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SOIL CARBON: THE RHIZOSPHERE PRIMING EFFECT AND SECOND GENERATION BIOFUELS

A dissertation submitted in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In

ENVIRONMENTAL STUDIES

By

Kelsey Forbush

June 2019

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SOIL CARBON: THE RHIZOSPHERE PRIMING EFFECT AND SECOND GENERATION BIOFUELS

Kelsey Forbush

Abstract

Anthropogenic driven climate change has the potential to dramatically reshape ecosystems worldwide. Rapid increases in atmospheric carbon dioxide and other greenhouse gases have increased global temperatures resulting in catastrophic losses of ecosystem services and biodiversity. Soils are the largest carbon reservoir in the terrestrial ecosystem, yet soil carbon is almost always excluded from global carbon models. Research in this field has been limited due to methodological and logistical difficulties. Understanding the key factors modulating soil carbon fluxes is essential for predicting future climatic conditions.

Soil organic matter (SOM) provides numerous benefits to the soil ecosystem including maintaining soil structure, improving water holding capacity, and increasing nutrient availability. Soils that have relatively low amounts of SOM are less productive and less resilient to environmental change compared to soils with high amounts of SOM. Furthermore, the most recalcitrant carbon has been found to remain in the soil for centuries, therefore is it important to investigate the biological and the physical mechanisms that determine the rate of SOM decomposition. The rhizosphere, the 2mm of soil surrounding roots, is a biological hotspot within the soil ecosystem. The effects of live roots on SOM stores can be quantified by measuring the rhizosphere priming effect (RPE) which is the change in rate of SOM decomposition due to the presence of roots.

The biological mechanisms driving the RPE have been studied in greenhouse and growth chamber experiments since the 1990s. However, much less is known about how the interactions between plant roots and soil structure dynamics effect the magnitude and direction of the RPE. In order to address this question, soybeans were grown at three different densities in a continuous ^{13}C-isotope labeling greenhouse. I measured three indicators of soil structure (1) the amount of clay particles in the leachate (2) the amount of dissolved organic carbon in the leachate and (3) aggregate size distribution. Greater rhizosphere activity was linked with increases in dissolved organic carbon and clay particles in the leachate. There was a greater proportion of large (>2mm) water stable aggregates in the higher density treatments. Together these results indicate that rhizosphere activity increases aggregate turnover, which may physically expose SOM to microbial attack and increase priming effects.

Evidence from greenhouse and laboratory experiments have indicated that plant roots and rhizosphere microbes jointly regulate the rate of SOM decomposition. However, due to methodological challenges, it is unknown how the RPE manifests at the ecological scale. Individual live roots from five woodies species were excavated from the field, inserted into chambers filled with native soil, and were incubated in the field for fifty days. A novel pulse trapping method was used to sample root and soil respiration in the field during a 48 hour period. Root and soil respiration were distinguished based on ^{13}C partitioning. Generally, greater root biomass at the end of the infield incubation was associated with higher priming effects. Species specific effects were also found. These results are consistent with laboratory studies, and

suggest that this method can be used in the future in order to gain a better understanding of root-soil interactions in near field conditions.

Preserving soil carbon stores through targeted agricultural practices has been a subject of interest in the U.S. since the 1930s. The Conservation Reserve Program recommends switchgrass for soil remediation due to its tolerance for saline and sodic soils and its extensive root system. Biofuel production in the U.S. has garnered increasing attention since the 1990s due to a national interest in energy independence and potential impacts of fossil-fuel-related climate change. Switchgrass, a grass native to the Midwestern United States, improves soil quality, stores carbon deep in the soil, and provides habitat for birds. Increasing the production of biofuel from switchgrass may possibly decrease carbon emissions, increase carbon sequestration, and improve rural economies. I summarize the current social, technological, and logistical impediments to second generation biofuel production. I then examine the role of uncertainty, both political and economic, and its role in the second generation biofuels industry. I conclude that without crop insurance for farmers growing biofuel crops and increases in subsidies, farmers are unlikely to grow enough switchgrass for biofuel to meet the federal mandate. Instead, it is likely that farmers will meet the cellulosic mandate by selling corn stover.

Acknowledgements

This work was supported by a grant from the U.S. National Science Foundation (Grant No.DEB-1354098). I thank Jon Eldon for his help in a pre-experiment related to Chapter One and Jenna Merrilees, for her laboratory assistance. I thank, Chris Barnard, Alex Paya, Shuxia Jia, and the KPBS field technicians, Patrick O'Neal and Jeffrey Taylor, for their invaluable help in field pre-experiments related to Chapter Two. I thank Peter Brommer, an undergraduate assistant, for his laboratory and field assistance. I also thank Dyke Andreasen and Colin Carney their work at the UCSC Stable Isotope Facility and Joy Matthews for her work at the UC Davis Stable Isotope Facility.

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JV: *J. virginiana*, RG: *Rhus glabra*, CD: *Cornus drummundii*, GT: *Gleditsia triacanthos*, and CO: *Celtis occidentalis*.

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*Indicates a disconnect between expectations and reality. In these cases, policies or incentives were put forth with the expectation of achieving specific outcomes. When implemented, these policies and incentives did not have the desired outcome. An example of this is the failure to meet the Renewable Fuel Standards.

This diagram illustrates areas of contracting, areas of standard and regulations, and areas of incentives within the switchgrass for biofuel industry. Contracts made between actors at different points in the production line are informed by regulations

and incentives. One of the greatest uncertainties is the availability of cellulosic biomass needed for second generation biofuel production (α). Federal fuel standards were created with the intent of driving the market towards alternative fuels, however the production volume standards have not been met (β). Farmers are incentivized to grow switchgrass on marginal and degraded landscapes through the CRP, however this biomass cannot be used for biofuel production. BCAP aims to increase switchgrass for biofuel production through further economic incentives, but enrollment has remained low. Farmer uncertainty regarding switchgrass profitability may be reduced through the creation of crop insurance programs (γ).

Without contracts clearly stating biomass quality requirements, it is difficult for contractors to guarantee the quality of the biomass shipped to refineries (A). Contracts specifying biomass quality characteristic may also impact transportation costs and contracting. The distance between the farm and the refinery as well as the density of biomass determines transportation costs. Farms that are closer to refineries and produce well dried biomass will have lower transportation costs (B). Factors such as water content, specific heat indexes, sulfur concentration, and ash content need to be standardized prior to biomass processing (1). Preprocessing biomass into more easily stored pellets may increase the efficiency of second generation biofuel production, but standards for this process have not been established (2).

CHAPTER 1: LINKING THE RHIZOSPHERE PRIMING EFFECT WITH SOIL AGGREGATES, DISSOLVED ORGANIC CARBON, AND LEACHABLE CLAY PARTICLES

Abstract

Aims: Plant roots play a major role in controlling soil organic matter (SOM) decomposition in terrestrial systems, which is termed the rhizosphere priming effect (RPE). The main question of this study is how the interactions between plant roots and soil structure dynamics modulate the RPE.

Methods: Soybeans were grown at three different densities in a continuous ^{13}C-isotope labeling greenhouse. I quantified the RPE, water stable aggregate fractions, dissolved organic carbon (DOC), and clay particle concentrations in the leachate at five and eight weeks after planting.

Results: The RPE at 8 weeks after planting was 15% and 65% above the unplanted control in the low density and the high density treatments, respectively. Between the first and second samplings, the proportion of large aggregates (>2mm) fell from 53% to 44% in the high density treatment and from 33% to 13% in the unplanted control treatment. Leachate DOC levels in the planted treatments were significantly higher than that in the unplanted control. Leachate clay particles increased with increasing planting density, suggesting that the aggregate turnover rate was greater in the high density treatment than that in other treatments.

Conclusions: The RPE was associated with DOC augmentation, aggregate dynamics, and mobilization of clay particles. These results demonstrate that root-

mediated soil structural dynamics, in addition to known biological mechanisms, play a crucial role in regulating the RPE.

Introduction

The global carbon cycle is largely dominated by the carbon exchanges between terrestrial ecosystems, the atmosphere, and the ocean (Schlesinger and Andrews, 2000). Land plants regulate the global carbon cycle both by photosynthesis and respiration above ground, and by root respiration and root-soil interactions belowground (Bais et al., 2006; Gougoulias et al., 2014). The rhizosphere may contribute up to 50% of terrestrial CO_2 efflux (Kuzyakov, 2010); however, this rhizosphere CO_2 flux remains one of the most uncertain parts of the global carbon cycle (Pausch et al., 2013). A better understanding of rhizosphere influences on belowground carbon cycling is crucial to improving global carbon flux estimates (Finzi et al., 2015).

The change in rate of soil organic matter (SOM) decomposition due to rhizosphere activity, referred to as the rhizosphere priming effect (RPE), is now recognized as a crucial factor in regulating SOM decomposition (Finzi et al., 2015; Kuzyakov and Blagodatskaya, 2015). Over a range of conditions, the presence of live roots has been shown to reduce SOM decomposition as much as 50% and accelerate SOM decomposition as much as 380% compared to unplanted controls (Zhu and Cheng, 2011). Results from a meta-analysis indicate that key factors determining the magnitude and direction of the RPE are plant species, soil texture, aboveground plant biomass, and the length of experimental duration (Huo et al., 2017).

The rhizosphere fosters microbial growth, which typically increases the rate of SOM decomposition in rhizosphere soil compared to fallow soil (Kuzyakov and Blagodatskaya, 2015). The RPE has been described as the result of interactions between living (soil microbes) and dead (recalcitrant carbon) SOM (Kuzyakov, 2010).

Soil microbes are primarily responsible for the mineralization of SOM; therefore, nutrient availability, water availability, and temperature are key determinants of priming effects (Gougoulias et al., 2014; Zhu and Cheng, 2011). Nutrient availability, particularly nitrogen availability, is thought to be one of the primary determinants of the RPE; two hypotheses have developed based on this connection. The competition hypothesis states that low nitrogen availability intensifies competition for nutrients between plants and microbes which reduces microbial activity in the rhizosphere and results in low priming effects (Kuzyakov, 2002). Conversely, the N-mining hypothesis posits that microbes use carbon rich exudates to extract nitrogen from SOM (Lu et al., 2018). Given this hypothesis, when inorganic nitrogen is readily available in the soil, microbes are able to quickly mineralize root exudates resulting in lower priming (Lu et al., 2018). Alternatively, it has been suggested that lower RPE's can also be the result of microbes preferentially consuming the higher quality root exudates instead of SOM (Kuzyakov, 2002). These hypotheses are based solely on biological reactions of soil microbes to changes in substrate and nutrient availability. There is a lack of research investigating the ways in which rhizosphere activities alter soil structure and how changes in soil structure affect nutrient availability (Kuzyakov, 2010).

In the absence of roots, chemical recalcitrance and bioavailability have been thought to be the main controls on the rate of SOM decomposition (Wershaw et al., 2004; Mikutta et al., 2006). Laboratory extractions from field samples have shown that SOM may be comprised of structurally complex, chemically recalcitrant macro-molecules (Dungait et al., 2012; Stockmann et al., 2012). These extractions show that the bioavailability of organic material is directly linked to the kinetic stability of SOM

(Mikutta et al., 2006), but there is limited evidence supporting this theory outside of the laboratory environment (Dungait et al., 2012). Over the past two decades, results produced by using new monitoring and sampling techniques have revealed the persistence of small organic molecules in the soil matrix, indicating that chemical recalcitrance alone is inadequate to explain the persistence of SOM (Schmidt et al., 2011; Lehmann and Kleber, 2015; Tipping et al., 2016). This theoretical shift, sometimes referred to as the emergent view of SOM, highlights the importance of soil texture and soil structure in the context of SOM decomposition (Six et al., 2004; Wang et al., 2015; Lehmann and Kleber, 2015). This emergent understanding of SOM asserts that complex interactions between soil physical, chemical and biological factors largely determine the fate of organic material within the soil matrix (Schmidt et al., 2011; Stockmann et al., 2012; Dungait et al., 2012).

Two physical controls on SOM accessibility are soil texture and macroaggregate stability (Six et al., 2000a, Planet et al., 2006; Tipping et al., 2016). Soil texture, specifically the clay content of the soil and the presence of amorphous aluminum and iron oxides, determines the extent to which SOM can sorb to these mineral surfaces (Cai et al., 2016; Baldock and Skjemstad, 2000). Stoichiometric analysis of SOM across a wide range of both temperate and tropical soils shows that nutrient rich SOM persists in the soil especially when adsorbed to mineral surfaces (Tipping et al., 2016). Soils with higher clay contents have a greater capacity to stabilize SOM (Plante et al., 2006).

Small organic molecules are also stabilized through physical occlusion within soil aggregate structures Schmidt et al., 2011). Roots, root hairs, and fungal hyphae

entangle and engulf soil particles thereby contributing to the physical stabilization of aggregates (Rillig et al., 2015; Tisdall et al., 2012; Ola et al., 2015). Specific root and fungal traits such as hyphal length, growth rate, and root/hyphal branching patterns determine aggregate stability (Lehmann and Rillig, 2015; Rillig et al., 2015). When these structures break apart, the previously occluded SOM becomes available for microbial mineralization (de Gryze et al., 2006). The rate of aggregate formation and destruction, or aggregate turnover, can therefore influence the rate of SOM released from these structures (Haynes and Beare, 1997; Eviner and Chapin, 2002).

Data from both laboratory and field experiments indicate a strong linkage between SOM decomposition and the rate of aggregate turnover (Six et al., 2000a; de Gryze et al., 2006; Cheng and Kuzyakov, 2005). Given that roots and rhizosphere activities together strongly modulate soil aggregate dynamics (Reid and Goss, 1981; Haynes and Beare, 1997; Eviner and Chapin, 2002), the interactions between the rhizosphere and soil structural dynamics are likely a crucial mechanism for regulating the RPE. However, existing hypotheses regarding potential mechanisms of the RPE have been predominantly microbiological and have rarely included any soil structural interactions with the rhizosphere (Kuzyakov, 2010). The possibility of soil aggregate dynamics modulating the RPE has been acknowledged, but there is a lack of experimental evidence (Cheng and Kuzyakov, 2005).

The complex interactions between the rhizosphere and the soil matrix are difficult to quantify (Gougoulias et al., 2014). The conceptual framework presented in Figure 1.1 illustrates several potential pathways of these complex interactions between rhizosphere priming and soil matrix. The green boxes indicate the direct measurements

taken in this experiment. The black boxes indicate linkages implicated in the literature. Biological mechanisms associated with the rhizosphere priming effects have been summarized in previous paragraphs, and therefore, not included in this framework. I here define the rhizosphere activities as the physical and chemical disturbances associated with the rhizosphere. The SOM physically and chemically made available to microbial attack due to rhizosphere activity is referred to as rhizosphere activated SOM. The RPE is considered a direct outcome of rhizosphere activated SOM.

Rhizosphere activities, primarily root exudates, increase the amount of microbially available carbon (Bais et al., 2006; Rasse et al., 2005). The dissolved organic carbon (DOC) from root exudates is thought to exist in the soil for only a matter of days due to its low molecular weight; however, these inputs create microbial hotspots within the rhizosphere and contribute significantly to soil carbon dynamics (Kuzyakov, 2010). Additionally, root exudates in the form of organic acids, in particular oxalic acid, has been shown to further make clay-associated SOM, organic material that is reversibly bonded to clay particles in the soil into clay particles, available for microbial mineralization (Keiluweit et al., 2015; Schmidt et al., 2011; Stockmann et al., 2013; Fig. 1.1 Arrows [2]→[5]→[9]). An increase in root density may consequently increase the amount of SOM-derived DOC in the soil matrix. In conjunction with root-microbial interactions, root-soil matrix interactions have the potential to cause priming effects.

Previous research has identified two types of protected carbon, aggregate occluded SOM and clay-associated SOM, that rhizosphere activity can make more accessible to microbial decomposition (Haynes and Beare, 1997; Eviner and Chapin,

2002; Stockman et al., 2013). Aggregate occluded SOM is exposed when roots physically disturb aggregate structures (Haynes and Beare, 1997; Cheng and Kuzyakov, 2005; Fig. 1.1 Arrows [1]→[4]). Similarly, clay particles and clay-associated SOM are added to the soil matrix when aggregates break apart (Cai et al., 2016; Six et al., 2000b; Fig. 1.1 Arrows [1]→[3]→[6] and [1]→[3]→[9]). Because the SOM contained in macroaggregates is younger and is more readily mineralized compared to carbon stored in microaggegates, the breakdown of macroaggregates is considered the structural determinant of soil carbon loss (Six et al., 2000b).

