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THE CONTROLS AND CONSTRAINTS OF FINE-ROOT LIFESPAN

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ABSTRACT

Despite fine roots accounting for up to 50% of global terrestrial net primary productivity and 60% of soil respiration, surprisingly little is known about their ecology. Much of our ignorance involving fine-root ecology stems from the difficulty in observing roots *in situ* without disturbing the environment they inhabit. As a result, the ecological study of roots is still in its infancy. Through the use of minirhizotrons and isotopic techniques, we are beginning to gain a better understanding of how long roots live. However based on the different methodological approaches employed, the answer to this seemingly basic question can differ by as much as five fold. Beyond these methodological discrepancies, a basic understanding of the controlling factors that govern root lifespan remains elusive. Marshall and Waring put forward one of the early hypotheses regarding the controls of fine-root lifespan. They hypothesized that fine roots are initially constructed with a static carbohydrate reserve and the use of this finite reserve to fuel the metabolic demands of the root dictates the root's longevity. In Chapter 2, we examine this hypothesis in greater depth by labeling *Sassafras albidum* trees with 99% $^{13}CO_2$ and tracking the fate of the label in fine roots that were at least two weeks old at the time of labeling. If a root's carbohydrate reserves truly are determined at initiation, than no ^{13}C labeled photosynthate should appear in the carbohydrate pools of existing, non-elongating roots. We found that both root non-structural and structural carbon pools incorporate carbon from current photosynthate and as a result we found no support for the underlying assumptions of hypothesis put forward by Marshall and Waring. In Chapter 3, we investigate another hypothesis concerning the control of fine-root lifespan, namely that root lifespan is dictated by some metric of the costs of building and maintaining the root compared to the benefits the root supplies in terms of nutrient or water

acquisition. Here we used a combination of minirhizotron tubes and in-growth cores fertilized with nitrogen to see if roots supplying greater levels of a limiting nutrient do indeed have extended lifespans. We found that for species with fine-root morphology, root lifespan was significantly extended by localized nitrogen fertilization, but this trend was not observed in species with coarse-root morphology. Finally, in Chapter 4 we investigated the role herbivory plays in fine-root lifespan. We know that herbivores and pathogens can significantly reduce root longevity, but how well roots are defended against such attacks remains unanswered. We therefore investigated the relationship between levels of fine-root soluble phenolics, a putative measure of chemical defenses against root herbivory, and specific factors that have been shown to be related to fine-root lifespan. Although we found significant correlations between fine-root phenolic concentrations and both root order and localized nitrogen availability, we were unable to find general utility in relating phenolic concentrations with factors that have been shown to extend fine-root lifespan. Combined, the research described in the following chapters represents a significant scientific contribution in furthering our understanding of the controls and constraints of fine-root lifespan.

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Preface

Funding for the following research was secured by David M. Eissenstat. The research was conceived by David M. Eissenstat and Thomas S. Adams. Thomas S. Adams, advised by David M. Eissenstat, installed the experiments, collected the data, analyzed the data, interpreted the results, and wrote the subsequent manuscripts. M. Luke McCormack assisted with the collection of the data used in Chapter 3, "Foraging strategies in trees of different root morphology: the role of root lifespan."

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Chapter 1

General Introduction

The roots of plants have aptly been described as the "hidden half" because they occur belowground and are not easily observed. As a result, direct root observation necessitates some level of disturbance of the environment in which roots grow. The difficulty in observing roots *in situ* has vexed scientists attempting to study roots and has led to overt displays of frustration. For example Pregitzer *et al*. (2002) states, "The fine roots of perennial plants are a royal pain to study." As a result of the difficulties involved with studying roots in their undisturbed environment, we know surprisingly little about them compared to aboveground plant tissues. We still cannot say with certainty how long roots live, when they proliferate, how many roots a plant maintains, what causes roots to die, and how these processes vary across species and across different environmental conditions. In stark contrast with roots, leaves are readily observable and ecologists have accordingly collected global datasets of leaf traits, which in turn have led to globally significant patterns relating specific leaf traits, such as leaf area and leaf nitrogen content to leaf lifespan (Wright *et al*., 1998; Reich *et al*., 1999). Yet, roots can account for up to 50% of global terrestrial net primary productivity (Jackson *et al*. 1997) and represent as much as 60% of total soil respiration (Pregitzer *et al*., 1998). Therefore, understanding the controls of root lifespan is critically important to understanding many community- and ecosystem-level processes such as carbon and nutrient cycling and is a key link to enhanced understanding of long-term changes in soil organic matter and ecosystem carbon balance (Norby and Jackson 2000, Guo *et al*. 2008).

With the advent of more advanced root observational techniques, such as minirhizotrons and isotopic tracers, we are slowly gaining an increased understanding of fine-root dynamics. Although overarching patterns governing root longevity remain elusive, specific root factors related to root lifespan are emerging.

Techniques for sampling fine roots and estimating fine-root lifespan

Root turnover and root lifespan estimates have generally employed one of three methods; soil coring, minirhizotron observation, and isotopic tracers. All three methods have strengths and shortcomings, and all three methods were utilized to a greater or lesser extent in the scientific studies that comprise the following chapters.

Traditionally, soil cores have been used to determine root standing crop by coring a known volume of soil and quantifying the roots within the core. Ingrowth cores are a modification of this soil coring process in which the core is taken, the existing roots are removed, and soil, without the roots, is returned to the hole formed by the coring. A smaller diameter core is then taken from within the original cored area at some later time. In this way, any roots found in the subsequent "ingrowth" core must have originated after the initial core was taken. Although time consuming, these soil coring methods allow for the quantification of root physical properties such as length, mass, nutrient status, mycorrhizal associations, etc. (Majdi, 1996). Additionally, the ingrowth core process allows for the manipulation of soil properties within the core and the study of root growth under varied soil conditions. Two main difficulties are associated with these destructive root coring techniques. First, soil coring does not allow for the observation of individual roots through time and therefore fine-scale measurements of temporal differences in root standing crop are not possible (Majdi, 1996). As a result, accurate

quantification of fine-root production, death, and resulting lifespan are impossible. Secondly, once excavated, it is difficult to accurately determine which roots were living verses dead prior to being severed from the parent plant (Majdi, 1996). As a result of these methodological limitations of destructive soil coring, rhizotrons and, later, minirhizotrons, have been employed to allow for the direct, nondestructive observation of individual roots *in situ*.

Rhizotrons are essentially subterranean viewing surfaces that allow for the direct nondestructive observation of roots *in situ*, thereby allowing individual fine roots to be monitored through time. This, in turn, allows for accurate assessment of root production, death, and subsequent lifespan. Initially rhizotrons consisted of large subterranean excavations with transparent windows into which researchers themselves would venture to observe roots (Hilton *et al*., 1969). As an obvious result, these rhizotrons were expensive to construct and did not allow for substantial replication. Root boxes are smaller rhizotrons, allowing observers to lie on the soil surface and access belowground information. By using thin transparent material, such as acetate film, for the root viewing surface, root growth can be observed and the same roots can later be excised for additional analyses by cutting through the viewing surface. Minirhizotrons are even smaller versions of the original rhizotron concept, consisting of clear tubes placed in the ground. Specialized cameras and root tracking software allow for repeated individual root observation through the minirhizotron tubes. A main limitation of the minirhizotron system is the inability to directly access the roots being observed (Majdi, 1996). As a result, the determination of many root physical properties, such as internal anatomy or nutrient composition, is not possible when using minirhizotrons. Additionally, the installation of the minirhizotron tubes themselves creates a substantial disturbance to the rhizosphere that may not return to equilibrium for multiple years (Joslin and Wolfe, 1999).

Relatively recently, isotopic techniques have also been employed to assess root lifespan and turnover (Gaudinski *et al*., 2000; Gaudinski *et al*., 2001). These techniques utilize an isotopic carbon source (usually bomb ^{14}C or FACE ^{13}C) to label roots and determine their turnover rate and/or lifespan based on differences in isotopic enrichment between the time of carbon incorporation during root production and present isotopic levels. The main advantage of these isotopic tracer techniques is that the determination of root lifespan involves no disturbance to the soil prior to root sampling. Once labeled, roots are excavated from the soil, prepared for analysis and then analyzed for isotopic content on a mass spectrophotometer. Bomb ^{14}C has the added advantage of utilizing the preexisting isotopic carbon label resulting from the thermonuclear weapons testing in the 1950's and 1960's and, as a result, the labeling process occurs naturally and therefore requires no additional resources or expenditures. However, because these isotopic techniques do not actually track the fate of individual roots, the sources and the timing of carbon deposition in newly formed roots is assumed to be constant. Such assumptions have recently been called into question from studies that appear to show older stored carbon is being incorporated into newly formed roots (Gaudinski *et al*., 2009; Vargas *et al*., 2009; Sah *et al*., 2011).

Methodological differences between minirhizotron and isotopic techniques in fine-root lifespan estimates result in root lifespan values that often vary by as much as five fold, with roots appearing to be longer lived using isotopic labeling (Strand *et al*., 2008; Guo *et al*., 2008). Isotopic techniques are biased towards roots of coarser diameter and higher branching order roots, simply because these roots are easier to separate from the excavated soil. These higher order coarser diameter roots have been shown to be longer lived (Wells and Eissenstat, 2001; Wells *et al*., 2002; Anderson *et al*., 2003; Guo *et al*., 2008a). Additionally, minirhizotron and

isotopic techniques are measuring different root properties. Minirhizotrons yield a number-based estimated of median root lifespan, whereas isotopic techniques yield a mass-based estimate of mean root lifespan (Strand *et al*., 2008; Guo *et al*., 2008b). As such, a clear consensus of how long roots live and what techniques are best suited to determine root lifespan remains elusive.

Factors that affect fine-root lifespan

Through the methodological advances discussed in the previous section, we are beginning to elucidate certain factors that appear to be related to fine-root lifespan. Although our understanding of the controls of fine-root lifespan is far from complete, the factors discussed below provide a foundation on which more robust hypotheses concerning the controls and constraints of fine-root lifespan can be constructed. We will discuss the current hypothetical explanations for the control of fine-root lifespan in the following section: Resource Optimization verses Predetermination.

Numerous studies have demonstrated that roots of greater diameter and higher branching order (sensu Pregitzer *et al*., 2002) tend to be longer lived than finer diameter, lower-order roots (Wells *et al*., 2002; Anderson *et al*., 2003; Gill *et al*., 2002; Majdi *et al*., 2001; Guo *et al*., 2008a, McCormack *et al*., 2012). Based on morphological constraints, it is not surprising that within a root system, roots of higher branching order should live longer than the lower order, more distal, roots they support. More interestingly, variation in root diameter between co-occurring species, which can be greater than 10 fold (Valenzuela-Estrada *et al*., 2008; McCormack *et al*., 2012), appears to be related to root lifespan (McCormack *et al*, 2012).

Mycorrhizal fungal associations can enhance root lifespan (Guo *et al*., 2008a) however this is not always the case (Chen and Brassard, 2013). Mycorrhizae, although traditionally

viewed as a mutualistic relationship between roots and fungi, appear to occupy a range of relationships from mutualistic to parasitic depending on the species involved, soil nutrient content, and temporal variation (Johnson *et al*., 1997; Jones and Smith, 2004; Koide *et al*., 2008). The dynamics of these relationships appear to be governed both by the fungus, which in the case of ectomycorrhizal fungi form a sheath around the root tip and appear to be able to regulate the flow of nutrients to the root (Jones and Smith, 2004) and by the root, which supplies carbon to the fungus, and may regulate fungal colonization, at least with arbuscular mycorrhizal fungi, via hypodermal passage cells (Sharda and Koide, 2008; Zadworny and Eissenstat, 2011). As such, it seems likely that future research will find that belowground root-fungal associations are far more complicated than a simple mutualistic relationship. Explanations for the enhanced root longevity seen from mycorrhizal associations range from enhance resource acquisition to protection from harmful soil organisms (Jones and Smith, 2004).

Roots growing at greater soil depths appear to live longer than those at shallower depths (Kosola *et al*., 1995; Wells and Eissenstat, 2001; Majdi *et al*., 2001; Anderson *et al*., 2003; Gill *et al*., 2002; Withington *et al*., 2006; Pritchard *et al*., 2008). This may result from less variable soil conditions in terms of temperature and moisture fluctuations, decreased herbivore pressure, or variation in fungal associations with increased soil depth (Anderson *et al*., 2003; Withington *et al*., 2006).

Faster growing species tend to have shorter lived roots than slower-growing, low-nutrient-adapted species (Ryser, 1996; Schläpfer and Ryser, 1996; McCormack *et al*., 2012). Studies comparing fast- and slow-growing species of similar evolutionary lineage indicate that fast-growing species typically have thinner roots (Comas *et al*., 2002; Comas and Eissenstat, 2004) and roots of lower tissue density (Ryser, 1996; Wahl and Ryser, 2000) which, in

combination, typically is reflected in higher specific root length (length/mass) (Comas *et al*., 2002; Comas and Eissenstat, 2004; Wright and Westoby, 1999). Roots of fast-growing species are therefore less costly to produce in terms of carbon input and also may exhibit lower allocation to defense compounds than their slow-growing relatives (Comas *et al*., 2002; Comas and Eissenstat, 2004). Thus, a number of the factors that are thought to control root longevity appear to be correlated with plant potential growth rate and the relationship between species growth rate, nutrient availability, and tissue lifespan has spawned numerous hypotheses attempting to relate nutrient availability and tissue defense (Grime, 1977; Coley *et al*., 1985; Bryant *et al*., 1983).

Root herbivory and defense

Allelochemicals produced by roots have the potential to help ameliorate the negative effects of many of the factors that are thought to control root longevity (Waterman and Mole, 1994). As such, the production of allelochemicals by roots may directly affect root longevity. Despite the potential importance of root allelochemicals, they are often not included in investigations of root longevity. This exclusion may in part stem from the uncertainty about what factors control the production of the many allelochemicals produced by roots, as well as the difficulty in assessing the effects of such allelochemicals (Kraus *et al*., 2004a).

Of the myriad of allelochemicals produced by various plant species, phenolic compounds seem to be the most ubiquitous (Harborne, 1997) and best studied (Waterman and Mole, 1994). Phenolic concentrations across plant species are known to vary widely (Jones and Hartley, 1999). Tannins, a specific class of polyphenols, are estimated to be the fourth most abundant compound produced by vascular plants (Hernes and Hedges, 2000), constituting as much as 40%

of leaf dry weight (Kuiters, 1990) and 35% of root dry weight (Moore *et al*., 2001). Phenolic compounds are thought to play a role in herbivore defense, litter decomposition, nutrient cycling, nitrogen sequestration, microbial activity (Kraus *et al*., 2004) and free radical scavenging (Close and McArthur, 2002). As a result, fine root phenolic production may play an important role in determining fine root longevity.

There are a variety of hypotheses describing the controls that account for variable production of phenolics by plants (Stamp, 2003). Examples of such hypotheses include the carbon-nutrient balance hypothesis (Bryant *et al*., 1983), the growth-differentiation hypothesis (Herms and Mattson, 1992), the resource availability hypothesis (Coley *et al*., 1985), and the protein competition model (Jones and Hartley, 1999). Despite, the persistence of these numerous hypotheses, at their core they all involve a tradeoff between the resources used in phenolic production and the synthesis of other compounds used for growth and/or reproduction. If this tradeoff is reduced to the level of nutrient supply (Bryant *et al*., 1983), these hypotheses generally predict that as the stoichiometric nutrient balance in plants changes, there should be an associate change in phenolic production. When plant C:N ratios are high, excess carbon is available to be allocated to the production of phenolic compounds. Conversely, with increased N availability, plant C:N ratios decrease and carbon allocation is shifted to growth and/or reproduction. This, in turn, decreases carbon availability for phenolic production. Although these hypotheses were originally conceived to describe the variation in leaf phenolic compounds, there is no reason they cannot be extended to describe the variation in root phenolic content. If we extend these stoichiometric hypotheses to the root system of a plant, then these hypotheses predict that plant roots growing in nitrogen-rich soil patches should contain a relatively smaller

proportion of phenolic compounds, compared with roots growing in the surrounding soil with lower nutrient availability.

