diethyl-ether, rotovaped and left under nitrogen to remove any residual solvent before loading it onto a Sephadex LH-20 column preconditioned in 50% ethanol. The column was washed with 50% ethanol to remove low molecular weight compounds, and the sorbed tannins were eluted with 75% acetone. The eluent was concentrated under vacuum, further evaporated under nitrogen to remove acetone, and the remaining aqueous solution was freeze dried to recover the purified
tannin. Tannins were characterized using solution-state $^{13}$C nuclear magnetic resonance as described previously (Norris et al., 2011; Tharayil et al., 2011; Table S1).

**Condensed Tannin Assay**

Purified tannin at a concentration 1000 μg ml$^{-1}$ was dissolved in 100% methanol for analysis. Proanthocyanidins were oxidatively cleaved by adding 3 ml of acid-butanol reagent [butanol and 12 N HCl (95:5) (v/v)] and 100 μl of Fe reagent (250 mg ferric ammonium sulfate to 10 ml of 12 N HCl) to 50 μl of a sample. The reaction mixture was incubated for 50 min at 95°C and cooled to room temperature before analyzing the cyanidin content at 550 nm. Multiple tannin concentrations were assayed and three analytical replicates were analyzed per concentration per species. Linear regression with zero intercepts were fit to these data and the slope [1000 x AU / (mg tannin)] were computed for comparison (Tharayil et al., 2011).

**Hydrolysable Tannin Assay**

The hydrolysable tannins were acid hydrolyzed to gallic acid units, which were then methylated to methyl gallate (Hartzfeld et al., 2002). Briefly, 100 μl of a sample (1000 μg of tannin in 1 ml of 100% methanol) was incubated with 2 ml of methanol and 400 μl of concentrated H$_2$SO$_4$ at 85°C for 15 h. The extract was analyzed to quantify the methyl gallate content using a high pressure liquid chromatography equipped with a photodiode array detector, as per the conditions described by Tharayil et al. (2011).
**Protein Precipitation Assay**

The radial diffusion assay characterizes the protein precipitation capacity of tannins based on the dimensions of opaque rings (precipitates) that are formed upon incubating tannins in an agar medium containing a model protein. Because of its ecological similarity to soil enzymes, almond β-glucosidase (ABG) was used as the model protein to determine the protein precipitation capacity in our study. The assay was performed in triplicate according to Norris et al. (2011) using tannin concentrations optimized in preliminary studies. Briefly, plates were prepared with 0.1 % ABG, and 14 µl aliquots of tannin were pipetted into each well and were incubated for 48 h at 25°C. The volumes of the opaque rings obtained were used to determine the amount of precipitated protein. The mass ratio of proteins to tannins (µg/µg; P:T) was used as a measure of the protein precipitation capacity of the tannins.

**Almond β-glucosidase Enzyme Inhibition**

The purpose of the almond β-glucosidase (ABG) inhibition assay was to compare the inhibition capacities of MTs and CTs in an isolated system with ample potential enzyme activity and minimal interference from soil. The difference in the potential ABG activity with and without tannins was monitored based on the formation of saligen through the cleavage of β-1,4 linkages of the substrate salicin (Juntheikki & Julkunen-Tiito, 2000; Tharayil et al. 2011). In short, 3000 µg ml⁻¹ of salicin and 8 µg ml⁻¹ of β-glucosidase were incubated with 0, 5, 10, 20, 40, and 80 µg ml⁻¹ tannins at 25°C for 20 min. The final volume of the reaction mixture across all tannin concentrations was kept the same, and
the reaction time was optimized based on preliminary experiments. The reaction was stopped by the addition of 500 μl of 6 N H₂SO₄. The amount of saligen produced was quantified using a high pressure liquid chromatography system equipped with a photo diode array detector (Shimadzu Corporation, Kyoto, Japan). Chromatographic separation was performed on a Gemini-C₁₈ column (250 × 4.6 mm; Phenomenex). The solvent conditions were as follows: 22.5% acetonitrile, run isocratically for 7 min at a flow rate of 0.8 ml min⁻¹. Quantitation was based on the peak area at 273 nm. The percent of enzyme inhibition was calculated based on the product formation in the presence of tannins relative to the product formation in the absence of tannin:

\[ \text{PEI} = \frac{\text{Control}_{\text{saligen}} - \text{Sample}_{\text{saligen}}}{\text{Control}_{\text{saligen}}} \times 100 \]  

(Eq. 1)

The results were further non-linearly regressed to a hyperbolic function similar to the Michaelis-Mention kinetics to acquire the half-saturation constant of inhibition (ki) and maximum inhibition [I_max; Sigmaplot v12.0. (Systat Software, Inc., San Jose, CA, USA)]

**Tannin Antioxidant Capacity**

The radical quenching capacity of the tannins was quantified to assess the ability of tannins to hamper potential peroxidase activity. A common means of measuring the antiradical capacity is to monitor the depletion of 2,2-diphenyl-1-picrylhydrazyl (DPPH•) spectrophotometrically when combined with antioxidant compounds. The scavenging capacity is defined as the ability to donate hydrogen or electrons to the radical, thereby causing the formation of a colorless compound (Chaillou & Nazareno, 2006). A working
concentration of DPPH• (38 μg ml⁻¹) was prepared in 100% methanol, and the tannins were prepared as 1000 μg ml⁻¹ stocks in 100% methanol. The appropriate volume of the tannin stock was added to DPPH• to prepare working tannin concentrations of 2.5, 5 and 10 μg ml⁻¹, and the volumes were adjusted accordingly with methanol. The experiment was optimized for 1.0 AU in the absence of tannins. The reaction mixture was shaken at room temperature for 15 min (26 rpm) and absorbance at 517 nm was measured immediately. The antioxidant capacity of the tannins was determined as follows:

\[
\% \text{ AAC} = \frac{\text{Control}_{\text{ABS}} - \text{Sample}_{\text{ABS}}}{\text{Control}_{\text{ABS}}} \times 100
\]  
(Eq. 2)

**Soil Enzyme Inhibition**

The potential activity of peroxidase (PER), β-1,4-glucosidase (BG), and β-1,4-N-acetylglucosaminidase (NAG) in the AQ and Pinus soils were monitored in the presence and absence of CTs and MTs. To allow comparison of enzyme inhibition across tannins and sites, the amount of soil slurry used in the respective assays were optimized to achieve equivalent potential enzyme activities in the Pinus and Acer/Quercus soils in the absence of tannins (amount of slurry varied < 6% between the two soils for various enzymes). The tannin concentrations tested varied from 5 to 160 μg ml⁻¹ which, based on the soil:buffer ratio, ranged from 2 to 64 μg g⁻¹ soil and is ecologically relevant (Kraus et al., 2003b; Joanisse et al., 2007).

The potential peroxidase enzyme activity and its inhibition were determined colorimetrically using 3,3’5,5’-tetramethylbenzidine (TMB) as the substrate (Johnsen & Jacobsen, 2008) at tannin concentrations of 0, 5, 10, 20, 40 and 80 μg ml⁻¹. The product
of the oxidation of this substrate has a higher molar absorptivity (Josephy et al., 1982) than the traditionally used L-DOPA and, hence, was found to be more sensitive for tracking tannin inhibition. Soil slurries were prepared by homogenizing 0.5 g of soil in 150 ml of 50 mM acetate buffer (pH 5.0). The appropriate volume of soil slurry was added to each reaction vial to provide similar potential enzyme activities between the two sites, and the slurry was incubated with various tannin concentrations. The final volume of the assay mixture was adjusted to 400 µl using acetate buffer. The tannin and slurry mixture was incubated on a rotatory shaker (26 rpm) for 20 min at 25 °C to provide ample time for tannin-enzyme complexation. The substrate, TMB (200 µl, 250 μg ml⁻¹), was then added, and the reaction mixture was further incubated on the rotary shaker. This reaction was stopped after 20 min by the addition of 1 ml of 5 % H₂SO₄. The sample absorbance was measured in a spectrophotometer at 450 nm.

Beta-glucosidase and NAG inhibition assays were performed using the same assay conditions described above, with the following modifications. The potential activities of BG and NAG were quantified by measuring the 4-methylumbelliferone (MUB) formed after the cleavage of the MUB-linked substrates 4-methylumbelliferyl-β-D-glucopyranoside and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide, respectively. Based on preliminary experiments that exhibited lower tannin inhibition of these hydrolases, the tannin concentrations were increased to 0, 20, 40, 80 and 160 µg ml⁻¹, and the substrate concentration was kept at 700 µg ml⁻¹. After incubation, the reaction was terminated by adding 1 ml of 100 % methanol to each reaction vial. Because the MUB fluorescence readings in the microplate reader varied greatly (especially in the presence
of tannins), the MUB content was quantified using the HPLC system described above, but with a fluorescence detector. The mobile phase consisted of 40% acetonitrile in 10 mM sodium acetate buffer (pH 5.0), which was run isocratically for 7 min. The MUB contents were quantified using an Ex-Em wavelengths of 315 nm and 475 nm, respectively, and the MUB retention time was 6.5 min. The percent inhibition of the enzymes was computed using Eq (1).

Statistical Analysis

The protein precipitation capacity of the tannin was analyzed using a mixed model analysis of variance with species as the fixed effect. For the remaining analyses, individual species were nested within the tannin type (MTs versus CTs) as a random effect. The half-saturation constant of inhibition ($k_i$) and maximum inhibition ($I_{max}$) of almond β-glucosidase were analyzed with a mixed-model analysis of variance with the tannin type as the fixed effect with species nested within tannin-type as a random effect and the individual species were compared by one-way ANOVA. The nature of interaction of tannins with proteins changes with respect to tannin: protein ratio (Siebert et al., 1996, Jöbstl et al., 2004, Poncet-Legrand et al., 2006, Pascal et al., 2007), and hence it proved to be less-robust to compare the overall inhibition across all tannin concentrations. Hence, in the soil enzyme assays, ANOVA was performed at individual tannin concentrations, and contrasted how the response to that particular tannin concentration varied across the tannin types and soils. The inhibition of the soil enzymes of BG, NAG, and PER was analyzed with a mixed model ANOVA with tannin type and site as fixed
effects and species nested into tannin-type as random effect, followed by Tukey’s HSD test. The antioxidant capacity was analyzed at each tannin concentration using a mixed model ANOVA with tannin type as the fixed effect. The antioxidant capacity was further regressed with the structural data obtained through $^{13}$C NMR analysis of CTs (Supplementary method) and the peroxidase inhibition at 10 μg ml$^{-1}$ in Pinus and AQ soils. All of the statistical analyses were conducted using SAS 9.2 (SAS Institute, Cary NC), and an alpha value of 0.05 was employed for all hypothesis tests.

2.3 Results

Tannin Characterization

The acid-butanol assay measurements were based on the susceptibility of the CT polymer to undergo oxidative cleavage in an acidic medium in the presence of a catalyst (Fe). The susceptibility to oxidative cleavage differed according to the molecular identity of the CT involved, including the chain length, PC/PD content and cis versus trans ratio of the tannins (Method S1). Overall, there was an eleven-fold difference in the value corresponding to the absorbance of procyanidins from the 8 investigated species (Table 1). The response of the acid-butanol assay on a per-weight basis was consistently higher for the tannins from the gymnosperms (pure CTs) compared to those from the angiosperms (MTs; $F_{1,6}$=14.87, $P$=0.008). The tannins from the angiosperms (Acer, Quercus and Betula) differed in their HT content, as determined by the acid hydrolysis assay ($F_{2,6}$=29.3; $P$ = 0.008). The Acer and Quercus tannins exhibited similar HT concentrations by mass, with a mean of 26%, while Betula presented 15% HT by mass ($P$
= 0.001; Table 2-1). Collectively, there was a 3-fold difference in the protein precipitation capacities of individual tannins (Table 2-1). Tannins from Vaccinium, Psuedotsuga (both pure CT species) and Quercus (MT species) showed higher protein precipitation capacities and precipitated an equivalent weight of protein per weight of tannin (P:T ~1.0). Overall, however, the protein precipitation capacities of the CTs and MTs did not differ statistically ($F_{1,6}=1.32; P = 0.2449$).
Table 2-1. Chemistry Characterizations of Mixed and Condensed Tannins.

<table>
<thead>
<tr>
<th>Species</th>
<th>Tannin type</th>
<th>Chemical Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HT % (^a)</td>
</tr>
<tr>
<td><em>Abies balsamea</em></td>
<td>CT</td>
<td>n.a. (^c)</td>
</tr>
<tr>
<td><em>Picea mariana</em></td>
<td>CT</td>
<td>n.a.</td>
</tr>
<tr>
<td><em>Vaccinium boreale</em></td>
<td>CT</td>
<td>n.a.</td>
</tr>
<tr>
<td><em>Pinus banksiana</em></td>
<td>CT</td>
<td>n.a.</td>
</tr>
<tr>
<td><em>Thuja plicata</em></td>
<td>CT</td>
<td>n.a.</td>
</tr>
<tr>
<td><em>Acer rubrum</em></td>
<td>MT</td>
<td>.26</td>
</tr>
<tr>
<td><em>Betula papyifera</em></td>
<td>MT</td>
<td>0.15</td>
</tr>
<tr>
<td><em>Quercus alba</em></td>
<td>MT</td>
<td>0.26</td>
</tr>
</tbody>
</table>

\(^a\) HT % by leaf litter weight.

\(^b\) Protein precipitation capacity, P:T means (±SE), mass of β-glucosidase precipitated per mass tannin. Similar letters represent statistical similarity by one-way ANOVA with the individual species.

\(^c\) n.a. not applicable.

\(^d\) Condensed Tannin reactivity. Similar letters represent statistical similarity by one-way ANOVA with the individual species.
Almond β-glucosidase inhibition

Tannins from *Betula papyrifera* exhibited the highest maximum inhibition (*I*\(_{\text{max}}\)) of almond beta-glucosidase (*I*\(_{\text{max}}\) ~ 80 %; Table 2-2) whereas *Picea mariana* exhibited the lowest maximum inhibition (*I*\(_{\text{max}}\) ~36 %). The highest half-saturation constant of inhibition (*k*\(_i\)) was observed for *Thuja plicata*, whereas *Quercus alba* exhibited the lowest *k*\(_i\) (~16.02). The maximum inhibition (*I*\(_{\text{max}}\)) of almond beta-glucosidase by MTs was 54 % greater than that of CTs (*F*\(_{1,6}\)=20.53; *P*= 0.004; Table 2-2). The half-saturation constant of MTs and CTs did not differ statistically (*k*\(_i\) ~30; *F*\(_{1,6}\)=0.466; *P*= 0.520).

Table 2-2. Tannin inhibition kinetic parameters of almond beta-glucosidase.

<table>
<thead>
<tr>
<th>Species</th>
<th>Tannin Type</th>
<th>Inhibition parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>K</em>(_i) (^a)</td>
</tr>
<tr>
<td><em>Acer rubrum</em></td>
<td>MT</td>
<td>23.81</td>
</tr>
<tr>
<td><em>Betula papyrifera</em></td>
<td>MT</td>
<td>40.27</td>
</tr>
<tr>
<td><em>Quercus alba</em></td>
<td>MT</td>
<td>15.86</td>
</tr>
<tr>
<td><em>Abies balsamea</em></td>
<td>CT</td>
<td>25.53</td>
</tr>
<tr>
<td><em>Picea mariana</em></td>
<td>CT</td>
<td>16.02</td>
</tr>
<tr>
<td><em>Pinus banksiana</em></td>
<td>CT</td>
<td>40.08</td>
</tr>
<tr>
<td><em>Vaccinium boreale</em></td>
<td>CT</td>
<td>24.50</td>
</tr>
<tr>
<td><em>Thuja plicata</em></td>
<td>CT</td>
<td>90.64</td>
</tr>
</tbody>
</table>

\(^a\) Half-saturation constant of inhibition by tannins. Similar letters represent statistical similarity by one-way ANOVA with the individual species.