In this study, I investigated the interactions between the rhizosphere and soil structural dynamics in a continuous ^{13}C-isotope labeling greenhouse experiment. I manipulated the intensity of rhizosphere activities by using three different planting densities and measured the levels of the RPE under these treatments at two sampling times. Because of the known difficulty in quantifying aggregate turnover, I measured the net change in aggregate size distribution as an indication of aggregate dynamics. Similarly, clay particles in the leachate were measured as an indication of clay-associated SOM disturbance. The primary objective of this experiment was to test the strength of two key linkages. First, I assessed the strength of the linkage between rhizosphere dynamics and the amount of leached DOC as demonstrated in Fig 1.1 Arrows [2]→[8] and [2]→[10]. I did this by measuring root respiration, root biomass, and the amount of DOC in the leachate. Second, I assessed the strength of the linkages between a change in rhizosphere activity and the net change in aggregate size distribution and the amount of clay in the leachate (Fig. 1.1 Arrows [1]→[3]→[6]).

Through these measurements I aimed to increase our understanding of the linkages between soil physical dynamics and the RPE.

Methods

Experimental Design

Soybeans (*Glycine max (L.)* Merr) were grown at three different planting densities: low (1 soybean per pot), medium (2 soybeans per pot), and high (3 soybeans per pot) in a sealed greenhouse and were continuously labeled with ^{13}C-depleted CO_2 (Dijkstra and Cheng, 2007a). The plants were grown in the greenhouse (73 m^2) from the time of seeding until the end of the experiment. The CO_2 concentration was monitored by an infra-red gas analyzer (Model LI-820, Li-COR, Lincoln, NE, USA) and was stabilized at 400 ± 5ppm by computer-controlled CO_2 injections from a tank filled with ^{13}C-depleted CO_2 ($\delta^{13}C$ of -38‰) (Pausch et al., 2013). The $\delta^{13}C$ value of the CO_2 in the greenhouse was maintained at −18‰ (± 0.3‰) by constant mixing of CO_2-free air injection and ambient air input (Cheng and Dijkstra, 2007). The CO_2-free air was produced by passing compressed air through six moistened soda lime (a mixture of NaOH and $Ca(OH)_2$) columns (20 cm in diameter, 200 cm in length). The CO_2-free air flow rate was set at a rate proportional to the leakage rate of the greenhouse. The CO_2-free air and the ^{13}C-depleted CO_2 ($\delta^{13}C$ of -38‰) were mixed with a fan within the greenhouse to insure uniformity in the CO_2 concentration and the $\delta^{13}C$ throughout the greenhouse.

The day to day variability of the $\delta^{13}C$ was monitored by trapping CO_2 in the greenhouse air by passing the air through a glass airstone immersed in 300ml of 0.5*M*

NaOH solution using an air pump. CO_2-free DI water and an excessive amount of 1*M* $SrCl_2$ were added to an aliquot of the CO_2 rich NaOH sample. The resulting $SrCO_3$ precipitate was washed with CO_2-free DI water until the precipitate solution reached a neutral pH (pH 7) according to the Harris et al., (1997) method. The remaining precipitate was dried at 105°C for 24 hours and analyzed for $\delta^{13}C$.

The soybeans were grown in PVC (polyvinal chloride) pots (15cm diameter, 40 cm height). Ten pots were assembled for each planting density including ten unplanted control pots. Each pot was fitted with an inlet tube at the bottom of the pot. This increased aeration at the bottom of the pot when attached to aquarium pumps and provided a CO_2 sampling outlet. Aquarium pumps were run twice every twenty-four hours during the dark hours in order to prevent contamination of the greenhouse $\delta^{13}C$ signal with that of the soil-derived CO_2. Surface soil (0-30cm) was collected from the organic farm at the Center for Agroecology and Sustainable Food Systems on the University of California Santa Cruz campus. The soil is a sandy loam (Mollisol) with pH of 6.4, a $\delta^{13}C$ value of -26.34‰, 0.92 C%, 0.10 N%, and a C:N ratio of 9.21. A kilogram and a half of sand was encased in a nylon mesh bag and placed at the bottom of each PVC pot in order to promote water drainage and aeration throughout the soil column. Seven kilograms of sieved (2 mm) air dried soil was added to each pot. Six soybean seeds were planted in each pot, and the plants were picked to the assigned density after emergence.

The soil moisture content was measured gravimetrically and was maintained at 70% water holding capacity throughout the experiment by frequent watering. In order to distribute water throughout the soil profile without impacting soil physical

conditions and aggregate integrity, water was added through a funnel attached to flexible plastic tubing and a buried perforated watering tube (0.32 cm inner diameter, 15 cm length). The pots were distributed randomly throughout the greenhouse (73 m^2) and were rotated every four days to ensure equivalent growing conditions. The air temperature within the greenhouse was maintained at 25°C during the day and 17°C at night by two air conditioning units. Artificial lighting (1100W lights P.L. Light Systems) was used to supplement the available natural light in order to maintain a twelve hour light period. The light intensity during the light period remained above 900 W m^{-2}. The relative humidity inside the greenhouse was kept at or below 45% using a dehumidifier (Kenmore Elite 70 pint).

The CO_2 efflux was sampled from half of the pots (5 pots per treatment) 5 weeks after planting. Following the CO_2 sampling, the soil was leached with DI water in order to quantify dissolved organic carbon (DOC) and clay particles in the leachate. The roots and the shoots of the plants were then separated and processed. The second half of the pots were sampled and processed 8 weeks after planting. Five weeks corresponded to the R1 stage of the flowering time (Casteel, 2010). Root growth increases significantly at this point in the growth cycle (Casteel, 2010). At eight weeks the plants were at the R3 stage of pod formation (Casteel, 2010). The rate of root growth tapers off at this point in the lifecycle (Casteel, 2010).

Measurements

Soil and root respiration rates were measured at five and eight weeks after planting. The CO_2 from soil and root respiration was sampled over the course of

twenty-four hours using a closed-circulation continuous trapping system (Cheng et al., 2003; Pausch et al., 2013). Before each sampling, the pots were sealed above the soil and around the base of each plant using a non-toxic silicone rubber (GI-1000, Silicones Inc., NC, USA). The CO_2 remaining in the pot was first removed by circulating the isolated air through a soda lime column for forty minutes. Then, during a twenty-four-hour period, air from the soil was circulated through a plastic bottle containing 300 ml of 0.5 *M* NaOH solution for thirty minutes every six hours. Previous studies have shown that the CO_2 trapping efficacy of this method was greater than 99% (Dijkstra and Cheng, 2007a; Cheng et al., 2005). Three blanks of CO_2 trapping systems were included at each sampling time in order to correct for the introduction of inorganic carbon due to possible contamination in the NaOH stock solution and from handling of the NaOH solution.

An aliquot of the NaOH solution in each CO_2 trap was analyzed for total inorganic carbon using a Shimadzu TOC-5050A Total Organic Carbon Analyzer. An excessive amount of $SrCl_2$ solution was added into another aliquot of NaOH solution from each CO_2 trap, and the resulting $SrCO_3$ was precipitated, repeatedly washed by using CO_2-free DI water until pH=7.0, and analyzed for $\delta^{13}C$ (Harris et al., 1997) after oven-dried at 105°C. Total soil respiration (as measured by the total CO_2 trapped in the NaOH solution) was partitioned into SOM-derived carbon (microbial respiration of SOM) and root-derived carbon (rhizosphere respiration = root respiration plus microbial respiration of root exudates) using a two-source mixing model (Zhu and Cheng, 2011).

$$C_{soil} = C_{total}(\delta^{13}C_{root} — \delta^{13}C_{total})/(\delta^{13}C_{root} — \delta^{13}C_{soil})$$

$$C_{root} = C_{total} — C_{soil}$$

Where C_{total} is the total respiration in the planted treatments, C_{soil} is the microbial respiration of SOM, and C_{root} is the rhizosphere respiration, $\delta^{13}C_{root}$, $\delta^{13}C_{soil}$, and $\delta^{13}C_{total}$ are the $\delta^{13}C$ values of the CO_2-C from rhizosphere respiration, SOM respiration, and total soil respiration respectively.

The $\delta^{13}C$ value of the root-derived CO_2 was corrected using a previously established fractionation factor (f) (Zhu and Cheng, 2011). The fractionation factor measured by Zhu and Cheng (2011) for *Glycine max* was -1.71‰. This was used to calculate the difference between the ^{13}C isotopic composition of root biomass and root-derived CO_2.

$$\delta^{13}C_{root\text{-}derivived} = \delta^{13}C_{root} + f$$

The rhizosphere priming effect was calculated by subtracting the CO_2 flux of unplanted soil from the SOM derived CO_2 from the planted treatment. The RPE is expressed as a percentage of the basal respiration of the unplanted soil. A positive RPE indicates a case when the SOM-derived CO_2 is higher than the CO_2 flux from the unplanted control. A negative RPE represents a situation when the SOM-derived CO_2 is lower than the CO_2 flux from the unplanted control.

$$RPE(\%) = [C_{SOM\text{- derived}}(\text{Planted}) - C_{SOM\text{- derived}}(\text{Control})] / C_{SOM\text{- derived}}(\text{Control}) \times 100$$

Leachate was collected from each pot by bringing each pot to water saturation and then applying a low vacuum to the sampling tube located at the bottom of the pot. The sponge fasted to the end of the sampling tube allowed passing of clay particles but prevented larger soil particles, such as sand and organic debris, from contaminating samples. The total amount of leachate collected was recorded for each sample. The samples were refrigerated overnight at 4°C. Whatman 42 filters were oven dried at 60°C for 24hr before use. The leachate was then gravity filtered through a prepared Whatman 42 filter.

The amount of clay in the leachate was determined using the clay particles caught by the filter. The clay particles and filters were oven dried at 60°C for 48 hours, and then the clay particles collected on the filter was weighed. A representative subsample of the leachate was stored in the freezer (-20°C) until it was analyzed for dissolved organic carbon (DOC) using a Shimadzu TOC-5050A Analyzer. The amount of clay particles in the leachate were used to infer the rate of large aggregate breakdown and general soil disturbance by roots. The concentration of DOC in the leachate indicates root exudation and may also indicate rhizosphere mediated liberation of occluded DOC.

Following the soil leaching, the pots were destructively harvested. The roots, shoots, and soils were separated for each sample. The plants were clipped at the base. The silicone sealant was removed, and the belowground biomass was removed from the soil. Fine roots were removed from the soil by hand picking. The soil was

homogenized and a subsample (approximately 1 kg) was stored at 4°C for further analysis. Root and shoot samples were dried in the oven at 60°C for 24 hours and then weighed to determine biomass. Soil moisture was determined gravimetrically by drying approximately 10g of soil in the oven at 105°C for 24 hours. Dried root, shoot, and soil samples were ground in a ball mill, and analyzed for $\delta^{13}C$ and $\delta^{15}N$ (Carlo Elba 1108 elemental analyzer interfaced to a Thermo-Finnegan Delta Plus XP isotope ratio mass spectrometer at the University of California Santa Cruz Stable Isotope Facility).

Soil microbial biomass C (MBC) was measured using a chloroform fumigation-extraction method (Vance et al., 1987). Total organic carbon in each extract was measured using a Shimadzu TOC-5050A Total Organic Carbon Analyzer. Microbial biomass C was calculated as the difference between the fumigated and non-fumigated samples and further divided by a proportionality coefficient, $K_{ec} = 0.45$ (Vance et al., 1987).

A subsample of the homogenized soil was used for aggregate analysis. Aggregate size distribution was measured using a modified version of the Beare 1994 method. Three sieves of decreasing mesh width (2000 µm, 500 µm, and 74 µm) were stacked and fastened together. Moist soil normalized to 100 grams dry weight was distributed evenly across the top sieve (2 mm). The soil and sieves were submerged in a bucket filled with deionized water for three minutes. The sieves were then oscillated thirty times over the course of two minutes while remaining submerged. The sieves were removed from the water and each aggregate fraction was washed from each sieve and placed into glass beakers. All aggregate fractions were then dried (in an oven at 105°C for 48 hours) and weighed.

Statistical Analysis

Significant differences between means were calculated using a two-way analysis of variance (ANOVA) between sampling time and plant density and pairwise multiple comparison procedures (Holm-Sidak method) for all datasets with the exception of the rhizosphere priming effect. Data sets, with the exception of mg C g root^{-1} day^{-1}, passed the Shapiro-Wilk Normality Test and the Equal Variance Brown-Forsyth test. Interaction effects between the sampling time and the planting density were found only for soil-derived C kg^{-1} soil d^{-1}. The differences between means for the RPE were calculated using individual student t-tests. All statistical analysis was done using SigmaPlot Version 14 from Systat Software, Inc. www.systatsoftware.com.

Results

CO_2 Efflux and Rhizosphere Priming Effects

Total CO_2 efflux increased as root biomass increased between the 5 weeks sampling time (5W) and the 8 week sampling time (8W). Total CO_2 efflux was the highest in the high density treatment at 8W measuring 24.03 ± 1.15 mg C kg^{-1} soil d^{-1}, increased from 13.99 ± 0.35 mg C kg^{-1} soil d^{-1} at 5W sampling (Table 1.2). Total CO_2 efflux in the low density treatment increased from 11.38 ± 0.46 at 5W to 15.04 ± 0.79 mg C kg^{-1} soil d^{-1} at 8W (Table 1.2). All planted treatments had higher CO_2 effluxes than the unplanted controls, and the release of CO_2 from the soil in the unplanted control remained statistically the same between 5W (8.31 ± 1.04 mg C kg^{-1} soil d^{-1}) and 8W (9.40 ± 1.36 mg C kg^{-1} soil d^{-1}) (Table 1.2). Carbon dioxide efflux per gram of root

biomass was the highest at 5W in the low density treatment (15.48 ± 2.38 mg C g^{-1} roots d^{-1}) and decreased as root biomass increased. By comparison, the high density treatment at 8W had 1.51 ± 0.06 mg C g^{-1} roots d^{-1} (Table 1.2).

Root-derived and soil-derived CO_2 were isotopically distinct due to the continuous isotopic labeling in the greenhouse (Table 1.1). Root-derived CO_2 approximately doubled between 5W and 8W for all planted treatments (Table 1.2). For the low density treatment, root-derived CO_2 increased from 2.84 mg C kg^{-1} soil d^{-1} at 5W to 4.26 mg C kg^{-1} soil d^{-1} at 8W, and from 4.23 at 5W to 8.55mg C kg^{-1} soil d^{1} for the high density treatment (Fig. 1.2). During this same period root biomass in the low density treatment increased from 0.57 g pot^{-1}g to 5.34 g pot^{-1} and from 1.48 to 10.33 g pot^{-1} for the high density treatment, indicating higher rates of root-derived CO_2 per root biomass in the low density treatments (Table 1.2). At 8W the high density treatment produced significantly more root-derived CO_2 compared to both the low and medium density treatments ($P<0.001$ and $P<0.003$ respectively).

SOM-derived CO_2, produced from the microbially mediated decomposition of SOM, increased for all planted treatments between 5W and 8W (Table. 1.1). The unplanted control treatments had a statistically insignificant change from 8.31 mg C kg^{-1} soil d^{-1} at 5W to 9.40 mg C kg-1 soil d^{-1} at 8W (Table 1.2, Fig. 1.3). The mid density treatment had the greatest increase in SOM-derived CO_2 between sampling times, from 6.62 ± 0.75 mg C kg^{-1} soil d^{-1} at 5W to 14.12 ± 1.04 mg C kg^{-1} soil d^{-1} at 8W (Table 1.2).

The rhizosphere priming effect increased between 5W and 8W for all treatments (Fig. 1.4). At the 5W sampling time, the RPE ranged from -11% in the mid density

treatment to 9% in the high density treatment (Table 1.2, Fig. 1.4). The low and high density treatments had slightly positive priming effects while the mid density treatment had a negative priming effect. The mid and high density treatments had much higher rates of priming at 8W. The high density treatment had a RPE of 65% at 8W compared to 9% at 5W. During the three weeks between sampling periods the mid density treatment shifted from a negative RPE to a positive RPE of 50% (Fig. 1.4).

Microbial Biomass

Neither sampling time nor root density significantly changed microbial biomass in the soil (Table 1.3). Microbial biomass remained consistent for all treatments between the first and second sampling time. Changes in the priming effect over this time period were not directly linked to changes in microbial biomass.

Aggregate Size Distribution

Larger aggregates (>2mm) are key components of soil structure and provide water and air passage through the soil profile (Tisdall & Oades 1982). For this reason, I used larger aggregates as evidence of soil structure stabilization. I use microaggregates (those caught in the (74µm sieve), the concentration of DOC in the collected leachate, and clay particles as evidence of macroaggregate breakdown. In order to compare aggregate size distribution across samples, I present the data as a percentage of the total soil dry weight (g $soil_{DW}$ fraction/ g $soil_{DW}$ total).

