

MORPHOLOGY AND DECOMPOSITION OF ROOTS
UNDER ORGANIC MANAGEMENT IN THE
SOUTHEASTERN U.S.

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ABSTRACT

A better understanding of the belowground function of forage mixtures in organic systems can contribute to management recommendations for organic farmers in relation to soil fertility and C sequestration. The productive capacity of an organic system depends on building soil quality and enhancing biotic interactions between plants and soil, perhaps more than conventional forages. Roots are an important location for these interactions: roots scavenge nutrients and water and then release organic compounds and minerals into the soil through rhizodeposition, root sloughing, and after plant death. In this study, our objectives were 1) to evaluate root biomass density, root length density, and root fineness in organic forage monocultures and mixtures at two soil profile depths, and 2) evaluate rates of C and N mineralization of roots from forages of three functional groups (legume, warm-season grass, cool-season grass) alone and in mixtures, as affected by soil N content. Soils and roots were sampled from two replicated trials on research farm sites in Tennessee and Kentucky, both conforming to USDA organic certification requirements. Roots were extracted from soil core samples taken at 0-15 and 15-30-cm depths, scanned and analyzed for root length and root fineness using WinRhizo, and total dry biomass determined. We evaluated C and N mineralization of roots from three forage species belonging to three functional groups: a legume, a cool-season C3 perennial grass, and a warm-season C4 annual grass alone and in mixtures, and at two soil N rates. We found that treatments containing orchardgrass (*Dactylis glomerata*) generally had higher root length density and biomass density, and treatments with annuals alone generally had lower root biomass density. While higher soil N rates increased C mineralization, there was no interaction observed with forage species treatment. Mixtures of red clover (*Trifolium pratense*) and crabgrass (*Digitaria* spp.) roots generally had greater C mineralization, whereas mineralization of C from red clover roots alone did not differ from that of soil alone.

TABLE OF CONTENTS

Chapter One: Introduction....	1
Chapter Two: Literature Review....	4
Chapter Three: Depth distribution of root biomass density, root length density, root-to-shoot ratio, and root C and N in organic species mixtures in the Southeastern US.....	20
Abstract.....	21
Works Cited.....	35
Appendix: Tables and Figures.....	40
Chapter Four: Soil nitrogen and forage root functional group interaction influences root carbon and nitrogen mineralization.....	55
Abstract....	56
Works Cited.....	72
Appendix: Tables and Figures.....	80
Chapter Five: Conclusions.....	94
Vita.....	96

LIST OF TABLES

Table 3-1: Species used in each forage mixture or monoculture treatment....40

Table 3-2: Mixed models analysis of variance for main effects and interactions for all root fineness, root biomass density (RBD), root length density (RLD), root C content, root N content, and root C:N ratio....41

Table 4-1: Analysis of root biochemistry, total C and N, lignin, starch, and carbohydrate fractions for each forage species.....80

Table 4-2: Treatment root biochemistry; nitrogen (N), carbon (C), lignin, C:N ratio and lignin:N ratio for each treatment....81

Table 4-3: Analysis of variance for effects on cumulative C mineralization at 0 to 21d, 0 to 49d, and 0 to 91d....82

Table 4-4: Analysis of variance for effects on water extractable carbon (C) and nitrogen (N) at days 49 and 91 days after initiation of incubation, at each N rate 83

Table 4-5: Analysis of variance for N mineralization 84

LIST OF FIGURES

Figure 3-1: Mean harvested aboveground forage biomass as affected by forage treatment, averaged across site....42

Figure 3-2: Mean root biomass density as affected by forage treatment, averaged across sampling season and sampling depth.....43

Figure 3-3: Mean root biomass density as affected by sampling season and sampling depth, averaged across forage treatment....44

Figure 3-4: Mean ratio of RBD to harvested biomass as affected by forage treatment....45

Figure 3-5: Mean root fineness as affected by sampling depth and sampling season, averaged across forage treatment.....46

Figure 3-6: Mean root fineness as affected by forage treatment, averaged across sampling season and sampling depth....47

Figure 3-7: Mean root length density as affected by forage treatment, averaged across sampling season and sampling depth....48

Figure 3-8: Mean root length density as affected by sampling season and sampling depth, averaged across forage treatment....49

Figure 3-9: Mean root carbon to nitrogen (C:N) ratio as affected by forage treatment, averaged across sampling season and sampling depth....50

Figure 3-10: Mean root carbon to nitrogen (C:N) ratio as affected by forage treatment and sampling depth, averaged across sampling season....51

Figure 3-11: Mean root carbon to nitrogen (C:N) ratio as affected by sampling season and sampling depth, averaged across forage treatments....52

Figure 3-12: Mean root tissue nitrogen (N) concentration as affected by forage treatment and sampling depth, averaged across sampling season....53

Figure 3-13: Mean root tissue N concentration as affected by sampling season and sampling depth, averaged across forage treatments....54

Figure 4-1: Total CO₂ mineralized across time as affected by forage root treatments....85

Figure 4-2: Effect of time and nitrogen rate on C mineralization rate, averaged across forage root treatments....86

Figure 4-3: The rate of C mineralization affected by root species treatment and averaged across time and soil nitrogen rate....87

Figure 4-4: Cold water extractable carbon as affected by treatment at low soil N rate....88

Figure 4-5: Hot water extractable carbon as affected by treatment and time, at low soil N rate, with means given for each day....89

Figure 4-6: Total water extractable carbon (TWEC) as affected by treatment and time, at low soil N rate, with means given for each day....90

Figure 4-7: Total water extractable carbon (TWEC) as affected by time and treatment, at high soil N rate, with means given for each day....91

Figure 4-8: Mineralized inorganic soil nitrogen at 21, 49, and 91 days after initiation of incubation....92

Figure 4-9: Hot water extractable nitrogen (HWEN) as affected by time and treatment, at low soil N rate, with means given for each day....93

CHAPTER ONE

INTRODUCTION

Soil contains both living and dead roots. Soil organic matter (SOM) is composed of living microbial biomass, dead bioresidues, and organic residues that have been processed into more stable organic compounds, often termed “soil humus” (Berg & McClaugherty, 2003). Roots are generally considered to be the highest contributor to soil organic carbon (SOC) (Brady & Weil, 2008; Rasse et al., 2005). Root decomposition concurrently mineralizes C (released as CO₂) and contributes to sequestered C pools through formation of stable organic compounds (Berg & McClaugherty, 2003; Puget & Drinkwater, 2001).

Decomposition of plant tissue in soil varies by plant species. Chen et al. (2017) reported that higher biodiversity contributes to slower decomposition of roots. In that paper, poor tissue quality such as N-poor grass roots slowed the decomposition of species mixtures. Although lack of available N is one impediment to decomposition, complex carbonaceous molecules are another, given that microbes would preferentially decompose simpler C structures such as carbohydrates. White et al. (2018) showed that plant root tissue had an initial high rate of decomposition that was followed by slower decomposition: cell components were the first to decompose but cell wall components such as lignin and suberin were more resistant.

According to DuPont et al. (2014), soil C increases when plants that have greater root length density and greater total root biomass are grown. Roots of perennial plants like those used for cover crops contribute to SOM due to their larger roots (Bolinder et al., 2002). SOM contributes to better soil physical conditions such as aggregation and improved tilth (Ricosky et al., 1995).

Immobilization or mineralization of carbon in soil depends on the N and C available, often expressed in terms of C:N ratio. Stewart et al. (2015) found that soils with a higher native N content led to increased decomposition of residues; however, at high temperatures (30° C) the rate of decomposition was decreased due to N immobilization (Stewart et al., 2015). The presence or absence of the plant functional group of legumes can lead to differences in soil N content, and one study reported that the presence of legumes led to decreased N limitation for microbial decomposers (measured by activity) as well as macrofauna decomposers (increased earthworm biomass) (Milcu et al., 2008).

Although there may be some differences in root placement between plant functional group (Ravenek et al., 2016), it is also possible that wild and domesticated plants of the same species also exhibit root morphology differences (Nakhforoosh et al., 2014). In a review of studies on root biomass density in biomes across the world, croplands were shown to have less root biomass density than grasslands (Jackson et al., 1996). Similarly, a study of wild and domesticated barley suggests that selection produces crop plants that selectively allocate more C to aboveground biomass to produce higher yields when conditions are optimal, while less-domesticated plants retain a greater root plasticity, thus enabling them to scavenge water and nutrients from soil when conditions are sub-optimal (Grossman & Rice, 2012). Root morphology is thus essential to building resilience in agricultural systems, a potential benefit when, as in organic production, inputs are low.

Root contribution to SOC has been studied in the context of biodiverse grasslands (Mommer et al., 2010; Ravenek et al., 2016) and in prairies of the Western U.S (Carrillo et al., 2014). Reviewing research on plant communities in grasslands where diversity leads to greater belowground outputs and where plant functional group influences C and N mineralization has

led to the objectives for the present study. This study focused on the belowground activity of an organic forage system, which is low-input and which varies in species diversity and plant functional group. Our objectives were (1) to evaluate root biomass density, root length density, root fineness, and root C:N ratio in organically-managed forage monocultures and mixtures at two soil profile depths, and (2) to evaluate rates of C and N mineralization of roots from forages of three functional groups (legume, warm-season grass, cool-season grass) alone and in mixtures, as affected by soil nitrogen content.

Given that limited information is available on root morphology and decomposition in organic mixed-species forages in the Southeastern U.S., we suggest that the performance of forages in the Southeastern U.S. will be dissimilar to agronomic croplands but more similar to species-rich, low-input grasslands and pastures. Therefore, these are our hypotheses: (1a) forage mixtures with a greater species richness will have higher aboveground biomass; therefore, we also expect higher root biomass, (1b) forage mixtures with perennials will have higher root length density, (2a) we expect that decomposition treatments with legume (red clover) roots will have higher C mineralization due to reduced N limitation, and (2b) we expect that higher soil N will increase mineralization of root C.

CHAPTER TWO

LITERATURE REVIEW

Part One: Root Morphology and its Impact on Soil N and C

Summary

Due to the nature of their occlusion underground, root morphology can be difficult to study. A compounding factor is the apparent phenological plasticity of roots depending on the soil in which they develop. At the extreme ends of the *r-K* life history spectrum (Jackson et al., 1996), cases can be found that would suggest that *K*-selected species develop deeper roots and *r*-selected species develop a fine network of shallow roots. DuPont et al. (2014) compared annual cropping systems with and without tillage to perennial grasslands and found that perennial grass roots are longer and thicker than either annual cropping system (DuPont et al., 2014). The root niche partitioning model of mixture overyielding (e.g., Mommer et al., 2010) is based on this theory. Root location in either deeper or shallower soil horizons and the fineness of roots may be correlated to yield by examining root-to-shoot ratios. Root length density (RLD), a measure of root length per weight of soil, can be understood as a measure of how much the plants are allocating resources to absorbing nutrients and water from soil.

Root Morphology in the Literature

Some aspects of root traits differ by plant functional group, making root structure one type of phylogenetic difference (Ravenek et al., 2016). Grasses (Poaceae) tend to have more and finer roots, leading to the appearance of a fibrous mass of roots underground (Taiz & Zeiger, 2010). These fine grass roots, with their combined high root length density (RLD), are generally considered to scavenge more nutrients from shallow soil layers, a function that is exploited in

the case of annual ryegrass (*Lolium multiflorum*) grown to scavenge nitrates after legumes (Santos et al., 2011). In contrast, some legumes and some non-legume forbs have a taproot of which the function is to gain water and nutrients from deeper soil horizons (Jackson et al., 1996). For example, alfalfa (*Medicago sativa*) can grow a taproot up to 4 m deep in order to reach water (Weaver, 1926). Yellow blossom sweetclover (*Melilotus officinalis*) is another legume with a deep taproot to 2 m (Bowman et al., 2012; Willard, 1927). However, a study of Brazilian savannahs with sparse non-leguminous trees (*Curatella americana*, *Byrsonima crassifolia*, and *B. coccolobifolia*) found that although roots were found throughout their studied strata to 1 m, 80% of grassland root biomass was found in the top 50 cm horizon (Barbosa et al., 2012).

Mueller et al. (2013) evaluated the relationship between diversity and root biomass density. In many situations, increased species diversity led to increased above-ground biomass productivity. Monocultures were compared with plots of increasing species richness (2, 4, 8, or 16 species) and species were randomly selected from a pool of species with broad functional group traits including grasses, legumes, and forbs. Grasses included 4 species of C₃ plants and 4 species of C₄ plants: C₄ is an adaptation in the photosynthetic system to increase efficiency at high temperatures and is common in warm-season grasses (Taiz & Zeiger, 2010). The root biomass values of the monoculture plots were used as “expected” values of root biomass present in the multispecies plots. Soil was divided into 3 profile depths (0-30, 30-60, and 60-100 cm). Monoculture plots of C₃ grasses had root systems shallower than the other monocultures. The plots with the most species richness (16 species) had more roots than their expected values at the deepest soil profile tested (60-100 cm). Specifically, when legumes and C₄ grasses were in the same plot, a greater-than-expected proportion of roots were found in deep soil than for plots containing only non-leguminous forbs and C₃ grasses. The fact that plots with high richness had

higher root biomass in deep soil than monocultures, along with a higher above-ground productivity, suggests that plants can allocate C to roots with some degree of plasticity. The authors suggest that increased biomass both above and belowground stems not only from niche partitioning (i.e., species that are naturally deep-rooted and species that are naturally shallow-rooted) but that species richness has some other effect on increasing biomass, such as functional group diversity or differences in seasonal growth patterns, which in turn drives the plants to allocate enough resources to the roots to uptake sufficient resources from soil at any depth (Mueller et al., 2013).

Other studies also assign more importance to plasticity than to niche differentiation by roots. Mommer et. al. (2010) used two grasses and two forbs (*Anthoxanthum odoratum*, *Festuca rubra*, *Leucanthemum vulgare*, and *Plantago lanceolata*) in mesocosm plots with a layer of poor, sandy soil either on top of or beneath a layer of richer soil. If species have significant plasticity in root growth, then root density between the treatments would not be much affected. On the other hand, if species' root depth was pre-determined by phenology, then species with shallow roots would have a disadvantage in the top-sand treatment but an advantage in the top-soil treatment. The results of this study showed some evidence for plasticity, as all species and mixtures had higher root biomass with rich soil on top than with sandy soil on top. Mixtures had an increased rooting depth and increased above- and below-ground biomass (overyielding) as compared to monocultures (Mommer et al., 2010).

Using ^{15}N labelled fertilizer, researchers in Switzerland found that a two-species mixture of shallow-rooted forage plants (*Lolium perenne* and *Trifolium repens*) had greater uptake of the labelled N in shallow soil layers than did two forage species with typically deep roots (*Chicorium intybus* and *Trifolium pratense*) (Husse et al, 2017). The authors used two leguminous (*T. repens*

and *T. pratense*) and two non-leguminous plants (*L. perenne* and *C. intybus*), one each of shallow- and deep-rooted. When shallow- and deep-rooted plants were combined in mixture, if the species were either both leguminous or both non-leguminous, there was no significant difference in aboveground biomass yield from monocultures. Only when plants differed both in rooting depth and in N-fixing capacity was there a significant increase in mixture biomass. Although biomass did not change for mixtures of deep- and shallow-rooted plants from the same N-fixing group, the proportion of N and phosphorus (P) in the dry tissues did increase, suggesting that the plants were able to scavenge these nutrients from all soil layers.

Although computer models exist to predict the root growth of a single plant (Bonneu et al., 2012), this model is not yet able to map the RLD of plant roots in mixture, due to the complex conditions and interactions occurring in mixtures. Both above-ground and below-ground competition were measured for two grass (*Hordeum vulgare*, barley, and *Triticum durum*, durum wheat) and two legume species (*Lupinus albus*, white lupin, and *Vicia sativa*, common vetch) by Mariotti et al. (2009). Although the authors found that some combinations exhibited signs of competition, the total above- and belowground biomass was always greater in combinations than in monoculture (Mariotti et al., 2009).

A component of mixture interaction is competition between plants when a resource is limiting. Ravenek et al. (2016) compared growth of four species of grasses (*Anthoxanthum odoratum*, *Festuca rubra*, *Phleum pratense* and *Poa pratensis*) to four species of non-legume forbs (*Centaurea jacea*, *Knautia arvensis*, *Leucanthemum vulgare* and *Plantago lanceolata*) in a greenhouse trial in Germany and found that root length density was a good predictor of a species' competitive ability, and that a high RLD was correlated to a high relative growth rate (RGR). The authors also measured selective root placement (SRP) as the ability of a plant to

selectively place its roots in an area of soil with higher nutrient availability. SRP was a trait that was typically higher for the forbs the authors used than for the grasses, showing a phenological difference between phylogenetic groups. However, SRP was not a predictor of competitive ability (Ravenek et al., 2016).

Overall, the literature shows that although there are some root traits which are specific to species, it is difficult to generalize about the role of phylogenetic or functional groups on root length, root biomass, or root fineness. Additionally, plant root structures have a built-in plasticity that allows them to adapt to different growing conditions.

Part Two: Decomposition of Root Tissue and its Impact on Soil C and N

Summary

Carbon (C) dynamics in soil become increasingly important as global climate change begins to show effects in the world. Aside from its atmospheric role, C has importance to soil function when it is present or absent in the soil. Soil organic C (SOC) has agricultural benefits such as increased soil nutrient and water retention, and as it is mineralized to support soil biological function, mineral nutrients become more available to plant acquisition (Berg & McClaugherty, 2003). SOC is derived from plant inputs, including rhizodeposition, incorporation of aboveground organic material, fine root turnover, and plant death. However, when comparing root inputs to inputs from aboveground material, roots contribute between 1.3 and 2.4 times as much to soil organic C (Rasse et al., 2005).

Perspectives on Root Tissue Decomposition

Experiments from the literature pertaining to nutrient cycling between roots and soil exist along a spectrum of small-scale or large-scale experiments, in-situ or in-vitro, and short or long

timescales. For the purposes of this work, special attention was given to literature related to grasslands and forages, as well as organically managed or similar low or reduced-input agricultural systems.

Organic forages and grazing systems are perhaps more similar to native grasslands and prairies because both ecosystems have relatively low external inputs of N and because both may be grazed by livestock or by wild megafauna. Due to the low amount of plant-available N in soil, root N concentration is a key contributor to residue decomposition rate. Johnson and Matchett (2001) examined root tissue quality and found that grasslands that had been grazed had a root tissue N concentration that was 30% higher in both lightly and heavily grazed subplots than in grazing exclusion plots. They also measured soil N and C mineralization and found that higher N from the lightly grazed prairies (N allocation by plants) led to greater mineralization of both N and C. Heavily grazed patches had a lower root mass, but the N inputs also increased soil N mineralization (Johnson & Matchett, 2001).

Different plant functional groups typically have different N concentrations that can lead to differences in mineralization of carbonaceous residues from soil. Legumes, because of their association with N-fixing rhizobia bacteria, obtain a large proportion of N from the atmosphere but also utilize N differently than other plants. Del Pozo et al. (2000) found that for a given amount of N, legumes produce a smaller amount of biomass. In legumes, C, not N, is often limiting for both aboveground and belowground growth. Some of the N is stored for seed production by the plant, giving the seeds a fitness advantage when quick germination and seedling development is important. Some of the N is invested in the structure of the plant, thus we might expect to find a more even C:N ratio in legume roots. However, this effect is typically less when the legumes are mobilizing N to fill seeds (Del Pozo et al., 2000). Researchers from

Estonia, in contrast to other studies, found that the roots of birdsfoot trefoil (*Lotus corniculatus*), alfalfa (*Medicago sativa*), large-leaved lupine, (*Lupinus polyphyllus*), and white sweetclover (*Melilotus alba*) grown as cover crops had a lower C:N ratio than the aboveground biomass (Talgre et al., 2017). They also found that although the decomposition rate of aboveground biomass was more strongly correlated to the C:N ratio than the decomposition of the roots, since the shoot residue released more mineralized C than root residue. A suggested cause for this finding is that the roots were lignified and thus the C was more recalcitrant. They found that red clover (*T. pratense*) decomposed more quickly than the other species, possibly because the other legumes used had more lignified roots.