\(^b\) Maximum inhibition capacity of almond-beta glucosidase by tannins. Similar letters represent statistical similarity by one-way ANOVA with the individual species.
Antioxidant Capacity

Overall, the radical quenching capacity of tannins was dependent on the tannin type (Fig. 2-1), and MTs exhibited 63%, 65% and 42% higher radical quenching capacities than CTs at 2.5, 5 and 10 µg ml⁻¹, respectively (F₁,₀=18.71; P = 0.002). The linear regression analysis relating the radical quenching capacity of CTs at 10 µg ml⁻¹ to their % PC content (Table S1) revealed a negative association (Fig 2-2a; R²= 0.711; P = 0.035). Additionally, there was a positive correlation between the radical scavenging capacity of tannins and their corresponding peroxidase inhibition at a 10 µg ml⁻¹ tannin concentration in the Pinus soils (Fig 2-2b; R²= 0.638; P = 0.017); however, the radical scavenging capacities of the tannins were not correlated with their inhibition of peroxidase in the AQ soils (Fig. 2-2c; P = 0.411).
Figure 2-1. The percent radical (DPPH•) quenching capacity of mixed-tannins (MTs, 3) and condensed-tannins (CTs, 5). The values represent the mean value of three analytical replicates of tannins from each species ±SD. A mixed-model analysis of variance followed by post-hoc Tukey’s HSD test was used to determine significant difference between the tannin types at each tannin concentration. At a given concentration, a significant difference ($P \leq 0.05$) in inhibition between the two tannin types is identified with an asterisk.
Figure 2-2. Linear regression analysis of the radical quenching capacity of tannins at a concentration of 10 μg ml⁻¹ with (a) the percent procyanidin content of condensed tannins, (b) the mean peroxidase inhibition levels in Pinus soil at a tannin concentration of 10 μg ml⁻¹, and (c) the mean peroxidase inhibition levels in Acer/Quercus soil at a tannin concentration of 10 μg ml⁻¹.
Soil Peroxidase Enzyme Inhibition

Depending on the tannin concentration, the inhibition of soil peroxidase was affected by site or site x tannin type interaction. At 5 μg ml\(^{-1}\), the inhibition of peroxidase by tannins was affected by the site (Fig. 2-3; \(F_{1,38}=58.34; P < 0.001\)), with tannins exhibiting 30% greater inhibition of the potential peroxidase activity in the AQ soils than in the Pinus soils (\(P < 0.001\)). At tannin concentrations of 10 and 20 μg ml\(^{-1}\), there was a site x tannin type interaction (\(F_{1,38}= 40.72; P < 0.001\)) for soil peroxidase inhibition. At 10 μg ml\(^{-1}\), the MTs and CTs exhibited similar levels of peroxidase inhibition in the AQ soil, which were 16% and 52% higher than the inhibition levels found in Pinus soil due to MT and CT, respectively (\(P < 0.001\)). Similarly, at 20 μg ml\(^{-1}\), the MTs and CTs showed statistically similar levels of peroxidase inhibition in AQ soil, which were higher than the inhibition levels of peroxidase in Pinus soils (22% and 34% higher due to MTs and CTs, respectively). At 40 μg ml\(^{-1}\), the inhibition of peroxidase was dependent on the site (\(F_{1,38}= 49.73; P < 0.001\)), with the inhibition of peroxidase being 9% higher in the AQ soils than in the Pinus soils (\(P < 0.001\)). At 80 μg ml\(^{-1}\), both tannin types exhibited 100% peroxidase inhibition in both of the soils.
Figure 2-3. Soil peroxidase (PER) inhibition as affected by different tannin types, concentrations and vegetation history. The bars represent the average inhibition (±SE) of mixed-tannin (from 3 species) and condensed-tannin (from 5 species) in *Pinus* and *Acer/Quercus* soils. Within a tannin concentration the asterisk indicates a significant site x tannin-type interaction at each tannin concentration, (s) denotes a main effect of site. Similar letters represents no significant difference at each tannin concentration (*P*≤0.05).
Soil Hydrolase Inhibition

Generally, the inhibition of hydrolases (BG and NAG) by MTs was similar to or greater than the inhibition by CTs in both soils. At 20 μg ml⁻¹, both MTs and CTs inhibited soil BG activity in both soils at approximately 28% (Fig. 2-4; P > 0.05). The inhibition of BG exhibited a significant site x tannin type interaction at 40 μg ml⁻¹ (F₁,₃₈=6.05; P = 0.018) and 80 μg ml⁻¹ (F₁,₃₈=19.22; P < 0.001). At 40 μg ml⁻¹, the CTs showed lower BG inhibition in AQ soils, (P = 0.015), whereas at 80 μg ml⁻¹, the CTs presented higher BG inhibition in Pinus soils (P < 0.001). At 160 μg ml⁻¹, the inhibition of BG was dependent on the site (F₁,₃₈=6.74; P = 0.013) and tannin type ( F₁,₆= 12.93; P = 0.011), and the BG activity in the Pinus soils was marginally more inhibited by tannins than the BG in the AQ soils (F₁,₁₄=4.15; P = 0.05). Furthermore, the MTs exhibited 33% greater inhibition of BG activity than the CTs (P = 0.003).

The inhibition of NAG showed a significant site x tannin type interaction at tannin concentrations of 40 μg ml⁻¹ (F₁,₃₈=4.86; P = 0.033) and 160 μg ml⁻¹ (F₁,₃₈=6.83; P = 0.012) (Fig. 2-5). Compared to CTs, MTs exhibited 40% inhibition of the NAG present in the Pinus soils.
Figure 2-4. Soil β-glucosidase (BG) inhibition as affected by different tannin types, concentrations and vegetation history. The bars represent the average inhibition (±SE) of mixed-tannin (from 3 species) and condensed-tannin (from 5 species) in Pinus and Acer/Quercus soils. Within a tannin concentration the asterisk indicates a significant site x tannin-type interaction at each tannin concentration, (t) denotes a main effect of tannin-type and (s) denotes a main effect of site. Similar letters represents no significant difference at each tannin concentration ($P \leq 0.05$).
Figure 2-5. Soil N-acetyl-glucosaminidase (NAG) inhibition as affected by different tannin types, concentrations and vegetation history. The bars represent the average inhibition (±SE) of mixed-tannin (from 3 species) and condensed-tannin (from 5 species) in *Pinus* and *Acer/Quercus* soils. Within a tannin concentration the asterisk indicates a significant site x tannin-type interaction at each tannin concentration. Similar letters represents no significant difference at each tannin concentration (P≤0.05).
2.4 Discussion

2.4.1 Comparison of enzyme inhibition by tannins produced by gymnosperms and angiosperms

In ecological studies, CTs are traditionally thought to possess a higher enzyme inhibition capacity than MTs, which is often corroborated through protein precipitation assays. However, the protein precipitation capacity may be less representative of the enzyme inhibition capacity of tannins (Julkunen-Tiitoo & Meier, 1992), and presence of HT units could enhance the overall reactivity of MTs. In the ABG assay, inhibition by tannins resembled a traditional hyperbolic curve, indicating saturation of the sorption sites on ABG that are accessible to tannins. Although the half-saturation constants of inhibition were similar among the two tannin-types, MTs exhibited a higher Imax than CTs indicating MTs were a more potent inhibitor of ABG. In the agar-based protein precipitation assay, the capacity of tannins to precipitate ABG was below unity (protein:tannin ratios of 0.92 for pure CTs and 0.65 for MTs; Table 2-1). In contrast, in the enzyme inhibition assays, a 1:1 protein-tannin ratio resulted in less than 15% inhibition of ABG, indicating that precipitation of the enzyme could take place prior to inhibition of its catalytic activity, and that a higher concentration of tannin was required to cause enzyme inhibition. This result indicates that the sorption of tannins to enzymes is not preferentially directed towards their catalytic sites. The observed enzyme inhibition at high tannin: protein ratio could partly be due to the conformational changes in protein
folding due to the excessive cross-links between the sorbed tannins and protein and/or physical blocking of catalytic sites, resulting in noncompetitive inhibition of the enzyme.

The disparity between enzyme inhibition and protein complexation is reflected well by the observation that even though the protein precipitation assay was not able to differentiate between the reactivity of CTs and MTs, in accordance with our hypothesis, MTs exhibited a higher $I_{\text{max}}$ of ABG inhibition compared to CTs. This higher inhibition capacity of MTs could have been due to their structural flexibility (Fletcher et al., 1976), which would enable MTs to cross-link with the protein at multiple binding sites. The tannins from *Acer rubrum* presented one of the highest ABG inhibition capacities among all of the examined tannins ($I_{\text{max}}$ of 77), but exhibited the lowest protein precipitation capacity. This result further bolsters the argument that the protein precipitation and enzyme inhibition, though operationally similar (caused by the binding of tannins on to proteins), could yield different functional results (enzyme inhibition and/or protein precipitation) depending on the composition and concentration of tannins. Additionally, the relatively high ABG inhibition capacity of MTs could be partly due to the differential affinity of HT and CT units for binding sites of the same enzyme. In MTs, the affinity of HT and CT subunits for ABG complexation differs among species (Method S2; Fig. S1), with the HT units in *Acer* tannins exhibiting a higher affinity for ABG complexation than those in *Quercus* tannins, possibly due to differences in structural linkages within and between these subunits.
2.4.2  Linking the antioxidant potential of tannins to their soil peroxidase inhibition

The redox potential of tannins is lower than that of H₂O₂ that facilitates the activity of lignin peroxidase (Wang, 2009) and that of peroxyl radicals (Hagerman et al., 1998b). Therefore, antioxidants such as tannins could readily quench or prevent the formation of radical intermediates of the peroxidase enzyme. Also, by chelating the soil Fe and Mn, tannins could prevent the Fenton reaction which generates hydroxyl radicals (Lopes et al., 1999), thus restricting the non-enzymatic oxidation of substrates. In accordance with our hypothesis, the MTs exhibited higher antioxidant capacities than the CTs, and this higher radical quenching capacity of MTs could be explained by three mechanisms. First, compared to the monomers of CTs (catechin), the monomers of HT (penta-O-galloyl-D-glucose) exhibit fifteen hydroxyl groups and a lower redox potential (Hagerman et al., 1998b), which increases their propensity for radical quenching. Second, the phenolic-coupling reaction that follows the abstraction of an electron by peroxidases is more favored in gallotannins, as it is an intra-molecular process in this case (resulting in the formation of ellagitannins), compared to CTs, for which it is an intermolecular process (Bors & Michel, 2002). These coupling reactions could, in turn, result in the reproduction of additional oxidizable phenolic moieties (Hotta et al., 2001; Bors & Michel, 2002) in the polymeric products, conferring a higher radical-scavenging capacity on these condensed HTs. Third, the hydroxyl group attached to the para position of the phenolic ring serves as the primary site of radical quenching, whereas the meta hydroxyl group is involved in the stabilization of the phenolic radical (Stojanovic et al., 2001; Bors & Michel, 2002). Thus, gallic acid with a para hydroxyl group would be more reactive than
procyanidins. This position-dependent antioxidant property of the phenolic ring is further evident from our observation that the antioxidant capacity of CTs was inversely related to the percent PC content. This result is also in agreement with the observation that the tannins that are abundant in prodelphinidins are more reactive than the tannins that are abundant in procyanidins (Nierop et al., 2006).

The radical quenching capacity of tannins was a robust predictor of their inhibition of the peroxidases present in Pinus soil, but not in AQ soil. This result would suggest that even though the peroxidases in the two soils are functionally similar, these enzymes could be dissimilar in the redox states of their intermediaries and, hence, their preference with respect to accepting electrons from tannins. Additionally, once these enzymes bind to tannins, the antioxidant capacity of the tannins is reduced by more than 50% (Riedl & Hagerman, 2001). Thus, the lack of correlation between the tannin-quenching capacity and the potential peroxidase activity in the AQ soils may also be attributed to the structural differences in the peroxidases from the two sites, which would alter their tannin-binding affinities. Because we normalized the potential enzyme activities of the two sites prior to the assay, the differences in the inherent redox potentials of the two soils systems should have had less of an influence on the above results. Irrespective of the tannin type and site history, at all tannin concentrations, the peroxidase inhibition was two-fold greater compared to the hydrolase enzymes. This result supports our hypothesis that the antioxidant properties of tannins could prove to be selectively inhibitory towards peroxidase. Although the potential role of antioxidants in soil organic matter dynamics has been studied previously (Rimmer, 2005; Rimmer &
Abbot, 2011), this is the first investigation to suggest an alternate mechanism of peroxidase inhibition in soil matrices. The complete inhibition of peroxidase observed in the present study at relatively low tannin concentrations (40 µg ml⁻¹) could help to explain the higher accumulation of litter and slower C mineralization found in polyphenol-rich ecosystems. This result also could provide a partial explanation for the general observation that litter layers that contain a high abundance of phenolic substrates often exhibit lower peroxidase activity (Sinsabaugh, 2010).

2.4.3 Effect of vegetation history on the susceptibility of native enzymes to inhibition by tannins

Because the substrate identity driven by plant community could be one of the many drivers of the microbial community composition, (Ayers et al., 2009, Strickland et al., 2009a, Strickland et al., 2009b), the microbial isoenzyme composition could also be acclimatized to the inhibitors co-occurring with the substrate. In parallel with the hypothesis presented above, the N-acetyl-glucosaminidases in the Pinus soils were more prone to inhibition by MTs than by CTs. The lower NAG inhibition by MTs detected in the AQ soils could be attributed to the acclimatization of the soil microbial community to MTs, resulting in the abundance of isoforms of NAG that was less susceptible to inhibition by tannins. The acclimation of microbial enzymes was further demonstrated by the finding that the BG activity in the AQ soils was less effectively inhibited by CTs because the microbes in the AQ soils were acclimatized to the CTs within MTs. Joanisse et al. (2007), similarly determined that the soil enzyme inhibition by CTs isolated from an
invasive plant to be greater than that of the native tannins, which bolsters our findings. Additionally, Bending and Read (1996b) observed that the proteases of the ericoid mycorrhizae maintained activity in the presence of tannic acid (purified HTs) while the enzymes of ectomycorrhizae were inhibited. Similarly, Ximines et al. (2011) found beta-glucosidase of *Asperelligus niger* was 5 times more resistant to inhibition by tannic acid than that of *Trichoderma reesei*. Combined, these investigations support our finding that even though enzymes are operationally similar they could exhibit different reactivity towards tannins in the soil based on the isoforms. These observed responses could also have been amplified due to differences in the catalytic efficiencies of isoenzymes in the two soils (Gerday et. al, 2000), the enzyme state (prior interaction with inhibitors reducing available tannin binding sites; Zimmerman & Ahn, 2011).

Irrespective of the tannin type involved, peroxidase activity in the AQ soils was consistently more inhibited than that of the peroxidases in the *Pinus* soils. Bending and Read (1996a) determined that the oxidoreductases of ericoid mycorrhizae, were up-regulated in the presences of tannins while those of ectomycorrhizae were not stimulated, again bolstering that the soil microbial composition could define oxidoreductase sensitivity to tannins. Within MTs, the differences in fine level structure could impose differential inhibition to enzymatic activity. For example Sinsabaugh et al. (2002) reported differential cumulative enzyme activity in decomposing maple and oak litter, with maple litter requiring a higher peroxidase and celllobiohydrolase activity and lower phenol oxidase and BG activity than oak litter per unit mass loss. This could be partially attributed to the differential sensitivity of enzymes to inhibitors in the litter.
Oxidative degradation pathways control the release of N from organic matter (Sinsabaugh et al., 2011; Northup et al., 1995). The formation of tannin-protein complexes, which are relatively resistant to degradation by enzymes (Adamczyk et al., 2009), limits the release of protein bound in the complexes. In the present study, the levels of peroxidase and NAG inhibition caused by tannins were inversely related to each other. This result is in agreement with the findings of Sinsabaugh et al. (2011), who reported that soil oxidoreductase activity was inversely related to N-acetylglucosaminidase activity but positively associated with protease activity. The inverse relationship observed for these enzymes suggests that the capacity of NAG to avoid enzymatic inactivation under poor potential peroxidase activity conditions would help to supply organic nitrogen through an alternative N source, such as chitin, under an otherwise nitrogen-limited environment.

2.5 Ecological Significance

Ecologically, the molecular properties of tannins and their structural diversity enable these compounds to interfere with multiple decomposition pathways. The present study demonstrates that MTs exhibit enzyme inhibition capacities that are equal to or greater than those of CTs. Moreover, the susceptibility to and the mechanism of inhibition are functions of the enzyme class and the presence of different isoforms of enzymes within a class. From an ecological perspective, these responses reveal that a shift in the tannin chemistry in an ecosystem could result in temporary accumulation of plant litter and hindrance of nutrient cycling until enzyme acclimatization occurs.
Similarly, because the tannin chemistry readily responds to the environment (Tharayil et al., 2011), the preferential degradation of litter in its parent ecosystem (i.e., the home-field advantage hypothesis; Ayres et al., 2009) may be explained in part by the soil isoenzyme response to a novel tannin (inhibitor) quality. Thus, our study provides an additional mechanism by which soil microorganisms could adapt to changing litter quality and inhibitors therein -through isoenzyme acclimation.
CHAPTER 3

IN-DEPTH CHARACTERIZATION OF THE TANNIN-PROTEIN INTERACTION AND IDENTIFICATION OF STRUCTURAL FEATURES LEADING TO THE INHIBITION HYDROLASES

3.1 Introduction

Tannins are water-soluble polyphenols produced through the phenylpropanoid pathway in plant tissues. Due to their structural nature, tannins readily interact with proteins (Hagerman et al., 1998a), and exhibit high radical quenching capacities (Hagerman et al., 1998b). Through these interactions, polyphenols in the foliage and plant detritus are linked with ecological and economic consequences. For example, tannins are implicated with attenuating plant herbivory and hence the quality of fodder, by deactivating digestive enzymes and through their prooxidant potential (Barbehenn et al., 2006). Similarly, tannins have been associated in retarding decomposition directly through microbial toxicity and indirectly through deactivation of soil extracellular enzymes and the protection of soil proteinaceous substrates (Northup et al. 1995; Kraus et al., 2003a).