Few studies have investigated the relationship between changes in plant root stoichiometry and phenolic concentration. Despite the functional importance of root order (Pregetizer *et al*., 2002; Guo *et al*., 2008a) no published studies have investigated the effect of root order on phenolic concentration. Entry *et al*. (1998) found that fine-root tannin concentrations of pine seedlings grown in pots were lower than tannin concentrations for lateral roots and tap roots; however, what constituted a "fine root" in that study remains unclear. Evidence from studies involving leaf phenolic concentrations with changing nitrogen availability has been mixed. Some studies show increased phenolic production with increased plant C:N (King *et al*., 2001; Castells *et al*., 2002), some studies show decreased phenolic production with increased plant C:N (Penulas *et al*., 1996; Tognetti and Johnson, 1999), and some studies show no effect of plant C:N on phenolic production (Kerslake *et al*., 1998; Bezemer *et al*., 1999; Booker and Maier 2001). This has led some investigators to call into question the validity of the overarching hypotheses concerning nitrogen availability and phenolic content (Hamilton *et al*., 2001; Close and McArthur, 2002).

Methodological difficulties also hinder studies investigating plant phenolic concentrations (Kraus *et al*., 2004a; Appel *et al*., 2001). Phenolic concentrations estimated using commercial standards such as tannic acid can be highly inaccurate (Appel *et al*., 2001). Appel *et al*., (2001) found that estimates of phenolic concentrations from five closely related oak species using commercial standards resulted in estimates that differed from the actual phenolic concentration by as much as twofold. As a result, most investigators suggest using standards derived from the specific tissue of study species of interest. This is particularly problematic

when investigating the phenolic content of fine-roots because the larger quantities of tissue that are required to create self-standards are rarely available.

Fine root lifespan: resource optimization or predetermination

Despite the numerous root traits have been correlated with root longevity, to date only two generalized hypotheses on the controls of root lifespan have been made in the literature; the Starch Depletion Hypothesis and the Resource Optimization Hypothesis. At their core, these two hypotheses differ dramatically in the mode of action controlling fine-root lifespan, with the Starch Depletion Hypothesis advocating passive control of fine-root lifespan by the plant and the Resource Optimization Hypothesis suggesting active control of fine-root lifespan by the plant.

Marshall and Waring (1985) contended that nonstructural root storage carbohydrates, such as starch, are deposited solely when the root is first formed and it is the rate of depletion of these reserves that determines root longevity. This "Starch Depletion Hypothesis" contends that the maximum lifespan a root can achieve is predetermined at root initiation and that environmental factors that alter the rate of starch depletion, such as temperature, determine the extent to which root lifespan will be diminished below this maximum threshold. As such, there is no active regulation of root lifespan by the plant. A number of subsequent studies have called into question the validity of the assertion by Marshal and Waring that root nonstructural carbohydrate reserves (e.g., starch) are solely created when a root is first born. For example, Nguyen *et al*. (1990) found that root carbohydrate levels increased late in the growing season, implying that root carbohydrate reserves were not solely laid down when a root is first formed. Additionally Kosola *et al*. (2002) found, in a study that tracked individual roots, that the root starch concentration of Eugenei hybrid poplars did not decline with root age. As a result, they

concluded that the starch reserves in the fine roots of the poplar were not determined at root birth and that these reserves were "labile and dynamic". Despite these contradictions, starch depletion is still cited as the mechanism by which root lifespan is controlled.

Yanai *et al*. (1995) postulated that roots supplying a greater benefit to the parent plant in terms of nutrient or water acquisition, when compared with their construction and maintenance costs in terms of carbon, should have increased lifespans. This cost-benefit based "Resource Optimization Hypothesis" implies an active level of control of root lifespan by the parent plant, which must assess the benefits provided by an individual root, or suite of roots, while deducting the costs incurred by the root. The processes by which such costs and benefits are assessed by a plant and the means by which root lifespan is subsequently controlled remain unexplained. As a result, scientific validation of this hypothesis remains elusive.

Summary

In the following chapters, utilizing a combination of soil coring techniques, minirhizotron observations, root box observations, and isotopic labeling we examine both of the prevailing hypotheses on the controls of root lifespan (The Starch Depletion Hypothesis and The Resources Optimization Hypothesis) as well as examining the relationship between fine root soluble phenolic content and various factors that have been shown to affect fine-root lifespan. In Chapter 2, the Starch Depletion Hypothesis is explicitly tested by labeling entire trees with $^{13}CO_2$ and tracking the fate of the isotopic label in the carbon pools of existing, non-elongating roots. In Chapter 3 the Resource Optimization Hypothesis is tested by creating sustained localized patches of enhance nitrogen availability to see if roots that are supplying a greater benefit, in

terms of limiting nutrients, are longer lived. In Chapter 4, the role of root chemical defenses is investigated by examining the relationship between fine-root soluble phenolic content and various factors that have been shown to affect fine-root lifespan.

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Chapter 2

The continuous incorporation of carbon into existing *Sassafras albidum* fine roots and its implications for estimating root turnover

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Summary

- Although understanding the timing of the deposition of recent photosynthate into fine roots is critical for determining root lifespan and turnover using isotopic techniques, few studies have directly examined the deposition and subsequent age of root carbon.
- To gain a better understanding of the timing of the deposition of root carbon, we labeled four individual *Sassafras albidum* trees with 99% ^{13}C CO_2. We then tracked whether the label appeared in roots that were at least two weeks old and no longer elongating, at the time of labeling.
- We found that not only were the non-structural carbon pools (soluble sugars and starch) of existing first-order tree roots incorporating carbon from current photosynthate, but so were the structural components of the roots, even in roots that were more than one year old at the time of labeling.
- Our findings imply that carbon used in root structural and nonstructural pools is not derived solely from photosynthate at root initiation and have implications regarding the determination of root age and turnover using isotopic techniques.

Introduction

The timing of the deposition of photosynthetically derived carbon into roots is of considerable scientific importance because it has implications both for factors controlling root physiology and lifespan and for the determination of root age and turnover using isotopic techniques. Despite this importance, and over two decades of work on the subject, there is still no clear consensus concerning the timing of the deposition of root carbon. Until recently, it has been assumed that both root nonstructural storage carbon and structural carbon are primarily deposited when a root is first initiated. Marshall and Waring [1] contended that nonstructural root storage carbohydrates, such as starch, are deposited solely when the root is first formed and it is the rate of depletion of these reserves that determines root longevity. Recent attempts to determine root age and root turnover using isotopic techniques, both bomb ^{14}C and ^{13}C labeling, have assumed that root structural tissue originates within the year that a root is formed and that no new carbon is subsequently added to the root [2-8]. However, to our knowledge, these assumptions concerning the timing of the origin of root structural and nonstructural carbon have not been adequately tested.

A number of studies have called into question the validity of the assertion by Marshal and Waring [1] that root nonstructural carbohydrate reserves (e.g., starch) are solely created when a root is first born. For example, Nguyen *et al*. [9] found that root carbohydrate levels increased late in the growing season, implying that root carbohydrate reserves were not solely laid down when a root is first formed. Additionally Kosola *et al*. [10] found in a study that tracked individual roots, that the root starch concentration of Eugenei hybrid poplars did not decline with root age. As a result, they concluded that the starch reserves in the fine roots of the poplar were not determined at root birth and that these reserves were “labile and dynamic”.

Structural carbon has also traditionally been viewed as originating from recent photosynthate during root formation [2-8]. But recent studies using bomb ^{14}C have found that the origin, and subsequent age, of root structural carbon may involve more complicated processes than previously assumed. Evidence now suggests that stored carbon fixed prior to root formation may be incorporated in the structural tissue of newly formed roots [3, 6, 8, 11] . Furthermore, the amount of stored carbon incorporated in the formation of larger diameter fine roots (1.5 -2 mm), presumably of higher root order [*sensu*12], is higher than in smaller diameter, lower order roots (<0.5mm) [6]. Because root age is positively correlated with root diameter [13-15], it is difficult to disentangle the relationship between root age assessed by direct observation, root diameter, and estimated age based on carbon residence time.

In an attempt to gain a better understanding of the timing of the deposition of root carbon, we labeled *Sassafras albidum* trees with 99% $^{13}CO_2$. We then tracked whether the label appeared in first-order roots that were at least two weeks old and no longer elongating at the time of labeling. Based on previous studies, we hypothesized that the ^{13}C label would appear in the nonstructural carbon pools of the existing first-order roots, but not in their structural carbon pool.

Materials and Methods

This study was conducted at a common garden planting (mixed species planting) located at the Russell E. Larson Agricultural Research Center, Pennsylvania State University, USA (40.8°N, 77.9°W) [see reference 16 for details]. For this study we labeled *Sassafras albidum* with 99% $^{13}CO_2$ (Cambridge Isotope Laboratories, Andover, MA USA) (Figure 1). We chose this species because of its comparatively small size (ca. 2.5 m tall) relative to other tree species

in the garden, and the easily recognizable, relatively large diameter, fine roots which emit a distinctive odor when harvested thereby precluding confusion with the roots of neighboring trees. Each species plot represented six trees planted in a double row with spacing of 3 m between trees within the row, 3 m between the double rows and 5 m between the six-tree plots.

Soils at the common garden are relatively fertile Hagerstown silt loam, well-drained, with a pH ranging from 6.1-6.5 and with some areas high in calcium. Previous to planting the trees, the site was used as a grass hayfield. The entire area was fenced to keep out deer. *Sassafras albidum* trees were collected in the early spring of 1996 from seedlings around State College, PA and therefore were approximately 13 years old at the time of the experiment. Understory vegetation was controlled within a half-meter of the tree using weed barrier cloth and gravel mulch and sprayed with glyphosate to a distance of about two meters from the trunk. Further from the trees, grass was mowed weekly or longer, depending on climatic conditions.

Root boxes were installed in the spring of 2008 and again in the spring of 2009[17]. Briefly, a 0.6 x 0.5 x 0.4 m hole was dug in the ground approximately 0.5 m in front of each study tree. A 0.6 x 0.5 x 0.4 m box constructed of 1.9 cm treated plywood, with a clear 0.127 mm thick (5-mil) acetate window, was then placed in the ground with the acetate window facing the tree (Figure 1B). A removable piece of 1.3 cm thick foam insulation board was placed against the window when not in use to minimize temperature differences between the soil and the interior of the root box. In this fashion, roots growing against the acetate window could be followed from birth by tracing the roots on the acetate using different colored paint pens (Marvy DecoColor pens, Uchida of America Corp., Torrance, CA, USA) on different tracing dates. Three small first-order roots per tree were harvested prior to labeling and pooled into a single pre-label sample per tree to assess background ^{13}C levels. After labeling the trees with ^{13}C,

preexisting roots of a known age were then excised by cutting through the acetate window and individual first-order roots were analyzed for ^{13}C in structural and nonstructural carbon pools.

Sassafras albidum trees, approximately 2.5 m in height, were labeled with 99% $^{13}CO_2$ by first surrounding the trees in a 2.4 x 1.2 x 1.2 m clear mylar balloon supported by a frame constructed of 1.9 cm internal diameter pvc pipe (Figure 1A). Mylar was also used to cover the soil surface to minimize non-target $^{13}CO_2$ uptake. During the labeling, air was constantly circulated in the balloon using three small battery powered fans hung within the canopy of the tree. Carbon dioxide concentrations within the balloon were measured during the labeling using a Li-Cor 6200 (Li-Cor Biosciences, Lincoln, NE, USA). The labeling itself occurred by releasing a short pulse of 99% $^{13}CO_2$ into the balloon and waiting until the CO_2 concentration in the balloon dropped back below background levels (~385ppm). This process took approximately 30 minutes per tree once the balloon was installed. Roots were collected immediately prior to labeling and at various times after labeling. Only first-order, distal, roots were sampled from the root boxes for ^{13}C analysis. All roots sampled from the trees were at least two weeks old at the time of labeling and were no longer actively elongating. Three trees were labeled on August 5th, 2008 and based on these results a fourth tree was labeled the following year on September 3rd, 2009 to enable the sampling of roots of greater age at the time of labeling as well as to examine in greater detail the fate of the label in both the structural and the non-structural root carbon pools. Only a single tree was labeled in 2009 due to a lack of additional suitably sized trees.

In 2008 all roots visible through the acetate windows were traced 19 days (d) prior to labeling for each of three trees labeled with 99% $^{13}CO_2$ and an unlabeled control tree. The unlabeled control tree was used to insure accurate background ^{13}C measurements. Root samples were excised from the root boxes immediately prior to labeling and three times after labeling (3,

6 and 19d). Additional larger diameter, higher-order roots (approximately 4^{th} or 5^{th} order) were dug from the soil directly beneath the three labeled trees and the unlabeled-control tree four days after labeling. The upper, non-distal, portion of two roots from each of these samples were then cut into segments and dissected under the microscope into three anatomical categories: late wood, early wood, and secondary cortex (Figure 2); where the late wood accounts for the inner most portion of the root cross section, surrounded by the early wood and finally the secondary cortex. Because these roots were not followed in the root boxes, their precise age was unknown, but based on the development of early and late wood; they were at least one year old at the time of labeling [18]. Individual sampled roots were freeze dried, ground, and then underwent acid-base-acid washing until the supernatant was clear to remove nonstructural carbon [4]. The remaining sample was oven dried at 60°C for 24 hrs, weighed, and placed into tin capsules.

In 2009, a single Sassafras tree was labeled with $^{13}CO_2$. Only one tree was labeled in 2009 and less $^{13}CO_2$ was used than in 2008 due to a decreased number suitable unlabeled trees. Because there was no statistical difference (P=0.40) in isotopic signature between the roots of the control tree and the roots sampled prior to labeling the labeled trees in 2008, we did not sample the roots of a second control tree in 2009. Roots were traced weekly prior to labeling, with three root flushes observed at 71d , 28d , and 16d prior to labeling. The weekly tracing interval did not allow for the determination of exact root ages in each flush, as roots could have been born on any of the previous seven days. For analysis, we assigned root birth as the day of initial observation, so a root might actually be as much as 7d older than the assigned birth date. Individual first-order roots were sampled immediately prior to labeling and twice after labeling, once at five days after labeling and once at 14 days after labeling. These individual sampled first-order roots were ground and then underwent a stepwise process to isolate soluble sugars,

starch and structural carbon. This involved first boiling the root samples in Millipore water (Millipore, Billerica, MA USA) and sampling the resulting supernatant for soluble sugar ^{13}C content. The remaining solid sample was then re-suspended in Millipore water and digested with 0.5 M sodium acetate and 5 units of amyloglucosidase and 2.5 units alpha amylase [19]. The resulting supernatant was then analyzed for starch ^{13}C content. Finally, the remaining root sample underwent the acid-base-acid cleaning mentioned above, leaving structural carbon for ^{13}C analysis. Solid samples were oven dried at 60°C for 24 hrs, weighed, and placed in tin capsules. Liquid samples were pipetted into pre-weighed tin capsules, oven dried at 60°C for 24 hrs, and then re-weighed.

All samples from both years of the study were analyzed for ^{13}C content at the UC Davis Stable Isotope Facility. Statistical analyses were conducted using SAS JMP 9.02 (SAS Institute Inc., Cary, NC, USA). Results from each year were analyzed using an ANOVA with sampling date, tree, and labeling treatment as factors in 2008 and sampling date, root age, root carbon pool, and labeling treatment as factors in 2009, where only one tree was labeled. Non-significant factors were removed from the model. Results from the final model were considered statistically significant at $P \leq 0.05$ using a one tailed T-test.