Total C contribution of roots to soil, including rhizodeposition and exudates, has been measured by ^{13}C labelling of plants (Puget & Drinkwater, 2001). Puget & Drinkwater (2001) found that root-derived C contributed more to soil C than did shoot-derived C. Therefore, soil C derived from root decomposition would be a fraction of the total SOC. By measuring the total carbon in root samples and measuring the CO_2 released as a result of decomposition, an estimate of the root contribution to more recalcitrant SOC can be made.

Carbon is central to many biochemical processes, such that its long residence as “humus” in the soil is favorable from a soil structure and a C sequestration perspective, though as mentioned before, its mobilization is related to the mineralization of N, a key source of plant nutrition. Carbon mineralization during decomposition is therefore the same as respiration by soil heterotrophs, the largest portion of which are bacteria and fungi. A study of an annual corn cropping system given a restoration treatment of planting diverse prairie plants showed that soil planted with prairie perennials had a higher ratio of fungal to bacterial biomass than the annual system (Herzberger et al., 2014), which implies that root chemical composition of perennials is

different than that of annuals, since it supports different organisms. Another study using an agronomic crop rotation with added species richness by means of cover crops showed that plant diversity across time also leads to a higher microbial biodiversity and functional diversity than a less-diverse plant mixture (Tiemann et. al, 2015). Tiemann et. al (2015) also suggested that rotational plant species richness leads to more nutrient utilization for low-input systems such as organic systems, due to higher soil C and N, and more soil aggregation.

Diversity of plant species is one contributing factor to rates of decomposition. Cong et al. (2015) found that shoot and root litter from an intercrop of corn and beans decomposed faster than expected under both laboratory and field conditions. They hypothesized that below-ground overyielding caused both soil C and soil N to be higher, thus making a more favorable environment for decomposition (Cong et al., 2015). DuPont et al. (2014) found that perennial plants contributed more to soil C because the perennial roots were larger and longer than annual wheat in the top 1-m soil profile. Perennials (such as big bluestem, *Andropogon gerardii*) were deeper in the soil profile and produced more above- and below-ground biomass with fewer inputs (DuPont et al., 2014).

Decomposition of root tissue is influenced by many factors, including plant functional group, plant species richness, and soil chemistry, specifically soil N. While some root C would be returned to the atmosphere by respiration, root C contributes more to recalcitrant SOC than other plant carbon. Increased SOC is desirable in agriculture systems both as a sequestered pool of C from the global C cycle and because SOC is beneficial to soil health and plant nutrition.

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CHAPTER THREE

DEPTH DISTRIBUTION OF ROOT BIOMASS DENSITY, ROOT LENGTH DENSITY, ROOT-TO-SHOOT RATIO, AND ROOT C AND N IN ORGANIC SPECIES MIXTURES IN THE SOUTHEASTERN US

Abstract

In order to evaluate forage species mixtures for organic forage systems, an important consideration is the contribution of each mixture to belowground biomass. Increased root biomass in soil has been shown to increase soil carbon, which in turn increases markers of soil health such as water-holding capacity and nutrient exchange, which are beneficial to forage system function. Plant species functional group and physiological traits can influence root parameters such as root length density and root biomass density. Forage mixture treatments included two monocultures and seven mixtures of plants from groups of perennial legumes, perennial grasses, cool-season annual grasses, cool-season annual legumes, warm-season annual grasses, and warm-season annual legumes, planted and managed organically on two replicated experimental sites in Tennessee and Kentucky, with four replicates at each location. Root core samples were taken four times in a year from 0-15 cm and 15-30 cm soil profile depth. Root biomass density (RBD) was measured by weighing roots extracted from soil cores and then dried, and root length density (RLD) and mean root fineness were determined by measurements of scanned images of all root biomass extracted from a known soil volume. Root carbon (C) and root nitrogen (N) were determined by combustion analysis of root samples from each experimental plot. A mixture of red clover (*Trifolium pratense*), orchardgrass (*Dactylis glomerata*), and tall fescue (*Festuca arudinacea*) had the highest RBD (507 mg roots kg⁻¹ soil) and the highest RLD (6,882 cm roots kg⁻¹ soil) among treatments. The lowest RBD was a crimson clover, annual ryegrass, sorghum sudangrass, cowpea mixture (166 mg roots kg⁻¹ soil) and the lowest RLD was a crimson clover, annual ryegrass, crabgrass, annual lespedeza mixture (3,248 cm kg⁻¹ soil). We found that RLD was highest in the spring, but for all seasons, RLD was higher for the 0-15 cm soil profile than the 15-30 cm soil profile. The presence of some

perennials, such as orchardgrass, tall fescue, and sometimes red clover, had a positive effect on the RBD and RLD of the treatments, indicating that perennial plots could increase SOC.

Introduction

The depth and abundance of roots of a plant species are based partly on species' phenology and partly on environment, including availability of belowground resources of nutrients and water, thus resulting in heterogeneous root systems even among plants of the same species (McCully, 1995). The amount of roots can be quantified in different ways, including the weight of the roots present in the soil (root biomass density, RBD) which is more closely representative of the amount of resources a plant has allocated to root production, and the length of roots in the soil (root length density, RLD) which can be extrapolated to suggest the amount of nutrient stress the plant is facing, given that a greater root surface area can gather more nutrients (McCully, 1995). Another factor in root nutrient uptake is average root diameter, given that the fine root area at the apex is classically associated with nutrient scavenging (Taiz & Zeiger, 2010). Plant roots are also host to a complex environment of mycorrhizal fungi and rhizosphere-associated fungi and bacteria, and this "rhizosphere sheath" acts as an extension of the root's water and nutrient-gathering function (McCully, 1995). Although rhizosphere microbes are not the focus of the current study, the rhizosphere cannot exist without the root, and thus the abundance of roots also gives key clues to the biodiversity and abundance of microbes in soil (Eisenhauer et al., 2017).

Carbon allocated to the roots by the plant can be lost as CO₂ by respiration of live roots, organic carbon can enter the soil as fine root turnover, or organic carbon can enter the soil through the processes known as rhizodeposition (Pausch & Kuzyakov, 2018). However, the

amount of C remaining in the roots is not insignificant and in perennial grasslands is an average of 16% of all assimilated C, whereas C remaining in roots is reported as only 10% for annual cropping systems (Pausch & Kuzyakov, 2018). Soil organic carbon (SOC) is well-known to have benefits ranging from better soil water retention to increased plant nutrient availability (Brady & Weil, 2008).

Previous studies have found that higher species richness in grasslands leads to greater nutrient utilization and productivity aboveground, which is known as overyielding and is one way that organic or low-input systems may be able to increase output without additional inputs (Tilman et al., 1996). Overyielding can occur in mixtures of plant species as compared to monocultures due to differences in nutrient acquisition such as leguminous or non-leguminous function, and season of growth (Husse et al., 2017). Sanderson et al. (2004) provide multiple drivers of overyielding, including facultative relationships such as hydraulic lift, shade and altered light radiation, lower soil temperatures, and more (Sanderson et al., 2004). Mueller et al. (2013) found that this relationship between productivity and diversity also extends to root depth and abundance. Ravenek et al. (2016) suggested that grasses are better suited to developing a high root length density (RLD) because they can create more roots with a smaller C allocation (specific root length or SRL). In Canada, alfalfa (*Medicago sativa*) and orchardgrass (*Dactylis glomerata*) were reported to have a lower root biomass density in the upper 30 cm of soil depth than other perennial species studied, such as switchgrass (*Panicum virgatum*), timothy (*Phleum pratense*), and red clover (*Trifolium pratense*) (Bolinder et al, 2002). Plant functional group can interact with N availability to increase root length density (RLD) because non-N-fixing plants must allocate more resources to growing roots than N-fixing plants under low N conditions (Sainju et al., 1998). Specifically, an annual rye cover crop was found to increase its root length

density early in the spring as temperatures increased, allowing it to scavenge NO_3^- and produce more biomass.

The objective for the current study was to evaluate root biomass density, root length density, root fineness, and root C:N ratio in species mixtures in organically-managed forage systems in the southeastern US. We hypothesized that: a) forage mixtures with a greater species richness will have higher aboveground biomass; therefore, we also expect higher root biomass, (1b) forage mixtures with perennials will have higher root length density.

Materials and Methods

Samples were taken from two experimental research sites, one in Knoxville, TN, USA at the Organic Crops Unit of the University of Tennessee, and the other in Lexington, KY, USA at the Organic Farming Unit at the University of Kentucky. The experimental sites in Knoxville and Lexington (UTK and UKY respectively) were planted in fall 2015 with nine forage treatments (Table 3-1), replicated in four blocks at each site in a randomized complete block experimental design and each with 36 plots. All seed used was organic or untreated. Experimental plots included monocultures of red clover (*T. pratense* cv. “Freedom!”) and orchardgrass (*D. glomerata* cv. “Persist”), plus treatment mixtures of red clover and orchardgrass, orchardgrass and alfalfa (*M. sativa* cv. “KingFisher OR401B”); red clover, orchardgrass, and tall fescue (*Festuca arundinacea* cv. “Lacefield MaxQII” non-toxic endophyte); red clover, orchardgrass, and large crabgrass (*Digitaria sanguinalis* cv. “Red River”); red clover, annual ryegrass (*Lolium multiflorum* cv. “Marshall”) and crabgrass; crimson clover (*T. incarnatum* cv. “Dixie”), annual ryegrass, sorghum sudangrass (*Sorghum bicolor* x *sudanese* cv. “BlackHawk 12 BMR”), and cowpea (*Vigna unguiculata* cv. “Iron & Clay”); and crimson

clover, annual ryegrass, crabgrass, and annual lespedeza (*Kummerowia striata* cv. “Legend”). The treatments with only annual species underwent tillage twice a year in such a way that the crimson clover and annual ryegrass were tilled under to provide a seedbed for sorghum sudangrass and cowpea or crabgrass and annual lespedeza in the summer, and in late fall, after the last forage harvest, the summer mixtures were tilled again so that planting of crimson clover and annual ryegrass could occur. The orchardgrass monoculture alone was fertilized in the spring, using feathermeal at a rate of 67.3 kg N ha⁻¹. Treatments included cool-season perennial legumes and grasses, cool-season annual legumes and grasses, and warm-season annual grasses and legumes (Table 3-1). These treatments were chosen to represent forage systems that could be used in Tennessee and Kentucky organic dairy systems.

Plots at UTK and UKY each measured 4m by x 8m. Soil type at UTK was a Dewey silt loam (fine, kaolinitic, thermic Typic Paleudult) and the soil type at UKY was a Bluegrass-Maury silt loam (Fine, mixed, active, mesic Typic Paleudalf). Aboveground growth at UTK and UKY was harvested at regular intervals with a forage harvester based on forage maturity. Biomass fresh and dry weights were measured, as well as the dry weight proportion of each species in mixture. Means and statistical analysis of the data from aboveground biomass measurements is presented to contextualize the root morphology data in terms of the root-to-shoot ratio and to discuss the influence of forage treatments on root measurements (Barbosa et al., 2012; Mariotti et al., 2009).

To evaluate root morphology, three soil cores were taken from each plot with a 1.75-cm internal diameter soil probe to a depth of 30 cm. Each soil core was divided to represent 0-15 cm and 15-30 cm soil profile depths and each depth was composited by plot. Soil cores were taken

seasonally four times during 2017-2018: winter (Nov-Dec 2017), spring (Apr-May 2018), summer (Aug-Sept 2018), and fall (Oct-Nov 2018).

Samples were held at 4°C until processing. Ten grams of soil from each sample was used for gravimetric water content measurement (105°C for at least 48 hours). Analysis of root biomass and morphology was based on a subsample of 25 g of field moist soil from each sample. Each subsample was treated with sodium hexametaphosphate 10%, a disaggregate solution (Bolinder et al., 2002), and then washed through a 500-µm sieve using tap water. Roots were separated from sand and debris by hand and suspended in deionized water. Roots were scanned and measured for length and average diameter using the WinRHIZO program. According to Pornaro et al. (2017), WinRHIZO accurately measured grass stolons when compared to manual measuring techniques with rulers and calipers. WinRHIZO uses a scanned root images to calculate root parameters including root length and root fineness and corrects for overlapping root images (Wang & Zhang, 2009). Data for average root diameter was used as-is for a representation of root fineness for each plot: data for root length was calculated as cm per kg based on the weight of the subsample.

Roots were then placed in a forced-air oven at 60°C for at least 24 hours until dry. Dry roots were weighed to calculate dry root biomass per field moist soil mass in units of mg roots per kg of soil (based on the weight of the subsample), which constitutes the root biomass density (RBD) measurement. Dried root tissue was analyzed for total C and N content by combustion, using a soil analyzer (Flash EA 1112 NC Soil Analyzer, Thermo Fisher Scientific Inc., Waltham). Final concentration of C and N constituents in root samples were calculated based on exact weight of analyzed root tissue.

Data analysis for RBD, RLD, average fineness, total concentration of C and nitrogen, and C:N ratio was completed using SAS 9.4 (Cary, NC) using mixed methods analysis of variance and means separated using Fisher's least significant difference ($P < 0.05$). Random effects included site (UTK or UKY) and block (nested within sites). Forage mixture (treatment), season, profile depth and their interaction were considered fixed effects. Statistical analysis of species was not included because monocultures only existed for RC and OG, although common species between mixtures are used for discussion purposes. For RBD, RLD, and root C:N ratio, a square root transformation was used to address equal variance and model homogeneity of variance assumptions. No transformation was used for the other response variables. Data analysis for average annual harvested biomass and harvest-to-RBD ratio was also completed using SAS 9.4 and using the same methods as the other data; however, the only fixed effect was treatment, as season and profile depth were not useful to a discussion of annual harvest-to-RBD ratio. The square root transformation was also used on these data for the same reasons as above.

Results and Discussion

Aboveground Biomass

With an average annual harvested biomass of 9,146 kg ha⁻¹, the crimson clover/annual ryegrass/sorghum sudangrass/cowpea treatment was most productive aboveground ($P=0.001$), which was statistically similar to the red clover monoculture (8,084 kg ha⁻¹), red clover, tall fescue & orchardgrass (8,010 kg ha⁻¹) and red clover & orchardgrass (7,855 kg ha⁻¹; Fig. 3-1). The least productive treatment was the orchardgrass monoculture, with an average biomass of 5,509 kg ha⁻¹ which was statistically similar to the red clover, orchardgrass, crabgrass treatment

(6,605 kg ha⁻¹). These results show that although an annual system was most productive, many perennial systems were as productive. Monocultures and mixtures represented both the top and bottom mean separation group, so there is no overwhelming evidence for aboveground overyielding related to plant species richness for the species mixtures in the environmental conditions of our study.

Averaged harvested biomass data does not represent the entire aboveground portion of the plant, but is an estimate that might constitute 80% of the aboveground biomass, excluding stubble and senesced leaves (Eichler Inwood et al., 2015; Rasmussen & Collins, 1991)

Root Biomass Density and Harvested Biomass-to-RBD ratio

Depth, treatment, and season were all significant main effects on root biomass density, and there was an interaction effect of depth and season (Table 3-2). The treatment of red clover, orchardgrass, and tall fescue had the highest RBD (282 mg kg⁻¹ soil) and was statistically similar to the orchardgrass monoculture and the mixtures of red clover and orchardgrass; alfalfa and orchardgrass, red clover, orchardgrass, and crabgrass; and red clover, annual ryegrass, and crabgrass (Fig. 3-2). All of these treatments have either red clover or orchardgrass in common. The lowest RBD was observed for the crimson clover, annual ryegrass, sorghum sudangrass, and cowpea mixture (166 mg kg⁻¹ soil). It was similar to the red clover monoculture and the crimson clover, annual ryegrass, crabgrass, and annual lespedeza mixture (Fig. 3-2). Soil fertility and aboveground yield would be expected to have an impact on root biomass density. Our hypothesis was that the treatments with higher species richness will have a higher root biomass density due to mixture overyielding (Oram et al., 2018). For the red clover, orchardgrass, tall fescue treatment, having three perennials in the mixture did lead to a high average aboveground

biomass productivity (7,655 kg ha⁻¹, Fig. 3-1). However, of the two annual treatments with tillage, one (crimson clover, annual ryegrass, sorghum sudangrass, cowpea) had much lower average RBD (166 and 233 mg roots kg⁻¹ soil, respectively, Fig. 2) in spite of large aboveground outputs expected from annual systems like these (Fig. 1) (e.g., Eichler Inwood et al., 2015). This could be due to the increased allocation from perennial species to build root systems (DuPont et al., 2014). Another factor could be tillage in the plots causing a shorter root-residence time. It is been known that roots add organic matter to no-till soils, and that tillage accelerates organic matter decomposition (Dimassi et al., 2014; Santos et al., 2011; Sauvadet et al., 2018).

The interaction of depth and season indicated that for each season when averaged across treatments, the RBD was greater at a shallower soil profile than the deeper one, but the difference in RBD between profile depths was not always significant (Fig. 3-3). Additionally, RBD was least in the dormant season (33.6 mg roots kg⁻¹ soil) and increased each season throughout the year at the 0-15 cm soil profile depth to a high of between 560 mg roots kg⁻¹ soil in summer and 680 mg roots kg⁻¹ soil in fall at the 0-15 cm profile depth (Fig. 3-3).

The ratio of RBD to harvested biomass was affected by forage treatment ($P < 0.001$). The orchardgrass monoculture has the highest average RBD to harvested biomass ratio at 0.325 (Fig. 3-4). RBD should not be considered to represent the entire root system of a plant due to sampling methodology, rather it is more a measure of the portion of soil that is comprised of roots. Because of this and the aboveground harvest methodology, this ratio should not be compared to root-to-shoot ratios from other studies. However, the implication of the orchardgrass monoculture's high ratio is that the orchardgrass monoculture invested a greater proportion of plant resources in belowground functions. This could be expected, since the input of N is low in the orchardgrass monoculture, with only 1 application of feather meal in the spring, and no

legume species in this treatment. In comparison with the orchardgrass monoculture, the red clover, orchardgrass, tall fescue treatment had an average 0.268 RBD to harvested biomass ratio and was in the same mean separation group (Fig. 3-2). Although this mixture has the ability to fix N and a higher average aboveground biomass than orchardgrass monoculture (Fig. 3-1), the investment in belowground function is still high relative to aboveground allocation. In contrast, the crimson clover, annual ryegrass, sorghum sudangrass/cowpea treatment had the lowest ratio of RBD to harvested biomass, at 0.072 (Fig. 3-2). This treatment therefore allocated much of its resources to aboveground production.

Average Root Fineness

Root fineness is the inverse of root diameter; therefore, when treatments or plots had a lower root diameter, they also had a higher root fineness. Treatment and season were both significant main effects on root fineness, and season had a significant interaction effect with depth (Table 3-2). The finest roots were found at the 15-30 cm depth in the summer (0.195 mm) and the coarsest roots were found in the 0-15 cm soil profile in the dormant season (0.226 mm, Fig. 3-5). Although finer roots were found at deeper soil profiles in dormant and spring seasons, the same was not true in the summer and fall (Fig. 3-5). Depth was not a main effect on average root fineness, despite other authors finding that finer roots were sometimes found at deeper soil profiles (Nakhforoosh et al., 2014). The red clover, annual ryegrass & crabgrass mixture had the coarsest roots on average (0.237 mm), with all other treatments not having a significant difference (0.199 to 0.215mm; Fig. 3-6).

The orchardgrass monoculture was not different in root fineness compared to the other mixtures despite the hypothesis that grasses would have grown more fine roots that were made

for scavenging nutrients: legumes can provide their own N nutrition and therefore might not have as great a need for fine roots. Experiments with wild and cultivated barley (*Hordeum vulgare*) showed that higher fertility levels led to roots that were not as fine (Grossman & Rice, 2012). Other factors influencing root diameter could include soil moisture and genotypic differences: Nakhforoosh et al. (2014) found that low soil moisture decreased root fineness in wheat but that the greatest differences were an effect of genotype.

