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

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Abstract

Aims: In addition to multiple above- and belowground abiotic factors, root herbivory can be an important determinant of root lifespan. In this study, 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 increased root lifespan.
Methods: In a common garden, utilizing nine temperate trees species, we examined the relationship between fine-root soluble phenolic content and fine-root branching order, diameter, rooting depth, localized nitrogen availability, and tree growth rate.
Results: Consistent with our hypothesis, fine-root soluble phenolic content significantly increased with increasing branching order ($P<0.001$). However phenolic content significantly decreased with increasing localized N enrichment ($P=0.002$), despite previous work indicating increased lifespan in N-enriched patches. We found no other significant relationships between fine-root soluble phenolic content and any of the other factors investigated.
Conclusions: 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 (Fig. 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, 1989; Stevens and Jones, 2006; Rasmann and Agrawal, 2008; Zvereva and Kozlov, 2012). 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*., 2008). 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 control and constrain 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.

Previous studies have shown that certain individual factors 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; Adams *et al*., 2013; Chen and Brassard, 2013). 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; Chen and Brassard, 2013). 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 below ground.

For this study 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 has been reported previously by McCormack *et al.* (2012).

In June, 2008 root in-growth cores were installed by pounding a 7.5 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 color coded 7.5 cm ID PVC pipe cut into 2.5 cm-high collars, which were placed on top of each in-growth core. The collars also served as reservoirs for the nitrogen solution as it soaked into the soil. Each in-growth core received 100 ml of 1 of the 3 levels of N weekly, which allowed for soil wetting 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 300 rpm 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) which is a modified Folin-Denis approach (Folin and Denis, 1912). Although, the Folin-Denis assay is the most commonly used method for measuring total phenolics in plant tissues (Waterman and Mole, 1994), it is not without limitations. Both interfering metabolites and structural variations in the phenolic compounds themselves can make precise quantification difficult (Appel *et al*., 2001).

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 additions consisting of 1L of 3 time 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.

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

Results

Root soluble phenolic content significantly increased with increasing branching order ($P<0.001$) (Fig. 2). However contrary to our hypothesis, phenolic content significantly decreased with increasing localized N enrichment ($P=0.002$) in the nine tree species we examined (Fig. 3). A significant decrease in fine-root soluble phenolic content associated with increased localized N addition was again observed in the *P. tremuloides* samples taken from the root box study examining the effect N on soluble phenolic content controlling for root age ($P=0.02$)(Fig. 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 content (Figs 4B and 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 content (Figs 4A and 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 content (*P. tremuloides* $P=0.85$) (Fig. 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). Despite occurring in relatively low concentrations in roots, phenolic compounds can still act as an important chemical defense mechanism against root herbivores (Potter *et al*., 2000; Stevenson *et al*., 2009). Additionally, due to high root turnover rates in many ecosystems, fine roots can contribute disproportionately high levels of phenolic compounds to the surrounding soil (Kraus *et al*., 2004). We therefore hypothesized that there would be a positive relationship between fine-root soluble phenolic content, 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 content. Fine-root soluble phenolic content was significantly positively correlated with root branching order (Fig. 2), despite species differences in phylogeny, root morphology, and mycorrhizal associations (i.e. arbuscular mycorrhizal versus 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 content were observed with increased root age in first-order roots (Fig. 4E). As a result, even though the

maximum age of the roots sampled from the in-growth cores was lower than the species specific median lifespans observed through minirhizotron tubes (McComack *et al.*, 2012), the soluble phenolic content of the in-growth core roots is likely representative of the roots of an older age structure growing outside the in-growth cores. Additionally, roots taken from the intact soil rather than in-growth cores (*A. negundo* Fig. 4b vs. Fig. 4e) had similar levels of soluble phenolics compared with roots taken from the in-growth cores. It therefore appears that 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. Intuitively this makes sense because as root branching order increases, the number of subordinate roots dependent on the higher order root also increases. As such, the loss of investment, in terms of carbon and other nutrients, associated with the mortality of higher order roots is far greater than the loss of the higher order root itself. It therefore stands to reason that roots of higher branching order should be better defended than the lower order roots they support.