Tannins are structural divided into condensed (proanthocyanidins) and hydrolysable tannins. Although the molecular classification of tannins is well established, a majority of investigations have characterized the biological relevance of condensed tannins. Meanwhile, the reactivity of hydrolysable tannins, and its subsequent ecological significance, is poorly understood. However, these investigations could gain more importance as in the advent of climate change the production of hydrolysable tannins in
the plant tissue could increase (Tharayil et al., 2011). Additionally, recently Triebwasser et al. (2012) elucidated that hydrolysable tannins may exhibit a greater ecological significance than previously anticipated through their high enzyme inhibition potentials. Therefore, exploring the biological reactivity of HTs is essential to understand their economic and ecological impacts.

The biological reactivity of tannins has been reported to be associated with the molecular chemistry (Kraus et al., 2003a; Triebwasser et al., 2012). Gymnosperms exclusively produce condensed tannins (CTs) whereas angiosperms more often produce both condensed and hydrolysable tannins simultaneously, resulting in mixed tannins (CT+HT; MTs). Generally, condensed tannins comprise of catechin or epi-catechin monomers that are C4-C8 or C4-C6 linked to produce polymers. Several factors can vary in the condensed tannin chemistry, including the hydroxylation patterning of the B ring, and cis vs. trans. Hydrolysable tannins comprise of galloyl moieties conjugated to a sugar molecule in the center (Kraus et al., 2003b). Hydrolysable tannins are further classified as gallotannins and ellagitannins depending on the absence or presence of C-C linkage between carbons on the gallic acid units respectively (Tharayil et al., 2011). While an overwhelming amount of research has been conducted on the biological reactivity of CTs and HTs independently, few studies have considered the interactions of CTs and HTs that are simultaneously generated within the plant tissues of mixed tannin producing species. In our past investigation, an experiment we conducted suggested that HTs and CTs could be covalently independent of each other. This was conducted by looking at the competition of HTs and CTs binding for amino acid sites along the protein. From this
experiment we recognized that few studies have considered if i) HTs and CTs generated in the tissues are covalently linked or mutually exclusive of each other and ii) understand how interactions between the two tannin-types affect protein binding capacities and enzyme activity. To further understand the biological relevance of hydrolysable tannins we functionally isolated tannins from tree species known to produce mixed tannin composition.

3.2 Materials and Methods

Tannin purification

Leaf litter of three mixed tannin species, *Acer rubrum* (Acer), *Quercus alba* (Q), and *Liquidambar styraciflua* (SG), were collected from the Clemson University Experimental Forest Fall 2011, air dried for one week and ground finely into a powder for tannin extraction. Eighty g of leaf litter was extracted three times with 70 % acetone and once with 100 % methanol. The extracts were pooled, rotovaped at 30 °C and left under nitrogen gas over night to evaporate residual solvent. The aqueous phase was mixed with diethyl ether three times to remove low molecular components, rotovaped and left under nitrogen overnight to remove residual diethyl ether before loading onto the Sephadex LH-20 column. The Sephadex LH-20 was preconditioned in 50 % ethanol before loading the extract and washed with 50 % ethanol to remove low molecular weight compounds. The remaining tannin extract was eluted with 75 % acetone followed by rotovaping and freeze-dried to recover the purified tannin. These tannins will be referred to as bulk tannins.
Tannin Fractionation

Two hundred mg of the purified freeze-dried tannins were loaded onto the Sephadex LH-20 column, preconditioned in 50 % ethanol, for tannin fractionation. The tannins interact with the column through hydrophobic mechanisms, hence elution of tannins from Sephadex LH-20 is dependent upon the affinity for the mobile phase. As such, individual tannins were fractionated on the Sephadex LH-20 column by utilizing the two mobile phases, 40 % and 75 % acetone to elute tannins into two fractions which functionally represents the polarity of each group. These fractions will be referred to as fraction 40 and 75 for each species respectively. The weight of Sephadex LH-20 used in the column for the fractionation was optimized to avoid oversaturation of the Sephadex by the tannins thereby limiting the contamination of the 40 % solvent fraction with tannin metabolites with lower polarity. Once eluted, the fractions were rotovaped to remove residual solvent, and freeze-dried to recover the isolated tannins.

Hydrolysable Tannin Quantification

The methanolsysis of hydrolysable tannins cleaves and methylates the galloyl units from the glucose moiety to form methyl gallate and ellagic acid. Briefly, 2 ml of methanol was combined with 200 μl of tannin stock prepared at 6 mg ml⁻¹ and 400 μl of 24 N H₂SO₄ and incubated at 85 °C for 15 hrs.. Methyl gallate and ellagic acid were analyzed by high pressure liquid chromatography with an onxy monolith C18 column (Phenomenex, 100 x 45 mm; Tharayil et al., 2011).The external standards were purchased from Sigma Aldrich. The mobile phases consisted of acetonitrile: water (5:95; v/v) with 0.01 % acetic
acid and acetonitrile: water (75:25; v/v). The compounds were separated using a binary gradient increasing the mobile phase 3 % min\(^{-1}\) for 14 min with a photo diode array detector set at 254 nm. The concentrations of methyl gallate and ellagic acid were converted to represent percent compound per weight tannin.

**Condensed Tannin Quantification**

Proanthocyanidin polymers undergo oxidative cleavage in the acid-butanol assay whereby the terminal units of the cleaved products are analyzed at 550 nm. The condensed tannin abundance is characterized by the chemical reactivity of each cleaved monomer. The tannin concentrations were optimized based on preliminary investigations using working tannin concentrations of 5, 10, 20, 40, 80 \(\mu\)g ml\(^{-1}\) and tannin stocks prepared at 3000 \(\mu\)g ml\(^{-1}\) in 50 % methanol. Methanol (50 %) was employed to accommodate the water solubility of the 40 % eluted fraction. Briefly, 3 ml of acid-butanol reagent prepared as 95:5 (v/v) butanol: 12 N HCl was combined with 100 \(\mu\)l of Fe reagent (250 mg ferrous ammonium sulfate dissolved in 10 ml 12 N HCl) and the appropriate volume of sample and 50 % methanol to control for the addition of water. The samples were incubated at 95 °C for 50 mins, allowed to cool at room temperature for 15 mins follow by analysis on the UV spectrophotometer at 550 nm. The chemical reactivity of the each tannin was determined by the slope of the linear function.

**Attenuated Total Reflectance- Fourier Transform Infrared Spectroscopy**

ATR-FTIR analysis was used for functional group characterization of the fractionated tannin samples. The samples were analyzed on a Thermo Scientific Nicoli
Spectrometer and the window used for the analysis was diamond with one reflection. Briefly, 5 mg tannin was combined with 25 mg KBr. Three analytical replicates were run of each tannin sample. The samples were scanned from 4000 to 700 cm\(^{-1}\) with a resolution of 4 cm\(^{-1}\). To generate the sample spectrum, 32 interferograms were co-added and averaged. The trace spectrum was generated by using the ratio of the sample spectrum to a KBr background and the baseline corrected. The final sample spectra was converted from % transmission to absorbance and normalized to ABS 1.0 for relative quantification of peak heights. Following data analysis the spectrums were processed by 2nd order derivatization to enhance the resolution of small peaks and minimize peak overlap (Lammers, 2008). The peak heights were measured from the tangential baseline.

The peak selected for spectral comparison across the species and fractions were with the range of 1000- 800 cm\(^{-1}\) according to Arshad and Siddiaqui, 1968, which is selective towards polyphenol oriented frequencies. The peaks selected were those that were common across all samples, and relative peak intensities were compared across the samples. If a particular wavelength was present in all samples but one fraction, the relative peak intensity for the sample absent of the peak was reported as zero.

**Protein precipitation Capacity**

The protein precipitation assay determines the amount of protein precipitated by the formation of the opaque ring. This assay was prepared as per with the plate prepared with 0.05 % almond β-glucosidase, 14 µl tannin pipette into each well and allowed to incubate for 48 hrs at room temperature (Tharayil et al., 2011). The tannin concentration
was optimized based on preliminary studies with 12,500 µg ml\(^{-1}\) in 50 % methanol (Tharayil et al. 2011; Norris et al. 2011). The volume of the opaque ring was used to determine the mass of protein precipitated to tannin (µg µg\(^{-1}\)).

*Enzyme Inhibition Capacity Assay*

Almond beta-glucosidase (ABG), saligen (enzyme substrate) and salicilin (enzyme product) were purchased from Sigma Aldrich. Tannin inhibition was conducted by comparing the enzyme activity in the absence and presence of purified tannins to determine the percent inhibition at multiple tannin concentrations. The purified tannin stock was prepared at 400 µg ml\(^{-1}\) in 5 % methanol, while ABG was prepared at 400 µg ml\(^{-1}\) acetate buffer 50 mM pH 5.0. For the inhibition assay, ABG was diluted to 8 µg ml\(^{-1}\) in 200 µl and combined with triplicate tannin concentrations of 0,5,10,20,40,80 µg ml\(^{-1}\) in volumes of 250 µl and placed on the rotary shaker at 26 rpm for 40 min, after which 500 µl of the mixture was combined with 500 ul of the substrate saligen (prepared at 3000 µg ml\(^{-1}\) in acetate buffer) and placed on the shaker for 20 min. The reaction was terminated by combining 500 µl of the slurry to 200 µl of 6 N HCl. The product, salicilene, was analyzed for by high pressure liquid chromatography using an onyx monolith C18 column using the photo diode array detector as 274 nm. An isocratic run of 22.5: 77.5 acetonitrile: water (v:v) was employed to elute the product from the column and quantification was determined with the externals standard salicilene. Percent enzyme inhibition was calculated as:

\[
\text{PEI} = \frac{(\text{ProductAB} - \text{ProductPre})}{\text{ProductAB} \times 100}
\]  

(EQ. 1)
ProductAB and ProductPre refer to product formation in the absence and presence of tannin respectively for each tannin concentration.

Statistical Analysis

The CT and HT quantification of the bulk tannins were analyzed by a one-way ANOVA with the fixed effect of species. Meanwhile, the CT and HT quantitation of the fractionated tannins and the protein precipitation were analyzed by a two-way ANOVA with the fixed effects of species, fraction and species x fraction. Both analyses were followed by post-hoc Tukey’s HSD test. The enzyme inhibition assay was analyzed by a two-way ANOVA with the fixed effect of species, fraction and species x fraction at each tested tannin concentration. This was followed by post-hoc Tukey’s HSD test. The results of enzyme inhibition and protein precipitation capacity were linear regressed against molecular features in the fractionated tannins (condensed tannin concentration, % methyl gallate, % ellagic acid) to associate tannin structure to enzyme inhibition. The results of the fourier-transform infrared analysis were analyzed by principle component analysis.

3.3. Results

Wet chemistry analysis of Bulk and Fractionated Tannins

In general in the wet chemistry analyses, the % methyl gallate, % ellagic acid and CT reactivity of the bulk tannins, differed between the tree species (Table 3-1). % methyl gallate exhibited a significant main effect of tannin species (F_{3,8}=6.34; P<0.0001) where Acer 75 contained the highest percentage with approximately 49.91 % methyl gallate per
weight basis tannin. Meanwhile, *Quercus* (Q) exhibited the lowest concentration with 3.94 %. Similarly, % ellagic acid exhibited a significant main effect of species (Table 3-2; F$_{3,8}$=129, P<0.0001). *Quercus* (Q) exhibited the highest percentage of ellagic acid, 15 %, while the concentrations recovered in the remaining species was statistically similar, approximately 2 % (P>0.05). For the CT chemical reactivity, *Acer* 40 exhibited the highest reactivity with 0.008 while *Acer* 75 contained the lowest CT reactivity rate of 0.004 (P<0.0001), meanwhile the CT reactivity of *Quercus* and Sweetgum (*Liquidumbar syraciflua*) were statistically similar (P> 0.05).

In general for the fractionated tannins fraction 75 was more abundant or exhibited higher chemical reactivity than the fraction 40 counterpart (Table 3-2). The % methyl gallate exhibited a significant species x fraction interaction (F$_{5,12}$=3089, P<0.0001), where A 75 exhibited the highest concentration, approximately 50 %, meanwhile *Acer* 40 contained 22.81 % (Table 3-2, P<0.0001). Similarly, SG75 contained 11.56 % methyl gallate whereas SG40 contained 0.87 % methyl gallate (P<0.0001). In contrast, the % methyl gallate in Q75 and Q40 were statistically similar to each other (P> 0.05).

Similar to above trends, % ellagic acid per weight basis tannin exhibited a species x fraction interaction (Table 3-2; F$_{5,12}$=105.81; P<0.0001) where Q75 contained the highest % ellagic acid per weight basis tannin with, 21.24 % ellagic acid, which was 175 % more than % ellagic acid present in Q40 (P<0.0001). In contrast, *Acer* and *SG* exhibited statistically similar percentages of ellagic acids amongst their fractions (P>0.05).
The CT chemical reactivity exhibited a significant species x fraction interaction (Table 3-2; F_{5,12}=73.61; P<0.0001), where A40 exhibited the highest CT reactivity, 0.004 and this was 200% greater than the CT reactivity observed by A75 (P<0.0001). Meanwhile, the CT reactivity of SG 40 and Q 40 were 66% lower than the CT reactivities present in SG 75 75 (P<0.001).

Table 3-1. Wet Chemistry Analysis of bulk tannins, similar letters represent no significant different (P>0.05).

<table>
<thead>
<tr>
<th>Tannin Species</th>
<th>Methyl Gallate (% weight)</th>
<th>Ellagic Acid (% weight)</th>
<th>CT slope</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acer 75</strong></td>
<td>49.91 a</td>
<td>2.15 b</td>
<td>0.004 c</td>
</tr>
<tr>
<td><strong>Acer 40</strong></td>
<td>22.81 c</td>
<td>1.40 b</td>
<td>0.008 a</td>
</tr>
<tr>
<td><strong>Quercus</strong></td>
<td>3.94 d</td>
<td>15.00 a</td>
<td>0.007 ab</td>
</tr>
<tr>
<td><strong>SweetGum</strong></td>
<td>29.01 b</td>
<td>2.094 b</td>
<td>0.006 b</td>
</tr>
</tbody>
</table>

Table 3-2. Wet Chemistry Analysis of fractionated tannins, similar letters represent no significant different (P>0.05).

<table>
<thead>
<tr>
<th>Tannin Fraction</th>
<th>Methyl Gallate (% weight)</th>
<th>Ellagic Acid (% weight)</th>
<th>CT slope</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acer 40</strong></td>
<td>22.81 b</td>
<td>1.40 c</td>
<td>0.004 a</td>
</tr>
<tr>
<td><strong>Acer 75</strong></td>
<td>49.91 a</td>
<td>2.15 c</td>
<td>0.002 c</td>
</tr>
<tr>
<td><strong>Q 40</strong></td>
<td>1.68 d</td>
<td>12.43 b</td>
<td>0.002 c</td>
</tr>
<tr>
<td><strong>Q 75</strong></td>
<td>1.91 d</td>
<td>21.24 a</td>
<td>0.003 b</td>
</tr>
<tr>
<td><strong>SG 40</strong></td>
<td>0.87 d</td>
<td>3.68 c</td>
<td>0.002 bc</td>
</tr>
<tr>
<td><strong>SG 75</strong></td>
<td>11.56 c</td>
<td>2.56 c</td>
<td>0.003 b</td>
</tr>
</tbody>
</table>
The principal component analysis on the deconvoluted relative IR peak intensities revealed changes in the FTIR as affected by fractionation and species (Fig. 3-1). The infrared frequencies were species specific thereby requiring species specific PCA.