Root Length Density

There was a significant main effect of forage treatment on root length density, and there was a significant interaction effect between season and depth (Table 3-2). The orchardgrass monoculture treatment; the red clover, orchardgrass, and crabgrass mixture; and the red clover, orchardgrass, tall fescue mixture were all in the highest mean separation group (5,689 to 6,882 cm kg⁻¹ soil) and not grouped with lower means (Fig. 3-7). Therefore, it is possible that orchardgrass contributes proportionately more to RLD than other species. This does lend credence to the hypothesis that species diversity contributes to higher RLD. Another possible explanation for this mixture's overyielding is that perennials may have the advantage when it comes to RLD simply due to growth starting from established root structures. Our hypothesis was that forage mixtures with perennials will have higher root length density.

The season and depth interaction indicated that RLD was higher in the 0-15 cm soil profile at an average of 6,885 cm kg⁻¹ soil, but that RLD in the 15-30 cm profile decreased slightly from the beginning to the end (dormant to fall) of the year (from 3,445 to 1,880 cm kg⁻¹ soil) (Fig. 3-8). Root length density in the 0-15 cm soil layer was significantly higher, at 10,430 cm kg⁻¹ soil, than other seasons in the spring. Cool-season plants, including the annuals, crimson

clover and annual ryegrass, were actively growing at the time of spring sampling. For treatments H and I (crimson clover, annual ryegrass, sorghum sudangrass, cowpea and crimson clover, annual ryegrass, crabgrass, lespedeza) (Table 3-1), the cool-season plants were replaced by actively-growing warm-season plants by the time of the summer sampling. Therefore, although some dead roots may still be present in the soil, sorghum-sudangrass, cowpea, crabgrass, and annual lespedeza would likely be responsible for most of the roots during the summer season. The abundant aboveground growth of sorghum-sudangrass could translate into increased belowground production during this season (Barbosa et al., 2012). Overall, however, in our study the treatment with sorghum-sudangrass did not have a significant separation from the lowest mean separation group among treatments and had an average RLD of 4608 cm kg⁻¹ soil (Fig. 3-7).

The treatment containing red clover, tall fescue, and orchardgrass, had the highest RLD (6882 cm kg⁻¹ soil) and the phenology of roots leads to the inference that this perennial mixture is effective at scavenging resources from the soil and using them to produce biomass (McCully, 1995). Additionally, these samples were taken 2 to 3 years after establishment of perennial plots, similar to a study that showed that RLD was higher in established perennial grasslands than in annual cropland (DuPont et al., 2014).

Root Carbon and Nitrogen

The carbon to nitrogen ratio (C:N ratio) of the root tissue represents its suitability for becoming mineralized by microbial activity: the lower the ratio, the easier it is for microbes to metabolize the tissue, given that microbial biomass stoichiometry is the general range of 5 to 7:1, and microbes generally have N needs that are higher than that present in many plant residues

(Spohn, 2015). Conversely, a higher or less even C:N ratio suggests that roots are responsible for sequestering carbon in the soil because less N availability is likely to limit and slow microbial decomposition. The C:N ratio represents only a partial picture of possible microbial activity. Additional factors include the lignin:N ratio and the amount of soil disturbances (Herzberger et al., 2014; Stewart et al., 2015).

Significant main effects on C:N ratio included treatment effect, depth effect, and interaction effect of treatment by depth and season by depth (Table 3-2). Although the C:N ratio was not significantly different at different soil profiles in the dormant and spring seasons, both summer and fall show a higher C:N ratio at deeper soil depths (39 and 88 C:N ratio, Fig. 11). The crimson clover, annual ryegrass, crabgrass, and lespedeza treatment had the highest C:N ratio both when considered as a whole (C:N ratio of 93) and when only the 0-15 cm soil layer was considered (C:N ratio of 43) (Fig. 3-9, Fig. 3-10). The depth effect indicated that the C:N ratio of root tissue from the deeper soil profile was higher (C:N ratio of 72) than the C:N ratio of roots from the shallower soil (C:N ratio of 40) although (except in the crimson clover, annual ryegrass, sorghum sudangrass, cowpea and crimson clover, annual ryegrass, crabgrass, annual lespedeza treatments) the difference between deep profile tissue and shallow was not significant (Fig. 3-10). Rasse et al. (2005) present several mechanisms that could be responsible for the preservation of C due to reduced decomposition at deeper soil profiles, including a lack of microbes, low nutrient density, and physico-chemical protection of soil minerals. However, the high C:N ratio in the annual treatments might be mostly related to the lower N levels in the 15-30 cm soil profile in these plots (8 and 9 g N kg⁻¹ root tissue, respectively, Fig. 3-12). The reason for low N in the annual treatments is unknown, because these treatments do contain N-fixing

legumes and therefore the mixtures should have access to the atmospheric N pool (Husse et al., 2017).

The differences in the C:N ratios between seasons and depths seem to be explained more by variations in the total N proportion of roots than by the C proportion, because there was not a significant effect of season on C (Table 3-2). This result is similar to results found by Gale and Cambardella, who found that both leaves and roots from oats (*Avena sativa*) had similar C constituents, as C concentration is unlikely to vary much in plant tissue (Gale & Cambardella, 2000).

Unlike root C, the constituent of N in root tissue was more variable and there were significant main effects of forage treatment, season, and depth on root N content (Table 3-2). There were also interaction effects of treatment by depth and season by depth (Table 3-2). The main effect of depth on root N content was such that the average proportion of N in roots from the 0-15 cm soil layer was 11.3 g N kg⁻¹ and the average proportion of N in roots from the 15-30 cm soil layer was 10.7 g N kg⁻¹ (Fig. 3-12). The season by depth interaction is such that while the root N decreases overall from spring to fall (124 to 84 g N kg⁻¹), the root N was lower in the 0-15 cm soil profile than the 15-30 cm soil profile during the dormant season, though not significantly (Fig. 3-13). In the fall, root N is significantly higher in roots from the 0-15 soil profile (93 g N kg⁻¹) than the roots from the 15-30 cm soil profile (75 g N kg⁻¹, Fig. 13). Root nitrogen may have decreased through the seasons due to mobilization to fill seeds in maturing plants (Del Pozo et al., 2000).

Conclusions

Root structures in organic forage mixtures can contribute to soil organic matter. Root biomass density helps us know how much resources the plant is allocating to belowground

functions. The mixture of perennials red clover, orchardgrass, and tall fescue had the greatest RBD although it was not significantly different than other mixtures, this mixture would be favorable for adding organic carbon to the soil and providing soil benefits while also generating forage aboveground, since this mixture was also in the highest mean separation group for harvested aboveground biomass. The highest ratio of RBD to harvested biomass was in the orchardgrass monoculture, since without a legume, the orchardgrass likely allocates more resources to the acquisition of nutrients. Between the two monoculture treatments, the orchardgrass monoculture had a higher RLD, confirming our hypothesis that treatments with grasses would have a higher RLD. As a type of low-input system, organic forage systems should use mixtures which are effective at scavenging nutrients. While the annual mixtures (crimson clover, annual ryegrass, sorghum sudangrass, cowpea and crimson clover, annual ryegrass, crabgrass, annual lespedeza) had a lower RBD to harvested biomass ratio, suggesting that this mixture had to invest less resources into roots to produce biomass, the annual mixtures contribute less to soil organic matter by leaving less root biomass in the soil. Seasonal differences in root length density, root fineness, and root C and N could be due to interacting factors of spring growth in perennials or cool-weather preferring annuals in mixtures.

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Appendix: Tables and Figures

Table 3-1: Species used in each forage mixture or monoculture treatment. Species used vary in functional group and season of growth, and plots vary in tillage status. Seeding rates in kg ha⁻¹ are given in subscript after each species common name.

Treatment species mixture	Tillage	-----Functional group-----					
		Cool-season		Warm-season			
		----Perennial---		----Annual-----		---Annual---	
		Legume	Grass	Legume	Grass	Legume	Grass
A. Red clover _{11.2}	No	1					
B. Orchardgrass _{16.8} (+ 67.3 kg N/ha annually as feather meal)	No		1				
C. Red clover ₉ + orchardgrass _{11.2}	No	1	1				
D. Alfalfa _{16.8} + orchardgrass _{11.2}	No	1	1				
E. Red clover ₉ + orchardgrass _{7.8} + tall fescue _{11.2}	No	1	2				
F. Red clover ₉ + orchardgrass _{11.2} + crabgrass _{4.5}	No	1	1				1
G. Red clover ₉ + annual ryegrass ₂₈ + crabgrass _{4.5}	No	1			1		1
H. Crimson clover ₂₈ + annual ryegrass ₂₈ + sorghum- sudangrass _{33.6} + cowpea ₂₈	Yes			1	1	1	1
I. Crimson clover ₂₈ + annual ryegrass ₂₈ + crabgrass _{4.5} + annual lespedeza _{16.8}	Yes			1	1	1	1

Table 3-2: Mixed models analysis of variance for main effects and interactions for all root fineness, root biomass density (RBD), root length density (RLD), root C content, root N content, and root C:N ratio. Means and interactions are considered significant at $P < 0.05$.

	Avg Fineness	RBD	RLD	Percent C	Percent N	C:N Ratio
Treatment	<0.001	<0.001	<0.001	<0.001	<0.001	0.006
Depth	N/S	<0.001	<0.001	<0.001	0.022	<0.001
Depth x Treatment	N/S	N/S	N/S	N/S	N/S	0.031
Season	<0.001	<0.001	<0.001	N/S	<0.001	<0.001
Depth x Season	0.003	0.001	<0.001	N/S	N/S	0.006

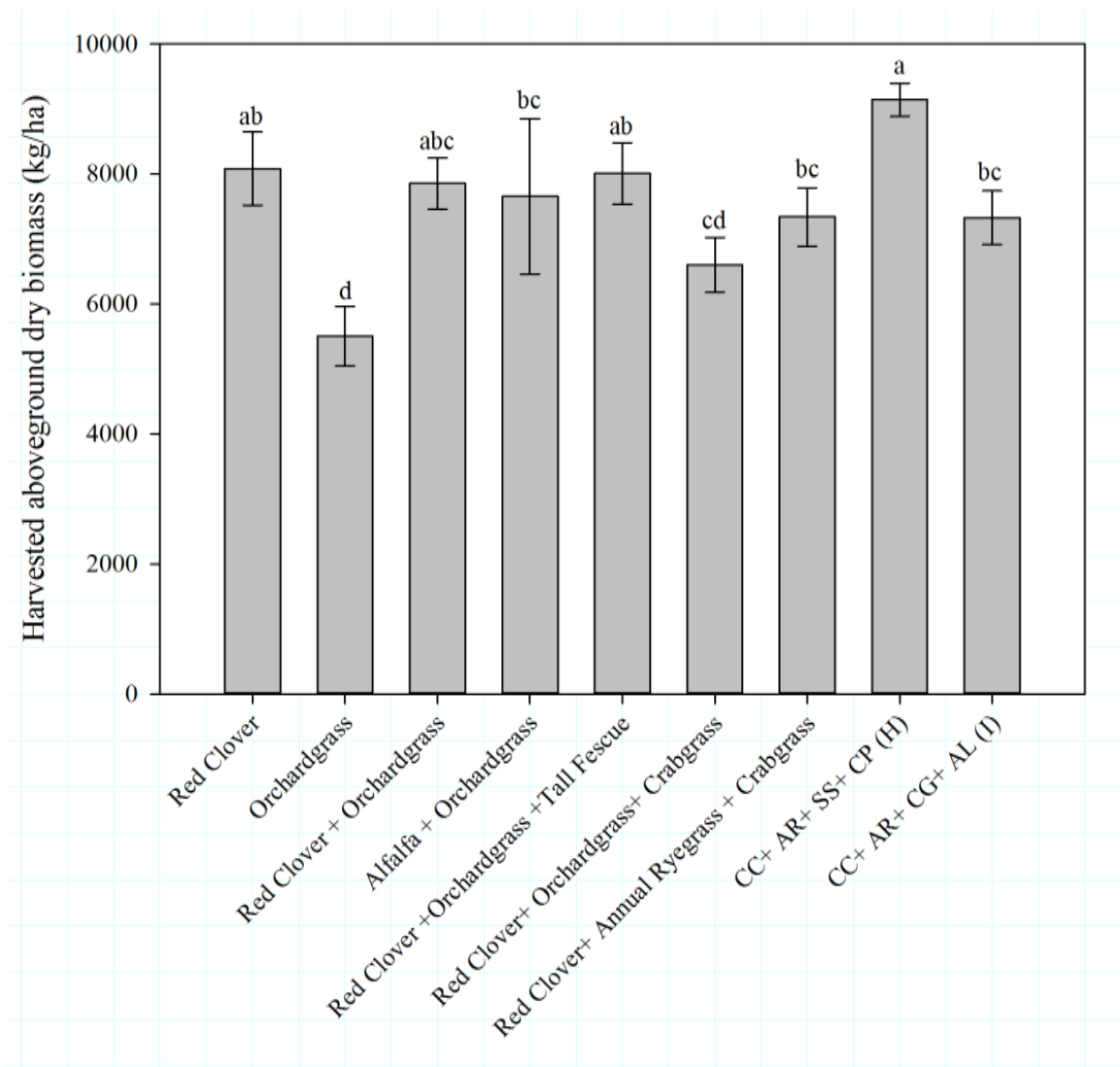


Figure 3-1: Mean harvested aboveground forage biomass as affected by forage treatment, averaged across site. Error bars represent the standard error of the mean. Bars with same letters are not significantly different at $\alpha = 0.05$. For species in treatments marked H and I, refer to Table 1. CC is crimson clover, AR is annual ryegrass, SS is sorghum sudangrass, CP is cowpea, CG is crabgrass, and AL is annual lespedeza.

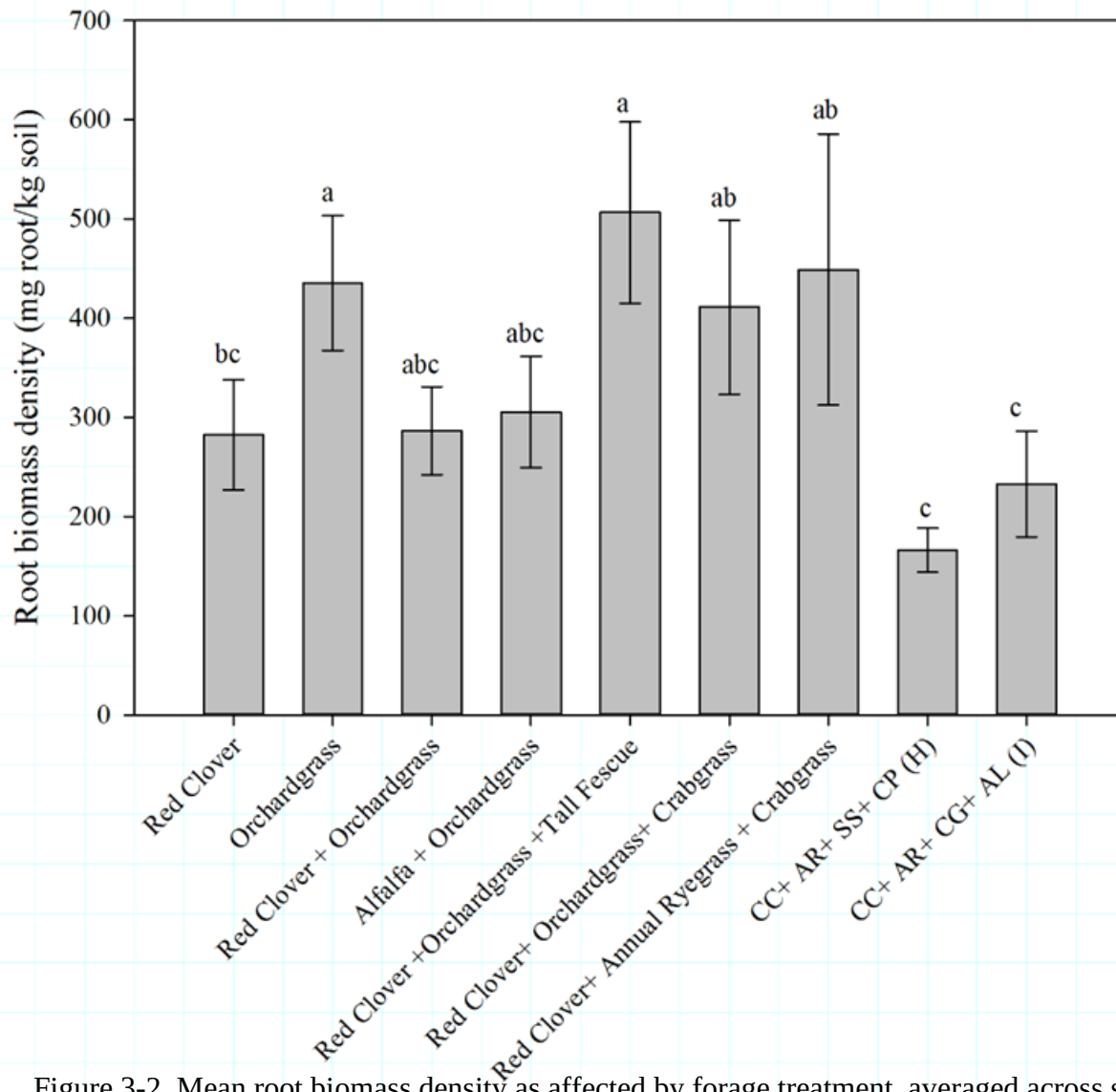


Figure 3-2. Mean root biomass density as affected by forage treatment, averaged across sampling season and sampling depth. CC is crimson clover, AR is annual ryegrass, SS is sorghum sudangrass, CP is cowpea, CG is crabgrass, and AL is annual lespedeza. Error bars represent the standard error of the mean. Bars with the same letter are not significantly different at $\alpha=0.05$.

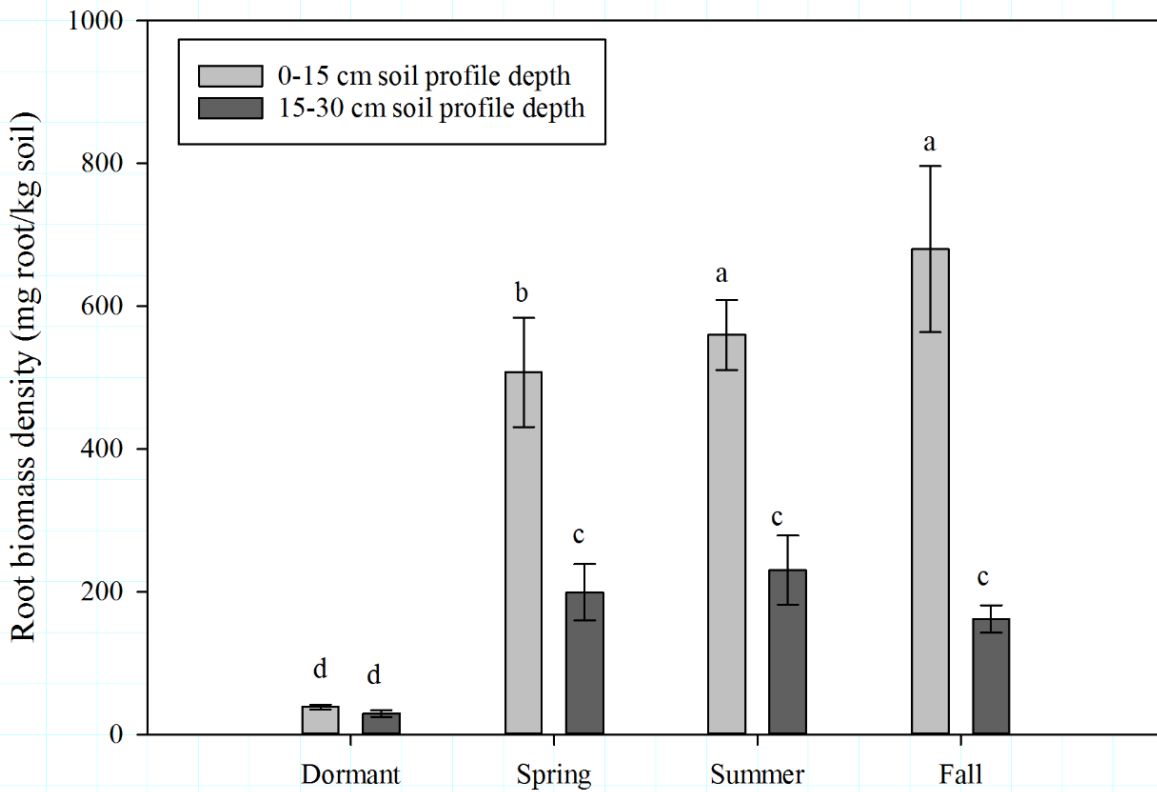


Figure 3-3: Mean root biomass density as affected by sampling season and sampling depth, averaged across forage treatment. Error bars represent the standard error of the mean. Bars with the same letter are not significantly different at $\alpha=0.05$.