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 content (Fig. 3), the opposite trend from our hypothesis. This negative relationship between fine-root soluble phenolic content and increased N availability may result from increased root growth constraining 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 enrichment 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, in some species roots produced under N enrichment could utilize nitrogen-based chemical defenses (Bryant *et al*., 1983) (e.g., alkaloids in *Acer* species and *L. tulipifera* (Barbosa and Krischik, 1987)) and as such reduced root phenolic contents my not equate to lower overall levels of chemical defense.

We found no other significant relationships between fine-root soluble phenolic content 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) (Fig. 4). Although there is ample aboveground evidence linking tissue phenolic content with reduced herbivory (Feeny, 1970; Hartley and Firn, 1989; Forkner *et al*., 2004; Fine *et al*., 2006), we found no general relationship between the factors that affect fine-root longevity and soluble phenolic content. 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 content 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 biochemicals 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, and despite the advances made in understanding aboveground plant-herbivore interactions using Folin-Denis-based soluble phenolic assays, we were unable to find general utility using the same assay to increase our understanding of the factors that have been shown to impact fine-root lifespan.

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Figure 1: The general drivers of optimal root lifespan.

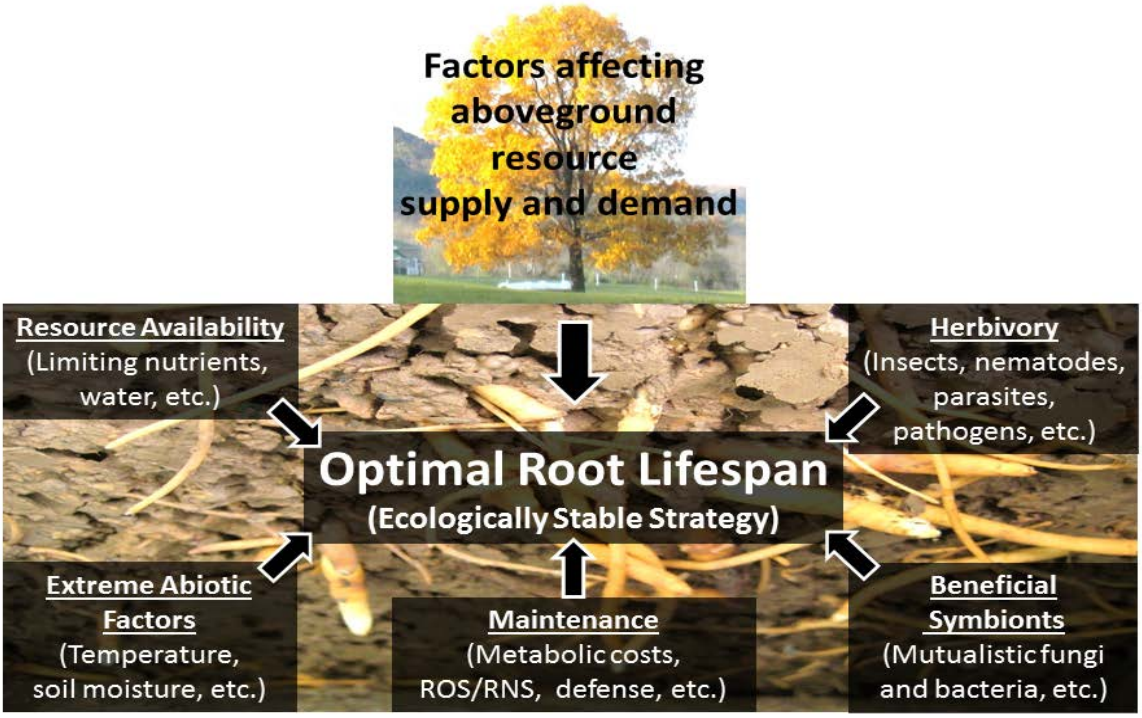

Figure 2: The relationship between root branching order and soluble phenolic content, as tannic acid (TA) equivelents (ug TA per mg root dry weight) from a modified Folin-Denis assay. Error bars denote standard error across blocks. $P \leq 0.05$ imply significant effects based on a two-tailed T-test. 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.