For *Acer*, PC axis 1 and 2 accounted for 40.6 % and 39 % of the variation respectively. Along PCA axis 1 and axis 2 the three fractions of tannin, Bulk, A75, and A40, were significantly different from each other (P<0.05). A75, most associated to the positive PC axis 1, was abundant in the wavenumbers 1685, 1517 and 969 cm\(^{-1}\). The 1685-1740 cm\(^{-1}\) represents C=O functional group, which is a functional group specific to HTs (Arshad and Siddiqui, 1968), and could indicate abundance of gallic acid conjugation to glucose moieties. Meanwhile, the 1517 and 969 cm\(^{-1}\) wavenumbers are representative of OH bending and/or ring skeletal vibration in HTs and CTs respectively. In contrast, A40 was most represented by the IR frequencies 1285, 1200, 1100 cm\(^{-1}\). 1200 and 1100 cm\(^{-1}\) are representative of C-O-C asymmetric stretching that be present in HTs and CTs (Arshad and Siddiqui, 1968).

For *SG*, PCA axis 1 and 2 described 65.9 % and 14.7 % of the variation respectively. Along PCA axis 1, B and SG75 were statistically similar to each other (P >0.05), meanwhile the SG Bulk and SG75 were statistically different from SG40 (P<0.05). Along PCA axis 2, all fractions were statistically different from each other (P <0.05). SG Bulk was abundant in the carbonyl functional groups 1685 and 1699 cm\(^{-1}\) respectively, suggesting different chemical environments for C=O groups. Meanwhile,
SG75 and SG40 were most abundant in the carbonyl functional groups 1685 and 1707 cm\(^{-1}\) respectively. Again, this is indicative of two chemical environments for the C=O in the fractions.

In the *Quercus* fractionations, the PCA axis 1 and 2 described 42.6 % and 35.5 % of the variation. Along PCA axis 1 and axis 2 each fraction was statistically different from each other (P<0.05). Q40 contained two carbonyl peaks at 1743 and 1726 cm\(^{-1}\) respectively; meanwhile Q75 was only abundant in 1726 cm\(^{-1}\). Meanwhile, Q75 was abundant in the peak 834 cm\(^{-1}\) which could be indicative of the \(\alpha\)-anomer of glucose. The Bulk tannin was most represented by the aromatic OH bending and ring skeletal vibration frequencies.

*Protein precipitation capacity of fractionated tannins*

The protein precipitation capacity of the fractionated tannins was found to have a significant species x fraction interaction (Fig. 3-2; \(F_{5,2}=211, P<0.0001\)). In comparing between the fractions of each species, the fraction 75 exhibited greater protein precipitation efficiency than fraction 40. For *Acer*, the protein precipitation capacity of A75 was 50 % greater than A40 (P<0.0001). Similarly, the protein precipitation efficiency of Q75 and SG75 were 1.76 and 2.80 mass protein precipitated per mass tannin, while Q40 and SG40 exhibited 0.92 and 1.24 protein precipitated per mass tannin respectively (P<0.0001). Linear regression analysis of P:T, comparing the structural features as characterized by wet chemistry to the P:T capacity, revealed no correlations between % methyl gallate (\(R^2=0.042; P=0.41\)), % ellagic acid (\(R^2=0.002; P=0.86\), and
CT reactivity ($R^2=0.0012; \ P=0.89$) with the protein precipitation capacity (data not shown).
Figure 3-1. Principal component analysis of the FTIR relative peak intensities of the fractionated and bulk tannins for each species. (a) represents PCA of *Acer*, (b) represents the PCA of Sweet Gum and (c) represents the PCA of *Quercus*. 
Figure 3-2. Protein Precipitation capacity (P:T ratio) of the fractionated tannins using almond beta-glucosidase as the model protein. The bars represent the mean (n=3) ±SE. Comparing the fractions for each species separately, similar letters represent no significant difference (P>0.05).

**Almond β-glucosidase inhibition dynamics by fractionated tannins**

In general, as the tannin concentration increased the inhibition of almond β-glucosidase (ABG) became dependent upon a species x fraction interaction (Fig. 3-3). At 5 µg ml\(^{-1}\) tannin, inhibition of ABG was dependent upon the main effect of species (Fig. 3-3; F\(_{5,12}=7.69\); P=0.0005) where Acer, with a percent enzyme inhibition of 6.17 %, was approximately two times greater inhibition capacity than SG and Quercus. At 10 µg ml\(^{-1}\) tannin, inhibition of ABG was dependent upon a species x fraction interaction (F\(_{5,12}=4.72\), P=0.0306), where A75 exhibited the greatest inhibition efficiency with 33 % inhibition (P<0.0001); meanwhile, the inhibition efficiency across the other species and
fractions remained statistically similar to each other (P > 0.05). Similarly, at 20 µg ml⁻¹ tannin concentration inhibition of ABG exhibited a species x fraction interaction (F₂,₁=9.60 P=0.0032). At this concentration A75 again exhibited the highest inhibition efficiency of 49 % (P<0.0001) whereas the inhibition efficiencies of A40, Q75, Q40 and SG 75 were statistically similar, approximating around 25 % for A40 and 15 % for Q75, Q40 and SG 75 respectively (P > 0.05). At 40 µg ml⁻¹ tannin concentration, inhibition of ABG was again dependent upon a species x fraction interaction where A75 exhibited approximately 60 % inhibition efficiency of almond β-glucosidase. Meanwhile, A40 with an inhibition efficiency of 25 % was statistically similar to SG 75 and Q75 that contained approximately 15 % inhibition capacities of almond β-glucosidase (P>0.05). Further, SG 40 continued to maintain an inhibition capacity of almond β-glucosidase of 6 % that was statistically lower than the remaining fractions (P>0.05). Last, 80 µg ml⁻¹ exhibited a species x fraction interaction where the inhibition dynamics observed at 80 µg ml⁻¹ resembled the interaction of 40 µg ml⁻¹ tannin concentration.

Linear regression analysis of % ABG inhibition against the wet chemistry features of the tannin fractions revealed that inhibition of ABG was positively associated to the methyl gallate concentration per weight basis tannin at the 20 and 40 µg ml⁻¹ (Fig. 3-4a and 3-4b; R²= 0.86; P<0.0001). A75, which was the most abundant % methyl gallate per weight basis tannin (Table 3-2), exhibited the highest inhibition efficiency of ABG followed by A40 which was the second most abundant in methyl gallate. The % ellagic acid nor CT reactivity were found not be correlated to the enzyme inhibition capacities of the tannin fractions (data not shown).
Figure 3-3. Inhibition of Almond beta-glucosidase by comparing tannin fractions and concentrations. The bars represent the average (n=3) ±SE. The asterisk represents a significant tannin fraction × species interaction, the (s) represent a significant main effect of species (n=3). At each tannin concentration, similar letters represent no significant difference (P<0.05).
Figure 3-4. Linear regression analysis of the % ABG inhibition against % Methyl gallate present in each tannin fraction. The black circles represent *Acer* 75, the white circles represent *Acer* 40, the black triangles are Sweet Gum 74, the white triangles are Sweet Gum 40, and the black and white squares are *Quercus* 75 and *Quercus* 40 respectively.
3.4. Discussion

3.4.1. Wet and FTIR characterization of Mixed Tannins

The distinct structural classes of tannins, divided as gallotannins, ellagitannins, and proanthocyanidins, result in differences in the biological reactivity, in term of efficiency and preferential mode of reactivity (review by Barbehenn and Constabel, 2011; Triebwasser et al 2012). For example, hydrolysable tannins preferentially interact with proteins through hydrophobic interactions whereas proanthocyanidins interact with proteins through hydrogen-hydrogen bonding (Hagerman et al., 1998). Consequently, the distinct mechanisms by which gallotannins and proanthocyanidins interact with proteins has different biological outcomes (Barbehenn et al., 2006). While gallotannins have a higher enzyme inhibition capacity, proanthocyanidins display a greater protein precipitation capacity that contributes to but is not directly associated to enzyme deactivation (Triebwasser et al., 2012). At the same time, evidence from our past investigation suggested that hydrolysable and condensed tannins present within a mixed tannin compete for binding sites along proteins which in turn affect the outcome of the total biological reactivity of the tannins (Triebwasser et al., 2012). Therefore, because the biological reactivity of a mixed tannin is the consequence in the interactions of gallotannin, ellagitannin, and proanthocyanidins it is important to understand how each tannin class contributes to the overall biological reactivity of the bulk tannin. Moreover, it is possible that the relative concentrations of each tannin class affect the overall biological reactivity and tannin-tannin interactions. To consider these questions, isolated
tannins from three tree species were functionally separated according to polarity on Sephadex LH-20.

The physical fractionation of tannins by Sephadex LH-20 further suggested that HTs and CTs are not covalently linked (Salminen et al., 2004), however further evidence is required for verification (Table 3-1). Moreover, in conjunction with past investigations (Hagerman et al., 1998a) HTs were generally found to be relatively more hydrophobic than condensed tannins, resulting in preferential accumulation of HTs in the 75 % acetone fractions. Similarly, condensed tannins of higher chemical reactivity accumulated in the 75 % acetone fraction. However, the relative contribution of gallotannins, ellagitannins, and condensed tannins to each solvent fraction was species dependent. Differences in the relative contributions of gallotannins and ellagitannins to the respective solvent fractions suggest that there are molecular differences in the polarity of gallo- and ellagitannins. In general, differences in molecular polarity are associated to the molecular size of the hydrolysable tannins (Moilanen et al., 2013), but could also be contributed by differences in the quantity of conjugated gallic acid units on each molecular species. Because the fractionation was conducted utilizing excess Sephadex LH-20 it is not likely that compound exclusion of Sephadex LH-20 binding sites was the mechanism leading to tannin fractions and most likely is associated with polarity.

Through the use of Fourier Transform infrared spectroscopy the breadth of the molecular heterogeneity of naturally produced tannin, specifically of the hydrolysable tannins was suggested. The carbonyl functional group (C=O) is selectively present in hydrolysable tannins and absent in the molecular structures of condensed tannins (Kraus et al., 2003b).
Through FTIR analysis it was shown that many of the tannins exhibit at least two distinct chemical shifts of the carbonyl groups that become prominent in the fractionated tannins. This suggests that the C=O bond resides in more than one chemical environment (Arshad et al., 1968). Meanwhile, that lack of overlapping in peaks in the FTIR across the species exemplifies the specificity in the tannin chemistry for each species.

3.4.2. Protein Reactivity of the Mixed Tannins

The reactivity of tannins may be defined by the protein precipitation efficiency or the enzyme inhibition efficiency. Few investigations have linked the molecular features of mixed tannins to their protein reactivity (Kraus et al., 2003a; Norris et al. 2011). Kraus et al. (2003a) reported no molecular feature, CT, % MG (methyl gallate), or % EG (ellagic acid), association to protein precipitation capacity. In contrast, the inhibition efficiency of the hydrolase enzyme, almond β-glucosidase, was positively associated to the % methyl gallate, in a concentration dependent manner. Gallotannins, the polymer from which the methyl gallate is derived, has been reported to exhibit higher binding constants to the model protein bovine serum albumin (ca. $10^5$) than ellagitannins (Deaville et al., 2007) and condensed tannins (ca. $10^{3-4}$; Frazier et al., 2010). This would suggest that the inhibition of hydrolases is driven by the higher binding constants of hydrolysable tannins. Alternatively, inhibition of hydrolases could also be driven by the selective binding of tannins to amino acids on the protein that alter the protein conformation, however this has not yet been shown.
3.5 Conclusion

The results indicated that within the natural isolated tannins from tree species hydrolysable tannins may exist in more than one chemical environment. It is difficult to explicitly discern characteristics of condensed and hydrolysable tannins from the FTIR spectra alone. Similarly, although tannins are predominately attributed to precipitating proteins, a molecular feature could not be associated to the protein precipitation capacity. In contrast, the % methyl gallate was found to positively link to the enzyme inhibition capacity, supporting the ecological function hydrolysable tannins.
CHAPTER 4

CHARACTERIZATION OF THE MICHEALIS-MENTEN KIENTICS AND
ACTIVATION ENERGY OF SOIL PEROXIDASE ACROSS ECOSYSTEMS OF
DIFFERING LITTER CHEMISTRIES

4.1 Introduction

Lignin, the second most abundant biopolymer on Earth (Glexiner, 2001), is traditionally thought to have a high biosequestration potential for atmospheric CO$_2$ due to its lower susceptibility to undergo microbial degradation (Prescott, 2010). However, recent investigations have suggested that lignin exhibits a shorter residence time in the soil than previously anticipated, with a turnover rate near to 5 years (Rasse et al., 2006; reviewed by Dungait et al., 2011; Schmidt et al., 2011), revealing that the factors regulating lignin decomposition in soils are less understood. One of the main factors that could control the rate of degradation of a polymeric substrate is the catalytic efficiency of the associated depolymerizing enzyme. For example, the rate-limiting step in the mineralization of proteins and polysaccharides is the catalytic efficiency of hydrolases, described by Michaelis-Menten kinetics, as well as their Activation Energy (Davidson et al., 2012; Wallenstein et al., 2011). Similarly, the substrate availability also regulates the production of hydrolases in soils, which in turn moderated the rate of degradation of polysaccharides (German et al., 2011). However, a similar interrelationship between substrate availability and the kinetics of oxidoreductases, the enzyme class that catalyzes the degradation of polyphenols, remains largely unexplored.
The linkages connecting monolignols to the polymeric lignin units contribute to the stability of lignin, and is one of the primary factors that governs its rate of degradation (Hedges et al., 1985; Boerjan et al., 2003; Talbot et al., 2010). Lignin may comprise of the three structural units: guaiacyl (G), syringyl (S), and hydroxyphenyl (H), which differ in their ring substitutions (Vanholme et al., 2010). The relative contribution of each lignin units in turn affects the relative abundance of the structural linkages in lignin. The β-O-4 bond is most abundant in S-S and S-G linkages and is more prone to cleavage than β-5, 5-5, and phenylcoumarin linkages, prevalent between G-G residues (Bahri et al., 2006). Additionally, compared to G rich lignin, the S and C rich lignins exhibit lower cross-linkages with cellulose fibers, resulting in faster mineralization of the respective litter (Talbot et al., 2010). Gymnosperms predominately produce G units, whereas angiosperms accumulate mixtures of G and S units, and grasses produce approximately equivalent mixture of G, S and H units (reviewed by Boerjan et al., 2003). Due to the differential susceptibility of intra-molecular linkages to degradation, the type and abundance of units could regulate the residence time of lignin in soil (Hedges et al., 1985; Bahri et al., 2006), which has been reported to vary across ecosystems (Hedges et al. 1985; Otto and Simpson, 2006; Thevenot et al., 2010), soil depth (Kaiser et al., 2004), and could change seasonally. The dynamics of the lignin chemistry across spatiotemporal and ecosystem scale could also in part regulate the production of oxidoreductases. However, it is less understood how lignin dynamics, namely the types and abundance of lignin units, in the soil affect the production and activity of oxidoreductases.
For hydrolases, the substrate availability is one of the primary drivers impacting enzyme production (German et al., 2011). However, unlike the mineralization mediated by hydrolases, the degradation of lignin generally provides a less direct metabolic gain to the microbial decomposers (Boyle et al., 1992; reviewed by Osono, 2007). The function of lignin decomposition is thought to liberate polysaccharide and proteinaceous resources chemically entrapped in the lignin matrices (Swift et al., 1979; Sinsabaugh, 2010), which are then degraded by hydrolases. Oxidoreductase activity has been reported to be elicited by polyphenols (Bending and Read, 1996) as well as by nitrogen limitations (Waldrop and Zak, 2006; Craine et al., 2007; Edwards et al., 2011), suggesting that multiple environmental and metabolic signals induce oxidoreductase production. Therefore in the soil, along with lignin chemistry, the production of oxidoreductases could be indirectly driven by microbial nutrient demands. Due to this potential complexity of oxidoreductase responses, exploring how oxidative enzyme activity is associated to ecosystems that sustain input of different litter chemistries would aid our understanding of the lignin decomposition in soils.

