

NEIGHBORING SPECIES IDENTITY AFFECTS THE
BELOWGROUND GROWTH AND PHYSIOLOGY OF TREES

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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January, 2015

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NEIGHBORING SPECIES IDENTITY AFFECTS THE BELOWGROUND GROWTH AND PHYSIOLOGY OF TREES

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Competition belowground is prevalent in both natural and managed landscapes. Competing individuals use unique species traits in order to acquire essential soil resources that vary temporally and spatially. Certain traits produce distinct phenotypes, such as shifts in root growth and architecture. These growth adjustments can take place along millimeter or meter-wide spatial scales. Other traits involve physiological mechanisms. Combined, these traits determine the competitive outcomes of interacting individuals. The sum of all species traits is likely to increase under inter-specific conditions due to the greater number of species, which in turn may alter competitive interactions compared to intra-specific conditions. As species assemble, either naturally or as a result of human intervention, it will be important to understand how belowground traits affect competitive interactions, which over time, affect the growth and productivity of tree-dominated landscapes.

BIOGRAPHICAL SKETCH

Alex Paya graduated *summa cum laude* from Ohio Wesleyan University in 2009 with a B.A. in botany and microbiology. While attending Ohio Wesleyan, Alex was engaged in student government, as well as independent scientific research projects centered on plant biology and physiology. Continued interest in research ultimately led him to pursue a graduate degree in plant sciences at Cornell University where he was conferred a masters of science in 2013 for his work on trees roots. Alex then continued on with his graduate work in pursuit of a PhD.

This collection of work is dedicated to all those who were, are, and will be driven to pursue science. Failure should never be discouraging.

“When standing at the edge of earth, don’t be afraid to jump.”

ACKNOWLEDGMENTS

I would like to acknowledge the invaluable support of the staff, students, researchers, and professors at the Technische Universität München (TUM). My four years of research at TUM's Kranzberger forest research station would not have been possible without their encouragement and friendship. I would like to thank the Mario Einaudi Center for International Studies small Grants program for funding the micro-CT portion of my dissertation. I would also like to thank the Graduate School and the Field of Horticulture for their financial support and confidence in my work throughout my time at Cornell University. I thank my family and friends for pulling me through the good times, and the bad. Lastly, I would like to thank Dr. Taryn Bauerle for all of her help and support.

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CHAPTER 1

UNDERSTANDING INTERACTIONS BETWEEN TREES: CONTEXT AND CHALLENGES

Competition among plants is a ubiquitous feature across cultivated and natural landscapes (Schwinning & Weiner 1998, Casper & Jackson 1998). Climate and competition for limited resources are the predominant factors affecting the growth and distribution of plants worldwide, and are therefore popular topics of study (Woodward & Williams 1987, Kelly & Goulden 2008). Unlike managed landscapes, natural landscapes are limited to environmental resource inputs, which places greater emphasis on positive (facilitation) and negative (competition) interactions as drivers of plant growth and community development (Brooker & Callaghan 1998). Forests and tree plantations constitute the largest natural or minimally managed landscapes on earth, with over 30% of earth's terrestrial surface covered in trees (Zhu & Waller 2003). Furthermore, trees provide invaluable natural resources as well as ecosystem services; therefore, it is in our best interest to better understand how economically and ecologically important tree species interact with each other, and whether interactions will change predictably with the coming rise in temperature, CO₂ concentrations, and changing patterns of precipitation.

While previous research efforts have concentrated on aboveground interactions in forests (Gaudio *et al.* 2008, Oberle *et al.* 2009), growing evidence has shifted focus, deeming belowground interactions more crucial in governing plant growth success

(Donald 1958, Casper & Jackson 1997, Coates *et al.* 2009). In forests, belowground interactions become increasingly more important once the canopy is closed, and competition shifts disproportionately from above to belowground. Factors affecting belowground interactions include environmental conditions, plant nutrient and C status, soil nutrient and water abundance, and competition with neighbor plants (Casper & Jackson 1997, Hodge 2003, Violle *et al.* 2009, Falik & Novoplansky 2003). Among these factors affecting belowground interactions, the effect of interspecific competition on root growth is least understood, and has many researchers questioning mechanisms, which tree species demonstrate inter- vs. intraspecific variation in root growth, and what effect variation in root growth may have on soil resource abundance, C budgets, competitive pressure, and overall plant growth.

Drawing from Casper and Jackson (1997) and Shenk (2007), belowground competition is defined here as a reduction in the abundance of an intermediary (water, nutrients, or soil space) to roots, caused by other roots. A reduction in the abundance of an intermediary results in nutrient/resource depletion zones; individual roots sense nutrient depletion zones as well as resource patches via ion/molecular feedback mechanisms at the fine root level (1st-4th order), and then respond either morphologically, physiologically, or both based on the relative abundance and distribution of resources (Fransen *et al.* 1998, Hodge 2003, Grams & Andersen 2009). Root morphological responses range from shifting fine root diameter, fine root abundance, fine root length, or simply the spatial distribution of fine roots. Alternatively, physiological responses to nutrient depletion zones or resource patches

include, but are not limited to altering fine root respiration and/or nutrient uptake kinetics.

Direct competition for resources is costly in terms of carbon spent in exchange for resources acquired (O'Brien *et al.* 2007). In order to circumvent the cost of competition, certain species of trees (Kaitaniemi & Lintunen 2010), annuals (Holzapfel & Alpert 2003), and perennial grasses (Schenk *et al.* 1999) tend to avoid competition with neighbors via root growth strategies which limit belowground interactions (Figure 1.1). Moreover, some of these species growing inter-specifically demonstrate greater net primary production (NPP) than the same species growing intra-specifically (Naeem *et al.* 1996, Costanza *et al.* 2007). This suggests that traits which reduce belowground competition also improve yield, and while this trend is not universal, the implications are far reaching in terms of greater carbon capture, yields in forestry, and ecosystem services.



Figure 1.1 Conceptual illustration of two interacting tree root systems. On the right, deciduous trees have greater fine root abundance within deeper soils compared to the evergreen trees on the left which demonstrate a more shallow rooting habit. The partitioning of inter- vs. intraspecific root systems may develop from differences in root architecture or variation in root growth due to either resource or non-resource cues. (drawing by Alex Paya, adapted from Rasmann *et al.* 2011)

Competition avoidance is traditionally grouped under non-resource competition; non-resource competition involves antagonistic root-root interactions in the form of root exudates and/or effects on resources (Schenk 2006). Recent examination of resource-independent interactions have produced novel and ecologically significant results; in terms of belowground competition, the most intriguing research deals with kin-recognition and object avoidance at the fine root level (Novoplansky *et al.* 2003, Falik *et al.* 2005, Bhatt *et al.* 2011). Like resource competition, non-resource competition is preceded by, and results in changes in root morphology and physiology (Falik *et al.*

2005). Because resource and non-resource competition can prompt similar growth responses in roots, determining which cue is more strongly effecting root growth is inherently difficult, but important as soil resource conditions continue to change, i.e. climate change (Eissenstat *et al.* 2000).

Tree dominated landscapes are particularly sensitive to seasonal and long-term changes in precipitation and temperature because of their long-lived nature and time to maturity (Hasenauer *et al.* 1999, Kirshebaum 2000). A changing climate will force trees to either respond plastically, or run the risk of being outcompeted by other, better adapted plant species (Aitken *et al.* 2008, *Reviewed in* Brassard *et al.* 2009). Determining whether belowground competitive pressure increases or decreases predictably based on neighbor identity (inter- vs. intraspecific), the soil resources they alter, or both would provide greatly improved parameters for future ecosystem and climate models.

Studying the interactions among trees is inherently difficult because of their large size, long life-span, and adaptability to changing environmental conditions. Researching inter- vs. intraspecific interactions belowground is further complicated by the interactions of roots with multiple species' roots, as well as soil pests and microbes (Young 1998). If we are to understand how inter- vs. intraspecific interactions affect the belowground competitive environment, an essential first step is taking a common garden approach where roots and belowground processes can be linked to an individual species, and often single individuals.

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CHAPTER 2

X-RAY VISION UNCOVERS ROOT-ROOT INTERACTIONS: QUANTIFYING SPATIAL RELATIONSHIPS BETWEEN INTERACTING ROOT SYSTEMS IN THREE DIMENSIONS

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2.1 ABSTRACT

Research in the field of plant biology has recently demonstrated that inter- and intra-specific interactions belowground can dramatically alter root growth. Our aim was to answer questions related to the effect of inter- vs. intra-specific interactions on the growth and utilization of undisturbed space by fine roots within three dimensions (3D) using micro X-ray computed tomography. To achieve this, *Populus tremuloides* (quaking aspen) and *Picea mariana* (black spruce) seedlings were planted into containers as either solitary individuals, or inter-/intra-specific pairs, allowed to grow for two months, and 3D metrics developed in order to quantify their use of belowground space.

In both aspen and spruce, inter-specific root interactions produced a shift in the vertical distribution of the root system volume, and deepened the average position of root tip production when compared to intra-specifically growing seedlings. Inter-

specific interactions also increased the minimum distance between root tips belonging to the same root system. There was no effect of belowground interactions on the radial distribution of roots, or the directionality of lateral root growth for either species. In conclusion, we found that most significant differences were observed between controls (solitary individuals) and paired seedlings (inter- or intra-specific), and only a few between inter- and intra-specifically growing seedlings. This would indicate that competition between neighboring seedlings was more responsible for shifting fine root growth in both species than was neighbor identity. However, significant inter- vs. intra-specific differences were observed, which highlights the importance of biological interactions in competition studies.

Keywords: micro-CT, interspecific interactions, belowground competition, *Picea mariana*, *Populus tremuloides*, 3D root system.

2.2 INTRODUCTION

Plants sharing a finite amount of space will inevitably interact with each other either above or belowground in the pursuit of essential resources. The outcomes of these interactions can range from positive (facilitation) to negative (competition), and are therefore highly relevant for the development of agricultural and ecological management practices (Grime 1979, Tilman 1987). Traditional parameters that quantify the effect of belowground interactions on root growth dynamics include diameter class, spatial/temporal deployment, growth rate, and fine root abundance (Casper & Jackson 1997, Eissenstatt & Yanai 1997, Eissenstatt *et al.* 2000, Kembell *et*

al. 2008, Hodge 2009). While parameters such as these differ across species, accurate *in situ* observations are inherently limited by the opaque and heterogeneous nature of soil matrices, and generally require a destructive harvest of roots (Joslin & Henderson 1982, Steingrobe *et al.* 2000), or visualization along a two dimensional (2D) surface (Gross *et al.* 1992, Majdi 1996, Eissenstatt *et al.* 2000).

However, recent advances in three dimensional (3D) imaging technology such as ground penetrating radar, laser imaging, nuclear magnetic resonance imaging (MRI), neutron radiography (NT), and X-ray computed tomography (CT) have made the observation of undisturbed root systems possible (Macfall *et al.* 1991, Butnor *et al.* 2001, Gregory *et al.* 2003, Kaester *et al.* 2006, Perret *et al.* 2007, Tracy *et al.* 2010, Moradi *et al.* 2011, Mairhofer *et al.* 2012). Innovations in software such as *Rootviz*, *Root track*, *RootReader3D*, and *Avizo* (Saoirse *et al.* 2010, Tracy *et al.* 2010, Clark *et al.* 2011, Mairhofer *et al.* 2012), and specific filtering algorithms (Perret *et al.* 2007) have helped improve 3D image resolution and stream-line the quantification of anatomical parameters such as lateral root length, lateral root number, root-system surface area, and volume of undisturbed root systems. However, accurately isolating roots from root-soil data is complicated by the continuum of water between soil particles, the root-soil interface, and within the root itself. As methods for isolating roots improve, steady technological advancements will, and have already increased the scope of viable research questions and objectives. For example, studies have already begun to explore the 3D spatial distribution of fine and coarse roots in forests (Pierret *et al.* 1999, Butnor *et al.* 2001), mechanical buckling in plant roots (Silverberg *et al.*

2012), and water uptake at the root-soil interface (Moradi *et al.* 2011).

As 3D imaging technologies become more widely available, questions about the occupation and exploration of space by interacting root systems can be better addressed, offering new insights to this important yet problematic component of root growth dynamics. For example, belowground interactions can result in whole root system segregation (*reviewed in* Schenk *et al.* 1999), stunted root elongation (Mahall & Callaway 1996, Falik *et al.* 2005, Bhatt *et al.* 2011), and/or over-yielding in response to spatially proximal self (roots belonging to self) and non-self roots (roots belonging to neighbor)(Gersani *et al.* 2001, Maina *et al.* 2002, Falik *et al.* 2003). An understanding of the mechanisms regulating the growth of roots driven by belowground interactions is still developing, however growing evidence suggests that traditional parameters including root biomass, root surface area, and diameter are insufficient in integrating spatially complex responses.

To our knowledge, the following experiment is the first attempt at observing and quantifying the effect of belowground interactions between two neighboring root systems in 3D. Our research employed micro-CT to capture the spatial distributions of both interacting (inter- vs. intra-specific) and control root systems (solitary) belonging to two-month-old tree seedlings. 3D models of root system architecture were developed from annotated CT image slices, and traditional belowground parameters such as root length, surface area, volume, and root tip counts were measured. Moreover, we also developed a series of belowground parameters that take advantage

of skeletonized 3D root system data, and quantified distances between root tips and the distribution of individual root volumes: a data set inaccessible with a 2D approach. Broadly, the goal of this work was to use these parameters to investigate the effects of both inter- and intra-specific interactions on the belowground growth of plants, and compare these effects to solitary individuals.

2.3 MATERIALS AND METHODS

2.3.1 PLANT GROWTH

Acrylic tubes (3.5 mm wall thickness, 64 mm inner diameter, 305 mm length) were covered with fine mesh (0.5 mm) along the base, capped, and secured to provide free drainage. Each tube was filled incrementally with polystyrene beads (1-3 mm), gently tamped throughout the filling process in order to reduce pore size and achieve greater bulk density, and then wrapped in aluminum foil to prevent light penetration. Polystyrene was used in place of peat, sandy loam, sand, or vermiculite based on trail experiments which demonstrated very high water retention in soil or soil like mediums. Additionally, contrast agents such as iodine containing compounds, barium sulfate (BaSO_4), gold chloride (Au_2Cl_6), and cow's milk were used, but sufficient contrast was not achieved.

Black spruce (*Picea mariana*) and quaking aspen (*Populus tremuloides*) were selected as “interacting” plant species based on differences in phylogeny, morphology, and the fact that they co-occur across northern latitudes of North America (DeByle &

Winokur 1985). Seeds from each species were germinated for five to seven days between two sheets of damp cellulose, and then transplanted into pre-wet hydroponic growth plugs (Rapid Rooster Grow plugTM, General hydroponics, Sebastopol CA) following radicle emergence (1-3 mm). Each acrylic tube received a single plug containing one individual of either spruce or aspen (control), or two plugs, each containing one individual, to simulate inter- vs. intra-specific interactions. A total of twenty-five tubes were prepared. There was five of each of the following tubes: solitary aspen, intra-specific aspen, inter-specific aspen/spruce, intra-specific spruce, and solitary spruce. Containers were randomly arranged on a hydroponic flood table modified to re-circulate nutrient solution for top-down irrigation. To prevent competition for light, a sheet of acetate was placed between interacting seedlings.

Plants were grown under greenhouse conditions (17 C° night, 20 C° day; KPL greenhouses, Cornell University, Ithaca NY) with supplemental lighting (12 hr days) for 60 days (April-June, 2012). Irrigation was fed from a 530 liter (140 gallon standard) reservoir to individual micro-spray emitters focused at the base of each plant (90°, 0.5 gph, Hydro FlowTM, Redmond WA). Irrigation provided each tree with a nutrient replete hydroponic solution balanced at 150 ppm N (Peter salts, 21-5-20, 382.8g/530 L; Epsom salts, MgSO₄ 7H₂O, 130.64g/530 L). Hydroponic nutrient solution was maintained at a pH of 5.5-5.8, and EC of 1.6-2.0 throughout the experiment, and automatically controlled by a programmable timer on a rotating schedule. Half way through the growth phase of the experiment, a pump malfunction resulted in the loss of multiple individuals, which produced an uneven number of

replicates for each species.

Irrigation was terminated after two months of growth. Plants were allowed to transpire residual water remaining in each container for two days prior to imaging in order to reduce imaging artifacts (Lontoc-Roy et al. 2006). Plants were then transported to Cornell's imaging facility for CT scanning.

2.3.2 MICRO-CT SCANNING

Whole seedling's root systems were imaged at Cornell University's Micro-CT facility. Due to a pump failure during the growth stage of the experiment, only thirteen out of twenty five tubes were imaged: 3 x solitary aspen, 1 x intra-specific aspen, 3 x inter-specific aspen/spruce, 3 x intra-specific spruce, and 3 x solitary spruce. Each scan was performed using a GE CT120 micro-CT scanner (GE Healthcare, London, Ontario, Canada). Initially, 10 bright-field images were acquired with no objects in the scanner, providing a correction for detector non-uniformity. Calibration and correction for signal non-uniformity was determined from measurement within a SB3 (GE Healthcare) water/bone phantom, scanned with the samples. Resulting image datasets were calibrated to the conventional scale of Hounsfield radiodensity units (HU), defined so that water and air have HU values of 0 and -1000, respectively. Each scan digitally acquired 720 projections at 0.5° intervals over 360° using 80keV, 32 ma, 32 ms exposure time and 100 µm x-y-z resolution. The obtained projections were used to reconstruct a CT dataset using a convolution back-projection algorithm implemented in 3D, giving a 70×70×50 mm³ volume of image data with 100 µm isotropic voxels.

Using a sequential stacking function (MicroView, GE Healthcare), three sequential image stacks ($70 \times 70 \times 50 \text{ mm}^3$) were taken from each tube and recompiled into a single $70 \times 70 \times 150 \text{ mm}^3$ data set with $100 \text{ }\mu\text{m}$ isotropic voxels. Using this function, we successfully increased the visible volume (i.e. visible rooting structure) three-fold: from 5 cm to 15 cm depth ($70 \times 70 \times 150 \text{ mm}^3$ scan = 1hr).

2.3.3 DESTRUCTIVE HARVESTING

Following X-ray scanning, plants were destructively harvested. Leaves/needles and petioles were removed from the main stem and scanned using a photo scanner (Epson Expression 10000XL, 2400 dpi, Epson America Inc., Long Beach CA). Directly following the removal of aboveground tissues, acrylic containers were inverted and tamped to release the polystyrene medium along with roots, which were gently rinsed under a 0.5 mm sieve. Polystyrene beads still attached to roots were removed using forceps. Individual roots were separated manually to prevent overlapping segments, placed on a photo scanner, and scanned. After scanning, above and belowground tissues were placed in separate paper bags, dried at 55 C for three days, and then weighed. Scanned images were analyzed for leaf surface area, root surface area, and total root length using *WinRhizo* (Winrhizo 2011, Regent Instruments, Canada). The number of root tips were counted manually using *ImageJ*.

2.3.4 IMAGE RECONSTRUCTION

Projections were exported from the GE CT120 micro-CT scanner as VFF format (Sun TAAC Graphic File) and converted to DICOM format using MicroView's DICOM

transfer tool. Image stacks were then imported one at a time into *ImageJ* using the *import, image sequence* function. Once the main taproot was found for each seedling, roots originating from a single individual seedling were given an arbitrary color code, and the entire cross sectional area was traced by hand for each root through each CT image slice (70 x 70 x 0.1mm) (Figure 2.1). This ensured that root diameter, surface area, and volume could be measured in the 3D model/reconstruction, and that root systems belonging to different individuals could be easily differentiated. Once a root came in contact with the container wall, tracing ceased because, 1) the roots were indiscernible from the container, and 2) the roots behaved atypically and tracked the container wall. Color-coded image stacks were then exported as an RGB TIFF stack, and opened in MATLAB® 2012b for three dimensional reconstruction and spatial quantification (The MathWorks Inc., Natick MA). In order to 3D render each root system, color codes were identified and isolated, which allowed for the subtraction of non-colored voxels; annotated circles representing root cross sections within each x-y plane were then stacked across the z-plane. This process effectively rendered each root system in 3D with little or no constraints on actual root system dimensions. In MATLAB each root cross section was also converted to a 3D binary matrix in order to measure spatially explicit parameters. Entries of these matrices were either 0 or 1, depending on whether that voxel was occupied by the root.

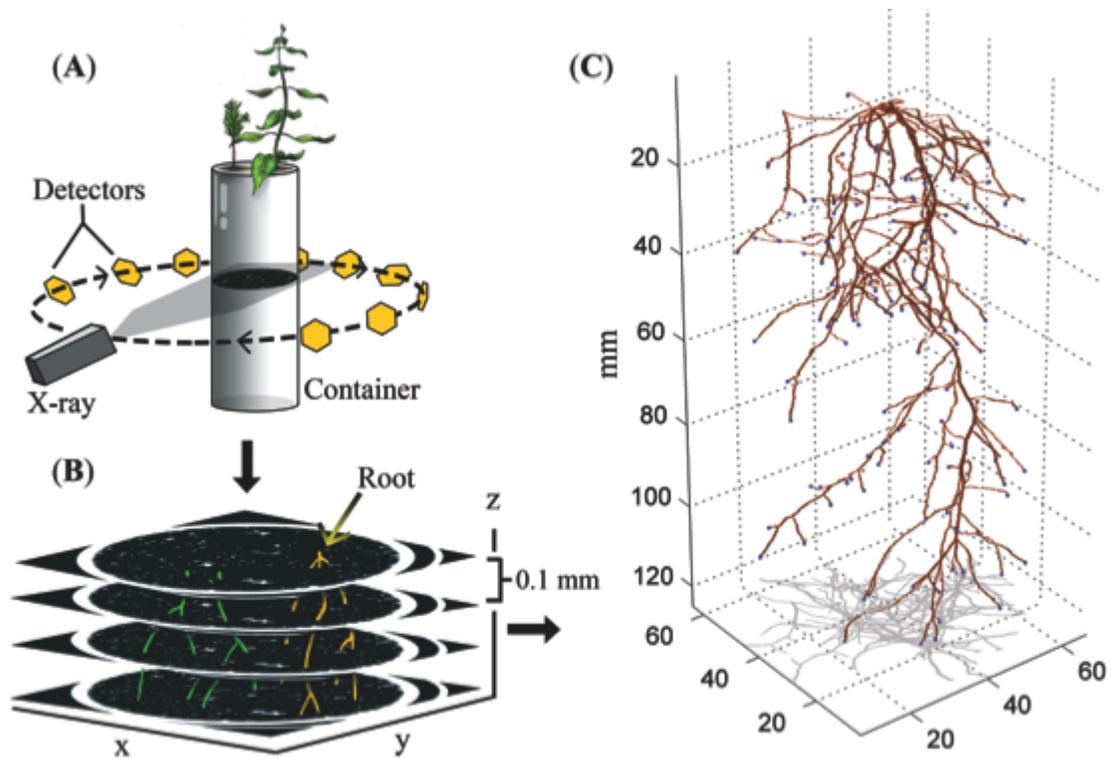


Figure 2.1 Experimental design and 3D output. A, drawing representing the principles of X-ray CT. X-rays are aimed at a container, and the signal attenuation of the X-ray beam is captured by a ring of detectors that integrate signal information into cross sectional images made of isotropic voxels (0.05 x 0.05 x 0.1 mm). B, identifiable roots are color coded, and then reconstructed in 3D. C, 3D reconstruction of a solitary aspen (*Populus tremuloides*) root system. Each terminal root tip is marked with a blue sphere to highlight root anatomy.