Results

In both 2008 and 2009 structural carbon of the roots of the labeled *S. albidum* trees, which were at least two weeks old at the time of labeling, were significantly (2008 n=35, P<0.0001; 2009 n=38, P=0.02) enriched in ^{13}C compared with roots sampled from the same trees prior to $13CO_2$ labeling (Figure 3, Table 1). The lower enrichment observed in 2009 likely resulted from less $^{13}CO_2$ being used in the labeling process. In 2008, no significant differences

were observed among the three labeled trees in the ^{13}C enrichment of the roots ($P = 0.44$) nor in timing of the root sampling post labeling ($P = 0.19$). Additionally, in 2008 all portions of the dissected roots (early wood (*ew*), late wood (*lw*), and secondary cortex (*sc*); were significantly enriched in ^{13}C by labeling with 99% $^{13}CO_2$ (Figure 4) (*ew*: $P = 0.01$, *sc*: $P = 0.01$, *lw*: $P = 0.04$). In 2009, in addition to root structural carbon, both root soluble sugars and root starch were also significantly enriched with ^{13}C by labeling ($P = 0.001$ and $P < 0.0001$) (Figure 5), with soluble sugars being most enriched followed by starch and then structural carbon. The timing of the root sampling post labeling significantly affected the ^{13}C enrichment of the root sugars ($P = 0.005$), with roots sampled later showing decreased ^{13}C enrichment. Root age at the time of labeling did not significantly affect ^{13}C enrichment of the root sugars ($P = 0.09$). Neither of these factors significantly affected the ^{13}C enrichment of the structural carbon pool (root age $P = 0.15$, sample date $P = 0.14$), nor of the starch pool (root age $P = 0.26$, sample date $P = 0.26$) (Table 1).

Discussion

Our results clearly demonstrate that the timing of the deposition of photosynthetically derived carbon into roots involves more complicated processes than has previously been assumed. Not only were the non-structural carbon pools (soluble sugars and starch) of existing first-order tree roots incorporating carbon from current photosynthate (Figure 5), but so were the structural components of the roots (Figures 3 and 4). Our results are in direct contradiction with the assertion of Marshall and Waring [1] that root carbohydrates associated with starch formation are derived only at root initiation. Additionally, the incorporation of current photosynthate into the structural carbon of roots which were at least two weeks old, and in some roots more than a

year old, at the time of labeling has implications regarding the determination of root age and turnover using isotopic techniques. For example, when using bomb ^{14}C analyses, if the incorporation of new carbon into the structure of existing roots continues to occur over sufficient time scales, an underestimation of the actual age of roots will result since the ^{14}C signature of the newly incorporated carbon would be depleted in ^{14}C compared to the carbon used to construct the root at initiation. Furthermore, in recent studies that have used the incorporation of ^{13}C from current photosynthesis into the structural carbon pools of labeled tree roots to assess root turnover [2, 5], if new structural carbon is continually added to existing roots, root turnover will appear to occur more quickly since the depletion in the ^{13}C signal will not be solely derived from the death of labeled roots and the initiation of new root growth.

Our results demonstrate that current photosynthate is incorporated into the structural tissue of higher-order roots (Figure 4) as well as first-order roots (Figures 3, 5). We also found evidence, when examining the full range of root ages sampled (16d to >1year), that this incorporation appears to decrease with root age (Figure 6). However, even the roots that were older than one year at the time of labeling never were depleted to the level of the non–labeled roots (Figure 6). Additionally, this trend of decreasing incorporation of C in structural tissues with age was not significant when just examining first-order roots sampled within a 2.5-month period.

Because roots of higher branching order must in general be older than the lower order roots they support, the diminished incorporation of current photosynthate with root age over longer time scales suggests that higher-order, longer-lived roots have fewer errors associated with estimates of root lifespans and turnover based on isotopic techniques. However, because roots of higher branching order, unlike lower-order roots, often undergo secondary development,

the potential for newly incorporated carbon to bias estimates of root lifespan and turnover may actually increase with increasing root order. One possible way to overcome this bias, based on our observation of decreased recent carbon assimilation in the older tissue of higher-order roots (Figure 4: late wood vs. early wood), would be to use the oldest, inner most root tissue of higher-order woody roots for isotopic analysis.

Although we did not specifically examine the physiological processes involved in the observed incorporation of recent photosynthate into the structural pools of existing roots, secondary cellular processes such as cell wall thickening could be responsible. In another tree species, first-order *Liriodendron tulipifera* roots of known age collected from the same common garden during the same time period showed significant secondary wall thickening between birth and 14 days in the hypodermal and endodermal cells of the primary tissue (Zadworny *et al*. unpublished data). Similar results have been observed among citrus rootstocks when comparing the primary tissue of first- with second-order roots [20]. Regardless of the exact physiological mechanism involved, we have clearly demonstrated that new carbon is incorporated into the carbon pools of existing first-order roots for months after birth and in higher order roots, for potentially years. Because this study involved only one species, further study is needed to determine if the observed assimilation of new carbon into existing non-elongating first-order roots is a broadly occurring process. Additionally, more work is needed to uncover the underlying mechanisms driving the observed assimilation as well as the exact extent to which new carbon may influence estimates of root age and turnover using isotopic techniques.

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<u>Appendix 2.1</u>: Calculation of *S. albidum* fine-root lifetime structural carbon incorporation from current photosynthate based on $^{13}CO_2$ enrichment results.

If we assume that the incorporation of recent photosynthate is constant and continues over the lifespan of a root (based on evidence of enrichment in roots over a year old), and we take the difference in the average δ value between the control and enriched samples from 2008 (i.e. -27.79‰ vs. -2.28‰ or a 13C/12C difference of 0.00029 13C/12C), the approximate mass of structural carbon in the average root sample (0.4 mg structural carbon in an average root sample size of 1mg dry weight), and the average time for the tree labeling process (i.e. 30 minutes) we can calculate the amount of new carbon assimilated into 1 mg of root structural carbon per minute (3.82×10^{-6} mg C min^{-1}). If we further assume the *S. albidum* trees are photosynthetically active for six hours per day, we can estimate the amount of new carbon assimilated into existing roots per day (1.38×10^{-3} mg C d^{-1}). If we then assume that *S. albidum* trees growing in our northern temperate region are likely only fully photosythetically active for about 3 months out of the year and we use minirhizotron-based estimates of median lifespan for *S. albidum* first-order roots of approximately one year (McCormack *et al.*, 2012), 0.12 mg or 12% of new carbon would be incorporated into the structural carbon pool of 1mg of root over its lifetime. Although 12% fine root structural carbon replacement is just a rough estimate, it none the less highlights the impact of deposition of newly derived carbon on estimates of C residence time over the lifetime of a root. Further study is needed to uncover the underlying mechanisms driving this assimilation as well as the exact extent to which new carbon may influence estimates of root age and turnover using isotopic techniques.

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Figure 2.1. Methods used in study. A. *Sassafras albidum* tree covered in a clear mylar balloon during the $^{13}CO_2$ labeling process. **B.** Example of a root box showing roots that were traced on a clear acetate window facing the study tree using different color paint pens.

Figure 2.2. *Sassafras albidum* root cross-section. Micrograph depicting how higher-order roots (approximately 4th or 5th order) were dissected prior to $\delta^{13}C$ analysis in 2008.

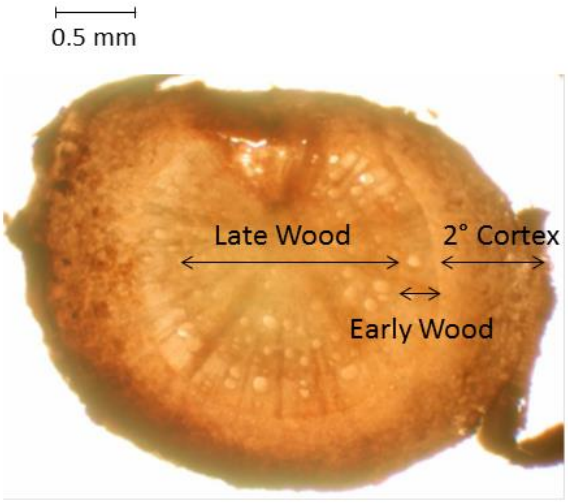

Figure 2. 3. Mean *Sassafras albidum* first-order root structural carbon $\delta^{13}C$ values. Data from an unlabeled control tree, and trees labeled in 2008 and in 2009. All roots were at least 2 weeks old at the time of labeling and subsequent sampling and some were older than 71 days. Black bars represent roots sampled prior to whole-tree labeling and grey bars represent roots sampled after whole-tree labeling. Note that less negative values indicate greater isotopic enrichment. Error bars denote standard error between individual sampled roots. Different lower case letters denote significant differences at $P \leq 0.05$ using a one tailed T-test between pre- and post-labeled roots.

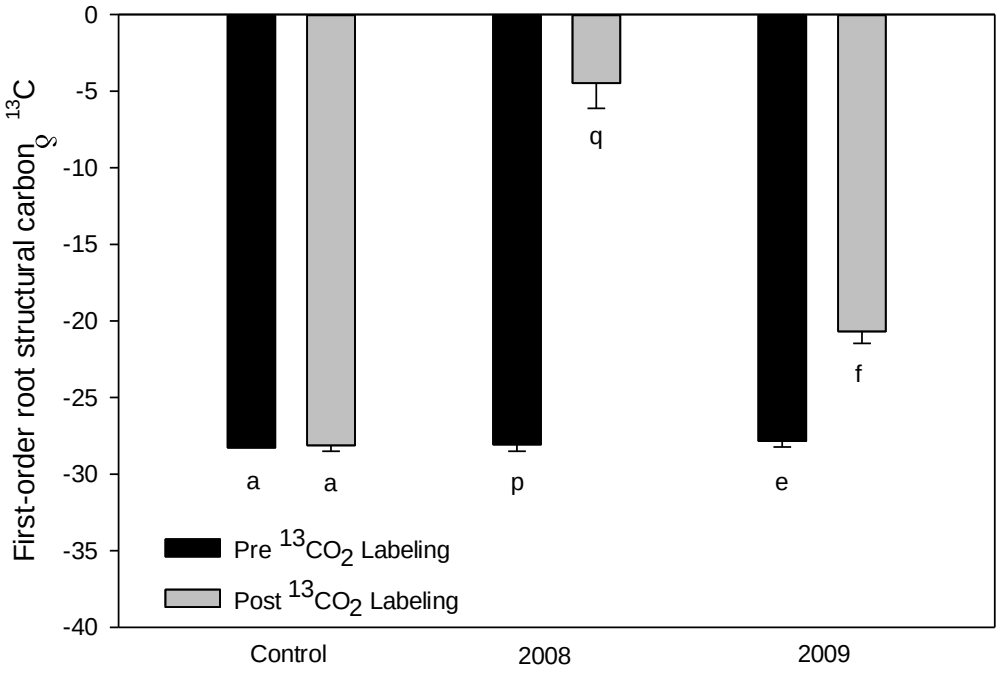

Figure 2.4. Mean *Sassafras albidum* root structural carbon $\delta^{13}C$ values from higher-order dissected roots. Trees were labeled and sampled in 2008. Control roots were sampled from unlabeled control trees and labeled roots were sampled from trees labeled with 99% $^{13}CO_2$. Late wood refers to the innermost portion of the dissected roots (see Figure 2). Early wood refers to the root tissue immediately surrounding the late wood. Secondary cortex refers to the outermost portion of the root surrounding the early wood. Note that less negative values indicate greater isotopic enrichment. Error bars denote standard error. Different lower case letters denote significant differences at $P \leq 0.05$ using a one tailed T-test between dissected root tissue from unlabeled control trees and labeled trees.

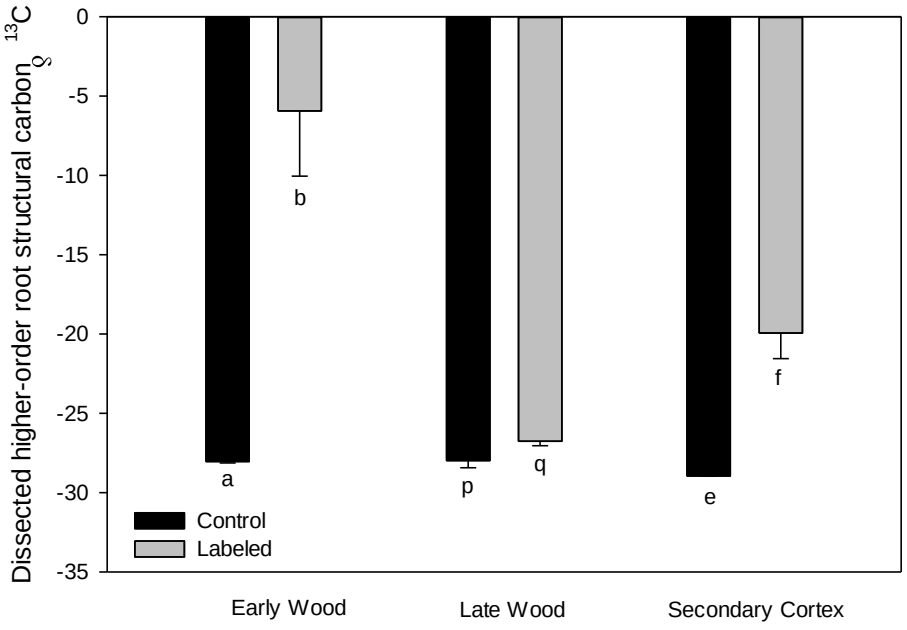

Figure 2.5. Mean *Sassafras albidum* first-order root carbon $\delta^{13}C$ values. Roots were at least two weeks old at the time of whole tree labeling in 2009. Black bars represent roots that were sampled immediately prior to whole-tree labeling. Light grey bars represent roots sampled after whole-tree labeling. Note that less negative values indicate greater isotopic enrichment. Error bars denote standard error. Different lower case letters denote significant differences at $P \leq 0.05$ using a one tailed T-test between pre- and post-labeled roots of each tissue type.

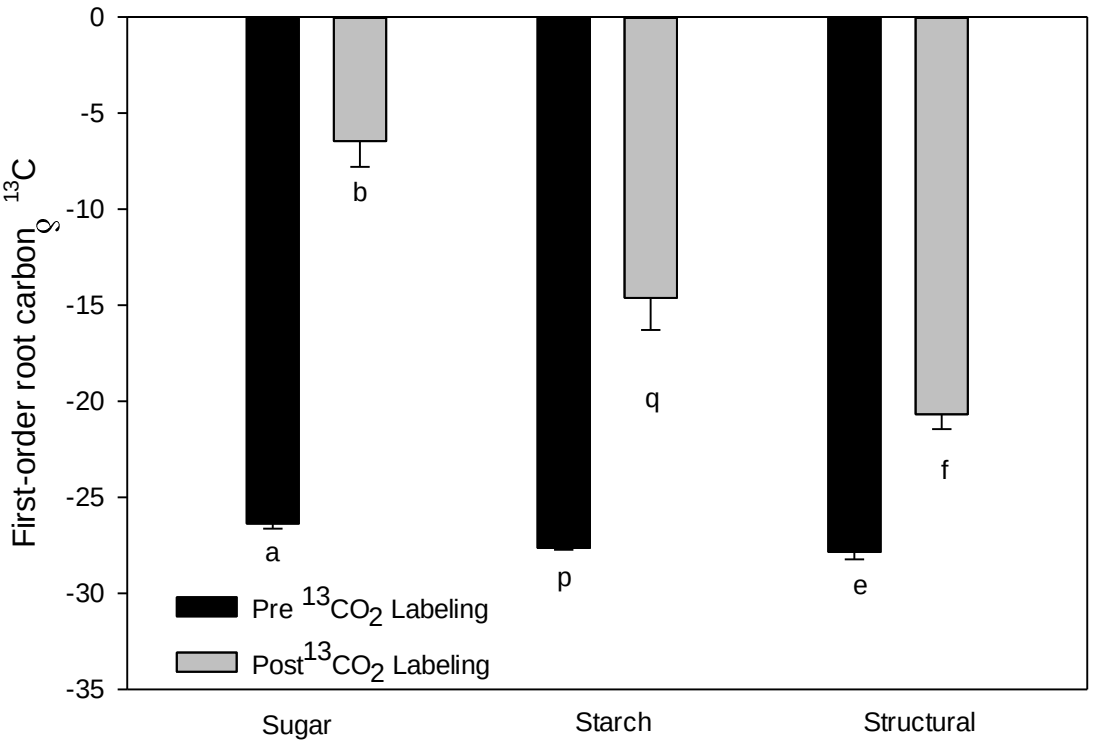

Figure 2.6. Mean *Sassafras albidum* root structural carbon $\delta^{13}C$ values verses root age at time of sampling. Circles represent roots sampled in 2009. The triangle represents the mean of the late wood (lw) structural root tissue from the 2008 dissection samples which were older than one year when sampled but the exact age is unknown. The grey horizontal bar shows the 95 percentiles for background $\delta^{13}C$ structural carbon values from 2008 and 2009. Note that less negative values indicate greater isotopic enrichment. Error bars denote standard error.