Along with the above factors that affect extracellular enzyme production, a secondary factor that could affect the rate of lignin decay is the catalytic efficiency of oxidative enzymes. The enzyme efficiency is characterized by the Michaelis-Menten kinetics, and secondarily by the Activation Energy (Wallenstein et al., 2011). In this study, the Michaelis-Menten kinetic constants are defined as i) the maximum rate of enzyme activity ($V_{\text{max}}$) and ii) the substrate concentration at 50% $V_{\text{max}}$ ($K_m$; Stone et al., 2011). Conceptually, the $K_m$ constant represents the efficiency of enzyme activity under
non-saturating substrate conditions (the slope; Fig. 4-1a) whereas the $V_{\text{max}}$ defines the enzyme efficiency under substrate saturating conditions (black dashed line; Fig. 4-1a). Combined, the most efficient enzyme would be considered the one that can achieve the highest $V_{\text{max}}$ at a lower $K_m$ (Fig. 4-1b). Alternatively, when comparing two enzymes with the same $K_m$ but differing $V_{\text{max}}$ (Fig. 4-1c; Marx et al., 2005), the enzyme with the higher $V_{\text{max}}$ is more efficient as it can achieve a higher velocity at higher substrate abundance beyond $K_m$. From an ecosystem perspective, the Michaelis-Menten kinetics of hydrolases has been shown to vary by soil depth, and seasons (Marx et al., 2005; Bell et al., 2010; Brzostek and Finzi, 2012). Given that the interaction of Michaelis-Menten kinetics and Activation Energy ultimately govern the rate of substrate depolymerization (Bengston and Bengtsson, 2007; Davidson et al., 2012), linking the kinetic behavior of oxidoreductases to the litter chemistry is pivotal to our understanding of lignin decomposition. However, due the complexity of the lignin chemistry, the chemical stability of lignin could also play a key role in determining the catalytic efficiency of soil oxidoreductases.
Figure 4-1. A schematic representation of the Michaelis-Menten kinetic parameters, $V_{\text{max}}$ and $K_m$, of a soil enzyme (a). Keeping $V_{\text{max}}$ constant, decreases in the $K_{m_0}$ (green dashed) to $K_{m'}$ (blue) increases the efficiency of the soil enzymes (b). Keeping the $K_m$ constant, increase in the $V_{\text{max}0}$ (green dashed) to $V_{\text{max}'}$ (blue) increases the efficiency of the soil enzyme (c). Schematic depiction of the relationship of the $V_{\text{max}}$ and $K_m$ parameters to generate a degradation efficiency term based on the slope (d). The blue line represents an enzyme of high efficiency, green represents moderate efficiency, and red represents low efficiency. Keeping $V_{\text{max}}$ constant, the black dashed line identifies a decrease in the degradation efficiency as the $K_m$ increases. Similarly, maintaining $K_m$ constant, the degradation efficiency increases with an increase in $V_{\text{max}}$. 
For this study, we characterized the Michaelis-Menten kinetics and Activation Energy of the oxidative enzyme, peroxidase. The low pH optima and high redox potential of peroxidase (Wang, 2009) enables this enzyme to oxidize aromatics more efficiently than phenoloxidases (Wang, 2009; Lundell et al., 2010), and therefore is an appropriate target enzyme for this study. Our first objective was to demonstrate the Michaelis-Menten kinetics of soil peroxidase using the substrate 3,3’,5,5’-tetramethylbenzidine (TMB). Secondly, we determined the degradation efficiency of soil peroxidase as influenced by soil polyphenol content across a pine, deciduous, and agricultural ecosystems. We hypothesized that the catalytic efficiency of peroxidase would be more associated to the lignin chemistry and the lignin quantity. We predicted that sites with lower total lignin content and higher guaiacyl units would have peroxidases with higher efficiency, potentially enabling the peroxidases to overcome the strain in degrading chemically stable lignin.

4.2 Methods and Materials

Collection and processing of soils

Three ecosystems (pine forest, deciduous forest, and cultivated agricultural field) that received qualitatively and quantitatively different litter inputs were selected for this study (Table 1). The soils of deciduous and pine forest were classified as Typic Kanhapludult, described as a pacolet fine sandy loam soil. The dominant species of the deciduous forest were White Oak (Quercus alba), Red Oak (Quercus rubra) and Mockernut Hickory (Carya tomentosa). The pine stand consisted of Viriginia Pine (Pinus virginiana). The
agricultural soil is described as thermic, Typic Udifluvent, typical of the alluvial soils, which has been in cultivation for at least 8 years and employs a corn and soybeans rotation. The selected sites were within 25km from each other and experienced similar climates. All the sites were sampled during first week of May 2012, last week of June 2012 and September 2012 to capture soil enzyme activities during spring, summer and fall seasons, respectively. At each site, three plots (6x6 m) were randomly selected for soil sampling that were at least 15 m apart, and from each sampling plot three soil cores (10 cm diameter) were taken at 5 cm intervals to a depth of 15 cm. The same plots were re-visited each season for sample collection. Upon collection, the soil samples were kept at 4 °C until arrival in the lab, where each sample was passed through a 2 mm sieve, and were stored at -20 °C until analysis.

**CuO Oxidation**

To determine the lignin chemistry we conducted CuO oxidation of lignins in the soil collected during the spring (Otto and Simpson, 2006; Kaiser et al., 2012). Briefly, soil equivalent to 25 mg soil C, was combined with 1 g CuO, 150 mg ferrous ammonium sulfate and 14 ml of 2 M NaOH that had been sparged for 20 min with argon in a 23 ml Teflon-lined acid digestion vessel. Further, each vessel was sparged with argon for an addition 15 min before closing. The samples were heated to 160 °C for 160 min and then allowed to cool to room temperature. Upon cooling, the supernatants were transferred to centrifuged tubes and 100 µl of the internal standards (cinnamic acid and ethylvanillin, 200 µg ml⁻¹ in 100 % methanol) were added to each sampled followed by 3 ml of 18N H₂SO₄. The samples were placed at 4 °C for 10 min, followed by centrifugation to settle
the humic fraction. Ten ml of the supernatant was extracted with 4 ml of ethyl acetate, the ethyl acetate fraction was collected and stored at -20 °C for further analysis.

In preparation for high pressure liquid chromatography (HPLC) analysis, 400 µl of the ethyl acetate was evaporated under N₂ and reconstituted in 25 % methanol. The samples were analyzed using a Shimadzu quaternary pump system (Shimadzu Corporation, Kyoto, Japan) coupled to a photodiode array detector on the Kinetex column (C₁₈, 100 x 4.6 mm; Phenomenex, Torrence, CA, USA). Each standard was analyzed at the wavelength that corresponded with their λₘₐₓ (Table S2). The mobile phases consisted of 0.05 % formic acid and 100 % methanol respectively using a binary gradient with a flow rate of 0.75 ml min⁻¹. The concentrations recovered were normalized to µg g⁻¹ of soil C and the total lignin concentration (the sum of the vanillyl, syringyl, and cinnamyl units) were calculated (Otto and Simpson, 2006; Kasier et al., 2012).

**Michaelis-Menten Kinetics**

The L-3,4-dihydroxyphenylalanine (L-DOPA) substrate is commonly used for the measurement of potential activity of soil oxidoreductases. Due to its higher polarity and molecular size L-DOPA rarely exhibits Michaelis-Menten kinetics with horseradish peroxidase (Rodríguez-López et al., 2000). Thus, for characterization of the Michaelis-Menten Kinetics (Vₘₐₓ and Kₘ) of soil peroxidase 3,3’,5,5’-tetramethylbenzidine (TMB) was used as the substrate (Johnsen and Jacobsen, 2008). TMB was prepared by dissolving 200 mg citric acid into 20 ml H₂O before adding 10 mg of TMB and vortexed until dissolution. The soil slurries were prepared by blending 200 mg of soil (fresh weight) with 100 ml of 50 mM acetate buffer (pH 5.0) for 1 min. The pH 5.0 was chosen
because it was mean pH across all the soil types (Table 1). To measure Michealis-Menten kinetics 200 µl of the working TMB concentrations (38, 62, 125, 250, 375, 500, 750, 1000 µM) was combined with 50 µl of H2O2 (2.7%) and 200 µl of the soil slurry in 2 ml micro-centrifuge tubes. The samples were incubated at 25 °C on a rotary shaker (26 rpm) for 30 min. The reaction was terminated with the addition of 1 ml of 5 % H2SO4, followed by centrifugation and the absorbance of supernatant was measured at 450 nm on a UV-VIS spectrophotometer.

The velocity of enzyme activity (µM min⁻¹ g⁻¹ soil) was calculated using the molar extinction coefficient of the yellow chromophore (representing oxidized TMB), 5.9 x 10⁴ M⁻¹ cm⁻¹ (Josephy et al., 1982). This was followed by nonlinear regression analysis to the Michaelis-Menten equation to acquire the \( K_m \) and \( V_{max} \) constants using GraphPad (La Jolla, CA, USA):

\[
V = \frac{V_{max} [S]}{K_m [S]} 
\]  
(Eq 1)

From preliminary experimentation, we were able to confirm the Michaelis-Menten kinetic responses of soil peroxidase using TMB as the artificial substrate in all soils (Fig. 4-2).
Figure 4-2. The observed Michaelis-Menten kinetics of soil peroxidases in the study systems. Substrate concentration is on the x-axis and the rate of enzyme activity is on the y-axis. Each data point represents the mean enzyme activity (n=3) at the respective substrate concentration ±SE. (red) refers to the deciduous soil, (yellow) refers to the pine soil, and (blue) identifies the agricultural soil.
Degradation Efficiency Term of soil peroxidase (PER\textsubscript{DE})

We generated a degradation efficiency term of peroxidase (PER\textsubscript{DE}) that incorporates the effects of the $V_{max}$ and $K_m$ into one parameter. For each replicate the $V_{max}$ and $K_m$ were linearly regressed through the origin and the slope defined the degradation efficiency term (Fig. 4-1d). The slope was multiplied by a unit of 1000. For interpretation, as the slope increases the peroxidase degradation efficiency increases. For example, keeping $V_{max}$ constant, Fig. 4-1d shows that the degradation efficiency declines as the $K_m$ value decreases. Similarly, keeping $K_m$ constant, as the $V_{max}$ increases the degradation efficiency increases. The caveat in using either the $V_{max}$ or $K_m$ alone is that it is difficult to compare the overall efficiency of enzymes when the Michaelis-Menten constants are analyzed separately. For example, the efficiency of an enzyme with a high $V_{max}$ could be counteracted with a high $K_m$, resulting in a poor efficiency under substrate non-saturating conditions. Also, the overall efficiency of an enzyme would be difficult to discern and compare across a set of data with numerous $V_{max}$ and $K_m$ values. Hence, the PER\textsubscript{DE} term provides a means to integrate and understand the net effect of the Michaelis-Menten kinetic constants of soil enzymes.

Activation Energy (PER\textsubscript{Ea})

To determine the temperature sensitivity of the peroxidase enzymes, the soil samples were tested at 10, 20, 30, and 40 °C. Briefly, the soil slurries were prepared as 100 mg soil in 100 ml acetate buffer (50 mM; pH 5.0), aliquoted to 2ml tubes and pre-incubated at the designated temperature in a shaker at 100 rpm for 30 min. The samples were then supplied with 200 µl of TMB (200 µg ml\textsuperscript{-1}), to obtain a working concentration of 1000
µM, 50 µl of H₂O₂ (2.7 %) and allowed to incubate an additional 30 min before terminating the reaction with 1 ml of 5 % H₂SO₄. As described above, the samples were analyzed spectrophotometrically at 450 nm. The absorbance values were converted to µM of the TMB oxidation product using the molar extinction coefficient, normalized to rate velocities µM min⁻¹ g⁻¹ soil and analyzed for the activation energies (PERₑₐ) according to the Arrhenius equation:

\[
k = A e^{-\frac{E_a}{RT}}
\]  
(Eq 2)

Where \( k \) is the rate constant (µM min⁻¹ g⁻¹ soil), \( A \) is the pre-exponential factor, \( E_a \) (J mol⁻¹) is the activation energy, \( R \) (8.134 Jk mol⁻¹) is the gas constant, and \( T \) is the temperature in Kelvin. Log transformation of the Arrhenius equation (Eq 2), expresses the equation as:

\[
\ln k = \left( -\frac{E_a}{R} \right) \left( \frac{1}{T} \right) + \ln A
\]  
(Eq 3)

The activation energy is the slope of the ln \( k \) (rate constant) plotted by \( 1/T \) (Parham and Deng, 2000). The \( Q_{10} \) of peroxidase was also analyzed (See Methods S3, Table S3).

Data analysis

Within each season the \( PER_{DE} \), and \( PER_{Ea} \) were analyzed by a mixed model analysis of variance with ecosystem, depth and their interaction as the fixed effects and replicate core within site as the random effect. This was followed by post hoc Tukey’s HSD test to compare between means. To compare the seasonal effects for each ecosystem independently, the fixed effects of season, soil depth and their interaction were tested in the fixed effects with the replicate core within site as the random effect, followed by post hoc Tukey’s HSD test. Within site variation was allowed to vary across sites to
accommodate observed heteroskedasticity. The total lignin concentrations was analyzed by a two-way ANOVA using the fixed effects of ecosystem, depth and their interaction, followed by *post hoc* Tukey’s HSD test. The \( \text{PER}_{DE} \) from the spring season was linearly regressed against the SCV/V, C/V and P/V ratios of lignin to discern relationships. The statistics were analyzed using SAS 9.2 (SAS Institute Inc., Cary, NC) and the linear regressions were generated with Sigma Plot 12.0.

### 4.3 Results

*Site Characteristics*

Excluding the agricultural site, the top soils (0-5 cm) in all sites exhibited the greatest C and N that ranged between 3.6-4.67 % and 0.14-0.16 %, respectively (Table 4-1). Due to tillage, the agricultural field site exhibited similar % C and %N across all soil depths. The soil pH remained constant across the seasons, however across the ecosystems, the soil pH was greatest in the agricultural soil (*ca.* 5.5), followed by the two forest soils (Table 4-1). The soil moisture in all sites varied with depth and seasons. In the deciduous and pine soils, the gravimetric moisture content was greatest in the spring and declined in the summer and fall. Meanwhile, in the agricultural soil, the soil moisture was greatest in the fall. In terms of total precipitation in the area, this region received the approximately 200 mm of rain in the spring months, greatest amount of precipitation in the summer, which declined in the fall (See Fig. S2).
Table 4-1. Soil Characteristics of ecosystems identified in the Clemson, South Carolina area.

<table>
<thead>
<tr>
<th>Site</th>
<th>% C</th>
<th>% N</th>
<th>pHe</th>
<th>% moistd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Spring</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Spring</td>
</tr>
<tr>
<td>Decid</td>
<td></td>
<td></td>
<td></td>
<td>Spring</td>
</tr>
<tr>
<td>0-5 cm</td>
<td>4.67 (0.90)</td>
<td>0.164 (0.03)</td>
<td>4.34</td>
<td>0.22</td>
</tr>
<tr>
<td>5-10 cm</td>
<td>1.95 (0.14)</td>
<td>0.073 (0.002)</td>
<td>4.95</td>
<td>0.25</td>
</tr>
<tr>
<td>10-15 cm</td>
<td>1.39 (0.16)</td>
<td>0.063 (0.003)</td>
<td>5.03</td>
<td>0.16</td>
</tr>
<tr>
<td>Pine</td>
<td></td>
<td></td>
<td></td>
<td>Spring</td>
</tr>
<tr>
<td>0-5 cm</td>
<td>3.63 (0.98)</td>
<td>0.142 (0.04)</td>
<td>4.72</td>
<td>0.21</td>
</tr>
<tr>
<td>5-10 cm</td>
<td>1.18 (0.20)</td>
<td>0.059 (0.01)</td>
<td>4.89</td>
<td>0.19</td>
</tr>
<tr>
<td>10-15 cm</td>
<td>1.06 (0.35)</td>
<td>0.055 (0.009)</td>
<td>5.22</td>
<td>0.17</td>
</tr>
<tr>
<td>Agric</td>
<td></td>
<td></td>
<td></td>
<td>Spring</td>
</tr>
<tr>
<td>0-5 cm</td>
<td>1.61 (0.04)</td>
<td>0.119 (0.004)</td>
<td>5.50</td>
<td>0.13</td>
</tr>
<tr>
<td>5-10 cm</td>
<td>1.58 (0.02)</td>
<td>0.114 (0.004)</td>
<td>5.58</td>
<td>0.14</td>
</tr>
<tr>
<td>10-15 cm</td>
<td>1.57 (0.01)</td>
<td>0.115 (0.001)</td>
<td>5.49</td>
<td>0.13</td>
</tr>
</tbody>
</table>

a The values represent the mean of C (n=3) with ±SE in the parentheses.
b The values represent the mean of N (n=3) with ±SE in the parentheses.
c Due to the little variation across the seasons, on the spring season soil pH is described.
d Due to the inherent variation in soil moisture, the mean (n=3) for each depth and season is reported.
Lignin profile

The total lignin concentrations of the soils was dependent upon an ecosystem x depth interaction (Fig. 4-3a; \( F_{4,4}=17.98; \ P<0.0001 \)). In the deciduous and pine site, the top 0-5 cm of soils in deciduous site had the highest concentration of lignin. Meanwhile, there was no change in the lignin concentration across the depths in the agricultural site. Across the ecosystems, in the top 0-5 cm the deciduous site exhibited the highest concentration of lignin followed by the pine site.