2.3.5 MORPHOLOGICAL AND ANATOMICAL ANALYSES

Root surface area was determined from the 3D data sets by sequentially analyzing each x-y cross-section with MATLAB's *bwtraceboundary* function. This identified the coordinates of the root perimeter from which we calculated the circumference of all roots passing through the plane. The circumference was multiplied by the cross-sectional thickness (100 μm) to estimate root surface area per image slice. This was performed for all cross-sectional images and the results summed to calculate root

system surface area. Root system volume was calculated by summing the total number of occupied voxels and multiplying by the volume per voxel, $10^{-3} \text{ mm}^3/\text{voxel}$.

Root tips were located by scanning through each cross-section and identifying terminal voxels. This generated a list of coordinates that were centered on root tips (Figure 2.1). With this information we were able to determine the depth-wise distribution along the z-axis (complete 3D information). To quantify the radial distribution of root tips (mm^2), root tip coordinate data was projected into the x-y plane. An ellipse whose circumference and orientation represents the occupied x-y area of the root system was then projected over the root tip coordinates. Multiplying π by both the major and minor axes of the ellipse provided the radial distribution of each root system. The ratio of the ellipse's major to minor axis is then a metric that defines how radially symmetric the root distribution is. In particular, if the ratio is 1, then the distribution is circularly symmetric. Values higher than 1 indicate the amount of asymmetric root growth in the plane that passes vertically through the ellipse's major axis.

2.3.6 STATISTICS

In order to validate our 3D rendering protocol, we used general linear regression to compare destructively harvested (2D) and 3D reconstructed root system data. Surface area and the number of root tips were chosen for regression because both were measurable in 2D and 3D, and could therefore be used to validate our manual tracing procedure of fine root cross sections through CT image slices. Differences in mean ranks between solitary, intra- and inter-specifically growing plants were analyzed

using the Kruskal-Wallis test on the following parameters: biomass, 2D/3D root length, 2D/3D surface area, SRA, SRL, manual and 3D root tip count, 3D root volume, root-root distance, major/minor axes, radial distribution, and rooting depth weighted by volume ($P = 0.05$, $H_0 = \text{mean ranks are equal}$). Where the null was rejected, post-hoc analyses were performed using the Wilcoxon each-pair test (non parametric multiple comparisons, $P = 0.0167$). It is important to note that the distribution of minimum Euclidean distances between root tips did not distribute normally, nor did the data behave normally post transformations (e.g. $\log x$, e^x , or x^{-1}). Instead, we used a two-parameter model for the probability distribution function that fit both species data where the ratio of the sum of squares of regression (SS_{reg}) to the total sum of squares (SS_{tot}) was between 0.75 and 0.99. This model was a good predictor of the fraction of root tips $f(x) dx$ separated by a minimum distance between x and $x+dx$ for control, inter-, and intra-specifically growing seedlings, where the *pdf* is given by:

$$f(x) = c_1 x e^{-x/c_2}. \quad \text{Eq. 1}$$

Non-linear regressions were fitted using Sigma Plot 11 (Systat Software, San Jose CA). All other statistics were done using JMP 10.0 (SAS Institute Inc., Cary NC).

2.4 RESULTS

2.4.1 DESTRUCTIVE ANALYSES

Analyses of each harvested seedling showed that belowground interactions had no significant effect on the aboveground growth of aspen or spruce ($P \geq 0.1487$, Figure

2.2 A and B). Belowground interactions had a measurable effect on aspen's root surface area, but not spruce ($P = 0.044$, Figure 2.2D). On average, inter-specific aspen root systems were reduced to 31% of the control samples surface area. Belowground interactions had no significant effect on either species' root biomass ($P \geq 0.061$, Figure 2.2C). Lastly, belowground interactions had a significant effect on fine root lengths in aspen ($P = 0.044$), but not spruce ($P = 0.21$).

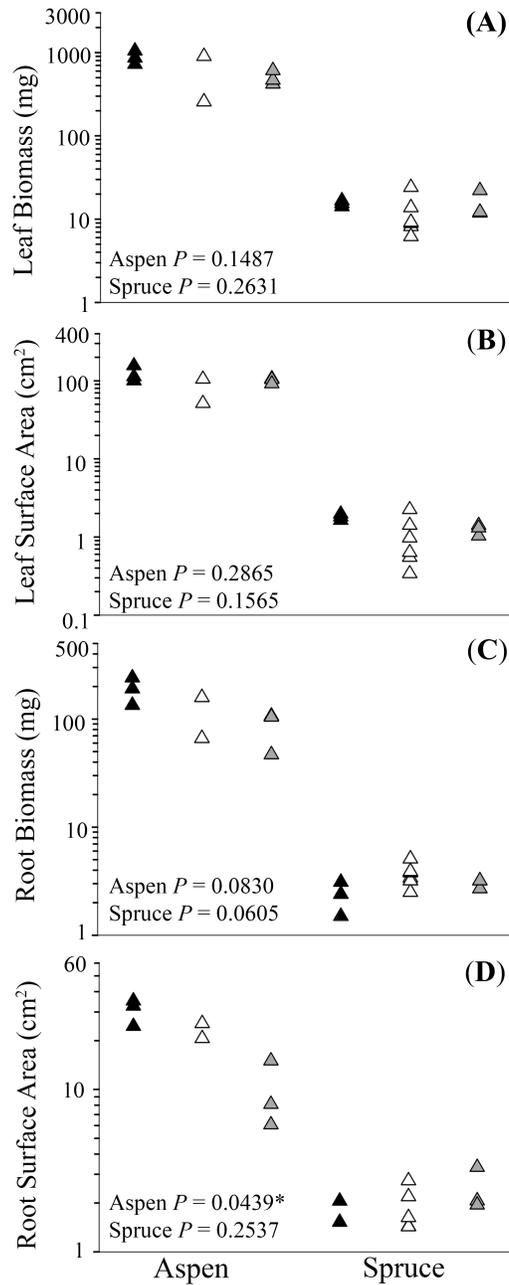


Figure 2.2 Comparing control, intra-, and inter-specific differences (black: control, white: intra-specific, grey: inter-specific) in aboveground (A, B) and belowground (C, D) biomass and surface area of aspen (*Populus tremuloides*) and spruce seedlings (*Picea mariana*) post destructive harvest. Each point (triangle) represents data from a single individual displayed on a log scale (y-axis); different letters denote significant differences between control, intra-, and inter-specific seedlings (Wilcoxon test, $P \leq 0.05$).

Two additional belowground parameters, specific root length (SRL) (cm mg^{-1}) and specific root area (SRA) ($\text{cm}^2 \text{mg}^{-1}$), were also calculated for both species. SRL and SRA depict the cost of root construction, and can be highly informative in establishing whether a treatment had an effect on root morphology. Control, inter-, intra-specifically growing spruce seedlings differed in terms of SRA ($P = 0.039$), but similar differences were not observed in aspen ($P = 0.078$, Table 2.1). Control, inter-, and intra-specific interactions also had a significant effect on the SRL of spruce seedlings ($P = 0.013$), but not aspen ($P = 0.21$).

Table 2.1 Belowground parameters of destructively harvested aspen (*Populus tremuloides*) and spruce (*Picea mariana*) seedlings grown under three experimental conditions: control, intra-specific, and inter-specific. Values listed are the median (maximum, minimum).

		n	Root Biomass (mg)	Root Surface Area (cm^2)	Number of Root Tips	Root Length (cm)	SRA (cm mg^{-1})	SRL ($\text{cm}^2 \text{mg}^{-1}$)
Aspen	Control	3	192 (241, 135)	32.7 (35.2, 24.5)	266 (315, 181)	693 (724, 528)	0.170 (0.180, 0.150)	3.79 (5.16, 2.19)
	Intra	2	113 (159, 66.4)	22.3 (23.9, 20.6)	173 (195, 152)	424 (443, 405)	0.230 (0.310, 0.150)	4.62 (6.68, 2.55)
	Inter	3	77.3 (106, 47.7)	8.11 (15.0, 6.07)	114 (142, 50)	168 (241, 107)	0.130 (0.140, 0.080)	2.26 (2.27, 1.61)
Spruce	Control	3	2.40 (3.10, 1.50)	1.52 (2.06, 0.529)	12.0 (15.0, 7.00)	19.3 (25.5, 14.2)	0.640 (0.660, 0.350)	8.23 (9.47, 8.06)
	Intra	6	3.60 (5.10, 2.50)	1.53 (2.75, 0.742)	15.0 (28.0, 6.0)	18.1 (28.5, 9.87)	0.415 (0.700, 0.300)	4.81 (7.32, 3.72)
	Inter	3	2.95 (3.28, 2.70)	2.07 (3.32, 1.95)	12.0 (22.0, 8.00)	27.7 (35.0, 22.2)	0.770 (1.04, 0.720)	10.2 (10.9, 8.21)

2.4.2 VALIDATION OF 3D RECONSTRUCTION

In order to validate our 3D reconstruction protocol, comparisons were made between destructive (i.e. 2D) and 3D root parameters. Surface area and the number of root tips were two parameters measured in both 2D and 3D, and were therefore chosen to

validate the 3D reconstruction by way of general linear regression. We found that 62% of the total number of root tips ($R^2 = 0.84$), and 76% of the total surface area ($R^2 = 0.82$) were successfully captured during 3D image reconstruction (Figure 2.3).

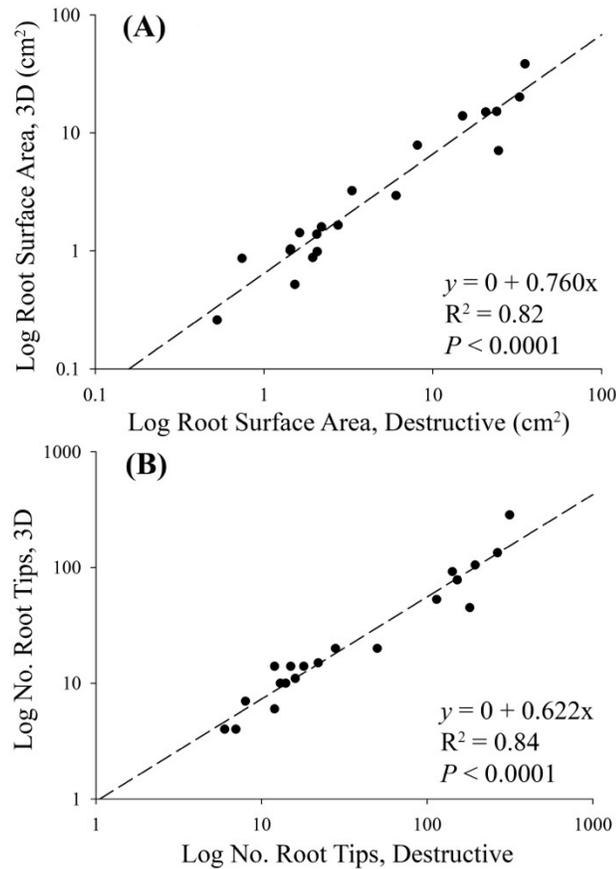


Figure 2.3 Correlation between destructive and 3D root surface area and the number of root tips of aspen (*Populus tremuloides*) and spruce (*Picea mariana*) seedlings. The slope of each regression indicates that 76% of root surface area, and 62% of root tips were successfully rendered in 3D, i.e. 24 - 38% of root systems were lost during the 2D annotation and 3D reconstruction phase of the experiment.

Examples of 3D root systems across all treatment and species combinations are presented in Figure 2.4.

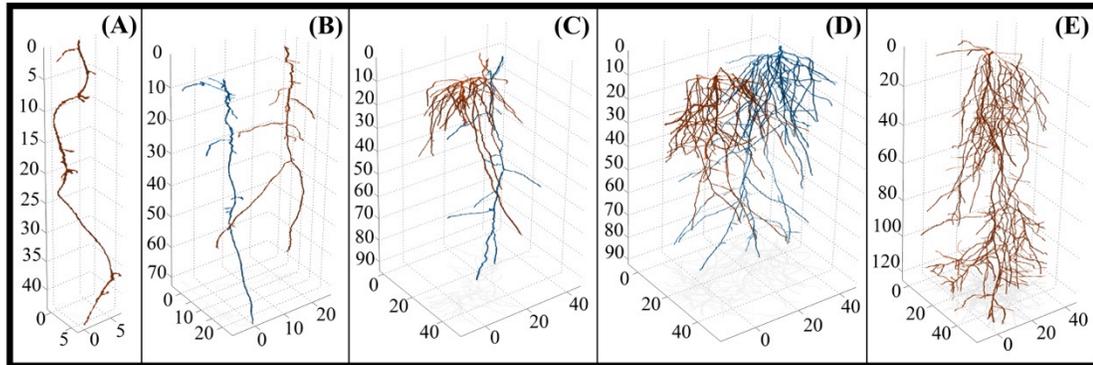


Figure 2.4 3D reconstruction of solitary and paired root systems (axes in 0.1mm increments). A, control spruce (*Picea mariana*) (n = 3 containers, 3 individuals). B, intra-specific spruce (n = 3 containers, 6 individuals). C, inter-specific aspen (*Populus tremuloides*) and spruce root systems (n = 3 containers, 3 individuals each species). Aspen is highlighted in orange, spruce is highlighted in blue. D, intra-specific aspen (n = 1 container, 2 individuals). E, control aspen (n = 3 containers, 3 individuals). Note the differences in X and Y axes.

2.4.3 3D UTILIZATION OF SPACE

The occupation of 3D space by individual root tips, and whole root systems was quantified via a set of five metrics: radial distribution of root tips, directionality (major/minor axes of radial distribution ellipse), minimum root-root tip distance, root system volume as a function of depth, and the vertical position of root tips. The radial distribution of root tips measured the radial expanse of a root system (mm^2), i.e. the area of an ellipse that encompassed the x/y distribution of all root tips projected along the z axis. The radial distribution of aspen roots ranged from 248-501, 500-522, and 212-325 mm^2 in the control, intra-specific, and inter-specific seedlings, respectively (Table 2.2). For spruce, the control, intra-specific, and inter-specific seedlings ranged from 16-43, 9-112, and 36-314 mm^2 , respectively (Table 2.2). We found no significant effect of belowground interactions on the radial distribution of roots.

Table 2.2 Measurements made in 3D of aspen (*Populus tremuloides*) and spruce (*Picea mariana*) seedlings grown under three experimental conditions: control, intra-specific, and inter-specific. Values listed are the median (maximum, minimum).

		n	Root Volume (mm ³)	Root Surface Area (cm ²)	Number of Root tips	Major/Minor Radii	Radial Distribution (mm ²)
Aspen	Control	3	230 (424, 84.0)	20.1 (38.4, 7.06)	134 (285, 45.0)	1.16 (1.38, 1.09)	484 (501, 248)
	Intra	2	159 (161, 157)	15.1 (15.2, 15.0)	91.5 (105, 78.0)	1.09 (1.10, 1.07)	511 (522, 499)
	Inter	3	88.5 (158, 33.4)	7.87 (13.9, 2.94)	53.0 (92.0, 20.0)	1.38 (1.57, 1.28)	263 (325, 212)
Spruce	Control	3	4.20 (11.7, 2.16)	0.519 (1.39, 0.259)	14.0 (14.0, 4.0)	1.54 (10.1, 1.19)	25.4 (43.4, 16.4)
	Intra	6	10.5 (21.0, 4.00)	1.23 (1.65, 0.863)	10.5 (15.5, 8.50)	3.37 (6.31, 1.78)	40.6 (112, 9.16)
	Inter	3	11.3 (37.7, 7.77)	0.984 (3.23, 0.878)	7.00 (15.0, 6.00)	2.70 (5.55, 1.46)	47.4 (313, 36.3)

The directional growth of roots was measured by dividing the major (transverse) by the minor (conjugate) axes of an ellipse that encompassed the radial distribution of each root system. Using this approach, we could determine whether a root system was concentric (major/minor = 1), or skewed/directional (e.g. major/minor >> 1). As was the case with the radial distribution of root tips, belowground interactions had no significant effect on the ratio of major/minor axes for either species. However, notable species trends were observed. The major/minor axes of spruce root systems ranged from 1-10, with an average of 4 across solitary and paired seedlings, indicating that spruce roots systems were relatively planar (Table 2.2). The major/minor axes of aspen root systems ranged from 1.1-1.6, with an average of 1.2 across control, inter- and intra-specific seedlings, indicating that aspen root systems were evenly distributed in all directions relative to each root system's center of mass.

The third metric, minimum root-root tip distance, measured the minimum distance between a root tip (x_1, y_1, z_1) and the next closest root tip (x_2, y_2, z_2) for every terminal

point in a root system. Belowground interactions had a significant effect on the minimum distance between root tips in both aspen ($P = 0.011$) and spruce ($P = 0.0002$). Post hoc analyses indicated that aspen roots grown intra-specifically (6.8 ± 0.28 mm) had wider distances between root tips compared to the controls (5.9 ± 0.13 mm) ($P = 0.0025$, Figure 2.5B). As for spruce, post hoc analyses indicated that the minimum distances between root tips in controls (6.0 ± 0.78 mm) were significantly less than inter-specific seedlings (11 ± 1.0 mm, $P < 0.0001$, Figure 2.5D). The minimum distances between root tips in intra-specific seedlings (7.8 ± 0.92 mm) were also significantly less than inter-specific seedlings ($P = 0.0007$, Figure 2.5D).

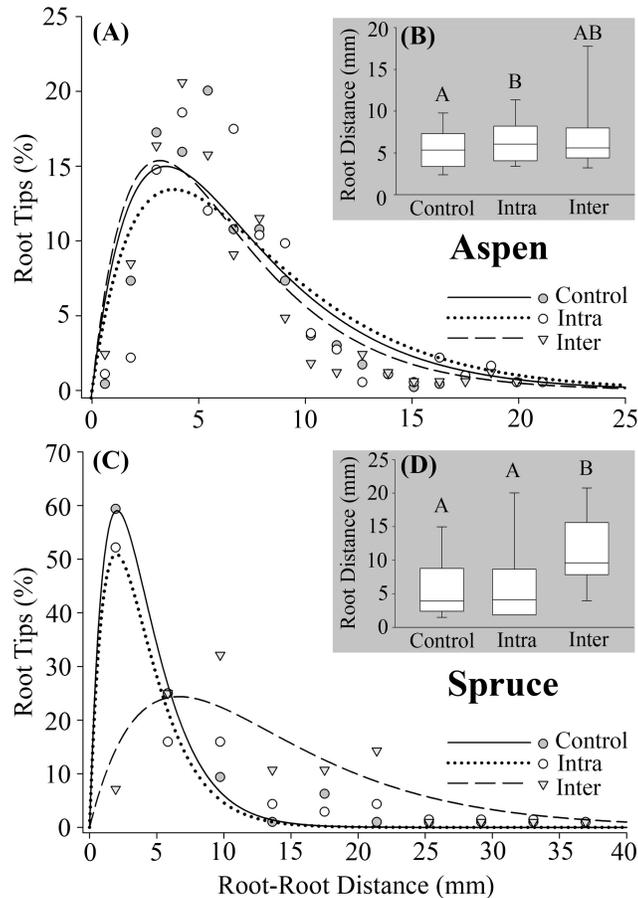


Figure 2.5 Minimum distance between each root tip (x_1, y_1, z_1) and the next closest tip (x_2, y_2, z_2) across all roots. A, aspen (*Populus tremuloides*) control ($SS_{reg}/SS_{tot} = 0.86$), intra-specific ($SS_{reg}/SS_{tot} = 0.77$), and inter-specific ($SS_{reg}/SS_{tot} = 0.86$). C, spruce (*Picea mariana*) control ($SS_{reg}/SS_{tot} = 0.99$), intra-specific ($SS_{reg}/SS_{tot} = 0.91$), and inter-specific ($SS_{reg}/SS_{tot} = 0.75$). B, D: box plots representing root-root distances for aspen and spruce, respectively. Each box displays the median value (straight line) and upper/lower quartiles; bars represent 10th/90th percentile. Differences between control, intra-, and inter-specific groups are indicated by different letters (Wilcoxon multiple comparisons, $P \leq 0.01667$). Note the differences in axes.

We also quantified differences between control, inter-, and intra-specifically growing plants in terms of their vertical placement of root system volume (Figure 2.6A and B). For both species, control, inter-, and intra-specific interactions were significant predictors of the depth of roots weighted by volume (Kruskal-Wallis test, $P < 0.0001$). In aspen, post-hoc analyses indicated that the largest difference was observed between

control (deep) and intra-specific (shallow) root systems ($P < 0.0001$). The second largest difference in rooting depth was observed between inter- and intra-specifically growing seedlings ($P < 0.0001$). The smallest difference was observed between inter-specific and control root systems ($P < 0.0001$). In spruce, post-hoc analyses indicated that the largest difference was observed between inter- and intra-specifically growing root systems ($P < 0.0001$). Inter-specific and control root systems also occupied significantly different rooting depths ($P < 0.0001$), while no significant difference was observed between intra-specific and control root systems ($P = 0.086$). The average rooting depth for control, intra-specific, and inter-specifically growing root systems for aspen was 56.2 ± 0.6 , 23.6 ± 0.6 , and 32.7 ± 0.7 mm, respectively, whereas spruce was 41.0 ± 2.4 , 34.4 ± 1.2 , and 28.6 ± 2.31 mm, respectively.