Overall, larger less-stable aggregates decreased in all treatments between the 5W and 8W sampling periods (Fig. 1.6). Macroaggregates broke down at higher rates

in the unplanted treatments compared to the planted treatments, with the high density treatment maintaining the highest proportion of macroaggregates. In the high density treatment, macroaggregates decreased from 53% at 5W to 44% at 8W compared to the decrease in the unplanted treatment from 33% at 5W to 13% at 8W (Fig. 1.6; Table 1.3). Correspondingly, microaggregates increased in all treatments with aggregates retrieved from the 500µm sieve remaining a relatively small proportion at both 5W and 8W. The aggregate fraction of >0.5 mm and <2 mm in the medium density treatment decreased from 17% at 5W to 11% at 8W, and in the high density treatment decreased slightly from 13% at 5W to 12% at 8W. The smallest aggregate fraction increased from 33% to 59% in the unplanted control treatment and from 24% to 35% in the high density treatment (Fig. 1.6, Tale 1.3).

Clay Particles and DOC

The amount of clay particles collected via gravity filtration from the leachate (mg clay kg^{-1} soil) increased with increasing root density. All planted treatments, irrespective of planting density, had greater amounts of clay in the leachate compared to the unplanted controls (Table 1.3). At 5W the high density treatment produced significantly more clay in the leachate (2.947 ± 0.72 mg clay kg^{-1} soil) as compared to the unplanted control (1.55 ± 0.81 mg clay kg^{-1} soil) (Fig. 1.5; Table 1.3). This pattern persisted at the 8W mark. The leachate from the high density treatment had 3.63 ± 0.72 mg clay kg^{-1} soil and the unplanted control had 1.43 ± 0.81 mg clay kg^{-1} soil (Fig. 1.5).

The presence of plant roots resulted in higher concentrations of DOC in the leachate compared to the unplanted control at both samplings ($P < 0.005$) (Fig. 1.7;

Table 1.3). At 5W the high planting density produced 5.47 ± 0.91 mg C kg^{-1} soil and the unplanted control produced 2.31 ± 0.15 mg C kg^{-1} soil (Table 1.3). This difference remained at the second sampling. There were slight increases in the concentration of leachate DOC in the low and medium planting densities between 5W and 8W. The concentration of DOC in the leachate in the low planting density treatment increased from 3.96 ± 0.36 mg C kg^{-1} soil at 5W to 4.35 ± 0.31 mg C kg^{-1} soil at 8W (Table 1.3). At the second sampling, there significantly more DOC was captured in the leachate in the mid density treatment compared to the unplanted control, 5.38 ± .025 mg C kg^{-1} soil compared to 2.57 ± 0.52 mg C kg^{-1} soil (Table 1.3). There was no significant change for either the high density treatment or the unplanted control between sampling periods (Fig. 1.7).

Discussion

In this experiment, I measured the rhizosphere priming effect, the net change in aggregate size distribution, and the amount of DOC and clay in leachate for three densities of soybeans at 5 and 8 weeks after planting. The soybeans were continuously labeled with ^{13}C-depleted C in a greenhouse so that the soybean tissue and the SOM had distinctive isotopic signatures, and this allowed us to distinguish SOM-derived CO_2 from root-derived CO_2 (Pausch et al., 2013). The measured RPEs ranged from -11% to 65% which falls within the range of previously measured RPEs (Cheng et al., 2014; Huo et al., 2017). The time elapsed since planting and the amount of plant biomass were the two most significant factors in determining the direction and magnitude of the RPE, which is consistent with the results from previous studies (Huo et al., 2017; Table

1.2). Plant growth is directly linked to root exudation which in turn stimulates the rhizo-microbial activities and produces priming effects (Bais et al., 2006; Kuzyakov, 2010). Furthermore, these results indicate that changes in soil physical structure, most significantly the increase in aggregate turnover as suggested by the simultaneous changes in aggregate fraction distribution, augmented DOC release, and enhanced clay particle concentrations in planted treatments, are linked to the RPE.

Roots and rhizosphere microbes have the potential to magnify DOC cycling in the soil directly through the addition of exudates or indirectly by accelerating aggregate turnover and the release of clay-associated DOC (Stockmann et al., 2013). Further research is needed in order to determine the proportion of root-derived DOC and SOM-derived DOC that becomes rhizosphere activated SOM (Fig. 1.1 [1]→[4]→[8] and [1]→[3]→[5]→[8]). Each mechanistic pathway augments DOC dynamics by adding rhizosphere activated SOM to the active SOM pool which ultimately results in priming effects (Fig. 1.1 Arrow [8]). Further analysis is also needed in order to determine the proportion of root-derived DOC and SOM-derived DOC in the leachate (Fig. 1.1 [2]→[8], [1]→[3]→[5]→[10], and [1]→[4]→[10]). The results from this experiment showed that planting density corresponded to increasing concentrations of DOC in the leachate at each sampling period (Fig. 1.7). DOC was more strongly linked to planting density than to the sampling time (Table 1.3). DOC is an extremely reactive fraction of SOM and can be readily metabolized by soil microbes (Kuzyakov, 2010), therefore it is unlikely that DOC would accumulate in the soil over a period of weeks (Mikutta et al., 2016).

I indirectly assessed the rate of aggregate turnover by measuring aggregate size distribution and clay particle concentration in the leachate. The net change in aggregate size distribution through time indicates the intensity of rhizosphere dynamics. Roots and rhizosphere microbes not only can improve aggregation through structural support and rhizodeposits (Rasse et al., 2005), but also can accelerate aggregate destruction (Eviner and Chapin, 2002). Aggregate formation and destruction together determine the overall aggregate turnover rate. Faster aggregate destruction and turnover rates enhance the release of clay particles and protected SOM into the soil matrix where it becomes rhizosphere activated SOM exposing to microbial decomposition or leach through the soil profile (Baldock and Skjemstad, 2000; Fig. 1.1 Arrows [1]→[3]→[6] and [1]→[3]→[9]). Therefore, by comparing the macroaggregate fraction and the clay particle concentration I can infer the rate of aggregate turnover. In the case that the macroaggregate fraction and the clay particle concentration are greater in the planted treatment compared to the unplanted treatment, I conclude that clay particles and SOM were released from macroaggregates during the destruction of the aggregates.

The most pronounced differences in macroaggregate fraction and in clay particle concentration were found between the high density treatment and the unplanted control treatment sampled at 8 weeks (Table 1.3). The macroaggregate fraction for the high density treatment was 44% compared to 13% in the unplanted treatment. At the same time, the high density treatment produced 3.63 ± 0.72 mg clay kg^{-1} soil and the unplanted treatments produced 1.43 ± 0.81 mg clay kg^{-1} soil. These results suggest that the aggregate turnover rate was greater in the planted treatment which supports the pathway illustrated in Fig. 1.1, arrows [1]→[3]→[6]. The data from this project in

conjunction with past studies suggest that plant roots are important for maintaining macroaggregates (Fig. 1.6; Six et al., 2000b; Abiven et al., 2009).

The processes of aggregate formation and destruction are likely accelerated in the presence of roots due to the increase in organic inputs and increased physical disturbance (Fig. 1.1 Arrow [1]). The unplanted treatments were not impacted by physical disturbance associated with root growth, yet the macroaggregate fraction decreased between 5W and 8W (Fig. 1.6). The maintenance of larger aggregates is vital to total soil porosity and SOM occlusion within aggregate structures (Mangalassery et al., 2013). As the proportion of large aggregates decreases aeration and macro pores also decrease, which impacts microbial access to SOM (Mangalassery et al., 2013). The protection and subsequent stabilization of SOM in microaggregate structures is one of the most influential factors controlling the rate of SOM decomposition (Schmidt et al., 2011; Dungait et al., 2012). Rhizosphere activities decreased the net change in aggregate size distribution, yet the increase in leached clay particles in the planted treatments suggests that roots play a key role in aggregate turnover (Cheng and Kuzyakov, 2005; Fig. 1.6). Following this logic, aggregate turnover may be linked to the magnitude of the RPE as I posit in Fig. 1.1 Arrows [1]→[7]. The high density treatment at 8W had the highest RPE and also had the greatest proportion of macroaggregates (Fig. 1.4; Fig. 1.6).

Clay particles in the leachate suggest the presence of clay-associated SOM in the soil matrix (Schmidt et al., 2011; Stockmann et al., 2013). Despite being reversible, these organo-clay complexes consist of a major component of stabilized SOM (Dungait et al., 2012). The total clay content of the soil is not always indicative of total soil

carbon (Plante et al., 2006), but the presence of clay increases the potential of clay-associated SOM accumulation (Cai et al., 2016). Clay/DOC interactions with rhizosphere compounds may play a larger role in soils with higher CECs (Baldock and Skjemstad, 2000). These organo-clay complex can be destabilized by root exudates and microbial activity (Stockmann et al., 2013; Keiluweit et al., 2015; Fig. 1.1 Arrows [2] → [5] → [9]). The high density treatment consistently had higher amounts of clay particles in the leachate compared to the unplanted treatments (Fig. 1.5), indicating that the high level of the RPE was connected with a higher release of clay-associated SOM. Both more clay particles and higher RPEs occurred in the high rooting density treatments, which suggested that the third pathway (Fig. 1.1 Arrows [1]→[3]→[9]) outlined in the Introduction also played a role.

This pathway may help to explain why the RPE has also been shown to preferentially impact long-term carbon (Dijkstra and Cheng, 2007b). The biological mechanisms associated with the RPE rely on the nutrient contents of the soil, root exudates, and soil organic matter (Kuzyakov, 2002; Finzi et al., 2015; Gougoulias et al., 2014; Bais et al., 2006). These explanations do not address the importance of the physical accessibility of SOM, which has been shown to play a key role in SOM stabilization (Dungait et al., 2012). Previous research has suggested that SOM stabilization is controlled in the long term (decadal rates) by soil structure and soil matrix bonds established between soil structures and SOM (Schmidt et al., 2011; Cotrufo et al., 2013). The clay in the leachate indicates disturbances in soil structure through the disruption of aggregate structures and the exposure of previously occluded organic material (Fig. 1.5). The clay in the leachate also indicates the disturbance of

the more stable clay-associated SOM by rhizosphere activities (Keiluweit et al., 2015). Both of these mechanisms help to explain how rhizosphere physical and chemical disturbance increases the RPE. Future research clarifying the contributions of each of these pathways is warranted.

Table 1.1. Shoot and root biomass, C:N ratio, root:shoot ratio, and $\delta^{13}C$ (‰) values in three planting density treatments (1, 2, and 3 soybean plants per pot) at two sampling times (5 and 8 weeks after planting). Each value is the mean of five replicates plus or minus one standard error. ANOVA results of the two main variables (planting density and sampling time) are given at the bottom of the table.

	Biomass			**$\delta^{13}C$ (‰)**			**C:N**	
Treatment	**Root**	**Shoot**	**Root:Shoot**	**Root**	**Shoot**	**Soil**	**Root**	**Shoot**
				5 Week Sampling				
1 plant/ pot	0.57 ± 0.05	1.73 ± 0.29	0.37 ± 0.05	-34.60 ± 0.07	-36.16 ± 0.14	-26.20 ± 0.06	16.89 ± 0.28	11.05 ± 0.65
2 plant/ pot	1.10 ± 0.18	2.64 ± 0.23	0.41 ± 0.04	-34.51 ± 0.10	-36.15 ± 0.04	-26.33 ± 0.03	17.22 ± 0.22	13.16 ± 0.53
3 plant/ pot	1.48 ± 0.10	4.22 ± 0.21	0.35 ± 0.02	-34.80 ± 0.14	-36.46 ± 0.26	-26.34 ± 0.04	19.39 ± 0.50	15.41 ± 0.67
control						-26.34 ± 0.01		
				8 Week Sampling				
1 plant/ pot	5.34 ± 0.43	1.35 ± 0.16	0.26 ± 0.02	-33.92 ± 0.14	-35.30 ± 0.18	-26.45 ± 0.07	20.50 ± 0.71	20.61 ± 1.10
2 plant/ pot	8.90 ± 0.32	2.26 ± 0.18	0.26 ± 0.02	-33.60 ± 0.12	-34.59 ± 0.18	-26.59 ± 0.10	20.54 ± 0.62	27.27 ± 1.14
3 plant/ pot	10.33 ± 0.43	3.16 ± 0.31	0.31 ± 0.03	-33.60 ± 0.16	-34.52 ± 0.19	-26.58 ± 0.13	19.89 ± 0.63	27.23 ± 1.12
control						-26.35 ± 0.03		
ANOVA (p-values)								
Sampling Time (S)	<0.001	0.01	0.003	<0.001	<0.001	0.004	<0.001	<0.001
Planting Density (P)	<0.001	<0.001	0.838	0.323	0.202	0.227	0.256	<0.001
S X P	<0.001	0.344	0.354	0.202	0.036	0.791	0.026	0.098

Table 1.2. Total CO_2-C, SOM-derived CO_2-C, Root-derived CO_2-C (mg C kg soil^{-1} day^{-1}) in three planting density treatments (1, 2, and 3 soybean plants per pot) and the unplanted control at two sampling times (5 and 8 weeks after planting). SPE represents Root Biomass Specific Priming Effect, which is calculated by dividing SOM-derived CO_2-C by gram root biomass (mg C g^{-1} root). The values in the table below are means of five replicates plus or minus one standard error. ANOVA results of the two main variables (planting density and sampling time) are given at the bottom of the table.

Treatments	Total CO_2-C	SOM-derived CO_2-C	Root-derived CO_2-C	SPE
		5 Week Sampling		
1 plant/ pot	11.38 ± 0.46	8.54 ± 0.53	2.84 ± 0.21	15.48 ± 2.38
2 plant/ pot	10.39 ± 0.74	6.62 ± 0.75	3.77 ± 0.75	8.23 ± 0.60
3 plant/ pot	13.99 ± 0.35	9.03 ± 0.29	4.23 ± 0.66	5.72 ± 0.10
control	8.31 ± 1.04	8.31 ± 1.04		
		8 Week Sampling		
1 plant/ pot	15.04 ± 0.79	10.78 ± 0.69	4.26 ± 0.34	2.06 ± 0.17
2 plant/ pot	19.12 ± 0.93	14.12 ± 1.04	5.00 ± 0.27	1.59 ± 0.12
3 plant/ pot	24.03 ± 1.15	15.48 ± 0.57	8.55 ± 0.89	1.51 ± 0.06
control	9.40 ± 1.36	9.40 ± 1.36		
ANOVA (p-values)				
Sampling Time (S)	<0.001	<0.001	<0.001	<0.001
planting Density (P)	<0.001	0.01	<0.001	<0.001
S X P	<0.001	0.01	0.16	<0.001

Table 1.3. The table below shows the results from post- CO_2 trapping processing. The concentration of clay in the leachate expressed as mg clay kg soil^{-1}. AFT: the aggregate fraction expressed as a percent of the total sample. MBC: microbial biomass carbon expressed as µg C g^{-1} soil. The values in the table below are means of five replicates plus or minus one standard error. ANOVA results of the two main variables (planting density and sampling time) are given at the bottom of the table.