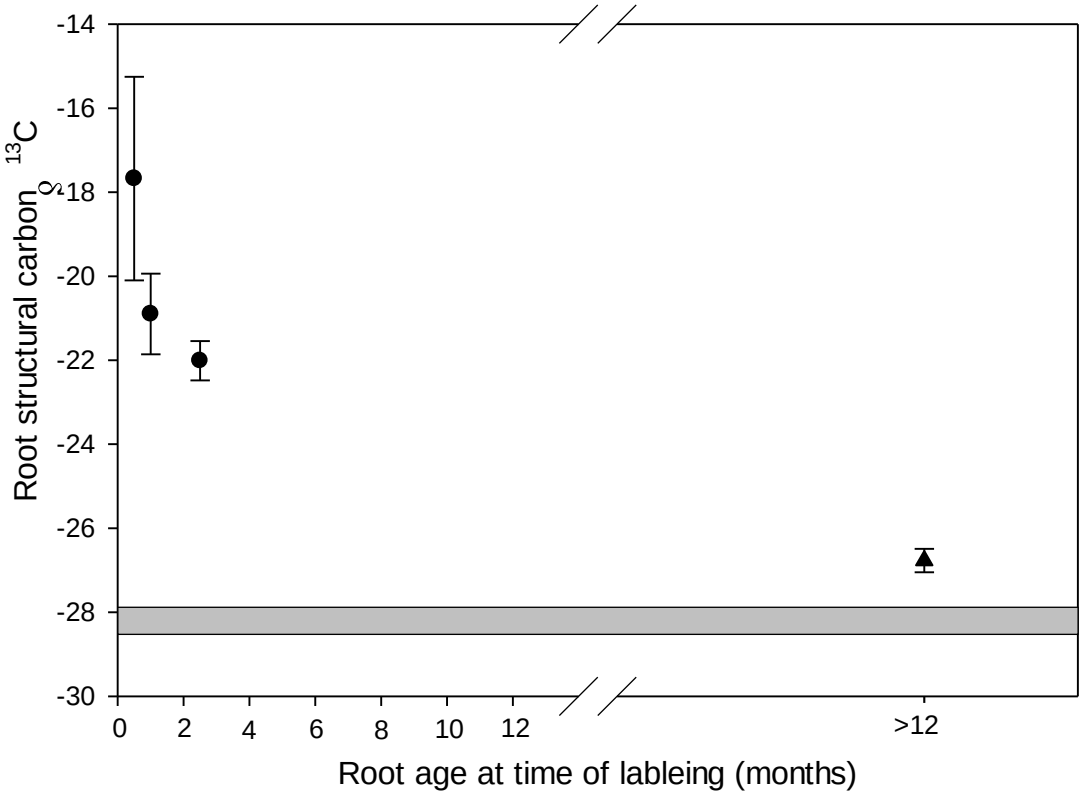

Table 2.1: *Sassafras albidum* δ^{13}C root data from trees labeled with 99% $^{13}CO_2$.

Data showing the number of samples (*n*) analyzed as well as the average δ^{13}C and associated standard error for each root age class, sampling date, and carbon pool from trees labeled with 99% $^{13}CO_2$ in 2008 (n=3) and 2009 (n=1), where less negative values indicate greater isotopic enrichment. $^{13}CO_2$ labeling significantly enriched the root structural carbon in 2008 (*n*=35, *P*<0.0001) and 2009 (*n*=38, *P*=0.02). Additionally, both the root starch and root sugars were significantly enriched in 2009 (*n*=38, *Pstarch*<0.0001, *Psugars* =0.001). The number of days sampling occurred post labeling did not significantly affect enrichment in root structural carbon in 2008 (*P*=0.19) nor root structural carbon nor root starch in 2009 (*Pstructural*=0.14, *Pstarch* =0.26). In 2009 root sugars were significantly affected by the days post labeling (*P*=0.005), with roots sampled later showing decreased enrichment. The age of the roots at the time of labeling did not significantly affect enrichment in any of the carbon pools in 2009 (*Pstructural*=0.15, *Pstarch*=0.26, *Psugars* =0.09).

Year	Root age (d)	Number of days post-labeling		
		3	**6**	**19**
Structural $\delta^{13}C$ *(Control* =-28.13 ± 0.24 (*n*=9))				
2008	**> 14**	-2.18 ± 3.38 (*n*=14)	-5.75 ± 1.28 (*n*=13)	-6.23 ± 3.89 (*n*=8)
2009		**5**	**14**	
Structural $\delta^{13}C$ *(Control* =-27.85 ± 0.38 (*n*=2))				
	16	-16.84 ± 3.73 (*n*=3)	-18.93 ± 3.76 (*n*=2)	
	28	-19.99 ± 1.46 (*n*=13)	-21.87 ± 1.22 (*n*=13)	
	71	-21.99 ± 1.91 (*n*=4)	-22.04 ± 1.87 (*n*=3)	
Starch $\delta^{13}C$ *(Control* =-27.64 ± 0.09 (*n*=2))				
	16	-16.66 ± 3.91 (*n*=3)	-12.17 ± 8.08 (*n*=2)	
	28	-14.90 ± 2.57 (*n*=13)	-15.50 ± 2.70 (*n*=13)	
	71	-17.38 ± 4.51 (*n*=4)	-5.41 ± 12.77 (*n*=3)	
Sugar $\delta^{13}C$ *(Control* = -26.38 ± 0.25 (*n*=2))				
	16	-0.68 ± 5.81(*n*=3)	-9.11 ± 1.98 (*n*=2)	
	28	-0.18 ± 1.37 (*n*=13)	-10.86 ± 1.77 (*n*=13)	
	71	-2.93 ± 9.11 (*n*=4)	-14.91 ± 1.04 (*n*=3)	

Chapter 3

Foraging strategies in trees of different root morphology: the role of root lifespan

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Summary

Resource exploitation of patches is influenced not simply by the rate of root production in the patches but also by the lifespan of the roots inhabiting the patches. We examined the effect of sustained localized nitrogen (N) fertilization on root lifespan in four tree species which varied widely in root morphology and presumed foraging strategy. The study was conducted in a 12-year-old common garden in central Pennsylvania using a combination of data from minirhizotron and root in-growth cores. The two fine-root tree species, *Acer negundo* and *Populus tremuloides*, exhibited significant increases in root lifespan with local N fertilization; no significant responses were observed in the two coarse-root tree species, *Sassafras albidum* and *Liriodendron tulipifera*. Across species, coarse-root tree species had longer median root lifespans than fine-root tree species. Localized N fertilization did not significantly increase the N concentration or the respiration of the roots growing in the N-rich patch. Our results suggest that some plant species appear to regulate the lifespan of different portions of their root system to improve resource acquisition while other species do not. Our results are discussed in the context of different strategies of foraging of nutrient patches in species of different root morphology.

Introduction

The lifespan of roots is of broad interest in ecology. Because fine roots account for as much as one third of global net primary productivity (Jackson *et al*. 1997), their lifespan is of major importance to carbon and nutrient cycles and is a key link to longer-term changes in soil organic matter and ecosystem carbon balance (Norby and Jackson 2000). Moreover, competition below ground occurs widely in plant communities and often dominates over competition above ground (Wilson 1988). Belowground competition may be largely associated with rapid exploitation of resource patches and control of the patches to the detriment of neighbors (Eissenstat and Caldwell 1988; Robinson *et al*. 1999)**.** While rapid root proliferation in nutrient-rich patches has been a major focus of research (Robinson 1996) , much less is known on how patch exploitation is influenced by root longevity.

Nutrient acquisition is normally positively correlated with the total root length available for resource acquisition, but total length at any point in time is not only a result of root production but also of root lifespan. Theoretically, root lifespan should be related to the lifetime efficiency of the root for resource uptake (Eissenstat and Yanai 1997; Yanai et al. 1995). Thus, root lifespan should be a function of the marginal benefits associated with resource acquisition relative to the marginal costs of maintaining the root that may also include evolutionary stable strategies which diminish the fitness of neighbors (e.g., O'Brien et al. 2007). Often the resource of interest is nitrogen (N), because it is commonly limiting for plants (Vitousek and Howarth 1991), its mobility in soil (especially for nitrate) causes root competition to be likely, and because its availability in the soil matrix can vary by as much as an order of magnitude over small spatial scales (Jackson and Caldwell 1993). Nitrogen uptake is a costly process which normally requires large energy expenditures associated with high protein turnover and

assimilation (Bloom *et al*. 1992; Bouma *et al*. 1996). Therefore, root lifespan may shift in N-rich patches depending on the relative benefits associated with greater uptake compared to the relative costs associated with root construction, ion uptake and maintenance. The influence of the N patch on root longevity may also depend on the duration of the patch, the degree the patch differs from the bulk soil in N availability and the size of the patch (Fitter 1994).

Although few in number, studies of the lifespan of fine roots growing in localized N patches have shown mixed results. For example, Pregitzer *et al.* (1993) found that localized N fertilization increased root lifespan in a mixed Northern hardwood forest, whereas Bai *et al*. (2008) found that localized N fertilization decreased root lifespan of the grass, *Leymus chinensis*. Additionally, Hodge *et al*. (2009) found that in the grass, *Lolium perenne*, the lifespan of roots inhabiting an N-rich patch can be extended or reduced in an unpredictable manner depending on the level of N enrichment. These variable results associated with the influence of localized N on root lifespan are exacerbated by methodological differences among studies, such as differences in the duration of the N application, differences in the plant functional groups examined, differences in the scale of the study systems, and differences in the determination of root lifespan. As a result, no clear picture has emerged concerning the role that localized nitrogen availability plays on the lifespan of fine roots.

In this study, we investigated the lifespan of fine roots growing in localized N-enriched patches that did not become depleted over time. The study was conducted in a common garden in central Pennsylvania using four Northeastern temperate tree species that varied widely in root morphology based on differences in root diameter and specific root length (SRL, root length-dry wt. ratio). Root diameter and SRL are directly linked to costs of construction to produce root length or surface area and have been linked to variation in both root proliferation (Eissenstat

1991) and root lifespan (Eissenstat *et al*. 2000; McCormack *et al.* 2012). For this study we contrasted species with coarse-diameter 1st and 2nd order roots (ordering classification follows a stream-based ordering system, Pregitzer et al. 2002) with those of fine diameter 1st- and 2nd-order roots, because of the possible links between root construction costs and root lifespan. Specifically, we hypothesized that the fine-root species would proliferate roots more quickly and to a greater extend in nutrient-rich patches but would have shorter lived roots compared with those of the coarse-root species. We expected all species to increase root longevity in fertilized compared to unfertilized soil.

Methods

All studies were conducted at a common garden planting located in central Pennsylvania, USA at the Russell E. Larson Agricultural Research Center, Pennsylvania State University (40.8°N, 77.9°W). The common garden consists of 16 species of trees that were planted mostly in 1996 as 1-yr-old liners in a randomized complete block design with 8 blocks. Each species was planted in groups of 6 trees in a double row of 3 trees with a spacing of 3 m between trees within the row, 3 m between the double rows and 5 m spacing between the 6-tree plots. We used 4 of the 16 trees species: 2 fine-root, high-specific root length (SRL) species (*Acer negundo,* ACNE and *Populus tremuloides,* POTR) and 2 coarse-root, low-SRL species (*Liriodendron tulipifera,* LITU and *Sassafras albidum,* SAAL). All four of the species at this site were principally colonized by arbuscular mycorrhizas (Zadworny and Eissenstat 2011).

Soils were relatively fertile Hagerstown silt loam, well-drained, with a pH ranging from 6.1-6.5. Previous to planting the trees, the site was used as a grass hayfield. The entire area was fenced to keep out deer. Blocking was used to control variation in soil characteristics. Plants were obtained from local native-plant nurseries, except for *Acer negundo* and *Sassafras albidum,*

which were collected from seedlings around State College, Pennsylvania. Understory vegetation was controlled within a half-meter of the tree using weed barrier cloth and gravel mulch and sprayed with glyphosate to a distance of about two meters from the trunk. Further from the trees, grass was mowed weekly or longer as needed. In June of 2005, two 45-cm long, 2.86-cm ID clear acrylic minirhizotron observation tubes were installed at an angle of 30 degrees from the vertical and a distance of 30 cm from the base of each study tree. This resulted in two tubes per tree spaced approximately 0.75 m apart. Tubes were installed in each of the 8 blocks, resulting in 16 minirhizotron tubes per tree species. The minirhizotron tubes were equipped with a 12-cm length of 1.6-mm-diameter irrigation tubing, which resulted in the irrigation tube running to a depth of 10 cm below the soil surface to enable localized fertilization (Eissenstat and Caldwell 1988). The portion of the minirhizotron tube above the soil surface was wrapped in black tape, stoppered and covered with a white aluminum can to minimize solar heating. One of the two tubes per tree was fertilized weekly throughout the growing season (roughly April through November), via the irrigation tube, with 10 ml of nutrient (NH_4NO_3) solution; the control tube was irrigated with 10 ml deionized water.

The nutrient solution (10 ml of 98.1 mg NH_4NO_3 L^{-1}; 3 times "available" soil solution N, see below) was added weekly to maintain a persistent localized nitrogen patch throughout the growing season of each year. The other tube of the pair received 10 ml of deionized water. In addition, 100 ml of deionized water was also applied monthly to all tubes throughout the growing season, to flush any potential salt accumulation. Two years passed between the tube installation and first imaging session to allow conditions to equilibrate from the disturbance of the tube installation. Minirhizotron images were taken using a Bartz 1.125" camera equipped with I-CAP version 4.01 imaging software (Bartz Technology, Carpinteria, CA, USA) at an

interval of approximately every three weeks throughout the growing season for 3 years, 2007-2009. Initially, a single indexing hole was drilled in each tube to allow imaging on the upper viewing surface. After one year of imaging (i.e. in 2008), a second indexing hole was drilled on the lower viewing surface of the tube, allowing for twice the number of observations per tube. Root data were collected from the minirhizotron images using RootFly 1.8.35 (Wells and Birchfield, Clemson University, SC, USA). Roots were considered dead when a root was observed to have shriveled to approximately half the original diameter. As such, some roots may have been functionally dead before they were classified as dead in our analysis, which could potentially lead to overestimations of root lifespans. However, we expect this overestimation to have been minimal as a result of relatively active decomposition rates under the warm, mesic conditions of the common garden site. Additionally, because death was based on the original diameter of the observed roots, this process minimized the potential for species bias in determining root lifespan. New roots falling outside the measured diameter range of the 1st and 2nd order roots of each species were excluded from analyses (McCormack *et al*. 2012) thereby controlling for encroachment of roots from neighboring species plots. Additionally, roots observed in the first imaging session were not included in the estimation of root lifespan because their birth date was unknown. The number of roots analyzed from this three-year study were as follows: POTR *n*=564, 337 from N-fertilized tubes and 227 from tubes receiving only water; ACNE *n*=346, 191 from N-fertilized tubes and 155 from tubes receiving only water; LITU *n*=149, 96 from N-fertilized tubes and 53 from tubes receiving only water; SAAL *n*=95, 45 from N-fertilized tubes and 50 from tubes receiving only water

To ascertain the level of localized N fertilization to be applied to the fertilized patches, we averaged the three highest values of soil solution N found in the common garden (n = 32).