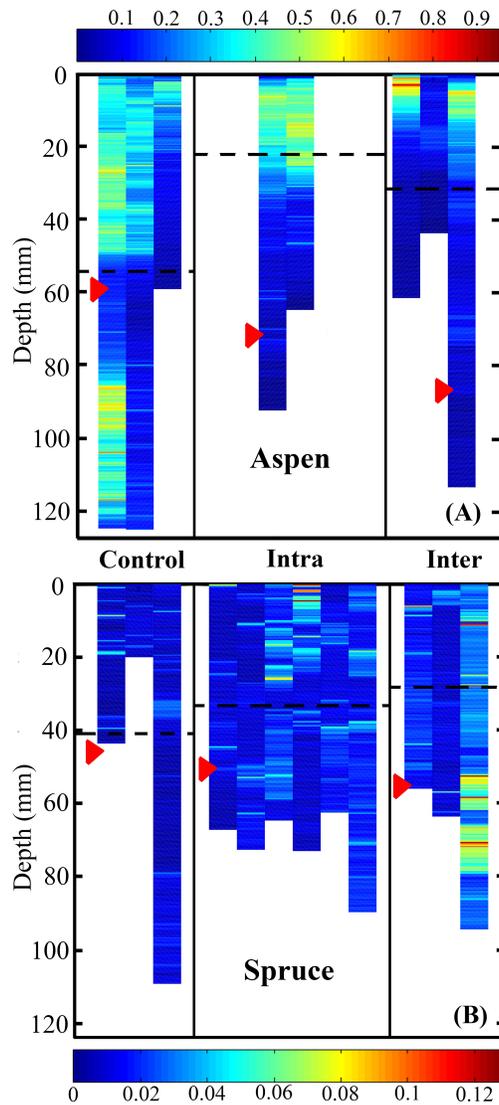


Figure 2.6 Heat map representing root system volume as a function of depth for aspen (A, *Populus tremuloides*) and spruce seedlings (B, *Picea mariana*). The average depths at which aspen and spruce root system volume was concentrated differed significantly between control, intra-, and inter-specifically grown seedlings ($P < 0.0001$). The average depth of aspen root tips also differed between control, intra-, and inter-specifically growing seedlings ($P \leq 0.0017$). Similar differences were not observed in the depth distribution of spruce root tips. Units are in mm³. Each striated column represents the full root volume of a single seedling. Red arrows indicate the average depth of root tips, while striped black lines indicate the average depth of root system volume.

The vertical distribution of aspen root tips differed significantly between control, intra-, and inter-specifically growing seedlings ($P < 0.0001$). Solitary aspen tended to distribute their root tips evenly across vertical space, and occupied an average depth of $58.6 \text{ mm} \pm 1.43 \text{ mm}$. Intra-specifically growing aspen root tips were predominately located between 300-1200 mm, and averaged $72.5 \pm 3.01 \text{ mm}$ in depth. Inter-specifically growing aspen root tips were concentrated between 400-1000 mm, and averaged $85.6 \pm 2.41 \text{ mm}$ in depth. Post hoc analyses indicated that inter-specific root tips were located significantly deeper than intra-specific root tips ($P < 0.0001$), as well as control root tips ($P < 0.0001$). Intra-specific root tips were also located significantly deeper than control root tips ($P = 0.0017$). The mean depths of spruce root tips did not differ between control, intra- and inter-specific seedlings, which were located at 45.2 ± 6.56 , 50.4 ± 2.86 , and $58.2 \pm 6.10 \text{ mm}$, respectively.

2.5 DISCUSSION

2.5.1 AFFECT OF BELOWGROUND INTERACTIONS ON ROOTS

In this experiment, each seedling was grown under full nutrient conditions without competition for light to minimize any variation between solitary and paired individuals that could be attributed to aboveground resource competition. The results of this study suggest that belowground interactions had measurable effects on both species' root system architecture and spatial distribution. For example in aspen, belowground interactions reduced belowground surface area, as well as SRL, which suggests that aspen's response to neighboring root systems was negative (i.e. competitive).

As mentioned above, belowground competition also shifted the distribution of aspen roots. One example was root-root tip distances; root tips under inter-specific conditions (7.5 mm) were spaced farther apart than controls (5.9 mm, Figure 2.5). While a 1.6 mm difference in spacing may seem small, phosphorus concentrations increase exponentially over a distance of 1mm from a root's surface (Hendriks *et al.* 1981). Therefore, relatively small adjustments in the spacing of fine roots could be the difference between high or low levels of competition for nutrients in soil (Hodge 2009). The observed variation in root-root spacing may also stem from fewer average root tips per inter-specific root system (102 tips) compared to intra-specific (173 tips) or controls (254 tips). Fewer total root tips are likely to be spaced farther apart given a constrained volume.

We also observed that the vertical placement of aspen's rooting volume differed between control, intra-, and inter-specific seedlings (Figure 2.6). This observation suggests that aspen can respond relatively quickly, and with high plasticity, to the presence of neighboring root systems by shifting the vertical placement of its rooting volume shortly after germinating. By changing the vertical distribution of root system volume, aspen may be capable of reducing levels of belowground competition with neighboring plants by occupying distinctly different vertical rooting zones (*reviewed in* Schenk *et al.* 1999). Alternatively, aspen may shift its root abundance as to spatially overlap with a neighboring plant in an attempt to outcompete them. Regardless of whether aspen's strategy is to outcompete, avoid competition, or some combination of both in response to a neighboring plant, we see that the presence of a neighboring

plant is sufficient to alter the vertical growth of aspen roots.

Belowground interactions also shifted the vertical positioning of root tips in aspen, and to a lesser extent in spruce. In both solitary and paired aspen seedlings, the mean depth of root system volume was shallower than the mean depth of root tips, which was most pronounced under inter-specific conditions (Figure 2.6). Under inter-specific conditions, the mean depth of root tips (85.6 mm) was markedly deeper than the mean depth of root system volume (32.7 mm). This apparent discrepancy between the average depth of root system volume, and the average depth of root tips is noteworthy, mainly because root volume and root tips are not functionally equivalent (Pregitzer *et al.* 1997). Especially in woody plants, a large proportion of root system volume is in the form of a long-lived, woody infrastructure that anchors the plant and supports essential transport functions, as opposed to the most distal part of the root system, the root tips, which are highly metabolically active and demonstrate the highest rates of nutrient and water uptake among all root classes (Pregitzer *et al.* 1997, Volder *et al.* 2005). By spatially segregating root volume from root tips, a plant can occupy an exclusive volume of space while simultaneously foraging for resources, all the while reducing competition with itself. Therefore, when quantifying root growth dynamics in 3D volumes, either in response to itself or non-self interactions belowground, special attention should be paid to the dynamic growth and placement of root tips independently of whole root systems.

As for spruce, the architecture of each root system was dominated by a main taproot

with minimal amounts of branching (Figure 2.4), which resulted in very few significant observations. However, there were some notable trends worth discussing. SRA and SRL tended to be higher under inter-specific conditions compared to intra-specific conditions, as well as the controls, indicating a lower cost of construction for spruce roots under inter-specific conditions. Also, inter-specific spruce roots tended to grow deeper, place root tips deeper, and were spaced farther apart when compared to controls- a response that was similar to aspen.

2.5.2 MODELING ROOT-ROOT INTERACTIONS

The use of mathematical models to describe biological phenomena is inherently complicated by the nature of organismal responses to heterogeneously distributed biotic and abiotic cues (Hodge *et al.* 2009). Belowground, this response can manifest in plants as a proliferation of roots into a nutrient rich patch (Robinson *et al.* 1999), as altered root morphology (Bolte & Villanueva 2006), or shifts in the direction of growth (Falik *et al.* 2005). Accurately modeling this type of non-random growth response is possible (e.g. Godin 2000), but requires data that is highly resolved, both spatially and temporally. In this study, we demonstrate high spatial resolution for a single point in time, which limits our ability to quantify dynamical growth processes. Nevertheless, 3D structural information, such as that captured by the micro-CT techniques used here, provides insights otherwise inaccessible with 2D destructive imaging. In turn, it can be expected that these findings can be used to verify or refute predictions of derived equations that incorporate the interactions between plants.

When modeling root-root distances, we discovered that a phenomenological exponential “growth and decay” model fit well for both species ($SS_{\text{reg}}/SS_{\text{tot}} = 0.75\text{-}0.99$). This model was chosen from a number of mathematical models that were developed to accurately describe the distribution of root-root distances. Alternative models that were generated but not included in this study tended to fit the data somewhat better, but were species specific. This 2-parameter model was adopted because it accurately described the distribution of data for both species across all belowground conditions, whereas other similarly simple models could not. When developing and applying such models, it is important to keep in mind not only the model’s fit, but the scope of the experimental question, the complexity of the model, it’s predictive value, and whether it can be applied universally to all species.

Also, based on similarities in nutrient, water, and light supplied to each container containing either one or two individual seedlings, and the equidistant orientation of paired individuals (intra- and inter-), we assumed that belowground interactions (intra- vs. inter-) would have the same effect on each paired seedling. There is no way for us to know with certainty that individuals were experiencing a treatment effect, or simply resource competition, which would result in similar belowground outcomes. However, as we mention, nutrient levels were maintained at an EC of 1.6-2.0 throughout the experiment, so resource competition would be kept to a minimum. Future experiments should aim to parse out the different effects of nutrient competition and non-resource interactions.

2.5.3 COMPARING METHODS AND CONSTRAINTS

Previous attempts to image undisturbed root systems have been met with mixed success. The benchmark for successfully rendering roots in 3D is set at roughly 90%. For example, Gregory *et al.* (2003) captured 90% of seven day old wheat roots that did not exceed 10 cm in total length. Kaester *et al.* (2006) reported that they could successfully capture 90% of roots larger than 0.18 mm, which they demonstrated on *Alnus incana* (alder) roots. However, alder roots had to first be removed from their growing medium and packed into quartz sand prior to imaging. In another experiment, Perret *et al.* (2007) captured around 87% of the total root segments, and 78% of the total root lengths in 21 day old chickpea.

In our experiment using *Picea mariana* (spruce) and *Populus tremuloides* (aspen), we successfully rendered between 62-76% of the actual root system architecture (Figure 2.3). We believe that roughly 30% of the root systems were lost in the annotation phase of the methodology because of the criteria we followed for each annotation. Specifically, roots that contacted the container wall were to be excluded on the basis that these roots will behave uncharacteristically, i.e. container circling. We would have predicted that based on aspen's larger root system size, a larger proportion of aspen roots would have been lost in the reconstruction phase when compared to spruce. However, data from both species were included in our general linear model (Figure 2.3). It appears that both species experienced a similar loss of roots during this phase of the experiment, which suggests that any bias introduced as a result of the annotation criteria was similar for both species.

Limitations of our methodology include 1) the use of synthetic growth medium, 2) manual identification of roots in the annotation process, and 3) the relatively small instrument aperture. Regretfully, we could not heed the call of Gregory and Hinsinger (1999), who argued that future advancements in research involving micro-CT and plant roots must focus on using natural soils in place of sand or hydroponics. Distinguishing between water within roots, and water in the medium is an often-reported limitation- one we experienced early on during method development. We attempted to circumvent this issue by growing plants in hydrophobic, synthetic “sand.” This way, the amount of water remaining in the container during imaging would be minimized, and identifying roots made easier. While this worked well for us, we cannot conclude that either species’ growth was unaffected by this growth method. Though residual water was minimized, there were still trace amounts that disrupted automated root tracking algorithms. Thus, the data required manually tracing each root through +1,400 cross sections, which required 6-8 hours per dataset. Future plant research employing micro-CT should strongly consider developing a robust root-tracking approach that is insensitive to artifacts imposed by residual water in the growth medium.

Lastly, the instrument’s aperture for accepting samples greatly limited our container size. Future work that employs micro-CT for phenotyping or quantifying belowground phenomena in an undisturbed space must consider the physical size constraints, and perhaps modify their experimental design to ensure that roots remain unimpeded by

the boundaries of the container. The containers used for this experiment were sufficiently long (~ 300 mm), but insufficiently wide (max ~70 mm). Had the container width not been limiting, it is likely that a fewer roots would have been lost during 3D reconstruction.

2.5.4 CONCLUSION

In this experiment, we could not conclude with any certainty that intra- and inter-specifically growing seedlings differed in terms of root system architecture and use of 3D space. We showed that, when compared to solitary individuals, inter-specific interactions could have the effect of reducing root production, shifting the depth of root tips, increasing spacing between root tips, and altering the distribution of root system volume over vertical space. Because predictable shifts in rooting depths, lateral root placement, and/or root abundance based on neighbor identity may have far reaching implications in terms of ecosystem function (Hooper *et al.* 2005), species coexistence (Grime *et al.* 1997, Stoll & Prati 2001, Bruno *et al.* 2003, Kembel *et al.* 2008), and plant evolution (Myers *et al.* 2000), interactions at the community level down to the individual and tissue level must be better understood. The future of this technique is in quantifying both very fine and coarse scale morphological and architectural shifts in root system growth. We demonstrate the ability to quantify spatial parameters and track multiple 3D root systems within a shared volume, which is an important advancement in the field of plant imaging. By coupling CT imaging with algorithms tailored to specific experimental conditions, a wide range of relevant architectural, morphological, and spatial parameters can be analyzed, and the effects

of belowground interactions better understood. It is our aim that the marriage of CT with novel algorithms will continue to pave the way toward understanding how plants sense, react, and respond belowground to neighboring plants, and shed light on this highly plastic, ecologically significant, and dynamic process that remains almost entirely unnoticed.

This work was made possible through intensive, cross-discipline collaboration. Individual contributions are as follows: AP for the experimental design, plant production, data analyses, and lead authorship. JS for 3D metrics, image construction, and major intellectual contributions. JP for intellectual contributions, data processing, and developing novel root-tracking algorithms. TB for intellectual contributions, including experimental design and critical revisions.

2.6 ACKNOWLEDGEMENTS

We acknowledge the invaluable assistance provided by Cornell's nano- and micro-CT facility run by Mark Riccio. We thank Professor Neil Matson for providing us with reservoirs and tools needed to grow our plants, professor Anthony Reeves for his assistance in developing preliminary root tracking algorithms, and Professor Joseph Fetcho for providing key software. We would also like to acknowledge Annika Kreye and Lindsey Saum for their help in designing and building hydroponic infrastructures, as well as Kim Goodwin and Kendra Hutchins for their help in the greenhouse. This work was partially supported by the Mario Einaudi Center for International Studies, and JLS was funded through an NSF GRFP fellowship.

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CHAPTER 3

INTER-SPECIFIC INTERACTIONS AFFECT FINE ROOT GROWTH, SURVIVORSHIP, AND RESPIRATION IN ADULT EUROPEAN BEECH (*FAGUS SYLVATICA*) AND NORWAY SPRUCE (*PICEA ABIES*)

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3.1 ABSTRACT

Competition belowground is a ubiquitous feature in both natural and managed landscapes. Competing individuals use unique species traits in order to acquire essential soil resources that vary temporally and spatially. Inter- vs. intra-specific interactions may differentially affect competitive outcomes due to differences in species traits used between competitors. To test this, we measured the effects of inter- and intra-specific interactions on the growth, placement, respiration, and survivorship of adult *Picea abies* and *Fagus sylvatica* tree roots growing in monotypic and mixed stands. Inter-specific interactions shifted the vertical placement, and diameters of both species' fine roots. Furthermore, we observed a significant effect of inter-specific interactions on root respiration in both species' roots, as well as the median lifespan of beech roots. Inter-specific fine root respiration in both species was lower in spring when compared to intra-specific fine root respiration. In beech, inter-specific roots

lived > 200 days longer than intra-specific roots. Also, the presence of mycorrhizae reduced the risk of mortality in beech and spruce roots by 65 and 32%, respectively. As the number of roots per area increased, the risk of fine root mortality decreased in both species as well. Our results indicate that survivorship as a foraging strategy may play a more important role than is currently assumed in competition models. We conclude that inter-specific interactions among dominant tree species can result in a reduction in belowground competition due to differences in species traits, but that research into more than just one species pair is required.

Key words: inter-specific, beech, spruce, survivorship, competition, root growth, species traits

3.2 INTRODUCTION

Across tree-dominated landscapes, interactions between neighboring individuals continuously shift the availability of above and belowground resources (Tilman 1980, Casper & Jackson 1997). Belowground, interactions result from the overlap of functionally equivalent tissues, i.e. roots. To date, the study of plant interactions has mostly centered on competition, both at the ecosystem and individual level (e.g. Wilson & Tilman 1991, DeAngelis 1992). Belowground resource competition begins once nutrient depletion zones overlap, which in turn affects an individual's growth and fecundity (Nye & Tinker 1977, Nambiar & Sands 1993). Based on the identity of neighboring individuals, the outcome of competitive interactions can vary depending on the belowground species traits each possess (Gaudet & Keddy 1988, Aerts 1999, Callaway *et al.* 2003). At an ecosystem level, over time, this could alter community

composition, nutrient availability, and ecosystem processes (Hooper & Vitousek 1997, Tilman *et al.* 1997, Grime 2006). By comparing inter- and intra-specific differences in root growth traits, we can begin to understand how the identity of a neighboring plant might impact belowground competition, and therefore long-term growth and productivity.

Because soil resources are often heterogeneously distributed, both spatially and temporally, trees are forced to constantly forage for essential resources or risk being outcompeted by neighboring plants (Casper & Jackson 1997, Farley & Fitter 1999, Ettema & Wardle 2002). Root foraging, an expression of phenotypic plasticity belowground, can take many forms (Tilman 1988, Hutchings & deKroon 1994, Hodge 2006). Root proliferation is essentially the most basic form of foraging, and often coincides with periods of increased resource availability and/or environmental favorability (Eissenstat & Caldwell 1988, Pregitzer *et al.* 1993, Hodge 2006). However, certain species will vary the timing of root production, which can have the effect of either preempting a resource or spatially partitioning its uptake (Chesson 2000, Stratton *et al.* 2000, McKane *et al.* 2002). Species can also differ in their relative growth rates of roots, where faster growing species have been linked to greater root turnover and resource acquisition (Kembel *et al.* 2005, Kembel *et al.* 2008, McCormack *et al.* 2013).

Additional foraging strategies include morphological and architectural adjustments to fine roots. Fine roots, defined here as 1-4th order roots less than 2mm in diameter, are

uniquely responsible for the uptake of soil resources, as well as for forming essential associations with soil microorganisms (Pregitzer *et al.* 1997, Pregitzer *et al.* 2002). Adjustments to fine root diameter or length, therefore, can have very real consequences on resource uptake and plant growth (Eissenstat 1992, Gill & Jackson 2000). Increasing root length per root mass, termed specific root length (SRL, cm g^{-1}), is an example of a morphological adjustment that effectively increases absorptive root area without shifting the C cost of root construction (Eissenstat 1991, Eissenstat & Yanai 2002, Ostonen *et al.* 2007). Examples of architectural adjustments include shifts in fine root branching angle (Mou *et al.* 1997, Pregitzer *et al.* 2002), as well as the formation of herringbone phenotypes upon sensing nutrient-rich patches (Drew & Saker 1978). However, differentiating between the effects of resource availability, inter-specific interactions, and/or alternate biotic cues on fine root morphology/architecture continues to complicate field experiments.

Field sites containing two or more monotypic groupings of plants that share a distinct boundary between species (common garden), but do not vary in resource abundance, have allowed researchers to draw comparisons between inter- and intra-specifically growing plants. For example, inter-specifically growing species may shift the average depth of fine roots when compared to either species' monotypic rooting depths, which could affect access to certain resources (e.g. Schmid & Kazda 2002). Competition for immobile nutrients (such as P, Ca, and Mg) is often greatest in near-surface soils where decomposing leaf and root litter, and coarse debris generate the largest quantities of plant-available cations (Jobbágy & Jackson 2001, Lynch & Brown 2001).

Other resources such as water are more homogeneously distributed, which would allow inter-specifically growing species that occupy distinctly different soil depths access to discrete resource pools (Caldwell *et al.* 1998, Williams & Ehleringer 2000). When discrete resource pools are unavailable due to a high proportion of root system overlap, certain tree species can trade off in the timing of water uptake (seasonal leaf phenology), as well as the depth of root activity (spatial), which allows for the coexistence of neighbors with very similar niche requirements (Meinzer *et al.* 1999, Stratton *et al.* 2000).

Not surprisingly, roots that proliferate in response to high levels of resources have been shown to persist longer in the soil than roots produced within relatively resource poor patches (Aber *et al.* 1985, Burton *et al.* 2000). It has been hypothesized that root persistence (longevity) may be an important yet undervalued foraging strategy (Eissenstat 1997). Individuals that maintain fine roots within resource patches for longer can effectively preempt soil space and also ensure a greater return on investment (Eissenstat 1997, Eissenstat 2000). Moreover, greater N availability in soils has been linked to greater fine root longevity, as well as fine root respiration (Aber *et al.* 1985, Volder *et al.* 2005). Therefore, inter-specific interactions between individuals that affect the availability of soil resources may consequently affect the persistence of roots and root respiration as well.

We present here an experiment aimed at characterizing inter- vs. intra-specific effects on the growth, placement, respiration, and survivorship of two co-occurring tree

species in Western Europe: European beech (*Fagus sylvatica*) and Norway spruce (*Picea abies*). We hypothesized that inter-specific over-yielding in beech/spruce stands can be attributed to a release from intense intra-specific competition resulting from a high degree of niche overlap (Pretzsch *et al.* 2012). We therefore predicted for both species that intra-specific fine roots would have smaller average diameters (morphological adjustment), greater fine root production, higher risk of mortality (lower survivorship), and lower rates of respiration when compared to fine roots growing inter-specifically.

3.3 MATERIALS AND METHODS

3.3.1 SITE CONDITIONS

The study was performed across ten randomized plots containing 70-75 year old Norway spruce (*Picea abies*) and European beech (*Fagus sylvatica*) trees located in the “Kranzberger Forst,” a research site near Freising, Germany (48°25’N, 11°39’E, 490 m a.s.l.). Rectangular plots (~ 5 x 20 m) contained monotypic groupings of spruce trees at one end, and at the opposite end, a monotypic grouping of beech trees (minimum of three individuals per species per plot). The central portion of each plot contained a “mixed” region with a high degree of interspecific root mixing (Mainiero *et al.* 2010, Häberle *et al.* 2012, Pretzsch *et al.* 2014). Soils were haplic luvisols, and replete in both nutrients and water. Long-term averages (1970-2000) of mean precipitation and air temperature were 786 mm and 7.6°C respectively (Matyssek *et al.* 2007). Stand basal area was 46.4 m² ha⁻¹ (Wipfler *et al.* 2005).

3.3.2 SITE INSTALLATION AND INFRASTRUCTURE

To prevent cross-plot root interactions and water flow between each of the ten experimental plots, plots were trenched in 2010 until a known clay-pan was reached (~ 1 meter), lined with low-density poly-vinyl plastic, and then backfilled (Häberle *et al.* 2012). During this time, in-ground plywood boxes (root box: 40x60x30 cm, H x W x D) containing two acetate windows were installed to view, trace, and sample roots of known species and age (Bouma *et al.* 2001). Three root boxes were installed per plot; one in each species' monotypic region, and one in the mixed species region. Viewing windows, when not in use, were insulated year round and a lid was screwed into place to prevent micro-climatic effects and exclude light penetration.

Time domain reflectometers (TDR) used to monitor volumetric soil water content were installed in 2010 across all ten plots at two soil depths: 0-10 and 10-30 cm (Cole, 1977). Soil volumetric water content was measured weekly from April through November, and monthly from December through March (TDR-100, Campbell Scientific, North Logan, Utah). Air temperature, relative humidity, and precipitation were collected continuously across 2011-2013; climate data was monitored at climate station "Weihenstephan," approximately 4 km from Kranzberger forst research station (Deutsche Wetterdienst, Offenbach, Germany).