Treatment	**mg clay kg soil^{-1}**	**mg C-DOC kg^{-1} soil**	**2mm AFT**	**500um-74um AFT**	**74um AFT**	**MBC (µg C g^{-1} soil)**
5 Week Sampling						
1 plant/ pot	1.15 ± 0.23	3.96 ± 0.36	44.40 ± 9.96	10.70 ± 2.91	29.00 ± 7.00	75.70 ± 9.31
2 plant/ pot	2.98 ± 0.44	3.76 ± 0.85	25.20 ± 9.96	16.70 ± 2.91	43.20 ± 7.00	88.39 ± 8.34
3 plant/ pot	2.947 ± 0.37	5.47 ± 0.91	53.70 ± 9.96	13.00 ± 2.91	24.50 ± 7.00	72.25 ± 9.31
control	1.55 ± 0.19	2.31 ± 0.15	33.33 ± .10.70	23.10 ± 3.25	33.00 ± 7.82	90.01 ± 10.75
8 Week Sampling						
1 plant/ pot	1.01 ± 0.20	4.35 ± 0.31	32.60 ± 9.96	16.10 ± 2.91	41.30 ± 7.00	93.11 ± 8.33
2 plant/ pot	1.60 ± 0.26	5.38 ± 0.25	19.99 ± 9.96	11.00 ± 2.91	58.60 ± 7.00	82.39 ± 8.33
3 plant/ pot	3.63 ± 1.33	5.10 ± 0.49	43.50 ± 9.96	12.50 ± 2.91	35.80 ± 7.00	84.59 ± 8.33
control	1.43 ± 0.19	2.57 ± 0.52	13.10 ± 10.70	18.00 ± 3.25	58.6 ± 7.82	76.33 ± 9.31
ANOVA (p-values)						
Sampling Time (S)	0.650	0.328	0.108	0.494	0.004	0.697
Planting Density (P)	0.025	0.006	0.037	0.065	0.024	0.860
S X P	0.561	0.487	0.905	0.234	0.772	0.300

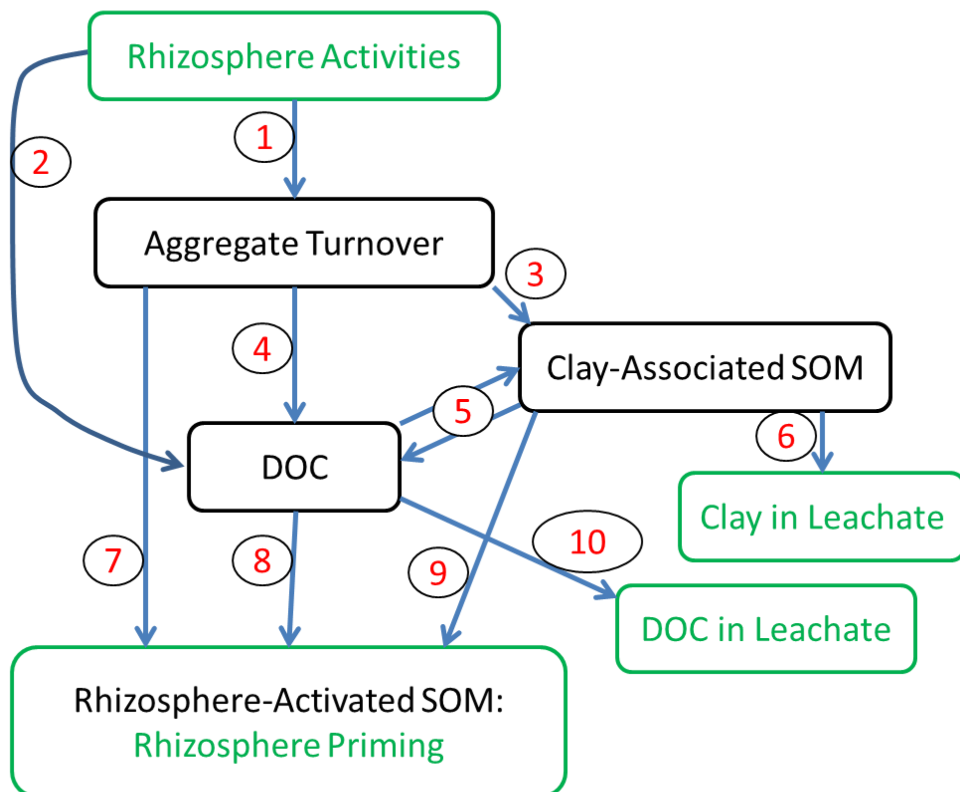


Figure 1.1.

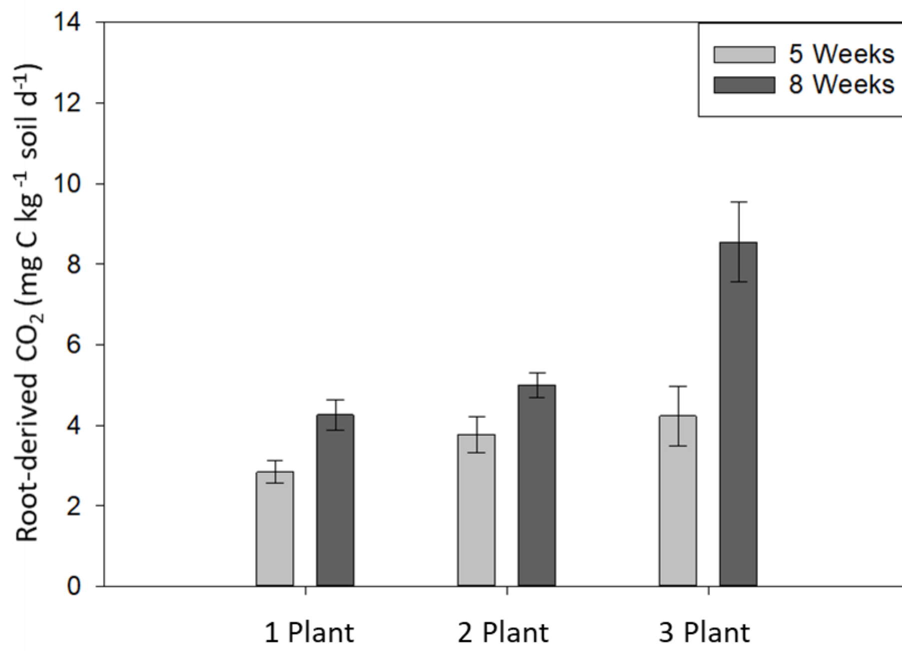


Figure 1.2.

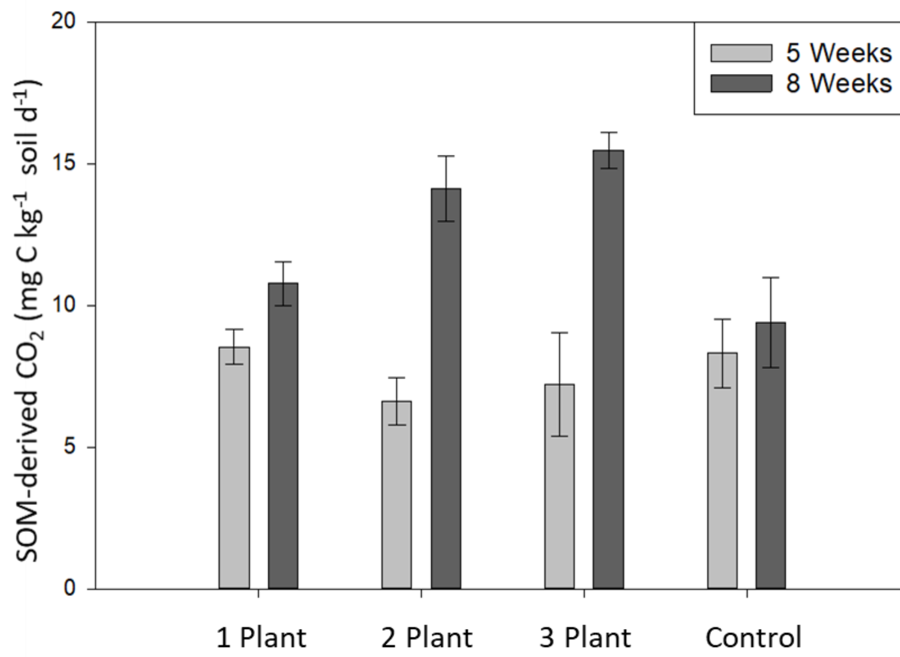


Figure 1.3.

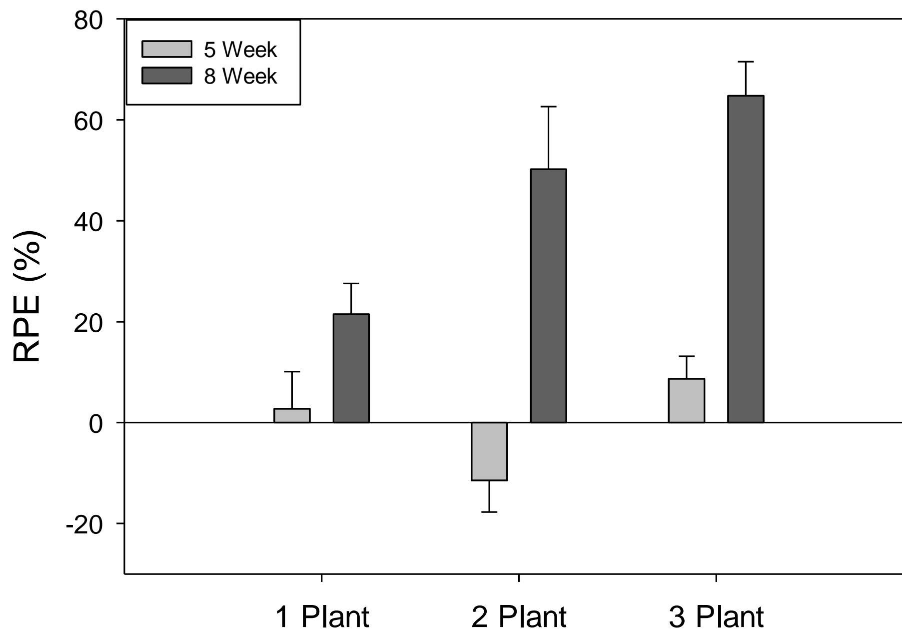


Figure 1.4.

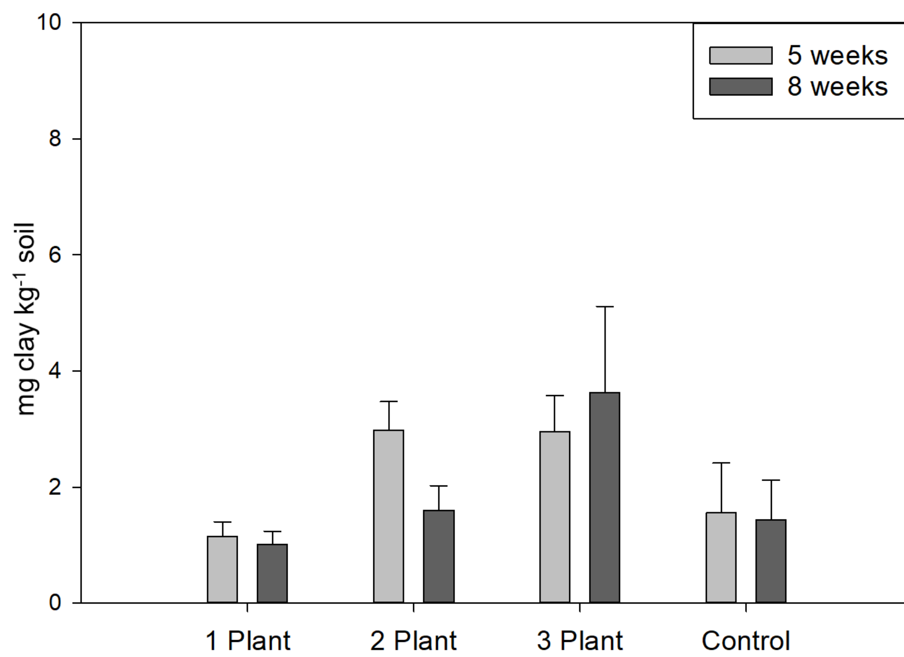


Figure 1.5.

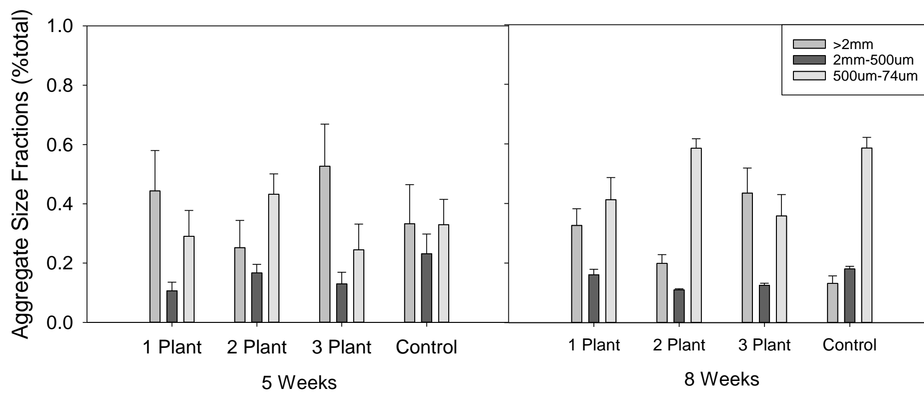


Figure 1.6.

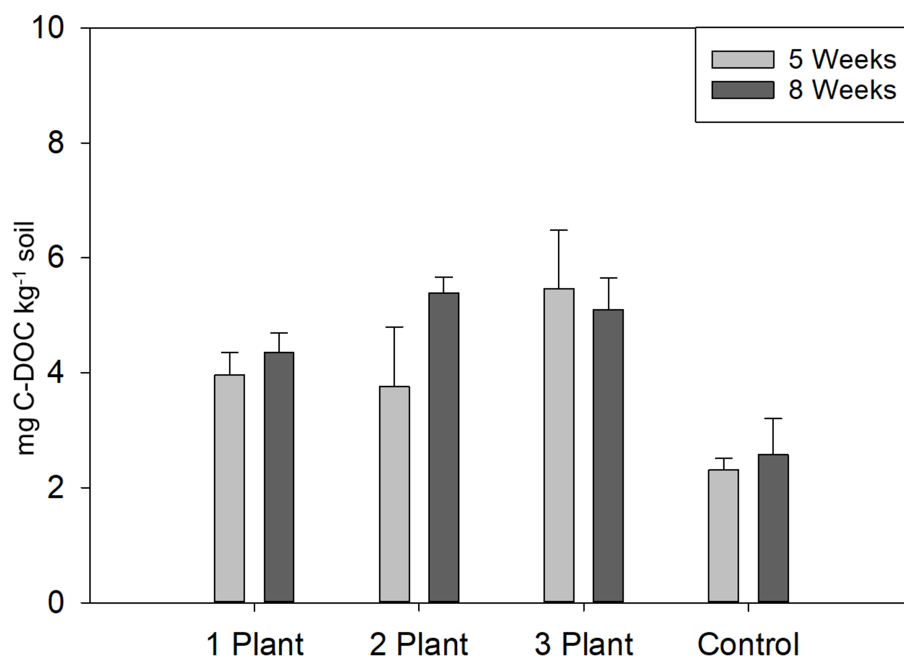


Figure 1.7.

CHAPTER 2: THE RHIZOSPHERE PRIMING EFFECT *IN SITU*

Abstract

Carbon dioxide released from the decomposition of soil organic carbon (SOC) at the global scale is the largest source of CO_2 among all CO_2 sources from the terrestrial component of the Earth, which therefore exerts a major control on the global carbon cycle. Emerging evidence from many laboratory studies indicates that plant roots and rhizosphere microbes together substantially regulate SOC decomposition, which is referred to as the rhizosphere priming effect (RPE). However, due to the lack of evidence from field studies, it is not possible to incorporate the RPE into a global models. This study investigated the RPE under field conditions using five woody plant species (all C_3 plants) which have recently encroached into native tallgrass prairie with vegetation of primarily C_4 grasses at the Konza Prairie Long-Term Ecological Research site. This special setting of C_3-plants growing into "C4-soils" permits the use of a natural ^{13}C tracer method to measure the RPE in field chambers. Over the course of two growing seasons, live roots from five C_3 woody species native to the Kansas tallgrass prairie were excavated, transplanted into root chambers filled with local C_4-derived soil, and incubated in the field for fifty days. More root growth corresponded to higher rates of soil organic matter decomposition compared to unplanted control chambers. *Cornus drummundii* had the most root biomass (1.22 ± 0.11 g), and the highest rhizosphere priming effect, 33%. *Juniperus virginiana* had the least root biomass (0.45 g), and reduced soil organic matter decomposition by 55% compared to the unrooted control chambers. Three other woody species, *Rhus glabra*, *Celtistsia occidentalis*, and *Gleditsia triacanthos*,

had priming effects close to zero. These results indicate that the direction and magnitude of rhizosphere priming by woody plant species is species specific, which reinforces findings from laboratory studies.

Introduction

Soil respiration, including root and rhizosphere respiration, is the greatest CO_2 flux in terrestrial ecosystems (Kuzyakov 2006), which is roughly ten times the magnitude of annual anthropogenic emissions (Raich and Schlesinger 1992). Rhizosphere activity directly influences root and soil respiration by modifying the form and abundance of soil nitrogen and carbon stocks through the chemical alteration of the rhizosphere environment (Brzostek et al. 2013; McKinley et al. 2011). Carbon additions from root activity enables soil microbes to mine nutrients from SOM thereby increasing the rate of SOM mineralization (Kuzyakov 2010; Fontaine et al. 2011). The change in rate of soil organic matter (SOM) decomposition in response to rhizosphere activity is termed the rhizosphere priming effect (RPE) (Kuzyakov 2002; Zhu and Cheng 2011). Growth chamber and greenhouse studies have found a wide range of priming effects conditional on abiotic conditions and biotic interactions (Huo et al. 2017). Variation in the RPE is attributed to soil temperature, soil moisture, CO_2 concentration, soil structure, and species-specific root-soil interactions (Kuzyakov 2010; Rewald 2014). Depending on these factors, rhizosphere activity can suppress SOM decomposition by as much as 50% or accelerate SOM decomposition as much as 380% compared to the no root control (Zhu and Cheng 2011).

