AN ABSTRACT OF THE THESIS OF

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David D. Myrold

The cycling of root-deposited photosynthate (rhizodeposition) through the soil microbial biomass is widely recognized as a critical component of ecosystem functioning. Little is currently known about the flow of photosynthate through the microbial biomass, however. My research goal was to examine if and how the flow of root-deposited photosynthate through the microbial biomass and individual phospholipid fatty acids (PLFAs) changes during two different developmental growth stages of greenhouse-grown annual ryegrass (*Lolium multiflorum* Lam.). Plants were labeled with $^{13}CO_2$ during one of two labeling periods: either during a growth stage in transition between active root growth and rapid shoot growth or during a growth stage dominated by shoot growth. In each labeling period, ^{13}C was followed throughout the plant/soil (rhizosphere and bulk soil) system during an 8-d chase period. PLFAs were analyzed on the first and last day of each labeling period.

The overall allocation of ^{13}C in the plant/soil system was similar in the two labeling periods. There were, however, fundamental differences between the two

labeling periods associated with the cycling of rhizodeposition through the microbial biomass and individual PLFAs. In each labeling period, the rhizosphere microbial biomass incorporated a greater percentage of ^{13}C-labeled rhizodeposition and turned over faster than the bulk soil microbial biomass. Additionally, in the first labeling period, rhizosphere and bulk soil microbial biomass incorporated more ^{13}C and turned over faster than in the second labeling period. The proportion of PLFAs associated with Gram-positive bacteria increased in the rhizosphere and bulk soils between the first and second labeling period. These Gram-positive organisms were less active in utilizing rhizodeposition in the second labeling period, however. There was a shift in activity within the Gram-negative bacterial community, with organisms containing 16:1ω5 utilizing rhizodeposition more actively in the second labeling period. Fungi utilized a greater proportion of ^{13}C-labeled rhizodeposition than bacteria in both labeling periods. These results suggest that some ecological/physiological changes had occurred within the plant/soil system between the first and second labeling periods, such as a change in the quality of rhizodeposition.

This is the first study to utilize ^{13}C pulse-chase labeling in combination with PLFA analysis to examine the dynamics of soil microbial communities actively involved in rhizosphere carbon cycling. Application of this approach under a wide range of environmental conditions has the potential to greatly enhance our understanding of rhizosphere carbon cycling.

MICROBIAL COMMUNITY DYNAMICS ASSOCIATED WITH RHIZOSPHERE CARBON FLOW

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CONTRIBUTION OF AUTHORS

Jessica L. Butler conducted research and wrote the manuscript. Dr. David Myrold reviewed the manuscript. Drs. David Myrold and Peter Bottomley were responsible for research development and assisted in interpretation of the results.

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CHAPTER 1

GENERAL INTRODUCTION

Introduction

The cycling of carbon and nutrients through terrestrial ecosystems has long been of interest to scientists. As primary producers, plants drive terrestrial ecosystems; however, the essential link between plants and the soil they grow in is only made possible through the presence of microorganisms (Brimecombe et al., 2001). Plants rely upon the microbially-mediated decomposition of organic matter for their supply of inorganic forms of nitrogen and other essential nutrients. It is well documented that root-deposited photosynthate serves as an important carbon source for microorganisms in the vicinity of growing plant roots. The subsequent cycling/turnover of this matter through the microbial biomass pool is critical to plant growth because it determines the availability of nutrients, through immobilization or mineralization. In 1904 Hiltner first used the term "rhizosphere" to describe the interaction between bacteria and legume roots (Lynch, 1990). Since that time, the term has evolved and is currently used more generally to describe the complex zone of high microbial activity at the root/soil interface. Today, a century later, the rhizosphere is still widely studied due to its recognized importance as a critical component of ecosystem functioning.

The intent of this literature review is to provide a general overview of the progress that has been made thus far in the study of rhizosphere carbon flow and the characterization of rhizosphere microbial communities. Emphasis will be placed mostly on examples utilizing the methodology employed in this thesis, which includes pulse-chase labeling and PLFA (phospholipid fatty acid) analysis.

Rhizosphere Carbon Flow

In the rhizosphere carbon flow literature, the term rhizodeposition is commonly used to represent all forms of root-derived compounds reaching the soil, irrespective of their mode of arrival or chemical nature. Rhizodeposition can consist of simple, soluble organic compounds, such as carbohydrates, organic acids, amino acids, vitamins, hormones, and proteins, and less-soluble compounds, such as lysates and mucilage (Hale and Moore, 1979). As much as 40% of plant primary productivity enters the soil as rhizodeposition, and a number of environmental and physiological factors have been shown to influence both the quality and quantity of rhizodeposition (Lynch and Whipps, 1990).

Evaluation of rhizodeposition quality and quantity has commonly been carried out in sterile nutrient solutions. By controlling other factors, this allows for the identification of a range of variables influencing rhizodeposition. For example, McDougall (1970) examined the influence of nutrient solution pH on rhizodeposition quantity and Chaboud and Rougier (1991) examined the effects of root density on the quality of maize rhizodeposition in sterile nutrient solution.

Several studies have documented that the presence of microorganisms increases the rates of rhizodeposition (Barber and Martin, 1976; Meharg and Killham, 1991; Merbach and Ruppel, 1992). It has also been suggested that the presence of soil particles affects rhizodeposition due to the mechanical force roots exert while moving through the soil (Barber and Gunn, 1974). Although important data have come from studies carried out in sterile soil or nutrient solution, they have also demonstrated the necessity of examining rhizosphere carbon flow in non-sterile soil, as it is the biological, physical, and chemical interactions present in the rhizosphere that make it such a complex environment. The rhizosphere is clearly not a static environment; it is highly variable in time and space, which makes its study extremely challenging.

Isotope Tracing Methodology

Interactions at the plant/soil interface are extremely complex, thus the use of carbon isotopes is helpful in any rhizosphere carbon cycling study. The radioactive isotope, ^{14}C, is the most commonly used as a tracer in carbon cycling studies, although, the use of stable isotope, ^{13}C, is increasing. Each of these isotopes has its advantages and disadvantages (Morgan and Whipps, 2001). ^{13}C has the advantages of not being radioactive and not being as discriminated against in photosynthesis as ^{14}C, whereas ^{14}C has an economic advantage in that samples can be analyzed rather cheaply. Analysis of ^{14}C does not require the use of a mass spectrometer, which is the major cost associated with ^{13}C analysis.

Depending upon the question(s) of interest, researchers employ either continuous or pulse-chase labeling techniques. Continuous labeling requires constant exposure of plant shoots to $^{14}CO_2$ or $^{13}CO_2$, whereas pulse-chase labeling only requires exposure to the label for a short time period, such as a few hours. Continuous labeling is ideal for examining the cumulative input of photosynthate to soil, which is often necessary in global carbon cycling studies (Meharg, 1994). In contrast, pulse-labeling is ideal for measuring the partitioning of recent photosynthate, which is often necessary for studies examining rhizosphere carbon flux. Another approach commonly used in carbon cycling studies, less so with the rhizosphere, utilizes the differences in natural abundances of ^{13}C known to exist between C3 and C4 plants. This approach will not be discussed here.

Carbon Allocation

The relative amount of photosynthate deposited in the rhizosphere is a function of source-sink relationships (Helal and Sauerbeck, 1984), which are tightly linked to several factors, such as plant developmental stage (Swinnen et al., 1994; Kuzyakov et al., 2001). The influences of plant growth stage on carbon distribution have been widely investigated. Increased allocation of photosynthate to shoots and decreased allocation to roots throughout developmental stages has been well documented in cereals, such as wheat and barley (Keith et al., 1986; Gregory and Atwell, 1991; Swinnen et al., 1994). Carbon allocation at different growth stages in perennial plants is much more variable. Several researchers have

reported significant decreases in belowground carbon allocation in perennial ryegrass with increasing plant age (Meharg and Killham, 1990; Kuzyakov et al., 1999). In contrast, other researchers have found increasing belowground carbon allocation with plant age (Dormaar and Sauerbeck, 1983; Miller et al., 1994; Kuzyakov and Domanski, 2000). At later stages of growth, seeds serve as the major carbon sink in annuals, whereas, in perennial plants carbon must be stored in root systems for the following growing season (Kuzyakov and Domanski, 2000). It is possible that the variability of carbon allocation belowground in perennials is a function of growing conditions, such as soil fertility. Although dependent upon a number of factors, the overall partitioning of carbon translocated belowground is similar in cereals and grasses (Kuzyakov and Domanski, 2000), with about 50% of carbon translocated belowground found in roots, 30% respired through root and microbial respiration, and the remaining 20% incorporated into soil microbial biomass and soil organic matter.

Any factor affecting growth conditions will likely influence plant carbon distribution dynamics (Lynch and Whipps, 1990); this recognition has lead to a number of studies examining the influences of a variety of environmental variables on rhizosphere carbon flow, such as light intensity, temperature, CO_2 concentration, soil fertility, and grazing. Exposure of perennial ryegrass (Hodge et al., 1997) and spring barley plants (Jensen, 1994) to high light intensities (1000 μmol m^{-2} s^{-1} compared to 350 μmol m^{-2} s^{-1}) led to increased carbon partitioning to the root systems, relative to the shoots; however, there was no effect on the amount

of rhizodeposition recovered in soil. In continuous labeling studies, temperature has been shown to affect carbon distribution within plants. In tall fescue, Prud'Homme et al. (1993) found increased allocation to root systems at lower temperatures (8/5°C day/night) relative to high temperatures (24/17°C day/night). They suggested that roots serve as storage organs during stressful times, such as periods of lower temperature. Although temperature did not appear to influence the quantity of carbon lost from the roots of 11 wheat cultivars, Martin and Kemp (1980) found a significant increase in the water-soluble fraction of rhizodepsoition with higher temperatures, suggesting qualitative changes in rhizodeposition. The effects of CO_2 concentration on carbon distribution have generally shown increased belowground allocation with increased CO_2 concentration in C3 plants (Kimball et al., 1993; Poorter, 1993; van Ginkel et al., 2000). In some cases the amount allocated belowground nearly doubled under elevated CO_2 (Rattray et al., 1995).

Soil nutrient status has been shown to affect carbon allocation. For example, Saggar et al. (1997) observed a 10% increase in belowground allocation in pasture plants growing in soil with lower levels of phosphorus. Grazing has been shown to increase carbon allocation to shoots (Johansson, 1993; Kuzyakov et al., 2002) and this aboveground allocation was enhanced with nitrogen fertilization (Kuzyakov et al., 2002).

Rhizodeposition Dynamics

It has been well documented that rhizodeposition can extend several millimeters from root surfaces. For example, in a $^{14}CO_2$ continuous labeling experiment, Helal and Sauerbeck (1983) found evidence of ^{14}C derived from maize roots 20 mm away from the root system; however, values of 3 to 5 mm are more commonly reported (Lynch and Whipps, 1990). Several studies have found that the percentage of soluble rhizodeposition decreases with distance from the rhizosphere, whereas the amount of insoluble rhizodeposition increases with distance into the bulk soil (Bokhari et al., 1979; Whipps, 1984, 1985, 1987). Although variable among plant species, the quality of rhizodeposition is also known to vary with plant age. In general, more simple sugars, carbohydrates, and proteins dominate at earlier stages, whereas more insoluble materials, such as sloughed-off cells dominate the later stages of growth (Hale and Moore, 1979; Bokhari et al., 1979). Furthermore, the amounts of rhizodeposition are known to vary with location on root systems. McDougall and Rovira (1970) found that the zone just behind lateral and primary root tips served as the zone of maximum rhizodeposition in wheat. Additionally, Norton et al. (1990) found that the young root tips of ponderosa pine seedlings served as a major sink for recent photosynthate, and that mycorrhizal fine roots contained more than 25% of the ^{14}C recovered in all plant material and contributed to high amounts of rhizodeposition reaching the bulk soil. Recently, Johnson et al. (2002) found that within the first 21 hr after pulse-labeling pasture plants, 5 to 8% of the respired ^{13}C had come from

mycorrhizae, thus demonstrating the role of mycorrhizae as a significant carbon sink. The presence of mycorrhizae is often neglected in rhizosphere studies, which is surprising given that the majority of plant species are mycorrhizal and that they can demand up to 20% of plant photosynthate (Sylvia, 1998). Although some investigations have traced rhizodeposition into the microbial biomass pool, overall, this has not yet been widely investigated (Kuzyakov et al., 2002).

Carbon moves through plants and into soil at phenomenal rates. It has been documented to occur is as little as 30 minutes (Cheng et al., 1993). Shortly after its arrival, this rhizodeposition gets incorporated into the soil microbial biomass. In a pulse-chase experiment, Rattray et al. (1995) found maximum incorporation of ^{14}C into the rhizosphere microbial biomass in 3 hr in perennial ryegrass. Seven days after labeling, they could no longer detect ^{14}C in the microbial biomass. Additionally, one week after pulse-labeling, Norton et al. (1990) observed a 50% decline in ^{14}C labeled microbial biomass associated with ponderosa pine seedlings. These results suggest that recent photosynthate cycles through the microbial biomass pool rapidly.

Rhizodeposition greatly influences carbon turnover in soil and can result in its accumulation or consumption due to its influences on microbial activity (Kuzyakov et al., 2001). Five days after pulse-labeling perennial ryegrass, Kuzyakov et al. (2001, 2002) found one-third of the soil ^{14}C present in the microbial biomass pool (between 0.5 and 1.7% of total recovered ^{14}C). Martin and Merckx (1992) found between 31 and 38% of soil ^{14}C incorporated into microbial

biomass after 63 d of exposing wheat to $^{14}CO_2$; however, because this was a continuous-labeling experiment, incorporation could have resulted from root decomposition and not just recent photosynthate. In a pulse-chase labeling experiment at different growth stages of perennial ryegrass, Kuzyakov et al. (2001) observed significant priming effects (stimulated decomposition of native soil organic matter) at later stages of plant growth. For example, after 100 d of growth, they found an additional 6 g C m^{-2} d^{-1} of native soil organic matter decomposed. Based on the assumption that the C:N ratio of the organic matter was constant, Kuzyakov et al. (2001) estimated high levels of nitrogen mineralization (6 kg of N ha^{-1} d^{-1}) resulting from this additional decomposition of native organic matter.

The influence of soil fertility on the incorporation of rhizodeposition into microbial biomass has been investigated and results suggest that microorganisms are often limited in mineral nutrients and not just carbon (Merckx et al., 1987; Liljeroth et al., 1990). Liljeroth et al. (1990) found increased incorporation of rhizodeposition into microbial biomass at higher nitrogen levels, suggesting that the microbial biomass at a lower level of nitrogen might have been nitrogen deficient. They also observed decreased decomposition of native soil organic matter in higher nitrogen soils. Similarly, Cardon et al. (2001) found that exposure of grassland communities to elevated CO_2 concentrations slowed the decomposition of organic matter as long as nutrients were abundant. Thus, a change in soil nutrient status has the potential to greatly influence the dynamics of rhizosphere carbon cycling as well as the cycling of other soil carbon pools.

Several researchers have examined the effects of increased CO_2 concentration on rhizosphere carbon dynamics (Rattray et al., 1995; Paterson et al., 1996). In perennial ryegrass (Rattray et al., 1995) and wheat (Paterson et al., 1996) significant increases in rhizodeposition occurred under elevated CO_2 concentrations; however, incorporation into the microbial biomass pool decreased significantly, relative to plants under ambient CO_2 concentration. The authors suggest that the microbial biomass was likely limited by available nitrogen either from the soil or from increased C:N ratios of rhizodeposition at elevated CO_2 concentrations, which has been observed in other studies (van de Geijn and van Veen, 1993).

Present and Future Considerations

Due to the many challenges faced in studying the rhizosphere, several researchers have focused their efforts on developing mathematical models (Darrah, 1991; Cheng et al., 1993; Swinnen et al., 1994; Kuzyakov et al., 1999, 2001; Kuzyakov and Domanski, 2002). Quantifying CO_2 efflux from soil is essential in many carbon cycling studies; however, it is not possible to directly distinguish between root respiration and microbial respiration in soil. In an attempt to overcome these challenges, Kuzyakov and Domanski (2002) developed an extensive simulation model based on the kinetics of $^{14}CO_2$ efflux from soil after pulse-labeling. Based on empirical evidence, the model assumes that $^{14}CO_2$ from root respiration occurs prior to microbial respiration of rhizodeposition, due to the

time required for exudation/secretion (rhizodeposition) and the subsequent uptake and utilization by microorganisms. The model appears to accurately predict $^{14}CO_2$ efflux from the soil (root derived separate from microbial derived) and ^{14}C dynamics in shoots, roots, and soil; however, the model has not yet been able to successfully describe the dynamics of ^{14}C in microorganisms (Kuzyakov and Domanski, 2002). The authors suggest that due to the simplicity of the model it is unable to account for the lower molecular weight rhizodeposition that gets incorporated into the microbial biomass within a few hours after assimilation. So far the model is only capable of predicting the incorporation of higher molecular weight compounds (e.g., polysaccharides) that leave the roots later due to the longer time required for synthesis of more complex compounds. Overall, the development of this model is a step forward, given the very limited research aimed at examining the flow of rhizodeposition through the microbial biomass.

Soil Microbial Communities

The majority of soil microorganisms are currently unknown and unculturable (Torsvik et al., 1990); therefore, any approach to studying the ecological aspects associated with soil microorganisms must be done at the community level. Because of the critical roles microorganisms play in key soil processes (nutrient cycling, organic matter decomposition, structure formation and stabilization), there has always been an interest in elucidating the factors that control their presence or activity. With the development of environmental

molecular biological methods, however, this interest has grown enormously in the last decade. One of the most commonly used methods today is PLFA analysis.

PLFA Methodology and General Application

It is generally accepted that specific phospholipid fatty acids serve as "biomarkers" (signatures) for certain microorganisms or groups of microorganisms (Zelles et al., 1992). Table 1.1 lists several common biomarker PLFAs and their associated groups of organisms. Furthermore, because different subsets (i.e., taxonomic groups) of a given microbial community contain different PLFA patterns, a change in the overall PLFA profile is indicative of a change in community structure. Phospholipids are the major constituents in the membranes of all living cells, are not present in storage compartments (Vestal and White, 1989), and are rapidly degraded upon cell death (Zelles et al., 1994). Therefore, by extracting and examining only the PLFAs in soil it is possible to examine the active fraction of the microbial community, whereas other methods that extract total soil fatty acids (e.g., Schutter and Dick, 2000) are unable to differentiate between the active and non-living biomass.

Throughout the last decade PLFA analysis has been successfully applied under a wide range of environmental conditions. Changes in microbial communities based on PLFA profiles, have been observed in response to liming of forest soils (Frostegård et al., 1993a), contamination of different heavy metals (Frostegård et al., 1993b, 1996) or alkaline dust (Bååth et al., 1992), different

growing conditions (field vs. greenhouse) (Ibekwe and Kennedy, 1998), seasonal changes along a fertility gradient (Bardgett et al., 1999), moisture stress (Wilkinson et al., 2002), removal of organic matter and compaction in forest soils (Ponder and Tadros, 2002), different laboratory handling procedures (Petersen and Klug, 1994; Schutter and Dick, 2000), and most commonly, different agricultural management (Zelles et al., 1992, 1994; Wander et al., 1995; Petersen et al., 1997; Bossio et al., 1998; Ibekwe and Kennedy, 1999; Calderón et al., 2001).