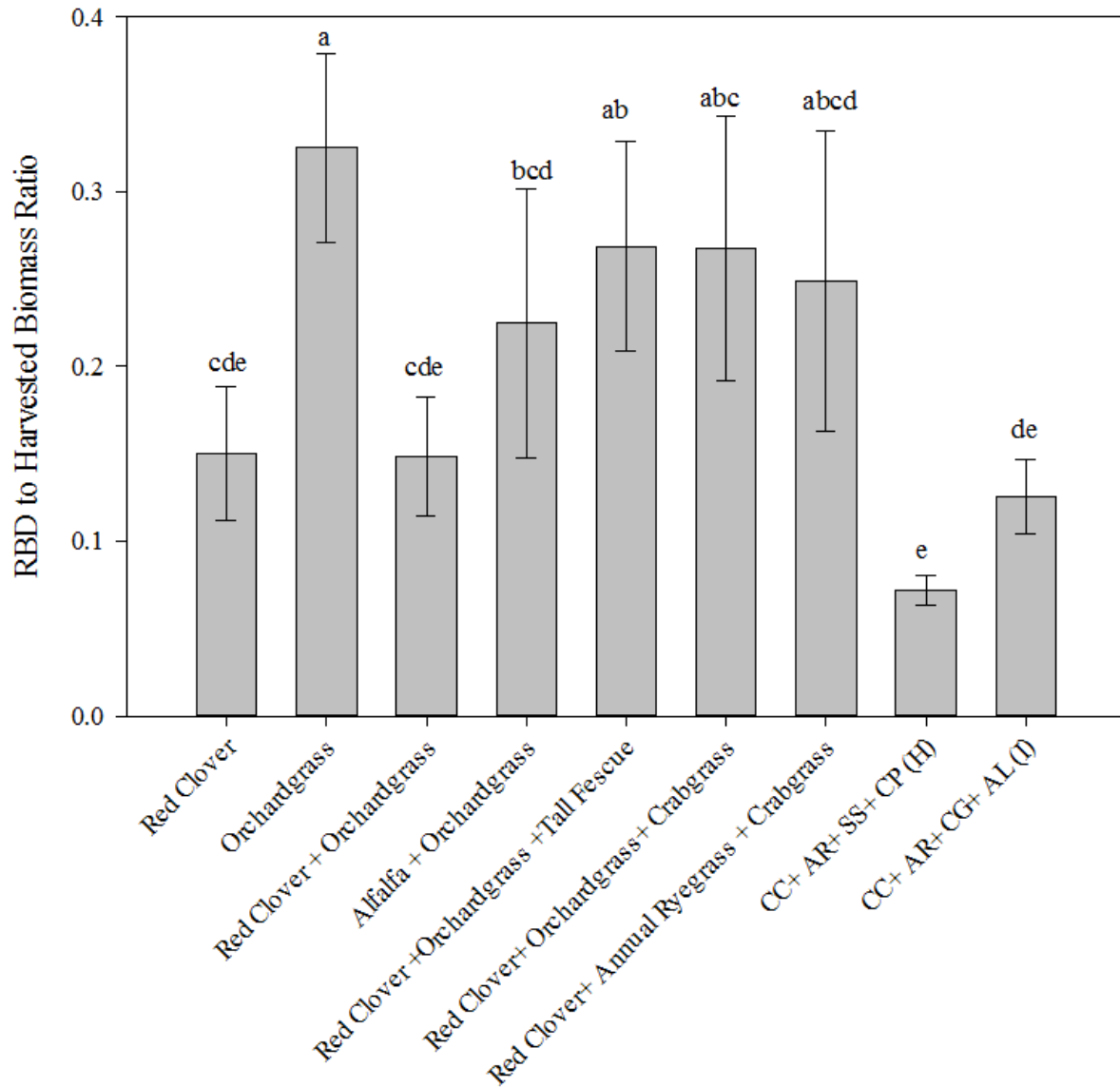


Figure 3-4: Mean ratio of RBD to harvested biomass as affected by forage treatment. Error bars represent the standard error of the mean. Bars with same letters are not significantly different at $\alpha = 0.05$. For species in treatments marked H and I, refer to Table 1. CC is crimson clover, AR is annual ryegrass, SS is sorghum sudangrass, CP is cowpea, CG is crabgrass, and AL is annual lespedeza.

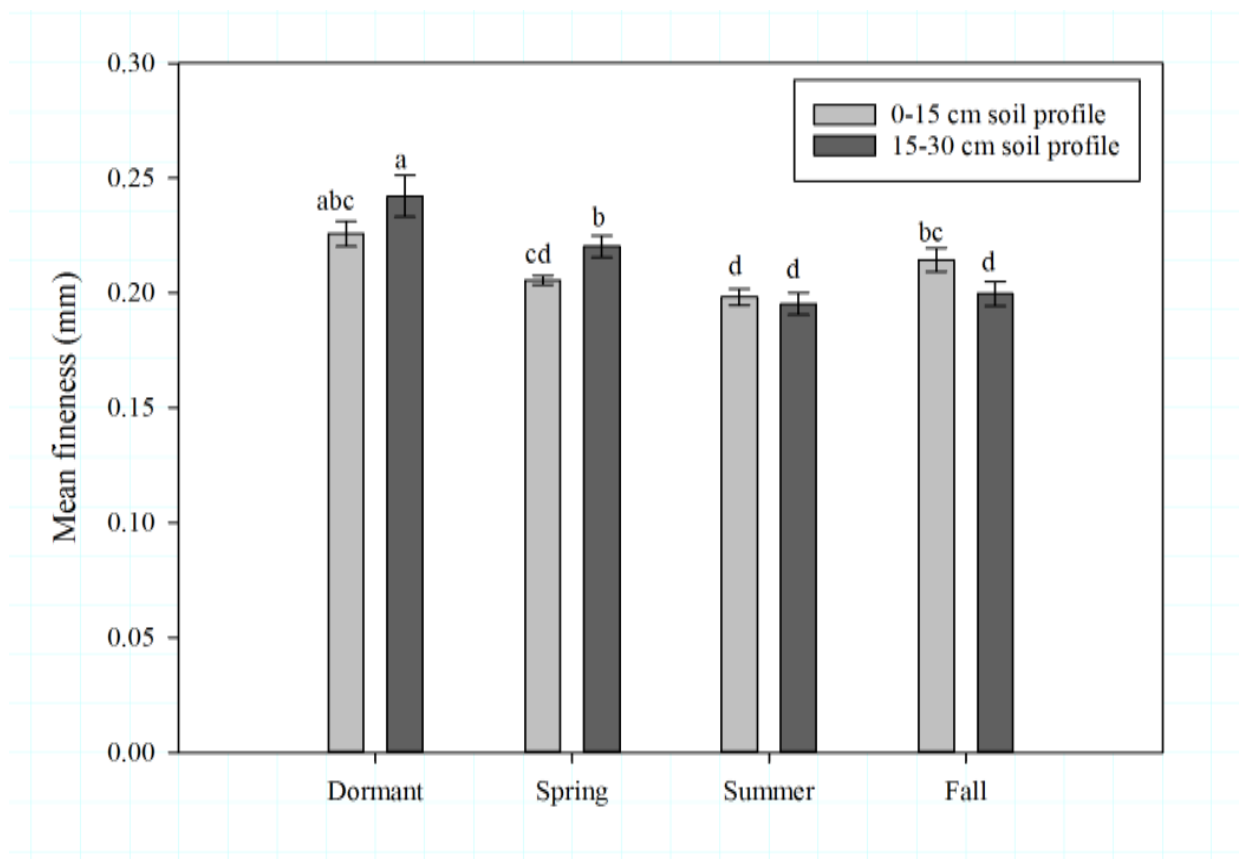


Figure 3-5: Mean root fineness as affected by sampling depth and sampling season, averaged across forage treatment. Error bars represent the standard error of the mean. Bars with same letters are not significantly different at $\alpha=0.05$.

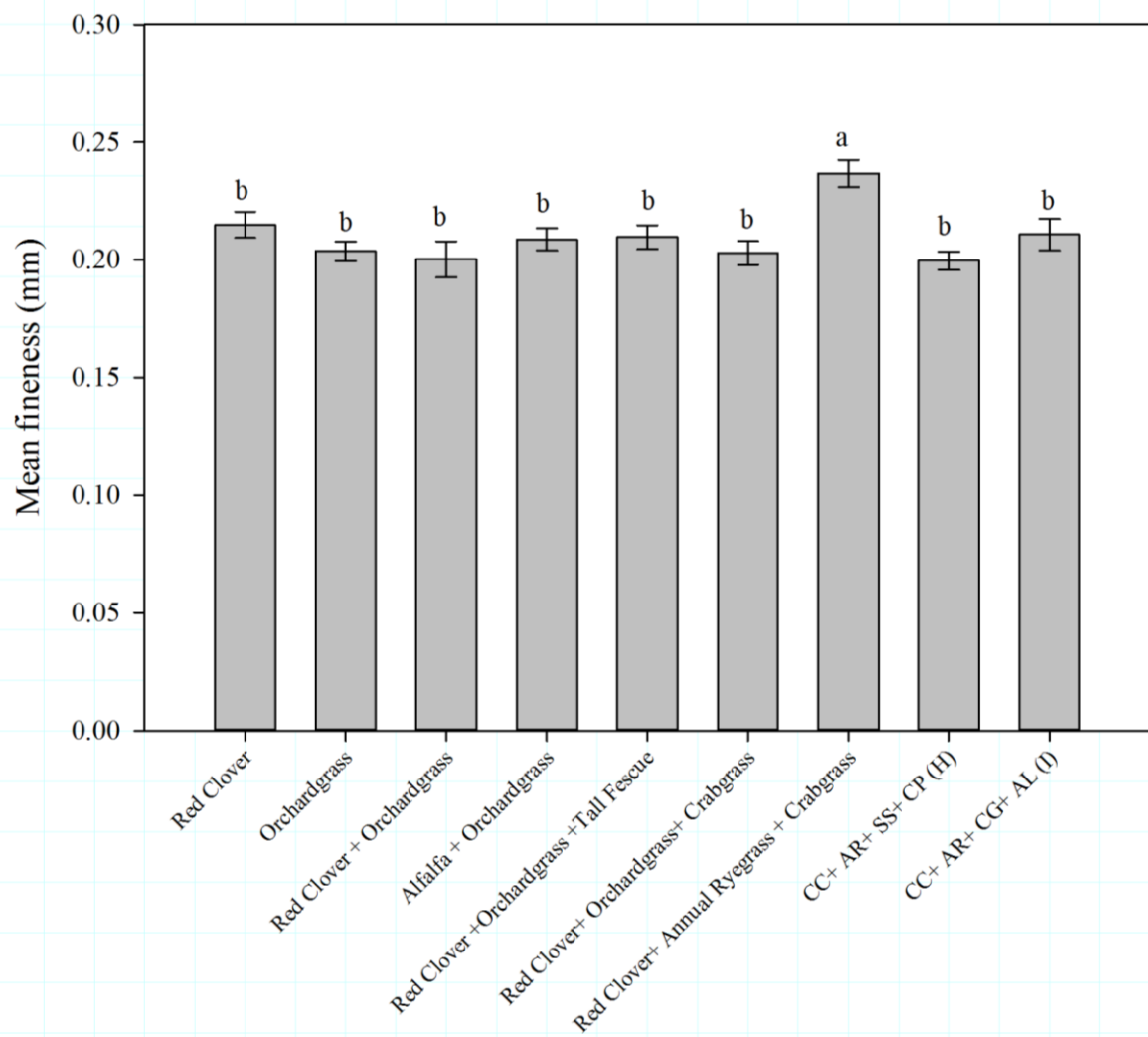


Figure 3-6: Mean root fineness as affected by forage treatment, averaged across sampling season and sampling depth. Error bars represent the standard error of the mean. For species in treatments marked H and I, refer to Table 1. Bars with the same letter are not significantly different at $\alpha=0.05$.

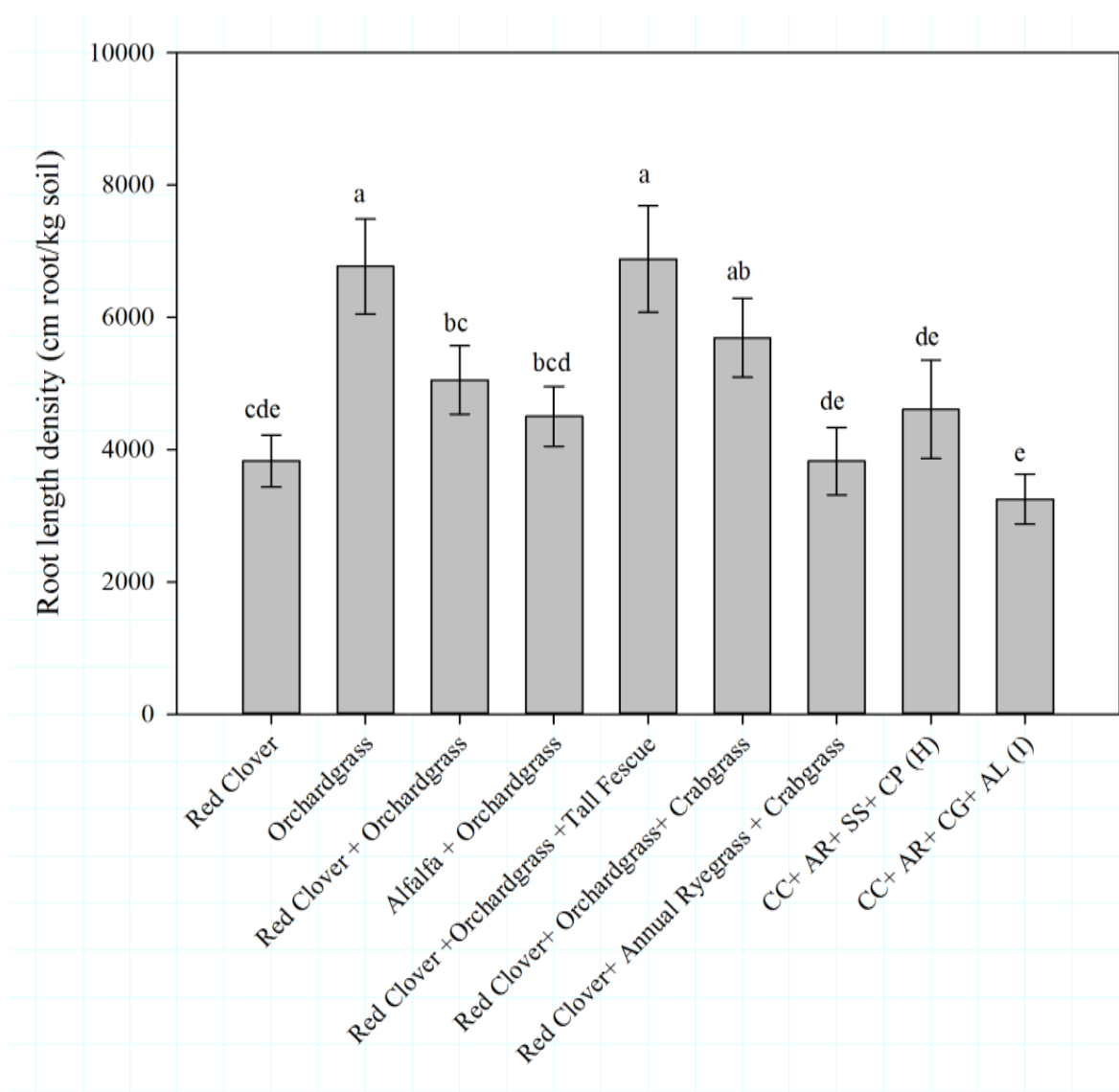


Figure 3-7: Mean root length density as affected by forage treatment, averaged across sampling season and sampling depth. Error bars represent the standard error of the mean. CC is crimson clover, AR is annual ryegrass, SS is sorghum sudangrass, CP is cowpea, CG is crabgrass, and AL is annual lespedeza. Bars with the same letter are not significantly different at $\alpha=0.05$.

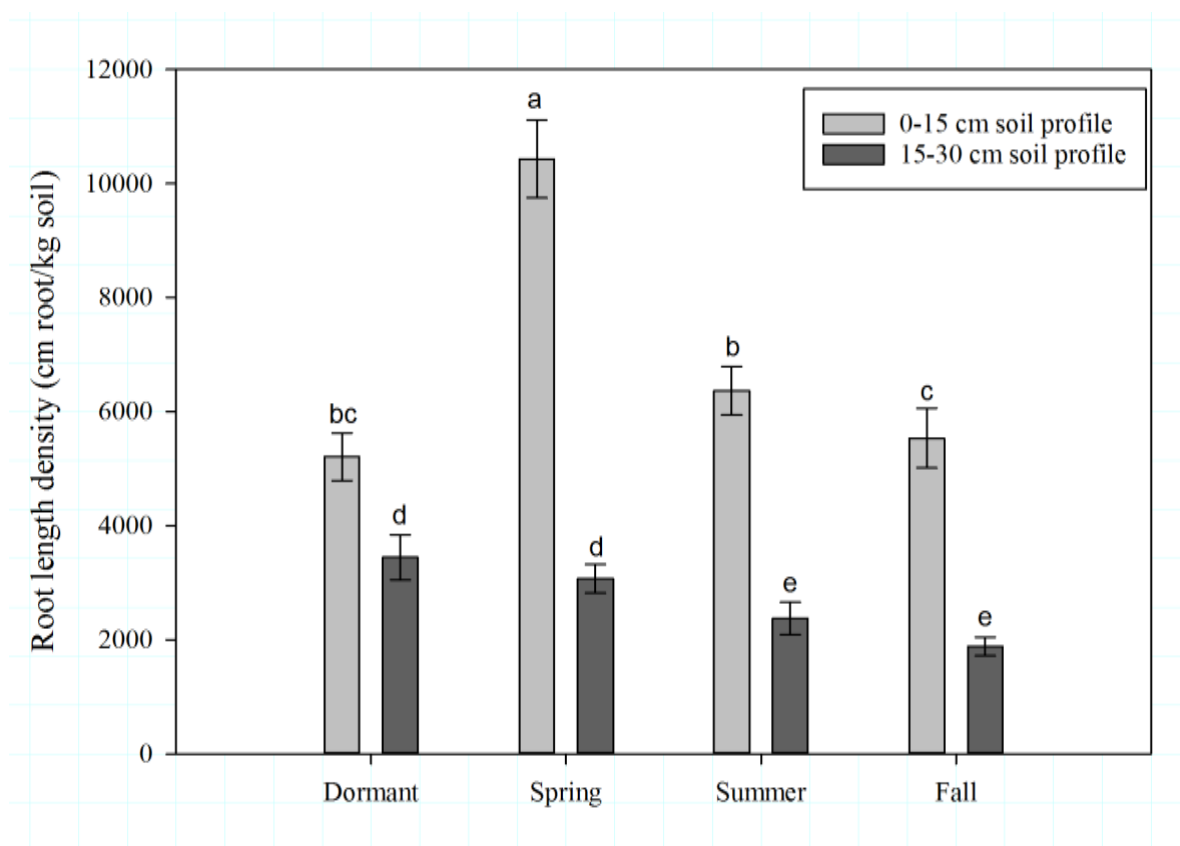


Figure 3-8: Mean root length density as affected by sampling season and sampling depth, averaged across forage treatment. Error bars represent the standard error of the mean. Bars with the same letter are not significantly different at $\alpha=0.05$.