A recent meta-analysis of 31 published papers on the RPE concluded that there is a 95% chance of positive priming effects in greenhouse and growth chamber experiments (Huo et al. 2017). Root exudates, low molecular weight organic compounds released from live roots, often stimulate the decomposition of SOM and

result in positive priming (Zimmerman and Ahn 2010; Bengtson et al. 2012, Shahzad et al. 2015). Plant species and soil texture have been identified as key qualitative factors that determine the magnitude and direction of the RPE (Huo et al. 2017). The most significant quantitative factors that determine the magnitude and direction of the RPE is the time elapsed between planting and sampling and the amount of shoot biomass (Bader and Cheng 2007; Huo et al. 2017). Long-term greenhouse studies with multiple respiration sampling points determined that although rhizosphere activity may initially suppress SOM mineralization, at later sampling dates rhizosphere activity accelerates SOM mineralization (Sallih and Bottner 1988; Kuzyakov 2002). Negative RPE's have been reported for studies lasting between 16 and 39 days, which suggests negative priming effects are short-term phenomena (Cheng et al. 2014).

Persistence of long-term soil carbon pools concomitant with positive priming effects indicate that other components of the biological carbon cycle are also determinants of the total soil carbon pool (Fontaine et al. 2004; Mukhopadhyay et al. 2016). In some cases, the addition of carbon sources has been shown to decrease the carbon pool overtime due to priming effects but these results depend on a number of biotic and abiotic factors (Fontaine et al. 2004). Both positive and negative priming are calculated in relation to an unrooted control soil, thus priming measures the relative change in rate of SOM mineralization due to rhizosphere activities (Zhu and Cheng 2011). The RPE is a means of mechanistically explaining a portion of soil CO_2 flux, and does not indicate whether the soil system is increasing or decreasing the total store of soil carbon (Kuzyakov 2010).

Ecosystem transitions, such as the gradual shift from tallgrass prairie to woodland, reapportion the distribution of biomass from belowground to aboveground (Van Auken 2009). This transition at the eastern edge of the Great Plains, driven by an increase in fire suppression throughout the region, has the potential to increase soil carbon storage (McKinley et al. 2011; Briggs and Knapp 1995). Aggregate stability, which increases SOM occlusion and persistence, in grassland soils is associated with higher plant diversity (Gould et al. 2016; Paustian et al. 2000). Specifically, the soil under *Juniperus virginiana* stands, an increasingly common tree in tallgrass prairie landscapes, has greater nitrogen and carbon stores compared to frequently burned grasslands (McKinley and Blair 2008). In general, regardless of species, areas that experience frequent fires are more nitrogen limited than less frequently burned areas (Blair 1997). The encroachment of woody species into prairie and shrubland is a widely reported phenomena, and so an improved understanding of the mechanisms responsible for belowground nutrient shifts is of global interest (Eldridge et al. 2011).

Thus far, mainly due to procedural challenges, experiments investigating the rhizosphere priming effect have been limited to growth chamber and greenhouse studies making it difficult to know how the RPE manifests in a natural system (Bird et al. 2011; Neuman et al. 2009). Infrequent sampling coupled with high spatiotemporal variation in root activity and soil conditions severely limits our understanding of rhizosphere activities under field conditions (Phillips et al. 2008; Bengtson et al. 2012). Because of this, there is no consensus on the impacts of species and climate on rhizosphere processes (Phillips et al. 2008). There is a clear

need for in field measurements of the RPE and rhizosphere impacts on soil carbon cycling overall (Bird et al. 2011).

In order to address this knowledge gap, I employed a field-based root chamber approach to measure the rhizosphere effects of five native woody tallgrass prairie species *in situ*. Root respiration has been measured in the field using chambers filled with native soil and a section of live root (Chen et al. 2009). These experiments estimate root respiration by comparing the respiration of an unrooted control chamber to the respiration from rooted chambers (Fu et al. 2008; Chen et al. 2009). Root respiration and rhizosphere respiration are often used interchangeably in ecological studies because of their inextricable nature (Andrews et al. 1999). An advantage to the root chamber method is that it enables researchers to collect data over the course of several months (Chen et al. 2009). However, these methods do not address root-soil interactions and the effect of these interactions on both root and soil respiration.

In this paper, I present a method that combines the isotopic ^{13}C natural tracer method with buried root chambers in order to assess root and root-soil interactions on rhizosphere respiration and the rhizosphere priming effect. I transplant sections of freshly-excavated live roots from five woody species with a C_3 photosynthetic pathway into root chambers filled with soil whose SOM has a C_4 $\delta^{13}C$ signature, commonly referred to as a "C_4" soil (Fu and Cheng 2002; Balesdent et al. 1988). The method was adapted from the buried root chamber system described in Cheng et al. (2005) with several key modifications. The goals of this study were to (1) measure species level variation of the RPE within an ecosystem; (2) investigate the potentials

and hindrances associated with *in situ* RPE measurement; and (3) present recommendations for future field rhizosphere measurements.

Methods

The buried chamber method described in this paper is adapted from Cheng et al.'s (2005) ^{13}C natural tracer *in situ* root chamber method. Methodological changes were made based on the findings from the 2005 experiment and additional pre-trial experiments in 2014 and 2015. The method used in this study differs in two key aspects. First, I use a modified 48-hour pulse trapping system in place of the continuous 24-hour trapping system described in Cheng et al. 2005. The increase from 24 to 48 hours increased the total amount of CO_2 trapped and reduced variability between samples. Using the pulse trapping method instead of the continuous trapping method reduced overnight interference from small mammals. Second, the soil used in this experiment had been incubated for six months prior to chamber installation in order to better detect the effects of live roots on recalcitrant SOM (McLauchlan et al. 2004).

Site Description

The experiment was conducted at the Konza Prairie Biological Station (KPBS), a 3487-ha tallgrass prairie Long-Term Ecological Research (LTER) site located 10km south of Manhattan, Kansas (Briggs et al. 2002). The KPBS site is divided into a number of experimental watersheds that have been subject to prescribed fire at different return intervals for decades in order to study the effects of

varying fire regimes on the tallgrass prairie (Briggs et al. 2002). The buried root chambers were installed in the Hulbert Plots, 10x25 m plots established between 1980 and 1982 that replicate some of the large-scale fire treatments across KPBS (Collins et al. 1995). Plots selected for this study either were burned every four years or had never been burned, and therefore had relatively high densities of the target woody species. The unrooted control chambers were distributed randomly throughout the same plots near rooted chambers.

Soil Preparation and Incubation

Shallow A-horizon soil (depth of 20cm) for use in the root chambers was collected from an annually-burned site at the KPBS. An annually burned site was chosen to maximize the cover of C_4 grasses and a distinct C_4 signature of the soil organic matter. The soil composited and mixed, then air-dried and sieved through 2mm screen. The soil was then rewetted and mixed and stored in aerated containers for five months. The purpose of the pre-incubation was to remove some of the labile SOM in order to better measure the impacts of live roots on recalcitrant SOM (McLauchlan et al. 2004). Because root exudates have a more pronounced effect on the decomposition of stabilized SOM, the direction and magnitude of priming effects is determined by the interactions between root exudates, mineral surfaces, and stabilized SOM (Bengtson et al. 2012; Zimmerman and Ahn 2010; Huo et al. 2017). The soil was then air-dried and separated into 650 gram portions for later use.

The native vegetation is dominated by perennial C_4 grasses and over time this has led to the accumulation of SOM with a "C_4" $\delta^{13}C$ (Briggs et al. 2002; Balesdent et

al. 1988). The soil is therefore named a "C_4 soil" since the SOM has a C_4 $\delta^{13}C$ signal (Staddon 2004). Due to fire suppression in some of the watershed and plot treatments, woody species have expanded into riparian and upland areas presenting the unique opportunity to study the effects of woody species rhizosphere carbon interactions using the ^{13}C natural tracer method (Briggs et al 2002, Dijkstra and Cheng 2007a, Staddon 2004). The invasive woody species have a "C_3" $\delta^{13}C$ signal, and the woody species have been established for less than 20 years, and there have not been major changes to the physical and chemical properties of the soil developed under C_4-dominated grassland vegetation (McKinley et al. 2011). As a result, it is possible to grow roots from C_3 woody species in "C_4" grassland soil while maintaining distinctive isotopic signatures for the CO_2 that originates from SOM decomposition and the CO_2 that originates from root respiration. Thus, it is possible to partition root-derived and soil-derived carbon from the total soil respiration measurements (Dijkstra and Cheng 2007a).

The ^{13}C natural tracer method takes advantage of the differential discrimination of the ^{13}C isotope by plants with different photosynthetic pathways during CO_2 assimilation (Fu and Cheng 2002). C_3 plants use the enzyme rubisco to assimilate CO_2 which leads to ^{13}C depletion in plant tissues relative to ambient air, approximately between $\delta^{13}C$ -20‰ and -35‰ (Kuzyakov 2006). Comparatively, C_4 plants use the enzyme phosphoenol pyruvate (PEP) carboxylase to assimilate CO_2 which discriminates to a much lesser degree against ^{13}C resulting in plant tissue $\delta^{13}C$ values between -7‰ and -15‰ (Kuzyakov 2006). The isotopic signatures for each of these plant types is great enough that plant tissue with a C_3 $\delta^{13}C$ and SOM with a C_4

$\delta^{13}C$ (or vice versa) can be accurately and reliably differentiated (Fu and Cheng 2002; Cheng et al. 2005; Kuzyakov 2006).

Root Chamber Construction

Root chambers were constructed out of PVC (polyvinyl chloride) pipe 41cm long, 5cm diameter, and 0.5cm wall thickness. The chambers were designed so that once the soil was added, approximately 8cm of air space remained. This air space was of particular importance during the closed loop respiration sampling because it increased the amount of oxygen rich air in the chamber and prevented the chambers from becoming anaerobic. Chambers designated to be rooted had a 2.5cm diameter hole in the side of the chamber. The chamber was capped at the bottom end with a rubber stopper. In the center of the stopper, plastic tubing was threaded through a 1/4" hole through which the root and soil respiration was sampled. During respiration sampling, an identical rubber stopper was used to seal the top of the chamber. In order to prevent the potential collapse of the flexible plastic tubing, Teflon tubing was inserted in at the connection point. This maintained air flow throughout the course of the experiment.

Root Isolation and Chamber Installation

Five woody plant species in total were used in the 2016 and 2017 field seasons: *Rhus glabra* (smooth sumac), *Cornus drummundii* (roughleafed dogwood), *Juniperus virginiana* (eastern red cedar), *Gleditsia triacanthos* (honey locust), and *Celtis occidentalis* (common hackberry). All five of the species are commonly found

in high densities throughout the tallgrass prairie and are representative of the plant community changes that occur when fire frequency decreases (Briggs et al. 2002). Each species possesses a unique root morphology that allowed us to isolate the root with confidence from the surrounding grass root mat. In 2016 'intact' roots from *Rhus glabra*, *Cornus drummundii*, and *Juniper virginiana* were carefully isolated and installed in buried chambers. In 2017, live roots from all five species were used; however due to particularly harsh weather conditions, *C. drummundii* and *J. virginiana* did not survive the root transplant process.

For the 2016 experiment, there were ten replicates per species and five unrooted control chambers. It was assumed based on pre-trial data that approximately 50% of all transplanted roots would die. The assumption proved functionally correct (Table 2.2). For the 2017 experiment, there were sixteen replicates per species and sixteen unrooted control chambers resulting in approximately eight successful chambers per treatment (Table 2.2). The unrooted soil controls were used to establish the rate of SOM decomposition in the absence of rhizosphere activity.

Spades were used to lift the root mat under the species of interest. Intact fine root systems were isolated by hand. Soil was shaken off from the roots, and roots were inspected for signs of damage (Fig. 2.1, B). The remaining soil on the roots was used as rhizo-microbial inoculum. Roots were then wrapped in wet damp paper towels. A narrow trench was dug for the PVC root chamber so that when the root was inserted the chamber would lie flush with the soil surface (Fig. 2.1, C). The selected root was removed from the wetted paper towels and inserted through the side of the chamber.

The chambers were gradually filled with 650 grams of the prepared soil and 200 grams of deionized water (Fig. 2.1, D).

Plastic wrap was then used to cover the root inlet in order to prevent mixing of the prairie soil and the chamber soil during the incubation period. Pre-experimentation showed that covering the root inlet with plastic wrap instead of the nontoxic silicon sealant during the infield incubation period resulted in visibly improved root growth. Further, past pre-experimentation showed that small mammals' attraction to the non-toxic silicon sealant inadvertently led to high root mortality rates during the infield incubation. Small mammals generally left the plastic wrapped chambers undisturbed.

Care was taken to bury the chambers in a way that left the sampling tube unbent so that air could pass through the tube with ease. Covering the chambers with surrounding soil insulated the chambers from extreme heat temperature fluctuation throughout the day and mimicked the natural protections the roots would experience in the surrounding environment (Fig. 2.1, F).

Chamber Incubation and Sampling

The chambers were left to incubate in the field for 50 days. Previous experiments have noted a pulse of carbon dioxide following soil disturbance, which makes it necessary to wait at least two weeks before respiration sampling (Six et al. 2000). This incubation period gives both the soil and the roots time to adjust to the new chamber environment so that during sampling I measure the impacts of rhizosphere activity on SOM decomposition without the disturbance playing a role in the sampling. During the incubation period, the chambers were examined for signs of

water loss and were aerated with air pumps in order to prevent anaerobic conditions at the bottoms of the chambers. Air pumps were attached to the sampling tube and ambient air was pumped into the bottom of the chambers for twenty minutes (Fig. 2.3). Aeration occurred 2, 4, and 6 days after installation in order to promote water distribution throughout the chamber and in order to avoid anaerobic conditions. 50 grams of water was added to the top of the chambers forty days after installation in order to increase soil moisture before respiration sampling.

Respiration Sampling

Soil and root respiration were measured 50 days after roots and chamber installation. Root and soil respiration were sampled over the course of 48 hours using a closed-circulation pulse trapping system adapted from Cheng et al. 2005. Notable changes in the sampling method included an extension of the sampling time from 24 to 48 hours and the shift from continuous respiration trapping to pulse respiration trapping. Extending the sampling period to 48 hours increased the amount of CO_2 collected for each sample, and this decreased measurement errors. The pulse trapping system was adapted in order to reduce rodent damage. When the CO_2 trapping system was left in the field overnight, rodents frequently chewed through air sampling tubes, which destroyed the sample. Since rodent activity was impossible to curtail, two one-hour samples were taken at 24 hours and 48 hours. The chambers were not exposed to ambient air during this closed-loop sampling period.

Before sampling, the plastic covering was removed from the root inlet and root inlet was sealed above the soil and around the base of each root using a non-toxic silicon

rubber (GI-1000, Silicons Inc. NC, USA). Once the sealant was dry (approximately 20 minutes after application), the chambers were recovered with soil in order to prevent direct sunlight from hitting the chambers. Stoppers were placed on top of the chambers, creating a closed system. Air was passed through soda lime columns for one hour in order to remove CO_2 from the closed- loop system. The air that remained in the chambers was considered CO_2-free air and the 48 hr sampling period commenced.

At 24hr and 48hr the sampling system was reconnected to the chambers. For one hour the air from the chamber was circulated through an airstone inside a plastic bottle containing 300 ml of 0.5 M NaOH solution. The CO_2 produced by the roots and soil in the chambers was absorbed into the 0.5 M NaOH solution. Three blanks containing the .5 M NaOH stock solution were processed in the same way as the rest of the samples in order to correct for possible contamination due to handling.

The total respiration for each chamber was measured by analyzing an aliquot of the NaOH solution using a Shimadzu TOC-5050A Total Organic Carbon Analyzer. The trapped CO_2 was precipitated out of the NaOH solution in the form of $SrCO_3$ using the Harris et al. (1997) method. An excess of $SrCl_2$ was added to a subsample of the NaOH solution from each chamber. The solution was mixed and the precipitate was left to settle at the bottom of the sample tubes. Once every 24hrs, the solution was removed from the precipitate, and CO_2 free DI water was added to the precipitate. After ten days, the solution had a neutral pH, ensuring that exposing the sample to ambient air would not contaminate the $\delta^{13}C$ signature. The $SrCO_3$ was then separated and oven dried at 105°C. A subsample of the $SrCO_3$ was mixed with the catalyst V_2O_5 to facilitate combustion and was sent to the UC Davis Stable Isotope Lab for $\delta^{13}C$ analysis

(Carlo Elba 1108 elemental analyzer interfaced to a Thermo-Finnegan Delta Plus XP isotope ratio mass spectrometer).