Table 1.1. Biomarker PLFAs and associated organisms. Adapted from Wilkinson et al. (2002)

Marker	Marker for
15:0i, 15:0a, 15:0, 16:0i, 16:1ω9, 16:1ω7, 17:0i, 17:0a, 17:0cy, 18:1ω7, 19:0cy	Bacteria
18:2ω6,9, 16:1ω5	Fungi, mycorrhizal fungi (16:1ω5)
Terminally branched 15:0i, 15:0a, 16:0i, 17:0i, 17:0a	Gram-positive bacteria (some Gram-negative bacteria)
10Me16:0, 10Me18:0	Actinomycetes
ω7 monounsaturated 16:1ω5, 16:1ω7, 18:1ω7	Gram-negative bacteria
Cyclopropyl 17:0cy, 19:0cy	
Polyunsaturated 18:3, 20:4	Micro-eukaryotes
Total cyclopropyl/ total ω7 monounsaturated	Nutritional stress

Application to the Rhizosphere

PLFA analysis has been applied to investigate changes in rhizosphere microbial communities in response to a number of factors, such as different cultivars of canola and wheat (Siciliano et al., 1998), different tree species planted in two different soils (Priha et al., 1999), different CO_2 concentrations (Ringelberg et al., 1997), different levels of root nematode herbivory (Denton et al., 1999), and agricultural management and seasonal variations under spring wheat (Petersen et al., 2002). With the exception of Petersen et al. (2002), all of these studies examined PLFA profiles at one point in time, which gives no information on the temporal variations in microbial community composition. Given the dynamic nature of the rhizosphere, time is likely to play a role in rhizosphere microbial communities. Other studies, however, have considered the effects of time as a major component of microbial community dynamics (Steer and Harris, 2000; Baudoin et al., 2002).

Steer and Harris (2000) examined PLFA profiles associated with the rhizosphere and bulk soils of *Agrostis stolonifera* during growth from 70 to 160 d. Overall, they found increasing proportions of Gram-negative bacterial PLFAs (16:1ω7, 16:1ω5, and 18:1ω7) and decreasing proportions of Gram-positive bacterial PLFAs (15:0i, 15:0a, and 16:0i) with increasing plant age from 90 through 160 d. Interestingly, they did not observe differences in PLFA profiles between the rhizosphere and bulk soils except after the 90-day sampling, which also corresponded with a period of intense root growth. During this time, rhizosphere

soils were significantly more abundant in cyclopropyl PLFAs, whereas the bulk soil was dominated (although not significantly) by PLFAs associated with Gram-positive bacteria (15:0i, 15:0a, and 16:0i). It is surprising that cyclopropyl PLFAs were higher in the rhizosphere during a time of active root growth, considering that the presence of these PLFAs is often interpreted as an indication of stress (Guckert et al., 1986), however, no explanations were given by the authors.

Recently, Baudoin et al. (2002) examined the effects of plant age (2-week-old vs. 4-week-old maize) on genetic (DNA) and metabolic profiles (Biolog) of microbial communities associated with three rhizosphere root zones known to be sources of rhizodeposition and non-rhizosphere soil. They found an increased amount of Biolog substrates utilized by all the rhizosphere-associated communities at the 4-week sampling; however, no differences were observed in the unplanted soil, suggesting that the quality of rhizodeposition might have changed between the two sampling dates. Principal component analysis of the DNA data revealed that time was more important in separating DNA fingerprints than were root locations or non-rhizosphere soil; however, non-rhizosphere soil did show some separation from rhizosphere soils at each sampling date. These findings clearly demonstrate the influences of plant age on microbial community dynamics, both functional and structural. Because it is a culture-based technique, Biolog is often questioned for its ecological significance, considering that a very small percentage of soil microorganisms are culturable (Konopka et al., 1998). Thus, better tools are needed to examine the in situ function of rhizosphere microbial communities.

In an attempt to assess the influence of rhizodeposition quantity on microbial dynamics, Griffiths et al. (1998) examined the effects of different amounts of synthetic root exudates added to soil on PLFA profiles. Overall, they found fungal PLFAs to dominate over bacterial PLFAs with higher amounts of substrate added. These findings show that substrate quantity affects microbial community structure, given that the only variation (treatment) in these soils was the amount of exudate added. This study was preformed in the absence of plants, which limits its application to real rhizosphere dynamics.

Stable Isotopes and PLFAs

Although PLFA analysis and other molecular tools are very useful in assessing rhizosphere microbial community structure and diversity, alone they provide little direct information with regards to the dynamics of the active microbial community involved in biogeochemical cycling. In the last few years, the combination of stable isotope techniques and molecular biological methods have made it possible to directly link groups of microorganisms with biogeochemical processes (Boschker et al., 1998; Radajewski et al., 2000; Boschker and Middelburg, 2002). Analysis of microbial communities using ^{13}C labeling in conjunction with analysis of microbial DNA or PLFAs has been limited, but the work that has been done demonstrates a very promising future for this approach. Using ^{13}C-labeled methanol and DNA extraction and density-gradient centrifugation, Radajewski et al. (2000) successfully identified methylotrophic

bacteria in forest soil. Hanson et al. (1999) utilized ^{13}C-labeled toluene and PLFA analysis to detect members of the microbial community capable of metabolizing toluene. Twenty-four hours after adding ^{13}C-labeled toluene to soil, 27% of the PLFAs became enriched in ^{13}C, whereas 96% of the PLFAs became enriched in ^{13}C from the addition of ^{13}C-labeled glucose. This approach has also been used in aquatic systems. For example, Boschker et al. (1998) utilized PLFA analysis together with ^{13}C labeling to examine two biogeochemical processes: sulfate reduction coupled to acetate oxidation and methane oxidation. This methodology has also been used to examine more general microbial processes. For example, Arao (1999) used PLFA analysis and ^{13}C-labeled acetate to examine bacterial and fungal activities in response to changes in pH.

Several studies have traced rhizodeposition into the total soil microbial biomass and several studies have examined the structure of microbial communities associated with the rhizosphere. To date, however, no studies have attempted to trace rhizodeposition into specific components of rhizosphere microbial communities (i.e., PLFAs). Although it is not possible to examine the significance of individual species in ecosystem processes, considering the functional redundancy that is known to exist among soil microorganisms (Konopka et al., 1998), a community-level approach is more practical to the study of microorganisms involved in major biogeochemical cycles, such as the cycling of rhizosphere carbon.

Conclusions

The study of rhizosphere biology has taken a dynamic course over the last several decades. Today there are hundreds of published studies on rhizosphere carbon dynamics and through constant methodological advancement this will only continue to grow. Because microorganisms are a vital component of nutrient cycling, knowledge of their activities in the rhizosphere is imperative to our understanding of ecosystem functioning. The application of molecular biological methods, such as PLFA analysis, in combination with the power of stable isotopes, has the potential to greatly enhance our understanding of the complex system, known as the rhizosphere. Chapter 2 of this thesis describes an experiment that was conducted to examine the structure of soil microbial communities actively involved in rhizosphere carbon cycling, through use of this new methodology.

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CHAPTER 2

MICROBIAL COMMUNITY DYNAMICS ASSOCIATED WITH RHIZOSPHERE CARBON FLOW

Abstract

The cycling of root-deposited photosynthate (rhizodeposition) through the soil microbial biomass has profound influences on plant nutrient availability. Currently, our understanding of microbial dynamics associated with rhizosphere carbon flow is limited. I used a ^{13}C pulse-chase labeling procedure to examine the flow of photosynthetically fixed ^{13}C into the microbial biomass and individual phospholipid fatty acids (PLFAs) in the bulk and rhizosphere soils of greenhouse-grown annual ryegrass (*Lolium multiflorum* Lam.). In order to assess the temporal dynamics of rhizosphere carbon flow through the microbial biomass, labeling took place during a stage of transition between active root growth and rapid shoot growth on one set of plants (Labeling Period 1), and 10 d later during the rapid shoot growth stage, on another set of plants (Labeling Period 2).

Overall, the distribution of ^{13}C in the plant/soil system was similar between the two labeling periods; however, the microbial cycling of rhizodeposition was not always similar between labeling periods. Within 24 hr of labeling, 10% of the ^{13}C retained in the plant/soil system resided in the soil, much of which had already been incorporated into the soil microbial biomass. Turnover of ^{13}C through the microbial biomass was faster in the rhizosphere soils relative to the bulk soils, and within rhizosphere and bulk soil, turnover was faster in Labeling Period 1 relative

to Labeling Period 2. Temporal differences in microbial community composition were much more apparent than spatial differences, with a greater relative abundance of Gram-positive PLFAs (15:0i and 15:0a) in the second labeling period. Although more abundant, Gram-positive organisms appeared to be less actively utilizing rhizodeposited carbon in the second labeling period, relative to the first labeling period. Gram-negative bacteria associated with the 16:1ω5 PLFA were more active in utilizing ^{13}C-labeled rhizodeposition in the second labeling period relative to the first labeling period. In both labeling periods, however, the fungal PLFA, 18:2ω6,9, was the most highly labeled. These results demonstrate the effectiveness of using ^{13}C labeling and PLFA analysis to examine the microbial dynamics associated with rhizosphere carbon cycling by focusing on the members actively involved.

Introduction

Because soil is the largest reservoir of organic carbon in the terrestrial biosphere (Cardon et al., 2001), worldwide efforts have focused on trying to understand the dynamics associated with soil organic matter in hopes of gaining insight into global carbon cycling and ecosystem functioning. Although the microbial biomass represents a relatively small portion of soil organic carbon, generally 1 to 3% (Anderson and Domsch, 1989), knowledge associated with the cycling of carbon and other nutrients through this pool is essential considering that most of primary productivity (plant material) passes through the microbial biomass

at some point in time (Ryan and Aravena, 1994). Quantifying the flow of root-deposited photosynthate through the soil microbial biomass is of great importance because of its profound influence on the nutrient supply for plant growth; however, current knowledge is limited. Thus, there is a fundamental need to gain more information on the microbial dynamics associated with carbon cycling in the rhizosphere.

The rhizosphere, a zone of high microbial activity in the vicinity of growing plant roots, has received considerable attention since Hiltner first coined the term in 1904 (see Hale and Moore, 1979). Through the use of the carbon isotopes, ^{13}C and ^{14}C, the flow of carbon from the above- to belowground plant parts, and the subsequent release of some of this photosynthate into the rhizosphere, have been widely investigated. Collectively referred to as rhizodeposits, these carbon compounds, which reach the soil from living roots, consist of a number of organic compounds that differ in their mode of arrival and their degree of complexity/degradability (Lynch and Whipps, 1990). Rhizodeposits include any component of the plant cell but are often conveniently classified into soluble and insoluble compounds. It has been estimated that as much as 40% of plant primary production may be lost through rhizodeposition (Lynch and Whipps, 1990) and several researchers have demonstrated that the flow of carbon into the rhizosphere is influenced by a number of physiological (e.g., plant growth stage, defoliation) and environmental (e.g., temperature, CO_2 concentration, soil fertility, and light intensity) factors (Martin and Kemp, 1980; Whipps, 1985). Studies examining the

influences of growth stage on carbon allocation belowground have found mixed results. For example, in most plants the amount of carbon allocated belowground has been shown to generally decrease with plant age; however, there have also been reports suggesting an increase in carbon allocation belowground in some perennials (see review by Kuzyakov and Domanski, 2000). Furthermore, researchers have also demonstrated that the quality of rhizodeposits entering the rhizosphere changes with age or developmental stage, plant species, and environmental conditions (Martin, 1977; Kraffcyzk et al., 1984). For example, the amount of proteins and carbohydrates in rhizodeposition decreases with age (Brimecombe et al., 2001).

The flow of photosynthate into and through the soil microbial biomass has not received nearly the attention that the partitioning of photosynthate within plants and soil has (Kuzyakov et al., 2002). Because microorganisms in the rhizosphere serve as both a source and sink of nutrients (Kouno et al., 2002), they directly influence plant growth and therefore play a critical role in maintaining ecosystem productivity. Thus, it is the interconnectedness of these two processes, the flow of carbon into the soil and the subsequent cycling of this matter through the microbial biomass that ultimately dictates ecosystem functioning.

An approach that has provided a great deal of information on soil carbon cycling through the microbial biomass is the application of isotopically labeled substrates (e.g., glucose and plant residue) to soil and subsequently monitoring their decomposition or turnover (Saggar et al., 1996; Kuzyakov and Demin, 1998; Aoyama et al., 2000; Kouno et al., 2002). Although these studies have revealed a

great deal of information on soil organic matter dynamics, the microbial biomass is generally considered a single entity, which reveals nothing of the structure of the microbial community actively involved in nutrient cycling.

Because of the great diversity of microorganisms in soil, most of which are unknown and unculturable (Torsvik et al., 1990), several molecular and biochemical methods have been developed over the past decade. For example, analysis of microbial DNA and phospholipid fatty acids (PLFAs) have proven extremely useful for describing the general structure of soil and aquatic microbial communities. Analysis of PLFAs has been useful in monitoring changes in microbial community structure in response several factors, such as agricultural management activities (Zelles et al., 1992; Petersen et al., 1997, 2002; Bossio et al., 1998), the addition of lime (Frostegård et al., 1993a), and heavy metal contamination (Frostegård et al., 1996). Analysis of PLFAs has also been utilized to examine the structure of rhizosphere microbial communities (Ibekwe and Kennedy 1998, 1999; Siciliano et al. 1998; Priha et al. 1999; Griffiths et al., 1999; Steer and Harris, 2000). Although these studies have provided considerable information regarding the dynamics of microbial community structure in rhizospheres, they did not reveal any information regarding the function of microbial communities associated with rhizosphere carbon cycling.

Only within the last few years has the use of carbon isotope techniques coupled with PLFA analysis been exploited. Because PLFA analysis reveals information on the active portion of the microbial community (Vestal and White,

1989), the labeling of individual PLFAs has the ability to directly link microbial processes with the groups of organisms involved (Boschker et al., 1998). So far this approach has only been used in a few studies. Boschker et al. (1998) first used this approach with ^{13}C-labeled acetate and methane to examine which members of aquatic microbial communities were involved in two processes: sulfate reduction coupled to acetate oxidation and methane oxidation. Subsequently, this approach has been used to trace ^{13}C-labeled carbon substrates into several cultured strains of bacteria and fungi (Abraham et al., 1998), to examine the activity of soil bacterial and fungal biomarkers through incorporation of ^{13}C-labeled acetate (Arao, 1999), and to link toluene degradation with specific PLFA biomarkers (Hanson et al., 1999).

A number of studies have used Biolog substrate utilization potential, a technique that assesses the metabolic capability of microbial communities, coupled with PLFA analysis or DNA based-techniques to attempt to link the functioning of microbial communities with their structure (Buyer and Drinkwater, 1997; Meyers et al., 2001; Schutter and Dick, 2001; Baudoin et al., 2002). Although these studies have provided information on the metabolic potentials of microbial communities, this culture-based technique does not give an assessment of *in situ* activity, which is a major benefit afforded by the ^{13}C-labeled PLFA methodology described above. Furthermore, the extent to which the quality and quantity of rhizodeposition influences microbial communities is still largely unknown (Griffiths et al., 1999). Thus, this new methodology has great potential to provide a wealth of information

on the structure of the microbial community members actively involved in the cycling of rhizodeposited carbon.

The overall objective of this study was to trace photosynthetically fixed carbon (^{13}C) through annual ryegrass (*Lolium multiflorum* Lam. var. Gulf) at two different stages of plant growth. The primary focus was on the flow of photosynthate into the microbial biomass and individual microbial PLFAs in rhizosphere and bulk soil. I hypothesized that the changes in carbon allocation that take place at the different growth stages would have an influence on both the quality and quantity of rhizodeposition, which would therefore influence both the activity and the turnover of the rhizosphere and bulk microbial communities. To test these hypotheses, I used a ^{13}C pulse-chase labeling procedure coupled with PLFA analysis to examine the flow of photosynthetically fixed ^{13}C into the rhizosphere and bulk soil microbial biomass and individual PLFAs.

Materials and Methods

Experimental Design

Twenty plants were labeled with $^{13}CO_2$ during each of two growth stages, and four replicate plants were sampled 1, 2, 3, 5, and 8 days after labeling. One unlabeled plant was also harvested at each sample period to serve as a control for background $\delta^{13}C$ values. In order to account for any autotrophic activity by soil microorganisms, four containers with just soil were included in each labeling period: two that served as unlabeled controls and two that underwent the labeling

process (one of each was harvested on Days 1 and 8 after labeling). Four extra plants were grown, two of which were used for plant biomass assessment prior to labeling and two were used to determine if plants were mycorrhizal at the time of each labeling period. All plant parts and soil were analyzed for total C and ^{13}C abundance. Microbial biomass carbon and associated $\delta^{13}C$ values were determined on all soil samples; PLFA profiles and associated $\delta^{13}C$ values were determined on a subset of soil samples.

Growing Degree Days

A preliminary plant growth study was conducted to determine when the developmental stages for active root growth and rapid shoot growth occur in annual ryegrass. At weekly intervals throughout a 10-week period, three replicate plants were harvested, dried and weighed to determine root and shoot biomass. I used growing degree days (GDDs), which is a simple and effective way of predicting the rate of plant development based on the amount of accumulated heat required for a plant to reach a certain stage of development (Griffith, 2000). Growing degree days were determined from the following equation:

$$GDD = ((T_{max} + T_{min})/2) - T_{base} \qquad \text{[Eq. 1]}$$

where T_{max} is the daily maximum temperature and T_{min} is the daily minimum temperature measured in °C. I used 0°C as the base temperature (T_{base}).

Soil

Soil was collected in the summer of 2001 from the top 10 cm of four replicate conventionally tilled, high residue incorporated, grass-seed research plots in Marion County Oregon (part of the Sustainable Grass Seed Cropping System Research Project established in 1992 by OSU and USDA-ARS scientists; see Gohlke et al., 1999 for a comprehensive site summary). This soil is classified as a Nekia silty-clay loam (clayey, mixed, mesic Xeric Haplohumult) with a pH of 5.1, 35% clay, and 3.7% carbon. Soil samples were air-dried, passed through a 4.75-mm sieve, homogenized, and stored at 4°C until the start of the experiment.

Growing Conditions

Black plastic containers (600 cm^3) were filled with 500 g of air-dried soil and wetted to 25% volumetric water content (approximate bulk density of 1.1 g cm^{-3}). Duct tape was placed over the holes in the bottoms of the containers to keep the soil in and discourage roots from growing out. Prior to planting, soil-filled containers were kept in a greenhouse for 4 weeks in order to allow any weed seeds to germinate. All germinated seeds were removed from the soil.

Seeds of annual ryegrass were germinated in the laboratory on moist filter paper in glass petri dishes. After 5 d, seedlings were transferred to the containers (two seedlings per container) in the greenhouse. Soil water content was adjusted every 2 d by weighing six randomly selected containers and determining the average amount of water necessary to maintain 25% volumetric water content.

Light intensity averaged 500 μmol m^{-2} s^{-1} with a 16-hr photoperiod. Plants were randomly moved at every watering period to reduce any potential bias in growing conditions. Maximum and minimum temperatures were recorded daily and converted into GDDs. Maximum daily temperatures ranged from 21 to 35°C; daily minimum temperatures ranged from 8 to 18°C.

Chamber and Labeling

A gas-tight labeling chamber (405 mm x 405 mm x 585 mm – length x width x height) was constructed out of 1/4-inch (6.4 mm) thick Plexiglass. The chamber was placed on a piece of Plexiglass (600 mm x 600 mm) that had the dimensions of the chamber lined with ¾-inch (19 mm) wide weather stripping to create a gas-tight seal. A hole drilled in the top of the chamber and was fitted with a rubber septum for use in labeling. A shelf (150 mm x 100 mm) was constructed inside the chamber to hold labeling supplies along with two, 60-mm square 12-volt fans to promote air circulation. Temperature and condensation were controlled by flowing water through a copper coil that was attached to a water bath maintained at 10°C. Temperature in the chamber averaged 27°C, with chamber temperature increasing throughout the day during each of the two labeling periods. The copper coil was wrapped around the perimeter of the bottom one-fourth of the chamber to avoid shading effects. Two holes were drilled on opposite sides of the chamber with ports to fit tubing for a LI-COR 6200 CO_2 analyzer (LI-COR Inc. Lincoln,

NE). Supplemental lighting was placed approximately 200 mm above the chamber to enhance photosynthesis during labeling.