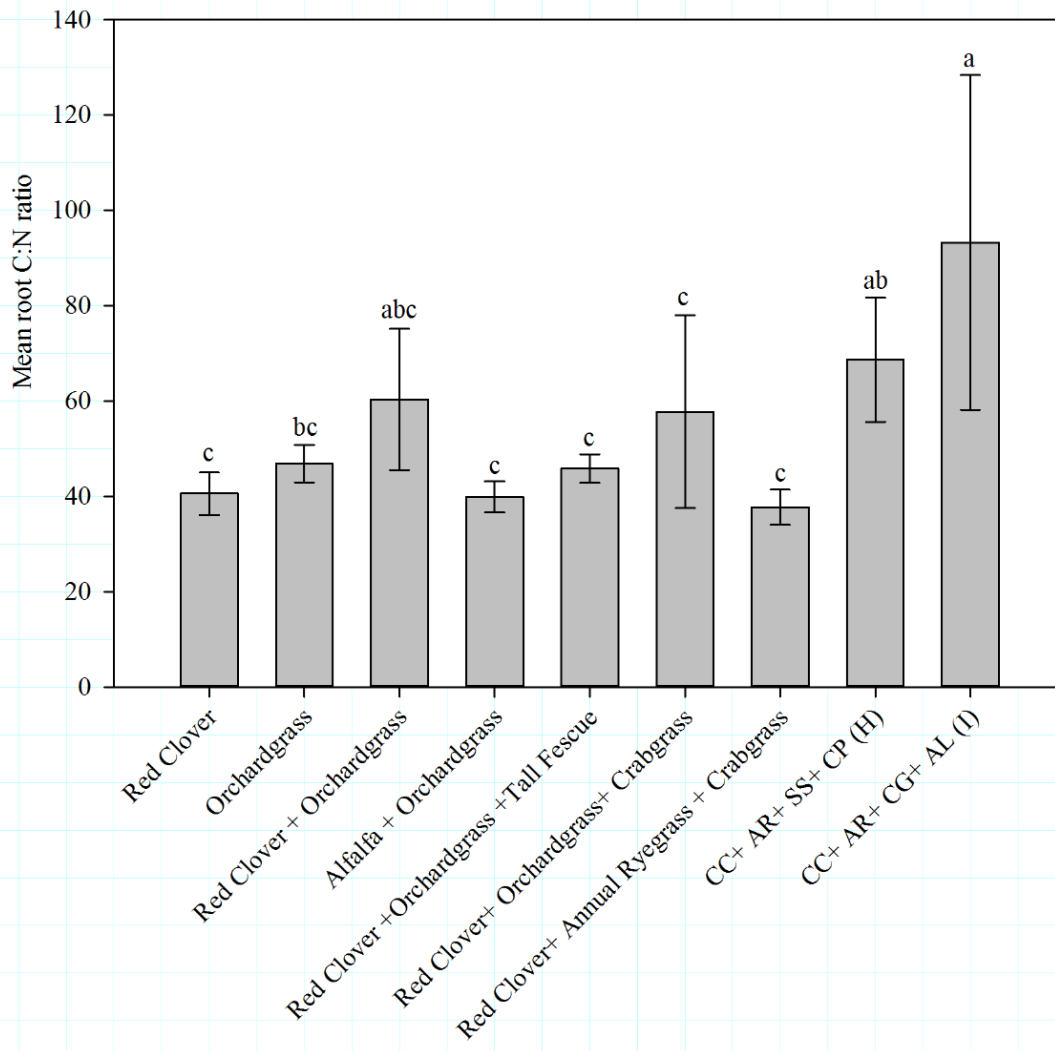


Figure 3-9: Mean root carbon to nitrogen (C:N) ratio as affected by forage treatment, averaged across sampling season and sampling depth. CC is crimson clover, AR is annual ryegrass, SS is sorghum sudangrass, CP is cowpea, CG is crabgrass, and AL is annual lespedeza. Error bars represent the raw standard error of the mean. Bars with the same letter are not significantly different at $\alpha = 0.05$.

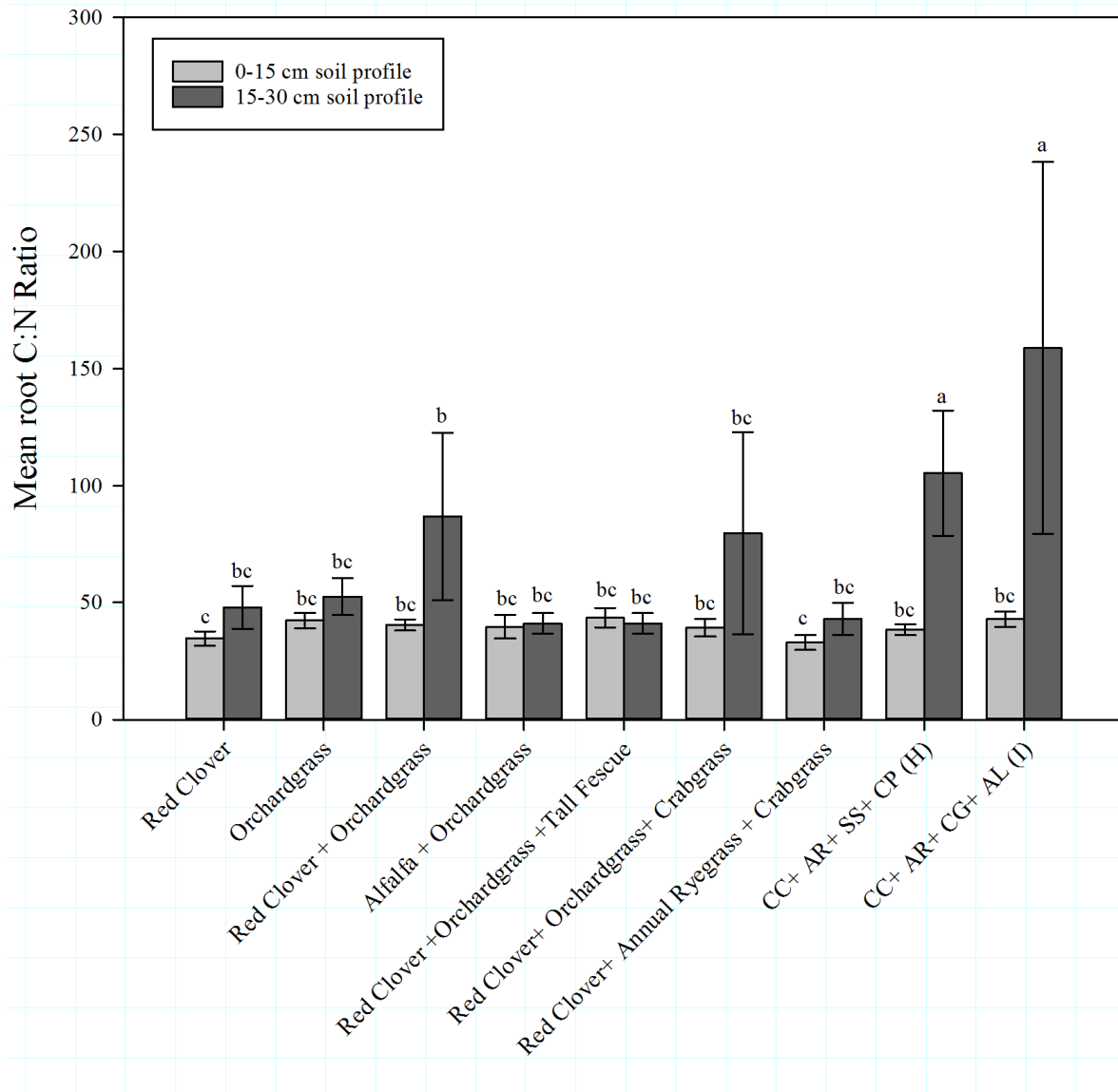


Figure 3-10: Mean root carbon to nitrogen (C:N) ratio as affected by forage treatment and sampling depth, averaged across sampling season. CC is crimson clover, AR is annual ryegrass, SS is sorghum sudangrass, CP is cowpea, CG is crabgrass, and AL is annual lespedeza. Error bars represent the raw standard error of the mean. Bars with the same letter are not significantly different at $\alpha=0.05$.

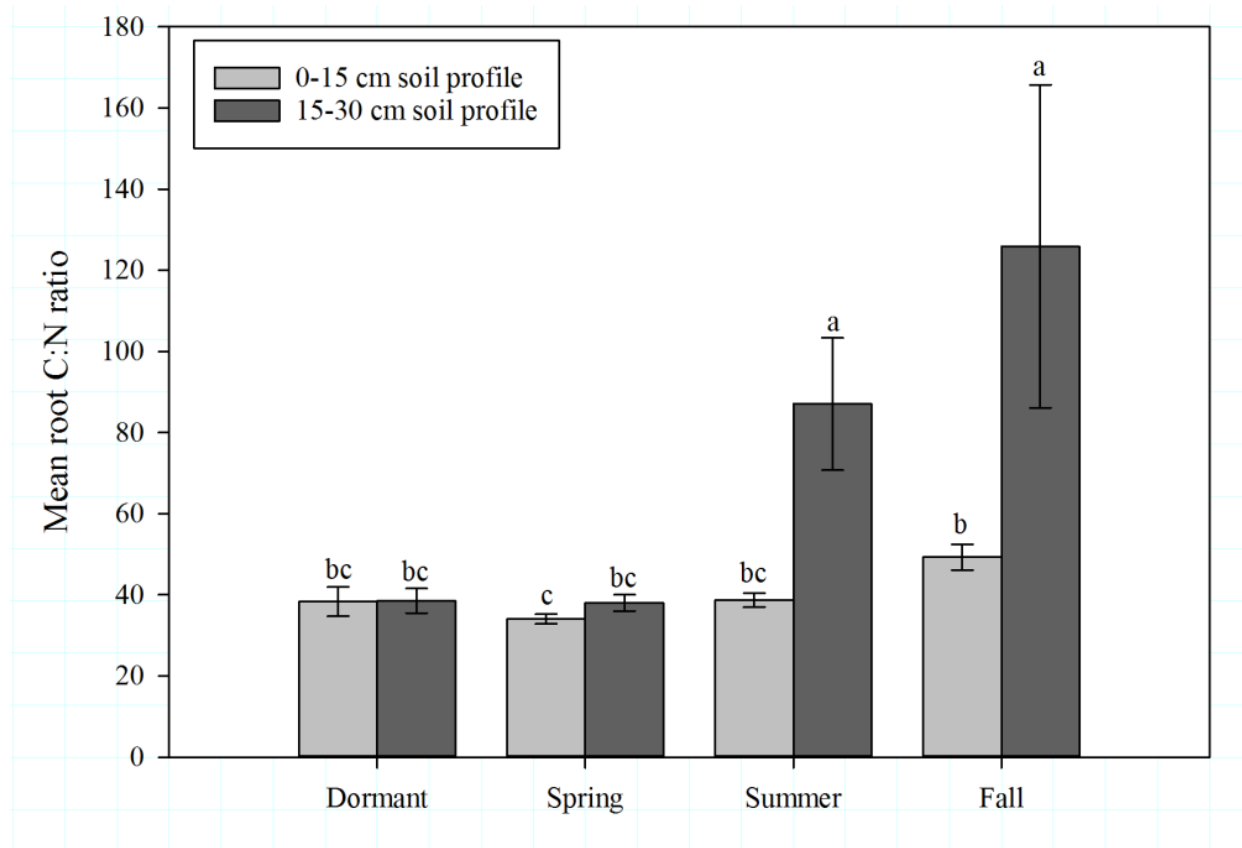


Figure 3-11: Mean root carbon to nitrogen (C:N) ratio as affected by sampling season and sampling depth, averaged across forage treatments. Error bars represent the raw standard error of the mean. Bars with the same letter are not significantly different at $\alpha=0.05$.

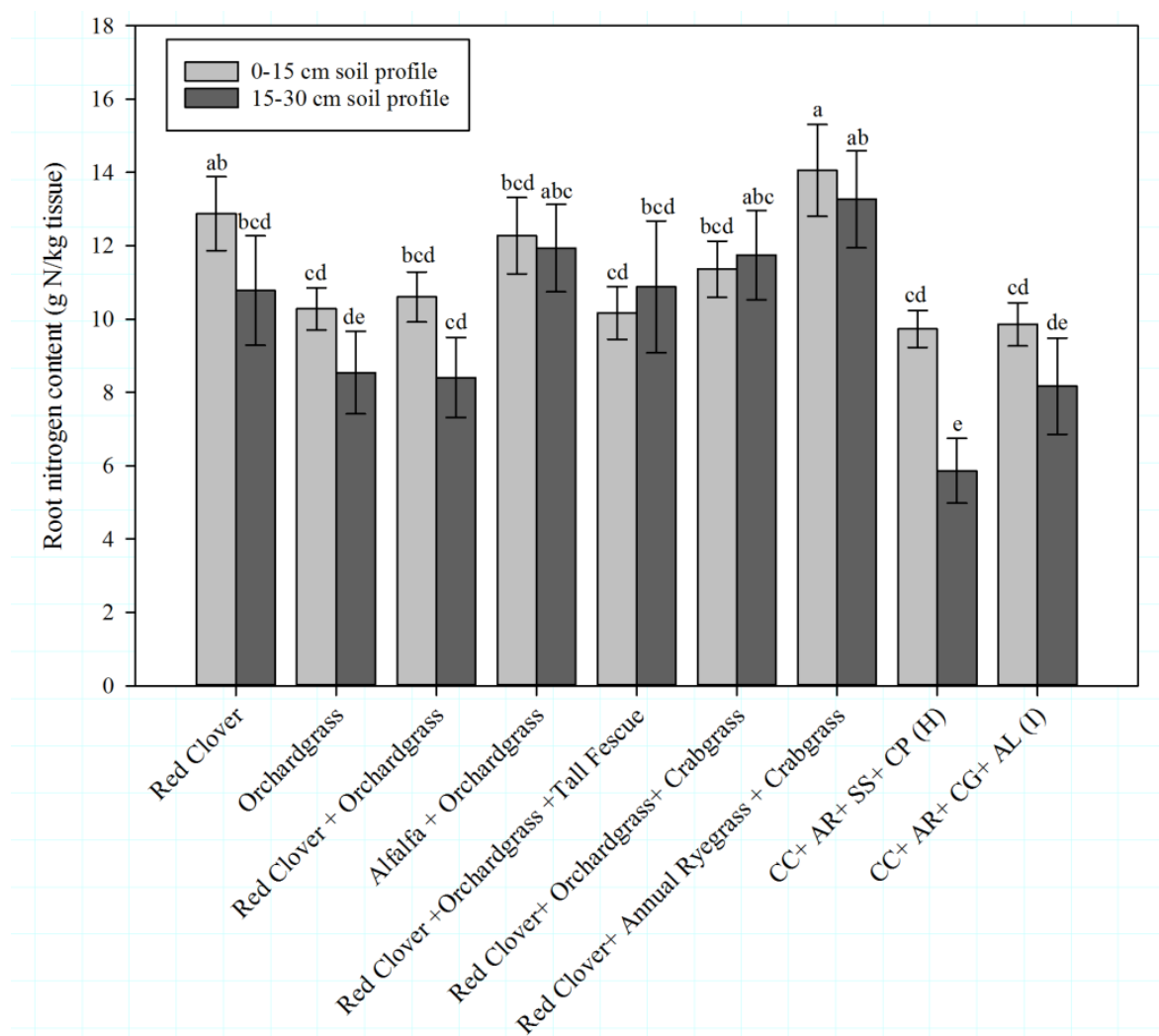


Figure 3-12: Mean root tissue nitrogen (N) concentration as affected by forage treatment and sampling depth, averaged across sampling season. CC is crimson clover, AR is annual ryegrass, SS is sorghum sudangrass, CP is cowpea, CG is crabgrass, and AL is annual lespedeza. Error bars represent the raw standard error of the mean. Bars with the same letter are not significantly different at $\alpha=0.05$.

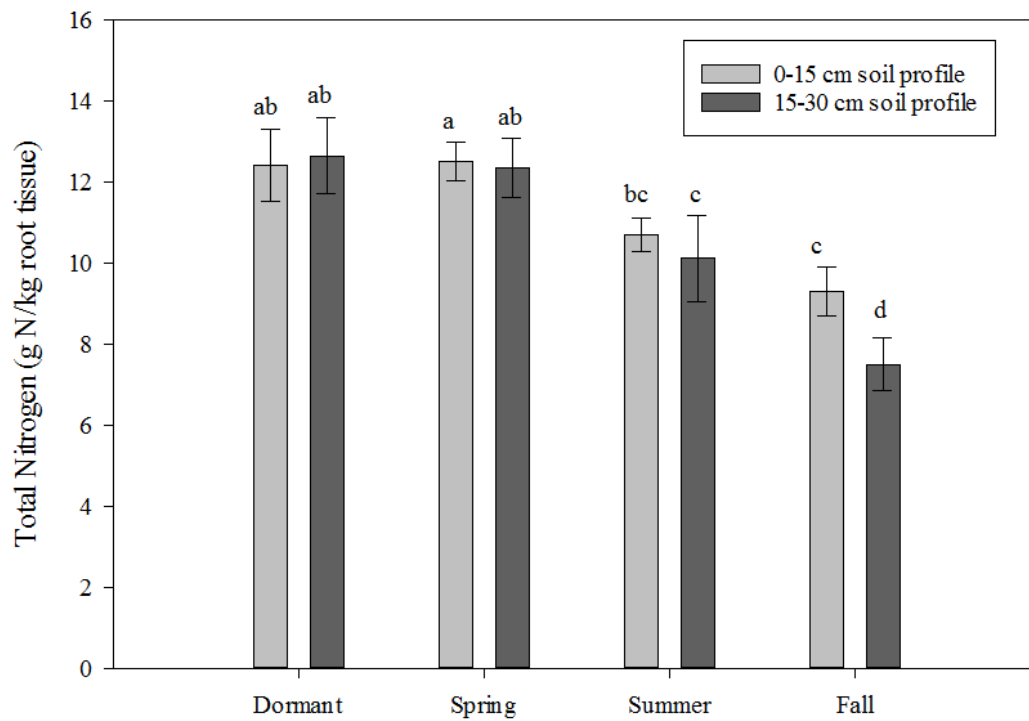


Figure 3-13: Mean root tissue N concentration as affected by sampling season and sampling depth, averaged across forage treatments. Error bars represent the raw standard error of the mean. Bars with the same letter are not significantly different at $\alpha=0.05$.

CHAPTER FOUR

SOIL NITROGEN AND FORAGE ROOT FUNCTIONAL GROUP INTERACTION

INFLUENCES ROOT CARBON AND NITROGEN MINERALIZATION

Abstract

Roots are the main plant contributor to soil carbon (C) and in forage systems, root decomposition dynamics influence the soil C cycle, which has implications for C sequestration, soil quality attributes, and crop nutrient availability. Nitrogen (N) addition increases the rate of C and N mineralization for C-rich residues such as root tissue. Using roots from three functional groups of forage plants including cool-season legume (red clover, RC; *Trifolium pratense* cv. Freedom!), cool-season grass (orchardgrass, OG; *Dactylis glomerata* cv. Persist), and warm-season grass (large crabgrass, CG, *Digitaria sanguinalis* cv. Red River), rates of CO₂ evolution, N mineralization, and water-extractable C and N from roots alone or in each possible combination were measured during incubation with field soil in mesocosms over a 91 day period. Forage root mixture treatments were evaluated under both low soil N and high soil N conditions. We expected that due to higher N content, both legume roots and soil with added N would increase rates of C mineralization from added root biomass. Red clover roots alone with soil did not have higher C mineralization rate than soil alone; however, red clover and grass roots together had higher C and N mineralization than red clover roots alone. Addition of inorganic N increased the initial C mineralization rate, but over the 91-d incubation root biochemistry was a better indicator of C mineralization. The mixture of red clover and orchardgrass roots, similar to the mixture of red clover and both kinds of grass roots, had the most cold water extractable C (CWECC), signifying that higher rates of labile C in the soil and root mixture.