3.3.3. ROOT OBSERVATION

Also in 2010, clear acrylic minirhizotron tubes (70 cm long, 6 cm outside diameter) were installed at an angle of 60° from the horizontal to a depth of 60 cm (51 vertical cm). A vertical depth of approximately 50cm was chosen based on previous research at Kranzberger forst which reported that $> 90\%$ of beech and spruce roots were located between 0-50cm (Häberle *et al.* 2012). Each plot contained six minirhizotron tubes: two in each species' monotypic regions, and two within the mixed species region (i.e. inter-specific region). Each tube was located a minimum distance of one meter from the plot boundaries, and in the case of inter-specific regions, tubes were installed equidistantly from both species. Before installation, minirhizotron tubes were capped at the base with plastic plugs lined with silicon caulk to reduce water infiltration. Tubes, when not in use, were covered with large plastic caps to prevent aboveground water infiltration and light penetration.

Beginning in May 2011 and ending in October 2013, continuous images were taken across the length of each tube using a specialized laparoscopic camera (BTC100X Camera, Bartz Technology, Carpinteria, California). Just prior to leaf emergence (April) and until leaf senescence (November), images were taken every 10-15 days. During the winter months, images were taken monthly. Images were approximately 15mm in height, and 18mm wide. All images were analyzed for fine root production, survivorship, standing crop, and morphology using *WinRHIZO Tron MF* (Regent Inc., Quebec, Canada). The date of root birth was calculated as the mid-point between the appearance of a new root tip and the preceding image session. Similarly, the death

date of a root was calculated as the mid-point between the disappearance or death of a root (black, shriveled) and the preceding image session (Comas *et al.* 2005). Roots that transected more than one observation window were noted and only counted once. Differences between species' roots were determined by visual inspection of cortex coloration (spruce: brown, beech: reddish white), along with root tip branching patterns (spruce: alternate branching, beech: herringbone and often opposite branching).

3.3.4 ROOT RESPIRATION

Root respiration was measured in the spring and fall of 2012 and 2013 (May 22 and October 6, 2012, and May 18 and October 7, 2013). Using the pre-installed root boxes in both intra- and inter-specific regions, fine root branches < 2mm in diameter containing 1st and 2nd order roots were traced along acetate windows using colored markers for a period of three weeks to ensure that fine root material was of a known species and age ($5 < x \leq 21$ days old). Pioneer roots, defined as first order roots exhibiting little or no branching, lower incidence of mycorrhizal colonization, and larger average diameter were excluded in this experiment (Zadworny & Eissenstat 2011). Highlighted roots were then excised from behind acetate windows using a razor blade, transferred less than 50 meters to a climate controlled hut, and placed into a cuvette containing oxygenated buffer (10 mM MES, 1mM CaSO₄, and 5.5 μ M K₂HPO₄, pH = 5.8; Volder *et al.* 2005) within an oxygen electrode system maintained at 21 ± 0.5 °C (Oxygraph, Hansatech, King's Lynn, UK). Oxygen consumption was then measured over a period of 15 minutes. After 15 minutes, root branches were removed from the cuvette, placed in a paper envelope, dried at 60⁰C for three days,

and then weighed. Respiration is presented in $\text{nmol O}_2\text{g}^{-1}\text{s}^{-1}$.

3.3.5 DATA ANALYSIS

Root production was calculated on a per plot basis as the total number of root tips produced per square meter of viewing window. Fine root standing crop was calculated by subtracting the number of dead roots from the total number of roots produced. Survivorship was analyzed using Cox's proportional hazards regression on pooled data from 2011-2013 (PROC PHREG, SAS Institute Inc. Cary, NC, USA). This type of analysis holds all covariates constant except for one, and tests the 'hazard' of that covariate, i.e. the risk of mortality of a fine root at time t , where t is the product of a baseline hazard function of k covariates. SAS's PROC PHREG uses the partial likelihood method of Cox (1972) to estimate a coefficient (β) for each of the covariates being tested, and then calculates a chi-square statistical test to evaluate the null hypothesis: $\beta = 0$.

Tested covariates were root diameter, root depth, root order, presence/absence of mycorrhizae, number of neighboring fine root tips, averaged volumetric soil water content the week of root tip birth, averaged volumetric soil water content the week of root tip death, and interaction (inter vs. intra-specific). Based on whether the covariate was continuous (diameter, depth, volumetric soil water content, or the number of neighboring fine root tips), or categorical (interaction, root order, or presence/absence of mycorrhizae) affected the interpretation of the parameter estimate and the hazard ratio. Negative parameter estimates indicated a decrease in mortality with each increase in the covariate (Wells & Eissenstat 2001). The percent change in the hazard

(mortality) per one unit change in soil depth (1 cm), volumetric soil water content (1 %), number of neighboring fine roots (1 root tip), or root diameter (1mm) can be estimated by subtracting one from the hazard ratio and multiplying that value by 100. As for categorical covariates, the model outputs are reported relative to the first category (i.e. 1 vs. 2, 3...). When interpreting the interaction, root order, or presence/absence of mycorrhizae, a positive parameter indicates greater risk or mortality for intra-specific roots, first order roots, and when mycorrhizae are visible, and estimated by subtracting one from the hazard ratio and multiplying that value by 100 while controlling for other covariates. Because there were very few observations of third or higher order roots, the analysis included first and second order roots, exclusively.

All remaining statistics were run using JMP 10.0 (SAS Institute Inc. Cary, NC, USA). The effects of year, season, species, and interaction on 1) root production, as well as 2) depth of production were tested using four-way ANOVA. The seasons are defined here as winter (December 01-February 28), spring (March 01-May 31), summer (June 01- August 31), and fall (September 01- November 30). The effect of year, species, and interaction on fine root respiration was tested using three-way ANOVA. Differences in soil water content across 2011-2013 among intra-specific spruce, intra-specific beech, and inter-specific regions was assessed using two-way ANOVA. Post-hoc analyses comparing means were performed using Tukey's HSD test ($\alpha = 0.05$).

3.4 RESULTS

3.4.1 SOIL WATER AND CLIMATE

Both interaction (inter- vs. intra) and sampling date were found to be significant predictors of soil water content between 0-10 cm ($P < 0.0001$), as well as 10-30 cm depths ($P < 0.0001$). Between 0-10 cm, average soil water content in mixed ($22.6\% \pm 0.1$) and beech-dominated soils ($21.9\% \pm 0.09$) was higher than spruce-dominated soils ($20.3\% \pm 0.09$; Figure 3.1A, $P < 0.0001$) across all three years. Between 11-30 cm, average soil water content in mixed ($28.5\% \pm 0.08$) and beech-dominated soils ($28.1\% \pm 0.08$) was also higher than spruce-dominated soils ($26.2\% \pm 0.08$, Figure 3.1B, $P < 0.0001$). During the month of August, beech, spruce, and inter-specific soil regions all reached similar minimum levels of soil water. Soil temperature was inversely related to soil water content, and did not vary significantly between beech and spruce dominated regions (Figure 3.1B).

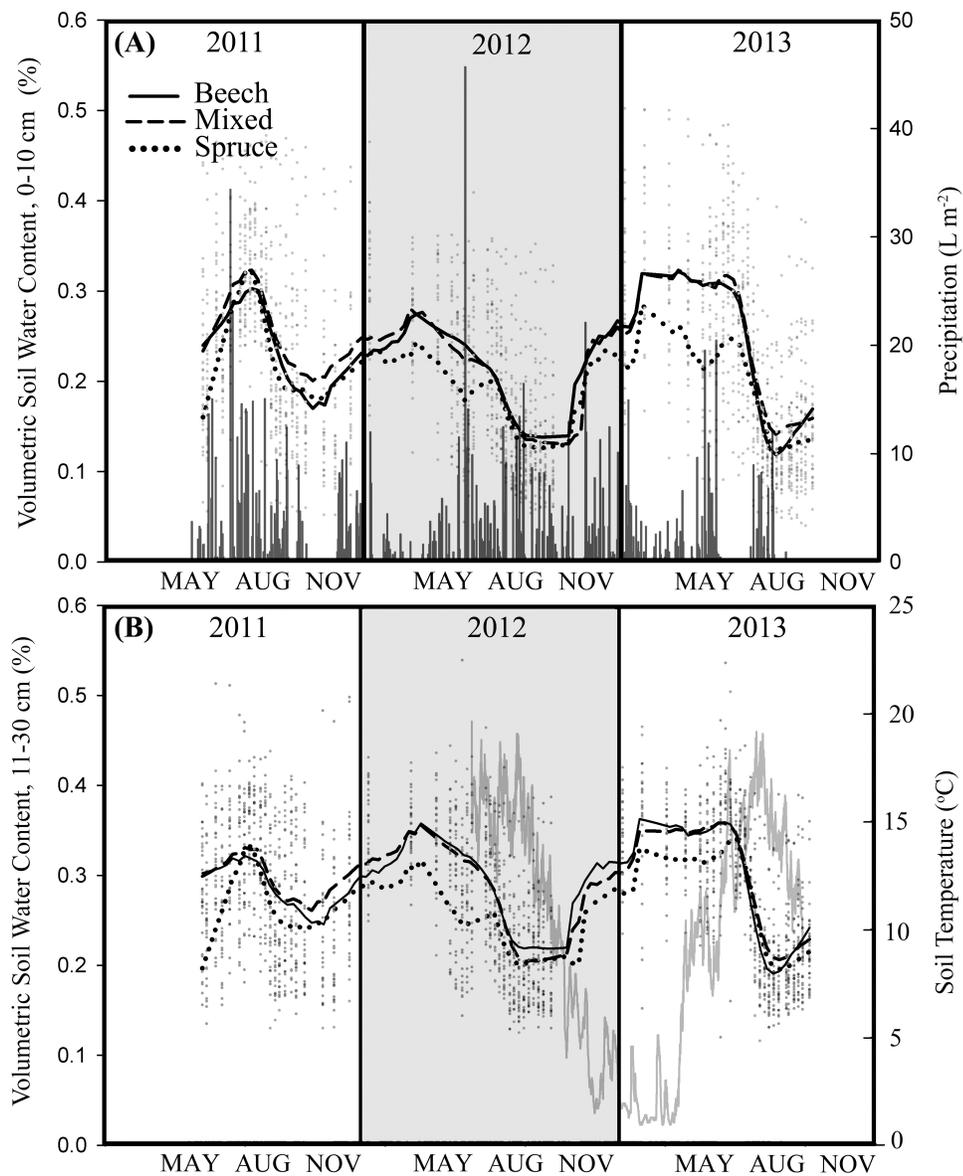


Figure 3.1 (A) Daily precipitation (grey bars) and soil volumetric water content 0-10 cm, and (B) soil volumetric soil water content 11-30 cm and soil temperature (grey line) at “Kranzberger forst” spanning from May, 2011 through September, 2013. Soil temperature was measured at a depth of 10 cm from June, 2012 through October, 2013. Soil volumetric water content was measured weekly during the growing season, and monthly during the winter. TDR probes were installed under beech dominated (solid line) and spruce dominated regions (dotted line), as well as between the two species monotypic regions in the mixed or inter-specific region (dashed line).

3.4.2 FINE ROOT PRODUCTION

Fine root production varied annually from 2011 to 2013 in both beech and spruce ($P < 0.0001$). Both species' root production was highest in 2011; production then decreased each year until observations ceased in fall, 2013 (Figure 3.2). Average annual fine root production for beech was 2473, 1136, and 426 roots m^{-2} in 2011, 2012, and 2013, respectively. Average fine root production for spruce was 769, 207, and 140 roots m^{-2} in 2011, 2012, and 2013, respectively. When evaluating root production on a seasonal basis, summer and fall production rates were the highest and statistically similar, while spring and winter production rates were the lowest and also statistically similar (Tukey HSD test, $P < 0.0001$). Between 2011 and 2013, inter- vs. intra-specific interactions had no effect on either species' root production ($P = 0.12$, Figure 3.2).

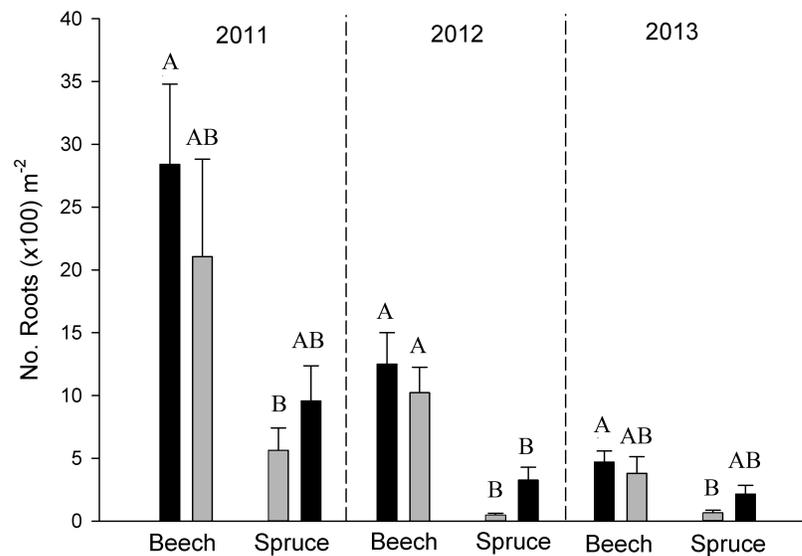


Figure 3.2 Annual production of *Fagus sylvatica* and *Picea abies* fine roots within intra-specific (black) and inter-specific (grey) soil regions within “Kranzberger forst” research station (bars represent the st. error). Production was calculated by dividing the number of newly produced root tips per area of viewing window down to 50cm. Fine root production was compared within each year using Tukey HSD test (No. of observed roots, 2011: [intra] beech = 1690, [inter] beech = 1253, [intra] spruce = 580, [inter] spruce = 335; 2012: [intra] beech = 744, [inter] beech = 688, [intra] spruce = 194, [inter] spruce = 21; 2013: [intra] beech = 180, [inter] beech = 227, [intra] spruce = 128, [inter] spruce = 39). Differences between means are denoted by different letters ($P < 0.05$).

Although belowground interaction (inter- vs. intra-) had no effect on the number of fine roots produced, it had a significant effect on the depth of root production in both beech and spruce ($P < 0.0001$, Figure 3.3). Additional predictors that significantly affected the depth of production were season ($P < 0.0001$) and species ($P < 0.0001$), but not sampling year ($P = 0.16$). Beech root production was the most shallow in the spring; roots were then produced significantly deeper as the seasons progressed (i.e. spring to winter). In spruce, the average depth for intra-specific roots was 20.5 cm, which was markedly deeper than inter-specific spruce roots (12.3 cm) (Figure 3.3). There were also weak seasonal trends in the depth of spruce root production: only

roots produced in winter were significantly shallower than other seasons ($P < 0.0001$).

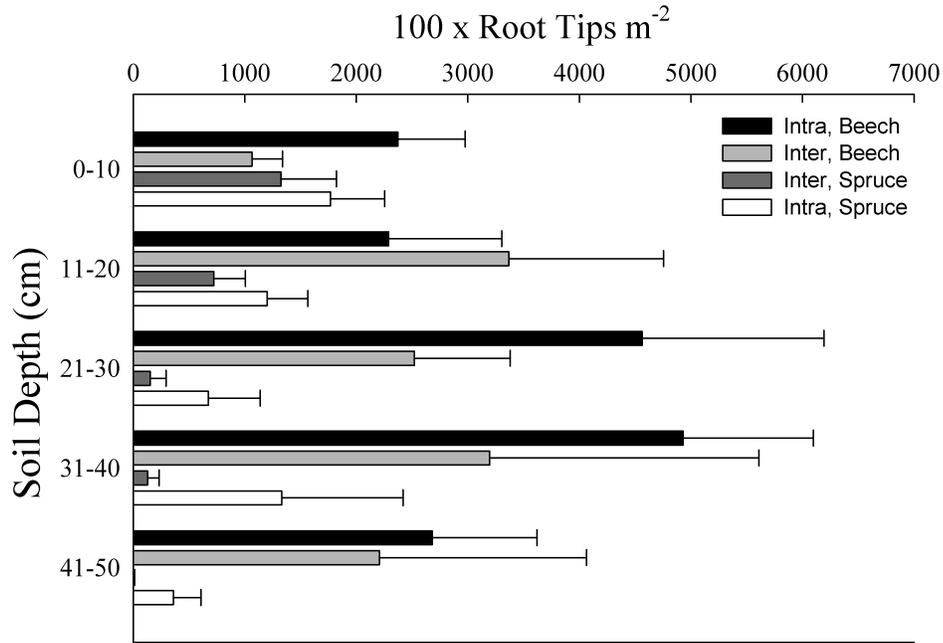


Figure 3.3 Mean number of *Fagus sylvatica* (beech) and *Picea abies* (spruce) roots produced per square meter of viewing window (st. error) between 2011-2013 at “Kranzberger Forst” research station. Belowground interactions (inter- vs. intra-specific) had a significant effect on both species’ rooting depth ($P < 0.0001$, ANOVA): inter-specifically growing roots were produced shallower than intra-specific roots. Beech root production was also significantly deeper than spruce across 2011-2013 ($P < 0.0001$, ANOVA).

Beech’s standing crop root population was significantly greater than spruce during all three years (Figure 3.4). In 2011, standing crop was greater within intra-specific rooting zones, but high winter mortality in 2012 shifted the larger root population from intra- to inter-specific rooting zones. In 2013, both inter- and intra-specifically growing beech roots converged toward a similar population size after high winter mortality within inter-specific soils, which suggests that competition may increase turnover. Unlike beech, the standing crop of intra-specific spruce roots was higher than inter-specific roots across all three years (Figure 3.4). Spruce root populations

were largest in 2011, and then declined steadily over the following two years. Additionally, both species' root populations in 2012 included a period of population growth in early to mid-May, then a short die-back of roots in mid to late summer, followed by a population growth spurt in the fall.

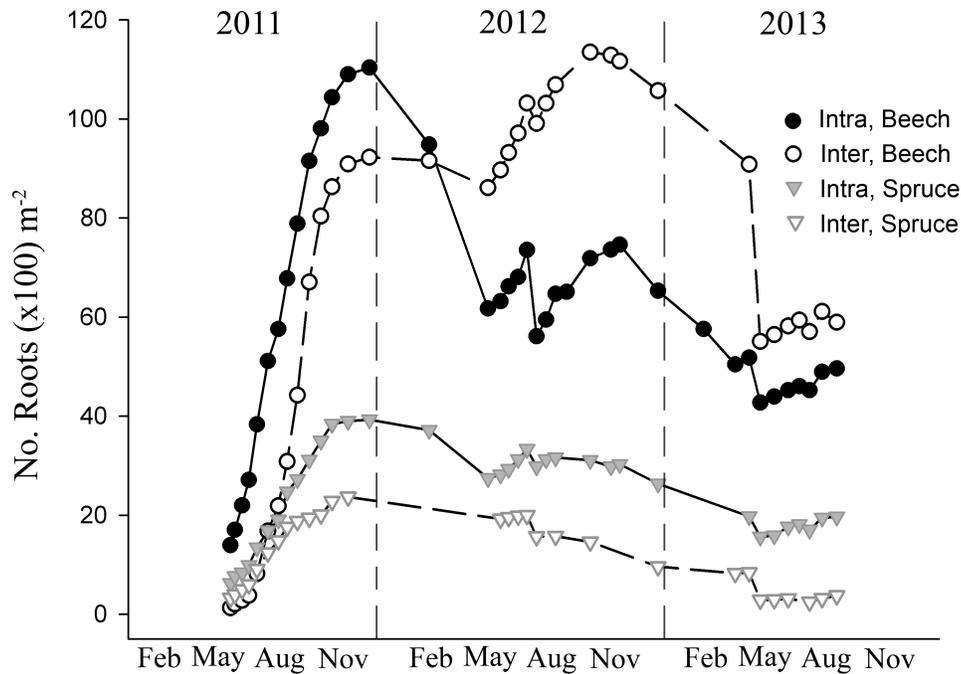


Figure 3.4 *Fagus sylvatica* (beech) and *Picea abies* (spruce) fine root populations (i.e. standing crop) between 2011-2013 at “Kranzberger Forst” research station. Each species’ fine root population was divided into two sub-populations based on the soil region (inter- vs. intra-specific) in which they were observed. Intra-specific beech: closed circles, inter-specific beech: open circles, intra-specific spruce: grey triangles, inter-specific spruce: open triangles. Population data is expressed as the number of living root tips per area of viewing window, and summed across all ten plots.

3.4.3 SURVIVORSHIP

When both inter- and intra-specific populations were combined and analyzed across the observed soil profile (0-50cm), the risk of mortality for beech roots was 48% greater than spruce roots, i.e. spruce roots tended to live longer than beech ($P <$

0.0001, hazard = 1.48, Figure 3.5). When analyzing spruce and beech separately, the following predictors significantly affected the risk of fine root mortality in both species: inter- vs. intra-specific interactions ($P = 0.034$), root order, soil depth, presence/absence of mycorrhizae, number of neighboring root tips, volumetric soil water content the week of root birth, and volumetric soil water content the week of root death ($P < 0.0001$ for all other covariates, Table 3.1).

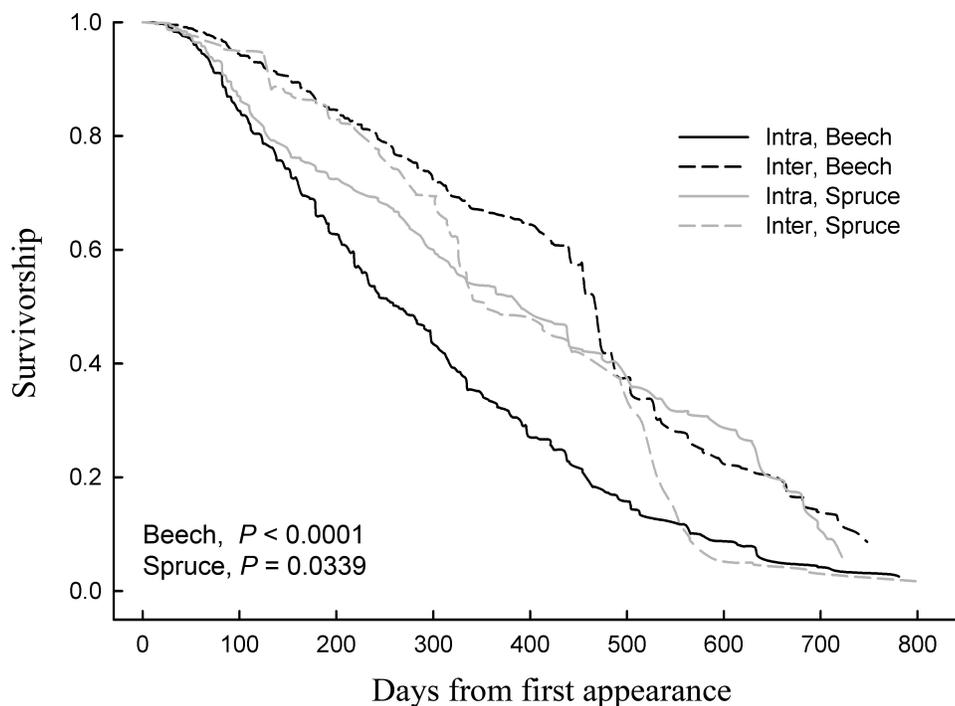


Figure 3.5 Cumulative survivorship of inter- (dashed line) and intra-specifically (solid line) growing *Fagus sylvatica* (beech, black) and *Picea abies* (spruce, grey) fine roots over three years of observation beginning in May, 2011. Species' P-values represent significant inter- vs. intra-specific differences in fine root survivorship (Cox's proportional hazards regression, $P \leq 0.05$). The median lifespan of intra-specifically growing beech and spruce roots was 260 and 382 days, respectively. The median lifespan of inter-specific beech and spruce roots was 466 and 352 days, respectively.