Prior to each labeling period 20 plants were randomly divided into two groups of ten, which allowed the plants to be spaced out and reduce any effects of shading on the evenness of $^{13}CO_2$ assimilation. Eleven containers (ten planted and one unplanted) were placed in the chamber and allowed to assimilate CO_2 until the concentration fell to 200 ppm (v/v). During this time the overall photosynthetic rate was determined and subsequently used to predict the rate of $^{13}CO_2$ assimilation. $^{13}CO_2$ cannot be directly monitored with the LI-6200 because the infrared range is set for $^{12}CO_2$ and only slightly overlaps with the range for $^{13}CO_2$ (Svejcar et al., 1990). To initiate $^{13}CO_2$ labeling, 1 mL of 1.5 M lactic acid was added to a beaker containing 22.4 mg $NaH^{13}CO_3$ (99.9 atom $\%^{13}C$ - $NaHCO_3$ – Cambridge Isotope Laboratories, Andover, MA), which increased the CO_2 concentration to about 600 ppm. Once the CO_2 concentration fell below 200 ppm, lactic acid was added to an adjacent beaker containing 22.4 mg of unlabeled $NaHCO_3$, which increased the CO_2 concentration by about 400 ppm. When the CO_2 concentration fell below 200 ppm the 11 containers were removed from the labeling chamber and placed in a similar Plexiglas chamber to maximize the assimilation of any respired $^{13}CO_2$ while the other set of containers was being labeled. Each set of 11 containers underwent this labeling procedure three times, however, the CO_2 concentration was only increased by about 200 ppm during the last labeling period (11.2 mg $NaH^{13}CO_3$). Care was taken to minimize any shading

effects or labeling biases by rotating the set of containers 180° between the first and second labeling and another 90° between the second and third labeling period. Once each set of plants had undergone all three labeling events, all 20 plants were placed back in the chamber for an additional 1.5 hr while sufficient $^{12}CO_2$ was evolved to increase CO_2 concentration by 400 ppm and assimilated three times in an effort to minimize loss of $^{13}CO_2$. Two days prior to the second labeling period, seed heads were removed from plants to enhance translocation of the ^{13}C label to the roots and associated soil. Once labeling had occurred, any new seed heads were left on the plants.

Based on the information obtained in the preliminary experiment, labeling of active root growth plants (Labeling Period 1) took place at 809 accumulated GDDs (41 calendar days) and harvesting of these plants continued through 992 accumulated GDDs (49 calendar days). Rapid shoot growth labeling (Labeling Period 2) took place at 1014 accumulated GDDs (50 calendar days; the day after the last set of plants labeled at rapid root growth had been harvested) and harvesting of these plants continued through 1179 accumulated GDDs (58 calendar days). Prior to the first labeling event, two plants were harvested, dried, and weighed (one at 574 GDDs and one at 742 GDDs) to assess whether the roots were growing at a similar rate as observed in the preliminary experiment.

Harvesting Procedure

On each sampling day, four replicate plants (previously determined through random number generation) were removed from their containers and weighed. The root/soil systems were sliced down the middle and shaken up in plastic bags until approximately four-fifths of the initial weight was in the bag – this portion was considered "bulk soil." The remaining one-fifth that was still attached to the root system was considered "rhizosphere soil." The rhizosphere soil was then carefully removed from the roots with a probe and forceps. Root fragments remaining in the bulk or rhizosphere soil were removed by passing through a 1-mm sieve. Any roots still present were removed with forceps. Soil samples that were not used for immediate analysis were stored in plastic bags at -20°C.

Plant and soil samples were dried in an oven at 65° C for 48 hr. Soil was ground to a fine powder with a mortar and pestle. During the grinding process, any roots were removed with forceps. Dried plant material was weighed, transferred into glass jars, and placed on a roller grinder with eight to ten stainless steel rods until ground to a powder (2 to 4 d).

Mycorrhizae Staining

A standard staining procedure was used to assess the presence or absence of mycorrhizae (Brundrett et al., 1996). Briefly, roots were rinsed in tap water, placed in Tissue-Tek plastic capsules (Fisher Scientific Co., Pittsburgh, PA), and steamed for 45 minutes at 100°C in a 10% KOH solution. The KOH solution was decanted

and the roots were thoroughly rinsed in tap water. Cleared roots were stained by steaming them in a solution of 0.5% trypan-blue in lactoglycerol. After staining, roots were rinsed in tap water and examined with a dissecting scope for the presence of diagnostic features of arbuscular mycorrhizae.

Microbial Biomass Carbon

Soil was analyzed for microbial biomass carbon (MBC) immediately following harvest. The fumigation-extraction procedure of Vance et al. (1987) was used, as modified by Bruulsema and Duxbury (1996) for ^{13}C analysis. Briefly, 15-g samples (wet weight) of fumigated (24 hr) and non-fumigated soils were placed on a shaker for 1 hr with 30 mL of 0.05 M K_2SO_4 and subsequently filtered through Whatman #40 filter papers. Two, successive 0.5-mL aliquots of each K_2SO_4 extract were pipetted onto acetone-rinsed tin squares (37 by 37 mm) (Environmental Microanalysis, Manchester, MA) held in depressions of ceramic well-plates. Well-plates containing the tins were placed in a drying oven at 60°C for 1.5 hr prior to adding the second 0.5 mL aliquot of K_2SO_4 extract. The following morning, the tin discs were balled up with gloved hands. Samples were analyzed for total C and ^{13}C abundance (see below). A K_C of 0.45 was used to convert chloroform flush carbon values into MBC (Vance et al., 1987). The following equation was used to determine the $\delta^{13}C$ value of MBC:

$$\delta^{13}C_{MBC} = (\delta^{13}C_{fum} \times C_{fum} - \delta^{13}C_{cont} \times C_{cont})/(C_{fum} - C_{cont}) \qquad \text{[Eq. 2]}$$

where C_{fum} and C_{cont} refer to the mass of C extracted from the fumigated and the non-fumigated, respectively, and $\delta^{13}C_{fum}$ and $\delta^{13}C_{cont}$ refer to their corresponding $\delta^{13}C$ values.

PLFA Analysis

Phospholipid fatty acids (PLFAs) were analyzed on frozen (-20°C) rhizosphere and bulk soils sampled 1 and 8 d following $^{13}CO_2$ labeling. Additionally, soils from Day 2 of the first labeling period were analyzed on freshly sampled soil. Lipids were extracted according to the procedure of Bligh and Dyer (1959). Briefly, soil (15 g wet weight) was shaken for 2 hr (200 RPM) in a single-phase mixture of chloroform, methanol, and 50 mM phosphate buffer (ratio of 1:2:0.8) and allowed to sit overnight. Lipids were extracted the following day by centrifuging (1000 RPM for 5 min) and filtering the supernatant through Whatman #1 filter papers. This step was repeated once after resuspending the remaining soil with additional chloroform and methanol (same 1:2 ratio). Following filtration, 15 mL of 2 M NaCl was added to the chloroform-methanol-buffer solution in order to separate the phases. The bottom (chloroform) phase was then pipetted into a 40-mL serum bottle and immediately dried under a stream of N_2. Phospholipids were separated from neutral and glycolipids on solid phase extraction columns containing 500 mg of silica (Supelco, Inc., Bellefonte, PA). Dried lipid extracts were re-dissolved in chloroform and transferred in three, 1-mL aliquots to columns that had been preconditioned with chloroform. Neutral lipids were eluted with two,

3-mL additions of chloroform, after which the glycolipids were eluted with two, 3-mL additions of acetone. Phospholipids were then eluted with three, 2-mL additions of methanol and immediately dried under N_2. Dried phospholipids were then converted to fatty acid methyl esters (FAMEs) through mild alkaline methanolysis by dissolving in 1 mL of 1:1 methanol:toluene and 1 mL of 0.2 M KOH and heating for 15 minutes in a water bath at 38 to 42°C. FAMEs were then extracted by adding 2 mL of de-ionized water, 0.3 mL of 1 M acetic acid, and 0.5 mL of hexane. The hexane/toluene phase was transferred to a fresh test tube. Two separate, 0.5-mL aliquots of hexane were then added to the original test tube and subsequently transferred to the test tube containing the hexane/toluene. All the hexane/toluene was then dried under N_2. Dried samples were then re-dissolved in three, 50-μL additions of hexane and transferred to glass inserts (200 μL) placed inside 2-mL amber vials (Agilent Inc., Palo Alto, CA) and analyzed by capillary GC-combustion-isotope ratio mass spectrometry (GC-C-IRMS).

The $\delta^{13}C$ values of individual PLFAs were determined with an Agilent 6890 gas chromatograph (Agilent Inc., Palo Alto, CA) equipped with a 30-m HP Innowax column (internal diameter, 0.25 mm; film thickness, 0.25 μm) connected to a Europa ORCHID on-line combustion interface in-line with a Europa 20-20 mass spectrometer (Europa Scientific, Cheshire, England). The carrier gas was He, and the oven temperature was ramped up from 120 to 260°C, at a rate of 5°C per min with a 5-min hold at 260°C. Carbon dioxide of known isotopic composition was injected at the beginning and end of each run. Individual PLFA peaks were

determined by comparison with retention times of authentic standards. PLFA peaks were confirmed by comparing peaks run on a 25-m HP Ultra-2 column (internal diameter, 0.2 mm; film thickness, 0.33 μm) with the MIDI system (Microbial ID, Inc., Newark, DE) and by GC-MS. In addition, PLFA extracts of similar soils were analyzed on the HP Innowax column and were then cross-referenced with a mixture of 37 fatty acid methyl esters (FAME 37 47885-4, Supelco, Inc, Bellefonte, PA), a mixture of 24 bacterial fatty acid methyl esters (P-BAME 24 47080-U, Supelco, Inc, Bellefonte, PA), 18:2ω6,9c, 10Me16:0, 10Me18:0 (Matreya, Pleasant Gap, PA), and MIDI standards (Microbial ID, Inc., Newark, DE). Standard curves were produced with tridecanoic FAME (Supelco, Inc, Bellefonte, PA). In the standard curves, isotopic discrimination was observed in peaks smaller than 52 pmol; these peaks were therefore excluded from the data set.

During the methylation step, when phospholipids are cleaved from fatty acids to form fatty acid methyl esters (FAMEs), an additional carbon atom is added to the fatty acid molecule. This additional carbon atom, of known $\delta^{13}C$ value (-44‰), was accounted for in the $\delta^{13}C$ values of the original PLFAs with the following equation:

$$\delta^{13}C_{PLFA} = [(C_{PLFA} + 1) * \delta^{13}C_{P\text{-}FAME} - \delta^{13}C_{MeOH}]/C_{P\text{-}FAME} \qquad [Eq. 3]$$

where C_{PLFA} and $\delta^{13}C_{PLFA}$ refer to the number of carbon atoms and the $\delta^{13}C$ value, respectively, of the PLFA; $C_{P\text{-}FAME}$ and $\delta^{13}C_{PFAME}$ refer to the number of carbon

atoms and the $\delta^{13}C$ value after derivitization, and $\delta^{13}C_{MeOH}$ refers to the $\delta^{13}C$ value of the methanol used for methylation.

Standard nomenclature is used to describe PLFAs. The number before the colon refers to the total number of carbon atoms; the number(s) following the colon refers to the number of double bonds and their location (after the 'ω') in the fatty acid molecule. Notations: "Me," "OH," "cy," "i," and "a" refer to methyl group, hydroxy, cyclopropane groups, and iso- and anteiso-branched fatty acids, respectively.

Twelve peaks, a total of 16 PLFAs, were used for data analysis; these 12 PLFAs accounted for approximately 70% of the total PLFA peak area. Although the HP Innowax column allowed for good separation of the fungal marker, 18:2ω6,9, some of the more common PLFAs were not separated by this column. These include: 16:1ω9, 16:1ω7, and 10Me16:0, which merged into one peak (denoted as 16:1ω7+), and 18:1ω9, 18:1ω7, and 10Me18:0, which also merged into one peak (denoted as 18:1ω7+).

Light Fraction

Because several of the common microbial PLFAs overlap with those found in plant material, it was necessary to determine if the obtained PLFA profiles and $\delta^{13}C$ values were representative of the soil microbial community and not plant material. To do this, 15-g soil samples were suspended in 20 mL of 50 mM phosphate buffer and centrifuged for 5 minutes at 10,000 RPM after which the

supernatant containing any litter was decanted and vacuum filtered through Whatman #1 filter papers. The centrifugation/filtering procedure was repeated twice and in between each repetition the soil was re-suspended prior to centrifugation. Phospholipids were extracted as described above. Litter material remaining on the filter papers was extracted for PLFAs or dried in an oven for 24 hr at 65°C, weighed, ground with a mortar and pestle, and analyzed for $\delta^{13}C$ abundance. The $\delta^{13}C$ values of the light fraction material were incorporated into a simple mixing model to determine the maximum amount of root material that could be residing in the so-called light fraction:

$$\% \text{ of } {}^{13}C \text{ derived from roots} = \qquad \text{[Eq. 4]}$$

$$100*((\delta^{13}C_{\text{Labeled LF}} - \delta^{13}C_{\text{Unlabeled LF}})/(\delta^{13}C_{\text{LabeledRT}} - \delta^{13}C_{\text{UnlabeledLF}}))$$

where LF and RT refer to light fraction and roots, respectively.

Isotopic Analysis

Plant, soil, light fraction, and K_2SO_4 extracts were analyzed for total C and $\delta^{13}C$ value on a PDZ Europa 20/20 isotope ratio mass spectrometer (IRMS) (Cheshire, England) interfaced with a Europa Roboprep Dumas combustion/reduction apparatus (Cheshire, England). Isotopic compositions were determined relative to Pee Dee Belemnite (PDB) from the following equation:

$$\delta^{13}C = [({}^{13}C/{}^{12}C_{\text{sample}})/({}^{13}C/{}^{12}C_{\text{standard}}) - 1] \times 1000 \qquad \text{[Eq. 5]}$$

Statistics

Analysis of variance (ANOVA) was used to evaluate all time effects using the SAS statistical software package (SAS Institute, 1996), with each labeling period analyzed separately unless otherwise noted. Overall effects of time on mole percentages and $\delta^{13}C$ values of PLFAs were determined by multivariate analysis of variance (MANOVA); labeling periods were analyzed together for mole percentage data and separately for $\delta^{13}C$ value data. For all analyses, rhizosphere and bulk soils were analyzed separately because they were not independent of each other. Differences between rhizosphere and bulk soil properties were evaluated using paired t-tests. Significant differences are reported at the $p \leq 0.05$ level. Data are reported as means of four replicates unless otherwise noted.

All PLFA community analyses were conducted with the PC-ORD statistical software package (McCune and Mefford, 1999). Non-metric multidimensional scaling (NMS; Kruskal, 1964; Mather, 1976) was used to provide a graphical representation of PLFA profile relationships. NMS is an ordination method that performs an iterative search for a placement of n entities on k dimensions (axes) that minimizes the stress of the k-dimensional configuration (McCune and Mefford, 1999). An advantage to NMS over other ordination procedures, such as principal component analysis (PCA), is that it does not assume the data is normally distributed.

PLFA mole percentage data underwent the "general relativization" option in PC-ORD to standardize individual PLFA across all samples. The "slow-and-

thorough" autopilot mode of NMS in PC-ORD used the best of 40 runs with a random starting configuration using the real data and 50 runs using randomized data for a Monte Carlo test of significance. Final stability was evaluated for each run by examining plots of stress (a measure of the dissimilarity between ordinations in the original p-dimensional space and in the reduced dimensional space) versus number of iterations. Sorenson distances were used to construct ordination plots.

Results

Plant Growth

In the preliminary GDD experiment, there was a clear distinction between the active and non-active root growth stages, with the active stage occurring between 800 to 1000 accumulated GDDs (Figure 2.1A). The active shoot growth stage overlapped with the active root growth stage; however, shoot growth continued throughout 1400 GDDs. Plant growth in the labeling experiment followed a similar trend, except that the start of the active root and shoot growth stages occurred around 600 GDDs (Figure 2.1B), compared to 800 GDDs in the preliminary experiment. It should be noted, however, that in the preliminary experiment, containers were seeded with only one plant, versus two in the labeling experiment, and flower/seed heads were not removed from shoots in the preliminary experiment, but they were in the labeling experiment (in order to favor translocation of some ^{13}C into the soil). The combination of these different conditions is reflected in the dissimilar amounts of plant biomass obtained on a

given GDD in each experiment. The difference in time required for reaching the active root growth developmental stage in the preliminary experiment compared to the labeling experiment could be the influence of season. Although plants were kept in the greenhouse, with a controlled photoperiod, they were still influenced by natural, outside light. The preliminary experiment took place during the winter months, whereas the labeling experiment took place during the spring months, with longer days. It is possible that increasing day length stimulated earlier development in the labeling experiment.

The first and second labeling periods began at 809 and 1014 accumulated GDDs, respectively. Because appropriate labeling times were based on the preliminary growth experiment, they ended up not taking place during the true active root and rapid shoot growth developmental stages, but rather during the transition root growth stage in Labeling Period 1 and the more active shoot growth stage in Labeling Period 2 (Figure 2.1B). It is evident that the shoots were more active than the roots during the second labeling period based on the increasing shoot-to-root ratios.

Neither of the plants harvested at the start of each labeling period appeared to be mycorrhizal. Although the diagnostic morphological features of active mycorrhizae (arbuscules and vesicles) were absent from the root systems, fungal hyphae in close contact with the root system were evident.

Carbon Flow/Distribution of ^{13}C

Regardless of the efforts made to alleviate potential $^{13}CO_2$ assimilation biases (i.e., rotating plants in the chamber), the labeling of plants was still quite variable. Figure 2.2 shows the amount of ^{13}C retained in the plant/soil system at each day throughout the entire 8-d chase period within each of the two labeling periods. Enough ^{13}C was added so that each plant would obtain 5.6 mg ^{13}C if 100% was assimilated. As seen in Figure 2.2, on the first day after labeling, the average amount of ^{13}C per plant was 3.1 mg (55% of added) in the first labeling period and 2.7 mg (48% of added) in the second labeling period. Because respiration was not accounted for, the difference between what was added and what was retained is the sum of the amount that was never assimilated and what was respired the first day. There were no statistically significant differences in the amount of ^{13}C retained on any of the days within or between labeling periods. Although not significant, there was quite a difference in the amount of ^{13}C retained on the second day of each labeling period, much of which is likely due to inherent plant-to-plant variability. In the first labeling period, the plants that were harvested on the second day were, on average, 25% smaller than the plants harvested on the first day and 35% smaller than those harvested on the third day (Figure 2.1B). It is possible that these plants simply did not have as much leaf area for photosynthesis compared to the rest of the plants, and therefore did not assimilate as much $^{13}CO_2$. In comparison, the plants that were harvested on the second day of the second labeling period were, on average, 6% bigger than the plants harvested the day

before them and 13% bigger than those plants harvested 1 d after them. These plants probably had more photosynthetic capacity than the plants harvested on Day 1, and therefore were able to assimilate a greater amount of $^{13}CO_2$ on the day of labeling.

Throughout the two chase periods, the $\delta^{13}C$ values in shoots and roots were all significantly higher than the shoots and roots of the unlabeled control plants (shoots: -28.6 ± 0.4‰; roots: -27.4 ± 0.3‰). In the first labeling period, the average shoot $\delta^{13}C$ value was 690‰ the day after labeling and declined to 380‰ by the eighth day (Figure 2.3). Consistent with a lesser amount of total ^{13}C in the plant/soil system (Figure 2.2), plants harvested on Day 2 of the first labeling period also had a lower average $\delta^{13}C$ value compared to plants harvested a day later. This provides further evidence that the plants harvested on Day 2, with lower total plant biomass, were not as photosynthetically active relative to plants harvested on Day 3. If these plants had assimilated the same amount as the others, then this would be reflected in their $\delta^{13}C$ value, which would have been higher, not lower, than those harvested on Day 3. Overall, the dynamics of $\delta^{13}C$ values in the roots followed a similar trend to the shoots; however, there were no significant differences between the root $\delta^{13}C$ values throughout the entire labeling period.