Introduction

The decomposition of root tissue is an important process given its contributions to the global carbon (C) cycle (Lal, 2004). Plants sequester C from the atmosphere through

photosynthesis and store in above and belowground tissues, both in structural and non-structural chemical compounds (Taiz & Zeiger, 2010). In forage systems, much of plant C is harvested through grazing or mechanical harvest, and remaining plant tissues (stubble, senesced leaves, roots, and extra-root compounds) are subject to microbial decomposition leading to production of less labile soil C compounds (Berg & McClaugherty, 2003; Stewart et al., 2015) and loss of fixed C by microbial respiration as carbon dioxide under aerobic decomposition, or methane under anaerobic decomposition (Pausch & Kuzyakov, 2018). Root components have been shown to have longer residence time in the soil because of chemical components such as polyphenols in legumes (Oglesby & Fownes, 1992), and lignin which increases with herbaceous plant maturity and in woody plants (Berg & McClaugherty, 2003).

A study on twenty species of agronomic crops in Brazil by Redin et al. (2014) showed that there were significant root chemical composition differences between species. They found that roots of forbs, including legume roots, had generally higher cellulose, lignin, and soluble fractions and also higher total N than roots of grasses (Redin et al., 2014). In contrast, grass roots were found to generally have a higher hemicellulose content. Redin et al. (2014) also analyzed C mineralization of roots of all 20 species and found that more C was mineralized from legume roots than grass roots. They suggested that it may have been due to higher N in the legume roots or because the legume roots had C compounds that were more soluble (Redin et al., 2014).

In the case of switchgrass (*Panicum virgatum*), a warm-season perennial grass, White et al. (2018) reported that decomposition of cell components of ground root tissue such as proteins and sugars primarily occurred in the first 30 d, whereas cell wall components of cellulose and lignin did not significantly decompose during a 270 d incubation. However, authors such as Stewart et al. (2015) have found that residues that are higher in lignin are decomposed more

completely due to less efficient use by microbes, leading to less SOM formation with high-lignin residues. When N-poor, C-rich plant residues are added to soil, the effect is a gross immobilization of N, in which inorganic N is quickly recovered and metabolized into organic forms by soil microbes. This reduced availability of inorganic N hinders the amount of microbial activity that can then occur (Recous et al., 1995). Hot-water extractable carbon (HWE) is one method to measure the labile pool of soil C. This pool is reported to contain 40-50% carbohydrates and HWE includes microbial soil biomass (Ghani et al., 2003). Cold-water extractable carbon (CWE) represents a fraction of soil C that is readily available for microbial uptake, such as labile C from plant residue or manures of grazing animals (Ghani et al., 2003). CWE is made up of simple carbohydrates and amines, while HWE contains microbial biomass and more complex carbohydrates. Cold-water extractable N is composed of inorganic N and highly labile forms of organic N which are readily-available for microbial assimilation. Hot-water extractable N is also available for microbial assimilation and can be broken down with microbial enzymes such as ureases, but it is a more stable pool (Norris et al., 2019; Ozlu et al., 2019).

Our objective was to evaluate decomposition of roots from forage species belonging to three functional groups (cool-season legume, cool-season grass, warm-season grass) alone and in mixture at two rates of soil inorganic N by evaluating CO₂ evolution and N mineralization from field soil mixed with roots in an aerobic laboratory incubation. Our hypotheses were that 1) red clover (cool-season legume) roots alone would show higher C and N mineralization and increase C and N mineralization when they were added to mixtures compared to orchardgrass (cool-season grass) and large crabgrass (warm-season grass) roots, and 2) that higher soil inorganic N would increase root decomposition, thereby increasing C mineralization and water-extractable C.

Materials and Methods

Field soil was collected from the Organic Crops Research Unit near Knoxville, TN, USA, a research facility of the University of Tennessee, which has been under organic management as defined by the USDA for 10 years. Specifically, the soil was gathered from a Dewey silt loam (fine, kaolinitic, thermic Typic Paleudult), a field site under forage management where multiple forage species were established as part of a forage mixture experiment beginning in 2015 (Leu et al., 2019). Soil was collected from across the site and composited to provide a representative sample. Bulk density was determined with an adapted core method to be 1.6 g cc^{-1} (Blake & Hartge, 1986). Soil was sieved ($<2 \text{ mm}$) to remove large plant residue and break up large soil aggregates. Sieved soil was pre-incubated for 7 days at room temperature (21°C) and at the moisture content at which it had been taken from the field. Then, moisture content of the soil was determined gravimetrically on three, 10-g samples (65°C for 48 hours).

Plant root tissue was obtained from the same field site from established plots. Roots were collected from forage species representing three functional groups: legume (RC), cool-season grass (OG), and warm-season grass (CG). Areas of conspecific plant matter for either RC, OG, or CG were targeted for digging and whole plants removed to collect root biomass. During one of the three trials of the experiment, crabgrass roots were not available in the field due to seasonality, so additional crabgrass was grown in a greenhouse environment using a sand and peat-based media. Greenhouse-grown crabgrass roots were used for the third trial of the experiment and field-grown crabgrass roots were used in trials 1 and 2. Soil containing roots was brought to the lab and soil soaked in 5% sodium hexametaphosphate, a dispersal agent, for 24 hours (modified from Bolinder et al., 2002). Roots were then washed from soil with tap water using a $500\mu\text{m}$ sieve and rinsed into a dish. Roots in the dish were then suspended in water and

separated from remaining sand and debris by hand. Cleaned roots were dried in a forced-air oven at 50°C until dry. Dried roots were held in airtight plastic bags at -18°C until use.

Soil C content had previously been determined at 10 g C kg⁻¹ soil, and the soil N content had previously been determined as 1.0 g N kg⁻¹ soil (by combustion, Flash EA1112, CE Elantech, Lakewood, NJ), a soil C:N ratio of 10:1. The preincubated native soil was used as a “low N” treatment, and 7 g of lab-grade KNO₃ was added to 1.1 kg of the preincubated soil to create a C:N ratio of ~5:1, to limit N deficiency for microbial growth (Redin et al., 2014).

Roots of each species were evaluated singly and in combination (i.e., RC, OG, CG, RC+OG, RC+CG, OG+CG, RC+OG+CG). Each root type by N treatment was established in a factorial design in a mesocosm consisting of a glass jar (approx. 1 L volume) containing three 20-mL vials each with 15 g of soil and a vial containing 3 mL of 2M NaOH as a CO₂ trap. This was a randomized complete block design and each treatment was replicated three times in each trial. Within trial, replicates for each treatment were treated as a block and arranged in close spatial proximity in the incubation chamber. There were three trials. Each trial also used blank mesocosms, which were empty except for a NaOH trap, to measure background CO₂ of the mesocosm at setup. At the initiation of each trial, vials were filled with 15 g of either high N or low N soil and roots were added at a rate of 3.2-g root tissue per kg of soil (Abiven et. al, 2005). This was equivalent to 0.05 g of roots to each vial: in multi-species vials, the total amount of roots in each vial was 0.05 g, with each species an equal proportion of the total weight. Roots were cut into pieces (<1-10 mm) with scissors to facilitate mixing in soil and placement in the vial. Roots were stirred into the soil of each vial such that roots were covered by and made contact with the soil. The NaOH, as with all reagents used during this experiment, was prepared using deionized water made CO₂-free by boiling. Parts of this protocol, including soil

preincubation, KNO₃ addition, and rate of plant tissue addition, were adapted from the protocol used by Abiven et al. (2005) to determine leaf and root decomposition of crop species including rice (*Oryza sativa*), soybean (*Glycine max*), sorghum (*Sorghum spp.*), brachiaria (*Brachiaria ruziziesens*), and wheat (*Triticum aestivum*). More CO₂-free water was added to the bottom of each mesocosm to maintain humidity and to facilitate sealing of the jar lid. Jars were tightened by hand to create an airtight seal and placed in a dark incubation chamber at 30°C.

Mesocosm treatments were removed from incubation for sampling at 21, 49, and 91 days after initiation of the trials. At the 21-day and 49-day time points, the NaOH trap was removed and precipitated using 3 mL of CO₂-free 1.5-M BaCl₂ to precipitate carbonates, and one vial of soil was removed from each mesocosm for further processing. Then a new (CO₂-free) NaOH trap was added, and the jar resealed by hand and returned to incubation. The 91-day timepoint was similar except no new NaOH trap was added and the trial terminated. The NaOH traps were titrated by a color end-point using 30-μL 1% phenolphthalein indicator with standardized 0.5-N HCl (Zibilske 1994). Mineralized C was calculated based on the titration of the NaOH trap and the weight of soil present in the mesocosm during the incubation period. The CO₂ mineralized was statistically represented as both a rate, the amount of CO₂ released per gram of soil, per day during the preceding interval of the titration date, and as the cumulative CO₂ released per mass of soil in that jar.

The removed vials of soil were destructively sampled for mineralized N by using 5 g of soil from each vial extracted with 1-M KCl for 30 minutes on a rotational shaker, then centrifuged (3500 rpm) and filtered (Whatman 42). Colorimetric analysis for soil inorganic N (NH₄-N and NO₃-N) was completed with a microplate spectrophotometer (Powerwave XS, Biotek, Winooski, VT, USA) as described by Sims (Sims et. al, 1995). To obtain water-extracted

C and N, subsamples of 4 g of soil from each vial with 40 mL of deionized (DI) water were shaken for one hour at 23°C. Samples were then centrifuged at 3500 rpm, filtered (Whatman 42), pH-adjusted with 0.75 mL of 2-M HCl, and stored at 4°C until analysis. Hot-water extractable C and N was determined by adding 40 mL DI water to the remaining sediments and incubating in a water bath for 16 h prior to being centrifuged, filtered, and pH-adjusted as described previously. The procedure for extracting C and N with water was adapted from Ghani et al. (2003), who used it to measure soil C and N effects of grazing intensity and drought in a pasture system (Ghani et al., 2003). Total organic C and N in the water extracts was measured using a total organic C analyzer (TOC-VCPH model, Shimadzu, Kyoto, Japan). Simple carbohydrates and other C compounds as represented by water extractable C represent catabolized C likely to be incorporated or respired by microbial biomass. Hot water extractable C (HWE C) includes C soil microbial biomass as well as simple soil C such as carbohydrates and amines (Ghani et al., 2003). Therefore, C will not have a long residence time in this pool (Rasse et. al, 2005). Cold-water soluble N (CWEN) represents a highly labile fraction of N containing inorganic N and substances such as uric acid that is immediately available for microbial assimilation, while hot-water soluble N represents N that is more stable, but still available for microbial assimilation (Cabrera et al., 2005; Norris et al., 2019). Water-soluble N is reported to predict potentially mineralizable N better than C:N ratio for crop residue (Cabrera et al., 2005). Subsamples of the red clover, orchardgrass, greenhouse-grown crabgrass, and field-grown crabgrass roots used in the study were analyzed for lignin, starch, water soluble carbohydrate, and ethanol soluble carbohydrate at the University of Georgia Feed and Forage Lab. Starch was determined following the method described by Holm and Karkalas (Holm et al., 1986; Karkalas, 1985). Water-soluble and ethanol-soluble carbohydrates were extracted according to Smith (1971) and

then carbohydrate was colorimetrically determined as described by Dubois (Dubois et al., 1956; Smith, 1971). The water-soluble carbohydrates fraction represents simple sugars plus fructans, while the ethanol-soluble carbohydrates includes negligible fructans. Lignin was determined as the amount of acid detergent fiber (ADF) that was not cellulose (not able to be dissolved in 24 N H₂SO₄) or ash: methods for extracting ADF were based on (Van Soest, 1963). Analysis of ADF to determine amounts of lignin present were carried out on an Ankom^{200/220} Fiber Analyzer (ANKOM Technology, NY) using F57 filter bags (ANKOM Technology, NY, using a protocol available from the Ankom Technology business (Ankom Technology, 2005). Additional root subsamples were analyzed for total N and total C by combustion using a soil analyzer (Flash EA 1112 NC Soil Analyzer, Thermo Fisher Scientific Inc., Waltham, MA). Final concentration of C and N constituents in root samples were calculated based on exact weight of analyzed root tissue.

Data of C mineralization, N mineralization, and water-extractable C and N were analyzed in SAS 9.4 (Cary, NC). We used mixed-models analysis of variance using PROC glimmix. Fixed effects included root species treatment, soil N rate, and time of measurement. Random effects included trial and block. Square root transformation was used where appropriate to ensure data conformed to model assumptions of normality and homogeneity of variance. Main effects and interactions were considered significant at $\alpha = 0.1$.

Results and Discussion

Root Biochemical Composition

Red clover roots had the highest N content (17.1 g kg⁻¹ tissue), but the roots of field-grown crabgrass also had relatively high N content (14.3 g kg⁻¹ tissue, Table 4-1), compared to orchardgrass and greenhouse-grown crabgrass (8.8 and 6.1 g kg⁻¹ tissue, respectively). Red

clover root chemistry was also different than grass root samples in terms of lignin composition (103 g kg⁻¹ tissue vs. 53 to 82 g kg⁻¹ tissue for the grasses, Table 4-1), and starch (44 g kg⁻¹ tissue vs. 4 to 20 g kg⁻¹ tissue for grasses). Starch is a relatively simple C compound which would be expected to degrade more quickly than lignin (Ibrahima et al., 1995). Water- and ethanol-soluble carbohydrates are two fractions of non-structural carbohydrates; that is, they are from inside the plant cell rather than the cell wall (Redin et al., 2014; White et al., 2018). Due to the similarities between the procedures for water-extractable carbohydrates from tissue and cold water-extractable carbon from soil, it is likely that the water-soluble carbohydrates from tissue are also simple carbohydrates and amines, which are readily available for incorporation into microbial biomass or for C mineralization through microbial respiration (Ghani et al., 2003; Smith, 1971). Field-grown crabgrass roots had the highest constituent of water-soluble carbohydrates (41 g kg⁻¹ tissue), which would be easily-degradable by microbes (Table 4-1). Red clover and orchardgrass roots both had only 8 g kg⁻¹ tissue, and therefore would have had less degradable tissues (Table 4-1).

When roots of different species were combined, the result was higher amounts of N than the case of grass roots alone. For example, the red clover roots alone treatment contained 0.85 mg N in root tissue in each vial and the orchardgrass roots alone treatment contained 0.44 mg N in root tissue in each vial (Table 4-2). However, the treatment with red clover and orchardgrass combined contained 0.65 mg N in each vial, thereby decreasing the C:N ratio to 30 from 42 for the orchardgrass alone treatment (Table 4-2). The combination of red clover and crabgrass had a slightly lower lignin:N ratio than red clover alone (5.8 vs 6.0, Table 4-2). Lignin:N ratio has been shown to be negatively correlated with N mineralization (Vahdat et al., 2011).

Cumulative C Mineralization

There were no significant differences in cumulative C mineralization ($\text{mg CO}_2 \text{ g}^{-1} \text{ soil}$) between root species treatments at 0-21 or 0-49 days after initiation of incubation, but there were significant differences between treatments at 0-91 days after incubation ($P=0.077$, Table 4-3), which was similar to that observed with CO_2 mineralization rates. At 0-49 days, trends in the data resembled the treatment differences at 0-91 days, but they were not statistically significant (Fig. 4-1). The treatment with soil with no roots had the lowest cumulative C mineralization at 91 days, with $4.2 \text{ mg CO}_2 \text{ g}^{-1} \text{ soil}$, and this treatment was statistically similar to red clover alone ($4.6 \text{ mg CO}_2 \text{ g}^{-1} \text{ soil}$) and orchardgrass alone treatments ($4.8 \text{ mg CO}_2 \text{ g}^{-1} \text{ soil}$, Fig. 4-1). Red clover root tissue may have had a lower decomposability due to higher lignin concentration (Table 4-1), or other quality factors such as the amount of polyphenols in legume roots (Oglesby & Fownes, 1992), whereas orchardgrass alone decomposition may have been slowed due to a high C:N ratio (Table 4-2).

The treatment of red clover and orchardgrass roots combined had the highest C mineralization at 91 days ($5.4 \text{ mg CO}_2 \text{ g}^{-1} \text{ soil}$) which was similar to the other mixtures of red clover and grasses (RC+CG, $5.4 \text{ mg CO}_2 \text{ g}^{-1} \text{ soil}$; RC+OG+CG, $5.1 \text{ mg CO}_2 \text{ g}^{-1} \text{ soil}$), the grass mixture (CG+OG, $5.2 \text{ mg CO}_2 \text{ g}^{-1} \text{ soil}$), and the orchardgrass alone and crabgrass alone treatments (4.8 and $5.2 \text{ mg CO}_2 \text{ g}^{-1} \text{ soil}$, respectively, Fig. 4-1). Although we hypothesized that red clover would have a higher C mineralization rate due to its higher N content and lower C:N ratio (Table 4-1), this was not supported by the data. The lignin in red clover roots may have hindered its decomposition when mixed alone with soil, but when combined with grass roots, the N from the red clover roots may have accelerated the decomposition of the non-lignified components of grass roots, given that the treatments combining red clover with orchardgrass and

red clover with crabgrass were in the highest mean separation group and not similar to other means (Fig. 4-1).

Rate of Carbon Mineralization

The rate of carbon mineralization ($\text{mg CO}_2 \text{ g}^{-1} \text{ soil day}^{-1}$) was significantly affected by forage root treatment ($P = 0.09$). There was also a significant main effect of incubation length and a significant interaction effect between incubation length and N rate. The mean C mineralization rate from 0-21 days at the high N rate was the highest at $0.11 \text{ mg CO}_2 \text{ g}^{-1} \text{ soil day}^{-1}$, and it was statistically higher than all other incubation lengths and N rates (Fig. 4-2). The high soil N rate did not translate into increased rate of C mineralization for the longer incubation lengths. In contrast, the mean C mineralization rate from the 49-91 day incubation interval and the high N rate was the lowest at $0.005 \text{ mg CO}_2 \text{ g}^{-1} \text{ soil day}^{-1}$, which was statistically less than all other days and N rates (Fig. 4-2). A high N rate could have increased microbial growth and led C to mineralize more rapidly in the first 21 days of the incubation, leaving less available C for mineralization during the later phase of the incubation (Recous et al., 1995). Differences in treatments for C mineralization rate are similar to the treatment effect on cumulative C mineralization at the 0-91 day interval (Fig. 4-3). The average C mineralization rate for the red clover roots alone treatment was the lowest, and was similar to the soil alone treatment ($0.06 \text{ mg CO}_2 \text{ gram}^{-1} \text{ day}^{-1}$), and the red clover and orchardgrass treatment was the highest ($0.07 \text{ mg CO}_2 \text{ gram}^{-1} \text{ day}^{-1}$), and was similar to every other treatment but the red clover alone and soil alone treatments.

Water Extractable Carbon

Cold water extractable C is similar to leaching loss from tissues and soils (Ibrahima et al., 1995) and the C represented is very labile and easily assimilated by microbes. Hot water C, in contrast, represents microbial biomass and carbohydrates (Ghani et al., 2003). For low soil N, there was a significant effect of forage root treatment on cold water extractable carbon (CWE), hot water extractable carbon (HWE), and total water extractable carbon (TWE, Table 4-3). Significant interaction effects between treatment and time of incubation also occurred for HWE and TWE (Table 4-3). At high soil N treatments, there were no significant effects of treatment on any category of water extractable C. There was a significant effect of time of incubation for CWE, HWE, and TWE, and there was also a significant interaction of root species treatment and time for TWE ($P=0.091$, Table 4-4).

At the low N rate, CWE was highest for the red clover and orchardgrass mixture (212 mg C kg⁻¹ soil) and was similar to the three-way mixture of red clover, orchardgrass, and crabgrass roots (209 mg C kg⁻¹ soil, Fig. 4-4). This shows that there was a relatively high concentration of C from these mixtures that was not yet mineralized to CO₂ but was labile and would likely mineralize over time. The HWE at the low N rate was highest for the soil without any roots at 49 days (514 mg C kg⁻¹ soil; Fig. 4-5) so it is likely there is a larger amount of degradable carbohydrates that have not been yet mineralized. From 49 to 91 days there was an average decrease in the amount of HWE across all treatments, suggesting lower microbial biomass and degradable C. On day 91, the lowest HWE at the low N rate was for orchardgrass roots only (205 mg C kg⁻¹ soil) and that was statistically similar to the crabgrass alone treatment (260 mg C kg⁻¹ soil) and the combination of crabgrass and orchardgrass (271 mg C kg⁻¹ soil, Fig.