In beech, intra-specific interactions increased the risk of beech fine root mortality by

147% (e.g. $[1-2.47] \times 100$) when compared to inter-specifically growing beech roots. Intra-specific interactions also increased the risk of spruce root mortality by 20% when compared to inter-specifically growing spruce roots (Table 3.1). The risk of first order root mortality in beech was 75% greater than second order roots, and in spruce, the risk of first order root mortality was 67% greater than second order roots (Table 3.1). Increasing fine root diameter in spruce roots had no significant effect on the risk of root mortality ($P = 0.062$, Table 3.1). However in beech, an increase in root diameter significantly decreased the risk of mortality: 93% reduction per 1mm increase in diameter ($P < 0.0001$, Table 3.1).

In both species, deeper roots lived significantly longer: with each 1cm increase in soil depth, the risk of fine root mortality decreased by 3% in beech, and 2% in spruce (Table 3.1). The presence of mycorrhizae decreased the risk of mortality by 65% in beech, and 32% in spruce (Table 3.1). For each additional neighboring root tip (i.e. +1 root tip per viewing window), the risk of root mortality decreased by 0.6% in beech, and 1.6% in spruce (Table 3.1). Volumetric soil water differentially affected the risk of fine root mortality in beech and spruce. In beech, we found that an increase in soil water during the birth-week of a root tip increased the risk of fine root mortality by 6%; increasing soil water during the week of root death also increased the risk of fine root mortality by 5%. In spruce, an increase in soil water during the birth-week of a root tip decreased the risk of fine root mortality by 3%, whereas increasing soil water during the week of root death increased the risk of root mortality by 5%.

Table 3.1 Statistical outputs from *Fagus sylvatica* and *Picea abies* fine root survival analyses (Cox Proportional Hazards model).

Species	Parameter	Parameter Estimate	St. Error	Significance	Hazard Ratio
Beech	Interaction	0.905	0.0420	< 0.0001	2.47
	Root order	0.558	0.0624	< 0.0001	1.75
	Root diameter	-2.66	0.260	< 0.0001	0.069
	Soil depth	-0.0278	0.00142	< 0.0001	0.973
	Mycorrhizae	-1.063	0.0386	< 0.0001	0.346
	No. of neighbors	-0.00554	9.27 E-4	< 0.0001	0.994
	Soil water, birth	0.0393	0.00437	< 0.0001	1.04
	Soil water, death	0.0482	0.00195	< 0.0001	1.05
Spruce	Interaction	0.183	0.0861	= 0.0339	1.200
	Root order	0.511	0.115	< 0.0001	1.67
	Root diameter	-0.561	0.301	= 0.0620	0.570
	Soil depth	-0.0247	0.00263	< 0.0001	0.976
	Mycorrhizae	-0.382	0.0677	< 0.0001	0.683
	No. of neighbors	-0.0158	0.00173	< 0.0001	0.984
	Soil water, birth	-0.0348	0.00649	< 0.0001	0.966
	Soil water, death	0.0469	0.00382	< 0.0001	1.05

The following covariates were tested: inter- vs. intra-specific interactions, root order (1st-2nd), root diameter (mm), soil depth (cm), presence vs. absence of mycorrhizae, number of neighboring root tips, volumetric soil water content the week of root birth (%), and volumetric soil water content the week of root death (%). Hazard ratios > 1 indicate a greater risk of mortality, while a hazard ratio < 1 indicates a decrease in the risk of fine root mortality. Significant results are highlighted in bold ($P \leq 0.05$).

3.4.4 MORPHOLOGICAL AND PHYSIOLOGICAL PLASTICITY

On average, beech fine roots were smaller in diameter (0.23 mm) than spruce fine roots (0.34 mm, $P < 0.0001$). Inter-specifically growing beech roots were also slightly larger than intra-specific roots (0.24 vs. 0.23 mm, $P < 0.0001$), while the opposite was true for spruce (0.31 vs. 0.35 mm, $P < 0.0001$). Additionally, the season in which roots were produced had a significant effect on the fine root diameter of both species: spring > summer > fall > winter ($P < 0.0001$).

Fine root respiration varied widely across years and seasons, but did not differ

significantly between species. Both species had lower respiration rates in 2012 than 2013 ($P < 0.0001$), and spring respiration was lower than fall ($P < 0.0001$). Respiration also differed between inter- and intra-specifically growing beech and spruce roots (Figure 3.6A and B). On average, intra-specific beech ($49.7 \pm 21 \text{ nmol O}_2\text{g}^{-1}\text{s}^{-1}$, mean \pm SD) and spruce roots ($44.2 \pm 22 \text{ nmol O}_2\text{g}^{-1}\text{s}^{-1}$) respired more across 2012 and 2013 when compared to inter-specific beech ($31.4 \pm 23 \text{ nmol O}_2\text{g}^{-1}\text{s}^{-1}$) and spruce roots ($31.7 \pm 17 \text{ nmol O}_2\text{g}^{-1}\text{s}^{-1}$, $P < 0.0001$). When looking at seasonal differences, inter-specific fine root respiration was significantly less than intra-specific root respiration in beech roots during the spring of 2012 and 2013, and in spruce roots during the spring of 2012 ($P < 0.0001$, Figure 3.6A).

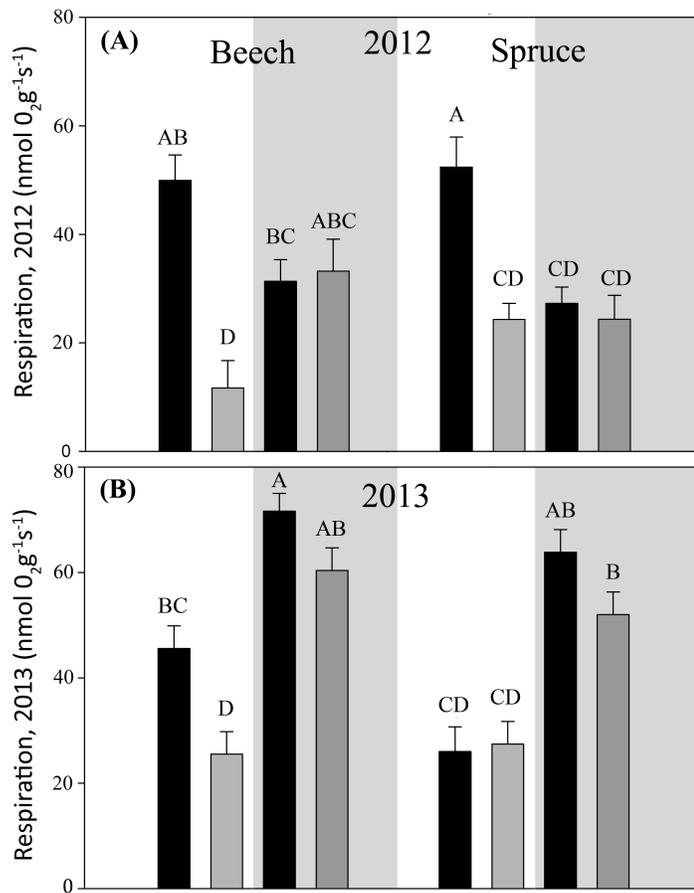


Figure 3.6 Respiration of 1st and 2nd order ($5 < x \leq 21$ days old) *Fagus sylvatica* (beech) and *Picea abies* (spruce) fine roots growing intra- (black bar) and inter-specifically (grey bar). Respiration was quantified via oxygen consumption during both the spring (white background) and fall (grey background) of 2012 and 2013 (A: 2012 beech $n = 56$, spruce $n = 56$; B: 2013 beech $n = 48$, spruce $n = 46$). Based on the observed annual variation in fine root respiration, comparisons were made within each year using Tukey HSD test ($P < 0.05$). Differences between means are denoted by different letters.

3.5 DISCUSSION

In our study, we found that inter-specific interactions affected the vertical placement of roots, reduced fine root respiration, altered fine root morphology, and dramatically decreased the risk of fine root mortality, which has broad implications for reducing belowground competition, ecosystem C budgets, and root ecology in mixed vs.

monotypic tree stands. Perhaps the most novel finding of this study came from the evaluation of inter- vs. intra-specific differences in fine root survivorship (Figure 3.5). The risk of intra-specific root mortality was significantly higher than inter-specific root mortality: 147 and 20% higher in beech and spruce, respectively. Moreover, survivorship and rooting depth appeared somewhat complimentary in beech, i.e. longer lived roots tended to occur in soil regions containing fewer overall roots. Based on a cost-benefit model of root longevity (Eissenstat 1992, Eissenstat *et al.* 2000), our findings indicate that roots growing inter-specifically achieve a greater, or perhaps a more continuous return on investment, thus warranting the prolonged maintenance (i.e. cost) of those roots. According to Tillman's model of resource competition (R^*), greater tissue longevity denotes greater competitive ability (lower R^*), which in this instance, indicates that both species' inter-specific roots are competitively superior to their intra-specific counterparts. Empirical studies have also tied greater median fine root life span to high soil N, lower respiration, and found that certain tree species moderate root longevity in order to improve resource acquisition (e.g. Adams *et al.* 2013). In summary, we confirmed that intra-specific roots experienced a higher risk of mortality. We also concluded that inter-specific soil regions at Kranzberger forst research station appear to constitute a less competitive belowground environment compared to either species' monotypic soil regions.

A relatively novel finding came when we analyzed how the number of observable roots in each of the minirhizotron's viewing window affected fine root mortality (i.e. no. of neighbor roots). In both beech and spruce, as the number of observable roots

increased per viewing window, the risk of mortality decreased (Table 3.1). We were initially surprised by this finding, as an increase in the number of neighbor roots should lead to greater inter-root competition for available soil resources. This would quickly deplete local nutrients and water; therefore, the cost of maintaining roots would be high and the return low. Under these circumstances, root lifespan would likely decrease, and resources reallocated toward the production of new roots within nutrient rich patches (Eissenstat *et al.* 2000). Conversely, an increase in fine root production and life-span may indicate the presence of nutrient rich patches of soil (Pregitzer *et al.* 1993, Hodge 2006), and explain why greater numbers of roots also had a lower risk or mortality when compared to solitary or sparsely grouped fine roots.

Other covariates affecting fine root survivorship included volumetric soil water content the week of root death and root birth. A negative correlation between soil water content (Figure 3.1) and fine root standing crop (Figure 3.4) supports the result that increasing soil water leads to increased mortality, albeit a small increase. However, high root mortality in winter due to lower soil temperatures could also explain the observed trends in fine root survivorship (Tierney *et al.* 2001). Interestingly, increasing soil water content the week of root birth decreased spruce root mortality; a result which differed from beech and from spruce the week of root death. This would suggest that spruce root production and survival, while clearly seasonal (Figure 3.4), might also be closely tied to patterns of precipitation.

Multiple studies have reported the effects of mycorrhizal colonization on fine root tip

mortality. Most authors report an increase in fine root survivorship when roots are mycorrhizal vs. non-mycorrhizal. For example, King *et al.* (2002) found that mycorrhizal fine roots lived an average of 341 days longer than non-mycorrhizal fine roots in *Pinus taeda* (507 vs. 166 days). Guo *et al.* (2008) found that first order *Pinus paulustris* roots colonized by mycorrhizae lived > 45% longer than their non-colonized counterparts. In our study, the presence of mycorrhizae decreased mortality by 65% in beech, and 32% in spruce, which is similar to what Guo *et al.* observed. Although we did not explicitly study the mechanisms, our observations support the theory that mycorrhizal colonization increases fine root lifespan, which was especially true for beech fine roots.

Between 2011 and 2013, both species' fine root production decreased by approximately 50% each year (75% total reduction, Figure 3.2). Alternatively, fine root population sizes changed at varying rates. These findings were surprising because 1) beech's inter-specific fine root populations grew predictably in 2012 across multiple plots while both populations of spruce roots steadily declined, and 2) environmental conditions across all three years were relatively favorable with only two exceptions. Spring 2012 was unseasonably hot and dry compared to other sampled years, while the spring of 2013 was significantly wetter (Figure 3.1). However, high vs. low levels of spring precipitation would likely have opposing effects on fine root growth, which was not observed. The observed decrease in production and population size may be explained by the trenching that took place in 2010. Trenching causes a large amount of disturbance to both fine and coarse roots, which may have produced a

compensatory growth response by both species in order to re-establish a sufficiently large fine root system to support aboveground transpiration. Over time, root growth would then normalize (decrease) to pre-disturbance levels. It is also possible that in early 2012, low levels of precipitation prompted greater beech root exploration into inter-specific soils, which we found to be wetter during pre-summer periods. However, because sampling year had no effect on the depth of root production across inter- and intra-specifically populated soils, nor did year affect the number of inter vs. intra-specifically produced roots for either species, it appears that the trenching “artifact” did not overtly affect our inter- vs. intra-specific comparisons. From these results, it is our belief that future studies involving extensive trenching of forest soils should consider not one, as was observed in our study, but a minimum of two years of normalization post trenching.

Although root production and root populations decreased significantly between 2011-2013, we were still able to quantify novel effects of inter-specific interactions on fine root growth dynamics. For example, we observed higher total fine root production (inter-specific beech + spruce) within mixed soil regions when compared to spruce in monotypic groupings (Figure 3.2), which was previously reported by Schmid and Kazda (2002). However, based on the observation that root production did not differ between inter- and intra-specific regions (Figure 3.2), we found no evidence of belowground over-yielding in response to inter-specific competition, or an increase in beech root growth as was observed in other studies.

Inter-specific interactions did however, have a significant effect on the depth of fine root production. We found that inter-specifically growing spruce produced shallower roots than monotypic groupings of spruce (Figure 3.3), and that inter-specific beech shifted the depth of root production depending on the season. We therefore provide further evidence that inter-specific interactions belowground are sufficient to induce a shift in the vertical placement of beech and spruce fine roots; however, the mechanism governing this shift in growth are still unknown. It is possible that the more uniform distribution of beech roots within mixed soil regions, as well as a significantly deeper average rooting depth of beech limits near-surface competition with spruce. Spruce growing in mixture may therefore be open to concentrate more roots within shallow soils that would otherwise be heavily occupied within monotypic spruce stands. It is also possible that non-resource cues, such as allelochemicals or rhizosphere effects, could explain an inter-specific shift in rooting depth; however, such mechanisms have yet to be identified (Schenk *et al.* 1999).

Inter-specific interactions also had an effect on beech and spruce root diameter: spruce responded to inter-specific interactions by decreasing fine root diameter, while the opposite was true for beech. Our results contradict what Bolte and Villanueva (2006) reported following a soil coring experiment: the authors observed a significant increase in beech specific root area within inter-specific soil regions, which typically requires that beech decrease root diameter per length of fine root. A reduction in fine root diameter is often followed by an increase in root construction efficiency (Eissenstat *et al.* 2000), which in our study, would confer a competitive advantage to

inter-specifically growing spruce. However, root system overlap among inter-specifically growing beech and spruce was relatively low, which suggests that a shift in spruce fine root diameter may simply indicate a lower investment by spruce into inter-specific root production. It may also indicate a strategy by spruce to mine less occupied mixed soil regions for resources using less C.

The median lifespan of intra-specifically growing beech and spruce roots was 260 and 382 days, respectively. As for inter-specifically growing roots, the median life-span of beech and spruce roots was 466 and 352 days, respectively. In a study by Withington *et al.* (2006), the authors detailed the median life span of a number of temperate trees, two of which were *F. sylvatica* and *P. abies*. In their experiment, the median life span of beech roots was 209 days, while the median lifespan of spruce was 259. In a similar study conducted at Kranzberger forst, Mainiero *et al.* (2010) reported a median life span of 77 days for beech, and 273 days for spruce. Because both species' median lifespan were somewhat greater in our study compared to other studies, we looked for differences in site characteristics to help explain the increase in fine root lifespan. The main difference between sites that could account for the differences in median lifespan was soil type. The median lifespan reported by Withington *et al.* came from observations of trees growing in nutrient-poor, sandy loam, whereas our observations came from trees growing in nutrient-rich clay loess. Previous reports that observed roots in nutrient rich patches living longer than roots in nutrient poor patches supports this theory (Aber *et al.* 1985, Adams *et al.* 2013). However, both our results and those of Withington *et al.* are irreconcilable with the findings of Mainiero *et al.* If we

compare the number of observations made on individual beech roots in this study (> 3000) to those made by Mainiero *et al.* (101), it is perhaps the limited number of observations, and only a single year of observations that led to their dramatic underestimation of beech's median life span (Brunner *et al.* 2013).

Volumetric soil water also differed between spruce and beech dominated soils, as well as between spruce and mixed soil regions. However, volumetric soil water in mixed soils did not differ from beech dominated soils, which was noted by Schume *et al.* (2004) in a mixed beech and spruce forest in Austria, indicating that mixed soil regions within Kranzberger forst were perhaps more influenced by beech than spruce root dynamics. Inter-specific beech root production was also significantly higher than spruce root production (mixture: 80% beech, 20% spruce), which further implicates beech as the more dominant species within mixed soil regions. However, mixed soil regions were found to contain the maximum observed volumes of water in 60% of the plots, which points to complementary water use, higher additions of water from beech stem flow, greater interception of water by spruce's canopy, and/or lower inter-specific root activity. This also indicates that inter-specifically growing spruce roots, at least for a period of time before reaching maximum depleted soil water levels in mid to late summer, may have access to greater volumes of water than spruce roots growing intra-specifically.

We also observed that inter-specific roots tended to respire less in the spring (Figure 6), which runs contrary to our predictions. It is important to note that longer lived

roots tend to respire less than short lived, more metabolically active roots (Volder *et al.* 2005), which we observed in the spring of 2012 and 2013. However, the mechanisms remain unclear. Without additional empirical data, we could only speculate that differences in canopy architecture and sub-canopy light penetration may explain why species' roots from inter-specific (more shaded) soil regions respired less. However, what remains uncertain is whether field differences in soil temperature would affect excised fine root respiration in a temperature-controlled chamber.

CONCLUSION

Inter-specific complementarity in water use has important implications for species survival during periods of drought, and based on our findings, may have further implications for ecosystem C budgets and C modeling. We demonstrate the effects of belowground inter-specific interactions on only two tree species, but there are likely multiple species that respond positively, neutrally and/or negatively to inter-specific interactions based on species traits, as well as environmental/resource conditions. What is important in light of changing climate and rising CO₂ is whether these shifts in growth result in positive or neutral outcomes, which would affect productivity and therefore positive C capture.

3.6 ACKNOWLEDGEMENTS

We are thankful for the invaluable technical support provided by Christof Seidler, Thomas Feuerbach, Peter Kuba, Sepp Heckmair, Annika Kreye, and Roman Meier. We would also like to thank Karl-Heinz Häberle for his help during the entirety of this project.

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CHAPTER 4

SEASONAL PATTERNS OF $\delta^{13}\text{C}$ IN TREE ROOTS: USING NATURAL ABUNDANCE MEASUREMENTS TO QUALIFY CARBON ALLOCATION BY ROOT ORDER

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4.1 ABSTRACT

The role of carbon reserves, and the mixing of multiple C sources during root respiration remains poorly understood in mature trees. We monitored the belowground C-supply-chain of two tree species, one evergreen (*Picea abies*) and one deciduous (*Fagus sylvatica*), by measuring the stable isotopic composition of respired C, labile C, and starch from 1st and 2nd order (< 21 days old) and 3rd and 4th order roots (> 21 days old) during periods of root production in spring and fall. In beech, the $\delta^{13}\text{C}$ of respired and labile C differed between 1st/2nd and 3rd/4th order roots; both fractions were also enriched in ^{13}C in spring compared to fall measurements. In spruce, there was no observed seasonality in $\delta^{13}\text{C}$ of the respired C, labile C, or starch. However, root order and climatic conditions strongly affected the $\delta^{13}\text{C}$ signature of both labile and respired C. Changes in $\delta^{13}\text{C}$ of C-rich fractions were unique to species, which we attributed to differences in functional traits. Patterns of C allocation in *F. sylvatica* were highly seasonal, and unaffected by moderate changes in climatic conditions.

Alternatively, C allocation in *P. abies* was affected by moderate changes in climatic conditions. Starch was also implicated as a major contributor to fine root respiration in both species.

Key Words: $\delta^{13}\text{C}$, *Fagus sylvatica*, fine root, *Picea abies*, respiration, sugar, starch

4.2 INTRODUCTION

Temperate forests consistently shift patterns of carbon (C) uptake, allocation, and rates of respiration in response to seasonal changes in resource and climate conditions (Schlesinger & Lichter 2001, Fahey *et al.* 2005, DeLucia *et al.* 2005, Hansen *et al.* 2010, Brüggemann *et al.* 2011). Once taken up, the majority of C is allocated toward three dominant pools: structural carbohydrates (e.g. cellulose and lignin), non-structural carbohydrates (e.g. sucrose and starch), and respired C (Farrar 1980, Chapin *et al.* 1990, Hoch *et al.* 2003). These three pools were historically monitored using a mass balance approach, where total C assimilation was compared against above and belowground biomass and respiratory loss of C over time (e.g. Giardina & Ryan 2002). A drawback of the mass balance approach is its inability to capture short-term trends in C allocation, including the quality of C used for growth and respiration; however, through monitoring the stable C isotopic composition ($\delta^{13}\text{C}$) of atmospheric C, plant organic C, and plant-respired C, the mechanisms controlling C uptake and loss in forests can be explored, and tree physiological processes better understood (Reviewed in Epron *et al.* 2012). For example, measuring the natural abundance of $\delta^{13}\text{C}$, as well as $\delta^{13}\text{C}$ following a pulse label has allowed scientists to more accurately calculate C residence times in living and dead organic matter (Steinmann *et al.* 2004,

Keel *et al.* 2012), quantify above to belowground C transfer (Kagawa *et al.* 2006, Grams *et al.* 2011), and measure the exchange of C between tree roots and associated fungi and bacteria (Steinmann *et al.* 2004, Högberg *et al.* 2008, Epron *et al.* 2011). However, a number of unanswered questions still remain regarding the temporal and spatial allocation of C in both deciduous and evergreen trees, especially in root tissues (Keel *et al.* 2012).