The degradation efficiency (\( PER_{DE} \)) of peroxidase in spring season was positively associated to the SCV/V ratio (Fig. 4-3b; \( R^2=0.79; \ P<0.0001 \)). In the summer and fall the relationship between \( PER_{DE} \) and SCV/V remained positive with an \( R^2=0.736 \) (\( P<0.0001 \); Fig S3e) and \( R^2=0.603 \) (\( P<0.0001 \); Fig. S3f). Similarly, the \( PER_{DE} \) was positively correlated with the C/V (Fig. 4-3c; \( R^2=0.7006; \ P<0.0001 \)). In the summer and fall, while the relationship between \( PER_{DE} \) and C/V remained positive, their association declined towards an \( R^2=0.60 \) (\( P<0.0001 \)) and \( R^2=0.51 \) (\( P<0.0001 \)) in each season respectively (Fig. S3a, S3b). Moreover, the relationship of the \( PER_{DE} \) with the C/V ratio exhibited a better trend then using the \( V_{max} \) or \( K_m \) (Fig. S4a, S4c). The \( PER_{DE} \) also exhibited a positive relationship with P/V ratio in spring (Fig. 4-3d; \( R^2=0.30; \ P=0.00018 \)), summer (Fig. S3c; \( R^2=0.39; \ P=0.0003 \)) and fall (Fig. S3c; \( R^2=0.27; \ P=0.003 \)). Similarly, the \( PER_{DE} \) exhibit better trend with P/V than the \( V_{max} \) or \( K_m \) constants (Fig. S4b, S4d).
**Figure 4-3.** The Total lignin concentrations of the soil (µg g⁻¹ soil C) across each ecosystem (a; the bars represent mean (n=3) ± SE). Linear regression of peroxidase degradation efficiency in the spring season against the SCV/V ratio (b), the C/V ratio (c), and the P/V ratio (d). The data was analyzed by a 2-way ANOVA using ecosystem, depth and ecosystem x depth as the effects followed by a post-hoc Tukey’s HSD test. Similar letters across the depths within each ecosystem (x,y,z), across the three ecosystems at 0-5 cm depth (A,B,C), at 5-10 cm depth (a,b,c) and at 10-15 cm depth (p,r,q) represent no significant difference (α=0.05).
Degradation Efficiencies (PER\textsubscript{DE}) of soil peroxidase

The \textit{PER\textsubscript{DE}} in the spring season across the ecosystems varied with depth (Table 4-2; \textit{F}_{4,12}=8.76; \textit{P}=0.0015). The \textit{PER\textsubscript{DE}} did not vary with depth in the agricultural soils (ca. 22.4; \textit{P}>0.05). Meanwhile, the \textit{PER\textsubscript{DE}} of the deciduous soil increased with depth where the \textit{PER\textsubscript{DE}} at 5-10 cm and 10-15 cm were 60\% (\textit{P}=0.0082) and 70\% (\textit{P}=0.0031) greater than the \textit{PER\textsubscript{DE}} at 0-5 cm. In contrast, in the pine soils the \textit{PER\textsubscript{DE}} decreased with depth. The \textit{PER\textsubscript{DE}} at 5-10 cm and 10-15 cm depths were 44\% (\textit{P}=0.0106) and 62\% (\textit{P}=0.0010) lower than the \textit{PER\textsubscript{DE}} at 0-5 cm, respectively. Comparing across the ecosystems at 0-5 cm depth, the agricultural soil exhibited the greatest \textit{PER\textsubscript{DE}} being 2.6- (\textit{P}<0.0001), and 5-fold (\textit{P}<0.0001) higher than that in the deciduous, and pine soils, respectively. At 5-10, and 10-15 cm depths, a similar trend persisted where the \textit{PER\textsubscript{DE}} in agricultural soil was approximately 1.5- and 8-fold higher than the deciduous, and pine soils (\textit{P}<0.0001), respectively.

In the summer, the \textit{PER\textsubscript{DE}} exhibited an ecosystem x depth interaction (Table 4-2; \textit{F}_{4,12}=9.73; \textit{P}=0.0010). In the deciduous soils, the \textit{PER\textsubscript{DE}} of the top soils (0-5 cm) were significantly lower than 5-10 cm (\textit{P}=0.0002) and 10-15 cm (\textit{P}<0.0001) depths. In the pine soils, the \textit{PER\textsubscript{DE}} at the 0-5 cm depth was 27\% higher than the 5-10 cm depth (\textit{P}=0.0361). The agricultural soil exhibited similar \textit{PER\textsubscript{DE}} across the three depths, however the \textit{PER\textsubscript{DE}} of this soils was 3-fold higher than in other ecosystems (\textit{P}<0.0001) across the three depths. Meanwhile, the \textit{PER\textsubscript{DE}} at 5-10 and 10-15 cm of the deciduous soils were both approximately 2-fold greater than in the pine soils (\textit{P}<0.05).
In the fall, the \( \text{PER}_{DE} \) varied with the ecosystems (Table 4-2; \( F_{3,8}=87.6; \ P<0.0001 \)). The \( \text{PER}_{DE} \) of the agricultural soil was 3.8-, and 4.5-fold greater than in the deciduous, and pine soils respectively (\( P<0.0001 \)).

Across the seasons, the \( \text{PER}_{DE} \) of the deciduous soils varied with depth (Table 4-2; \( F_{4,12}=3.31; \ P=0.047 \)). In the top soils, the \( \text{PER}_{DE} \) did not differ among the seasons. Meanwhile, at 5-10 cm the \( \text{PER}_{DE} \) in the fall season was significantly lower than the spring (\( P=0.0014 \)) and summer seasons (\( P=0.0334 \)). At 10-15 cm, the \( \text{PER}_{DE} \) of spring season was 52 % higher than in the fall (\( P=0.0026 \)). The \( \text{PER}_{DE} \) of pine soils varied across seasons (\( F_{2,12}=7.91; \ P=0.0064 \)). The \( \text{PER}_{DE} \) in the spring was 43 % and 53 % lower than in the summer (\( P=0.0191 \)) and the fall (\( P=0.0022 \)), respectively. Similarly, the \( \text{PER}_{DE} \) of the agricultural soil exhibited a main effect of season (\( F_{2,12}=36.3; \ P<0.0001 \)). In the agricultural site, the \( \text{PER}_{DE} \) in spring was lower than the summer (\( P<0.0001 \)) and fall (\( P<0.0001 \)). Similarly, the \( \text{PER}_{DE} \) in summer was significantly lower than the fall season (\( P=0.001 \)).
Table 4-2. The degradation efficiency ($PER_{DE}$) of soil peroxidase across the ecosystems, soil depths and seasons. Values represent means (n=3) of the slope ± SE in the parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Spring</th>
<th>Summer</th>
<th>Fall</th>
<th>REML&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5 cm</td>
<td>8.7 (0.8)</td>
<td>8.5 (0.6)</td>
<td>9.3 (1.6)</td>
<td>D</td>
</tr>
<tr>
<td>5-10 cm</td>
<td>13.9 (1.5)</td>
<td>11.6 (1.3)</td>
<td>8.3 (0.8)</td>
<td></td>
</tr>
<tr>
<td>10-15 cm</td>
<td>14.8 (1.5)</td>
<td>11.9 (1.1)</td>
<td>9.7 (1.2)</td>
<td></td>
</tr>
<tr>
<td>Pine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5 cm</td>
<td>5.5 (1.5)</td>
<td>7.4 (1.2)</td>
<td>10.3 (0.9)</td>
<td>S</td>
</tr>
<tr>
<td>5-10 cm</td>
<td>3.1 (0.6)</td>
<td>5.8 (0.4)</td>
<td>7.3 (1.5)</td>
<td></td>
</tr>
<tr>
<td>10-15 cm</td>
<td>2.1 (0.4)</td>
<td>6.1 (1.0)</td>
<td>5.5 (2.9)</td>
<td></td>
</tr>
<tr>
<td>Agric</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5 cm</td>
<td>22.3 (1.2)</td>
<td>29.5 (1.8)</td>
<td>40.0 (2.4)</td>
<td>S</td>
</tr>
<tr>
<td>5-10 cm</td>
<td>22.7 (1.3)</td>
<td>28.6 (0.7)</td>
<td>33.4 (3.7)</td>
<td></td>
</tr>
<tr>
<td>10-15 cm</td>
<td>22.4 (1.3)</td>
<td>28.5 (2.0)</td>
<td>32.6 (1.3)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>REML represent the restricted maximum likelihood analysis of the degradation efficiency term for each season independently. Significant differences among the means is not reported in the table.

<sup>b</sup>REML analysis of the degradation efficiency term for each ecosystem independently. (S) represents the main effect of season, (D) represents the main effect depth and (S x D) represents season x depth interaction. Significant differences among the means is not reported in the table.
Activation Energy (PER<sub>Ea</sub>) of soil peroxidase

The PER<sub>Ea</sub> in the spring exhibited an ecosystem x depth interaction (Table 4-3; F<sub>4,12</sub>=4.03; P=0.0269). In the deciduous soils, the PER<sub>Ea</sub> in the 5-10 cm depth was 14.8 % and 7.7 % higher than the PER<sub>Ea</sub> of the 0-5 cm (P<0.0001) and 10-15 cm depths (P=0.0031) respectively. The pine site exhibited a similar trend, the PER<sub>Ea</sub> of the 5-10 cm depth was 25 % and 42 % greater than the 0-5 cm (P=0.0077) and 10-15 cm depths (P=0.0005). Meanwhile, in the agricultural site, the PER<sub>Ea</sub> of the 0-5 cm depth was 20 % and 13 % lower than the 5-10 cm (P=0.0016) and 10-15 cm depths (P=0.0369) respectively. Across the sites, the PER<sub>Ea</sub> of the pine site at the 0-5 cm depth was greater than the agricultural (P=0.0291) site. Similarly, the PER<sub>Ea</sub> of the pine soil in the 5-10 cm depth was significantly greater than the agricultural soil (P=0.0115) as well as the deciduous soil (P=0.0014).

Similarly, the PER<sub>Ea</sub> in the summer varied by depth across the ecosystems (F<sub>4,12</sub>=8.20; P=0.0020). In the deciduous soil, the PER<sub>Ea</sub> in the 0-5 cm depth was 10 % greater than the PER<sub>Ea</sub> of the 5-10 cm (P=0.0043) and 10-15 cm (P=0.0064) depths. Meanwhile, in the pine soil the PER<sub>Ea</sub> at 5-10 cm was 15% lower than the 0-5 cm and the 10-15 cm depths (P<0.0001). At 0-5 cm depth, the PER<sub>Ea</sub> of the pine soil was significantly greater than the other sites (P<0.05). Meanwhile, at 5-10 cm, the PER<sub>Ea</sub> of the deciduous soil was significantly lower than the pine and agricultural soil (P<0.05). This trend persisted in the 10-15 cm depth, and the PER<sub>Ea</sub> of the pine soil was significantly greater than the
agricultural soil ($P<0.05$). In contrast to the past seasons, the $PER_{Ea}$ of the fall season exhibited no statistical differences.

Across the seasons, the $PER_{Ea}$ of the pine soil exhibited an season x depth interaction ($F_{4,12}=5.89; P=0.0074$). At 0-5 cm, the $PER_{Ea}$ in summer was 24% and 20% higher than that of the spring ($P=0.0209$) and fall ($P=0.0424$), respectively. Meanwhile, in the 5-10 cm, the spring $PER_{Ea}$ was 16% greater than the summer ($P=0.0499$) and 27% greater than the fall ($P=0.0132$). In contrast, at 10-15 cm the spring $PER_{Ea}$ was 29% lower than the summer ($P=0.002$) and 18% lower than in the fall ($P=0.0499$). Similarly, the $PER_{Ea}$ of the agricultural soil exhibited a season x depth interaction ($F_{4,12}=6.88; P=0.0041$). The $PER_{Ea}$ of the summer top soil was 30% and 10% greater than the spring ($P<0.0001$) and fall ($P=0.022$) soils respectively. Similarly, the $PER_{Ea}$ in the fall was 16% greater than in the spring ($P=0.0045$).
Table 4-3. The spatiotemporal changes of the Activation Energy of soil peroxidase ($PER_{Ea}$) across the three ecosystems, soil depths, and seasons. Values represent means (n=3) of the $PER_{Ea}$ ± SE in the parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Spring $PER_{Ea}$ (kJ mol$^{-1}$)</th>
<th>Summer $PER_{Ea}$ (kJ mol$^{-1}$)</th>
<th>Fall $PER_{Ea}$ (kJ mol$^{-1}$)</th>
<th>REML$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5 cm</td>
<td>25.66 (0.865)</td>
<td>27.35 (0.198)</td>
<td>24.23 (3.989)</td>
<td>NA$^c$</td>
</tr>
<tr>
<td>5-10 cm</td>
<td>29.49 (0.446)</td>
<td>24.64 (1.175)</td>
<td>26.66 (1.862)</td>
<td></td>
</tr>
<tr>
<td>10-15 cm</td>
<td>27.38 (0.490)</td>
<td>24.80 (0.292)</td>
<td>27.23 (1.356)</td>
<td></td>
</tr>
<tr>
<td>Pine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5 cm</td>
<td>29.47 (0.602)</td>
<td>36.54 (1.850)</td>
<td>30.50 (0.291)</td>
<td>S, S x D</td>
</tr>
<tr>
<td>5-10 cm</td>
<td>36.93 (2.346)</td>
<td>31.13 (2.482)</td>
<td>29.20 (1.396)</td>
<td></td>
</tr>
<tr>
<td>10-15 cm</td>
<td>25.98 (1.852)</td>
<td>36.44 (2.058)</td>
<td>31.78 (2.765)</td>
<td></td>
</tr>
<tr>
<td>Agric</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5 cm</td>
<td>24.53 (1.721)</td>
<td>31.76 (0.407)</td>
<td>28.65 (0.664)</td>
<td>S, S x D</td>
</tr>
<tr>
<td>5-10 cm</td>
<td>30.77 (0.564)</td>
<td>29.54 (1.454)</td>
<td>29.20 (0.170)</td>
<td></td>
</tr>
<tr>
<td>10-15 cm</td>
<td>28.15 (0.351)</td>
<td>30.23 (0.341)</td>
<td>29.86 (0.167)</td>
<td></td>
</tr>
<tr>
<td>REML$^a$</td>
<td>E, D, E x D</td>
<td>E, D, E x D</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

$^a$For each season, a REML statistical analysis was conducted using the main effects of ecosystem (E), depth (D) and ecosystem x depth (E x D). Significant differences among the means is not reported in the table.

$^b$For each individual ecosystem, a REML statistical analysis was conducted using the main effects of season (S), depth (D) and season x depth (S x D). Significant differences among the means is not reported in the table.

$^c$NA indicates that there is no significant main effect or interaction.
4.4 Discussion

To date, only few studies have attempted to elucidate the kinetics of oxidoreductases, and to our knowledge, none of the studies have characterized the peroxidase kinetics in soils. Given that peroxidases facilitate the degradation of complex biopolymers including lignin, the characterization of soil peroxidase kinetics could help in predicting the rate of carbon and nitrogen cycling in ecosystems. Generally, the $V_{\text{max}}$ or $K_m$ are used as independent variables for defining the enzyme activity (Saiya-Cork et al., 2002; Freeman et al., 2004; Stone et al., 2011). Although the $V_{\text{max}}$ was high in lignin-rich Pinus soils, the associated increase in $K_m$ resulted in a lower $\text{PER}_{DE}$ in these soils, whereas the lignin-poor agricultural soils that had the lowest $V_{\text{max}}$ and $K_m$ exhibited the highest $\text{PER}_{DE}$. Thus, an interactive parameter of $V_{\text{max}}$ and $K_m$ better described the total efficiency of the soil enzymes in our study sites. Overall, we observed that the catalytic efficiency of peroxidase across the depths and seasons was ecosystem specific, and the degradation efficiency of peroxidase was higher in soils with low lignin content. The results also indicate that apart from soil phenolic content, a myriad of soil biotic and abiotic parameters might contribute to the complexity of peroxidase kinetics in ecosystems.