We averaged the highest soil solution N values found, under the assumption that plants would forage preferentially for the higher N. Soil solution N was determined using a saturated paste approach. Briefly, in July of 2007, 25-g soil samples were collected to a 10-cm depth in two blocks for each of the 16 species at a distance of 50 cm from the trunk of the tree. Soil was moistened with water until the surface glistened and then centrifuged (10,000 rpm for 20 min). The supernatant was analyzed for nitrate and ammonium using a Lachat Quikchem 8500 autoanalyzer (Hach Co., Loveland, CO, USA). Using this approach we determined that the available nitrogen at the common garden in naturally occurring high-N patches averaged 11.4 mg N L^{-1}. Previous studies on tree seedling growth on similar soil at a site adjacent to the common garden found low soil nitrogen values (0.13 to 0.15 mg nitrate per kg soil) and low seedling foliar N content, leading the investigators to conclude that nitrogen was limiting at the site (Harpster 2011).

In June, 2007 root in-growth cores were installed by pounding a 7-cm internal diameter (ID) steel tube into the ground to a depth of 30 cm. Soil was removed from the core, sieved of existing roots, and returned to the hole. Five in-growth cores, corresponding to 5 N fertilization levels (0, 3, 10, 20, and 30 times soil solution N (11.4 mg N L^{-1})), were created in 4 blocks of 3 species (*A. negundo*, *P. tremuloides*, and *L. tulipifera*) resulting in 60 in-growth cores total. *Sassafras albidum* was not used in the in-growth core portion of the experiment due to the low root densities observed for this species which would have prevented collection of adequate root samples for analyses. In-growth cores were marked with color coded 7.5-cm ID PVC pipe cut into 2.5-cm-high rings, which also served as reservoirs for the fertilization solution. Each in-growth core received 100 ml of 1 of the 5 levels of N weekly which allowed for soil saturation to roughly 30 cm. After approximately 3 months, a smaller 5-cm ID steel tube was pounded inside

the existing core to a depth of 20 cm. Immediately after coring occurred, one to two small intact first- and second-order root branches (where first-order roots are distal) were dissected from the total root pool, rinsed in DI water to remove any attached soil and analyzed for respiration using a Clark-type oxygen electrode (Hansatech Oxygraph, King's Lynn, UK). The use of intact first- and second-order root branches minimized root wounding and any resulting effects on root respiration. After the respiration measurement, roots were frozen, freeze dried, weighed to obtain dry mass, and then ground with a mortar and pestle for N:C analysis (Fisons EA 1108 CNS-O Analyzer, Fisons Instruments, Mt. Pleasant, NJ, USA). Roots used to determine specific root length (SRL) and root diameter by branching order were dug directly from the soil beneath the species of interest from 4 blocks in the spring of 2009. Roots were cleaned of soil with water, dissected to order, and scanned on an Epson Perfection 4490 desk top scanner. Root length and diameter was then obtained from the scanned images using WinRhizo software (Regent Instruments, Quebec City, Quebec, Canada). After scanning, the roots were oven dried at 60°C for 24 hrs and weighed. Specific root length ($m\ g^{-1}$) was calculated by dividing the length of the root sample by its dry mass.

Statistical analyses and root lifespan determination were conducted using SAS JMP 9.02 (SAS Institute Inc., Cary, NC, USA). Log-rank tests were used to determine the significance of fertilization on root lifespan for each species (Fig. 1). Cox proportional hazards tests (Cox, 1972) were used to identify fine root traits that had a significant effect on fine root lifespan (Table 1). These traits included rooting depth, number of neighboring roots, season of birth, and any resulting interactions with the N fertilization treatment. Rooting depth was determined by the depth of the root observed through the minirhizotron tube. The number of neighboring roots refers to the number of additional roots observed in same viewing pane of the minirhizotron tube.

Season of birth refers to a categorical assignment based of root birth date, where roots born between April and June of any year were assigned to one category, roots born between July and September were assigned to a second category, and roots born after September were assigned to a third category. Differences in cumulative root length from the in-growth core samples were determined by conducting a two-way ANOVA comparing the cumulative root length produced on the final minirhizotron image session across blocks (Fig. 2). Two-way ANOVAs with interactions were run to determine differences in root respiration, root length, and root N:C between species and treatment (N Fert. or H_2O) (Fig. 3). Results were considered statistically significant at $P \leq 0.05$.

Results

Root lifespan was significantly increased by N fertilization in the two fine-root species, *P. tremuloides* (POTR *P*=<0.0001, 1st order root SRL = 65.2 ± 2.1 m g^{-1}, diameter = 0.17 ± 0.003 mm) and *A. negundo* (ACNE *P*=0.0009, 1st order root SRL = 44.5 ± 2.7 m g^{-1}, diameter = 0.23 ± 0.007 mm), but had no significant effect on the lifespan of the two coarse-root species, *L. tulipifera* (LITU *P*= 0.14, 1st order root SRL = 8.9 ± 1.3 m g^{-1}, diameter = 0.67 ± 0.01 mm) and *S. albidum* (SAAL *P*=0.79, 1st order root SRL = 13.8 ± 0.9 m g^{-1}, diameter = 0.54 ± 0.05 mm: Figure 1). However, the non-significant result of localized N fertilization on LITU root lifespan may be related to the reduced statistical power compared to the fine-root species because of the smaller number of roots observed for LITU. Median root lifespan of the fertilized fine-root species increased by 48% or from 69 d to 102 d for POTR and 40% or from 113 d to 188 d for ACNE. Median root lifespan of the coarse-root species for water control and fertilized roots were 284 d and 309 d for LITU (9% increase) and were 304 d and 310 d (2% increase) for SAAL (*P*= 0.14 and 0.79, respectively).

The effect of N fertilization on root production was assessed in two ways: by direct observation of the fertilized and unfertilized minirhizotron tubes and by measuring the total root mass of roots in the in-growth cores receiving the 5 levels (0 through 30 times background N) of nitrogen fertilization and converting root mass to root length. Nitrogen fertilization did not significantly affect cumulative root length production observed using the minirhizotron tubes (Figure 2: N Fert. Effect: P=0.35; Species effect: P = 0.28, Species x N Fert. Interaction: P=0.94.). We also examined root growth responses using in-growth cores (Figure 3, c). In-growth root mass was converted to length using species-specific, first-order-root SRL values and assuming first-order roots accounted for 50% of the total root mass in the in-growth cores which corresponded reasonable well observations of the dissected root samples. Localized nitrogen fertilization caused a significant increase in root length (P=0.0052; P-values were obtained from differences across blocks in the cumulative root length produced on the final minirhizotron image session), with fertilization levels of 30 times soil solution N resulting in 2.5 times more root length in LITU, 3 times more root length in POTR, and almost 5 times more root length in ACNE compared to the 0 or 3 times solution N (no data available for SAAL). Tree species differed in in-growth core root length consistently across all N levels with LITU having the shortest length and POTR the longest (Species effect: P<0.0001). In all three species there was no significant difference in in-growth core root length between 0 and 3 times N fertilization, which was consistent with the lack of significance in cumulative root length production observed in our minirhizotron tubes (Figure 2).

Contrary to expectations, N fertilization did not affect the N:C ratio (used as a proxy for N concentration because N:C ratio avoids errors in dry wt. estimation from soil contamination on the root surfaces) of the 1st- and 2nd-order roots from the in-growth cores (Figure 3, b: N Fert.

Effect: P= 0.15). However, root N:C ratio differed among tree species, with the lowest N:C ratio in LITU and the highest in ACNE (Species effect: P<0.0001).

Nitrogen fertilization did not significantly increase root respiration across species (Figure 3, a: N Fert. Effect: P= 0.22). In addition, no significant correlation was found between root N:C and root respiration across all treatments (P=0.54). As with root N:C , there were significant species differences in respiration, with LITU exhibiting the fastest root respiration and POTR the slowest (Figure 3, a: Species effect P= 0.01).

In addition to examining the main effect of localized N fertilization on root lifespan, we also examined the effect of rooting depth, number of neighboring roots, season of birth, and any resulting interactions on root lifespan using Cox proportional hazard analyses (Table 1). Root diameter was not included in this analysis since our strict diameter criteria (see methods section) for roots to be included in our data set precluded the inclusion of a wide range of root diameters. Rooting depth only significantly affected root lifespan for two of the four study species (ACNE and POTR), with roots living deeper in the soil having a decreased risk of death and therefore living longer (i.e. having a hazard ratio less than one). The lack of a consistent effect of rooting depth on root lifespan across all species studied may be an artifact of the relatively short minirhizotron tubes (i.e. 45 cm, allowing for root observations of the upper 20 cm of soil) used in this study. Number of neighboring roots also significantly affected lifespan for two of the four species investigated (ACNE and POTR), with roots living with more neighbors having shorter lifespans. These species also had the highest root density observed through the minirhizotron tubes. As such, competition among neighboring roots may negatively affect lifespan at high root densities. The seasonality of root birth significantly affected root lifespan for one of the four

species investigated (POTR), with roots born later in the season having longer lifespans than those born earlier in the season.

In addition to the main effects on root lifespan, there was a significant interaction between localized nitrogen fertilization and the season of root birth in both fine-root species (ACNE and POTR, Table 1). For ACNE, fertilized roots had significantly longer lifespans when born early (April- June) or late (after September) in the season but not over the summer (July – September). For POTR, fertilized roots had significantly longer lifespans when born during the spring (April- June) and the summer (July – September), but not later in the season (after September). For POTR, there was also a significant interaction between N fertilization and the number of neighbors. The number of neighboring roots only significantly affected survivorship when one to three, or greater than six, neighboring roots were present, but not when zero or four to six neighbors were present. In general, although competition from neighboring roots may interact with the N fertilization treatments resulting in a significant interaction, we are unsure how the exact number of neighboring roots causes significant differences in this interaction.

Discussion

In this common garden study, we created sustained, localized, nitrogen-rich patches in which root lifespans could be observed and root samples could be collected to estimate shifts in N concentration and respiration. We found that the root lifespan of the fine-root species was significantly increased by localized N fertilization, whereas the root lifespan of the coarse-root species was either virtually unchanged by N fertilization (SAAL) or possibly modestly increased for which we lacked the statistical power to detect (LITU) (Figure 1). Surprisingly, we observed no increases in root length in response to a three-fold increase in localized N fertilization, but did

observe root length increases at higher levels of N fertilization (Figure 3, c). In addition, we found that neither root N:C nor root respiration increased with N addition (Figure 3, a and b).

Assuming resource optimization, we hypothesized that root lifespan should maximize the lifetime root efficiency or the lifetime benefits relative to the lifetime costs (Yanai *et al*. 1995). Therefore, roots growing in the N-rich patches that do not become depleted should be longer lived because they presumably are supplying more of the limiting resource, N, than roots elsewhere on the tree that are foraging in less fertile soil. Additionally, resource optimization predicts that species with coarse, low-SRL roots should have longer root lifespans because these roots are more costly, in terms of carbon, to construct for the deployment of length or surface area (Yanai *et al*. 1995). In both cases we found support for the resource optimization hypothesis. Species with coarse, low SRL roots had longer median lifespans than species of high SRL (Figure 4) and high-SRL species had significantly longer median lifespans in N fertilized verses unfertilized patches (Figure 1). At the same time, the cost of maintaining these roots, as measured by respiration, was not significantly altered by fertilization (Figure 3a). However, because the result of increased root lifespan with N fertilization was not seen across all species, additional factors beyond rapid adjustments in root system resource optimization might be influencing root lifespan in some species.

Our results suggest that a key difference in root lifespan between species in response to N fertilization is in variation in plasticity of the physiological traits that control root lifespan. High-SRL species clearly responded to localized N fertilization by extending their lifespan, in contrast to the low-SRL species; where we saw no response in one species (SAAL) and a relatively small, non-significant response in the other species (LITU) (Figure 1). This plasticity of the fine-root species may offset the limitations of their shorter median root lifespans, thereby

conferring an increased ability to utilize resource heterogeneity. Based on these findings, there appear to be clear species-specific differences in plasticity in root lifespan. The mechanisms behind these observed differences in root lifespan plasticity were not directly studied here, but may arise from differences in the ability to mobilize defense compounds, differences in colonization of mycorrhizal fungal symbionts, or differences in the ability to scavenge reactive oxygen and reactive nitrogen species.

Although plasticity in plant traits has been studied in aboveground structures, relatively little experimental work has been conducted examining root plasticity under field conditions (see Hodge 2004, 2009), and to our knowledge no studies have examined the relationships among root lifespan, diameter, plasticity, and localized nutrient availability. Our results suggest that a tradeoff may exists between phylogenetically constrained root morphology (Comas and Eissenstat 2009; Chen et al. 2013) and root plasticity. Although we did not set out to test the positive relationship between species growth rate and root plasticity (Grime 1977), in our study system it does not appear that the root plasticity we observed is correlated with species growth rate since all of the species examined have high relative growth rates. In fact, LITU is one of the fastest growing trees in the common garden in terms of height and trunk diameter growth; yet the lifespan responses of LITU roots to fertilization were relatively modest and not statistically significant. Additionally, since the tree species investigated co-occur naturally and are known to inhabit moderately fertile soils, the differences in root morphology and the corresponding differences in root lifespan plasticity were not related to adaptations to different soil fertility levels.

Root proliferation in response to N fertilization was generally low in this study and relative responses were fairly similar across species. Localized N fertilization rates at three

times that of the high range of naturally occurring available soil solution N caused no enhanced proliferation in either the in-growth cores or minirhizotrons, despite the strong lifespan responses in the fine-root species. At higher rates of N addition in the in-growth core portion of our study, we observed an increase in root length density for all tree species, with a non-significant tendency for fine-root species to exhibit slightly more proliferation than coarse-root species (P= 0.40). The greater root proliferation in disturbed soil of fine- root species compared to coarse-root species had been previously observed in a common garden study of citrus roots with a common shoot cultivar (Eissenstat 1991); however, the influence of N fertilization was not examined. In a study in a nearby mixed forest stand near our common garden experiment, fine-root species had greater root proliferation in disturbed soil patches compared with coarse-root species, but again fine-root species only showed a tendency of greater root proliferation to fertilization than coarse-root species (Eissenstat *et al*. unpubl. data; P = 0.19). Collectively these field studies suggest that while root morphology may influence plasticity in root proliferation in response to N fertilization, the response is generally weak compared to the natural variability in proliferation.

In conclusion, our study clearly indicates that plant species vary in root lifespan responses to nutrient-rich patches. We found that root lifespan was clearly increased by N-rich patches in tree species with fine root morphology and was negligibly increased in one of the two species with coarse root morphology. Additionally, the low-SRL species, whose roots are more costly to construct, in general had longer lifespans than high-SRL species. Additional studies are needed to confirm the potential linkages of root morphology with root proliferation and root lifespan.

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Figure 3.1: Survival probability curves of roots in localized patches of four tree species that varied widely in root morphology (*P values* indicate significance of fertilization effect for each species analyzed separately). Tree species were *Acer negundo* (ACNE), *Populus tremuloides* (POTR), *Liriodendron tulipifera* (LITU), and *Sassafras albidum* (SAAL). The sustained N-fertilized patch treatment (N Fert., closed circles with solid line) was three times background soil solution N, with the control patch (H_2O, open circles with dashed line) contained no additional N.