Roots are major C sinks (Nadelhoffer & Reich 1992). Carbon allocated to root growth and root-associated bacteria and fungi can range from 20-63% of the total C fixed over the life of a tree (Litton *et al.* 2007). Nearly one half of forest soil respiration, which constitutes as much as one half of ecosystem respiration, can also be traced to roots (Taneva *et al.* 2006, Trumbore *et al.* 2006, Brüggemann *et al.* 2011), but the origin of C used for root growth, especially the finest roots (1st-4th order, < 2mm diameter) that are uniquely responsible for the acquisition of soil resources, is less certain (Pregitzer *et al.* 1997, Trumbore *et al.* 2006).

The fine root fraction of the total root system constitutes the largest biomass, length, and surface area fractions of belowground tissues (Persson 1984), and therefore has a high C demand during periods of peak root production. This high C demand is met by a steady supply of both rehydrolyzed C stores and recent photosynthates (Gaudinski *et al.* 2009), the proportions of which can shift according to root order/diameter and time (Howarth *et al.* 1994, Kagawa *et al.* 2006, Keel *et al.* 2012). For example, a recent study by Keel *et al.* (2012) observed significant differences between fine roots of <

1mm and fine roots of 1-3mm in terms of the proportion of C allocated to labile sugars, starch, or structural C in *Pinus sylvestris*, with the proportions of C allocated to each pool differing during early vs. late season growth. The authors concluded that the observed differences in C allocation were a result of functional differences among higher and lower order roots. Alternatively, Gaudinski *et al.* (2009) measured C allocated to new root growth directly using a ^{14}C tracer, and concluded that 55% of new annual root growth in deciduous oak forests came from starch. Within stands of *Picea abies*, Andersen *et al.* (2010) observed little change in the $\delta^{13}\text{C}$ of soil respiration measured within the two weeks following labeling the canopy with depleted $^{13}\text{CO}_2$, which suggests a major role for stored C in *P. abies* root respiration. Contrary to what Gaudinski *et al.* observed in oak, or what Andersen *et al.* observed in spruce, Lynch *et al.* (2013) determined that while 25% of fine root respiration in mature stands of *Liquidambar styraciflua* came from starch, recent photosynthates were exclusively used in the construction of new root tissue. It appears then, that patterns of C allocation in belowground tissues are highly variable, and that C reserves can play both a major and a minor role in fine root respiration depending on the species, time of year, and diameter class of roots.

To date, the intricate physiological process of allocating recently fixed vs. stored C toward fine root growth remains poorly understood, but determining the temporal patterns of $\delta^{13}\text{C}$ across functionally different tissues should help us better understand patterns of C allocation, and how the belowground C-supply-chain operates. To do this, multi-year studies must first focus on to what degree patterns of belowground C

allocation are specific to species or functional groups, whether these patterns shift predictably across a growing season and/or across specific tissues, and finally how environmental and climatic conditions alter patterns of C allocation.

Here, we describe a two year experiment monitoring natural abundance $\delta^{13}\text{C}$ of fine root respired C, labile sugars, and starch within a mixed forest stand of mature evergreen and deciduous trees. We address the following questions regarding seasonal and root order trends in fine root $\delta^{13}\text{C}$: 1) does $\delta^{13}\text{C}$ of fine root respired C, labile sugars, and starch shift between root order and season, and are these patterns consistent across years? and 2) can we compare different C pools, e.g. C source vs. C product, as a means of tracking the temporal allocation of C across various root orders within the root system? With regards to $\delta^{13}\text{C}$, we hypothesized that C allocation between higher (3rd and 4th) and lower (1st and 2nd) order fine roots will differ between deciduous and evergreen tree growth strategies, with root order variation in $\delta^{13}\text{C}$ greater in deciduous tree species due to seasonal fluctuations between stored C, and recent photosynthates used for growth and respiration. We also hypothesized that in deciduous species, respired C will closely resemble starch's isotopic signature in spring, but not in the fall, indicating a transition from using stored C toward more recently fixed photosynthates. In evergreen species, we predict that respired C will resemble a mixture of photosynthates and starch throughout the growing season.

4.3 MATERIALS AND METHODS

4.3.1 SITE CONDITIONS

The study was performed across eight randomized plots containing 65-70 year old European beech (*Fagus sylvatica*) and Norway spruce (*Picea abies*) trees located in the “Kranzberger Forst,” a research site near Freising, Germany (48°25’N, 11°39’E, 490 m a.s.l.). Rectangular plots (~ 5 x 20 m) contained monotypic groupings of spruce trees at one end, and at the opposite end, a monotypic grouping of beech trees (for details see Pretzsch *et al.* 2014). Soils were haplic luvisols, and replete in both nutrients and water. Long-term averages (1970-2000) of mean precipitation and air temperature were 786 mm and 7.6 °C respectively (Matyssek *et al.* 2007). Stand basal area was 46.4 m² ha⁻¹ (Wipfler *et al.* 2005).

4.3.2 PROJECT INSTALLATION

In order to accurately assess root physiology and gain access to roots of known species and age, in-ground plywood boxes (root box: 40x60x30 cm, H x W x D) containing two acetate windows were installed under monotypic groupings of each species in May 2010 (Bouma *et al.* 2001). Viewing windows, when not in use, were insulated year round and a lid was screwed into place to prevent micro-climatic effects and exclude light penetration.

Time domain reflectometers (TDR) used to monitor volumetric water content were also installed in 2011 across all eight plots at two soil depths: 0-10 and 10-30 cm. Soil volumetric water content was measured weekly from April through November, and

monthly from December through March (TDR-100, Campbell Scientific, North Logan, Utah). Air temperature, relative humidity, and precipitation were collected during the week prior to each sampling period; climate data was monitored by DWD at climate station “Weihenstephan,” approximately 4 km from Kranzberger forst research station (DWD Offenbach, Germany). Beginning in the last week of May, 2012, soil temperature at 10cm was continuously logged every 4 hours using HOBO temperature pendants (HOBO Pendant® Temperature/Alarm Data logger, Bourne MA, USA).

4.3.3 FINE ROOT RESPIRED C

Root respired C was measured during spring and fall seasons of 2012 and 2013 (2012: May 25 and October 4; 2013: May 14 And October 2). Early and late season measurements were made to coincide with periods of active root production (Mainiero *et al.* 2010). One to three weeks prior to sampling, root growth was tracked along the clear acetate windows within the root boxes to ensure sampled root material would contain newly produced root tips less than 21 days old. Pioneer roots, defined as first order roots exhibiting little or no branching, lower incidence of mycorrhizal colonization, and larger average diameter, were excluded in this experiment (Zadworny & Eissenstat 2011). During sampling, roots were chosen from eight randomly selected root boxes: four within each species’ monotypic regions. Three to four fine root branches (1st-4th order, < 2mm) including roots less than 21 days old were excised from behind the acetate windows as whole root branches, placed in damp cellulose, and transported directly to Technische Universität München (TUM) for

analysis.

Root segments from each species were carefully rinsed to remove soil particles; 10 mg of either 1st and 2nd order (< 21 days old), or 3rd and 4th order (> 21 days old, indeterminate age) fresh root fragments were placed into 12ml exetainer vials (Labco Limited, High Wycombe, England), which were then capped, flushed with CO₂ free air, and allowed to respire in the dark for approximately two hours (Werner *et al.* 2007). After two hours, the ^{13/12}C ratio of respired gas was determined using a front-end gas auto-sampler (Gilson 221 XL, Gilson Inc., Middleton, USA) coupled to an isotope ratio mass spectrometer (IRMS, GVI-Isoprime, Elementar, Hanau, Germany). Carbon isotope ratios are expressed in δ-notation using Vienna PeeDee Belemnite (VPDB) as the standard. The following was used to calculate δ¹³C (Eqn 1);

$$(R_{\text{sample}}/R_{\text{VPDB}})-1 \times 1000$$

where R_{sample} and R_{VPDB} were ^{13/12}C ratios of the sample and VPDB, respectively. Repeated standard gas measurements varied ± 0.05 ‰ (SD, n = 24).

4.3.4 δ¹³C FINE ROOT STARCH AND LABILE SUGARS

Root sampling for starch and labile sugar stable isotopic analysis took place on May 26 and October 8, 2012, and May 17 and October 5, 2013 from the same root boxes used to sample roots for δ¹³C_R. Fine root growth was tracked for a period of one to three weeks prior to sampling, then fine root branches that included roots younger than 21 days old were excised from behind acetate windows, gently rinsed with deionized

water, patted dry, and frozen on site in liquid N₂. Both species' roots were freeze dried at TUM, carefully packaged, and transported to Cornell University (Cornell University, Ithaca, NY) for ^{13/12}C analysis of C-rich fractions.

Freeze dried root branches were pooled according to species and plot of origin, dissected into 1st and 2nd order, or 3rd and 4th order root fragments, and pulverized inside 2 ml metal bead beater tubes (MoBio Laboratories, Carlsbad, California). A small proportion (< 5 mg) of pulverized root material was placed into tin capsules and ^{13/12}C of bulk root material was analyzed using IRMS at Cornell's COIL facility (Thermo Delta V Advantage IRMS, Thermo Scientific). A protocol developed by Wanek *et al.* (2001) was followed for extracting labile sugars and starch from the remaining pulverized beech and spruce root material. Extracted starch and labile sugars were then allocated to tin capsules, and analyzed for ^{13/12}C using IRMS. Starch and labile sugars are expressed in δ-notation.

4.3.5 ISOTOPIC MIXING

A suppositional mixing model was developed in order to estimate the relative proportions of starch (i_s) vs. recent photosynthates (i_p) contributing to fine root respiration in higher and lower order roots. Standard isotopic mixing models require ^{13/12}C of both sources and products in order to calculate the contributions of the former to the latter (Phillips *et al.* 2005). In our model however, the stable isotopic composition of one source (recent photosynthates, δ¹³C_p) was not measured directly. Instead, δ¹³C values of labile sugars (δ¹³C_L) from each species' roots were used. Based

on the assumption that labile sugars from fall-produced roots consist of high proportions of recent photosynthates (*reviewed in Epron et al. 2012*), which is especially true for deciduous tree species, average $\delta^{13}\text{C}_L$ values from fall measurements were used in place of $\delta^{13}\text{C}_P$ in our mixing model (beech: -28.9‰ , spruce: -27.2‰ , *see discussion*). However, initial outputs from our mixing model produced proportions greater than 1, as well as negative proportions, prompting a closer evaluation of the mixing model and our model assumptions. We found that $\delta^{13}\text{C}_R$ was enrichment by approximately 1.0‰ in the spring compared to starch, and $> 3\text{‰}$ compared to bulk organic matter (OM). In a recent review on isotopic discrimination in leaves vs. roots by Ghashghaie and Badeck (2013), the authors report a range of $1\text{-}5\text{‰}$ enrichment of respired C when compared to OM, starch, and sucrose in root tissues of woody species. Based on the fact that our differences between $\delta^{13}\text{C}_S$ and $\delta^{13}\text{C}_R$ were within this reported range (0.6 to 1.9, see Figure 4.4a and b), we incorporated a correction factor for apparent respiratory discrimination, Δ_R . We then performed a sensitivity analysis (uncertainty analysis) to determine how our model outcomes varied with changes in Δ_R (Table S4.1) (Eqn 2):

$$i_p = \frac{\delta^{13}\text{C}_R - (\delta^{13}\text{C}_S - \Delta_R)}{\delta^{13}\text{C}_P - (\delta^{13}\text{C}_S - \Delta_R)}$$

Where R: respiration, S: starch, P: mean photosynthates (2011), and Δ_R : respiratory discrimination.

4.3.6 STATISTICAL ANALYSES

All statistical analyses were performed with JMP 10.0 (SAS Institute, Cary, NC, USA). Data was grouped by species, and the effect of year, season, and root order on

$\delta^{13}\text{C}_R$, $\delta^{13}\text{C}_R - \delta^{13}\text{C}_L$, and $\delta^{13}\text{C}_R - \delta^{13}\text{C}_S$ was analyzed using three-way ANOVA. Post-hoc analyses of mean $\delta^{13}\text{C}$ values were performed using Tukey's HSD t-test, $P = 0.05$. Due to the low number of replicates, non-parametric statistics were used to analyze the effect of year, season, and root order on species' $\delta^{13}\text{C}_L$ and $\delta^{13}\text{C}_S$ (Wilcoxon/Kruskal-Wallis Test). Variation between soil and environmental parameters and $\delta^{13}\text{C}_R/\delta^{13}\text{C}_L$ was tested using general linear models.

4.4 RESULTS

4.4.1 FINE ROOT RESPIRATION

Both beech and spruce fine roots exhibited consistent temporal and spatial (root-order) trends in $\delta^{13}\text{C}_R$ over the 2012 and 2013 growing season (Table 4.1). In beech, $\delta^{13}\text{C}_R$ differed significantly between years ($P = 0.016$), season ($P < 0.0001$), and root order ($P < 0.0001$). For spruce, year ($P = 0.0004$), season ($P = 0.0012$), and root order ($P < 0.0001$) also had a significant effect on $\delta^{13}\text{C}_R$.

Table 4.1 Mean $\delta^{13}\text{C}$ of respired C, labile C, and starch in 65-70 year tree roots grouped according to species (*Fagus sylvatica* & *Picea abies*), year (2012 & 2013), season (spring & fall), and root order (1st/2nd & 3rd/4th).

Species, Year	Season	Order	n	Respired C		n	Labile Sugar		Starch		
				Mean (‰)	St. Error		Mean (‰)	St. Error	Mean (‰)	St. Error	
Beech, 2012	Spring	1st & 2nd	16	-25.6	0.379	2	-28.0	0.354	-27.4	0.46	
		3rd & 4th	17	-25.8	0.215	2	-27.9	0.327	-27.7	0.305	
	Fall	1st & 2nd	20	-28.5	0.082	2	-27.9	0.059	-26.9	0.01	
Beech, 2013	Spring	1st & 2nd	20	-25.8	0.176	4	-27.9	0.143	-26.2	0.372	
		3rd & 4th	15	-25.5	0.142	4	-29.6	0.0463	-26.6	0.518	
	Fall	1st & 2nd	16	-28.0	0.249	4	-29.0	0.08	-26.4	0.167	
		3rd & 4th	13	-26.4	0.276	4	-30.3	0.444	-26.6	0.571	
	Spruce, 2012	Spring	1st & 2nd	16	-24.6	0.248	2	-26.4	0.192	-25.3	0.6
			3rd & 4th	16	-26.4	0.248	2	-26.6	0.315	-25.2	0.095
Fall		1st & 2nd	20	-26.3	0.125	2	-26.3	0.289	-25.5	0.205	
Spruce, 2013	Spring	1st & 2nd	20	-26.3	0.365	4	-28.1	0.144	-25.7	0.304	
		3rd & 4th	15	-27.4	0.154	4	-28.6	0.285	-25.3	0.533	
	Fall	1st & 2nd	13	-26.0	0.276	4	-27.7	0.036	-25.3	0.102	
		3rd & 4th	15	-27.5	0.257	4	-28.3	0.123	-24.9	0.166	

Mean values are expressed in δ -notation (‰). Two separate methods were used for measuring $\delta^{13}\text{C}$ of respired C vs. starch/labile C, hence two separate columns for replicates (n). Respired C was measurable via cuvette incubation using < 5mg fresh roots, which allowed for a greater number of replications. Labile sugar and starch were extracted from either 2 x 100 mg, or 4 x 100 mg of dry roots, which greatly reduced the number of possible replicates.

Seasonal $\delta^{13}\text{C}_R$ trends showed that spring was significantly enriched compared to fall measurements for both beech and spruce. However, within a given season, trends in $\delta^{13}\text{C}_R$ between 1st/2nd and 3rd/4th order roots were unique to species. In spruce, $\delta^{13}\text{C}_R$ of 1st/2nd order roots were significantly enriched compared to 3rd/4th order roots in both spring and fall (Figure 4.1b). Conversely, $\delta^{13}\text{C}_R$ in beech did not differ between root orders in the spring, but 1st/2nd order fall roots were significantly depleted when compared to 3rd/4th order fall roots (root order*season, $P < 0.0001$, Figure 4.1a). Significant two-way interactions between year and season were also observed in both species (beech: $P = 0.019$, spruce: $P = 0.0004$), which is intuitive considering annual variation in $\delta^{13}\text{C}_R$ should result from the sum of seasonal differences. Across the 2012 and 2013 growing season, $\delta^{13}\text{C}_R$ for both species varied widely, but remained within

the currently known range of $\delta^{13}\text{C}$ for C3 plants: -21 to -35 (Bowling *et al.* 2008). The range of values for $\delta^{13}\text{C}_R$ across sampled years and seasons was 6.2 and 6.7 ‰ for beech and spruce, respectively.

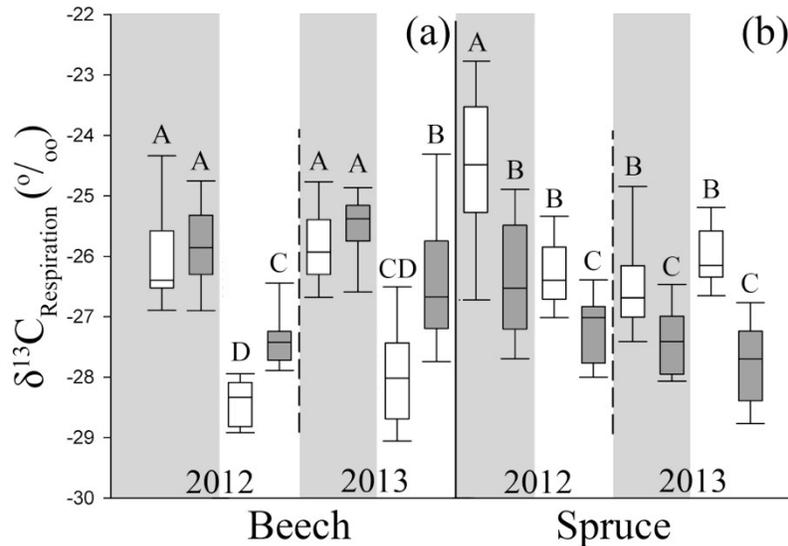


Figure 4.1 $\delta^{13}\text{C}$ of fine root respired C from 1st/2nd order (white) and 3rd/4th order (dark grey) beech (a) and spruce (b) roots. Measurements were taken during the spring (shaded background) and fall (un-shaded background) of 2012 and 2013. Each box displays the median value (straight line), and upper/lower quartiles; bars represent 10th/90th percentile. $\delta^{13}\text{C}_R$ was compared within species using Student's t test ($n = 13$ -20 per root order). Different letters denote significant differences between means, $P \leq 0.05$.

4.4.2 FINE ROOT LABILE C AND STARCH

Similar to $\delta^{13}\text{C}_R$, $\delta^{13}\text{C}_L$ in fine roots shifted predictably across season and root order during the 2012 and 2013 growing seasons (Figure 4.2a and b). In beech, year ($P = 0.0036$), season ($P = 0.046$), and root order ($P = 0.030$) had significant effects on $\delta^{13}\text{C}_L$, whereas in spruce, only year ($P < 0.0001$) had an effect on $\delta^{13}\text{C}_L$ (Figure 4.2b). In both beech and spruce roots, $\delta^{13}\text{C}_L$ was depleted in 2013 compared to 2012, and in beech, spring $\delta^{13}\text{C}_L$ (-28.33 ± 0.32) was enriched by nearly one per mill when

compared to fall (-29.2 ± 0.30).

Root order differences were observed in the 2013, but not the 2012 cohort of beech and spruce roots (Figure 4.2a and b), which was likely due to low replication in 2012. Root order differences in $\delta^{13}\text{C}_L$ were observed in the fall for both beech ($P = 0.029$) and spruce roots ($P = 0.030$), but only beech displayed differences between higher and lower order roots in the spring ($P = 0.030$). The difference in minimum and maximum $\delta^{13}\text{C}_L$ across both seasons and years was 4.3 and 3.5 ‰ for beech and spruce, respectively.

In contrast to respired and labile C, little variation was observed in $\delta^{13}\text{C}_S$. For both beech and spruce, $\delta^{13}\text{C}_S$ did not vary between seasons or root orders (Figure 4.2c and d). However, year had a significant effect on $\delta^{13}\text{C}_S$ in beech ($P = 0.0044$); $\delta^{13}\text{C}_S$ in 2012 (-27.25 ± 0.16) was significantly depleted compared to $\delta^{13}\text{C}_S$ in 2013 (-26.42 ± 0.20), but no such difference was observed in spruce. The difference in minimum and maximum $\delta^{13}\text{C}_S$ across both seasons and years was 3.0 and 2.1 ‰ for beech and spruce, respectively.

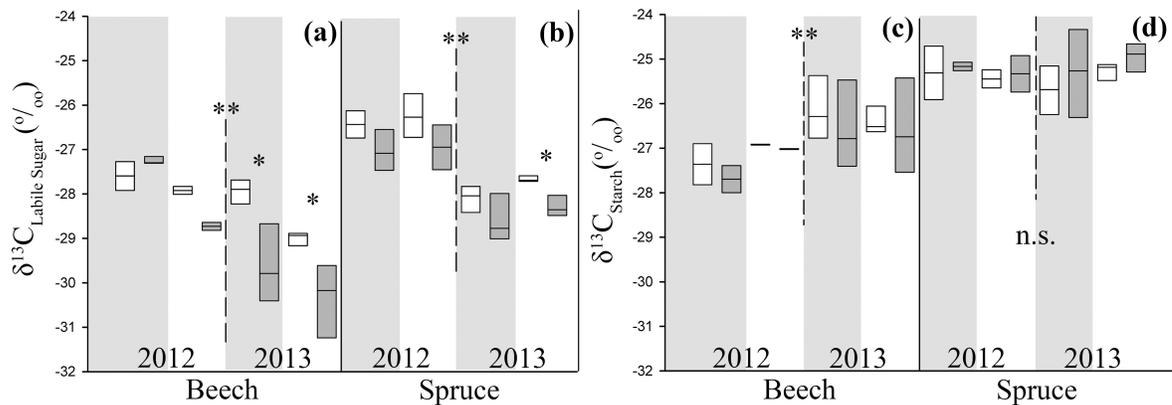


Figure 4.2 $\delta^{13}\text{C}$ of fine root non-structural carbohydrates from 1st/2nd order (white) and 3rd/4th order (dark grey) beech (a, c) and spruce (b, d) fine roots. Measurements were taken during the spring (shaded background) and fall (un-shaded background) of 2012 and 2013. Each box displays the median value (straight line), and upper/lower quartiles. Root order differences in $\delta^{13}\text{C}_L$ and $\delta^{13}\text{C}_S$ were tested using Wilcoxon's non-parametric test; significant differences are indicated by *, $P \leq 0.05$ ($n = 2$, 2012 and $n = 4$, 2013). Annual differences in $\delta^{13}\text{C}_S$ and $\delta^{13}\text{C}_L$ are indicated by ** (Tukey HSD test, $P \leq 0.0125$).