4-5), suggesting that microbial decomposition may have been limited by N in these grass only treatments.

Trends in TWEC at the low N rate are similar to those for HWE C, as it includes the simple sugars of C WEC and the slightly more stable carbohydrates and microbial biomass of HWE C (Fig. 4-6). Again, the highest amount of TWEC was from the soil alone treatment, which also had the lowest C mineralization rate (Fig. 4-3). It may be that there was more unmineralized yet available C in this treatment because the addition of root tissue created a priming effect on soil organic matter (Abiven et al., 2005).

At the high soil N rate, there was no significant effect of treatment on C WEC or HWE C alone, but when combined as TWEC, there was a main effect of time ($P < 0.001$) and an interaction effect of treatment and time ($P = 0.091$, Table 4-4). At day 49, the TWEC was highest for the crabgrass and orchardgrass mixture at 808 mg C kg⁻¹ soil, and this was statistically similar to the orchardgrass (712 mg C kg⁻¹ soil) and crabgrass (780 mg C kg⁻¹ soil) alone treatments as well as the mixture of red clover and crabgrass (713 mg C kg⁻¹ soil, Fig. 4-7). The grass root treatments have significantly greater TWEC than some of the red clover combination treatments at the high N soil rate, in contrast to low N TWEC, where red clover added to grass roots were some of the treatments with higher TWEC. This is likely because in the high N treatments, C decomposition was not as dependent on the organic N in red clover roots (Table 4-1).

Mineralized Soil Nitrogen

Measured N mineralization was strongly affected by the rate of N added at the beginning of incubation (Table 4-5). There was also an interaction effect between N rate and days after

incubation ($P < 0.001$, Table 4-5). The lowest amount of mineralized N was found in soil from the low N rate at day 49 after incubation ($39.1 \text{ mg N kg}^{-1} \text{ soil}$) but this was not statistically different from the amount found in soil from the low N rate at day 21 ($39.8 \text{ mg N kg}^{-1} \text{ soil}$, Fig. 4-8). The highest amount of inorganic N was found in the soil from the high N rate 21 days after initiation of incubation ($812.6 \text{ mg N kg}^{-1} \text{ soil}$; Fig. 4-6).

At the low N rate, N mineralization increased at the first two observation points (21 and 49 d) after initiation of incubation to the third timepoint (91 d; Fig. 4-8). This was not mirrored at the high soil N rate, in which the amount of inorganic N decreased from 21 to 49 days after initiation of incubation, and then increased to the 91-day timepoint (Fig. 4-8). Assimilation and release of mineralized N by microbes is likely the mechanism in the changes observed across time. Recous et al. (1995) suggested that there is a high demand for N while microbes are mineralizing simpler C compounds but a lower demand for N when microbes are mineralizing more recalcitrant forms of C in tissues, possibly due to a population shift from bacteria to fungi as the dominant decomposers. The addition of $1 \text{ g N kg}^{-1} \text{ soil}$ may have been higher than needed to evaluate effects of N release from soils and roots by microbes, and other studies have used lower rates to decrease N limitation for microbes (Redin et al., 2014). Recous et al. (1995) added 20 to $90 \text{ mg N kg}^{-1} \text{ soil}$ to measure carbon and nitrogen fluxes from maize residue, and Redin et al (2014) added approximately $70 \text{ mg N kg}^{-1} \text{ soil}$ to prevent N limitation.

Water Extractable Nitrogen

Due to the magnitude of difference between water extractable N and uneven variance between high and low N rates, we evaluated the effects of root treatment and time separately at each N rate. The effect of N rate was such that the mean total water extractable N from high N

soil was 1,265 mg N kg⁻¹ soil and mean total water extractable N from low N soil was 114 mg N kg⁻¹ soil. At the low soil N rate, there were no significant effects of root species treatment on any category of water extractable nitrogen (Table 4-4). For hot water extractable nitrogen (HWEN) and total water extractable nitrogen (TWEN), there was a significant interaction effect of treatment and time of incubation when soil N was low (Table 4-4). The highest amount of HWEN in low N soil was measured 49 days after initiation of incubation in the crabgrass and orchardgrass roots treatment (49 mg N kg⁻¹ soil, Fig. 4-9). Statistically similar treatments included the mixture of red clover and orchardgrass roots (45 mg N kg⁻¹ soil) and the mixture of red clover, orchardgrass, and crabgrass roots (45 mg N kg⁻¹ soil, Fig. 4-9). The lowest HWEN at day 49 was observed in the red clover and crabgrass roots treatment (37 mg N kg⁻¹ soil) which was statistically similar to the red clover alone, orchardgrass alone, crabgrass alone, and soil alone treatments (Fig. 4-9). After 91 days of incubation, the highest HWEN was observed in the red clover and orchardgrass roots treatment, which was similar to red clover alone, crabgrass alone, and crabgrass and orchardgrass combined treatments (Fig. 4-9). Mineralizable N is correlated to water-soluble N, and HWEN has been shown to be a better predictor of N mineralization than C:N ratio (Cabrera et al., 2005).

Among soils with N added, there was a significant effect of time of incubation on HWEN and TWEN, but there were no effects of root species treatment, likely due to the overwhelming effect of the high rate of soil N (Table 4-4). Both HWEN and TWEN were higher at day 49 than at day 91, with 157 mg N kg⁻¹ soil for HWEN and 1342 mg N kg⁻¹ soil for TWEN at day 49 and 86 mg N kg⁻¹ soil for HWEN and 1247 mg N kg⁻¹ soil at day 91. Between day 49 and day 91, it is possible that this N could have been mineralized and as such it would no longer be in the HWEN pool, shifting to the CWEN pool (Quemada & Cabrera, 1995).

Conclusions

Forage roots differ in biochemical composition between species, which affects decomposability by soil microbes. Another factor leading to differential decomposition is the availability of N. Although N is present in forage roots, especially legume roots, added inorganic N is more accessible. With the addition of inorganic N, the rate of C mineralization increased from 0-21 days after initiation of incubation, but then slowed from 49-91 days after initiation of incubation, possibly because of limitations of C available for microbial growth, or because the remaining C was more recalcitrant, due to a high proportion of lignin. Without added inorganic N, there was a stronger root species treatment effect on water extractable C, with mixtures of red clover and orchardgrass and red clover, orchardgrass, and crabgrass having the most CWEC at the low N rate. These two treatments generally had lower lignin:N ratios compared to the other treatments.

Because the C mineralization from the treatment with red clover alone was not higher than the soil with no roots, red clover in monoculture may increase short-term soil C sequestration compared to grasses, but only if belowground allocation of biomass is similar. Red clover roots contain more N than other residues, but during decomposition of red clover roots, lignin and polyphenols reduce the rate of C loss from soil. Vahdat et al. (2011) found that lignin resists decomposition from microbes and binds with protein, thus physically protecting cell materials and reducing carbon mineralization. Adding roots of red clover to grass roots led to higher C mineralization than red clover alone, emphasizing the interactions among forage root species decomposition when forage species are grown in mixture. It is likely that the increased availability of N from red clover roots increased decomposition of more available organic

compounds present in grass roots. We did not observe any apparent differences in decomposability between cool- and warm-season grasses in our study.

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Appendix: Tables and Figures

Table 4-1: Analysis of root biochemistry, total C and N, lignin, starch, and carbohydrate fractions for each forage species.

	Lignin	Starch	Water Soluble Carbohydrates	Ethanol Soluble Carbohydrates	Non- Structural Carbohydrates	Total N	Total C
	-----g/kg tissue-----						
Red clover	103	44	8	29	52	17.1	398.7
Orchardgrass	53	6	8	4	14	8.8	366.5
Greenhouse- grown crabgrass	68	4	1.2	22	16	6.1	420.9
Field-grown crabgrass	82	20	41	14	61	14.3	355.7

Table 4-2: Treatment root biochemistry; nitrogen (N), carbon (C), lignin, C:N ratio and lignin:N ratio for each treatment.

	N	C	lignin	C:N ratio	lignin:N ratio
	-----mg/vial-----				
RC†	0.86	19.9	5.2	23	6.0
OG	0.44	18.3	2.7	42	6.0
CG	0.72	17.8	4.1	25	5.7
RC+ OG	0.65	19.1	3.9	30	6.0
RC+ CG	0.79	18.9	4.6	24	5.9
CG+ OG	0.58	18.1	3.4	31	5.8
RC+ CG+ OG	0.68	19.1	4.0	28	5.9
CGgg	0.31	21.0	3.4	69	11.1
RC + CGgg	0.58	20.5	4.3	35	7.4
CGgg+ OG	0.37	19.7	3.0	53	8.1
RC+ CGgg+ OG	0.54	20.2	3.8	37	7.0

† RC = red clover, OG = orchardgrass, CG = crabgrass (field grown; trial 1 and 2), CGgg = greenhouse-grown crabgrass (trial 3).

Table 4-3: Analysis of variance for effects on cumulative C mineralization at 0 to 21d, 0 to 49d, and 0 to 91d. Random effects were trial and block, nested within trial. Main effects and interactions were considered significant at $\alpha < 0.1$. N/S represents that the effect or interaction was not significant.

Cumulative CO₂ (mg per gram)			
	Days 0-21	Days 0-49	Days 0-91
	-----p-values-----		
Root species treatment	N/S	N/S	0.077
Soil N	0.001	N/S	N/S
Root species x soil N	N/S	N/S	N/S

Table 4-4: Analysis of variance for effects on water extractable carbon (C) and nitrogen (N) at days 49 and 91 days after initiation of incubation, at each N rate. Main effects and interactions were considered significant at $\alpha < 0.1$. N/S represents that the effect or interaction was not significant.

	Cold water extractable C	Hot water extractable C	Total water extractable C	Cold water extractable N	Hot water extractable N	Total water extractable N
	-----Low N, <i>P</i> -values-----					
Root species treatment	0.046	0.095	0.020	N/S	N/S	N/S
Time	<0.001	<0.001	<0.001	0.002	<0.001	<0.001
Root species x time	N/S	0.029	0.094	N/S	0.043	N/S
	-----High N, <i>P</i> -values-----					
Root species treatment	N/S	N/S	N/S	N/S	N/S	N/S
Time	0.013	<0.001	<0.001	N/S	<0.001	0.028
Root species x time	N/S	N/S	0.091	N/S	N/S	N/S

Table 4-5: Analysis of variance for N mineralization. Random effects were trial and block, nested within trial. Main effects and interactions were considered significant at $\alpha < 0.1$. “N/S” represents that an effect or interaction was not significant. The three-way interaction of root species, soil N, and time had no significant effect on mineralized N, so it was excluded from the model.

Mineralized nitrogen	
	P-values
Root species treatment	N/S
Soil nitrogen	<0.001
Time	<0.001
Root species x soil N	N/S
Root species x time	N/S
Soil N x time	<0.001

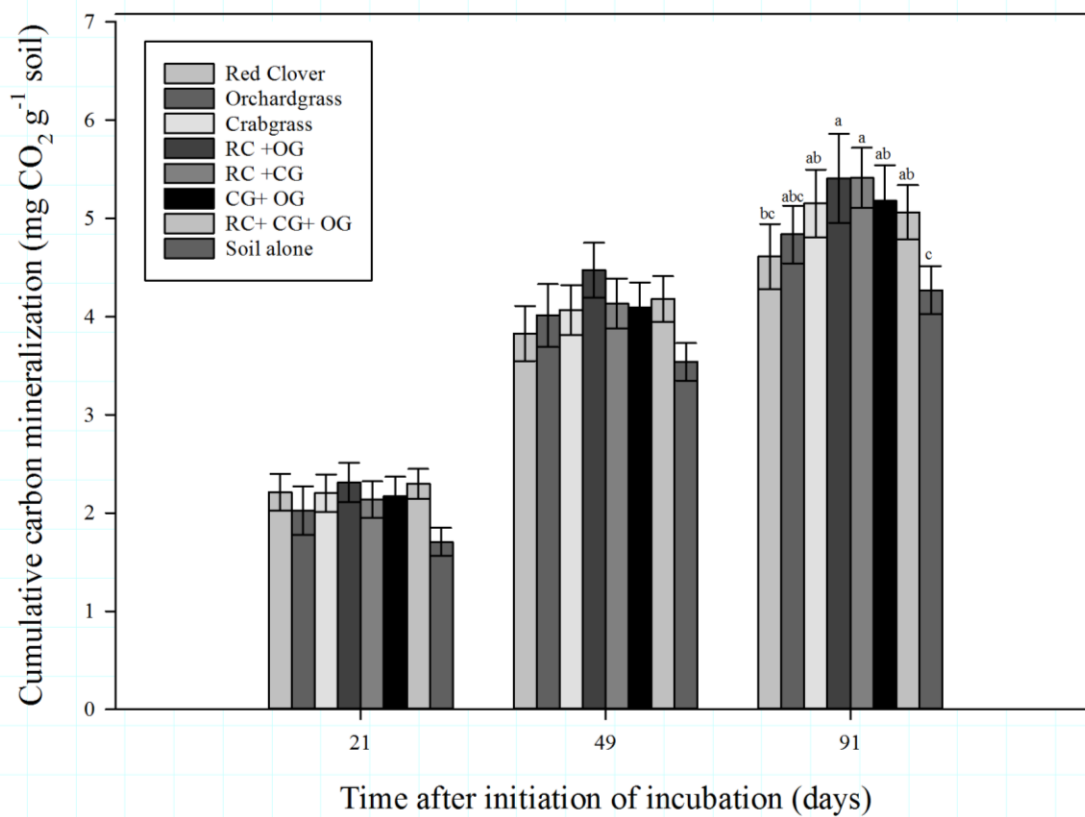


Figure 4-1: Total CO₂ mineralized across time as affected by forage root treatments. Error bars represent the standard error of the mean. Within day, bars with the same letter are not significantly different at $\alpha=0.1$.

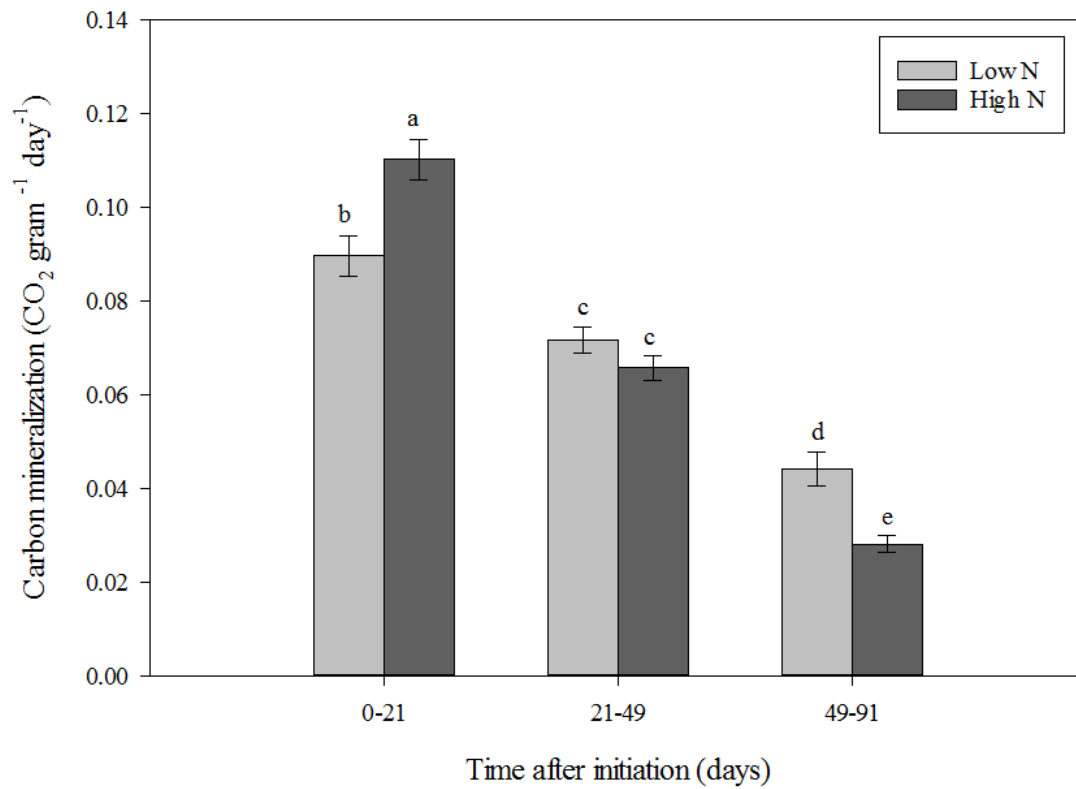


Figure 4-2: Effect of time and nitrogen rate on C mineralization rate, averaged across forage root treatments. Error bars represent the standard error of the mean. Bars with the same letter are not significantly different at $\alpha=0.1$.

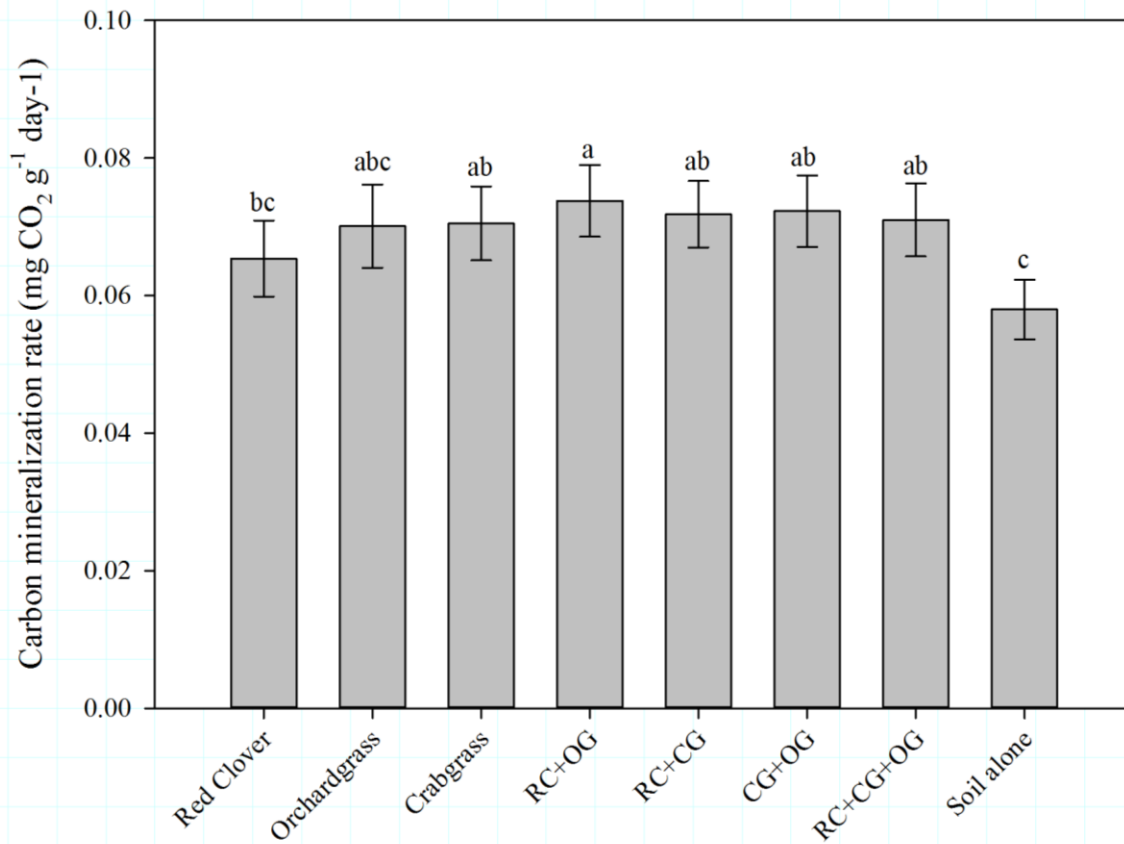


Figure 4-3: The rate of C mineralization affected by root species treatment and averaged across time and soil nitrogen rate. Error bars represent the standard error of the mean. Bars with the same letter are not significantly different at $\alpha=0.1$.