In the second labeling period, shoots had an average $\delta^{13}C$ value of 500‰ 1 d after labeling. By Day 8, the average shoot $\delta^{13}C$ value had declined to 350‰; however, there were no significant differences in shoot $\delta^{13}C$ values throughout the entire chase period. There appeared to be some shoot-to-root translocation between

Days 1 and 2. Following Day 2, root $\delta^{13}C$ values generally declined, although there was a subtle increase between Days 5 to 8, which was not significant and is likely the result of initial labeling variability.

Throughout each chase period, the $\delta^{13}C$ values of both the rhizosphere and bulk soils were significantly higher than the unlabeled, planted control soils (rhizosphere: -27.2 ± 0.03‰; bulk: -27.2 ± 0.04‰). Aside from the variability associated with Day 2 of the first labeling period, the $\delta^{13}C$ value in the rhizosphere soil declined steadily during the 8 d, from an average of -21.6‰ on Day 1 to -25.3‰ by Day 8 (Figure 2.4). Conversely, the $\delta^{13}C$ value in the bulk soil did not significantly change during the 8 d. In the second labeling period, the $\delta^{13}C$ values in the rhizosphere soil also showed an overall decreasing trend from an average of -23.8‰ on the first day to –25.1‰ on Day 8; however, there were no significant differences with time. Similar to the first labeling period, the $\delta^{13}C$ values in the bulk soil in the second labeling period did not significantly change with time.

Overall, the total amount of ^{13}C retained in each labeling period was similar, as was the distribution of the label within each of the four compartments (shoots, roots, rhizosphere soil, and bulk soil). Including the amount of ^{13}C residing in each of the four compartments on each day throughout the entire 8-d chase period: 46% of the added ^{13}C was retained in the first labeling period, whereas 48% was retained in the second labeling period. Table 2.1 shows the distribution of the amounts retained in each of the four compartments on Days 1 and 8 of each labeling period. It appears as though labeling period had a slight, but

not significant, influence on carbon allocation from the shoots to the roots, with more carbon being allocated to the roots in the first labeling period. Overall, plants from each labeling period transferred about 10% of their photosynthate to the soil, with slightly higher amounts transferred to the rhizosphere than the bulk soils in Labeling Period 1 and vice versa in Labeling Period 2. Because rhizosphere soil was operationally defined as the 100 g of soil most closely associated with the roots, there may have been some sampling variability in the second labeling period when roots were occupying a greater portion of the container. This could explain why more ^{13}C reached the bulk soil in the second labeling period.

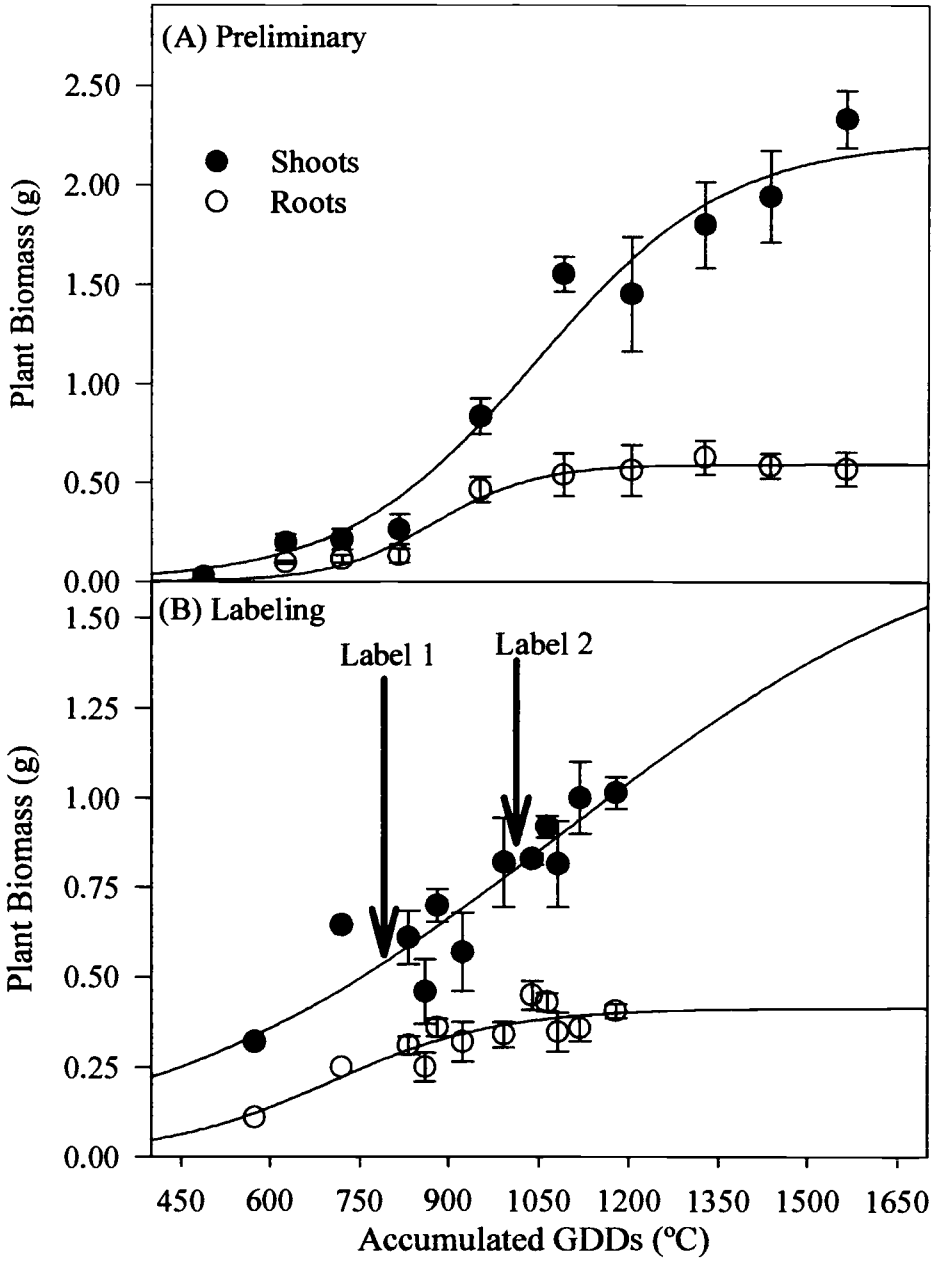

Figure 2.1. Change in root and shoot biomass with accumulated growing degree days (GDDs) (with SE, n = 4) in (A) the preliminary growth study and (B) the labeling experiments. Arrows indicate when labeling took place.

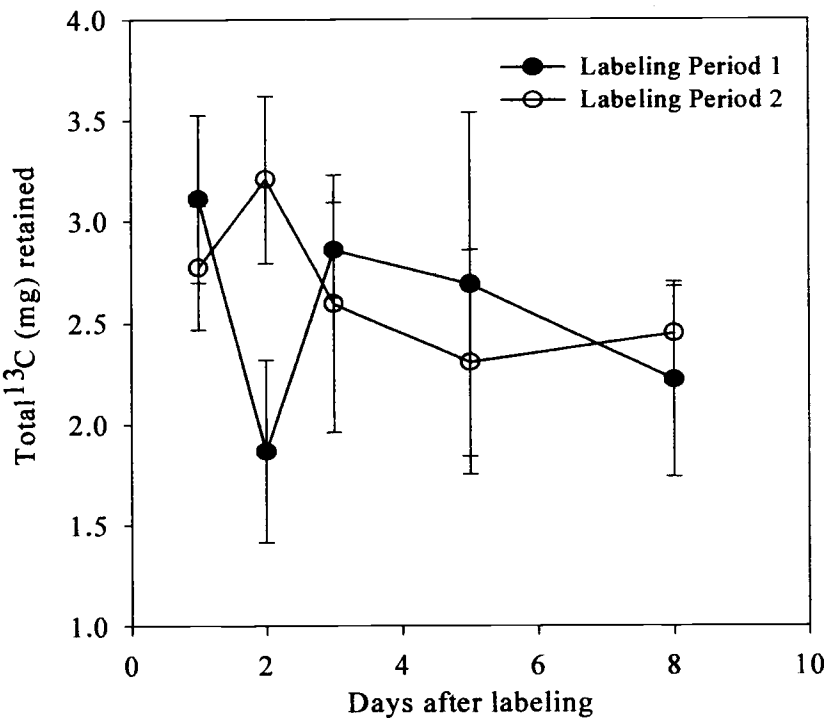

Figure 2.2. Average ^{13}C retained in each compartment (shoots, roots, and soil) throughout 8-d chase period of each labeling period (with SE, n = 4). Approximately 5.6 mg of ^{13}C was added per plant.

Table 2.1. Distribution of the ^{13}C retained on Days 1 and 8 of each labeling period (mean with SE, n = 4). In each labeling period, within shoots, roots, rhizosphere (rhiz.) soil, or bulk soil, dissimilar letters 'a and b' indicate significant differences between Day 1 and Day 8. Significant differences between Label Period 1 and 2 within shoots, roots, rhizosphere (rhiz.) soil, or bulk soil for that day are indicated with 'x and y.'

	Labeling Period			
	1		2	
	Day 1	Day 8	Day 1	Day 8
% in Shoots	68.8 (1.2)	67.8 (3.7)	72.1 (4.9)	68.5 (3.5)
% in Roots	18.9 (1.6)	23.2 (2.6)	15.7 (2.8)	19.8 (2.9)
% in Rhiz. Soil	7.2 (0.9)a	3.7 (0.6)b	5.0 (0.7)	3.7 (0.3)
% in Bulk Soil	5.2 (0.8)	5.3 (1.0)y	7.1 (1.6)	8.0 (0.4)x

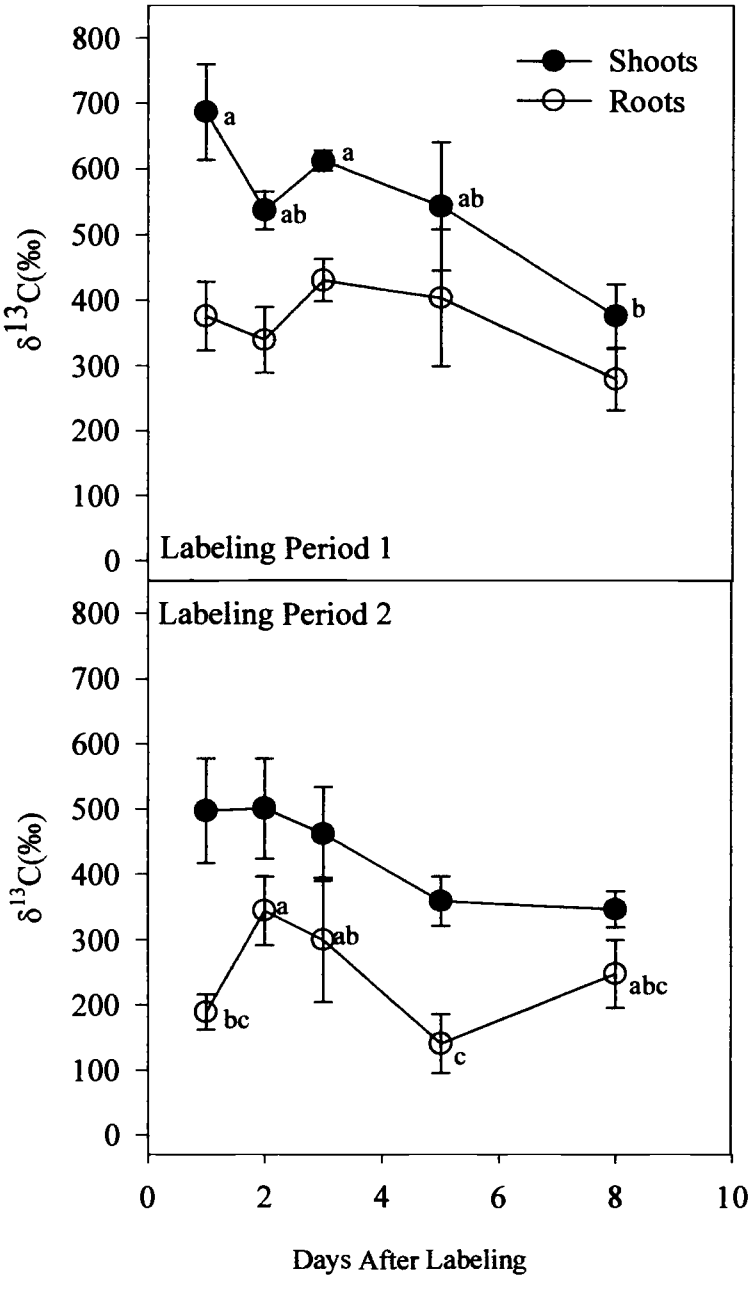

Figure 2.3. Average $\delta^{13}C$ values for shoots and roots throughout the 8-d chase period in each labeling period (with SE, n = 4). Within shoots or roots of each labeling period, dissimilar letters represent significant differences.

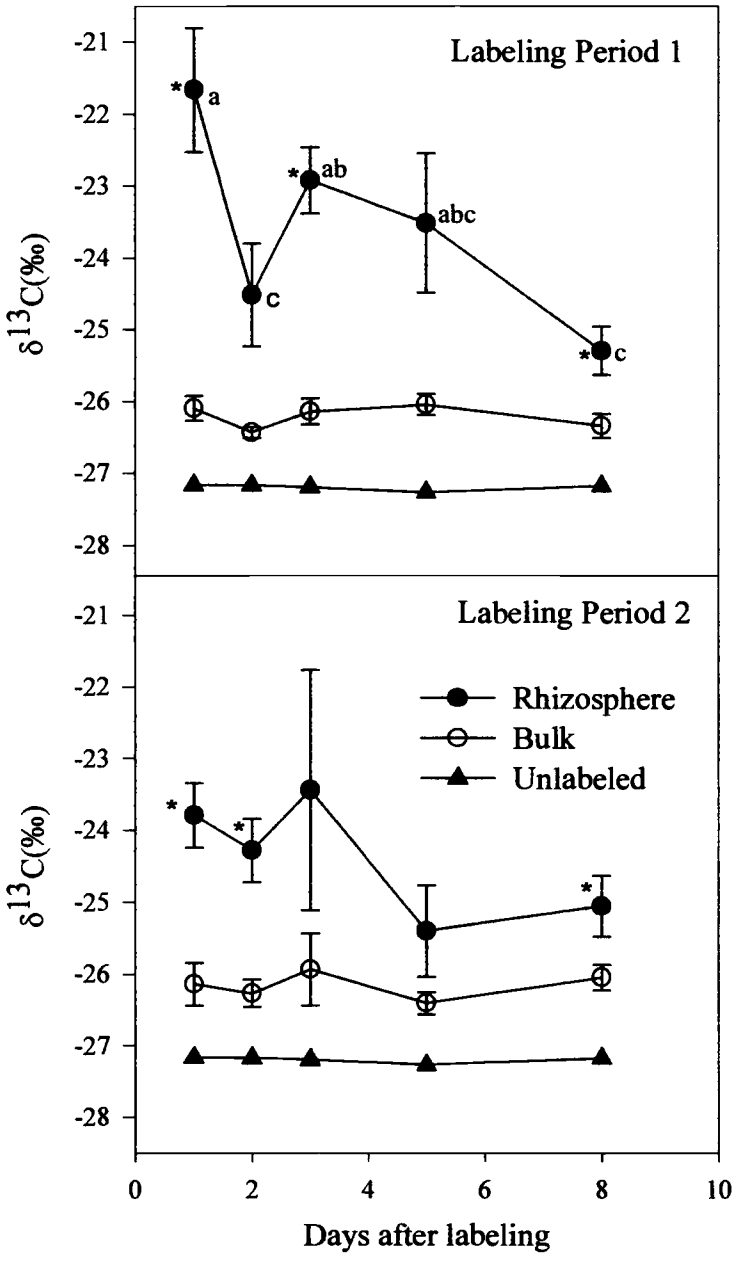

Figure 2.4. Average $\delta^{13}C$ values of rhizosphere, bulk, and unlabeled control soils (rhizosphere and bulk averaged for control) throughout the 8-d chase period in each labeling period (with SE, n = 4). The '*' to the left of rhizosphere soil indicates a significant difference between the $\delta^{13}C$ values in the rhizosphere and bulk soil. Within rhizosphere or bulk soils of each labeling period, dissimilar letters indicate significant differences.

^{13}C Incorporation into Microbial Biomass

Soluble carbon (unfumigated K_2SO_4 extracts) in both the rhizosphere and bulk soils of the first labeling period showed a downward trend throughout the 8-d chase period; whereas in the second labeling period, no clear trend was evident (Figure 2.5). In the first labeling period, soluble carbon was higher in the rhizosphere than in the bulk soil throughout the 8 d; however, these differences were only significant on Days 1, 5, and 8. In the second labeling period, soluble carbon was significantly higher in the rhizosphere compared to the bulk soil on Days 1, 2, and 5. Overall, there did not appear to be a clear distinction between the amount of soluble carbon in the rhizosphere and bulk soil.

Microbial biomass carbon (MBC) varied throughout the experiment (Figure 2.6). In the first labeling period there was a significant decline in the rhizosphere MBC between the first and second sampling days, followed by a steady increase through Day 8. The bulk MBC followed a similar trend but appeared to lag behind the rhizosphere MBC by 1 d. In the second labeling period, the rhizosphere MBC also declined between Days 1 to 2, along with the bulk MBC declining a day later. These differences were not significant, however. There was a significant difference between MBC in the rhizosphere and bulk soils throughout the first labeling period, with the exception of Day 2 when they were equal. There was a trend of higher MBC in the rhizosphere relative to the bulk soil in the second labeling period; however, this difference was only significant on the first day.

Except for in the rhizosphere on Day 1 and Day 2 in Labeling Period 1, the $\delta^{13}C$ values of soluble carbon pools did not significantly change throughout the entire chase periods (Figure 2.7). Soluble carbon in one of the four containers sampled on the first day of the first labeling period had a $\delta^{13}C$ value of 220‰ in the rhizosphere compared to an average of 22‰ for the other three plants sampled that day. This point was therefore removed from the data set prior to data analysis. The significance of this point is not known, but may be the result of plant-to-plant variability. In each labeling period soluble carbon in the rhizosphere had significantly higher $\delta^{13}C$ values relative to the unlabeled, planted and unplanted control soils (-26.5 ± 0.1‰). In the first labeling period, the bulk soil $\delta^{13}C$ value was only significantly higher than the control from Days 2 through 8, whereas in the second labeling period, the bulk $\delta^{13}C$ value was significantly higher throughout the entire chase period. Throughout the entire chase periods, soluble carbon in the rhizosphere soil had higher $\delta^{13}C$ values relative to the bulk soil; in the first labeling period this difference was significant on every sampling day, whereas it was only significant on Days 2 and 8 of the second labeling period.

Figure 2.8 shows the ^{13}C incorporation into the MBC pool. Throughout each of the chase periods, the $\delta^{13}C$ values of the rhizosphere and bulk MBC were significantly higher than the $\delta^{13}C$ values of the unlabeled, planted control MBC (-24.7 ± 0.04‰). Furthermore, there was no evidence of substantial autotrophic activity, as revealed by the $\delta^{13}C$ values of the microbial biomass in the unplanted control soils that underwent the labeling treatment (-24.5 ± 0.1‰). Initial

incorporation of rhizodeposited ^{13}C into the MBC occurred within the first 24 hr of labeling, as is illustrated by the high $\delta^{13}C$ values 1 d after labeling in of each labeling period (Figure 2.8). In the first labeling period, rhizosphere MBC $\delta^{13}C$ values had a downward trend throughout the 8-day chase period (from 460‰ on Day 1 to 50‰ on Day 8). The bulk soil MBC also declined from 64‰ on Day 1 to 1‰ on Day 8. Similar trends were evident in the second labeling period. The $\delta^{13}C$ values of the MBC declined in the rhizosphere soil from 220‰ on Day 1 to 40‰ on Day 8 and in the bulk soil from 60‰ on Day 1 to 10‰ on Day 8. Rhizosphere microbial biomass was significantly more labeled than the bulk microbial biomass on Days 1, 3, and 8 of the first labeling period and Days 1, 2, 5, and 8 of the second labeling period.