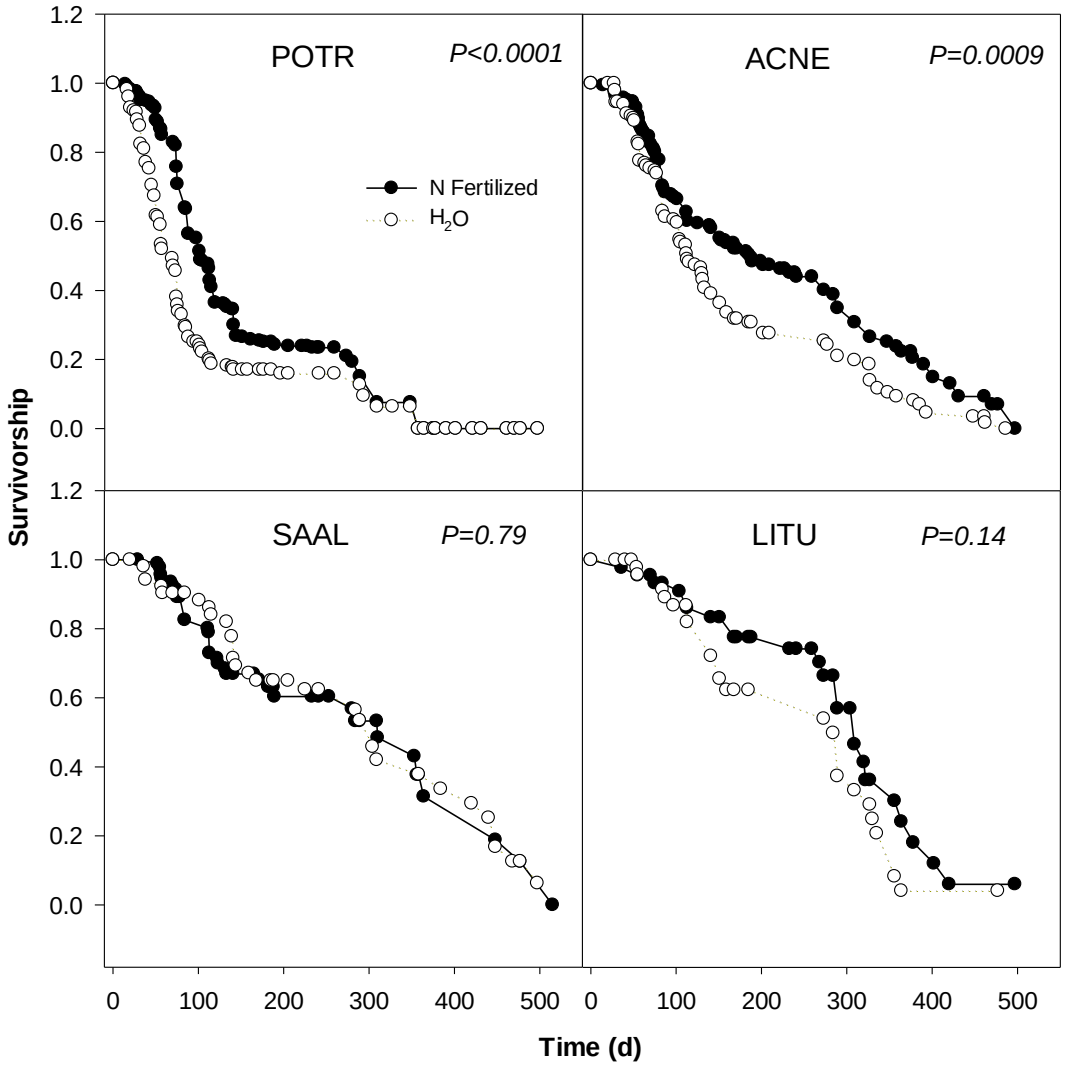

Figure 3.**2**: Cumulative root length production assessed with minirhizotrons over three years and, of four tree species that varied widely in root morphology. The N fertilization treatment (N Fert., closed symbol) was three times soil solution N, with the control (H_2O, open symbol) not containing additional N. P-values obtained from differences across blocks in the cumulative root length produced on the final minirhizotron image session. Tree species were *Acer negundo* (ACNE), *Populus tremuloides* (POTR), *Liriodendron tulipifera* (LITU), and *Sassafras albidum* (SAAL).

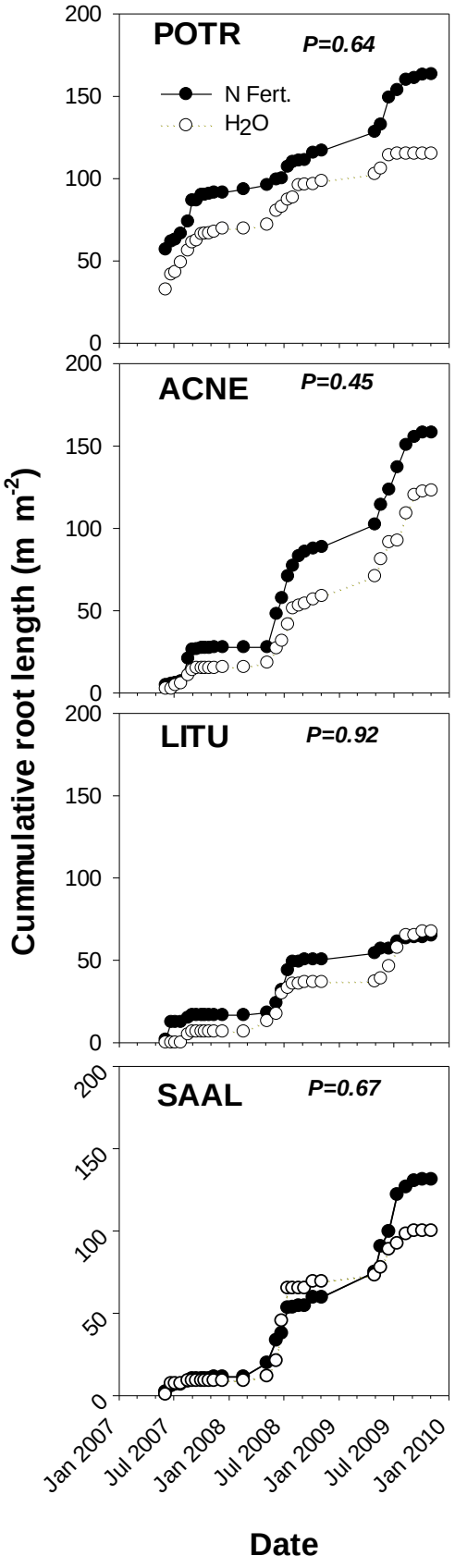
POTR
P=0.64
N Fert.
H2O
ACNE
P=0.45
LITU
P=0.92
SAAL
P=0.67
Cummulative root length (m m-2)
0
50
100
150
200
Jan 2007
Jul 2007
Jan 2008
Jul 2008
Jan 2009
Jul 2009
Jan 2010
Date

Figure 3.3: Effects of multiple levels of N addition on root respiration, N:C ratio and root length from ingrowth cores of three tree species that vary widely in root morphology**.** Tree species denoted as: POTR = squares, ACNE = triangles and LITU = circles. **Panel a:** the relationship between N fertilization level (as a multiple of soil solution N) and root respiration (species effect *P=0.01*, N fertilization effect *P=0.23*). **Panel b**: the relationship between N fertilization level, as a multiple of soil solution N, and root N:C (species effect *P<0.0001*, N fertilization effect *P=0.15*). **Panel c**: the relationship between N fertilization level and root length which was calculated from root mass using species specific first order SRL values (species effect *P<0.0001*, N fertilization effect *P=0.005*). Error bars denote standard errors.

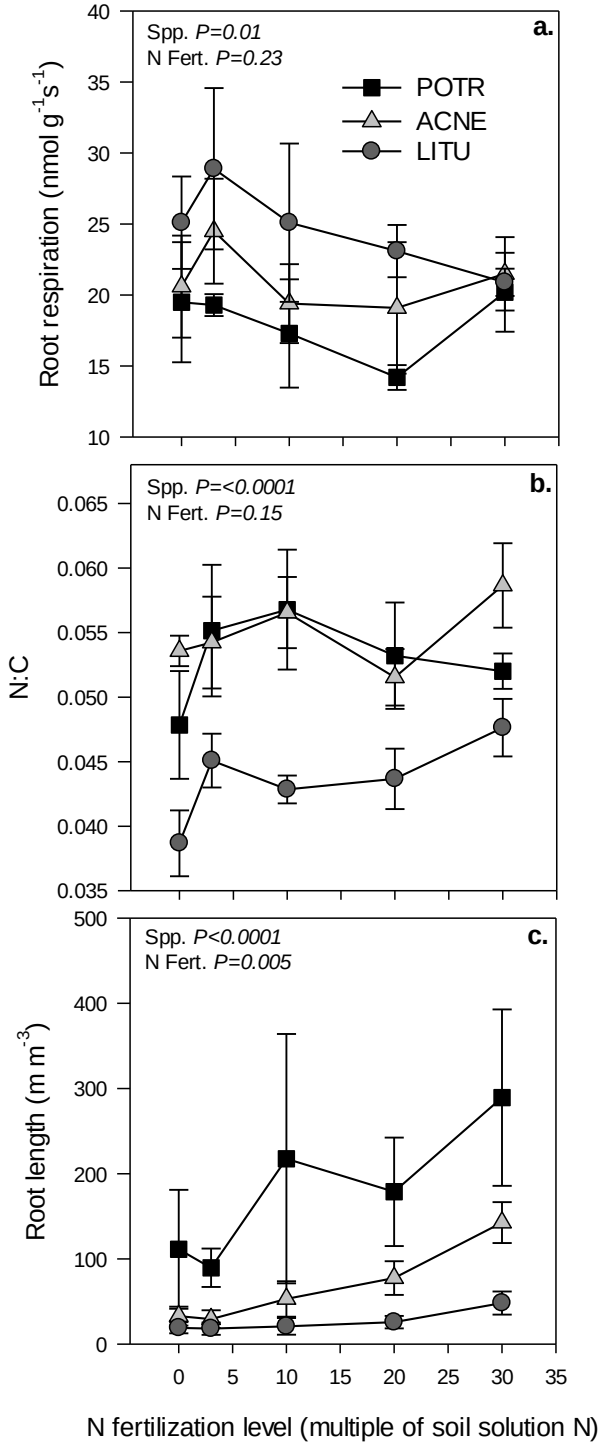
a.
Spp. P=0.01
N Fert. P=0.23
POTR
ACNE
LITU
Root respiration (nmol g-1s-1)
40
35
30
25
20
15
10
b.
Spp. P=<0.0001
N Fert. P=0.15
N:C
0.065
0.060
0.055
0.050
0.045
0.040
0.035
c.
Spp. P<0.0001
N Fert. P=0.005
Root length (m m-3)
500
400
300
200
100
0
0
5
10
15
20
25
30
35
N fertilization level (multiple of soil solution N)

Figure 3.4: Relationship of median root lifespan with root diameter of four tree species that varied widely in root morphology. Tree species denoted as: POTR = squares, ACNE = triangles, LITU= circles and SAAL = hexagons. The N fertilization treatment (N Fert., closed symbol) was three times soil solution N, with the control (H_2O, open symbol) not containing additional N. Regression line equation: y = 508.43x + 6.02. Error bars denote standard errors.

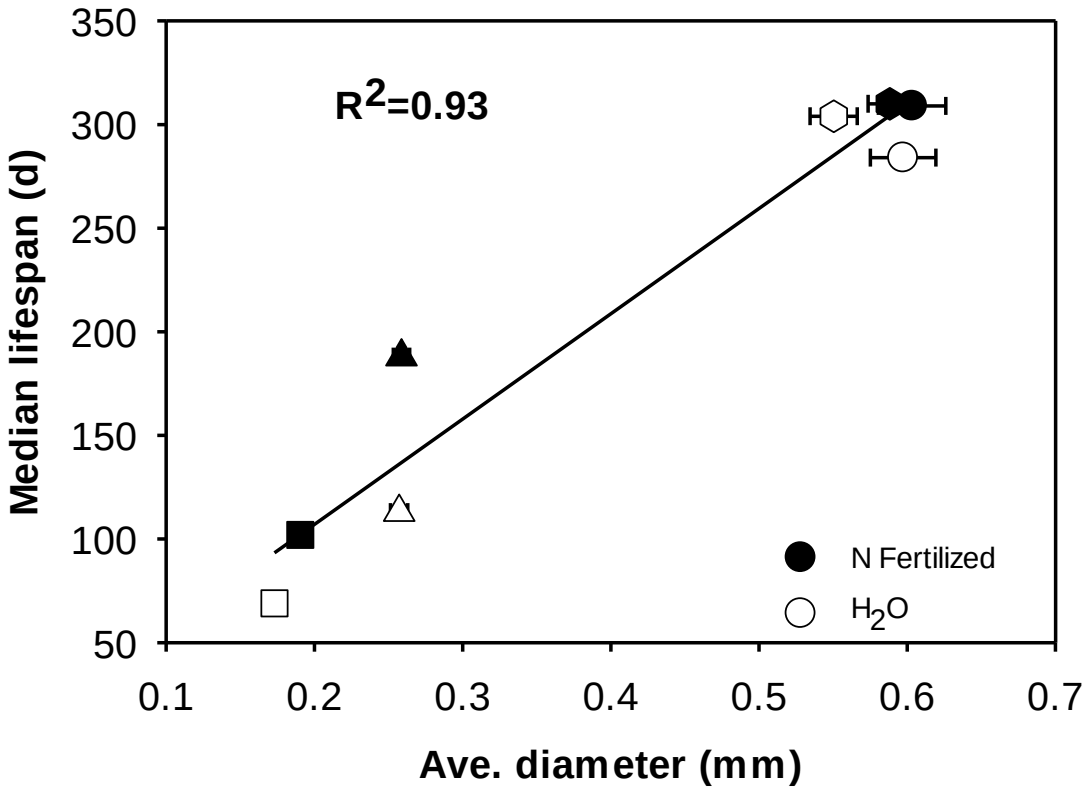

Table 3.1: Data table showing *P*-values with Cox proportional hazard risk ratios in parentheses for the four tree species studied and four factors (N fertilization, rooting depth, number of neighbors, and season of birth) plus resulting interactions. Season of birth has three risk ratios per species corresponding to the three birth categories used (i.e. April-June, July-September and after September). Significant results ($P \leq 0.05$) are in bold.

	ACNE	POTR	LITU	SAAL
N Fertilization	**0.0256** (0.73)	**<0.0001** (0.53)	0.2145 (0.69)	0.8608 (0.96)
Rooting depth	**0.0131** (0.97)	**<0.0001** (0.92)	0.0791 (0.95)	0.0573 (0.94)
# Neighboring roots	**0.0060** (1.06)	**0.0133** (1.01)	0.2534 (0.82)	0.2478 (1.12)
Season of Birth	0.2500 (0.94, 1.36, 1.44)	**<0.0001** (0.60, 0.21, 0.34)	0.7175 (1.14, 1.90, 1.66)	0.3117 (0.69, 1.13, 1.64)
N Fertilization X Rooting depth	0.6056	0.5542	0.0987	0.0593
N Fertilization X # Neighboring roots	0.1375	**0.0007**	0.9337	0.4182
N Fertilization X Season of Birth	**0.0088**	**0.0010**	0.6714	0.9284

Chapter 4

On the controls of root lifespan: assessing the role of soluble phenolics

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Summary

- Despite the importance of fine-root lifespan to many community- and ecosystem-level processes, a comprehensive understanding of the actual controls of fine-root lifespan, beyond a general framework of life history strategy and optimality, remains elusive.

- In addition to multiple above- and belowground abiotic factors, root herbivory can be an important determinant of root lifespan.