4.4.1. Response of Michaelis-Menten Kinetic constants of soil peroxidase and $\text{PER}_{DE}$ of soil across ecosystems.

We found that the relationship between $V_{\text{max}}$ and $K_m$ varied with ecosystems (Table S4). For example, the agricultural soils exhibited the lowest $V_{\text{max}}$ and $K_m$, which resulted in the highest $\text{PER}_{DE}$. Meanwhile, across the three ecosystems the $V_{\text{max}}$ was
greater in *Pinus* and deciduous sites, demonstrating higher potential of peroxidase activities in these soils. However, these sites also had a high $K_m$ resulting in an overall lower $PER_{DE}$. The degree of peroxidase activity would vary with the quantity and chemical identity of lignin monomers, and hence with the vegetation history of a site. For example, *Pinus* litter is composed predominantly of vanillyl units that form C-C linkages and which are difficult to enzymatically breakdown (Talbot et al., 2010). Hence, we expected the peroxidases adapted to the *Pinus* ecosystem to exhibit a lower $K_m$ and higher $V_{max}$, thus a higher $PER_{DE}$. In addition to the vanillyl monomers, the deciduous trees and grasses also carry syringyl and cinnamyl monomers that form more labile linkages (C-O-C) which are less refractory (Talbot et al., 2010). Therefore, peroxidases from these ecosystems could exhibit a lower $PER_{DE}$. However, the observed positive relationship of the $PER_{DE}$ to the SCV/V, C/V, and P/V ratios (Fig. 4-3) suggests that the increase in relative abundance of vanillyls was accompanied by a corresponding decrease in $PER_{DE}$. This lower $PER_{DE}$ in the *Pinus* soils was contributed by higher $K_m$ values indicating that peroxidases from these soils have a less affinity for substrates. The higher $K_m$ could partly be due to a greater presence of inhibitors such as tannins that could act both as redox buffer, and block the catalytic sites of enzymes (Triebwasser et al., 2012). Excess of simple phenolic compounds in soil could also outcompete TMB for the active site of peroxidases. This inhibitory effect of phenolic compounds, especially those arising due to substrate competition, could partly be overcome at higher concentration of TMB, and thus could result in the higher $V_{max}$ observed in the *Pinus* soils.
The agricultural soils which had the lowest lignin concentration exhibited the highest \( PER_{DE} \), however the degradation efficiency did not follow the lignin concentration in soils from \( Pinus \) and deciduous forests. Substrate availability serves as metabolic cues that elicit microbial production of soil enzymes (German et al., 2011) which in turn could affect the \( PER_{DE} \). For example, nitrogen deposition in forest ecosystems have shown to reduce oxidative enzyme activities (Waldrop et al., 2004; Waldrop and Zak, 2006; Grandy et al., 2008), whereas nutrient deficiency and resource heterogeneity could induce peroxidase activity. Alternatively, the addition of nitrogen to agricultural soils has been shown not to alter the activity of peroxidases (Henry et al., 2005; Stursova et al., 2006; Zeglin et al., 2007). This suggests that the \( PER_{DE} \) could facilitate resource mining in C and N limited environments (Sinsabaugh, 2010; Allison et al., 2011) such as forested soils, but may be less relevant in resource rich agricultural soils. The ecosystem specific function of peroxidases to microbial C and N demands is further corroborated by lack of a relationship of the \( PER_{DE} \) with labile C, described as the permanganate oxidizable C (Fig. S5a), or with total soil N (Fig. S5b). Collectively, this suggests that peroxidase production could be elicited and sustained by multiple factors that are ecosystem specific such that peroxidase could facilitate multiple ecological roles.

The ecosystem specific responses of the \( PER_{DE} \) could also be the consequence of several biotic and abiotic factors. The pH and moisture have also been reported to mediate oxidoreductase activity in soil (Sinsabaugh et al., 2008; Sinsabaugh, 2010; Fuji et al, 2013). However, in this investigation while the pH and moisture varied across the ecosystems and depths (Table 4-1), they were not associated to the kinetic constants (data
not shown), suggesting that these abiotic factors were not the primary driver of the observed kinetics in our study system.

4.4.2. Spatiotemporal dynamics of the peroxidase temperature kinetics

The activation energy ($PER_{Ea}$) and temperature sensitivity ($Q_{10}$) are important parameters that determine the stability of enzyme and enzyme-substrate complex (Farrell et al., 1994; Trasar-Cepeda et al., 2007; Wallenstein et al., 2011). The apparent slow-turnover of lignin is thought to be a consequence of its thermal stability that chemically hampers its decay (Bosatta and Ågren, 1998). Consequently, under warming due to the availability of excess energy, substrates with high activation energies, such as lignin, are anticipated to exhibit greater thermal sensitivity to degradation. Also, the reaction kinetics of soil enzymes could be accelerated under elevated temperatures (Steinweg et al 2013; Davidson et al 2012), resulting in an overall accelerated degradation.

Due to the structural complexity of lignin, we anticipated that the activation energy of peroxidases would be similar to phenol oxidases and greater than for hydrolases. Moreover, due to the refractory nature of vanillyl units, we expected that the peroxidases from the Pinus soils to exhibit higher activation energy compared to other two ecosystems that are low in vanillyl lignins. In our study, the $PER_{Ea}$ (Table 4-3) and $Q_{10}$ values (Table S3) were within the ranges reported from other ecosystems (Trasar-Cepeda et al., 2007; Steinweg et al., 2013). However, contrary to our predictions the $PER_{Ea}$ ($ca. 36$ kJ mol$^{-1}$) and $Q_{10}$ ($Q_{10} < 2$) of peroxidase exhibited lower temperature sensitivity than reported for soil phenol oxidases and hydrolases (Steinweg et al., 2013).
Generally, the activation energy of an enzyme indicates the reliance of the enzyme kinetics on an external energy supply for substrate conversion. Therefore, the low activation energies of peroxidases could suggest that peroxidases are less reliant upon external energy for its catalytic activity, resulting in this enzyme being more efficient than phenol oxidase and hydrolases. Seasonal variation in soil enzyme isomers could also affect the activation energy and substrate affinity of enzymes. Hence, the observed seasonal variation of \( PER_{Ea} \) could be due to the seasonal variation in the \( K_m \) of the enzyme isomers (Di Nardo et al., 2004; Davidson et al., 2012).

Similarly, the \( PER_{Ea} \) did not exhibit substantial variation across the ecosystems, which could implicate that \( PER_{Ea} \) is less-driven by lignin monomer composition or the availability of lignins in the soil. The limited variation observed in the \( PER_{Ea} \) across the ecosystems of differing plant litter chemistry could be due to the similarity in the activation energy of lignins between gymnosperms and angiosperms (Santos et al., 2011), hence an adaptation of the \( PER_{Ea} \) to lignin chemistry would be less likely. Collectively, the low \( PER_{Ea} \) and \( Q_{10} \) suggest that peroxidase activity would not be sensitive to warming in our study systems, and hence, would not be a major factor contributing to the accelerated rate of lignin degradation under rising temperatures.

Alternatively, the observed responses of \( PER_{Ea} \) could be specific to the substrate, TMB, used to quantify \( PER_{Ea} \). Although the TMB substrate can capture potential peroxidase activity, the structural simplicity of TMB in comparison to lignins could result in lower activation energy of the enzyme-TMB complex. In turn, this could affect the
apparent activation energy of peroxidases in our study system. Experiments using L-DOPA as a substrate also reflected a lower $PER_{Ea}$ values (Steinweg et al., 2013). However, lignin degradation is demonstrated to increase with increase in temperature (Feng et al., 2008; Conant et al., 2008). Combined, this suggests that the temperature sensitivity of the degradation kinetics of soil peroxidases could be primarily driven by the activation energies of the substrates than that of the enzyme.

4.5 Conclusion

The Michaelis-Menten kinetics and Activation energy of soil enzymes are considered rate-limiting steps in the soil decomposition process and assist in predicting soil respiration (Davidson et al., 2012). To date, many investigations have elucidated the trends of hydrolase kinetics and the mechanisms moderating those responses. However, this is the first study to identify and characterize kinetics of soil peroxidases. Additionally, we determined that the $PER_{DE}$, described as the integrative term of $V_{max}$ and $K_m$, best associated to the vanillyls of lignins in the soils rather than the N availability or substrate quantity alone. Collectively, this study highlights the importance of how the lignin chemistry may be moderating soil peroxidase kinetics, in addition to the myriad of factors reported to affect peroxidase activity. Moreover, due to the significant differences in the efficiency of peroxidases across the ecosystems, this study suggests that ecosystems of differing plant litter chemistries could exhibit unique mean residence times of lignin in the soil. Because the kinetics of soil oxidoreductases has been relatively unexplored, the implementation of the Michaelis-Menten kinetics technique into future
empirical investigations would enable to better grasp the factors regulating oxidoreductases and its relationship to soil organic matter chemistry.
CHAPTER 5

CONCLUDING REMARKS AND FUTURE INVESTIGATIONS

Through this research we were able to glimpse into the external life of phenolics in the soil. Unfortunately, for many, and as is my circumstance, we leave our programs with as many questions as we began with. While I have non-regrettably spent countless hours reading, and practicing on analytical techniques and theory in conjunction with ecological theory, what I strived to accomplished was to identify chemical mechanisms that could described ecological phenomena. All the while I focused on questions that were not only interesting but would be novel to the field and relevant. However, looking back after the past 2 years of work, while I am content that this work has been accomplished there is still an over-shadowing sensation that the work could have been better and that we face new questions to consider. Because of the objective of a researcher is to not only address current questions but also to reflect on new directions to pursue this chapter will focus on summarizing what we have learned and expanding on new paths to follow.

5.1 Chapter 2

In Chapter 2 we aimed at elucidating how the tannin chemistry could affect soil enzyme activity. In this investigation we expanded on this idea further by considering that the protein chemistry, governed by the soil isoenzyme chemistry, could also concomitantly affect the tannin-enzyme interaction, collectively resulting in enzyme class and ecosystem specific responses. Most importantly, our work highlighted the ecological relevance of hydrolysable tannins in soil decomposition studies. In addition, we discerned
that tannins could also interfere with oxidoreductase activity through the antioxidant potential exhibited by the tannins. Therefore, what questions from this study remain to be addressed?

In this study, we focused on the immediate result of the tannin-enzyme interactions focusing on tannin concentrations that were ecologically relevant. There were a number of limitations and interesting questions to consider from this study. Firstly, we measured enzyme inhibition of $V_{\text{max}}$. However, from Chapter 4 we learned that $K_m$ and the $E_a$ are also pertinent to the degradation efficiency of enzymes. Therefore, the next step could be to test the inhibition effects of tannins on soil enzyme $K_m$ and $E_a$. This would involve testing enzyme inhibition at multiple enzyme concentration, tannin concentrations, and temperatures. Because tannins are proposed to inhibit enzyme by non-competitive means, a particular enzyme $K_m$ may not be significantly impacted. However, from a soil perspective where multiple isoenzymes are present and exhibit modified affinities towards tannins could result in the generation of a modified soil $K_m$. Moreover, the tannin-protein complex could further stabilize soil enzymes resulting in changes in the thermal sensitivity of the enzyme.

Meanwhile, re-emphasizing that we investigated the immediate consequences of tannins in the soil, it would be interesting to monitor the recovery time of enzyme activity upon the introduction of tannins to the soil. For example, we discerned that hydrolysable tannins are more effective inhibitors of soil enzymes. However, would this effect last longer than condensed tannins? Hydrolysable tannins have been reported to be broken
down faster than condensed tannins, therefore, in the long run condensed tannins could more effective inhibitors of soil enzyme activity. Another question that stems from this would be to re-asses ecosystem specific responses of additional factors in the decomposition process linked to enzyme activity, in other words carbon and nitrogen mineralization. Enzymes are tied to the depolymerization and/or transformation of biomolecules in the soil. Therefore, upon the introduction of tannins to the soil, understanding ecosystem specific responses in soil carbon and nitrogen mineralization would benefit our understanding of tannins in the soil.

Upon their introduction to the soil they are promptly lost and thus far are not recoverable. One reason that could be leading to the limited recoverability of tannins in the soil is their rapid transformation into modified metabolites that are not identical to the parent molecules. Using tannins that are isolated, of individual mass, and the molecular structure has been extensively characterized it would be interesting to understand the enzymatic transformations of tannins in aqueous conditions that exclusively contain the oxidoreductase and the tannin. Moreover, if these studies could be expanded further to concentrate oxidoreductases of natural fungal variants in the soil, this could assist in understand the transformations of hydrolysable and condensed tannins in the soil.

Lastly, tannins are proposed to hamper the decomposition of the leaf litter. However, few studies have investigated the impacts of tannins in litter decomposition or if there is a significant decrease in decomposition due to the introduction of tannins at ecological relevant concentrations. Further, few studies have monitored the molecular
transformations during the decomposition process, in particular hydrolysable tannins. Understanding how long tannins persist, and where they go would benefit our ability to gain insight in the ecological relevance of tannins in the soil organic matter and leaf litter decomposition process. Above all, due to the ecological relevant of hydrolysable tannins in enzyme deactivation, it is essential that we expand our understanding of hydrolysable tannins in the soil. Limited investigations have considered the impacts, turnover and overall legacy of hydrolysable tannins in the soil decomposition process.

In summary:

- How are soil enzyme kinetics, $K_m$ and $E_a$, impacted?
- What is the lifespan of tannins in the soil? How promptly does enzyme activity recover?
- What are ecosystem specific responses in carbon and nitrogen mineralization?
- What are the enzyme-mediated transformations of hydrolysable and condensed tannins?
- How long do tannins persist in the leaf litter and what molecular transformations do they undergo?
- Ecological relevance of hydrolysable tannins?

5.2 Chapter 3

From an experiment conducted in Chapter 2 we discerned that the HTs and CTs compete for amino acids sites along the protein and that this appears to be a species
specific response. In Chapter 3 we pursued this idea further by fractionating mixed tannins (tannins containing both hydrolysable and condensed tannins) according to their polarity. We were interested in determining i) are the hydrolysable and condensed tannins structural independent species ii) what molecular structure can predict enzyme inhibition of hydrolases and oxidoreductases iii) by what mechanism do mixed tannins inhibition enzymes- competitive HT vs. CT binding to the protein amino acid sites or restricting conformational movement of the protein.

We did find additional evidence suggesting that hydrolysable and condensed tannins are structurally independent molecular species the evidence was not conclusive. Further analysis of the fractionated tannins by mass spectrometry, ESI-MS/MS and MALDI-TOF, would assist elucidating if HTs and CTs are covalently linked or independent. Additionally describe the average molecular masses within each fractionated class. Secondly, we were able to identify that the gallotannins of hydrolysable tannins are linked to the catalytic deactivation of hydrolases. However, further characterization of the redox potential of tannins and its association in preventing oxidoreductase activity was not accomplished. There were a number of analytical limitations, primarily the tannins were not soluble in 100% organic solvent preventing the use of the antioxidant potential assay designed in chapter 2. Optimizing cyclic voltammetry would aid in identifying how the redox potential is associated to the tannin chemistry and oxidoreductase activity.
Lastly, to address how tannins inhibit the catalytic activity of hydrolases we were interested in conducting isothermal titration calorimetry to investigate the tannin-protein interaction further. By monitoring the heat release or heat absorption when two metabolites interact we can understand the binding constant of tannin towards the protein (i.e. number of tannin molecules bind per unit protein), and the Gibb’s free energy of the interaction. The Gibb’s Free energy is an interesting constant because it describes the spontaneity of the reaction. Hence, this constant describes the affinity of tannins towards the protein. Therefore, though this technology, with the mixed tannins, we would be able to begin to elucidate how tannins inhibit enzyme activity. Is it the high binding constants of tannins towards to the protein? Or do hydrolysable tannins have higher affinity or is binding to the protein hydrolysable and condensed tannin concentration specific? However for accurate assessment of the tannin binding constants to proteins and the Gibb’s free energy, we would require the average molecular masses of each fraction. In summary:

- Asses the redox potential of tannins, finding a link between the redox potential and hydrolysable tannin chemistry
- Optimizing mass spectrometry of mixed tannins, identify the molecular masses of hydrolysable tannins
- Identifying the mechanism by which mixed tannins, namely the hydrolysable tannins, inhibition hydrolase activity.
5.3 Chapter 4

In chapter 4, we took a step back from tannins and focused on how lignin and the lignin chemistry could indirectly manipulate the enzyme kinetic and activation energy of soil peroxidase. Most importantly, this was the first investigation that identified the presence of Michaelis-Menten kinetics in a soil oxidoreductase. Moreover, we learned that the degradation efficiency of peroxidase was associated to dynamics of lignin chemistry including the p-hydroxy : vanillyl ratio (P/V) and cinnamyl : vanillyl ratio (C/V), showing how the peroxidase degradation efficiency can be driven by several factors. However, this was an observational study that did not empirically demonstrate the relationship of peroxidase kinetics to lignin and polyphenol chemistry. There are several factors that could affect the output of oxidoreductases in the soil. The investigation conducted in Chapter 4 is a stepping stone towards understanding how oxidoreductase kinetics mediate the soil decomposition process.