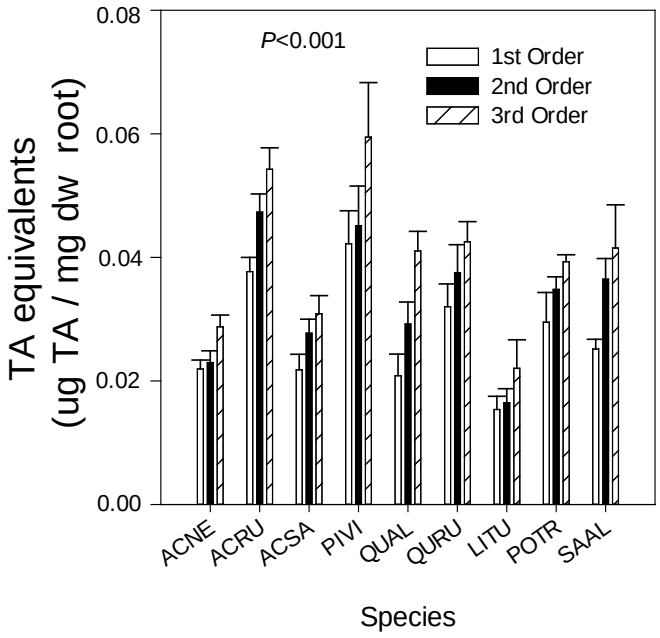

Figure 3: The relationship between nitrogen enrichment level and root soluble phenolic content, as tannic acid (TA) equivelents (ug TA per mg 1st order root dry weight) from a modified Folin-Denis assay. Error bars denote standard error across blocks. $P \leq 0.05$ imply significant effects based on a two-tailed T-test. Four letter species codes are explained in Methods Section. Open bars denote no N enrichment, black bars denote a N enrichment level of 11.4 mg N L^{-1} (3 times soils solution N), and hatched bars denote a N enrichment level of 114.0 mg N L^{-1} (30 times soil solution N).

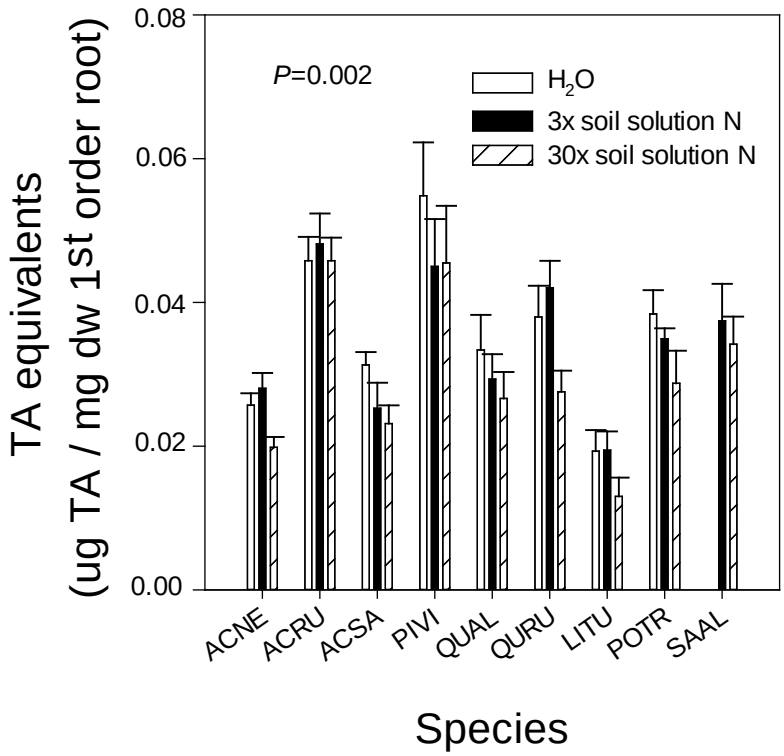

Figure 4: The relationship between factors that have been shown to effect fine-root lifespan and first-order root soluble phenolic content, as tannic acid (TA) equivelents (ug TA per mg 1st order root dry weight) from a modified Folin-Denis assay. Error bars denote standard error across blocks. $P \leq 0.05$ 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 11.4 mg N L^{-1} enrichment (3 times soil solution N) (black bars)