4.4.3 FINE ROOT ORGANIC MATTER

Both species demonstrated unique patterns in the stable isotopic composition of fine root OM. In beech, the $\delta^{13}\text{C}_{\text{OM}}$ differed significantly between root orders ($P < 0.0001$). With only one exception (Fall, 2013), 3rd/4th order roots were significantly depleted by 1-2 ‰ compared to 1st/2nd order roots (Figure 4.3a and b). We also observed significant two-way interactions between year and root order ($P < 0.0001$), indicating that differences in $\delta^{13}\text{C}_{\text{OM}}$ between lower and higher order roots can shift considerably from year to year. Lastly, a significant three-way interaction was observed between root order, year, and season ($P < 0.0001$), indicating a high degree of temporal variability across root orders in $\delta^{13}\text{C}_{\text{OM}}$.

As for spruce, root order ($P < 0.0001$), season ($P = 0.014$), and year ($P = 0.0008$) were

all significant predictors of $\delta^{13}\text{C}_{\text{OM}}$. Just as beech, 1st/2nd order roots were significantly enriched compared to 3rd/4th order roots (Figure 4.3b). Also, fall roots tended to be more enriched in ^{13}C compared to spring, and 2013 more depleted than 2012. No significant interactions between predictors were observed.

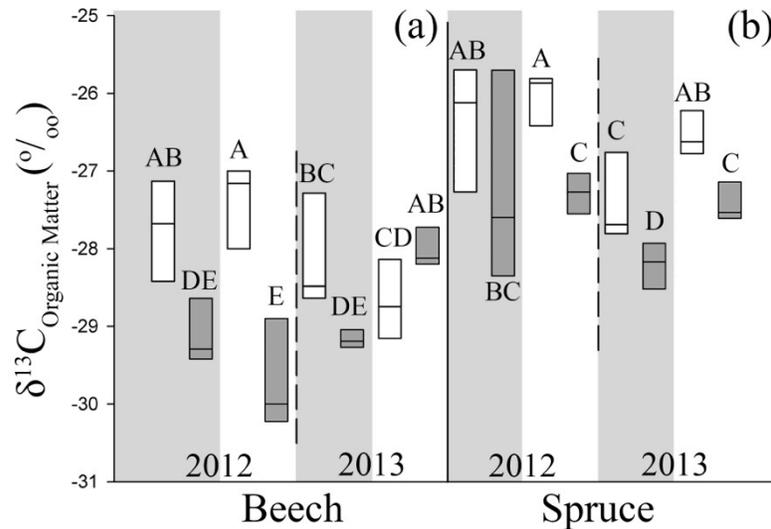


Figure 4.3 $\delta^{13}\text{C}$ of 1st/2nd order (white) and 3rd/4th order (dark grey) beech (a) and spruce (b) root organic matter. Measurements were taken during the spring (shaded background) and fall (un-shaded background) of 2012 and 2013. Each box displays the median value (straight line), and upper/lower quartiles. $\delta^{13}\text{C}_{\text{OM}}$ was compared within species using Student's t-test ($n=5$, 2012; $n=8$, 2013). Different letters denote significant differences between means, $P \leq 0.05$.

4.4.4 SOIL WATER AND CLIMATE

The following parameters were measured in order to determine whether any observed variation in $\delta^{13}\text{C}$ of roots was attributable to environmental conditions: volumetric soil water content (Table 4.2), relative humidity, precipitation, and temperature (Table 4.3). Soil water content did not help explain variation in $\delta^{13}\text{C}_{\text{R}}$ and $\delta^{13}\text{C}_{\text{L}}$, instead, climatic variables predicted variation better, including vapor pressure deficit (VPD). For spruce, the coefficient of determination between $\delta^{13}\text{C}_{\text{R}}$ and relative humidity ($R^2 =$

0.98, $P = 0.0086$), as well as temperature ($R^2 = 0.99$, $P = 0.005$) were high. Relative humidity ($R^2 = 0.77$) and temperature ($R^2 = 0.76$) were also good determinants of beech $\delta^{13}\text{C}_L$, though trends were also statistically insignificant. Lastly, increasing VPD correlated positively with $\delta^{13}\text{C}_L$ in spruce ($R^2 = 0.82$, $P = 0.0021$).

Table 4.2 Volumetric soil water content averaged over two weeks: one week prior to, and the week of sampling from intraspecific regions of each experimental plot (n= 8).

Species, Year	Season	0-10 cm		11-30 cm	
		Soil H ₂ O (%)	St. Error	Soil H ₂ O (%)	St. Error
Beech, 2012	Spring	21	2	30	2
	Fall	13	1.3	21	1.3
Beech, 2013	Spring	30	2	34	1.4
	Fall	16	1.2	23	0.8
Spruce, 2012	Spring	15	1.5	21	1.7
	Fall	14	2	20	2
Spruce, 2013	Spring	21	2.6	29	1.4
	Fall	14	2	21	1.6

Table 4.3 Total precipitation, average minimum humidity, average maximum temperature, and vapor pressure deficit (VPD) inside Kranzberger forest one week prior to sampling.

Year	Season	Precipitation (mm)	Relative Humidity (%)	Temperature (°C)	VPD (KPa)
2012	Spring	1.4	39.2 (4.83)	22.3 (2.78)	1.41 (0.37)
	Fall	12.7	75.9 (16.6)	14.7 (1.6)	1.11 (0.56)
2013	Spring	6.5	74 (20.3)	14.6 (4.8)	0.66 (0.69)
	Fall	12.6	82 (9.7)	13.25 (2.17)	0.74 (0.48)

Spring 2012 stands out as markedly drier, and hotter compared to other years and seasons. With the exception of precipitation, values are listed as means (\pm standard deviation).

4.4.5 C SOURCES VS. C PRODUCTS

Comparisons between $\delta^{13}\text{C}$ of labile C, starch, and respired C gave rise to unique, and fairly consistent trends over time and across different root orders (Figure 4.4a and b). Because $\delta^{13}\text{C}_L$ was found to differ significantly across season and root order, mean $\delta^{13}\text{C}_L$ was calculated independently for each root order across season, and year.

Alternatively, $\delta^{13}\text{C}_\text{S}$ did not differ between root orders or season, only between 2012 and 2013 in beech roots (Figure 4.2c).

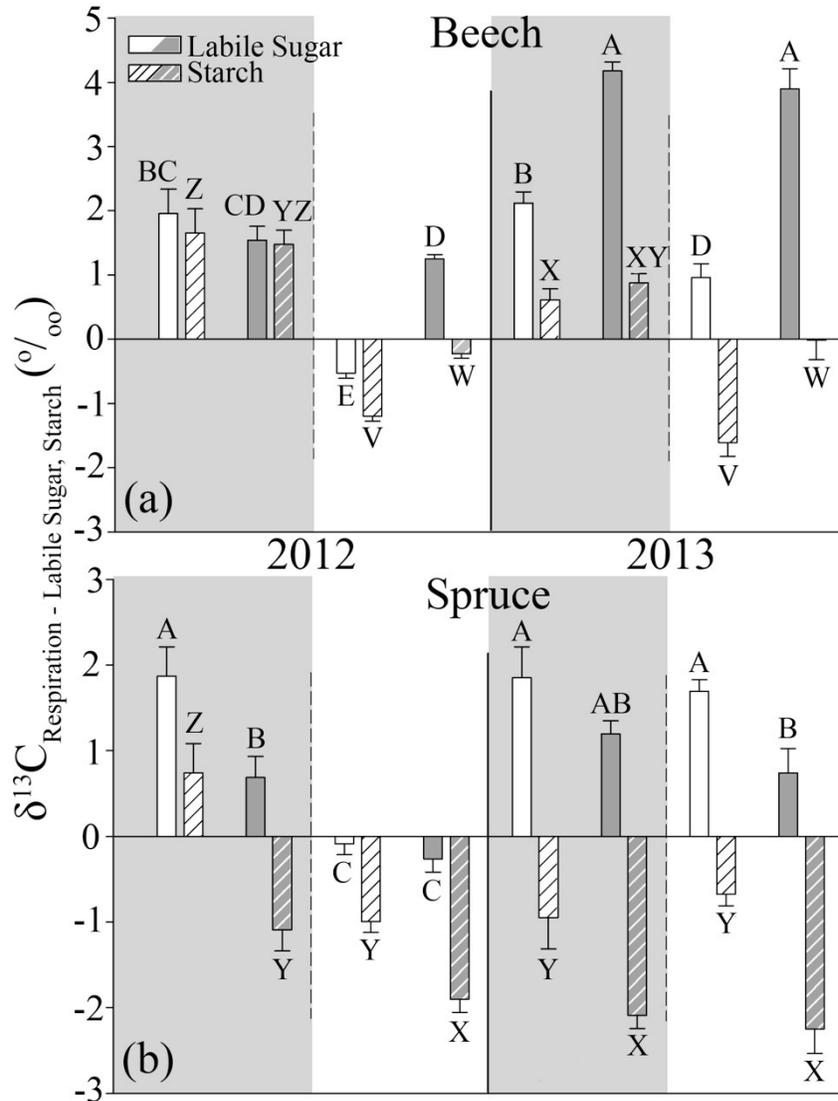


Figure 4.4 Relative differences between $\delta^{13}\text{C}_\text{R}$ and $\delta^{13}\text{C}_\text{S}$ (striped) or $\delta^{13}\text{C}_\text{L}$ (solid) of 1st/2nd order (white), and 3rd/4th order (dark grey) beech (a) and spruce (b) roots. Measurements were taken during the spring (shaded background) and fall (unshaded background) of 2012 and 2013. Student's t-test was used to determine significant differences between means ($n = 13\text{-}20$ per root order), indicated here by different letters, $P \leq 0.05$. Starch and labile C were tested separately, hence the different lettering scheme (Labile sugar: A-E, starch: V-Z). Note the differences in y-axis. Relationships between product ($\delta^{13}\text{C}_\text{R}$) and predicted substrates ($\delta^{13}\text{C}_\text{L}$ and $\delta^{13}\text{C}_\text{S}$) appear unique to species.

4.4.6 COMPARING RESPIRED C AND LABILE SUGARS

The following predictors had significant effects on $\delta^{13}\text{C}_R - \delta^{13}\text{C}_L$ in beech: root order, season, and year ($P < 0.0001$, each predictor). Respired C was enriched compared to labile C in 3rd/4th order roots (2.64 ± 0.11), and slightly less enriched in 1st/2nd order roots (1.08 ± 0.10); root order differences in $\delta^{13}\text{C}_R - \delta^{13}\text{C}_L$ were also more enriched in the spring compared to fall ($P < 0.0001$), and in 2013 compared to 2012 ($P < 0.0001$). Lastly, a three-way interaction between root order*season*year ($P = 0.034$) significantly effected $\delta^{13}\text{C}_R - \delta^{13}\text{C}_L$ in beech, which indicates that the interaction between labile C and respired C in beech roots is highly dynamic, both spatially and temporally.

In spruce, root order, season, and year were also significant predictors of $\delta^{13}\text{C}_R - \delta^{13}\text{C}_L$ ($P < 0.0001$, each predictor). Greater differences between $\delta^{13}\text{C}_R$ and $\delta^{13}\text{C}_L$ were observed in 1st/2nd order roots (1.27 ± 0.13) compared to 3rd/4th order roots (0.59 ± 0.13); root order differences did not translate across seasons or years. $\delta^{13}\text{C}_R - \delta^{13}\text{C}_L$ was greater in spring compared to fall, and in 2013 compared to 2012.

4.4.7 COMPARING RESPIRED C AND STARCH

The following predictors had significant effects on $\delta^{13}\text{C}_R - \delta^{13}\text{C}_S$ in beech: root order ($P < 0.0001$), season ($P < 0.0001$), and year ($P = 0.0031$). On average, $\delta^{13}\text{C}_R$ in 3rd/4th order roots was significantly enriched compared to starch (0.58 ± 0.11), whereas the opposite was true of 1st/2nd order roots (-0.16 ± 0.10). $\delta^{13}\text{C}_R - \delta^{13}\text{C}_S$ in the fall was significantly depleted (more negative) compared to the spring, and enriched in 2012 compared to 2013. A two-way interaction between root order*season ($P < 0.0001$) was

also found when comparing $\delta^{13}\text{C}_R$ and $\delta^{13}\text{C}_S$ in beech roots, indicating that starch's contribution to respiration differs between the spring and fall across root orders.

When comparing $\delta^{13}\text{C}_R$ and $\delta^{13}\text{C}_S$ in spruce, root order ($P < 0.0001$), season ($P = 0.0012$), and year ($P = 0.0003$) were also significant predictors. On average, respired C from 3rd/4th order roots was highly depleted compared to starch in 3rd/4th order roots (-1.82 ± 0.13), and slightly less depleted in 1st/2nd order roots (-0.51 ± 0.13). $\delta^{13}\text{C}_R$ - $\delta^{13}\text{C}_S$ in the fall was significantly depleted compared to spring, and further depleted in 2013 compared to 2012. A two-way interaction between year*season ($P = 0.0004$) was also found when comparing $\delta^{13}\text{C}_R$ and $\delta^{13}\text{C}_S$, indicating that seasonal trends will vary between years.

4.4.8 C MIXING

Regarding the proportions of starch used in fine root respiration, we found that beech exhibited consistent seasonal and root order trends, while spruce exhibited poor seasonal, but consistent root-order trends. Using Eqn. 2, starch's contribution to fine root respiration in beech varied between 86-97% in the spring, and 28-100% in the fall. In the spring, there were no apparent root-order trends in the proportion of starch contributing to respiration (i_s), whereas in the fall, there were distinct differences between i_s in 1st/2nd vs. 3rd/4th order roots: starch contributed 28-35% toward fine root respiration in 1st/2nd order roots, and 87-100% in 3rd/4th order roots in the fall. Starch's contribution to fine root respiration in spruce varied between 0-91% in the spring, and 0-65% in the fall. There were also clear root order differences observed during spring and fall in spruce: i_s varied between 50-91% and 47-65% in 1st/2nd order spring and

fall roots, respectively; and in 3rd/4th order roots, i_s varied between 0-42% in the spring, and was approximately 0% in the fall (Table 4.4).

Table 4.4 Relative proportions of starch (S) and photosynthates (P) contributing to fine root respiration as estimated by Eqn. 1, grouped according to species (*Fagus sylvatica* and *Picea abies*), year (2012 and 2013), season (spring and fall), and root order (1st/2nd and 3rd/4th).

Species, Year	Season	Root Order	Starch	Photosynthate*	Mean Respired C	Δ_R	P	S
Beech, 2012	Spring	1 st & 2 nd	-27.3	-28.9	-25.6	-2	9.6	90.4
		3 rd & 4 th	-27.3	-28.9	-25.8	-2	14.2	85.8
	Fall	1 st & 2 nd	-27.3	-28.9	-28.5	0	72.1	27.9
		3 rd & 4 th	-27.3	-28.9	-27.5	0	13.3	86.7
Beech, 2013	Spring	1 st & 2 nd	-26.4	-28.9	-25.8	-1	11.2	88.8
		3 rd & 4 th	-26.4	-28.9	-25.5	-1	3.4	96.9
	Fall	1 st & 2 nd	-26.4	-28.9	-28.0	0	64.9	35.1
		3 rd & 4 th	-26.4	-28.9	-26.4	0	0.4	99.6
Spruce, 2012	Spring	1 st & 2 nd	-25.3	-27.2	-24.6	-1	9.3	90.7
		3 rd & 4 th	-25.3	-27.2	-26.4	0	57.9	42.1
	Fall	1 st & 2 nd	-25.3	-27.2	-26.3	0	53.2	46.8
		3 rd & 4 th	-25.3	-27.2	-27.2	0	100.5	-0.5
Spruce, 2013	Spring	1 st & 2 nd	-25.3	-27.2	-26.3	0	50	50
		3 rd & 4 th	-25.3	-27.2	-27.4	0	110**	-10
	Fall	1 st & 2 nd	-25.3	-27.2	-26.0	0	35.5	64.7
		3 rd & 4 th	-25.3	-27.2	-27.5	0	118.4**	-18.4

Mean starch, photosynthate (* = average fall $\delta^{13}C_L$), and mean respired C is shown in δ -notation (‰). Proportions are expressed in percent ($100 \times i_p = \% P$, $100 \times i_s = \% S$). $\delta^{13}C_R$ varied widely across root order, season, and year. $\delta^{13}C_S$ varied between years in beech samples only. A correction factor (Δ_R : 0, -1, or -2) for respiratory discrimination was included after performing a sensitivity analysis (Table S4.1). Proportions that did not fall within range of 0 and 1 (**) resulted from photosynthate δ -values that were enriched compared to respired C (Table S4.2).

4.5 DISCUSSION

4.5.1 TEMPORAL VARIATION IN RESPIRED C

It is generally believed that by late spring, photosynthates containing low $^{13/12}C$ are transported from aboveground tissues toward various C sinks, mix with rehydrolyzed starch (enriched by about 2 ‰ relative to OM), and steadily deplete $\delta^{13}C_R$ over a growing season (Keel *et al.* 2007, Kuptz *et al.* 2011). For example, *Larix gmelinii*,

Populus euramericana, and *Fagus sylvatica* tend to allocate photosynthates in spring toward aboveground growth, and by fall, shift allocation belowground (Horwath *et al.* 1994, Kagawa *et al.* 2006, Kuptz *et al.* 2011), which may explain why $\delta^{13}\text{C}_R$ in beech roots was depleted in fall measurements, and why $\delta^{13}\text{C}_R$ was enriched in spring.

Unlike beech, spruce continues photosynthesis into late fall and the winter months under mild weather conditions. The rapid mixing of old and new C throughout a growing season, which has been proposed in *Fagus*, *Quercus*, and *Picea* (Keel *et al.* 2007, Kuptz *et al.* 2011), could account for spruce's lack of seasonality with regards to $\delta^{13}\text{C}_R$ (Dannoura *et al.* 2011). Our observations that $\delta^{13}\text{C}_R$ is neither enriched compared to $\delta^{13}\text{C}_S$, nor depleted compared to $\delta^{13}\text{C}_L$ is also evidence in favor of high mixing between C pools (Figure 4.4). We should note that 2012 was a unique year for spruce: we credit the strong enrichment of $\delta^{13}\text{C}_R$ in spring to the high vapor pressure deficit (1.41 KPa) recorded the week preceding our measurements. High VPD could enrich $\delta^{13}\text{C}_R$ by a.) reducing leaf-level gas exchange and prompting the breakdown of further C reserves (Lange *et al.* 1971), and/or b.) photosynthesis at low stomatal aperture and low internal CO_2 concentration. Noting 2012 as an exception, the observed temporal patterns in $\delta^{13}\text{C}_R$ appear to support our predictions regarding seasonal changes in belowground C allocation in beech, and high mixing among photosynthates and starch in spruce.

4.5.2 TEMPORAL VARIATION IN LABILE SUGAR AND STARCH

The enrichment of $\delta^{13}\text{C}_L$ in spring vs. fall measurements further supports our prediction that beech transitions between distinct early and late season C sources. In

spruce, the lack of seasonal variation in $\delta^{13}\text{C}_L$ further reinforces the supposition that spruce readily mixes new and old C throughout the season.

Annual variation in $\delta^{13}\text{C}_L$ in beech and spruce can likely be explained by the low precipitation, low relative humidity, and high maximum temperature observed in 2012. Interestingly, the significant effect of year on beech $\delta^{13}\text{C}_L$ in spring was not apparent in $\delta^{13}\text{C}_R$ (Figure 4.2a vs. Figure 4.1a). It is also possible that $\delta^{13}\text{C}_L$ and $\delta^{13}\text{C}_R$ are partially uncoupled, which was previously observed by Lynch *et al.* (2013) in *Liquidambar styraciflua*. Discrimination against the heavier ^{13}C isotope during respiration could also explain the uncoupling of $\delta^{13}\text{C}_L$ and $\delta^{13}\text{C}_R$ (Cernusak *et al.* 2009).

The $\delta^{13}\text{C}$ of starch varied annually in beech roots, but not spruce (Figure 4.2c and d). This suggests that starch in 1-4th order beech roots < 2mm is relatively young, with a turnover time < 1 year. As for spruce, the lack of annual variation in $\delta^{13}\text{C}_S$ could be attributed to either longer turnover times (> 1 year), or active mixing between new and old C.

4.5.3 ROOT ORDER VARIATION IN RESPIRED C

Temporal shifts in $\delta^{13}\text{C}_R$ have been reported in forests at the soil, plant, and ecosystem level (e.g. Fessenden & Ehleringer 2003, Scartazza *et al.* 2004), so our observations of strong seasonal shifts in $\delta^{13}\text{C}_R$ in a mixed soft/hardwood forest are not all together surprising. However, by categorizing roots into size classes, interesting trends in the

belowground C-supply-chain emerge. One example of root order and temporal variability in C allocated to fine roots < 1mm and 1-3mm was recently demonstrated by Keel *et al.* (2012) in *Pinus sylvestris* roots using a $^{13}\text{CO}_2$ label on whole trees. They concluded that differences in patterns of C allocation resulted from functional differences among fine roots.

The current study was conducted on 1st/2nd order roots including newly produced root tips, along with 3rd/4th order roots $\leq 2\text{mm}$. For both species, natural abundance $\delta^{13}\text{C}_R$ differed significantly between higher and lower order roots (Table 4.1). We put forward three explanations for root order differences in $\delta^{13}\text{C}_R$ that are by no means mutually exclusive; 1.) variation in PEPc activity between higher vs. lower order roots, 2.) post-photosynthetic discrimination at the fine root level, or 3.) allocation of different proportions of isotopically distinct C sources to higher vs. lower order root respiration.

The first explanation, one of many published by Cernusak *et al.* (2009) to try and explain discrepancies in $^{13/12}\text{C}$ between heterotrophic tissues and leaves, suggests that elevated PEPc activity in heterotrophic tissues can result in the relative enrichment of root organic matter (OM) compared to $\delta^{13}\text{C}_L$ or leaf OM (Gessler *et al.* 2009). If PEPc activity varies according to root age or function (i.e. across different root orders) then it follows that variation in PEPc could explain the observed root order variation in $\delta^{13}\text{C}_R$. However, beech and spruce exhibited opposite root order trends in $\delta^{13}\text{C}_R$. Considering that 1st/2nd order roots in both species were <21 days old, and highly

metabolically active (Pregitzer *et al.* 1997), it is unclear whether actively growing roots with similar functions would exhibit such differences in PEPc activity, but species variation in PEPc at the root level can not be excluded.

Post photosynthetic discrimination in heterotrophic tissues has been reported across a host of plant species, for example during starch/cellulose biosynthesis and nighttime respiration (Gleixner & Schmidt 1997, Gessler *et al.* 2008). Our mixing model outcomes were also greatly improved after including Δ_R , and while this is evidence of post photosynthetic discrimination occurring in belowground tissues, including the finest roots, it does not necessarily explain root order differences in $\delta^{13}C_R$. Based on the observation that spring respiration in beech did not differ among root orders, there is perhaps greater evidence for the third explanation.