The production of oxidoreductases by soil microbes is known to be elicited by nutrient limitations experienced by the microbial community. Therefore, a field investigation that could be conducted would be to characterize how soil nitrogen and carbon additions affect the kinetics of soil peroxidase across multiple ecosystems. Similarly, Talbot et al., 2011 generated three mutant lines of *arabidopsis* that exhibit differences in the quantity of the three monolignols, vanillyl, syringyl, and cinnamyl. Placing the litter of these three mutant lines across ecosystems of differing litter chemistries and/or nitrogen treatments, it would be interesting to discern if there is a
relationship between the lignin chemistry and the elicited peroxidase kinetics. A myriad of studies could be conducted to understand the biotic and abiotic mechanism moderate peroxidase kinetics and how this enzymatic process is pertinent to soil decomposition.

- What mechanisms moderate peroxidase kinetics- lignin chemistry, microbial nutrient demands (carbon and nitrogen), microbial community composition?
- How are enzyme kinetics affects in global change events- climate change, N deposition?
- Is oxidoreductase activity and their kinetics relevant to understanding the transformation of phenolics in the soil?
- Does oxidoreductase activity mediate the mineralization and respiration of phenolic derived CO₂?

5.4 Conclusion

Collectively, there have been both failures and successes during the development of this master’s research. Nevertheless, there are many questions of soil decomposition to address. Such research is essential for implementation into agroecosystems and well as predicting future environmental conditions.
SUPPLEMENTARY INFORMATION

Methods S1 $^{13}C$ Nuclear Magnetic Resonance

Molecular characterization of the CTs was conducted by solution state $^{13}C$ NMR as reported by Norris et al. (2011). Briefly, the CTs were analyzed at 75.47 Hz on a Bruker MSL 300 spectrometer (Bruker Instruments Inc., Karlsruhe, Germany). Each sample was prepared by dissolving 150 mg of tannin in 3 ml 1:1 deuterated water and acetone. The acquisition parameters included inverse gate decoupling, 45° pulse width, 0.5 sec FID acquisition time, 3.6 s relaxation delay. The FID was processed with baseline correction, and line broadening of 8 Hz. The chemical shifts are reported using tetramethylsilane and acetone as the primary and secondary standards respectively. The Acer and Quercus tannins were analyzed using 250 mg tannin prepared in 2 ml 70% deuterated acetone on a Bruker 300 MHz with similar acquisition and processing parameters.

The proanthocyanidin content of each tannin was determined by the NMR spectral interpretation as per Kraus et al. (2003a). In short, the proanthocyanidin content (% PC) relative to prodelfidins was calculated based on the ratio of peaks 116 to 107. The chain lengths were determined using the peak ratios of 65-69 ppm to 69-75 ppm.
Table S1. Nuclear Magnetic Resonance Characterization and Quenching Capacity of Tannins.

<table>
<thead>
<tr>
<th>Tannin-Type</th>
<th>Tannin</th>
<th>NMR Spectral Interpretation</th>
<th>Chain Length</th>
<th>PC (%)</th>
<th>cis (%)</th>
<th>HT (%)</th>
<th>Que(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>Abies</td>
<td></td>
<td>4.5</td>
<td>21</td>
<td>72</td>
<td>n.a.</td>
<td>70</td>
</tr>
<tr>
<td>CT</td>
<td>Picea</td>
<td></td>
<td>5.4</td>
<td>86</td>
<td>76</td>
<td>n.a.</td>
<td>48</td>
</tr>
<tr>
<td>CT</td>
<td>Vaccinium</td>
<td></td>
<td>3.7</td>
<td>94</td>
<td>94</td>
<td>n.a.</td>
<td>45</td>
</tr>
<tr>
<td>CT</td>
<td>Pinus</td>
<td></td>
<td>5</td>
<td>23</td>
<td>71</td>
<td>n.a.</td>
<td>63</td>
</tr>
<tr>
<td>CT</td>
<td>Thuja</td>
<td></td>
<td>3.8</td>
<td>27</td>
<td>38</td>
<td>n.a.</td>
<td>54</td>
</tr>
<tr>
<td>MT</td>
<td>Acer</td>
<td></td>
<td>2.5</td>
<td>85</td>
<td>n.a.a</td>
<td>67</td>
<td>89</td>
</tr>
<tr>
<td>MT</td>
<td>Betula</td>
<td></td>
<td>n.a.</td>
<td></td>
<td></td>
<td></td>
<td>73</td>
</tr>
<tr>
<td>MT</td>
<td>Quercus</td>
<td></td>
<td>n.a.</td>
<td></td>
<td></td>
<td></td>
<td>81</td>
</tr>
</tbody>
</table>

a n.a. no determination due to interference by hydrolysable tannins.
b n.a. HT structure interferes with cis analysis, therefore are not characterized.
c Percent Quenching of DPPH• radical.
Methods S2 Intrapolymer Competition of tannins for proteins

The purpose of this experiment was to determine whether the HTs and CTs within a mixed tannin differed in their affinity for protein binding. Using tannins from *Acer* and *Quercus*, we optimized the ratio of tannin to protein to form a tannin-protein precipitate that could be easily separated from the supernatant but contained limited protein (sorption sites) so as to induce competition between tannins units (HT vs. CT) if present. Briefly, 10 mg of tannin was dissolved in 1.2 ml of 15 % methanol to which 2 mg of bovine serum albumin (BSA) was added. The mixture was shaken on the orbital shaker for 2 h and the tannin protein complexes were isolated by centrifugation (14000 rpm for 10 min). A sub-sample of the supernatant was analyzed for CT and HT concentrations using the acid-butanol assay and acid-methanolysis, respectively.
Fig. S1. The intrapolymer tannin (HT versus CT) competition for protein binding sites of the mixed tannin species- *Acer* and *Quercus*. The bars represent the mean methyl gallate:procyanidin ratio of three replicates before and remaining in solution after complexation with BSA (±SE). The Student t-test for each species was performed for each species. Significance is indicated with an asterisk, $P \leq 0.05$ was considered significant.
**Table S2.** Compound identification for lignin derived phenolics and their corresponding $\lambda_{\text{max}}$ used for analysis by high pressure liquid chromatography-DAD

<table>
<thead>
<tr>
<th>Compound</th>
<th>Type$^a$</th>
<th>$\lambda_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-hydroxybenzaldehyde</td>
<td>P$^b$</td>
<td>284</td>
</tr>
<tr>
<td>p-hydroxyacetophenone</td>
<td>P</td>
<td>275</td>
</tr>
<tr>
<td>p-hydroxybenzoic acid</td>
<td>P</td>
<td>255</td>
</tr>
<tr>
<td>3,5-dihydroxy-benzoic acid</td>
<td>P</td>
<td>307</td>
</tr>
<tr>
<td>Vanillin</td>
<td>V</td>
<td>309</td>
</tr>
<tr>
<td>Acetovanillone</td>
<td>V</td>
<td>304</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>V</td>
<td>260</td>
</tr>
<tr>
<td>Syringaldehyde</td>
<td>S</td>
<td>308</td>
</tr>
<tr>
<td>Acetosyringone</td>
<td>S</td>
<td>300</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>S</td>
<td>274</td>
</tr>
<tr>
<td>p-coumaric acid</td>
<td>C</td>
<td>309</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>C</td>
<td>323</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>IS$^c$</td>
<td>310</td>
</tr>
<tr>
<td>Ethyl-vanillin</td>
<td>IS</td>
<td>276</td>
</tr>
</tbody>
</table>

$^a$Type refers to monolignol class, Vanillin (V), Syringyl (S), and Cinnamyl (C)

$^b$The Type (P) refers to phenolics

$^c$IS refers to Internal Standard, these compounds are not lignin derived phenolics
Methods S3: $Q_{10}$ Calculation
To assess the temperature sensitivity further by $Q_{10}$, the data was also fitted an exponential function (Sigmaplot 12.0, Cary, NC):

$$k = Ae^{bT}$$  \hspace{1cm} (Eq 1)

where $k$ is the rate constant (uM min$^{-1}$ g$^{-1}$ soil), $A$ is a constant, $T$ is temperature and $b$ is the temperature sensitivity. The $Q_{10}$ was calculated as (Sigma Plot 12.0, Location):

$$Q_{10} = e^{10b}$$  \hspace{1cm} (Eq 2)

Table S3. $Q_{10}$ Analysis of soil peroxidase. To compare $Q_{10}$ across sites, 2-way REML was conducted where the fixed effects of ecosystem, depth and the interaction of ecosystem x depth were considered, followed by post-hoc Tukey’s HSD test. To compare changes of Q10 in an individual ecosystem across the seasons, a 2-way REML was conducted where the fixed effects were season, depth and the interaction of season x depth, followed by post-hoc Tukey’s HSD test. Ecosystem (E), Depth (D), Season (S) Ecosystem x Depth (E x D), Season x Depth (S x D).

<table>
<thead>
<tr>
<th>Ecosystem</th>
<th>Spring $Q_{10}$</th>
<th>Summer $Q_{10}$</th>
<th>Fall $Q_{10}$</th>
<th>REML</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deciduous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5cm</td>
<td>1.40</td>
<td>1.44</td>
<td>1.47</td>
<td>S</td>
</tr>
<tr>
<td>5-10 cm</td>
<td>1.43</td>
<td>1.39</td>
<td>1.48</td>
<td></td>
</tr>
<tr>
<td>10-15cm</td>
<td>1.43</td>
<td>1.38</td>
<td>1.45</td>
<td></td>
</tr>
<tr>
<td>Pine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5 cm</td>
<td>1.48</td>
<td>1.58</td>
<td>1.48</td>
<td>S x D</td>
</tr>
<tr>
<td>5-10 cm</td>
<td>1.52</td>
<td>1.45</td>
<td>1.45</td>
<td></td>
</tr>
<tr>
<td>10-15 cm</td>
<td>1.50</td>
<td>1.56</td>
<td>1.58</td>
<td></td>
</tr>
<tr>
<td>Agric</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5 cm</td>
<td>1.39</td>
<td>1.57</td>
<td>1.53</td>
<td>S, S x D</td>
</tr>
<tr>
<td>5-10 cm</td>
<td>1.51</td>
<td>1.53</td>
<td>1.53</td>
<td></td>
</tr>
<tr>
<td>10-15 cm</td>
<td>1.47</td>
<td>1.52</td>
<td>1.54</td>
<td></td>
</tr>
<tr>
<td>REML</td>
<td>E, D</td>
<td>E, D</td>
<td>E, ExD</td>
<td></td>
</tr>
</tbody>
</table>
Fig. S2. Mean annual temperature and precipitation in the Clemson Area from May, 2013 through October, 2013. Precipitation is represented by the grey bars and temperature is represented by the line.
Fig. S3. Linear correlations of the degradation efficiency of peroxidase (PER$_{DE}$) in the summer and fall against the C/V, P/V and SCV/V ratio of lignin. (a) summer PER$_{DE}$ against the C/V, (b) fall PER$_{DE}$ against the C/V, (c) summer PER$_{DE}$ against the P/V, and (d) fall PER$_{DE}$ against the P/V, (e) summer PER$_{DE}$ against the SCV/V ratio and (f) fall PER$_{DE}$ against the SCV/V ratio.
**Fig S4.** $V_{\text{max}}$ (Spring) against the C/V ratio (a), $V_{\text{max}}$ (Spring) against the P/V ratio (b), $K_m$ (Spring) against the C/V ratio (c), and $K_m$ against the P/V ratio (d).
Method S2: Permanganate Oxidizable Carbon POXC

The POXC analysis was based according to Culman et al., 2012.

2.5 gram of air dried soil was combined with 2 ml 0.2 M KMnO₄ and 18 ml of water, shaken for 2 min on a rotary shaker. The sediment was allowed to settle for 10 min before diluting 0.5 ml of the sample into 50 ml water. Each sample was then processed on a UV-VIS spectrophotometer at 550 nm. The concentrations of the standards used for the analysis were 0.0005, 0.01, 0.015 and 0.02 M. The POXC was calculated according to Weil et al., 2003:

$$\text{POXC (mg kg}^{-1}\text{ soil)} = [0.02 \text{ mol L}^{-1} - (a + b \times \text{ABS})] \times (9000 \text{ mg C mol}^{-1}) \times (0.02 \text{ L solution/ Wt})$$

![Figure S5](image-url)

**Figure S5:** The relationship of the $PER_{DE}$ of the spring season to the Permanganate Oxidizable Carbon of the spring soils (a) and total soil N (b).
Table S4. Enzyme kinetics, $V_{\text{max}}$ and $K_m$ of soil peroxidase according to the soil depth and season in each ecosystem. The values represent mean (n=3) ±SE in the parentheses. Significant differences among the means are not reported in the table.

<table>
<thead>
<tr>
<th>Deci</th>
<th>Spring</th>
<th>Summer</th>
<th>Fall</th>
<th>REML $^a$</th>
<th>REML $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km (µM)</td>
<td>Vmax (µM min$^{-1}$ g$^{-1}$ soil)</td>
<td>Km</td>
<td>Vmax</td>
<td>Km</td>
<td>Vmax</td>
</tr>
<tr>
<td>0-5 cm</td>
<td>888 (103)</td>
<td>7.62 (0.71)</td>
<td>772 (93)</td>
<td>6.54 (0.74)</td>
<td>335 (7)</td>
</tr>
<tr>
<td>5-10 cm</td>
<td>420 (88)</td>
<td>5.59 (0.65)</td>
<td>435 (31)</td>
<td>4.96 (0.29)</td>
<td>395 (12)</td>
</tr>
<tr>
<td>10-15 cm</td>
<td>279 (21)</td>
<td>4.13 (0.55)</td>
<td>322 (15)</td>
<td>3.83 (0.38)</td>
<td>284 (18)</td>
</tr>
<tr>
<td>Pine</td>
<td>0-5 cm</td>
<td>1310 (272)</td>
<td>7.09 (2.39)</td>
<td>643 (143)</td>
<td>4.52 (0.73)</td>
</tr>
<tr>
<td>5-10 cm</td>
<td>1997 (506)</td>
<td>6.56 (2.54)</td>
<td>550 (76)</td>
<td>3.28 (0.70)</td>
<td>501 (22)</td>
</tr>
<tr>
<td>10-15 cm</td>
<td>2035 (412)</td>
<td>8.34 (4.05)</td>
<td>356 (13)</td>
<td>2.21 (0.45)</td>
<td>412 (72)</td>
</tr>
<tr>
<td>Agric</td>
<td>0-5 cm</td>
<td>134 (7)</td>
<td>2.98 (0.01)</td>
<td>87 (4)</td>
<td>2.57 (0.05)</td>
</tr>
<tr>
<td>5-10 cm</td>
<td>136 (12)</td>
<td>3.05 (0.12)</td>
<td>101 (3)</td>
<td>2.91 (0.16)</td>
<td>99 (3)</td>
</tr>
<tr>
<td>10-15 cm</td>
<td>137 (13)</td>
<td>3.08 (0.12)</td>
<td>110 (3)</td>
<td>3.18 (0.28)</td>
<td>106 (5)</td>
</tr>
<tr>
<td>REML $^c$</td>
<td>E</td>
<td>E, E x D</td>
<td>E, D, E x D</td>
<td>E, D, E x D</td>
<td>E, E x D</td>
</tr>
</tbody>
</table>

$^a$REML represent the restricted maximum likelihood analysis of $K_m$ for each ecosystem independently. (S) represents the main effect of season. (D) represents the main effect depth and (S x D) represents season x depth interaction. Least significant difference between means is not reported in the table.

$^b$REML: restricted maximum likelihood analysis of $V_{\text{max}}$ for each ecosystem independently.

$^c$For each season, the $K_m$ and $V_{\text{max}}$ were analyzed by REML using the fixed effects of ecosystem (E), depth (D), and ecosystem x depth (E x D) interaction for each variable separately.

$^d$Na refers to significant main effect or interaction for the designated variable.


Grabber, J. H. (2005). How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies this paper was originally presented at the lignin and forage digestibility symposium, 2003 CSSA annual meeting, denver, CO