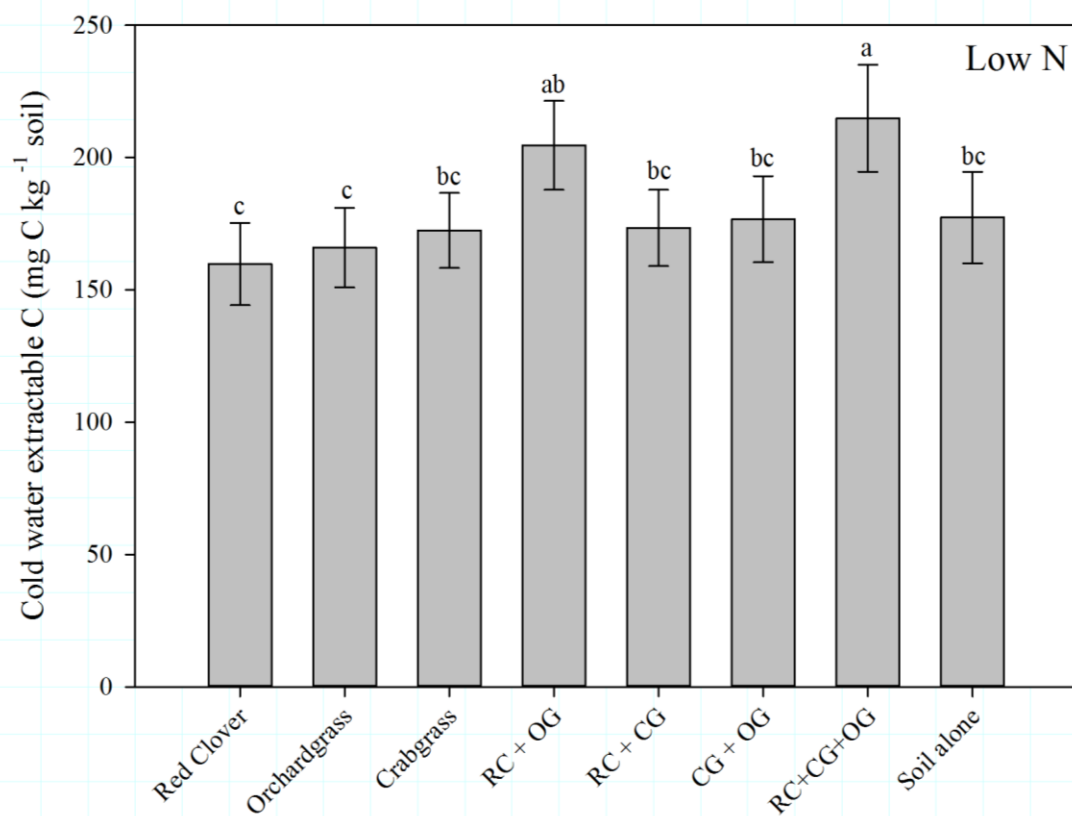


Figure 4-4: Cold water extractable carbon as affected by treatment at low soil N rate. Bars represent the standard error of the mean. Bars with the same letter are not significantly different at $\alpha=0.1$.

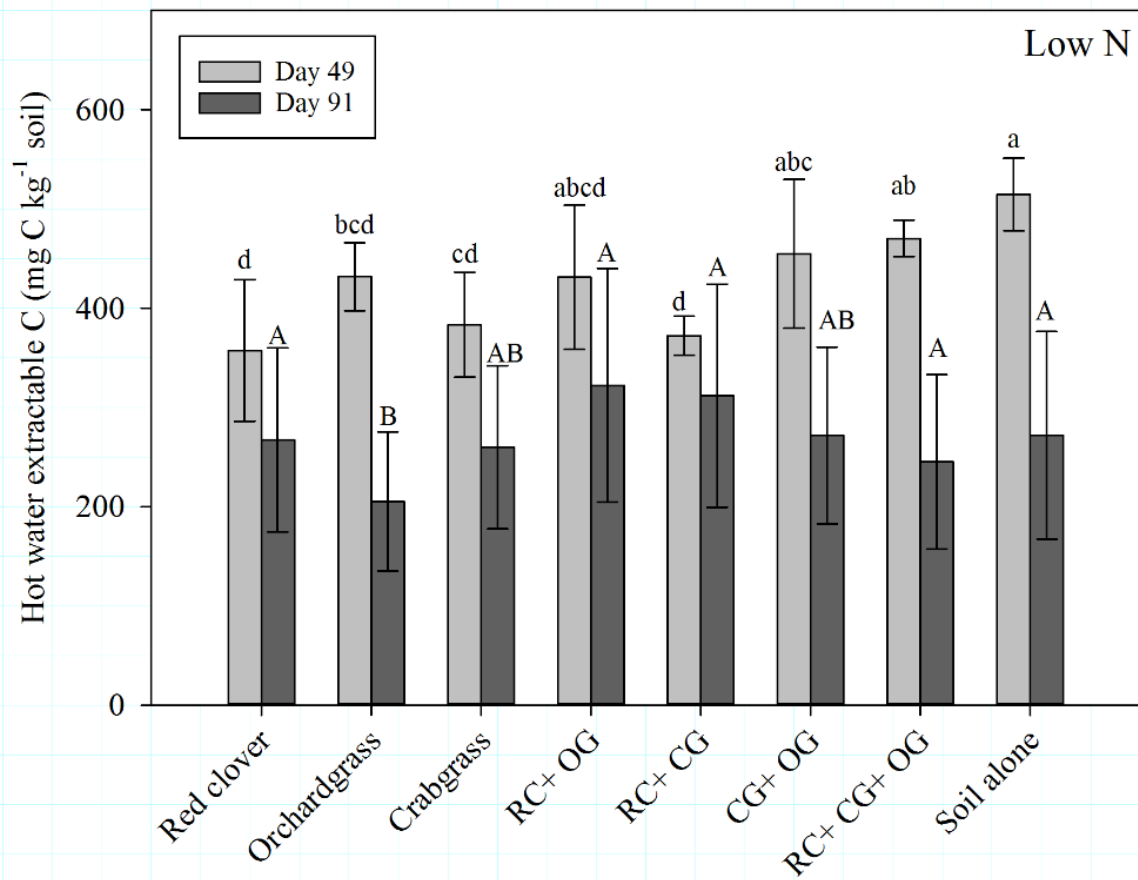


Figure 4-5: Hot water extractable carbon as affected by treatment and time, at low soil N rate, with means given for each day. Bars represent the standard error of the mean. Within day, bars with the same letter are not significantly different at $\alpha=0.1$.

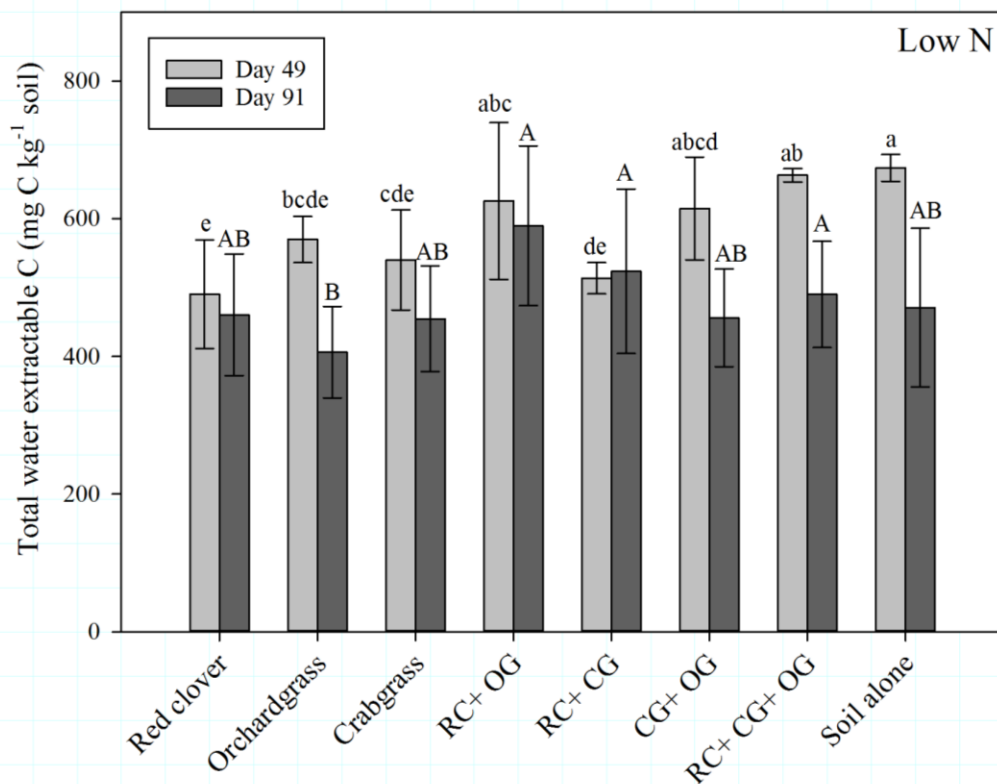


Figure 4-6: Total water extractable carbon (TWEC) as affected by treatment and time, at low soil N rate, with means given for each day. Bars represent the standard error of the mean. Within day, bars with the same letter are not significantly different at $\alpha=0.1$.

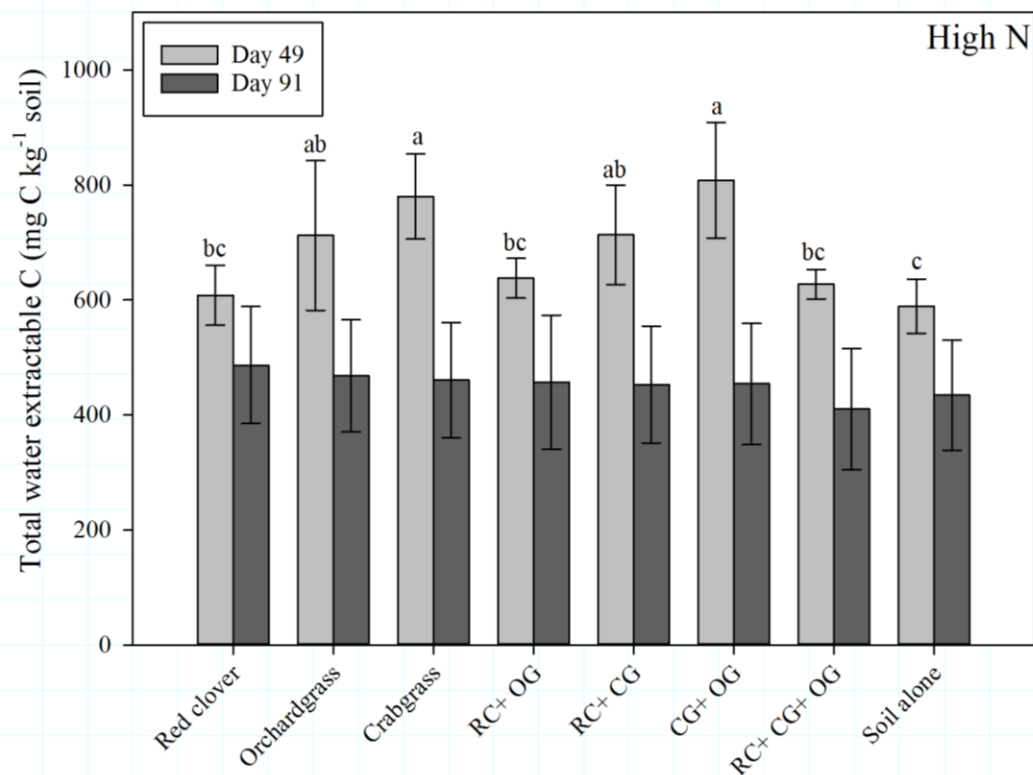


Figure 4-7: Total water extractable carbon (TWEC) as affected by time and treatment, at high soil N rate, with means given for each day. Bars represent the standard error of the mean. Within day, bars with the same letter are not significantly different at $\alpha=0.1$.

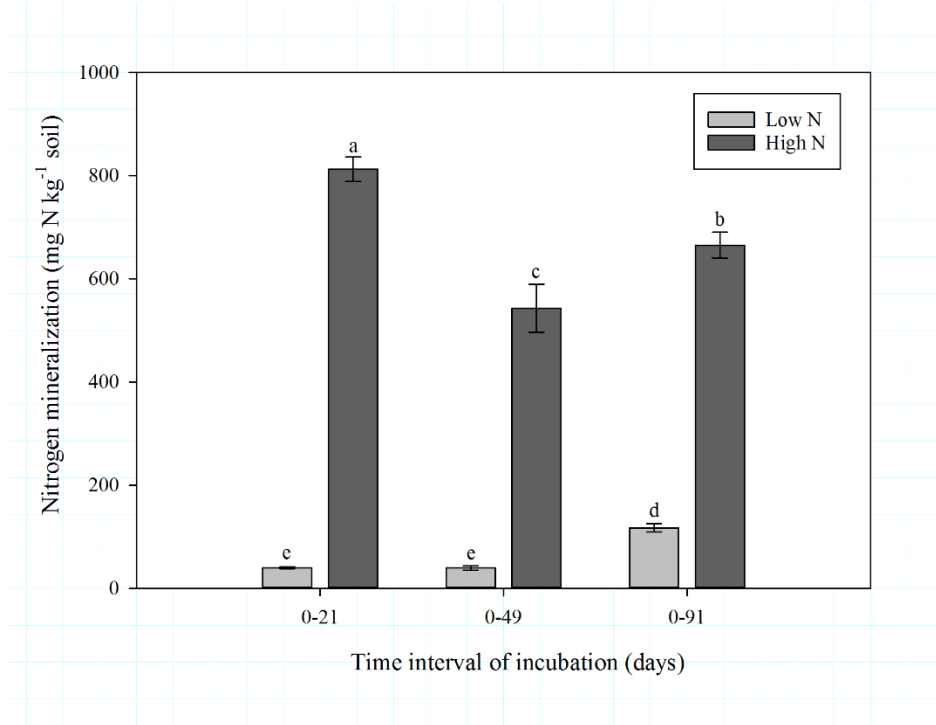


Figure 4-8: Mineralized inorganic soil nitrogen at 21, 49, and 91 days after initiation of incubation. Error bars represent the standard error of the mean. Bars with the same letter are not significantly different at $\alpha=0.1$.

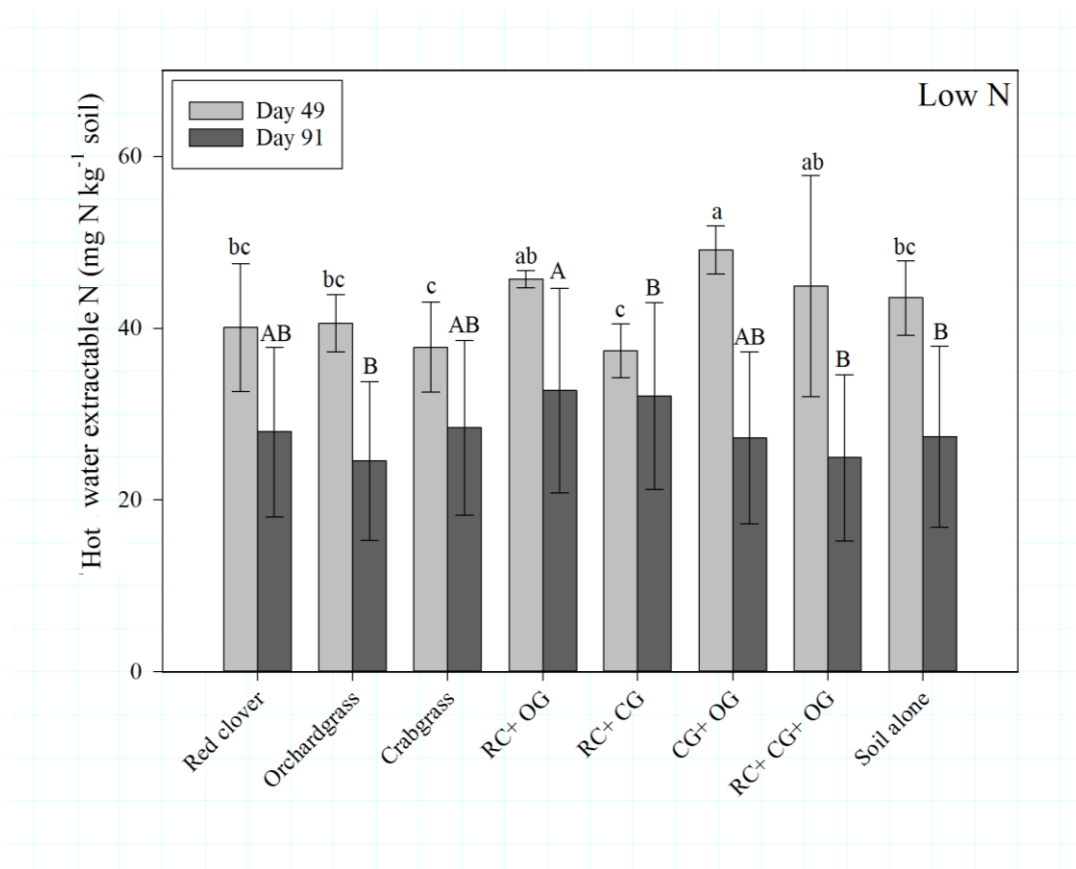


Figure 4-9: Hot water extractable nitrogen (HWEN) as affected by time and treatment, at low soil N rate, with means given for each day. Bars represent the standard error of the mean. Within day, bars with the same letter are not significantly different at $\alpha=0.1$.

CHAPTER FIVE

CONCLUSIONS

Plant belowground dynamics are complex, and plant growth depends on interactions between plant roots and the soil environment. Characteristics of root morphology such as root biomass density and root length density can help us understand the amount of resources the plant allocates to belowground function. Root morphology as represented by root length density and average root fineness represent the amount of root surface area and thus root nutrient and water uptake function. An all-perennial mixture of red clover, orchardgrass, and tall fescue had the greatest RBD, statistically similar to some other perennial mixtures, suggesting that perennial systems provide the soil quality benefits of soil organic carbon (SOC) sequestration while also having the resiliency to provide aboveground yields. The highest ratio of RBD to harvested biomass was from the orchardgrass monoculture. The orchardgrass monoculture thus allocated more resources to nutrient acquisition, since without a legume to provide N, this treatment was likely limited by N availability. Organic forage systems, as a type of low-input agriculture, should use mixtures which are not only efficient at scavenging nutrients, but which can also provide aboveground biomass yield. While some of the highest aboveground biomass amounts were harvested from annual mixtures, these annual mixtures did not have a high ratio of RBD to harvested biomass. This suggests that annual mixtures may not contribute as much to SOC compared to perennials with similar aboveground productivity. Plant growth is affected by seasons, and seasonal fluctuations in RBD, RLD, and root fineness could be due to growth

patterns of warm- and cool-season species. Seasonal differences in root C and N could also be caused by plant seasonal growth.

In our study of root decomposition rates among forage functional groups and mixtures, addition of inorganic N increased the amount of C mineralization from roots during the first 21 days but by the last 42 days of incubation, the C mineralization rate was lower in treatments with added N. The treatment with red clover roots alone did not have a cumulative C mineralization rate different than a soil-alone control, showing that although the amount of root-derived N in that treatment would be higher than a grass-alone treatment, other biochemical factors may have limited decomposition of red clover roots. However, when red clover roots were mixed with roots of grasses, cumulative C mineralization rates were the highest among treatments. The mixed residues may have decomposed more because microbes were able to access both the N from legume roots and the more available carbohydrates from grass roots. Although we used both a warm-season grass (crabgrass) and a cool-season grass (orchardgrass) in our study, we did not observe any significant differences in decomposition between the two.

In low-input systems such as organic forages, mixtures of perennial legumes and grasses may provide the best belowground plant function for nutrient and water acquisition, as well as allowing C mineralization of belowground inputs, which is related to nutrient cycling and plant nutrient availability.

VITA

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