The total respiration was separated into SOM-derived and rhizosphere derived carbon using a two-source mixing model as described in Cheng et al 2005.

$$C_{soil} = C_{total}(\delta^{13}C_{root} — \delta^{13}C_{total})/(\delta^{13}C_{root} — \delta^{13}C_{soil})$$

$$C_{root} = C_{total} — C_{soil}$$

Where C_{total} is the total respiration in the rooted treatments, C_{soil} is the microbial respiration of SOM, and C_{root} is the rhizosphere respiration. And where $\delta^{13}C_{root}$, $\delta^{13}C_{soil}$, and $\delta^{13}C_{total}$ are the $\delta^{13}C$ values of the CO_2-C from rhizosphere respiration, SOM respiration, and total soil respiration respectively. $\delta^{13}C_{root}$ is distinct for each species.

$$\% \ RPE = C_{SOM\text{-} derived} (CO, CD, GT, RG, JV) - C_{SOM\text{-} derived} (Control) / C_{SOM\text{-} derived} (Control) *100$$

The rhizosphere priming effect is calculated by first subtracting the SOM-derived CO_2 produced by the rooted chamber from the SOM-derived CO_2 produced by the unrooted control chamber. This difference is then divided by the SOM-derived CO_2 produced by the unrooted control chamber and then multiplied by one hundred so that it is expressed as a percent. The rhizosphere priming effect is the percent change in SOM decomposition between the rooted and unrooted chambers.

Destructive sampling

Following the pulse trapping period, the chambers were removed from the field by cutting the roots at the base of the silicon sealant. Roots and soil were separated by hand. The soil was homogenized and stored at 4°C for further analysis. Root samples were washed and dried in the oven at 60°C for 24 hours and then weighed to determine biomass. Soil moisture was determined by drying approximately 10g of soil in the oven at 105°C for 24 hours. Root and soil samples were ground in a ball mill, and analyzed for $\delta^{13}C$ and $\delta^{15}N$ (Carlo Elba 1108 elemental analyzer interfaced to a Thermo-Finnegan Delta Plus XP isotope ratio mass spectrometer at the University of California Davis Stable Isotope Facility).

Statistical Analysis

The values presented in the tables are the treatment means plus or minus one standard error. Significant differences between means were calculated using a one-way analysis of variance (ANOVA) and pairwise multiple comparison procedures (Holm-Sidak method) for all datasets with the exception of the rhizosphere priming effect. The ANOVA was calculated using data from both field seasons. The differences between means for the RPE were calculated using individual student t-tests. All statistical analyses were performed using SigmaPlot Version 14 from Systat Software, Inc. San Jose, California USA, www.systatsoftware.com.

Results

Root Survival

Weather conditions played a role in determining root transplant success. Roots from species excavated on hotter days with less shade cover from canopy had higher root mortality (Table 2.2). In 2016 all the chambers were installed on one day with a temperature high of 32 °C. In 2017 the chambers were installed over a period of seven days with an average temperature high of 37 °C. The *C. dummundii* chambers were installed on a day with a temperature high of 40°C, and this treatment experienced 100% mortality. Roots isolated under tree canopies were the most likely to survive through the transplant process. There was a broad canopy covering the soil under *C. occidentalis* and this treatment had a 50% survival rate on a day with a temperature high of 40 °C. Comparatively, the soil under *R. glabra* was exposed to direct sunlight and had a 31% survival rate on a day with a temperature high of 35 °C. The weather throughout the chamber incubation was similar between 2016 and 2017 (Fig. 2.3). The results from this study suggest that the weather during installation is much more important than the temperature throughout the incubation. If possible, root chamber installation is likely to be most successful on cooler days and under canopy cover or shade.

Root Biomass

During chamber installation, roots of approximately the same size were chosen to be installed in each chamber for each species. Variation in total root biomass between species is attributed to natural variation in root growth. Intra-

treatment variation in root biomass is attributed to differences between randomly selected roots. All survived roots showed signs of good health and new growth when removed from the chamber after respiration sampling (Fig. 2.2). The final root biomass for *J. virginiana* was 0.45 ± 0.08 g $chamber^{-1}$, which was significantly less biomass compared to the other species (Table 2.1). *C. drummundii* had the greatest biomass, 1.22 ± 0.11 g per chamber (Table 2.1). *R. glabra* had similar amounts of final root biomass during both the 2016 and 2017 field seasons, 0.92 ± 0.23 g and 0.72 ± 0.07 g per chamber, respectively (Table 2.1).

Soil moisture

Soil moisture in the harvested chambers was similar among all rooted chambers in each field season, though soil moisture varied between seasons, averaging 23% water content in 2016 and 13% water content in 2017 (Table 2.2). The unrooted control chambers had 37% water content in 2016 and 32% water content in 2017 (Table 2.2). Notably, the differences between the rooted chambers and the non-rooted control chambers was greater than the differences among any of the rooted treatments. The buried chambers were subjected to several severe storms common to the Midwest. Depending on the orientation of the chambers and the direction of water runoff, some chambers became saturated with water. Once flooded, the roots were generally able to remove excess water whereas the control chambers were more impacted. Better moisture control in future iterations of this approach is desirable.

$\delta^{13}C$ Partitioning

In all chambers, the roots and soil had a distinct $\delta^{13}C$ signal. The soil used in 2016 had an average $\delta^{13}C$ of -16.24 ± 0.02‰ and the soil in 2017 had a $\delta^{13}C$ of -17.00 ± 0.02‰ (Table 2.2). The viability of roots in each chamber was assessed using the $\delta^{13}C$ of the $SrCO_3$ precipitate. Using this method, I determined that *J. virginiana* and *C. drummundii* in 2017 had no active roots at the time of respiration sampling. The average $SrCO_3$ $\delta^{13}C$ for these species was -14.40 ± 0.45‰ and -16.14 ± 0.24‰ respectively (Table 2.1). By comparison, cores with *R. glabra* in 2017 had a $SrCO_3$ $\delta^{13}C$ value of -19.32 ± 0.64‰ demonstrating that the roots in these chambers were alive and contributing to the total sampled respiration (Table 2.1).

Respiration

Total respiration of the chambers varied depending on whether or not the chambers had roots or not and the plant species. Generally, higher root biomass correlated to high rates of total respiration. The final root biomass for *C. drummundii* was greatest among the treatments and this treatment also produced the greatest respiration 71.08 ± 8.28 mg CO_2-C $48hr^{-1}$ $chamber^{-1}$ (Table 2.1). *C. occidentalis* and *J. virginiana* produced the lowest respiration, 46.77 ± 3.56 mg CO_2-C $48hr^{-1}$ $chamber^{-1}$ and 46.22 ± 9.67 mg CO_2-C $48hr^{-1}$ $chamber^{-1}$ respectively (Table 2.1). There was no significant difference between the amount of respiration produced by the unrooted control chambers in the 2016 and 2017 field seasons. The *R. glabra* treatment in 2016 produced slightly higher total respiration during the 2016 compared

to the 2017 field seasons, 69.21 ± 7.93 mg CO_2-C 48hr^{-1} chamber^{-1} and 54.86 ± 9.03 mg CO_2-C 48hr^{-1} chamber^{-1} (Table 2.1).

Priming

The $\delta^{13}C$ of both the soil and the plant roots were each distinct from one another so that the root and soil respiration could be partitioned using the ^{13}C natural tracer method. The incubated soil had an average $\delta^{13}C$ of -17.04‰ and for example, *G. triacanthos* roots had an average $\delta^{13}C$ of -27.38 ± 0.34‰ (Table 2.1, Table 2.3). The $\delta^{13}C$ of the total respiration as measured by the $SrCO_3$ precipitate was significantly different between the rooted and unrooted chambers. For example, the unrooted control chambers had a $SrCO_3$ $\delta^{13}C$ of -17.42 ± 0.29‰ compared to the $SrCO_3$ $\delta^{13}C$ of *C. occidentalis* 19.25 ± 0.40‰ (Table 2.1).

Lower root biomass was associated with lower SOM-derived CO_2. *J. virginiana* had both the lowest root biomass and the lowest respiration of SOM-derived CO_2 (Table 2.1). C. *drummundii* had the greatest root biomass and the highest amount of SOM-derived CO_2 (Table 2.1). The amount of primed carbon per gram root biomass was statistically similar for all species excluding *J. virginiana* (Fig. 2.5). *C. drummundii* primed 9.54 ± 4.61 mg C g^{-1} root and *G. triacanthos* primed 13.71 ± 15.80 mg C g^{-1} root (Table 2.3). Comparatively, *J. virginiana* primed -59.91 ± 18.44 mg C g^{-1} root (Table 2.3).

Root biomass and species partially determined the direction and magnitude of the RPE. *C. drummundii* had the highest root biomass and accelerated the rate of SOM decomposition by 33% compared to the unrooted control treatment (Table 2.1,

Fig. 2.3). Correspondingly, *J. virginiana* had the lowest biomass of any species and was the only species to produce a negative priming effect, -55% RPE (Fig. 2.3, Fig. 2.5). Each of the other three species, *R. glabra*, *C. occidentalis*, and *G. triacanthos* had priming effects close to zero during the 2017 field season (Fig. 2.3). The presence of roots of these three species neither significantly accelerated nor retarded the rate of SOM decomposition. Compared to the unrooted control chambers, *R. glabra* suppressed the rate of SOM decomposition by 3% during the 2016 field season, and increased the rate of SOM decomposition by 8% during the 2017 field season (Fig. 2.3). Although there was a clear species effect on priming, the amount of root-derived CO_2 per gram of root biomass had no significant species effect (Fig. 2.6). For *R. glabra* in 2016 and *G. triacanthos* in 2017 there was high intra species variability, but this did not result in significant differences between species.

Discussion

This experiment used a ^{13}C natural tracer root chamber method to measure the rhizosphere priming effect *in situ* (Cheng et al. 2005). Due to methodological challenges, there have been few studies able to quantify rhizosphere effects in the field (Huo et al. 2017; Neuman et al. 2009). The RPE measured using the buried root chamber method ranged from -55% to 33%, which is consistent with past greenhouse and growth chamber studies, and was dependent both on species and on year (Zhu and Cheng 2011).

Advantages and Reliability

There are several advantages to this system. (1) The materials are portable and are suited to remote locations. The equipment used to measure respiration are battery powered and organized into easily transportable boxes. (2) Root branches can grow in the chambers for months. In this experiment, the roots remained in the field for 50 days following chamber installation. Some considerations may need to be made based on what is known about root growth rates. For example, *G. triacanthos* filled the chamber with roots during the allotted field incubation period (Fig. 2.2). Larger chambers may need to be used if the species is known to have high root growth rate and a longer infield incubation time is desired. (3) It is possible to partition the rhizosphere and soil respiration using the natural tracer method. In all cases, the difference between soil and root $\delta^{13}C$ values was significant enough to reliably distinguish root and soil respiration (Table 2.1).

The reliability of successful root chamber installation and measurement is dependent on the weather during the time of installation and the rodent activity in the area during the respiration sampling time. Roots from species excavated on hotter days with less shade cover from canopy had higher root mortality (Table 2.2). *C. dummundii* was planted on a day with an average temperature of 33 °C and a high of 41°C and experienced 100% mortality (Table 2.2). Roots isolated under tree canopies were the most likely to live through the transplant process (Table 2.2). *C. occidentalis* and *R. glabra* were transplanted on days with similar weather and yet had vastly differing transplant success (Table 2.2). If possible, root chamber installation is likely to be most successful on cooler days and under canopy cover or shade. The implementation of the 48hr pulse trapping method and the use of live

rodent traps in the surrounding area drastically reduced the number of samples lost due to rodent activity.

Adaptability

The use of the silicon sealant makes this method most suited to plant species that have roots with flexible suberized bases and to plant species with easily identifiable root morphology. The ^{13}C natural tracer method is most suited for systems that naturally have C_3 species growing in historically C_4 soils or vice versa (Fu et al. 2008; Cheng et al. 2005). However, greenhouse experiments have routinely planted C_3 species in non-native C_4 soils and have provided valuable results (Fu and Cheng 2002; Dijkstra and Cheng 2007b). Therefore, future experiments transplanting C_3 roots into root chambers containing C_4 soil are expected to provide mechanistically relevant information.

Soil Water Content

There are two main challenges associated with the implementation of this method. Soil water content was much lower in the rooted chambers compared to in the unrooted chambers (Table 2.2). The unrooted chambers maintained desired soil water contents during the experiment (Table 2.2). At the time of harvest soil moisture ranged from 9% soil water content in one *C. occidentalis* chamber (0.59g root biomass, treatment average 12.17 ± 0.85% soil water content) to 39% soil water content in several of the control chambers (Table 2.2). Rooted chambers had significantly lower soil moisture contents than the control chambers due to the live

roots water uptake capacity (Table 2.2). Between the rooted treatments, *J. virginiana* had significantly higher soil moisture (Table 2.2). Notably, the differences between the rooted chambers and the non-rooted control chambers was greater than the differences between any of the rooted treatments (Table 2.2).

It has been reported that up to 70% of the variation in soil respiration can be attributed to variations and interactions between soil water and temperature (Wildung et al. 1975). Better moisture control in future iterations of this approach is desirable. *R. glabra* was the only species to survive both field seasons and so can provide some insight into how variation in water availability impacted the priming effect. The soil water content for *R. glabra* was 21.86 ± 4.15% in 2016 and 11.11 ± 0.25% in 2017, and the unrooted control chambers had a soil water content of 36.77 ± 3.22% in 2016 and 32.36 ± 0.97% in 2017 (Table 2). The priming effect was negative for *R. glabra* in 2016 and positive in 2017 (Table 2.1). However, root biomass and root respiration were higher in 2016 (Table 2.1, Table 2.3). Scheduled watering throughout the infield incubation based on rainfall patterns and temperature may help to maintain the soil water content in the rooted chambers.

Species Specific Priming Effects

The levels of the RPE reported in this study are consistent with previous growth chamber and greenhouse studies (Huo et al. 2017; Cheng et al. 2014). In both years higher RPEs were correlated with greater total biomass and higher rates of root respiration (Table 1, Fig. 4). However, when the amount of primed SOM was standardized between species by calculating the amount of primed SOM per gram

root biomass, the species effect on the RPE was less apparent (Fig. 2.5). The RPE was highest for *C. drummundii*, but *C. occidentalis* and *G. triacanthos* had statically similar rates of priming per gram biomass (Fig. 2.5, Table 2.3).

Juniperus virginiana had the lowest priming effect and the least root biomass of all the treatments. Yet, root respiration per gram biomass was statistically similar for all plant species (Fig. 2.6). It follows that the RPE was driven by root exudation in the case of *J. virginiana*. Negative priming effects have found exclusively in short-term studies such as this (Cheng et al. 2014). The negative priming effect reported here may have been accentuated by the low final root biomass for *J. virginiana* (Table 2.1). The other species included in this study had greater amounts of final root biomass and were therefore able to physically and chemically disturb the soil to a greater degree than *J. virginiana*, potentially leading to higher priming effects (Table 2.1; Kuzyakov 2002). The preferential substrate hypothesis may best apply here; in the absence of easily accessible SOM, the rhizosphere microbes rely more heavily on root exudates (Dijkstra et al. 2013). As root biomass and soil disturbance due to root growth increase, the RPE increases as well (Table 2.1, Fig. 2.4).

Conclusion

The buried root chamber method described here is a more ecologically relevant method of measuring root-soil dynamics than previous methods of measuring the RPE including greenhouse studies and root chambers (Phillips et al. 2008, Neuman et al. 2009). While previously utilized root chamber methods (Fu et al. 2008, Chen et al. 2009) can provide ecologically scaled data under realistic soil

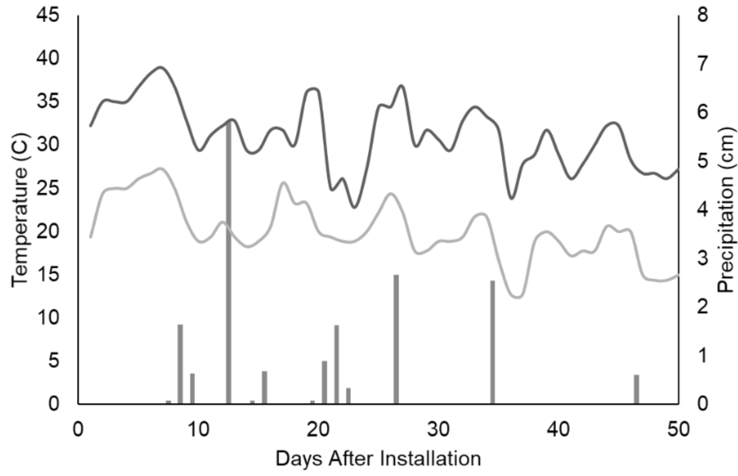
conditions for root respiration, these methods do not provide rhizosphere and soil respiration partitioning or rhizo-soil interactions as this method is able to do. Because the roots remain attached to the woody species in this method, the roots are able to grow, die, and interact with the soil as would happen naturally in the field (Cheng et al. 2005). I conclude that species and root biomass are key determinants of the magnitude and direction of the rhizosphere priming effect.

Figure 2.1.