There was a strong correlation between the $\delta^{13}C$ values of the rhizosphere and bulk microbial biomass in each labeling period (Labeling Period 1: $R^2 = 0.77$; Labeling Period 2: $R^2 = 0.82$). The rhizosphere MBC was six times more highly labeled than the bulk MBC in the first labeling period, whereas in the second labeling period, the rhizosphere MBC was only, on average, three times more highly labeled than the bulk MBC.

The proportion of the ^{13}C in the soil that resided in the microbial biomass pool on each sample day is shown in Figure 2.9. On Day 1, 97% of the ^{13}C in the rhizosphere soil resided in the microbial biomass pool in the first labeling period, compared to 85% in Labeling Period 2. In the bulk soil, 88% of the ^{13}C label resided in the MBC pool in Labeling Period 1, whereas 68% resided in this pool in

the second labeling period. By the end of the 8-d chase period in Labeling Period 1, 42% of the rhizosphere soil ^{13}C resided in the rhizosphere MBC, whereas 36% of the bulk soil ^{13}C resided in the bulk MBC pool. In the second labeling period, 27% of the rhizosphere soil ^{13}C resided in the rhizosphere MBC and 23% of the bulk soil ^{13}C resided in the bulk MBC pool. There were no significant differences between the percentages of soil ^{13}C in the rhizosphere MBC compared to the bulk MBC in either chase period.

Figure 2.10 shows the proportion of the total amount of ^{13}C retained in the plant/soil system, on each sample day, that resided in the rhizosphere and bulk soil microbial biomass pools. In the first labeling period, the rhizosphere and bulk soil MBC initially represented 7% and 4% of the total ^{13}C, respectively on Day 1; by Day 8 the rhizosphere and bulk MBC each represented only 1 to 2% of the total ^{13}C in the plant/soil system, respectively. Similar trends were observed in the second labeling period, where 4.2% of the total ^{13}C was initially found in the rhizosphere and bulk MBC pools. The only significant difference between the percentage of ^{13}C in the rhizosphere and bulk MBC was on the first day of Labeling Period 1, whereas Days 5 and 8 of the second labeling period had a significantly higher percentages of total ^{13}C in the bulk MBC than in the rhizosphere MBC.

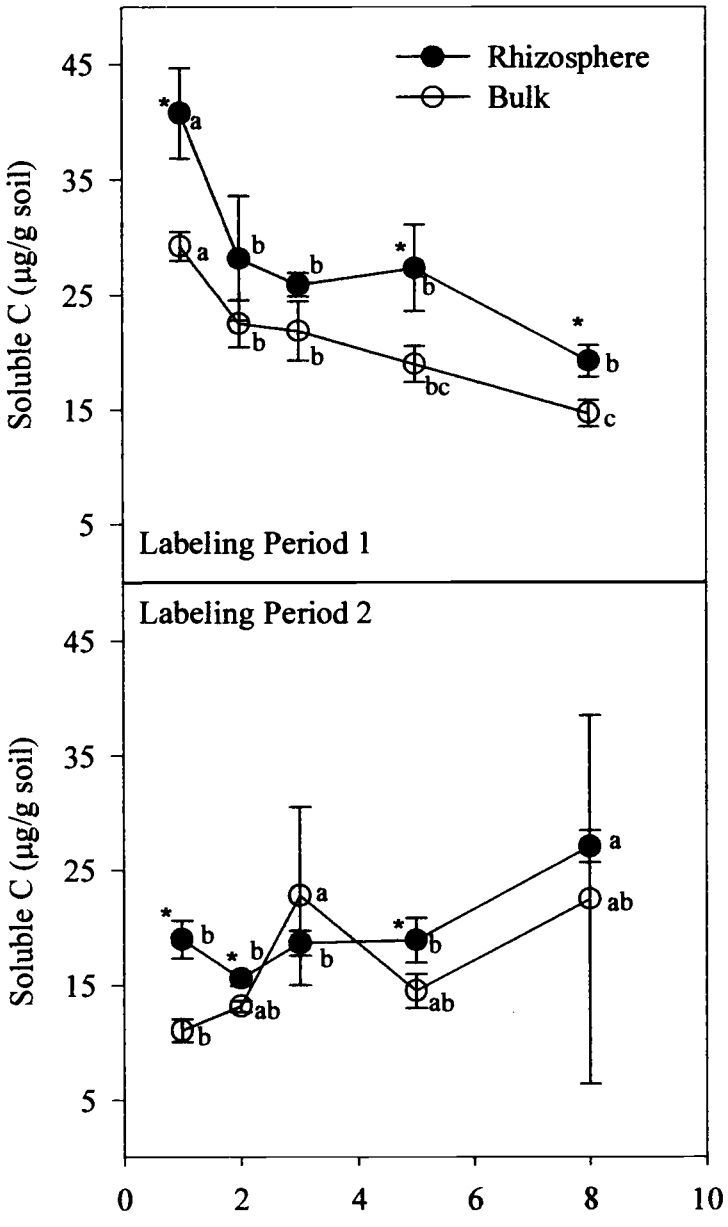

Figure 2.5. Soluble carbon (µg/g soil) in the rhizosphere and bulk soil throughout the 8-d chase periods (means with SE, n = 4). Within the rhizosphere or bulk samples, dissimilar letters indicate significant differences. The '*' to the left of rhizosphere samples indicate significant differences between the rhizosphere and bulk samples on that day.

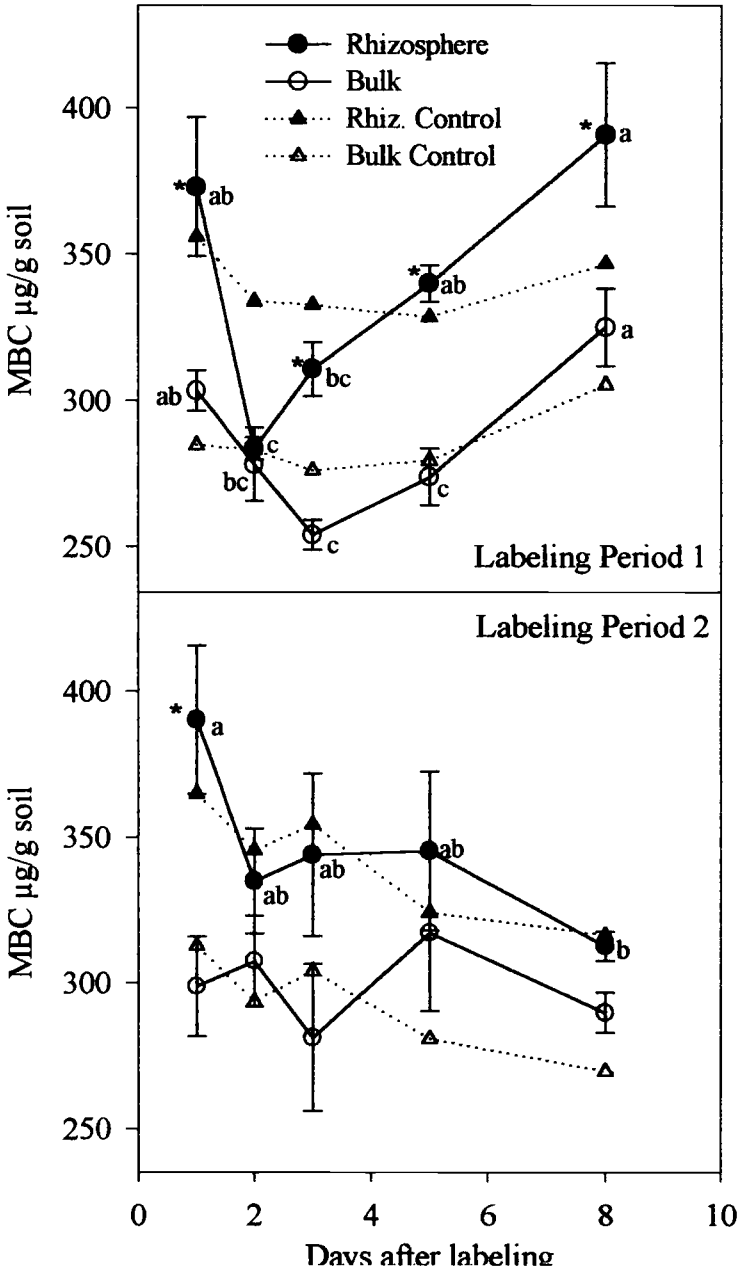

Figure 2.6. Microbial biomass carbon (MBC; µg/g soil) in rhizosphere and bulk soil throughout the two 8-d chase periods (mean with SE, n = 4). 'Rhiz. Control' and 'Bulk Control' were not subjected to labeling (n = 1). Within labeled rhizosphere or bulk samples, dissimilar letters indicate significant differences. '*' indicates significant differences between rhizosphere and bulk samples on that day.

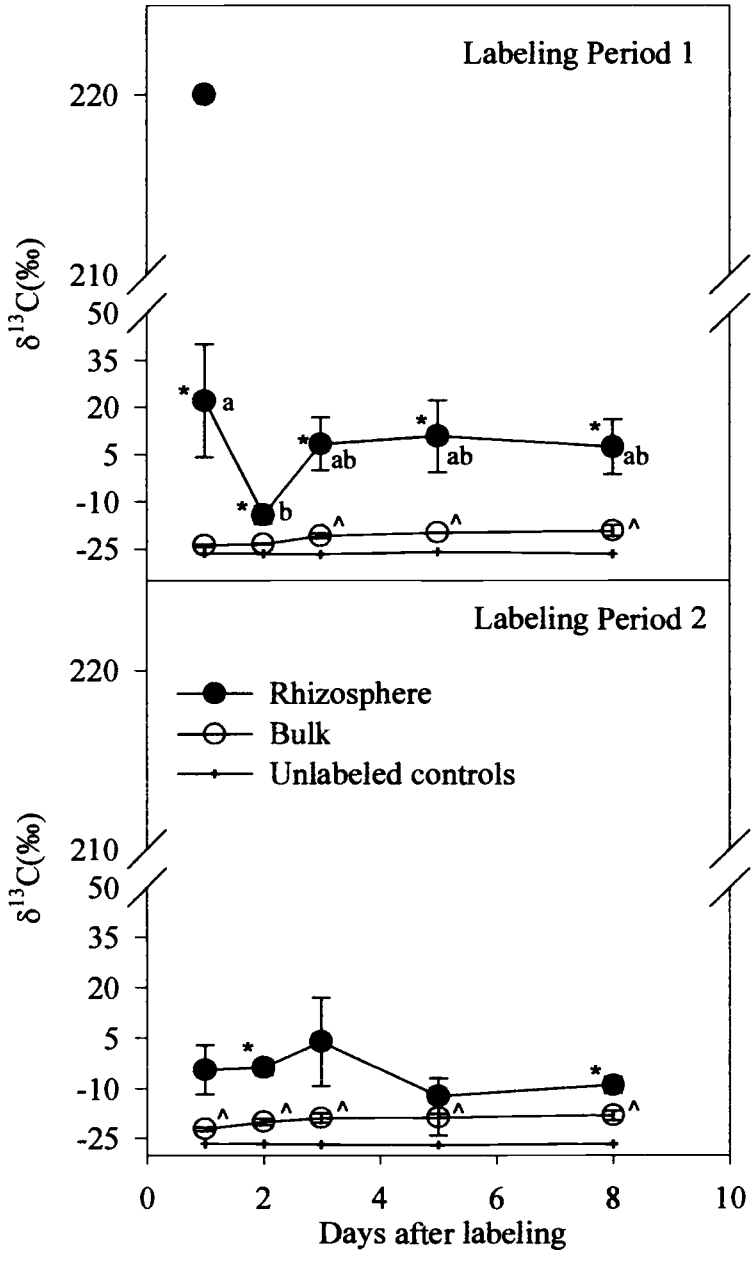

Figure 2.7. Average $\delta^{13}C$ values of soluble carbon in rhizosphere and bulk soils throughout the two 8-d chase periods (with SE, n = 4). Within rhizosphere or bulk samples, dissimilar letters indicate significant differences. The outlier in the rhizosphere of Labeling Period 1 was removed prior to data analysis. The '*' indicate significant differences between the rhizosphere and bulk samples and the '^' indicate significant differences between the bulk samples and the unlabeled control samples.

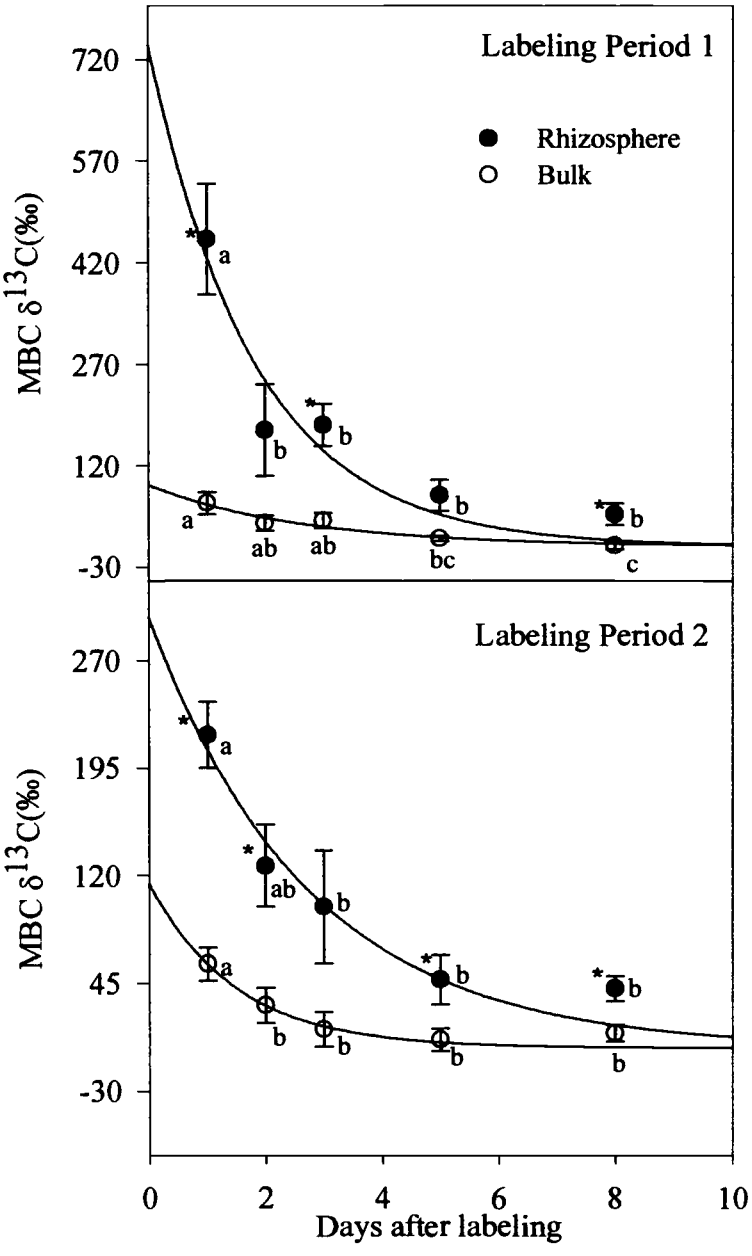

Figure 2.8. Average $\delta^{13}C$ values of rhizosphere and bulk microbial biomass (MBC) throughout the 8-d chase periods (with SE n = 4). Dissimilar letters within rhizosphere or bulk samples indicate significant differences. The '*' to the left of the rhizosphere samples indicate significant differences between the rhizosphere and bulk samples. The R^2 for the negative exponential line fit in Label Period 1 was 0.87 in the rhizosphere and 0.91 in the bulk soil. In Label Period 2 the R^2 was 0.93 in the rhizosphere and 0.93 in the bulk soil.

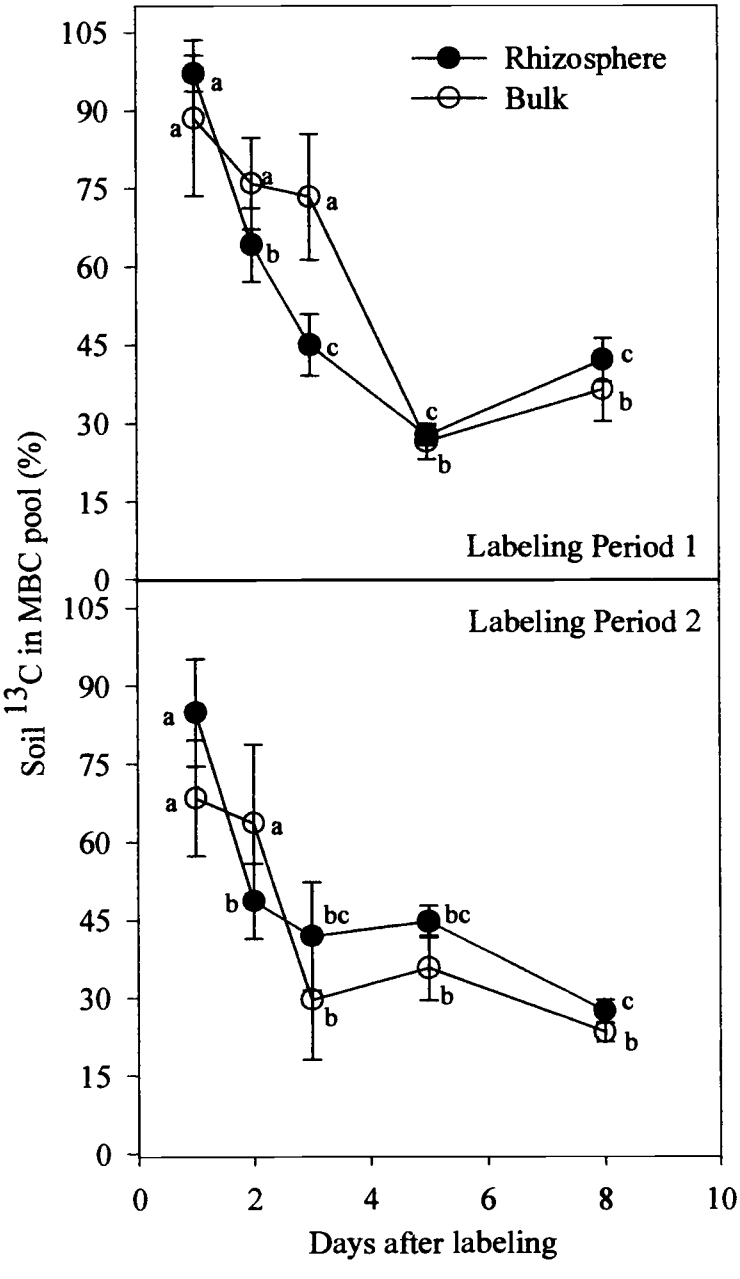

Figure 2.9. Percentages of soil ^{13}C residing in the microbial biomass (MBC) pool, for rhizosphere and bulk samples throughout the two 8-d chase periods (mean with SE, n = 4). Within rhizosphere or bulk samples, dissimilar letters indicate significant differences.