- In this study, utilizing nine temperate trees species in a common garden setting, we investigated the relationship between fine-root soluble phenolic content, a putative measure of chemical defense against herbivory, and explicit factors that have previously been related to fine-root lifespan.

- We hypothesized that fine-root soluble phenolic content would be positively related to factors previously shown correlated with increase root lifespan including increased fine-root branching order, diameter, rooting depth, localized nitrogen availability, and tree growth rate.

- Consistent with our hypothesis, root soluble phenolic content significantly increased with increasing branching order ($P<0.001$). However phenolic content significantly decreased with increasing N fertilization ($P=0.002$) in the nine tree species we examined, despite previous work indicating increased lifespan in N-enriched patches. Moreover, we found no other significant relationships between fine-root soluble phenolic content and any of the other factors investigated.

- While this study provides detailed information of sources of variation in soluble phenolic content in roots, we were unable to find general utility in using a Folin-Denis-based soluble-phenolic assay to increase our understanding of the factors associated with fine-root lifespan.

Introduction

As much as one third of global terrestrial net primary productivity is devoted to the production of fine roots (Jackson *et al.*, 1997), with root respiration accounting for up to 60% of total soil respiration (Pregitzer *et al*., 1998). As a result, understanding the factors that control fine-root lifespan is critically important to understanding many community- and ecosystem-level processes. Multiple above- and belowground drivers affect fine-root lifespan (Figure 1). Aboveground abiotic and biotic conditions can influence resource allocation between shoots and roots which in turn can affect fine-root production, maintenance, and lifespan (Eissenstat and Duncan 1992; Reich, 2002; Enquist and Niklas, 2002). Belowground, abiotic and biotic factors also influence fine-root lifespan. Extreme soil conditions outside the physiological tolerances of fine roots can affect root lifespan. For example, prolonged drought can cause reduced root lifespan or death (Huang and Nobel, 1992; Espeleta and Eissenstat, 1998; Meier and Leuschner, 2008; Bauerle *et al*., 2008). Like other plant tissues, fine roots are also susceptible to attack by herbivores and pathogens, which can cause considerable damage leading to root and whole-plant mortality (Stanton, 1988; Kosola *et al*., 1995; Eissenstat *et al*., 2000; Wells *et al*., 2002). Belowground herbivory can have greater deleterious effects on plant fitness than aboveground herbivory and can act as an important determinant of fine-root lifespan (Brown and Gange, 1990; Stevens and Jones, 2006; Rasmann and Agrawal, 2008; Zvereva and Kozlov, 2011). Symbionts, such as mycorrhizal fungi, can also influence root lifespan (Guo *et al.*, 2008). Root fitness can be enhanced or diminished by ecotmycorrhizal associations which range from biotrophic to saprotrophic (Koide *et al*., 2006). Furthermore, maintaining existing roots represents significant carbon costs via metabolic (Lambers *et al*., 1996) and defense allocation; both mobile (Kraus *et*

al., 2004) and structural (Zadworny and Eissenstat, 2011). Additionally root metabolism can result in the formation of potentially damaging free radicals such as reactive oxygen / nitrogen species (ROS/RNS) that may, in turn, affect root longevity (Smithwick *et al*., 2013). Individually and in concert, these above- and belowground abiotic and biotic forces influence fine-root lifespan. Although it has been hypothesized that optimal fine-root lifespan is governed by some ecologically stable strategy (Smith and Price, 1973; Dybzinski *et al*., 2011) whereby the cost of maintaining a root is weighed against the benefits the root provides (Yanai *et al.*, 1995; Eissenstat and Yanai, 1997), a comprehensive understanding of the actual controls of fine-root lifespan, beyond this general framework, remains elusive.

With the increased use of minirhizotrons to study fine-root lifespan, certain individual factors have been shown experimentally to influence fine-root lifespan. For example, roots that are of coarser diameter or of higher branching order typically live longer than finer diameter or lower-order roots (Majdi *et al*., 2001; Gill *et al*., 2002; Wells *et al*., 2002; Anderson *et al*., 2003; Guo *et al*., 2008). Additionally, roots growing at greater soil depths typically live longer than those at shallower depths (Kosola *et al*., 1995; Wells and Eissenstat, 2001; Majdi *et al*., 2001; Gill *et al*., 2002; Anderson *et al*., 2003; Withington *et al*., 2006, Pritchard *et al*., 2008). Increased localized nitrogen (N) availability can increase root lifespan (Pregitzer *et al*., 1993; Adams *et al*., 2013). Also, faster growing species tend to have shorter lived roots than slower growing species (Ryser, 1996; Schläpfer and Ryser, 1996; McCormack *et al*., 2012). These explicit factors that have been shown to affect fine-root lifespan provide us with a starting point for more detailed investigations of the general drivers of fine-root lifespan mentioned above.

Polyphenols are the most widely distributed class of plant secondary metabolites (Hattenschwiler and Vitousek, 2000) and phenolic compounds have been studied extensively in

the context of herbivore defense in aboveground plant tissues for decades (Feeny, 1970; Cates and Rhoades, 1977). Recent studies have also investigated the role that phenolics play in plant physiology, soil nutrient dynamics, plant-plant interactions, and plant-mycorrhizal interactions (Kraus *et al*., 2003). Phenolic compounds are ubiquitous in the environment, are found in all plants (Appel, 1993), and can account for up to 40% of the dry weight of leaves and bark (Kraus *et al.*, 2003). In general, levels of phenolic compounds observed in fine roots are lower than that of leaves (Kaplan *et al*., 2008) but still act as an important chemical defense mechanism against root herbivores (Potter *et al*., 2000; Stevenson *et al*., 2009). Despite this, relatively few studies have investigated the role of phenolic compounds in roots.

For this study we considered fine-root lifespan in the context of herbivory/parasitism. Specifically, we looked at the relationship between fine-root soluble phenolic content, a putative measure of chemical defense against herbivory, and explicit factors that have previously been related to fine-root lifespan. Across nine northern temperate tree species, we hypothesized that fine-root soluble phenolic content would be positively related to increased fine-root branching order, diameter, rooting depth, and localized nitrogen availability, factors previously shown correlated with increase root lifespan. We also examined the relationship between whole-tree growth rate and fine-root soluble phenolic content. Collectively these comparisons allowed us to evaluate the strength of the linkages between soluble phenolic content and patterns of variation in fine root lifespan.

Methods

All studies were conducted at a common garden planting that minimized abiotic environmental variation across tree species and allowed for a well-replicated experimental design. The garden was located in central Pennsylvania, USA at the Russell E. Larson Agricultural Research Center, Pennsylvania State University (40.8°N, 77.9°W). The common garden consists of 16 species of trees that were planted mostly in 1996 as 1-yr-old liners in a randomized complete block design with 8 blocks. Details about the common garden can be found in McCormack *et al.* (2012) and Adams *et al.* (2013). Data used in this study regarding first-order root diameter and tree growth rate, expressed as ten-year diameter growth at breast height (dbh), for the common garden tree species have been reported previously by McCormack *et al.* (2012).

In June, 2008 root in-growth cores were installed by pounding a 7-cm internal diameter (ID) steel tube into the ground to a depth of 30 cm approximately 0.5 m from the base of the study tree. Soil was removed from the core, sieved of existing roots, and returned to the hole. Three in-growth cores for each of 3 N fertilization levels (0, 3, and 30 times soil solution N (11.4 mg N L^{-1})) (Adams *et al.*, 2013), were created in 4 blocks in each of 6 species (*Acer negundo* (ACNE), *Acer rubrum* (ACRU), *Acer saccharum* (ACSA), *Quercus rubra* (QURU), *Quercus alba* (QUAL) and *Pinus virginiana* (PIVI)). In-growth cores were marked with 7.5-cm ID PVC pipe cut into 2.5-cm-high rings, which also served as reservoirs for the fertilization solution. Each in-growth core received 100 ml of 1 of the 3 levels of N weekly, which allowed for soil saturation to roughly 30 cm. After approximately 3 months, a smaller 5-cm ID steel tube was pounded inside the existing core to a depth of 20 cm. Root in-growth cores were again installed in June, 2009 for 3 additional species (*Liriodendron tulipifera* (LITU), *Populus tremuloides*

(POTR), and *Sassafras albidum* (SAAL)) following the same procedure described above. The resulting soil cores were placed in labeled plastic bags and kept frozen. The three in-growth cores per N fertilization treatment per block were pooled for adequate root sample size. The soil cores were later rinsed with water using a 2-mm sieve to isolate the roots. The roots were then dissected to branching order, freeze dried, and ground with a mortar and pestle. The resulting samples were weighed on a microbalance and placed in capped disposable 50ml tissue culture tubes with 1ml of 50% acetone. Samples weighing less than 5mg were not used. The tubes were then placed on a shaking rack at 300rpm for 24hrs. A volume of 0.1ml of the resulting supernatant was used to measure total soluble phenolic content as tannic acid equivalents using the Hach Tanniver method (method no. 8193, Hach, Loveland, CO, USA) (see Jonsson *et al.*, 2006) which is a modified Folin-Denis approach.

In May 2009 soil cores were taken 0.5 m from the base of three *L. tulipifera* trees in four blocks to a depth of 60cm using 5cm internal diameter Giddings soil corer (Giddings Machine Co., Windsor, CO, USA). Individual cores were divided into 10 cm depth increments. Roots were cleaned of soil and analyzed for phenolic content as described above. This process was repeated in 2013 for *A. negundo* in seven blocks. Also in May, 2009 "pioneering" and fibrous first-order roots were sampled from existing *L. tulipifera* root boxes (see Zadworny and Eissenstat, 2011). Root boxes were again utilized in 2012 to sample *P. tremuloides* roots of known age to examine the relationship between root age and soluble phenolic content. In this experiment, root boxes were placed between two *P. tremuloides* trees in eight blocks with one viewing window facing each of the two trees. One window per box received weekly N fertilization consisting of 1L of 3 times soil solution N (11.4 mg N L^{-1}) and the other window received 1L of water. The age of the roots growing against the viewing windows was assessed

by weekly tracing using different colored paint pens (Zadworny and Eissenstat, 2011). All roots sampled from both the soil cores and the root boxes underwent the same process of rinsing with water, freeze drying, grinding, and Hach Tanniver method to determine total soluble phenolic content as described above.

Results from each study were analyzed using an ANOVA using SAS JMP 9.02 (SAS Institute Inc., Cary, NC, USA) and were considered statistically significant at $P \leq 0.05$.

Results

Root soluble phenolic concentration significantly increased with increasing branching order ($P<0.001$) (Figure 2). However contrary to our hypothesis, phenolic concentration significantly decreased with increasing N fertilization ($P=0.002$) in the nine tree species we examined (Figure 3). A significant decrease in fine-root soluble phenolic concentration associated with increased localized N fertilization was also observed in the *P. tremuloides* samples taken from the root box study examining the effect N on soluble phenolic concentration controlling for root age ($P= 0.02$)(Figure 4E). Neither rooting depth (*L. tulipifera* $P=0.40$, *A. negundo* $P=0.41$) nor the type of root sampled (pioneering vs fibrous) (*L. tulipifera* $P=0.52$) significantly affected root soluble phenolic concentration (Figure 4B, D). Additionally in the nine tree species examined, we found no evidence that the species-specific growth rate, expressed as the 10-year dbh ($P=0.53$), nor the diameter of first-order roots across these species ($P=0.21$) significantly affected root soluble phenolic concentration (Figure 4A, C). Finally, we also found no evidence that age of first-order roots over the 1.5 month study duration significantly affected root soluble phenolic concentration (*P. tremuloides* $P=0.85$) (Figure 4E).

Discussion

The controls of fine-root lifespan are poorly understood, but certain factors such as rooting depth, root branching order, root diameter, species growth rate, and localized nitrogen availability commonly have been shown to affect fine-root lifespan (Adams *et al*., 2013; Chen and Brassard, 2013; McCormack *et al*., 2012). Additionally, root herbivory can be a significant driver of fine-root lifespan in many systems (Wells *et al*., 2002; Stevens and Jones, 2006; Rasmann and Agrawal, 2008; van Dam, 2009). We therefore hypothesized that there would be a positive relationship between fine-root soluble phenolic concentration, a general metric of chemical defense against herbivory, and factors that have been shown to enhance fine-root lifespan.

Across the diverse species we investigated, we found strikingly consistent patterns of variation in fine-root soluble phenolic concentration. Fine-root soluble phenolic concentration was significantly positively correlated with root branching order (Figure 2), despite species differences in phylogeny, root morphology, and mycorrhizal associations (i.e. arbuscular mycorrhizal verses ectomycorrhizal fungi). Although roots of higher branching order must be older than the lower order roots they support, the increase in phenolic content with branching order does not appear to be explicitly driven by root age as no significant differences in soluble phenolic concentration were observed with increased root age in first-order roots (Figure 4E). Consistent with our hypothesis, roots of higher branching order have enhanced chemical defenses against herbivory compared to lower-order roots which in turn may be related to the observed increased longevity of roots of higher branching order.

Despite evidence linking increased fine-root longevity with localized N availability for some species in our study system (Adams *et al.*, 2013), we found a significant negative

relationship between N availability and fine-root soluble phenolic concentration (Figure 3), the opposite trend from our hypothesis. This negative relationship between fine-root soluble phenolic concentration and increased N availability may result from increased root growth at the expense of secondary metabolite production as predicted by the Carbon:Nutrient Balance Hypothesis (Bryant *et al*., 1983). In a study examining the effects of localized N availability on fine-root lifespan, using the same levels of N fertilization in the same common garden setting, root growth significantly increased with increased N availability in all of the species examined (Adams *et al*., 2013). Although not measured, the negative relationship between fine-root soluble phenolic concentration and localized N availability could also represent a shift from carbon- to nitrogen-based chemical defenses (i.e. from phenolics to alkaloids) (Bryant *et al*., 1983).

We found no other significant relationships between fine-root soluble phenolic concentration and any of the other factors investigated (i.e. rooting depth, species specific first-order root diameter, root type (pioneer vs. fibrous) and tree growth rate) (Figure 4). Although there is ample aboveground evidence linking tissue phenolic content with reduced herbivory (Feeny, 1970; Hartley and Firn, 1987; Forkner *et al*., 2004; Fine *et al*., 2006), we found no general relationship between the factors that affect fine-root longevity and soluble phenolic concentration. Based on our findings, either root herbivory is not a major driver of the variability in fine-root lifespan in our study system, soluble phenolic concentration is not an adequate measure of fine-root chemical defense against herbivory, or the Folin–Denis assay of soluble phenolics is not sufficiently robust to capture subtle variations in mobile carbon-based root defenses. As an example, simultaneous increases and decreases in the multiple compounds that comprise the total fine-root soluble phenolic pool could occur without an overall change in

magnitude of the phenolic pool itself (Appel *et al*., 2001; van Dam, 2009). Additionally, fine roots could be utilizing other means of herbivore deterrence or avoidance. Perhaps differences in structural defenses such as increased hypodermal cell layers with thickened tangential cell walls or decreased passage cell numbers as seen in pioneering roots (Zadworny and Eissenstat, 2011), rather than chemical defense levels, are mediating herbivory and influencing root lifespan. It is also plausible that increased root lifespan may reflect differences in herbivore pressure rather than actual defense against herbivory. For example, roots inhabiting deeper soils may have longer lifespans simply because herbivore / parasite abundance, and subsequent pressure, can decrease with soil depth (Steinberger and Loboda, 1991; Verschoor *et al*., 2001; Jumpponen *et al*., 2010); irrespective of any defense mechanism employed. Regardless of the underlying reason, we were unable to find general utility in using a Folin-Denis-based soluble-phenolic assay to increase our understanding of the factors that have been shown to impact fine-root lifespan.

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Figure 4.1: The general drivers of optimal root lifespan.