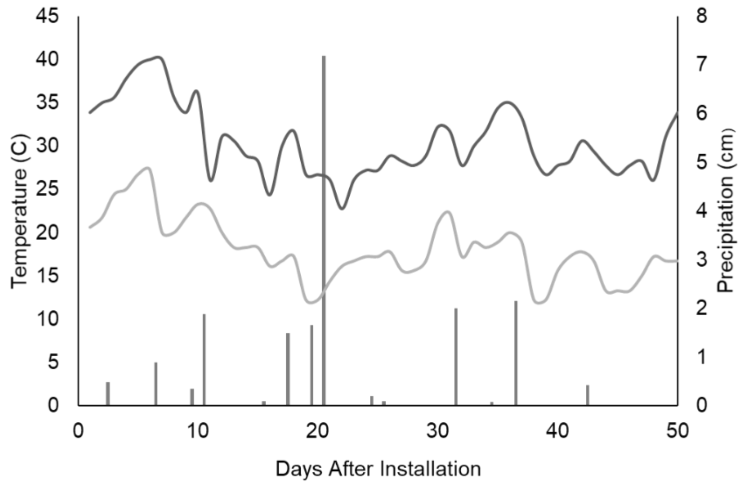
A)

B)

Figure 2.2.



A)



B)

Figure 2.3.

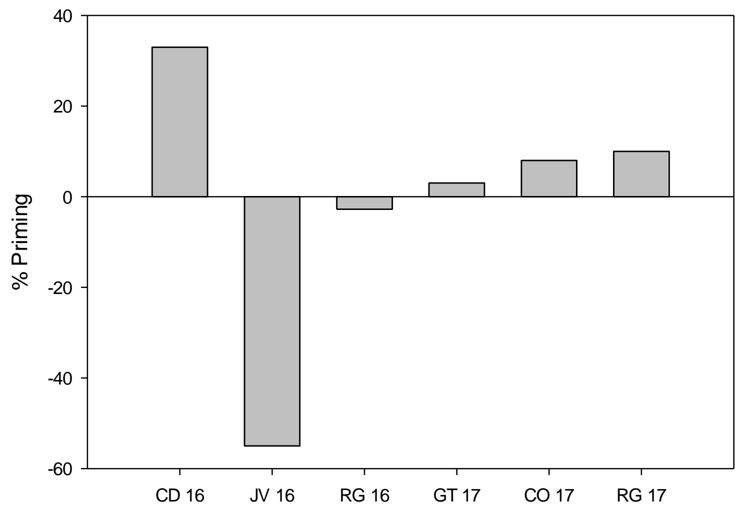


Figure 2.4.

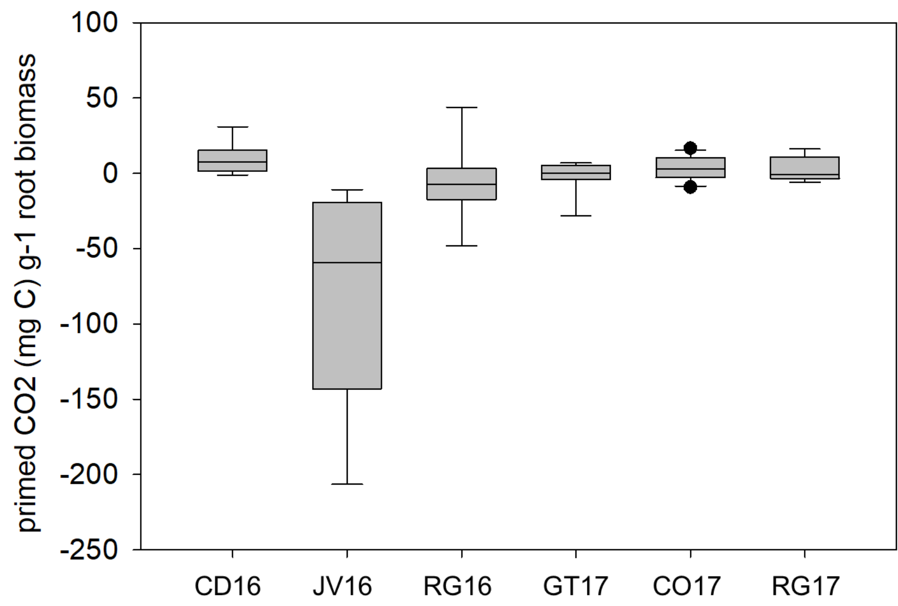


Figure 2.5.

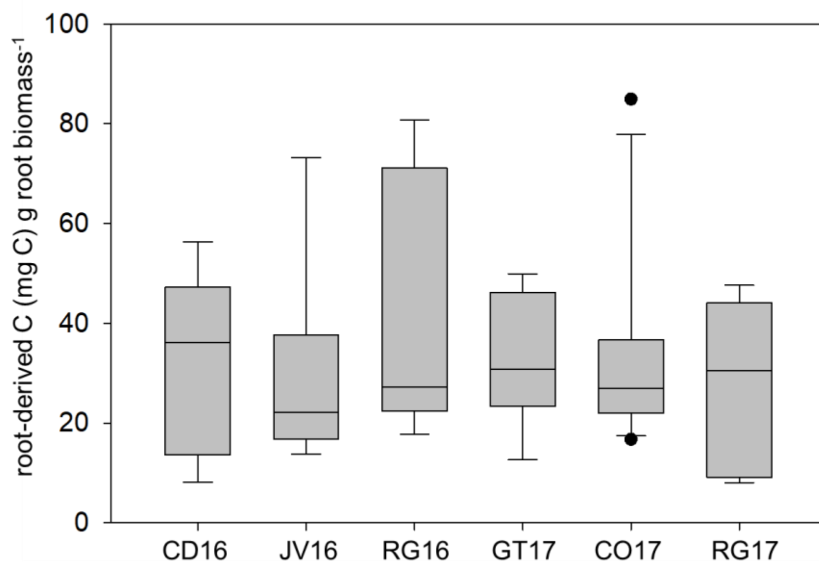


Figure 2.6.

Table 2.1. Respiration and Root Characteristics. Respiration characteristics were measured 50 days after chamber installation using the 48 hour pulse trapping method. Root characteristics were measured during the destructive harvest after respiration sampling. Each presented value is the treatment average plus or minus one standard error. For each column, significant differences are indicated by different letters (*post hoc* Holm-Sidak, P<0.05).

*Based on isotopic signatures and visual appearance, these roots were determined to be dead.

RG: *Rhus Glabra,* JV: *Juniperus virginiana,* CD: *Cornus drummundii,* GT: *Gleditsia triacanthos,* CO: *Celtis occidentalis,* NR: unrooted control

2016						
	Respired δ^{13}C (‰)	**root biomass**	**total respired CO_2-C 48hrs^{-s}**	**soil-derived CO_2-C 48hrs^{-1}**	**soil-derived CO_2-C 48hrs^{-1} g^{-1} root biomass**	**total respired CO_2-C 48hrs^{-1} g^{-1} root biomass**
RG	-20.08 ± 0.21^{a}	0.87 ± 0.24^{b}	69.21 ± 7.93^{b}	21.25 ± 2.90^{b}	38.29 ± 12.99^{a}	69.21 ± 7.93^{b}
JV	-19.67 ± 0.52^{a}	0.40 ± 0.12^{a}	46.22 ± 9.67^{a}	9.83 ± 2.20^{a}	22.73 ± 5.00^{a}	48.04 ± 7.75^{ab}
CD	-20.79 ± 0.44^{a}	1.22 ± 0.13^{a}	71.08 ± 8.28^{b}	28.97 ± 2.37^{b}	24.69 ± 2.77^{a}	83.12 ± 6.29^{b}
NR	-16.35 ± 0.20^{b}		21.85 ± 0.53a	21.85 ± 0.53^{b}		26.02 ± 0.95^{a}
2017						
GT	-19.25 ± 0.40^{a}	0.84 ± 0.19^{b}	52.65 ± 4.54^{ab}	25.02 ± 1.56^{b}	35.51 ± 6.09^{a}	64.14 ± 3.34^{b}
RG	-19.32 ± 0.64^{a}	0.72 ± 0.07^{ab}	54.86 ± 9.03^{ab}	26.24 ± 1.84^{b}	40.94 ± 8.12^{a}	65.64 ± 2.75^{b}
CO	-19.91 ± 0.47^{a}	0.65 ± 0.12^{ab}	46.77 ± 3.56^{a}	26.76 ± 1.15^{b}	46.88 ± 6.12^{a}	55.92 ± 2.95^{ab}
JV*	-14.40 ± 0.45^{c}	0.14 ± 0.03^{a}				
CD*	-16.14 ± 0.24^{b}	0.33 ± 0.08^{a}				
NR	-17.42 ± 0.29^{b}		24.28 ± 1.11^{a}	24.28 ± 1.11^{b}		33.33 ± 2.15^{a}

Table 2.2. Soil Characteristics measured at the end of the infield incubation, 50 days after chamber installation. The values presented are treatment averages plus or minus one standard error. For each column, significant differences are indicated by different letters (*post hoc* Holm-Sidak, P<0.05). The success rate of each rooted treatment was assessed based on the $SrCO_3$ $\delta^{13}C$ values and the appearance of the root during the destructive harvest. The success rate of unrooted chambers was based on whether or not the chambers were anaerobic at the time of harvest. For all measurements, only the successful chambers were included in the calculations therefore the number of samples in each treatment varies.

RG: *Rhus Glabra,* JV: *Juniperus virginiana,* CD: *Cornus drummundii,* GT: *Gleditsia triacanthos,* CO: *Celtis occidentalis,* NR: unrooted control

2016				
	$\delta^{13}C$ (‰)	**C:N**	**Water Content (%)**	**Success Rate (%)**
RG	-16.30 ± 0.02^{cb}	12.06 ± 0.14^{a}	21.86 ± 4.15^{b}	80
JV	-16.52 ± 0.08^{b}	12.02 ± 0.04^{a}	23.04 ± 2.24^{cb}	50
CD	-16.28 ± 0.18^{cb}	12.46 ± 0.20^{ab}	24.28 ± 2.05^{cb}	50
NR	-16.24 ± 0.02^{c}	11.94 ± 0.07^{a}	36.77 ± 3.22^{c}	100

2017				
GT	-17.08 ± 0.03^{a}	12.63 ± 0.08^{b}	14.26 ± 0.85^{ab}	63
RG	-16.92 ± 0.02^{ab}	12.77 ± 0.03^{cb}	11.11 ± 0.25^{a}	31
CO	-17.17 ± 0.02^{a}	12.95 ± 0.07^{c}	12.17 ± 0.85^{ab}	50
NR	-17.00 ± 0.02^{ab}	12.17 ± 0.03^{ab}	32.36 ± 0.97^{cb}	50

Table 2.3. Respiration characteristics as measured during the 48 hour pulse trapping. Values are presented as treatment averages plus or minus one standard error. For each column, significant differences are indicated by different letters (*post hoc* Holm-Sidak, P<0.05).

RG: *Rhus Glabra,* JV: *Juniperus virginiana,* CD: *Cornus drummundii,* GT: *Gleditsia triacanthos,* CO: *Celtis occidentalis,* NR: unrooted control

	primed CO_2 (mg C) chamber^{-1}	root-derived CO_2 (mg C) chamber^{-1}	primed CO_2 (mg C) g^{-1} root biomass
2016			
RG	-3.53 ± 3.07[ab]	41.86 ± 8.61 [a]	-6.72 ± 3.26[b]
JV	-13.45 ± 2.15[a]	31.41 ± 9.54 [a]	-59.91 ± 18.44[a]
CD	11.22 ± 4.29[bc]	37.61 ± 6.02 [a]	9.54 ± 4.61[b]
2017			
GT	1.56 ± 2.38[b]	31.07 ± 4.43 [a]	13.71 ± 15.80[b]
RG	0.92 ± 1.84[b]	27.37 ± 7.09 [a]	2.91 ± 3.40[b]
CO	1.15 ± 1.07[b]	33.12 ± 5.86 [a]	5.84 ± 4.25[b]

CHAPTER 3: SECOND GENERATION BIOFUELS: POTENTIAL AND OBSTACLES

Introduction

Biofuel production in the U.S. has garnered increasing attention since the 1990s due to a national interest in energy independence and potential impacts of fossil-fuel-related climate change (Searchinger et al. 2008). Increasing the production of biofuels may possibly address both issues. Politically, biofuels present an opportunity to increase the domestic energy supply, provide new markets for farmers, and reduce national dependence on imported oil (H.R. 6 2007). Biofuel's actual contribution to greenhouse gas reduction is debated (Searchinger et al. 2008), but its contributions to energy independence and local farm economies are not. Governmental support in the forms of research grants and price subsidies for cellulosic biofuels increased between 2009 and 2016 (Chite 2014).

Yet there remains uncertainty associated with the future of the biofuel industry (EPA 2019). Biofuel production continues to miss government-mandated production targets (EPA 2019). Inertia has been shown to play a role in public opinion, consumers and producer alike tend to prefer the status quo over any change, and without clear success in the second generation biofuels industry, continued public funding for biofuels could decline (Klein et al. 2010).

Innovative technological development that reduces the cost of production is integral to the further expansion of the biofuel industry (Abengoa Bioenergy 2011). The NREL, USDA, DOE, and coordinated state biofuel research programs are working towards the same goals of improved biomass pre-processing and increased

efficiency in switchgrass pyrolysis in order to increase the efficiency of cellulosic biofuel production (NREL 2018). Joint research efforts are yielding new methods of cellulosic material enzymatic pretreatment which have dropped the theoretical production cost of cellulosic fuel (NREL 2018). Biofuel refineries have distinguished themselves based off of their unique enzymatic pretreatment, and because of this companies refuse to share this proprietary information (Abengoa Bioenergy 2011). Meanwhile, governmental labs, like NREL, have developed their own methods of producing ethanol and biodiesel, but these methods have not yet reached refinery operators (NREL 2018).

New technologies that improve efficiency are an important part of the future of biofuel. Other aspects of biofuel markets also need attention. This paper examines market, institutional, and communications aspects of biofuel markets. It reviews the literature on economic barriers to expansion of second generation biofuels, with special attention paid to switchgrass as a potential fuel source. The term “economic barrier” has a variety of definitions. Economic barriers are defined in this paper as “factors that make entry unprofitable while permitting established firms to set prices above marginal cost, and to persistently earn monopoly return" (Ferguson, 1974). Barriers can also be identified as benefits industry insiders have over potential entrants (Demsetz 1982; Fee et al. 2004). Because the switchgrass economy has yet to launch, I instead define economic barriers more broadly as any encumbrance on market exchange, whether technical, legal, contracting, or otherwise. I consider these many categories of constraints to ultimately be “economic” because the logic of market exchange is to

engage in profitable transactions and that is the ultimate decision participants make: whether a transaction will be profitable or contribute to future profitability.

I first discuss the current status of cellulosic biofuel production in the United States by addressing the roles of federal and state governments, including multiple governmental agencies, most prominently, the Environmental Protection Agency and the Department of Energy. I then estimate the potential of switchgrass as a bioenergy source within the United States based on the existing land available and the supply needed to reach the U.S. Department of Energy's set goals for bioenergy. Next, I summarize the current social, technological, and logistical impediments to biofuel production. I then examine the role of uncertainty, both political and economic, and its role in the cellulosic biofuels industry.

Cellulosic Biofuel in the United States

Since the 1990s, the United States Environmental Protection Agency (EPA) and the United States Department of Energy (USDOE) have created incentive programs for alternative energy development with the intent of reducing greenhouse gas (GHG) emissions from energy production and increasing domestic energy self-reliance. One source of energy that has gained attention since the mid-2000s is bioenergy: fuel and electricity derived from living organisms. Bioenergy has been hailed as a means of simultaneously reducing carbon emissions and increasing energy independence within the United States (Tyner et al. 2010). Bioenergy encompasses all types of energy and fuel derived from biological feedstocks. Biofuels typically are liquid fuels design for use in the transportation sector.

Renewable Fuel Standards were first created as a part of the *Energy Policy Act of 2005* and were expanded in the *Energy Independence and Security Act of 2007* (H.R. 6 2017). These mandates were meant to increase the amount of alternative fuels incorporated into the United States' national fuel supply (Figure 3.2.β). The carbon emissions associated with alternative fuels is regulated so that advanced biofuels must be produced in a way that generates no more than half of the GHG emissions of petroleum liquid fuel production, and cellulosic biofuels must achieve a 60% GHG emissions reduction (EPA 2019). The assessment of fuel life cycles varies depending on the method used, and so there remains some debate about the estimated carbon emission mitigation impacts associated with biofuel production (Searchinger 2008).