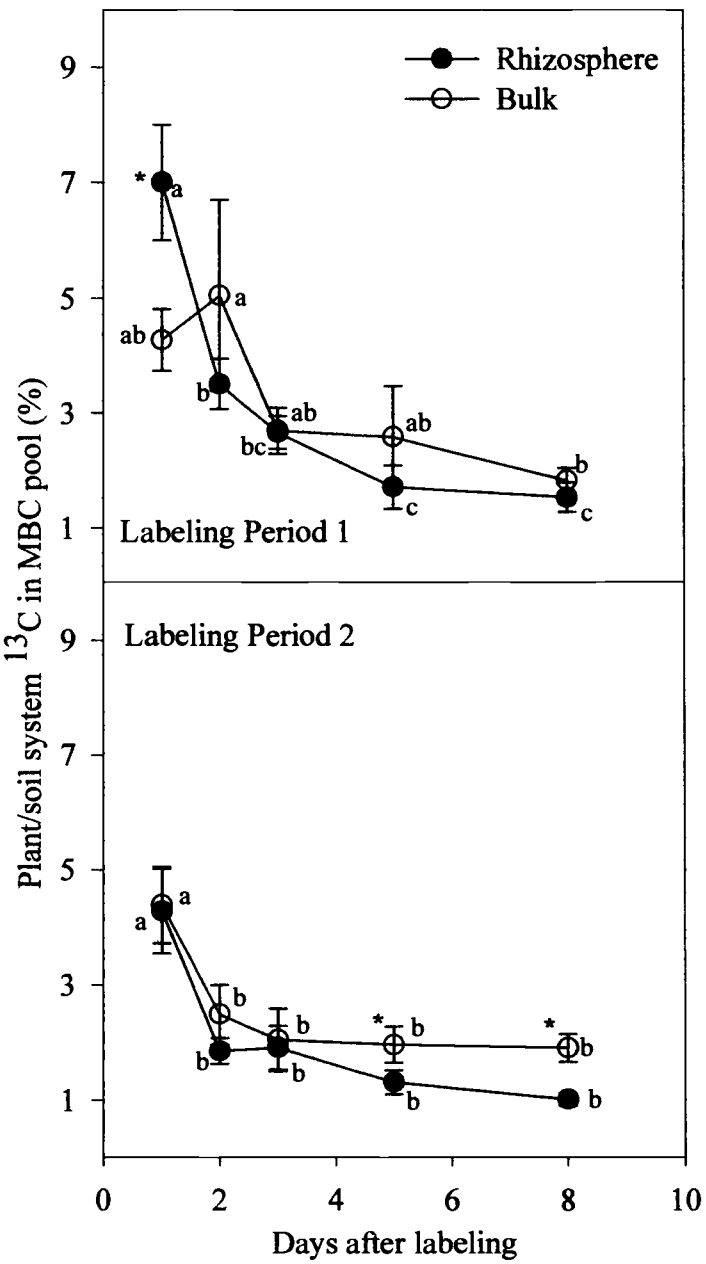

Figure 2.10. Percentage of plant/soil system ^{13}C that resides in the microbial biomass (MBC) pool throughout the two 8-d chase periods (mean with SE, n = 4). Within rhizosphere or bulk samples, dissimilar letters indicate significant differences. The '*' to the left of rhizosphere samples indicate significant differences between the rhizosphere and bulk samples.

PLFAs

Because recovery rates were not determined through use of an internal standard, PLFA data are expressed on a mole percentage basis. PLFA mole percentage data from Days 1 and 8 of Labeling Periods 1 and 2 were analyzed together, with rhizosphere and bulk samples analyzed separately. Because samples from Days 1 and 8 of each labeling period were extracted on frozen soil, whereas samples from Day 2 of the first labeling period were handled differently by extracting freshly sampled soil, data from Day 2 were removed from the data set. Data from Day 2 was subsequently analyzed with Labeling Period 1 data only and will therefore be discussed with reference to the first labeling period only.

A list of the PLFAs used in the data analysis, along with their mole percentages is shown in Table 2.2. Figure 2.11 (A) shows the non-metric multidimensional scaling (NMS) plot of PLFA community profile data for rhizosphere and bulk soils sampled on Days 1 and 8 of each labeling period; Figure 2.11 (B) shows the corresponding weights of the individual PLFAs. Generally, the lower molecular weight fatty acids have negative values on both axes, and the higher molecular weight PLFAs have positive values on both axes, with the exception of the 18:1ω7+ having a slightly negative value on axis one. Symbols with a 'c' signify the unlabeled control soils for that given time period (Figure 2.11A). Because the control samples group with their labeled counterparts, for the most part, I assumed that the actual labeling treatment (i.e., sitting in a chamber for

1 d) did not affect overall PLFA profiles. Consequently, the control samples were removed from the data set prior to further data analysis.

There appeared to be a trend with age: the majority of the samples from the first labeling period had positive axis two values and the majority of samples from Labeling Period 2 had negative axis two values. Axis 1 separated those samples that had higher amounts of 18:0 and 18:2ω6,9. There was a subtle separation between the rhizosphere and bulk samples on the first day of the first labeling period, with the bulk samples appearing more closely related to samples in the second labeling. There were very few significant differences in mole percentages of individual PLFAs between the rhizosphere and bulk soils (Table 2.2). On Day 1 of the first labeling period, 15:0a and 16:1ω7+ were significantly higher in the bulk soil, whereas 19:0cy was significantly higher in the rhizosphere soil. On Day 1 of the second labeling period, 17:0cy was significantly higher in the bulk soil; whereas on day 8, 16:1ω7+ was significantly higher in the bulk soil and 16:0i and 19:0cy were significantly higher in the rhizosphere soil. There were few time effects on the overall PLFA profiles in the bulk soils. However, 16:1ω7+ decreased significantly with time in the bulk soil, whereas 17:0cy increased from the first day of Labeling Period 1through Day 1 of the second labeling period. In the rhizosphere samples, time/age had several significant effects on PLFA profiles. Generally, 15:0i and 15:0a increased with time and 16:1ω7+ and 18:1ω7+ decreased with time. Although only significant between the eighth days of each sampling period, 16:1ω5 also decreased with time. Cyclopropyl PLFA, 19:0cy

decreased significantly from the first day of Labeling Period 1 through the first day of Labeling Period 2, but then increased significantly on Day 8 of the second labeling period.

Figure 2.12 (A) shows the NMS plot of PLFA profile data from Labeling Period 1 with the second day included; Figure 2.12 (B) shows the corresponding weights of the individual PLFAs. Samples from the second day of this labeling period all have positive values on both axes, whereas the majority of samples from Days 1 and 8 have negative or slightly positive values on each axis. Similar to the PLFA weights in Figure 2.11 (B), the majority of the higher molecular weight PLFAs are grouped together. In this case, however, the separation of the cyclopropyl PLFAs 17:0cy and 19:0cy was much more evident. The proportion of these cyclopropyl PLFAs were significantly higher in both the bulk and rhizosphere soils sampled on Day 2 relative to Days 1 and 8, whereas 16:1ω7+ was higher in the rhizosphere and 15:0i, 15:0a, 16:0, and 16:1ω7+, were higher in the bulk soils sampled on Days 1 and 8 (Table 2.3).

Table 2.2. Average mole percentages of individual PLFAs from Days 1 and 8 of each labeling period (with SE, n = 4). Dissimilar letters within rhizosphere or bulk samples indicate significant time differences. The '*' denotes a significant difference between rhizosphere and bulk soil samples on that day. The '+' indicates that more than one PLFA contributes to that peak: 16:1ω7+ includes 16:1ω7, 16:1ω9 and 16:0Me; 18:1ω7+ includes 18:1ω7, 18:1ω9 and 18:0Me.

	Labeling Period 1								Labeling Period 2							
	Day 1				Day 8				Day 1				Day 8			
PLFA	Rhizosphere		Bulk		Rhizosphere		Bulk		Rhizosphere		Bulk		Rhizosphere		Bulk	
	mol%															
15:0i	9.0	(0.2)b	9.8	(0.5)	9.2	(0.6)b	10.6	(0.6)	11.2	(0.3)a	10.2	(1.1)	10.9	(0.6)a	11.5	(1.3)
15:0a	*7.1	(0.2)b	7.7	(0.1)	7.7	(0.4)b	8.2	(0.3)	8.7	(0.2)a	7.9	(0.5)	7.9	(0.4)ab	8.3	(0.5)
16:0i	5.7	(0.1)	6.1	(0.7)	5.7	(0.5)	6.3	(0.4)	6.5	(0.4)	6.2	(0.2)	*6.7	(0.1)	5.9	(0.2)
16:0	12.6	(0.2)	12.7	(0.2)	12.4	(0.7)	12.7	(0.1)	13.1	(0.2)	11.9	(0.6)	12.3	(0.2)	11.9	(0.6)
16:1ω7+	*9.7	(0.1)b	10.1	(0.1)a	9.8	(0.4)b	9.7	(0.2)ab	9.7	(0.2)b	9.1	(0.5)b	*8.4	(0.3)a	9.3	(0.2)b
16:1ω5	4.4	(0.2)ab	4.6	(0.2)	5.0	(0.3)a	5.0	(0.2)	4.8	(0.2)ab	4.5	(0.1)	4.4	(0.2)b	4.5	(0.3)
17:0i	4.0	(0.3)	4.3	(0.03)	4.6	(0.7)	4.1	(0.3)	4.1	(0.1)	4.5	(0.03)	4.5	(0.3)	4.0	(0.2)
17:0cy	6.8	(0.4)	7.1	(0.4)b	7.5	(0.2)	7.5	(0.1)b	*7.3	(0.4)	8.6	(0.3)a	7.9	(0.2)	7.8	(0.5)ab
18:0	4.5	(0.2)	4.3	(0.2)	4.5	(0.6)	4.4	(0.2)	4.4	(0.5)	4.7	(0.6)	5.3	(0.3)	5.3	(0.4)
18:1ω7+	20.2	(0.7)a	19.1	(0.3)	18.9	(0.5)a	17.2	(1.2)	16.6	(0.4)b	16.6	(0.2)	15.6	(0.9)b	16.8	(1.0)
18:2ω6,9	6.1	(0.3)	5.5	(0.3)	4.5	(0.6)	5.3	(0.3)	5.2	(0.3)	6.9	(1.3)	5.9	(0.4)	6.3	(0.9)
19:0cy	*9.9	(0.1)a	8.7	(0.2)	9.2	(0.3)b	8.9	(0.3)	8.6	(0.2)b	9.0	(0.7)	*10.1	(0.2)a	8.4	(0.1)

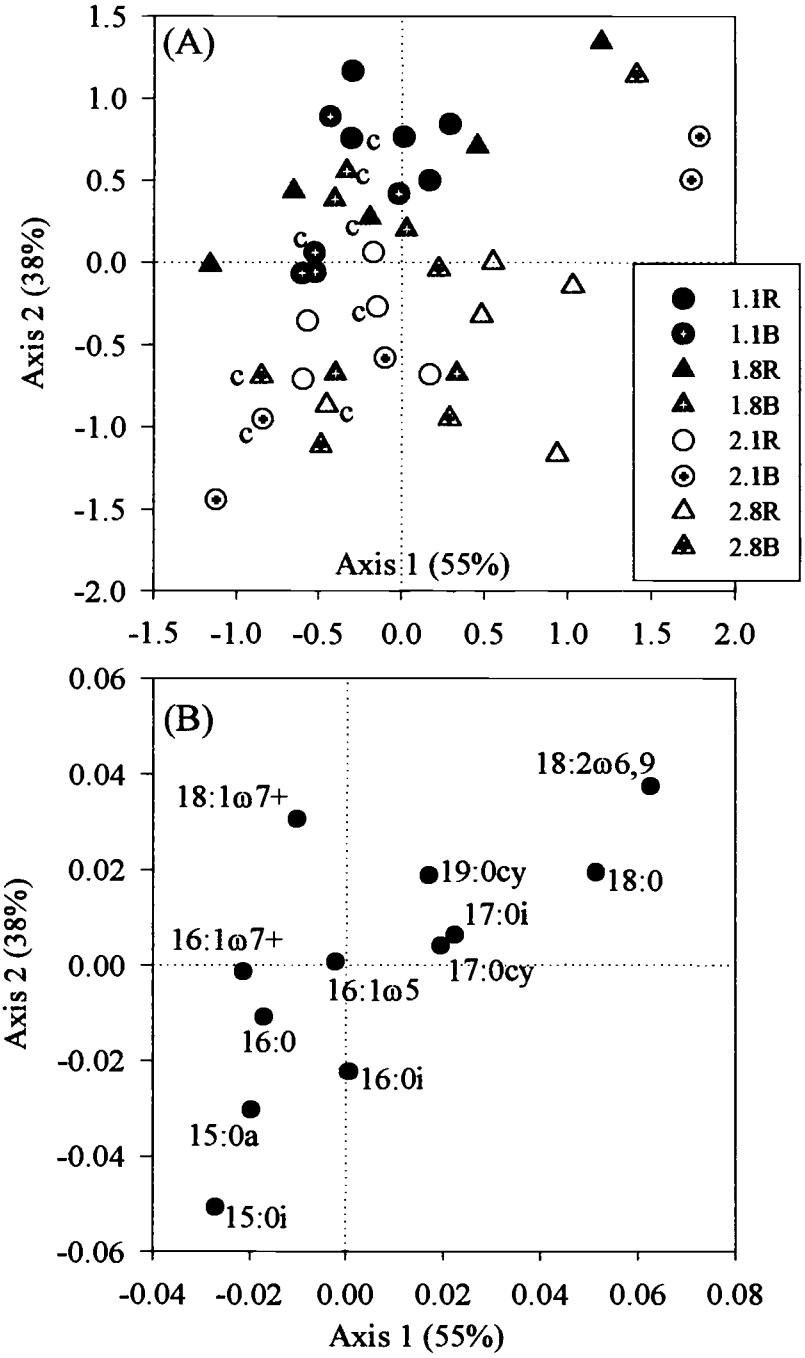

Figure 2.11. (A) Nonmetric-multidimensional scaling plot of PLFA profiles from rhizosphere and bulk soils sampled Days 1 and 8 of each labeling period. (B) Corresponding weights for individual PLFAs. Points with a 'c' are unlabeled, planted controls. (A) Legend: the first number indicates labeling period, followed by day and 'R' and 'B' indicate rhizosphere and bulk. The proportion of variance explained by each axis is indicated in parentheses.

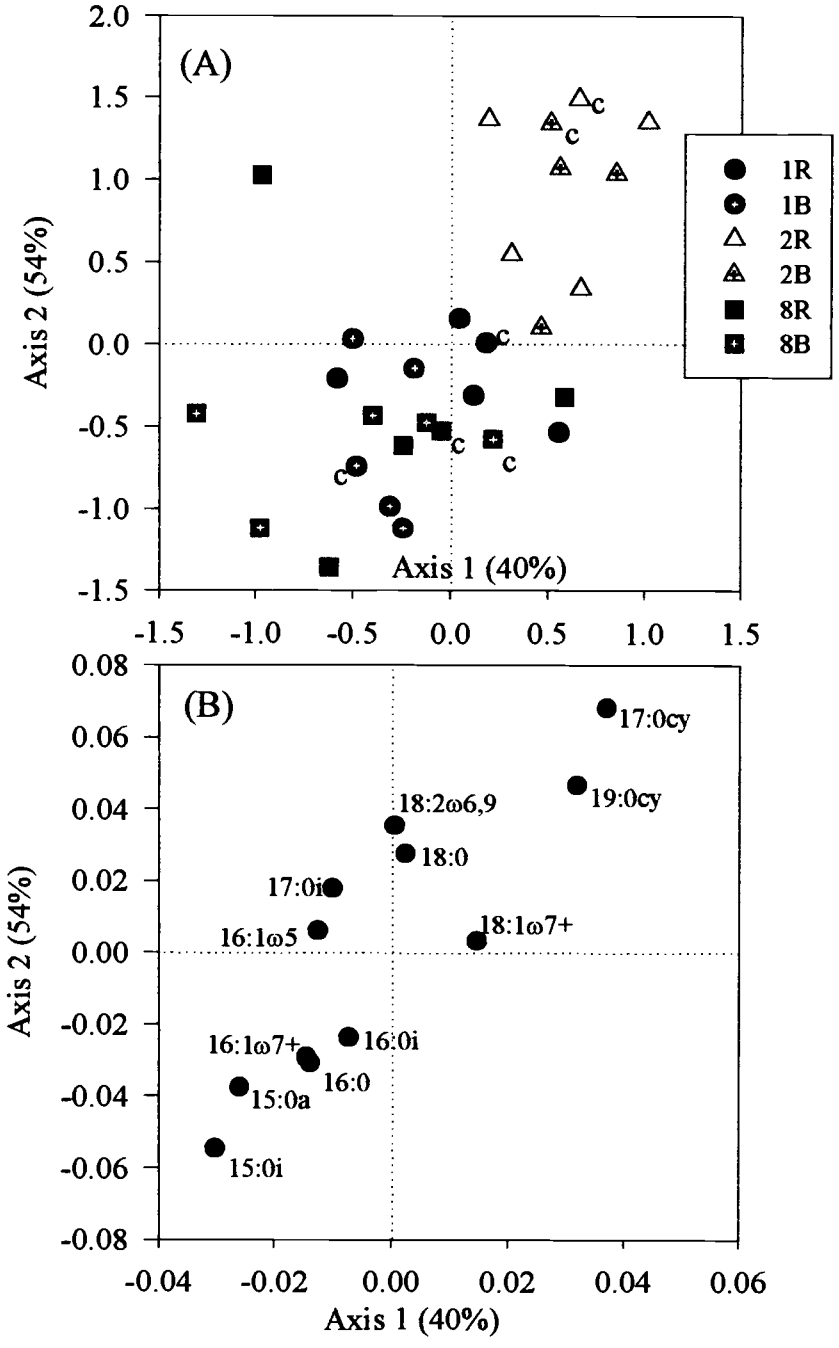

Figure 2.12. (A) Non-metric multidimensional scaling plot of PLFA profiles from Labeling Period 1, including Days 1, 2, and 8. (B) Corresponding weights for individual PLFAs. Points with a 'c' are unlabeled, planted controls. (A) Legend: the number refers to the day after labeling and 'R' or 'B' indicate rhizosphere or bulk soil. The proportion of variance explained by each axis is indicated in parentheses.

Table 2.3. Average mole percentages and $\delta^{13}C$ values of PLFAs from freshly sampled soil on Day 2 of Labeling Period 1 (with SE, n = 4 for rhizosphere samples and n = 3 for bulk samples.)

	mol%		$\delta^{13}C$ (‰)	
PLFA	Rhizosphere	Bulk	Rhizosphere	Bulk
15:0i	7.7 (0.4)y	7.9 (0.3)xy	*-21.5 (2.3)x	^-29.1 (1.1)xy
15:0a	6.4 (0.2)y	6.5 (0.2)xy	-18.7 (5.2)x	^-27.4 (1.4)x
16:0i	5.2 (0.3)	5.4 (0.1)	-11.4 (8.4)	^-30.7 (1.5)x
16:0	11.5 (0.4)	10.9 (0.3)xy	57.4 (21.6)y	-4.8 (2.2)
16:1ω7+	8.5 (0.3)xy	8.7 (0.2)xy	-21.5 (4.9)x	^-28.4 (2.0)x
16:1ω5	4.7 (0.1)	4.7 (0.1)	6.5 (2.1)	-6.7 (5.6)
17:0i	4.0 (0.02)	4.4 (0.1)	-14.2 (9.1)	^-24.8 (5.9)
17:0cy	10.4 (1.0)xy	9.4 (0.9)xy	-24.7 (2.8)x	^-30.9 (1.0)
18:0	4.5 (0.2)	4.5 (0.4)	5.0 (8.8)	-20.4 (2.6)
18:1ω7+	19.5 (0.2)	20.0 (0.5)y	1.1 (7.8)x	-18.7 (1.5)x
18:2ω6,9	6.2 (0.4)	5.7 (0.3)	292.3 (95.6)	59.5 (20.6)
19:0cy	11.5 (0.5)xy	12.1 (0.4)xy	-8.8 (9.75)	^-31.8 (2.1)

Rhizosphere and bulk samples were analyzed separately. Within rhizosphere or bulk samples in mol% or $\delta^{13}C$ columns, 'x' indicates a significant difference from Day 1 and 'y' indicates a significant difference from Day 8 of Labeling Period 1 (see Tables 2 and 5 for data on Days 1 and 8). The '*' indicates a significant difference between rhizosphere and bulk samples and '^' indicates no significant difference from unlabeled control soils in the '$\delta^{13}C$' column.

^{13}C Incorporation into Microbial PLFAs

The $\delta^{13}C$ values of PLFAs in the unlabeled, planted control soils varied. Within each unlabeled control plant there were no consistent trends in the $\delta^{13}C$ values for each of the PLFAs in rhizosphere soil compared to bulk soil. Furthermore, 8 of the 12 peaks were not significantly different between the rhizosphere and bulk soil within all unlabeled plants; thus I used all 10 unlabeled control samples (rhizosphere and bulk soils from the five sampling days) to determine the statistical significance of the labeled samples (Table 2.4).