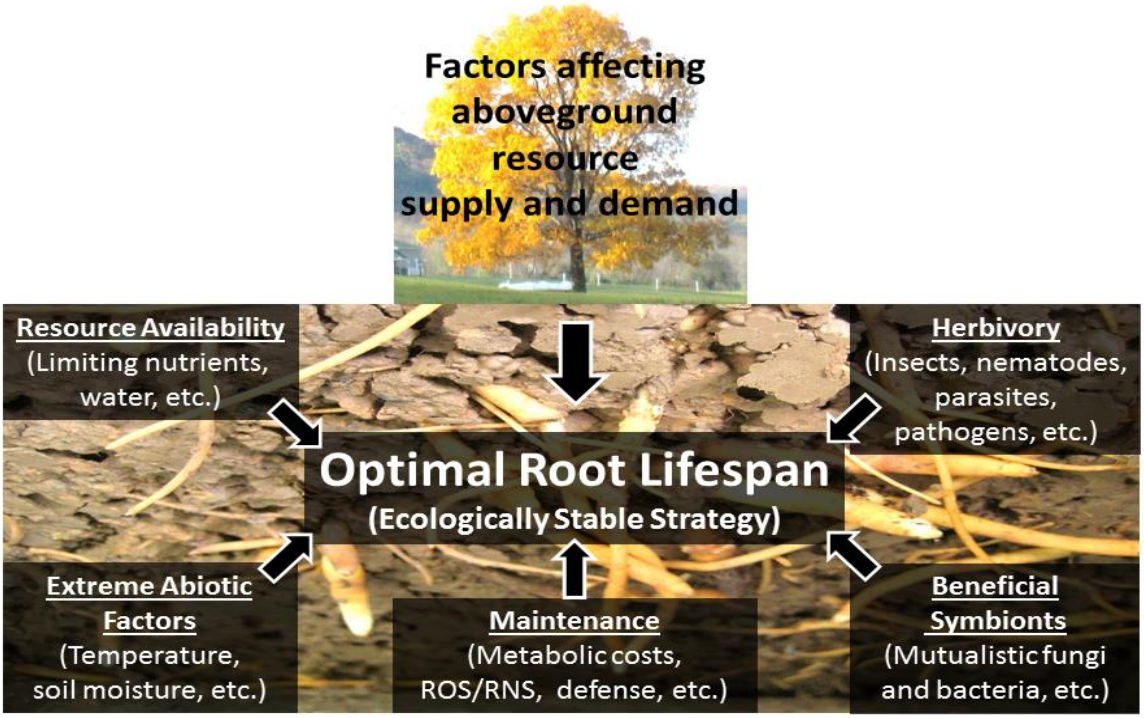


Figure 4.2: The relationship between root branching order and soluble phenolic concentration, as tannic acid (TA) equivelents (µg TA per mg root dry weight) from a modified Folin-Denis assay. Error bars denote standard error across blocks. $P \leq 0.05$ imply significant effects based on a two-tailed T-test. Four letter species codes are explained in Methods Section. Open bars denote first-order roots, black bars denote second-order roots, and hatched bars denote third-order roots.

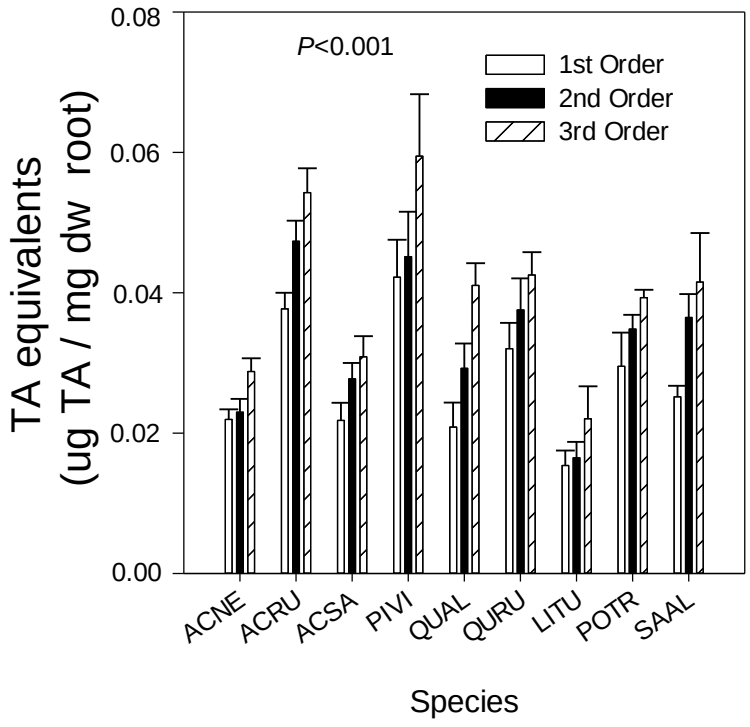


Figure 4.3: The relationship between nitrogen fertilization level and root soluble phenolic concentration, as tannic acid (TA) equivelents (µg TA per mg root dry weight) from a modified Folin-Denis assay. Error bars denote standard error across blocks. $P \leq 0.05$ imply significant effects based on a two-tailed T-test. Four letter species codes are explained in Methods Section. Open bars denote no N fertilization, black bars denote a N fertilization level of 3 times soils solution N, and hatched bars denote a N fertilization level of 30 times soil solution N.

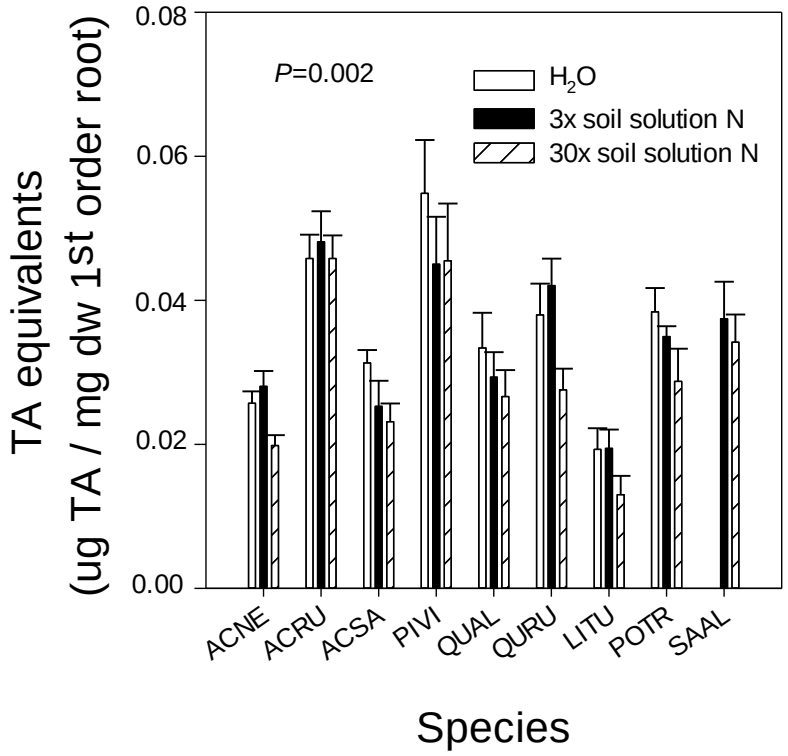
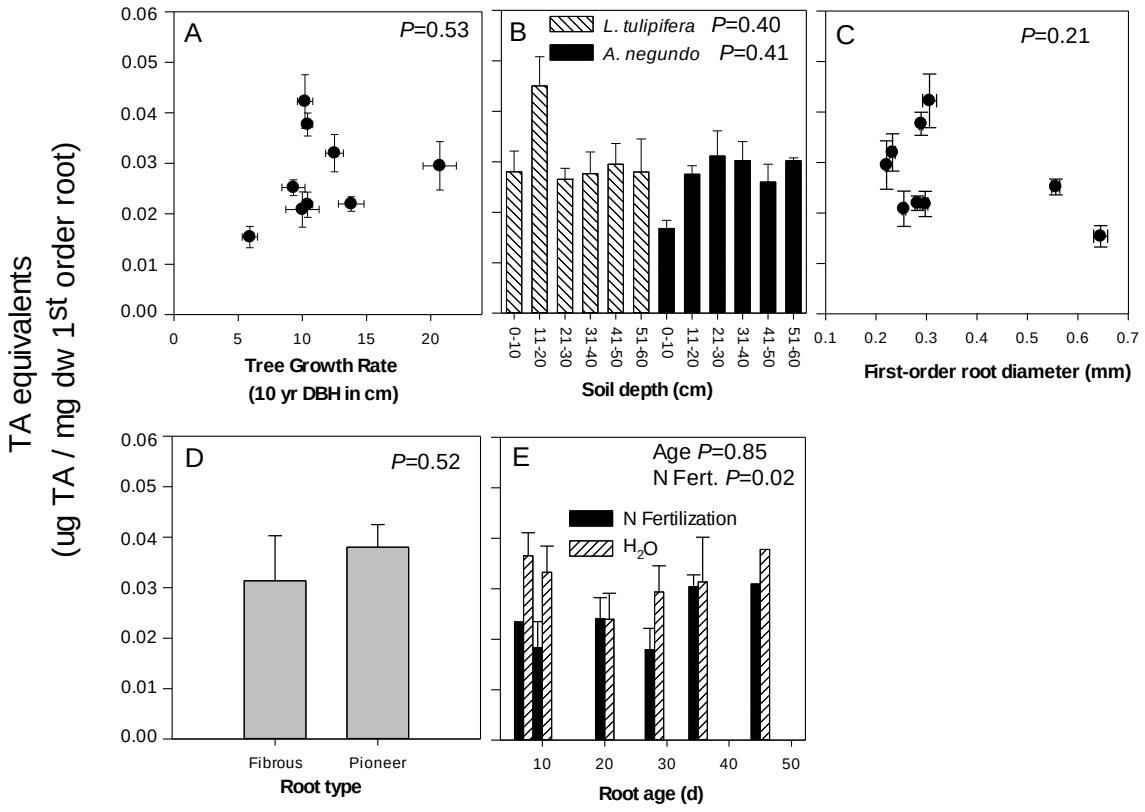


Figure 4: The relationship between factors that have been shown to effect fine-root lifespan and first-order root soluble phenolic concentration, as tannic acid (TA) equivelents (µg TA per mg root dry weight) from a modified Folin-Denis assay. Error bars denote standard error across blocks. $P \leq 0.05$ imply significant effects based on a two tailed T-test. **A:** The relationship between tree growth rate, as measured by the 10 year diameter at breast height (dbh), and first-order root soluble phenolic content. **B:** The relationship rooting depth and first-order root soluble phenolic content. Grey bars denote *L. tulipifera* roots and black bars denote *A. negundo* roots. **C:** The relationship between first-order root diameter and soluble phenolic content. **D:** The relationship between first order root type (fibrous vs. pioneer) in *L. tulipifera* and soluble phenolic content. **E:** The relationship between root age and soluble phenolic content of *A. negundo* roots sampled from root boxes that either received water (grey bars) or 3 times soil solution N fertilization (black bars)



Chapter 5

Synthesis

The preceding chapters represent a body of work attempting gain a better understanding of the controls and constraints of fine-root lifespan. While far from complete, significant progress to this end was made and many interesting scientific contributions were revealed. Although many of the results were non-significant or counter to our expectations, no shortage of effort went into such revelations.

In Chapter 2, the underlying assumption of Starch Depletion Hypothesis (Marshall and Waring, 1985), namely that fine roots are initiated with finite starch reserves and it is the consumption of these reserves that dictates fine-root lifespan, was explicitly tested by labeling *Sassafras albidum* trees with a ^{13}C isotopic tracer. By showing that carbon from current photosynthate is being incorporated into the non-structural carbon pools of existing, non-elongating roots, we were able to show that roots are not solely born with a finite reserve of sugars and carbohydrates and therefore root lifespan is not simply dictated by their consumption. As such, we found no support for the assumptions upon which the Starch Depletion Hypothesis is based (Marshall and Waring, 1985). Furthermore, the continued incorporation of current photosynthate into the structural pools of existing fine roots implies that root age determination using bomb ^{14}C isotopic tracer methods likely underestimates the actual age of the sampled roots, thereby further exacerbating the discrepancies in estimating fine-root lifespan between isotopic and minirhizotron based approaches (Strand *et al*., 2008, Guo *et al*., 2008b).

In Chapter 3, we investigate the other prevailing hypothesis of the control of fine-root

lifespan; resource optimization. Here we examined the hypothesis put forward by Yanai *et al*., (1995) that root lifespan is optimized to maximize resource acquisition while minimizing carbon expenditures. By creating sustained localized nitrogen patches around minirhizotron tubes we were able to assess if roots supplying greater benefit to the parent tree in terms of a limiting nutrient had increased longevity as predicted by the resource optimization hypothesis. We found support for resource optimization in tree species with fine-root morphology but no support in species with coarse-root morphology. Based on this partial support for resource optimization, we hypothesized that plasticity in root lifespan may in part be mediated by root morphology.

In chapter 4, we looked closer at the role herbivory plays in controlling fine-root lifespan. Specifically, we examined the relationship between fine-root soluble phenolic concentration, a putative general measure of defense against herbivory, and factors that have been previously shown to affect fine-root lifespan; namely root order, root diameter, root type (fibrous verses pioneer), rooting depth, localized nitrogen availability, and tree growth rate. We hypothesized that if herbivory was playing a role in root lifespan, the factors that enhance root lifespan would be associated with higher fine-root soluble phenolic concentration. Across all of the factors we investigated only root order and localized nitrogen availability were significantly correlated with fine-root soluble phenolic concentration. Root order was positively correlated with soluble phenolic concentration across all nine species studied. Higher order roots tend to be longer lived than the lower order roots they support (Wells *et al*., 2001; Anderson *et al*., 2003; Gill *et al*., 2002; Majdi *et al*., 2001; Guo *et al*., 2008a, McCormack *et al*., 2012) and so the trend of increasing soluble phenolic concentration with increasing root order supported out hypothesis. However, fine-root soluble phenolic concentration was negatively correlated with localized nitrogen availability. In chapter 3 we showed that root lifespan increased with localized

nitrogen availability, at least for species of fine-root morphology, and therefore we hypothesized that fine-root soluble phenolic concentration would be positively correlated with localized nitrogen availability; the opposite trend of what we observed. These conflicting results, combined with the lack of significant correlation between fine-root soluble phenolic concentration and the other factors we examined, lead us to conclude that in our study system there is not a general utility in using fine-root soluble phenolic concentration to explain variation in fine-root lifespan.

In the preceding chapters we explicitly tested the two prevailing hypotheses on the controls of fine-root lifespan. Based on our results we can reject the Starch Depletion hypothesis (Marshall and Waring, 1985) as the sole means by which root lifespan is controlled, but further study is necessary to fully validate the Resources Optimization Hypothesis (Yanai, 1995; Yanai and Eissenstat 1997). Additionally, although we elucidated interesting patterns of variation in fine-root soluble phenolic concentration across species, we did not find general utility in using fine-root soluble phenolic concentration to explain variation in fine-root lifespan.

Fine roots truly are the, "hidden half" and Kurt Pregitzer (2002) aptly describes the challenges in studying fine roots as a "royal pain". Yet fine roots can account for up to a 50% of annual global net primary productivity (Jackson *et al.*, 1997), so our understanding of many ecological processes is hindered by our lack of understanding of the controls and constraints of fine-root lifespan. The difficulties in studying fine roots *in situ*, in conjunction with the heterogeneous environment they inhabit and the complex biotic relationships they form, likely will force progress in their study to continue to lag behind that of other plant organs. The challenge therefore is to convince future researchers that the difficulties associated with the study of fine roots are an opportunity rather than a hindrance. Basic questions about fine roots remain

unanswered and it is only through incremental advances, such as those presented in the preceding chapters, that the "black box" that represents the ecology of fine-roots will be illuminated.

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