The third explanation- that different proportions of isotopically distinct C sources are allocated to higher vs. lower order root respiration, was recently reported by Keel *et al.* (2012). In our study, $\delta^{13}C_R$ of 1st/2nd order beech roots was depleted compared to 3rd/4th order roots in the fall. This could indicate greater proportions of a depleted ^{13}C source (photosynthates) was being used in high proportions for 1st and 2nd order root respiration (Lynch *et al.* 2013). Because starch was likely the dominant, if not exclusive C pool available for root growth and respiration in the spring, we wouldn't expect to see root-order differences in $\delta^{13}C_R$ (Figure 4.1a).

As for spruce, Andersen *et al.* (2010) pulse labeled adult *Picea abies* trees in late

summer with ^{13}C depleted CO_2 , and observed little to no measurable labeled C in spruce root respiration, indicating low utilization of photosynthates in root growth and respiration in late summer/early fall. Instead, starch would be the main source of root available C (Fahey *et al.* 2013). This could explain the relative enrichment in $\delta^{13}\text{C}_\text{R}$ of 1st/2nd order roots compared to higher order roots (Figure 4.1b). In higher order roots, the depletion of starch reserves during root respiration would increase their C sink strength, thus stimulating the influx of C from the trunk, stems, and canopy. If higher order spruce roots received greater proportions of recent photosynthates compared to lower order roots, then the metabolism of large proportions of photosynthates would deplete $\delta^{13}\text{C}_\text{R}$ and $\delta^{13}\text{C}_\text{OM}$ in 3rd/4th order roots relative to 1st/2nd order roots (Figure 4.1b, Figure 4.3b).

4.5.4 CHANGES IN LABILE SUGARS

Both species exhibited consistent trends in $\delta^{13}\text{C}_\text{L}$ across seasons and years: 3rd/4th order root $\delta^{13}\text{C}_\text{L}$ was consistently depleted relative to 1st/2nd order roots (Figure 4.2a and b). Higher order roots are generally implicated in the transport and storage of C (Pregitzer *et al.* 1997); by incorporating more ^{12}C into structural and storage compounds in higher order roots that spatially precede lower order roots in the C-supply-chain, $\delta^{13}\text{C}_\text{L}$ as well as $\delta^{13}\text{C}_\text{OM}$ in 1st/2nd order roots would become enriched in ^{13}C (explanation 2), which was observed in both species (Figure 4.2a and b; Figure 4.3a and b). Also, relatively high contribution of starch toward growth and respiration of 1st/2nd vs. 3rd/4th order roots would further enrich $\delta^{13}\text{C}_\text{L}$ (explanation 3). We conclude that mechanistic differences in the C-supply-chains of beech and spruce

could account for root-order differences in $\delta^{13}\text{C}_R$ and $\delta^{13}\text{C}_L$, which we discuss below.

4.5.5 C MIXING AND THE C-SUPPLY-CHAIN

Estimates generated by our suppositional mixing model are inherently coarse (Table 4). For example, the $\delta^{13}\text{C}$ of phloem sugar ($\delta^{13}\text{C}_P$) was not measured directly - instead we used mean root $\delta^{13}\text{C}_L$ values from fall measurements. The use of mean $\delta^{13}\text{C}_L$ values in place of $\delta^{13}\text{C}_P$ did not outwardly detract from our model, as our mean $\delta^{13}\text{C}_L$ values were within ± 1 ‰ of reported $\delta^{13}\text{C}$ values from mid-summer phloem sugars measured at the same site (Kuptz *et al.* 2011). We also found that manipulating $\delta^{13}\text{C}_S$ and not $\delta^{13}\text{C}_P$ values produced more outputs that were within range (i.e. 0-1), which suggests that respiratory discrimination (Δ_R) occurs specifically during starch hydrolysis (Table S4.2). If this is the case, the inclusion of a Δ_R factor in any model that aims to predict starch's contribution to respiration is inherently complicated, for starch can be a major or minor contributor to respiration depending on the time of day (night vs. day), the season (spring vs. fall), and the species studied (evergreen vs. deciduous).

In circumstances where $\delta^{13}\text{C}_R$ values were more depleted than $\delta^{13}\text{C}_P$, such as with 3rd/4th order spruce roots in 2013 (Table S4.1), modifying Δ_R did not bring the model outcomes within range of 0-1. In such instances, it was theorized that spruce's labile sugars from the fall contained high proportions of carbohydrates originating from hydrolyzed starch (enriched in ^{13}C compared to photosynthates). Evidence for this theory was tested by substituting fall $\delta^{13}\text{C}_L$ values with more depleted $\delta^{13}\text{C}$ values

(Table S4.2), which effectively produced model outputs within range of 0 and 1.

Based on our final model outputs (Table 4.4), beech and spruce allocate C differently to 1st-4th order roots. For spruce, starch played a major role in 1st/2nd order root respiration, whereas photosynthates were more implicated in 3rd/4th order root respiration. This would indicate that proximal C sources provide energy to root tips, and the aboveground supply of C is less involved in root tip respiration. In beech, spring root respiration was almost exclusively fueled by starch, whereas in the fall, photosynthates were predominately respired in 1st/2nd order roots (Lynch *et al.* 2013). Therefore beech, especially during the late growing season, was highly dependent on photosynthates for the growth and respiration of fine roots, which raises questions concerning beech's susceptibility to late-season drought.

Based on our results, we conclude that beech and spruce exhibit distinctly different temporal patterns in $\delta^{13}\text{C}$ across their C-rich fractions. Root order differences suggest that, independent of species identity, 1st/2nd order roots are distinguishable from 3rd/4th order roots in terms of their stable isotopic composition, and that these differences most likely result from differences in the C-supply-chain. Therefore, special attention must be paid to isotopically distinct tissues when conducting future research into fine roots.

4.6 ACKNOWLEDGEMENTS

We are thankful for the invaluable technical support provided by Christof Seidler,

Thomas Feuerbach, Peter Kuba, Sepp Heckmair, Roman Meier, Kim Sparks, and the staff at COIL. We would also like to thank Carsten Müller for his help with sample drying, and the support and guidance of Karl-Heinz Häberle during the entirety of this project. Funding was partially provided by the Mario Einaudi Center for International Studies, small grants program.

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Table S4.1 Sensitivity analysis of Δ_R (0, -1, or -2) used in Eqn 2. Outputs are the relative proportions of starch (S) and photosynthates (P) contributing to fine root respiration, grouped according to species (*Fagus sylvatica* and *Picea abies*), year (2012 and 2013), season (spring and fall), and root order (1st/2nd and 3rd/4th).

Species, Year	Season	Root Order	Starch	Photosynthate	Mean Respired C	Δ_R	P	S
Beech, 2012	Spring	1 st & 2 nd	-27.3	-28.9	-25.6	0	-100.0	200.0
		3 rd & 4 th	-27.3	-28.9	-25.8	0	-89.8	189.8
	Fall	1 st & 2 nd	-27.3	-28.9	-28.5	0	72.1	27.9
		3 rd & 4 th	-27.3	-28.9	-27.5	0	13.3	86.7
	Spring	1 st & 2 nd	-27.3	-28.9	-25.6	-1	-24.5	124.5
		3 rd & 4 th	-27.3	-28.9	-25.8	-1	-18.2	118.2
	Fall	1 st & 2 nd	-27.3	-28.9	-28.5	-1	82.6	17.4
		3 rd & 4 th	-27.3	-28.9	-27.5	-1	46.0	54.0
	Spring	1 st & 2 nd	-27.3	-28.9	-25.6	-2	9.6	90.4
		3 rd & 4 th	-27.3	-28.9	-25.8	-2	14.2	85.8
	Fall	1 st & 2 nd	-27.3	-28.9	-28.5	-2	87.4	12.6
		3 rd & 4 th	-27.3	-28.9	-27.5	-2	60.8	39.2
Beech, 2013	Spring	1 st & 2 nd	-26.4	-28.9	-25.8	0	-24.6	124.6
		3 rd & 4 th	-26.4	-28.9	-25.5	0	-35.5	135.5
	Fall	1 st & 2 nd	-26.4	-28.9	-28	0	64.9	35.1
		3 rd & 4 th	-26.4	-28.9	-26.4	0	0.4	99.6
	Spring	1 st & 2 nd	-26.4	-28.9	-25.8	-1	11.2	88.8
		3 rd & 4 th	-26.4	-28.9	-25.5	-1	3.4	96.6
	Fall	1 st & 2 nd	-26.4	-28.9	-28	-1	75.0	25.0
		3 rd & 4 th	-26.4	-28.9	-26.4	-1	29.0	71.0
	Spring	1 st & 2 nd	-26.4	-28.9	-25.8	-2	31.0	69.0
		3 rd & 4 th	-26.4	-28.9	-25.5	-2	25.0	75.0
	Fall	1 st & 2 nd	-26.4	-28.9	-28	-2	80.6	19.4
		3 rd & 4 th	-26.4	-28.9	-26.4	-2	44.9	55.1
Spruce, 2012	Spring	1 st & 2 nd	-25.3	-27.2	-24.6	0	-38.4	138.4
		3 rd & 4 th	-25.3	-27.2	-26.4	0	57.9	42.1
	Fall	1 st & 2 nd	-25.3	-27.2	-26.3	0	53.2	46.8
		3 rd & 4 th	-25.3	-27.2	-27.2	0	100.5	-0.5
	Spring	1 st & 2 nd	-25.3	-27.2	-24.6	-1	9.3	90.7
		3 rd & 4 th	-25.3	-27.2	-26.4	-1	72.4	27.6
	Fall	1 st & 2 nd	-25.3	-27.2	-26.3	-1	69.3	30.7
		3 rd & 4 th	-25.3	-27.2	-27.2	-1	100.3	-0.3
	Spring	1 st & 2 nd	-25.3	-27.2	-24.6	-2	32.6	67.4
		3 rd & 4 th	-25.3	-27.2	-26.4	-2	79.5	20.5
	Fall	1 st & 2 nd	-25.3	-27.2	-26.3	-2	77.2	22.8
		3 rd & 4 th	-25.3	-27.2	-27.2	-2	100.3	-0.3
Spruce, 2013	Spring	1 st & 2 nd	-25.3	-27.2	-26.3	0	50.0	50.0
		3 rd & 4 th	-25.3	-27.2	-27.4	0	110.0	-10.0
	Fall	1 st & 2 nd	-25.3	-27.2	-26	0	35.3	64.7
		3 rd & 4 th	-25.3	-27.2	-27.5	0	118.4	-18.4
	Spring	1 st & 2 nd	-25.3	-27.2	-25.6	-1	67.2	32.8
		3 rd & 4 th	-25.3	-27.2	-25.8	-1	106.6	-6.6
	Fall	1 st & 2 nd	-25.3	-27.2	-28.5	-1	57.6	42.4
		3 rd & 4 th	-25.3	-27.2	-27.5	-1	112.1	-12.1
	Spring	1 st & 2 nd	-25.3	-27.2	-25.6	-2	75.6	24.4
		3 rd & 4 th	-25.3	-27.2	-25.8	-2	104.9	-4.9
	Fall	1 st & 2 nd	-25.3	-27.2	-28.5	-2	68.5	31.5
		3 rd & 4 th	-25.3	-27.2	-27.5	-2	109.0	-9.0

Table S4.2 Sensitivity analysis of $\delta^{13}\text{C}_\text{P}$ (Recorded range of $\delta^{13}\text{C}$ from mid-summer phloem sugars, Kuptz *et al.* 2011) used in Eqn 2. Outputs are the relative proportions of starch (S) and photosynthates (P) contributing to fine root respiration, grouped according to species (*Fagus sylvatica* and *Picea abies*), year (2012 and 2013), season (spring and fall), and root order (1st/2nd and 3rd/4th).

Species, Year	Season	Root Order	Starch	Photosynthate*	Mean Respired C	Δ_R	P	S
Beech, 2012	Spring	1 st & 2 nd	-27.3	-28	-25.6	0	-220.00	320.00
		3 rd & 4 th	-27.3	-28	-25.8	0	-197.47	297.47
	Fall	1 st & 2 nd	-27.3	-28	-28.5	0	158.67	-58.67
		3 rd & 4 th	-27.3	-28	-27.5	0	29.33	70.67
	Spring	1 st & 2 nd	-27.3	-29	-25.6	0	-94.29	194.29
		3 rd & 4 th	-27.3	-29	-25.8	0	-84.63	184.63
	Fall	1 st & 2 nd	-27.3	-29	-28.5	0	68.00	32.00
		3 rd & 4 th	-27.3	-29	-27.5	0	12.57	87.43
	Spring	1 st & 2 nd	-27.3	-30	-25.6	0	-60.00	160.00
		3 rd & 4 th	-27.3	-30	-25.8	0	-53.85	153.85
	Fall	1 st & 2 nd	-27.3	-30	-28.5	0	43.27	56.73
		3 rd & 4 th	-27.3	-30	-27.5	0	8.00	92.00
Beech, 2013	Spring	1 st & 2 nd	-26.4	-28	-25.8	0	-38.54	138.54
		3 rd & 4 th	-26.4	-28	-25.5	0	-55.70	155.70
	Fall	1 st & 2 nd	-26.4	-28	-28	0	101.90	-1.90
		3 rd & 4 th	-26.4	-28	-26.4	0	0.63	99.37
	Spring	1 st & 2 nd	-26.4	-29	-25.8	0	-23.60	123.60
		3 rd & 4 th	-26.4	-29	-25.5	0	-34.11	134.11
	Fall	1 st & 2 nd	-26.4	-29	-28	0	62.40	37.60
		3 rd & 4 th	-26.4	-29	-26.4	0	0.39	99.61
	Spring	1 st & 2 nd	-26.4	-30	-25.8	0	-17.01	117.01
		3 rd & 4 th	-26.4	-30	-25.5	0	-24.58	124.58
	Fall	1 st & 2 nd	-26.4	-30	-28	0	44.97	55.03
		3 rd & 4 th	-26.4	-30	-26.4	0	0.28	99.72
Spruce, 2012	Spring	1 st & 2 nd	-25.3	-27	-24.6	0	-42.94	142.94
		3 rd & 4 th	-25.3	-27	-26.4	0	64.71	35.29
	Fall	1 st & 2 nd	-25.3	-27	-26.3	0	59.41	40.59
		3 rd & 4 th	-25.3	-27	-27.2	0	112.35	-12.35
	Spring	1 st & 2 nd	-25.3	-28	-24.6	0	-27.04	127.04
		3 rd & 4 th	-25.3	-28	-26.4	0	40.74	59.26
	Fall	1 st & 2 nd	-25.3	-28	-26.3	0	37.41	62.59
		3 rd & 4 th	-25.3	-28	-27.2	0	70.74	29.26
	Spring	1 st & 2 nd	-25.3	-29	-24.6	0	-19.73	119.73
		3 rd & 4 th	-25.3	-29	-26.4	0	29.73	70.27
	Fall	1 st & 2 nd	-25.3	-29	-26.3	0	27.30	72.70
		3 rd & 4 th	-25.3	-29	-27.2	0	51.62	48.38
Spruce, 2013	Spring	1 st & 2 nd	-25.3	-27	-26.3	0	55.88	44.12
		3 rd & 4 th	-25.3	-27	-27.4	0	122.94	-22.94
	Fall	1 st & 2 nd	-25.3	-27	-26	0	39.41	60.59
		3 rd & 4 th	-25.3	-27	-27.5	0	132.35	-32.35
	Spring	1 st & 2 nd	-25.3	-28	-25.6	0	35.19	64.81
		3 rd & 4 th	-25.3	-28	-25.8	0	77.41	22.59
	Fall	1 st & 2 nd	-25.3	-28	-28.5	0	24.81	75.19
		3 rd & 4 th	-25.3	-28	-27.5	0	83.33	16.67
	Spring	1 st & 2 nd	-25.3	-29	-25.6	0	25.68	74.32
		3 rd & 4 th	-25.3	-29	-25.8	0	56.49	43.51
	Fall	1 st & 2 nd	-25.3	-29	-28.5	0	18.11	81.89
		3 rd & 4 th	-25.3	-29	-27.5	0	60.81	39.19

CHAPTER 5

5.1 SYNTHESIS

The body of work presented here demonstrates that inter-specific interactions among naturally co-occurring species can cause a shift in fine root growth and physiology of adult trees and seedlings, and that these shifts in growth can take place across a range of spatial scales. Furthermore, these experiments were conducted on species pairs at markedly different age and developmental stages, which suggests that growth shifts in response to inter-specific interactions are not constrained by tree age. Overall, our results improve our understanding of how plants, specifically trees, respond to belowground interactions with both conspecific and inter-specific neighbors. Moreover, we demonstrate that certain species mixtures can affect soil water conditions, as well as the longevity of fine root tissues, which may be modifying the belowground competitive environment in forests.

Because our experiments focused on only two pairs of species however, we are conservative in our attempts to extrapolate our results to other temperate species growing in mixture. Nor can we be sure that inter-specific interactions among three or more species, and not just two, would produce similar results. However, in light of the many benefits of increasing species richness in tree-dominated landscapes (Hooper & Vitousek 1997, Hooper *et al.* 2005, Zhang *et al.* 2012), we encourage researchers to continue to identify unique combinations of species and their traits that shift under inter-specific conditions. Defining a set of “core” species traits could aid landscape

management and forest plantings as a means to increase species richness without a loss in productivity, and thus generate highly productive, ecologically rich landscapes.

We also demonstrated phenological differences in the belowground C-supply-chain of one deciduous and one evergreen tree species. We provided evidence that implicates starch as a major contributor to fine root respiration in both deciduous and evergreen species (Gaudinski *et al.* 2009), and demonstrate the strong seasonality of C allocation in deciduous trees using natural abundance measurements of $^{13/12}\text{C}$ combined with a basic isotopic mixing model. In our evergreen species (*Picea abies*), we observed a relay of C within the fine root system. Specifically, we observed larger proportions of C that resembled recent photosynthates (a) being shuttled to higher order roots (b). Higher order roots (b) were then shuttling larger proportions of C resembling starch toward the growth and respiration of fine root tips (c). Graphically, the C-supply-chain of spruce can be viewed as: **a → b**, **b → c**. As for beech, spring growth and respiration was fueled almost exclusively by C resembling starch (**b → c**), whereas in the fall, greater proportions of C that resembled recent photosynthates was shuttled to newly produced root tips (**a → c**), while higher order root respiration was fueled by C resembling starch (**b → c**). Overall, we found that starch was a large contributor to fine root respiration in both deciduous and evergreen species, but that more research is required to understand how post-photosynthetic fraction may influence natural abundance measurements and our interpretations via mixing models. Furthermore, knowing how C is allocated throughout the fine root system under ideal conditions will hopefully help future researchers interpret any changes in the C-supply-chain

once exposed to stress.

Practical implications

This research has far reaching practical implications in terms of landscape management, forestry, ecosystem restoration, and arboriculture. Determining which unique species combinations interact neutrally or positively, either above or belowground, could serve to increase productivity directly through facilitation, or indirectly by limiting competition. For centuries, scientists and farmers alike have known how to limit aboveground competition for light; however, there are opportunities to further limit competition belowground and/or reduce the leaching of essential resources. Our research indicates that mixed soil regions may constitute a less competitive environment when compared to either species monotypic soil regions, especially when the two interacting species are shallow vs. deeply rooted. However, while inter-specific interactions can induce a shift in root growth and longevity, the practical importance lies in whether shifts in root growth and life span can be tied to a rise in inter-specific productivity.

We also discovered that the use of emerging technologies, especially those emerging from medical fields, can and will continue to improve what we know about plants. In our set of micro-CT experiments, we found that morphological and architectural adjustments to fine roots can take place at millimeter scales, including the spatial separation of root system volume from root tips, which has not previously been described. Furthermore, in none of our experiments did we test directly for

interference (non-resource) competition; however, there was evidence of non-resource based interactions in both natural (forest) and controlled (greenhouse) settings. For this reason, future work should focus on the whether inter-specific interactions in forests can be mediated by bacterial, mycorrhizal, and/or root exudate signals.

Regarding Norway spruce and European beech, we found that inter-specifically growing adult trees may experience lower levels of belowground competition compared to either species growing in monoculture, which supports the continued planting of both species in mixture across central and western Europe. Our results also indicated that belowground competition was more asymmetric than symmetric (Meinen *et al.* 2009). We also observed that intra- vs. inter-specific competition resulted in faster turnover rates for both the evergreen and deciduous species studied. Regarding black spruce and quaking aspen, our results suggest that both species growing inter-specifically from seed experience high levels of above and belowground competition. However, based on the observed inter-specific interactive effects of both species, co-planting mature individuals that aren't forced to compete for light could benefit both species in mixture.

5.2 FUTURE RESEARCH

C-supply-chain

In this collection of research, we evaluated the stable C isotopic composition of C-rich fractions from the roots of one deciduous and one evergreen species. We observed

distinct species differences in the C-supply-chain, and these differences seemed to stem from obvious differences in phenology (Keel *et al.* 2012). We cannot make any generalization past these two species, however. The next logical step would be to evaluate the $\delta^{13}\text{C}$ of C-rich fractions from a number of economically and ecologically important deciduous and evergreen tree species. With that information, we could begin to relate trends in the C-supply-chain to specific environmental conditions, as well as phylogeny. Additionally, higher resolution could be achieved by separating each individual root order as opposed to grouping fine roots into 1st/2nd and 3rd/4th order categories. Furthermore, the temporal resolution of the study conducted here is relatively poor- future work should attempt bi-monthly sampling at a minimum in order to capture all relevant changes in $\delta^{13}\text{C}$. Lastly, $\delta^{13}\text{C}$ can vary widely across tissues and time; therefore, $\delta^{13}\text{C}$ of phloem sugars should be sampled and evaluated directly in order to achieve a mass-balance. Not only would this type of information be helpful in predicting the affects of climate change on C allocation in trees, but from the standpoint of better understanding stable isotope applications, this type of research would help to improve what we known of post photosynthetic discrimination in belowground tissues, which will be critical for the development of better ecosystem and biogeochemical level C models (Badeck *et al.* 2005).

Inter-specific effects on roots

The use of minirhizotrons, and the subjectivity of data analysis has been a controversial topic since the method was first developed (*Reviewed in* Johnson *et al.* 2001). However, the minirhizotron method is highly effective in capturing long-term

temporal trends in root growth and mortality. Recently, the application of stable C isotopes has also proven to be an effective method at determining C-residence times, root turnover, and the timing of root production (*e.g.* Gaudinski *et al.* 2010). An interesting avenue for future research would combine unique species combinations with ^{13}C labeling in order to determine whether root growth and belowground C dynamics are altered by inter-specific interactions, and/or whether there is complementarity in C allocation patterns.

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