In the first labeling period, all of the peaks were significantly enriched in ^{13}C to some extent (Table 2.5), with the exception of 17:0cy in the rhizosphere and 19:0cy in the bulk soil. Incorporating the most label in both the rhizosphere and bulk soils in the first labeling period were 18:2ω6,9 (rhizosphere: 274 ± 35‰; bulk: 48 ± 16‰) and 16:0 (rhizosphere: 77 ± 12‰; bulk –0.8 ± 1.3‰). Just as there were significant differences in the $\delta^{13}C$ values in the rhizosphere and bulk microbial biomass, there were also significant differences in the labeling of PLFAs within the rhizosphere relative to the bulk soil in the first labeling period. With the exception of 16:1ω5, 17:0i, and 17:0cy, all other PLFAs were significantly more enriched in ^{13}C in the rhizosphere compared to the bulk soil. By the eighth day of the chase period, the $\delta^{13}C$ values in the rhizosphere had declined significantly in 15:0i, 15:0a, 16:0i, 16:0, 16:1ω7+, 18:1ω7+, and 18:2ω6,9; whereas in the bulk soil, only the 16:0 showed a significant decline relative to Day 1. Furthermore,

only 16:0, 18:1ω7+, 18:2ω6,9 were still significantly more enriched in ^{13}C in the rhizosphere soil relative to the bulk soil on Day 8.

Much like the first labeling period, the PLFAs that incorporated much of the label in the rhizosphere and bulk soils in the second labeling period were, 18:2ω6,9 (rhizosphere: 127 ± 21‰; bulk: 18 ± 11‰) and 16:0 (rhizosphere: 28 ± 8‰; bulk: -10 ± 2‰); however, 16:1ω5 was also highly enriched and actually had a higher $\delta^{13}C$ value than 16:0 (rhizosphere: 43 ± 6‰; bulk: 22 ± 6‰) (Table 2.6). In the first labeling period, all PLFAs in the rhizosphere and bulk soil, with the exception of the cyclopropyl PLFAs, had incorporated ^{13}C 1 d after labeling. In the second labeling period, several of the PLFAs did not incorporate label on Day 1 (relative to unlabeled controls), including: 15:0i, 17:0i, 17:0cy, and 19:0cy in the rhizosphere and bulk soil and 15:0a, 16:0i, and 16:1ω7+, in the bulk soil only. On Day 8 of the second labeling period, the only PLFAs in the rhizosphere that were not significantly enriched in ^{13}C, relative to the unlabeled control soils, were the cyclopropyl PLFAs 17:0cy and 19:0cy. Similar trends were observed in the bulk soils on Day 8 of the second labeling period. Several PLFAs were more enriched in ^{13}C in the rhizosphere relative to the bulk soil, including 16:0, 16:1ω5, 18:0, and 18:2ω6,9. Compared to the first labeling period where several of the PLFA $\delta^{13}C$ values declined substantially between Days 1 and 8 in the rhizosphere, in the second labeling period, only 16:1ω5 declined significantly. Conversely, the bulk soil showed a slight but significant increase in the $\delta^{13}C$ values of 15:0i, 15:0a,

16:1ω7+, and 18:1ω7+, and a decrease in the $\delta^{13}C$ values of 18:0 and 16:1ω5. The decline in 16:1ω5 and 18:0 were not significant, however.

Including data from Day 2 in Labeling Period 1, several PLFAs had already showed a significant decline in $\delta^{13}C$ values relative to Day 1, including 15:0i, 15:0a, 16:1ω7+, and 18:1ω7+ in the rhizosphere and bulk soil, and 16:0i in the bulk soil only (Table 2.3). The cyclopropyl PLFAs 17:0cy and 19:0cy showed a slight increase in their $\delta^{13}C$ values in the rhizosphere on Day 2, relative to Day 1; however, the increase was only significant for 17:0cy. Relative to Day 8, only 16:0 had a significantly higher $\delta^{13}C$ value in the rhizosphere on Day 2. Although none of the PLFAs were significantly more enriched in the bulk soil on Day 2 relative to Day 8, 15:0i was slightly, but significantly, more enriched in the bulk soil on Day 8 relative to Day 2, but not relative to Day 1.

Table 2.4. Average $\delta^{13}C$ values of PLFAs in unlabeled, planted control soils. Rhizosphere and bulk samples were averaged from each time period (with SE, n = 10).

PLFA	$\delta^{13}C$ (‰)	PLFA	$\delta^{13}C$ (‰)
15:0i	-30.1 (0.5)	17:0i	-34.5 (1.6)
15:0a	-29.1 (0.6)	17:0cy	-32.0 (1.6)
16:0i	-30.6 (1.1)	18:0	-31.9 (2.7)
16:0	-33.5 (0.5)	18:1ω7+	-32.3 (0.6)
16:1ω7+	-35.2 (1.0)	18:2ω6,9	-28.6 (2.5)
16:1ω5	-30.6 (1.6)	19:0cy	-34.2 (0.7)

Table 2.5. Average $\delta^{13}C$ values of individual PLFAs extracted from rhizosphere and bulk soils on Days 1 and 8 of Labeling Period 1 (with SE, n = 4).

Label 1	Day 1				Day 8			
PLFA	Rhizosphere		Bulk		Rhizosphere		Bulk	
	----------$\delta^{13}C$ ‰----------							
15:0i	*-8.5	(4.1)a	-21.8	(0.9)	-21.5	(1.2)b	-23.6	(0.6)
15:0a	*5.4	(8.3)a	-20.7	(1.7)	-17.4	(3.5)b	-23.5	(1.2)
16:0i	*11.4	(9.2)a	-17.4	(3.5)	-17.6	(6.3)b	^-26.5	(3.9)
16:0	*76.8	(11.9)a	-0.9	(1.3)a	*10.1	(3.7)b	-9.8	(2.4)b
16:1ω7+	*-2.7	(3.9)a	-21.1	(1.1)	-20.0	(0.8)b	-23.0	(2.1)
16:1ω5	1.0	(8.3)	-10.0	(6.2)	-2.1	(4.8)	-9.4	(4.7)
17:0i	-6.4	(5.9)	-9.6	(4.6)	-17.6	(4.4)	-19.0	(4.8)
17:0cy	^-33.3	(1.9)	^-27.5	(2.7)	^-30.0	(1.7)	-24.9	(1.9)
18:0	*18.3	(9.7)	-15.4	(4.9)	-9.0	(5.8)	-20.0	(6.1)
18:1ω7+	*15.4	(4.6)a	-14.1	(0.4)	*-8.7	(1.1)b	-16.1	(1.6)
18:2ω6,9	*274.0	(34.9)a	48.1	(15.7)	*117.4	(17.1)b	49.2	(6.2)
19:0cy	*-18.4	(2.4)	^-30.5	(1.9)	-24.3	(2.9)	^-30.9	(1.3)

Within rhizosphere or bulk samples, dissimilar letters indicate significant time differences. The '*' denotes a significant difference between rhizosphere and bulk samples on that day. The '^' indicates no significant difference from unlabeled controls. The '+' indicates that more than one PLFA contributes to that peak: 16:1ω7+ includes 16:1ω9 and 16:0Me; 18:1ω7+ includes 18:1ω9 and 18:0Me.

Table 2.6. Average $\delta^{13}C$ values of individual PLFAs extracted from rhizosphere and bulk soils on Days 1 and 8 of Labeling Period 2 (with SE, n = 4).

Label 2	Day 1				Day 8			
PLFA	Rhizosphere		Bulk		Rhizosphere		Bulk	
	----------$\delta^{13}C$ ‰----------							
15:0i	^-25.6	(1.5)	^-28.6	(0.5)b	-19.6	(3.3)	-24.6	(1.3)a
15:0a	-21.2	(2.2)	^-26.5	(0.3)b	-20.2	(1.5)	-21.0	(1.7)a
16:0i	-21.1	(3.6)	^-25.2	(0.9)	-16.9	(2.5)	^-24.8	(2.7)
16:0	*28.1	(7.7)	-7.8	(1.9)	8.7	(6.9)	-8.1	(3.0)
16:1ω7+	-24.1	(4.2)	^-30.2	(1.1)b	-16.6	(5.4)	-20.2	(3.0)a
16:1ω5	*42.8	(5.7)a	21.4	(5.9)	12.0	(5.9)b	-4.1	(4.2)
17:0i	^-26.2	(3.2)	^-26.9	(1.7)	-16.7	(9.0)	^-31.2	(5.6)
17:0cy	^-31.0	(1.6)	^-30.1	(0.8)	-23.6	(2.6)	-23.8	(3.4)
18:0	*-4.1	(3.8)	-18.3	(1.2)	^-30.8	(17.1)	^-27.0	(6.1)
18:1ω7+	-8.7	(5.3)	-21.3	(1.4)b	-7.6	(3.8)	-9.8	(2.4)a
18:2ω6,9	*127.1	(20.7)	17.6	(11.4)	73.6	(38.8)	46.5	(9.4)
19:0cy	^-26.9	(2.9)	^-34.9	(1.0)	^-29.9	(3.2)	^-34.9	(3.6)

Within rhizosphere or bulk samples, dissimilar letters indicate significant time differences. The '*' denotes a significant difference between rhizosphere and bulk samples on that day. The '^' indicates no significant difference from unlabeled controls. The '+' indicates that more than one PLFA contributes to that peak: 16:1ω7+ includes 16:1ω9 and 16:0Me; 18:1ω7+ includes 18:1ω9 and 18:0Me.

Discussion

Results from this study demonstrate that the coupling of ^{13}C pulse-chase labeling and PLFA analysis serves as a useful tool for obtaining information on the structure of the microbial communities actively involved in the cycling of rhizodeposition. Although there was a slight, but not significant, shift from more ^{13}C allocated to the roots in Labeling Period 1 to more ^{13}C allocated to shoots in the second labeling period, in general, the ^{13}C distribution was similar between the two labeling periods. This similarity is likely because plants were labeled during a stage of transition into active shoot growth and then 10 d later during a more active shoot growth stage (Figure 2.1) rather than during two distinct growth stages (i.e., active root growth and rapid shoot growth). Nevertheless, several fundamental differences associated with the microbial cycling of ^{13}C-labeled rhizodeposition were apparent, which suggests that some ecological/physiological changes had occurred within the plant/soil soil system between the first and second labeling periods.

Carbon Flow

Several studies have documented that maximum shoot and root respiration occur within the first 24 hr after pulse-labeling (Gregory and Atwell, 1991; Swinnen et al., 1994; Kuzyakov et al., 2001; Kuzyakov and Domanski, 2002). For example, in a $^{14}CO_2$ pulse-chase labeling experiment with wheat and barley, Gregory and Atwell (1991) observed that on 50-day-old plants, 15 to 25% of the

assimilated $^{14}CO_2$ was respired within the first 24 hr, the majority of which occurred in the first 7.5 hr. Others have reported shoot respiration rates as high as 50% shortly after assimilation (Warembourg and Morral, 1978). These findings, along with the possibility that the plants did not assimilate all of the $^{13}CO_2$ on the day of labeling could explain why only 50% of the added ^{13}C was retained in the plant/soil system 1 d after labeling. This trend was consistent between the two labeling periods, suggesting that they responded similarly.

Kuzyakov and Domanski (2000) put together an excellent review on above- and belowground carbon distribution in pulse-chase labeling experiments. From this review, it appears that on average, 50% (and as high as 80%) of plant-assimilated carbon is transferred belowground in pasture plants. Of this 50%, half remains in roots (25% of total assimilated) and the other half is incorporated into microbial biomass and soil organic matter or respired by roots and microorganisms. It is difficult to compare my carbon allocation results with other studies given that I am not certain what percentage of the $^{13}CO_2$ was initially assimilated or how much was respired. Overall, however, I found an average of 20% retained in roots and an average of 10% retained in soil (Table 2.1), suggesting that the belowground distribution of ^{13}C in my study was similar to previous reports.

The fact that in each of the two labeling periods there were no significant changes in the total amount of ^{13}C retained in the plant/soil system with time (although there was a decreasing trend; Figure 2.2) and that the only significant change in the distribution of ^{13}C occurred within the rhizosphere (Table 2.1)

suggests that, for the most part, the label had stabilized in the plant system within the first 24 hr. Although there might have been some subtle shifts in carbon allocation within the plant, the most dynamic flow of carbon throughout the rest of the chase period was clearly evident within the microbial biomass pool.

Through use of mathematical models, Kuzyakov et al. (2001) demonstrated that maximum $^{14}CO_2$ efflux from the soil after pulse-labeling occurs in two main phases: the first of which is root respiration, occurring during the first day after labeling, followed by the second phase, occurring between the second and fifth days, which is dominated by microbial respiration of rhizodeposits. Although I can only speculate about root respiration in my study, the latter part of these findings is generally consistent with my findings, as is shown by the declining proportion of soil ^{13}C in the microbial biomass throughout the 8-d chase periods (Figure 2.9).

After pulse-labeling 3-week-old wheat plants with $^{14}CO_2$, Cheng et al. (1993) found evidence of ^{14}C in the soil in less than 1 hr, suggesting that recently assimilated carbon moved through the system very rapidly. Additionally, Rattray et al. (1995) found maximum incorporation of ^{14}C into the microbial biomass in the rhizosphere of perennial ryegrass within 3 hr of pulse-labeling. By the seventh day of the chase period, they could no longer detect ^{14}C in the microbial biomass. Although I did not sample until 24 hr after labeling, translocation of ^{13}C into the soil and the subsequent incorporation into the microbial biomass happened rapidly (within 24 hr) in my system as well. This rapid flux is also shown by the low $\delta^{13}C$ values of the soluble carbon pools the first day after labeling (Figure 2.8).

Although ^{13}C-labeled rhizodeposition appeared in similar proportions in each of the two labeling periods (Table 2.1), differences in both the incorporation of ^{13}C-labeled rhizodeposits into the MBC pool and the subsequent turnover of ^{13}C-labeled microbial biomass were evident. The fact that only 85% of the rhizosphere soil and 70% of the bulk soil ^{13}C were incorporated into the MBC on Day 1 of the second labeling period, whereas 95% of rhizosphere ^{13}C and just under 90% of the bulk soil ^{13}C were incorporated into the MBC in the first labeling period (Figure 2.9), suggests that the quality of exudates might not have been as readily available to the microbial biomass in Labeling Period 2 as in Labeling Period 1. It has been documented that the amounts of easily metabolized proteins and carbohydrates in rhizodeposition generally decrease with age (Brimecombe et al., 2001). Thus there could have been more complex, less soluble forms of rhizodeposition entering the soil, such as sloughed-off root cap cells. Alternatively, it is possible that rhizodeposits had lower C:N ratios in Labeling Period 1 and that nitrogen availability was influencing the amount of rhizodeposition that got incorporated into the microbial biomass, which has been documented in other studies (Merckx et al., 1987; Liljeroth et al., 1990). I did not measure soil nitrogen availability, however.

Bokhari et al. (1979) found 4.5 times more soluble sugar in the rhizosphere of blue grama, whereas 1.3 times more insoluble sugar was found in the non-rhizosphere soil. Additionally, decreasing ratios of soluble-to-insoluble rhizodeposits with increasing distance from the roots has also been observed in

wheat, barley, maize, tomato and pea plants (Whipps 1984,1985,1987) thus, it is not surprising that I observed slower incorporation of rhizodeposition into the MBC in the bulk soils, relative to the rhizosphere soils.

Five days after pulse-labeling perennial ryegrass with $^{14}CO_2$, Kuzyakov et al. (2001, 2002) found between 0.5 and 1.7% of the total system ^{14}C incorporated into the microbial biomass pool, which corresponded to approximately one-third of the total soil ^{14}C (presumably the entire container was considered rhizosphere soil with nine plants and 2.2 kg soil). Similarly, in my study, between 1 and 3% of the plant/soil system ^{13}C on Day 5 resided in each of the microbial biomass pools. Although there were slight variations between the labeling periods, these values generally correspond to approximately one-third of the ^{13}C in the soil (Figure 2.9).

During the 8-d chase periods, the decline of ^{13}C in the microbial biomass carbon pool followed an exponential relationship with time (Figure 2.8). Average decay rates and turnover times for rhizosphere and bulk microbial biomass pools in each of the two labeling periods were estimated by fitting the data to a negative exponential model:

$$F^* = F_o^* e^{-kt} \qquad \text{[Eq. 6]}$$

where F_o^* is the atom fraction excess ^{13}C at time t = 0, F^* is the atom fraction excess ^{13}C at time t, and k is the decay rate. Average atom fraction ^{13}C of the soluble carbon pool was used to determine the atom fraction excess ^{13}C of MBC because I assumed that the majority of rhizodeposited ^{13}C entering the MBC pool had originated from readily available compounds in the soluble carbon pool.

Furthermore, this calculation of average turnover time is based on the assumption of steady-state conditions (i.e., constant microbial biomass pool size). Although not strictly true (MBC varied significantly at a few time points; Figure 2.6), given the high variability and random up/down fluctuations, this would seem a reasonable assumption.

Average decay constants (k) and associated turnover times (the inverse of the decay constant, k) for the rhizosphere soils were, 0.32 ± 0.07 d^{-1}, with a turnover of 3.2 d for Labeling Period 1, and 0.24 ± 0.05 d^{-1} for Labeling Period 2, with a turnover time of 4.2 d. Bulk values were: 0.18 ± 0.02 d^{-1} with a turnover time of 5.5 d in Labeling Period 1, and 0.14 ± 0.07 d^{-1} and a turnover time of 7.1 d. The turnover times were nearly twice as fast in the rhizosphere compared to the bulk soil in each labeling period. This suggests that the microorganisms in the rhizosphere were more active than in the bulk soil, which would be expected.

Faster turnover rates are generally observed in the rhizospheres of younger root systems (Kuzyakov et al., 2001), thus it is likely that I would have seen turnover rates faster than 3 d had I labeled in earlier stages of root growth. Furthermore, the slower turnover times observed in both the rhizosphere and bulk soils of Labeling Period 2 suggest that the microbial biomass was more stable at the later stage of plant growth. Norton et al. (1990) estimated 9 d for the turnover of ^{14}C labeled microbial biomass utilizing rhizodepositon under ponderosa pine seedlings. In a soil incubation study following ^{14}C-labeled glycine and ^{14}C-labeled glucose into the microbial biomass, Kuzyakov and Demin (1998) observed half-

lives of 0.6 d for glycine and 2.9 d for microbial biomass labeled with ^{14}C glucose, suggesting that simple substrates flow through the microbial biomass pool at very rapid rates.

Microbial Community Dynamics

Because many PLFAs are present to some degree in most, if not all organisms (e.g., 16:0), relating PLFAs to specific groups of organisms must be done with caution. Nevertheless, several PLFA biomarker trends have been recognized in the literature (Zelles, 1999). Additionally, because I was unable to separate some of the more common PLFAs (16:1ω9, 16:1ω7, and 10Me16:0; 18:1ω9, 18:1ω7, and 10Me18:0), I am limited in my ability to interpret the ecological significance of these PLFAs in this experiment.

The fact that there were very few differences between the rhizosphere and bulk PLFA profiles throughout the experiment could arise from my operational definition of rhizosphere soil (one-fifth of the soil most closely associated with the root system). It is possible that if I had sampled the 50 g closest to the roots more differences might have been seen. Steer and Harris (2000), however, used compartmentalized pots to separate roots of *Agrostis stolonifera* from bulk soil and did not find a separation between rhizosphere and bulk soil microbial communities until the plants were 90 days old and even then, the separation was not great.

There were clearly temporal influences on PLFA profiles; the most significant of which was the increasing proportion of branched PLFAs, 15:0i and

15:0a, which are generally synthesized by Gram-positive bacteria (O'Leary and Wilkinson, 1988). Although evident in both the rhizosphere and bulk soil, this finding was more pronounced in the rhizosphere soil. In contrast to my findings, Steer and Harris (2001) observed a decrease in the proportion of PLFAs associated with Gram-positive bacteria with increasing plant age; this was coupled with an increase in several monounsaturated fatty acids that are commonly found in Gram-negative bacteria (16:1ω7, 16:1ω5, and 18:1ω7). My data showed a decreasing trend in the proportions of 16:1ω7+ and 18:1ω7+ with time, but it is not clear if that resulted from a decline in PLFAs associated with Gram-negative bacteria or other organisms associated with the overlapping peaks (e.g., actinomycetes). An explanation for the different findings observed in this experiment compared to that of Steer and Harris (2000) is that root growth had basically ceased in my plants by the time of sampling, whereas their roots were still actively growing, especially between 90 and 120 d.

^{13}C PLFAs

Phosphoplipids are rapidly degraded upon cell death and they are not found in storage lipids (Zelles et al., 1992), therefore they serve as useful indicators of microbial growth. The incorporation rate of rhizodeposits into PLFAs was slower in the second labeling period (e.g., several of the PLFAs were more enriched in ^{13}C on Day 8 compared to Day 1) compared to the first labeling period, which suggests that the growth rate of the organisms incorporating the label was slower. This is

consistent with the lower amount of the soil ^{13}C incorporated into the total MBC pool on Day 1 of the second labeling period relative to the first labeling period, suggesting that rhizodepositon in the second labeling period was comprised of less readily available substrates compared to Labeling Period 1.

Because the 16:0 PLFA is ubiquitous, the $\delta^{13}C$ values of 16:0 were used to normalize the $\delta^{13}C$ values of the other peaks in the two labeling periods. Table 2.7 shows the average percentages of ^{13}C incorporated into each PLFA peak relative to the $\delta^{13}C$ value of 16:0 for Day 1 of each labeling period. For each peak, percentages were determined by dividing the difference between the $\delta^{13}C$ value in the labeled and unlabeled samples by the difference in $\delta^{13}C$ value of the labeled and unlabeled 16:0 PLFA. As seen in Table 2.7, on Day 1 of Labeling Period 1, with the exception of the cyclopropyl PLFAs, all PLFA peaks were at least 20% as labeled as 16:0. In contrast, only four rhizosphere and six bulk soil PLFA peaks were at least 20% as labeled as the 16:0 PLFA on Day 1 of Labeling Period 2.

Table 2.7. Average percentages of ^{13}C incorporated into each PLFA peak relative to the amount of ^{13}C incorporated in the 16:0 PLFA in rhizosphere and bulk soils on Day 1 of each labeling period. Numbers greater than 100 indicate that those peaks were as labeled or more highly labeled than 16:0. 'NL' stands for no label, where values were negative.

	Labeling Period			
	1		2	
PLFA	Rhizosphere	Bulk	Rhizosphere	Bulk
	----------	----------	%----------	----------
15:0i	20	25	7	6
15:0a	31	26	13	10
16:0i	38	40	15	31
16:0	-----	-----	-----	-----
16:1ω7+	30	43	18	20
16:1ω5	29	63	119	203
17:0i	26	76	14	30
17:0cy	NL	14	2	7
18:0	46	51	45	53
18:1ω7+	43	56	38	43
18:2ω6,9	274	235	253	180
19:0cy	14	12	12	NL

Hanson et al. (1999) found that the majority of PLFAs were enriched in ^{13}C when incubated with ^{13}C-labeled glucose (4 mg g^{-1} soil), a readily available carbon source often found in rhizodeposition. This suggests that the entire community was stimulated from the addition of a plentiful amount of glucose. In contrast, Arao (1999) found only a few PLFAs enriched in ^{13}C after incubating soil with a lesser amount of ^{13}C-acetate (1 mg g^{-1} soil), also an easily metabolized substrate. These dissimilar findings could be the result of differences in substrate quality, but more

likely the result of differences in substrate quantity, with much more glucose added compared to acetate.

The fact that 18:2ω6,9, a prominent fungal marker (Frostegård and Bååth, 1996), not associated with arbuscular mycorrhizal fungi (Olsson et al., 1998), was the most enriched in ^{13}C in each of the labeling periods suggests that the fungal populations in the rhizosphere and bulk soils were most actively utilizing rhizodeposition. This is consistent with the presence of fungal hyphae growing in close association with the root systems I examined. Griffiths et al. (1999) observed an increase in 18:2ω6,9 with increasing amounts of synthetic root exudates added to soil. Additionally, after a 24-hr incubation of two different soils amended with ^{13}C-labeled acetate, Arao (1999) found that ^{13}C was mainly incorporated into 18:2ω6,9, 16:0, and 18:1ω9. Both of these studies and mine suggest that fungal organisms are very successful at competing for simple carbon compounds and incorporating them into membrane lipids. In agreement with Arao (1999), I also found significant amounts of the ^{13}C label incorporated into16:0 and 18:1ω9. I am limited in interpreting the significance of label incorporation into 18:1ω9 because it overlaps with two other PLFAs, 18:1ω7 (commonly found in Gram-negative bacteria; Frostegård and Bååth, 1996) and 10Me18:0 (commonly found in actinomycetes; Lechevalier, 1977). Due to their ubiquity, incorporation of ^{13}C into the 16:0 and 18:0 PLFAs does not provide very useful information about the types of microorganisms involved.

A fundamental difference between the two labeling periods was the incorporation of ^{13}C into the 16:1ω5 PLFA (Table 2.7), which is found in Gram-negative bacteria (Frostegård et al., 1993b) and arbuscular mycorrhizal fungi (Olsson et al., 1995). Arbuscular mycorrhizal fungi do not appear to be a major contributor to the 16:1ω5 peak because my plants were not infected with mycorrhizae. Although it is surprising that my plants were not mycorrhizal, given that it is generally recognized that the majority of plant species (80%) form mycorrhizae (Bonfante and Perotto, 1995), it is possible that the greenhouse light intensity was not sufficient enough to maintain photosynthetic rates that would support mycorrhizae. Mycorrhizae can demand up to 20% of the total carbon assimilated by its plant partner (Sylvia, 1998), and light intensity has been shown to greatly influence mycorrhizal growth on sudangrass (Graham et al., 1982). Thus it appears that Gram-negative bacteria were the major contributors to the 16:1ω5 peak in my study.

Gram-negative bacteria associated with 16:1ω5 incorporated some ^{13}C labeled rhizodeposition in each of the labeling periods, however, there was a dramatic difference in their activity between labeling periods. In Labeling Period 1, 16:1ω5 was labeled only 29% relative to 16:0 in the rhizosphere and 63% relative to 16:0 in the bulk soil, whereas in Labeling Period 2, 16:1ω5 was labeled slightly higher than 16:0 in the rhizosphere and was twice as ^{13}C enriched as 16:0 in the bulk soil (Table 2.7). It is possible that a change in rhizodeposition quality led to a change in the activity of the Gram-negative bacterial community; thus

resulting in the Gram-negative organisms associated with 16:1ω5 actively utilizing more rhizodeposited carbon in Labeling Period 2. Interestingly though, these Gram-negative bacteria were likely utilizing the more readily available rhizodeposition in the second labeling period considering they incorporated ^{13}C into PLFAs within the first 24 hr after labeling. This is also shown by the significant decline in the 16:1ω5 $\delta^{13}C$ value in the rhizosphere and bulk soil between Days 1 and 8 of Labeling Period 2 (Table 2.6), suggesting rapid turnover of these organisms. In contrast, the $\delta^{13}C$ values of 16:1ω5 in Labeling Period 1 were not different between the rhizosphere and bulk soils, nor did they change between Days 1 and 8 (Table 2.5), suggesting that there was little turnover of these Gram-negative bacteria in the first labeling period.

Clearly, 16:1ω5 represented the biggest difference in rhizodeposition cycling among the Gram-negative bacterial community in the two labeling periods. Because of overlapping peaks, I was unable to differentiate between changes in the activity of Gram-negative bacteria associated with the 16:1ω7+ or 18:1ω7+ peaks. The cyclopropyl PLFAs, also associated with Gram-negative bacteria, are formed from the precursors 16:1ω7 and 18:1ω7 during periods of stationary growth (Guckert et al., 1986). These cyclopropyl PLFAs, 17:0cy and 19:0cy were the least labeled relative to 16:0 (Table 2.7), and this was similar in each of the two labeling periods. Thus, ^{13}C-labeled rhizodeposition was used to synthesize cyclopropyl PLFAs, however, this was not a major component of the overall activity of the Gram-negative bacterial community.

In the first labeling period, Gram-positive bacteria were actively utilizing ^{13}C-labeled rhizodeposits, as shown by the labeling of the 15:0i, 15:0a, 16:0i, and 17:0i PLFAs relative to 16:0 in the rhizosphere and bulk soils on Day 1 (Table 2.7). In contrast, in the second labeling period, these Gram-positive PLFAs were only enriched slightly, and in many cases they were not significantly different from the unlabeled control soils (Tables 2.6 and 2.7). Although there was an increase in Gram-positive organisms (mol% 15:0i and 15:0a; Table 2.2) between the two labeling periods, it appears that Gram-positive bacteria in the second labeling period either did not compete well for rhizodeposited carbon or simply utilized soil organic matter preferentially. Furthermore, it is possible that the increase in Gram-positive organisms in the second labeling period resulted from some sort of priming effect (an accelerated decomposition of soil organic matter), which often results from rhizodeposition in the later stages of plant development (Kuzyakov et al., 2001). It has been suggested that the presence of certain groups of microorganisms in the rhizospheres of different plant species is related to the variations in exudates produced (Grayston et al., 1998). Thus, temporal variations in rhizodepositon within one plant species are also likely to influence the activity and presence of certain microorganisms.

Day 2 PLFAs (Mol% and $\delta^{13}C$ Values)

It is not completely clear why the freshly extracted soils sampled on Day 2 of Labeling Period 1 exhibited such different PLFA profiles (high proportions of

cyclopropyl PLFAs 17:0cy and 19:0cy on Day 2) than Days 1 and 8 of Labeling Period 1 (Figure 2.12). A ratio of cyclopropyl PLFAs to their precursor (the cis conformation of 16:1ω7 and 18:1ω7) greater than 0.1 is generally recognized as a stationary growth phase resulting from some type of stress, such as a decrease in pH, temperature, or starvation (Guckert et al., 1986; Ponder et al., 2002). Because I could not separate out the precursor peaks, the ratios of 17:0cy to 16:1ω7c or 19:0cy to 18:1ω7c are not known. A few possible explanations for the high proportions of cyclopropyl PLFAs on Day 2 include: (1) the plant/soil system had been stressed from the high temperatures endured in the labeling chamber, (2) the plants harvested on Day 2 could have been less photosynthetically active, which led to less rhizodeposition and therefore induced a starvation or nutritional stress on the microbial community, or (3) storage could have altered the PLFA profiles of Days 1 and 8.

My initial explanation for the high mol% of cyclopropyl PLFAs on Day 2 of Labeling Period 1 was that the system had experienced some stress from the high temperatures endured in the labeling chamber, which led to the synthesis of cyclopropyl PLFAs. Because the unlabeled, planted control soils that never went in the labeling chamber, also had high proportions of cyclopropyl PLFAs, this explanation is not valid and the cyclopropyl PLFAs were probably present prior to labeling.

Although I did not measure photosynthetic activity of the unlabeled control plant harvested on Day 2, I do know that on average, the labeled plants harvested

on Day 2 tended to be smaller, were less photosynthetically active on the day of labeling than the plants harvested on Day 1 or Day 3 (Figures 2.1, 2.2, and 2.3), and supported a lower MBC (Table 2.6). Thus, the presence of these cyclopropyl PLFAs could be the result of nutritional stress induced by a decrease in photosynthetic activity, which subsequently led to a reduction of substrates available in the soil. The control plant did not have as low MBC as the labeled plants, thus it is possible that the actual labeling treatment did cause a decline in MBC; however, it does not appear to have altered the overall PLFA profiles relative to the control plant. The fact that the cyclopropyl PLFAs are not highly labeled (Table 2.3) also suggests that these PLFAs were likely present prior to labeling.

Two studies have examined the effects of storage on fatty acid profiles (Petersen and Klug, 1994; Schutter and Dick, 2000); however, neither study is directly applicable to my situation. Shutter and Dick (2000) used a method to extract total soil fatty acids (not just phospholipids) and Petersen and Klug (1994) tested the effects of storing at temperatures higher than freezing; whereas I extracted PLFAs from soil stored at -20°C. Schutter and Dick (2000) observed that profiles did change compared to those of freshly extracted soil when stored at -20°C for 1 month (similar to my samples); however no clear trends were evident and results varied among soils. Petersen and Klug (1994) found an increase in the mol% of cyclopropyl fatty acids after incubating soil for 3 weeks at 25°C, but not when incubated at 4.5 or 10°C. Overall, no clear trends demonstrating the

influences of storage protocols on fatty acid profiles were found in either study. Thus more research is needed in this area, considering that it is often impossible to extract freshly sampled soil.

PLFAs and Light Fraction

Although a considerable amount of effort went into removing all visible root material from soil during harvest, it was possible that the highly labeled PLFAs (i.e.,18:2ω6,9 and 16:0) were coming from root material remaining in the soil, as 18:2ω6,9 and 16:0 are two of the most abundant PLFAs in plant material. This concern was addressed by extracting PLFAs from light fraction (i.e., the litter material removed from soil) and soil with the light fraction removed to see how the $\delta^{13}C$ values of PLFAs would be affected. Light fraction samples that were removed from labeled soil were also analyzed for their $\delta^{13}C$ values.

A dominant PLFA in plants and soil, 16:0, was used to determine if plant derived PLFAs were present and thus contributing to the $\delta^{13}C$ values observed in soil. Table 2.8 shows the $\delta^{13}C$ values of 16:0 in soil with and without the presence of light fraction. The $\delta^{13}C$ value in all of the rhizosphere soils increased upon removal of the light fraction material, whereas bulk soils either stayed the same or declined by just 1 to 2‰, providing evidence that the labeling of PLFAs in soil was not coming from plant derived PLFAs. It is not clear why the $\delta^{13}C$ values in the rhizosphere increased upon removal of the light fraction. A possible explanation

could be that there were more unlabeled organisms associated with the light fraction in the rhizosphere and they were lost during the centrifugation process.

An average of 15.8 mg of light fraction was separated from each of the 15-g soil samples during centrifugation. When the light fraction was extracted for PLFAs, all peaks were below the detection limit, therefore, no PLFA data were obtained. Table 2.9 shows the maximum amount of plant material that could potentially be influencing the $\delta^{13}C$ value of the light fraction material. Considering that <0.64% of the bulk soil light fraction and 1.2 to 1.8% of the light fraction in the rhizosphere could be coming from root material provides further evidence that the soil PLFA $\delta^{13}C$ values are not coming from living roots. Furthermore, this calculation provides a liberal estimate of root material that could be in the light fraction given that the $\delta^{13}C$ values of the light fraction could very likely be the result of microbial biomass or labeled organic matter without any root material at all. Nevertheless, it is extremely important to remove any root material from soil prior to PLFA extraction, as the presence of highly labeled plant derived PLFAs has potential to severely alter data interpretation.

Table 2.8. $\delta^{13}C$ values of the 16:0 PLFA before and after removal of light fraction (LF) material (n = 1).

Labeling Period	Soil	Days after labeling	Soil with LF $\delta^{13}C‰$	Soil without LF $\delta^{13}C‰$
1	Rhizosphere	1	48.6	58.2
		8	7.1	23.2
	Bulk	1	0.1	-0.9
		8	-11.3	-9.4
2	Rhizosphere	1	34.4	45.5
		8	14.7	19.7
	Bulk	1	-6.2	-6.7
		8	-5.7	-7.4

Table 2.9. $\delta^{13}C$ values and amount of light fraction (LF) separated from 15 g soil samples (same amount as used in PLFA analysis). All soils were taken from Labeling Period 1 (n = 1). Rhizosphere 'R' and Bulk 'B' soils were analyzed from the same plant for unlabeled control and from Days 1 and 8 following labeling. Data in the "% of root in LF" column were determined using Equation 4; in this column, NA, not applicable, refers to unlabeled control samples and NL, no label, refers to samples that were less than zero, indicating no ^{13}C label.

Sample	$\delta^{13}C$ Soil+LF	$\delta^{13}C$ Roots	$\delta^{13}C$ LF	LF in 15 g soil (mg)	% of root in LF
Control R	-27.3	-26.7	-28.5	16.9	NA
Control B	-27.2	-26.7	-27.9	17.4	NA
Day 1 R	-23.9	172.6	-24.9	16.4	1.8
Day 1 B	-26.2	172.6	-26.6	14.7	0.7
Day 8 R	-25.0	267.0	-24.8	15.9	1.3
Day 8 B	-26.0	267.0	-28.8	13.6	NL

Conclusion

Results from this experiment confirm that recently assimilated carbon moves through the plant/soil system at a very rapid pace. Within 24 hr after labeling, approximately 10% of the total ^{13}C retained in the plant/soil system resided in the soil, much of which had already been incorporated into the soil microbial biomass. Although the overall carbon distribution patterns were similar in the two labeling periods, my results show fundamental differences in the way in which rhizodeposition was cycled through the total microbial biomass and individual PLFAs during a transition stage of plant development and in a stage more dominated by shoot growth. Thus, the use of PLFA analysis coupled with ^{13}C pulse-chase labeling appears to be a promising and effective approach for examining the microbial dynamics associated with rhizosphere carbon cycling. Application of this methodology to a range of plant species, developmental growth stages, and environmental conditions has the potential to greatly enhance our knowledge of rhizosphere processes by focusing on the members actively involved in the cycling of nutrients within this complex system.

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CHAPTER 3

SUMMARY

The flow of root-deposited carbon (rhizodeposition) through the soil microbial biomass is a key factor influencing the nutrient availability for plant growth. Due to methodological constraints imparted by the complexity of the soil environment, our knowledge of microbial dynamics associated with rhizosphere carbon flow is currently very limited. Several researchers have investigated the flow of photosynthate into the soil microbial biomass and several researchers have examined the structure of rhizosphere microbial communities; however, until now, no studies had attempted to examine the flow of rhizosphere carbon into specific members or taxonomic groups of the microbial community. A fundamental step to understanding rhizosphere carbon cycling is linking the structure of soil microbial communities with those members actively involved.

The goal of this thesis was to investigate the dynamics of rhizosphere and bulk soil microbial communities associated with the cycling of root-deposited carbon. This was accomplished through the use of recently developed methodology: ^{13}C pulse-chase labeling techniques coupled with phospholipid fatty acid (PLFA) analysis. In order to examine temporal variations of rhizosphere carbon cycling, annual ryegrass plants were pulsed with $^{13}CO_2$ during one of two labeling periods: a transition stage between active root growth and rapid shoot

growth (Labeling Period 1) or during a growth stage dominated by shoot growth (Labeling Period 2). During the 8 d following labeling in each labeling period, ^{13}C was traced through shoots, roots, soil, microbial biomass, and PLFAs.

The overall above- and belowground distribution of ^{13}C was similar between the two labeling periods. In each labeling period, ^{13}C-labeled rhizodeposition cycled through the rhizosphere microbial biomass more rapidly than the bulk soil microbial biomass. Additionally, cycling of rhizodeposition occurred more rapidly in Labeling Period 1 relative to Labeling Period 2. A likely explanation for these results is the spatial and temporal differences in the quality/availability of rhizodeposition. The temporal aspect of this was further shown by the differences in microbial community structure and differences in the microbial community members actively utilizing rhizodeposition between the two labeling periods. Overall, the results show that time/plant age has a significant influence on both the structural and functional dynamics of microbial communities associated with rhizosphere carbon flow. Furthermore, this study illustrates both the need and ability to go beyond the microbial community "black box" in order to obtain a more complete understanding of rhizosphere carbon cycling.

Because the cycling of rhizosphere carbon through the microbial community has such profound influences on soil organic matter dynamics and nutrient cycling, there remains a fundamental need to examine the structure of microbial communities actively cycling root-deposited carbon under a wide range of environmental conditions. This thesis demonstrates a promising potential for the

future use of stable isotopes in combination with PLFA analysis to directly link the structure of the microbial community with those members actively involved in rhizosphere carbon cycling.

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