The rate of adoption of biomass-based liquid fuel and electricity generation has lagged expectation in the U.S. (Figure 3.1). The biofuel industry has repeatedly missed the production goals established by the EPA (EPA 2019). Following the Great Recession (2007-2009), rising oil prices made biofuels comparatively affordable, and corn ethanol was incorporated at higher rates into the national fuel supply than it had been in previous years (Fig. 3.1). By 2010 the U.S. crude oil price per barrel of oil was $74.71. By 2016 that price was down to $38.29 per barrel (U.S. Energy and Information Administration 2018).

The National Renewable Energy Laboratory (NREL), founded by the DOE in 1974, collaborates with the USDA in order to research production methods for biofuel and bioenergy crops. One of the main goals of the NREL in relation to biofuels is to reduce the price of ethanol to $2.15/gallon (NREL 2018). The NREL claimed to have met this target in 2012, however current estimates of ethanol range from $2.15-$4.55

per gallon. The variation in cost is a result of differences in production methods. The NREL is also working on creating biofuel products that are virtually indistinguishable from gasoline and diesel products. The more easily cellulosic biofuel products are able to be blended with conventional oil products, the more valuable the biofuel products.

Three categories of biofuel feedstocks are used in United States. First generation biofuels refer to fuels that are produced from starch, sugar, animal fats and vegetable oil (Dragone et al. 2010). Examples of these are corn kernels, sugar cane, and used cooking oil. Fuel crops such as corn and sugar cane have high concentrations of starch and sugar; starch makes up 70-72% of corn by dry weight, and the sugar cane residues used in biofuels contain 135kg sucrose per ton (Bothast 2005; Dhaliwal et al. 2011). Energy dense sources such as these can be fermented and distilled into fuel ethanol with few intermediate steps, an advantage compared to other biofuels. Corn ethanol produced in the United States and sugar cane ethanol produced in Brazil are currently the most commonly used biofuels.

Second generation biofuels, also known as advanced biofuels, are fuels that are produced from rapidly replenished biomass. Feedstocks include various types of prairie grasses (switchgrass, *Panicum virgatum*, and Miscanthus), agricultural byproducts (corn stover and wheat residue), and fast-growing trees (eucalyptus and poplar) (Dragone et al. 2010; White et al. 2013). Corn stover is comprised of the leaves and stems of corn plants, which typically remain on the field after corn harvests, and can be used to produce biodiesel much in the same way as switchgrass and other cellulosic products.

Second generation biofuels, which include cellulosic biofuel, are of particular interest in the United States due both to the capacity of the midwestern and southeastern United States to produce these feedstocks (White et al. 2013; Tyner et al. 2010) as well as the United States' government interest in diversifying the national energy supply (USDOE 2017; EPA 2019). Research funded in part by the United States Department of Energy (USDOE 2017) has focused on increasing the efficiency of cellulosic bioenergy production with the goal of creating an economically viable industry by 2020 (USDOE 2017; EPA 2019). In addition to research support, U.S. and state governments have established production mandates and subsidies to encourage the economic expansion of the biofuels industry. The Revised Renewable Fuel Standard (RFS2), the Farm Bill, and other federal legislation impact the development the biofuels industry by increasing the demand for biofuel products and encouraging entrance into the market. In 2011 the USDOE set a goal of producing 36 billion gallons of biofuel annually by 2022 as part of the Green Energy Initiative. This goal is consistent with the Energy Independence and Security Act of 2007, which mandated 16 billion gallons of cellulosic feed stock and 36 billion gallons of renewable fuel by 2022. It is estimated that 114 million gallons of second generation biofuel was produced throughout the U.S. in 2016 (USDA 2019), which is below the Renewable Fuel Standard target of 260 million gallons of cellulosic biofuel (EPA 2019). At the current rate of growth and production in the cellulosic biofuel industry, it was unlikely that producers will meet the EPA's 2017 cellulosic biofuel goal of 311 million gallons (EPA 2019). In response to the failure to meet repeated mandates, the Renewable Fuel Standard mandate for

cellulosic biofuels is 288 million gallons for 2018, lower than the mandate established for 2017 (EPA 2019).

Third generation biofuels are derived from genetically engineered low maintenance crops, such as algae (Dragone et al. 2010). Still in development, it is unclear whether third generation biofuels will become commercially viable (Searchinger et al. 2008). If developed, these fuels are expected to have fewer environmental consequences and a lower carbon footprint than first or second generation biofuels (Dragone et al. 2010). However, mass production of these fuels is not yet feasible, and due to multiple technological hurdles, third generation biofuels are not likely to be commercially viable until after 2020 (Dragone et al. 2010).

As a part of an effort to increase bioenergy technology, the 2014 Farm Bill Energy Title IX provided $700 million for bioenergy technology research, educational outreach, and biorefinery construction (Chite 2014). The federal government through its Biomass Infrastructure Partnership (BIP) has awarded a nearly $1 billion across 20 states, and the 2008 Farm Bill provided a $1.01 per gallon subsidy for biofuel (Dwivedi et al. 2009; USDA 2019). Additionally, the U.S. government has created programs, such as the biomass crop assistance program (BCAP) to incentivize farmers to grow bioenergy crops on their land. The program was established in 2014 when the most recent Farm Bill was approved, and it is designed to provide loans and advice to farmers growing bioenergy crops (White et al. 2013; Chite 2014). Programs like BCAP and BIP, in addition to state and federal production mandates, indicate that there is strong political will to create a cellulosic biofuels industry within the United States. Depending on environmental conditions and prices, the amount of land in production

for commodity crops and bioenergy crops fluctuates. Land that may be considered too poor for traditional cash crop cultivation may be put into production according to changes in supply, demand, and price (Skevas et al. 2016).

The Potential of Switchgrass as a Biofuel

Switchgrass, *Panicum virgatum*, is a perennial warm season deep-rooting bunch grass native to North America. Since the Dust Bowl era, the federal Conservation Reserve Program, previously known as the Soil Conservation Service, has encouraged farmers to plant switchgrass on marginal and degraded land in order to improve water infiltration to aquifers and decrease soil erosion and soil salinity. Perennial grasses grow quickly, are resistant to many pests and disease, provide local fowl with habitat, and have deep rooting systems that are both able to survive in salt affected soils and to access deep water resources (Perlack et al. 2011).

Switchgrass can be grown relatively easily especially in the midwestern and southeastern United States, but there is not at present sufficient economic incentives for planting the crop (White et al. 2013). Interviews show that farmers are generally uninterested in even leasing their land for devoted biofuel crop production (Skevas et al. 2016). The midwestern United States and the southeastern United States are considered the regions where switchgrass grows most easily because they have consistent rainfall, high humidity, and nutrient rich soils that are likely to produce the highest switchgrass yields with the least amount of inputs (Mitchell et al. 2012). Kentucky, southern Virginia, and Tennessee in particular have a high potential for switchgrass production (Jensen et al. 2007; Wen et al. 2009). Although switchgrass

grows well under harsh conditions, higher yields can be obtained under preferable soil and weather conditions. A survey of Missouri farmers with lands in the CRP and lands that are susceptible to erosion are unlikely to grow switchgrass (Burli et al. 2019). Farmers are more likely to invest in switchgrass if they have lands under pasture or forest/woodlands (Burli et al. 2019).

Unlike annual crops, such as corn, perennial grasses sequester carbon in their root systems over the course of decades (Garten and Wullschleger 1999, Perlack et al. 2011). As individual roots slough off and die, the biomass is broken down by microbial populations and a portion is eventually occluded within the soil structure where the carbon remains for hundreds of years. Improved soil structure from increases in soil organic carbon and deep root channels increases water infiltration and can ameliorate sodic soils (Garten and Wullschleger 1999). Switchgrass has also been shown to reduce nitrogen pollution caused by runoff when planted near waterways (Woodbury et al. 2018). The many environmental benefits of established switchgrass stands are the reasons that it has been studied by agricultural scientists and advocated for by conservationists since the 1930s.

In the 1980s, the renewable fuels industry took an interest in switchgrass because of its high yield and capacity to grow on marginal lands (Perlack et al. 2011). Farmers can harvest the upper portions of stands, leaving roots and roughly one foot of stems in place to regrow. The same stand can be harvested up to two times per year (Wu, 2017). On average switchgrass yields 6-8 tons/acre on marginal lands including reclaimed mine lands (Jensen et al. 2007; Scagline-Mellor et al. 2018). These yields vary according to variety, soil conditions, and fertilization rates (Jensen et al. 2007).

Up to 11 tons/acre have been recorded under favorable conditions (Schmer et al. 2008). The variation in yields is partially due to a lack of established farming practices.

If baled, switchgrass is valued at approximately $55/ton farm gate, the market value of switchgrass not including selling costs (Burden 2012). As of 2012, the cost of producing switchgrass is estimated to be between $40 and $60/ton, creating a tight margin of profitability. Unlike first generation biofuels, switchgrass is a low-density biofuel, which means that the energy per unit mass ratio is lower than other high-density crops, such as corn or soy (Perlack et al. 2011). Without government incentives, the potential profitability of switchgrass as a bioenergy source is considered to be low (Burden 2012).

However, preprocessing of bioenergy inputs has the potential to increase the value of switchgrass biomass for farmers. When switchgrass biomass is processed into pellets, a favored medium of biofuel plants, the biorefineries would pay approximately $200/ton (USDA). The cost of processing switchgrass into pellets is roughly $88/ton, machinery and labor costs not included (Ciolkosz 2015). This again results in a narrow margin of potential profitability, and is therefore unlikely to be adopted widely by farmers.

Due to the relatively low energy density of switchgrass, biofuel refineries tend to have low energy production capacities compared to other types of fuel plants. A 25 million gallon per year (MGY) plant will use approximately 1100 dry tons of biomass daily if the plant is operating at full capacity (Mitchell et al. 2012). To minimize transportation costs, the biomass used to fuel the plant must come from surrounding areas (USDA 2010). The limited distance that the biomass should optimally travel

places restrictions on the areas where a new cellulosic biofuel plant can be established. For example, if a 25 MGY plant is supplied by a 25-mile radius area, 5% of the land would need to be devoted to switchgrass production (Mitchell et al. 2012). This would not dramatically alter the agricultural landscape, but it does require that several farmers in the same area agree to grow the biofuel crop.

Barriers to the Expansion of Second Generation Biofuel

The development of the biofuels industry has been much slower than entrepreneurs and lawmakers anticipated. One study reports that only 26% of the interviewed Kansas farmers would consider producing cellulosic feedstock (Fewell et al. 2016). Other farmer interviews similarly concluded that farmers were uninterested in investing in biofuels (White et al. 2013; Jensen et al. 2007).

Barriers to market expansion can be divided between refinery-focused and farmer-focused categories. Starting with refineries, since the mid 2000's, the technology required to efficiently produce cellulosic biofuels has been slow to develop (Yang et al. 2011; Gustafsson et al. 2015). One of the main goals of bioenergy research is to decrease production costs by increasing process efficiency (Dwivedi et al. 2009). For example, each type of biomass has specific pretreatment and processing requirements, which makes it difficult for refinery plant operators to utilize heterogeneous materials (Figure 3.2.1). Plant operators prefer fuel sources of standard form and quality, such as fuel pellets with a specific range of available BTUs. Preprocessing of agricultural products can occur in agricultural fields following harvesting or in nearby facilities (Figure 3.2.2). This helps to create a uniform product,

which makes it easier to sell at a higher price (Dexter et al.1994). This strategy has been shown to work towards the expansion of other agricultural products especially grains (USDOE 2017, Dexter et al. 1994).

Biofuel refineries, the United States Department of Agriculture, and the United States National Renewable Energy Laboratory have spent considerable time and money developing efficient refinery processes (NREL 2018; Ripplinger et al. 2012). Second generation fuels require complex multistep pyrolysis, separation, fermentation, and distillation processes with each phase contributing to overall cost (Yang et al. 2011). Improving the efficiency of pyrolysis, the process of using heat to breakdown the biomass structure in order to extract cellulose and hemicellulose, is a point of focus among refinery operators (Gustafsson et al. 2015). Companies have sought to improve this process by the addition of specialized enzymes that increase the speed of structural degradation (Yang et al. 2011). Cellulosic products, unlike the corn kernels used in first generation biofuel, have a variety of structurally complex carbohydrates that makes this initial step difficult (Yang et al. 2011).

In 2014, Abengoa (https://www.energy.gov/lpo/abengoa-bioenergy), an owner of several biofuel plants, cited their particular blend of enzymes as one of the main reasons supporting the possibility of large-scale biofuel processing (Abengoa Bioenergy 2011). The specific types of enzymes and the particular processes used by pilot plants are rarely disclosed in order to protect trade secrets and comparative economic advantage (Abengoa Bioenergy 2011). Nevertheless, individual companies have succeeded in reducing the production cost per gallon from $4.13 to $2.35 per gallon. For comparison, corn ethanol is produced at a cost of $1.60-1.90 per gallon.

Another barrier to commercialized second-generation fuel production is the cost of transportation and storage of the low-energy-density cellulosic biomass (Figure 3.2.B; Pantaleo et al. 2013; White et al. 2013). Transportation costs are determined by distance from farm to refinery as well as additional factors including site accessibility and road tortuosity (Pantaleo et al. 2013). Transportation costs are one of the greatest costs associated with ethanol production and are one of the key determinants of the costs of ethanol production (Figure 3.2.B; Ma and Eckhoff 2012). Establishing multiple smaller scale bioenergy refineries closer to fields may decrease transportation costs. Pantaleo et al. (2013) suggest that under this scenario, improvements in transportation costs may be outweighed by losses in refinery efficiency. The increased efficiency associated with larger refineries must be balanced with the increased costs of longer transportation routes (Ma and Eckhoff 2012).

The USDA estimates biofuel production capacity for new biorefineries based on the available farm land in a hundred-mile radius, the radius established by cost-benefit analysis (Ripplinger et al. 2012; USDA 2010). Unlike other cost categories, transportation and storage are considered to be predictable elements of the supply chain (USDA 2010). Rather than focusing on reducing transportation costs, market actors have concentrated on researching technological improvements in the efficiency of the pyrolysis process that will ultimately produce greater cost savings (USDA 2010, Yang et al. 2011).

Turning to farm-based obstacles, refineries struggle to secure adequate feedstock from farmers (Fewell et al. 2016; Weitzman 1980). The high cost associated with producing cellulosic biofuel appears to be a major obstacle for many potential

entrants (Gustafsson et al. 2015). Corn stover and other primary agricultural resources may be used in place of devoted energy crops in order to meet the RFS2 (Varvel et al. 2008; Perlack et al. 2011). Compared to switchgrass, corn stover is an inexpensive cellulosic energy fuel source that farmers may prefer (Varvel et al. 2008).

Farmer knowledge

Information failures result in decreased market efficiencies and market failures (Allen 2000). Bioenergy surveys done in Kansas, southern Virginia, and Tennessee showed that only 20% of farmers in these areas knew that switchgrass could be used for bioenergy production (Figure 3.2.α; Wen et al. 2009; Jensen et al. 2007; White et al. 2013). Meanwhile, officials in the USDA extension offices and the other outreach programs reported they had full knowledge of governmental assistance programs for all types of bioenergy production as well as an understanding of the uses and best management practices for switchgrass production (White et al. 2013; Wen et al. 2009). Farmers are often classified as independent land managers (White et al. 2013), and since economic decisions depend in part on the manager's own goals and access to information (Allen 2000), this gap in knowledge transfer likely has an impact on current farmer decisions. One survey of Missouri farmers concluded that farmers who were shown successful switchgrass demonstration plots at USDA extension offices were more likely to be interested in growing switchgrass in the future (Burli et al. 2019). Further research is needed in order to understand if farmers are truly resistant to growing switchgrass for biofuels or if incomplete information is contributing to the lack of interest.

Crop Insurance

Even when the land being converted is marginal or otherwise non-arable, the establishment of a devoted biofuel crop is considered by farmers to be risky (Skevas et al. 2016; Burli et al. 2019). Crop insurance is a possible government response to reduce such risk. The Federal Crop Insurance Corporation was established in 1938 in response to the economic devastation farmers faced during the Dust Bowl (1930-1936) (Yu and Wu 2016). The Federal Crop Insurance Act of 1980 increased the number of crops included in the program, and by 1994 farmers were required to be enrolled in the program in order to qualify for a certain loans and price support systems (USDA 2017). In 1996, modifications were made to the farmer mandate so that all eligible farmers were no longer required to enroll in the program; however, farmers were still required to enroll in the program in order to receive disaster relief, effectively maintaining a high level of participation, (USDA 2017). Overall, the program has encouraged farming under riskier conditions; research suggests that farmers are less likely to take additional risk avoidance strategies if they are covered by either crop or revenue insurance (Glauber et al. 2002). In 2013, 295 million acres across the United States were protected by crop insurance programs (Yu and Wu 2016). Evidence shows that crop insurance programs influence farmer crop-choice decision making. For example, in 1999 revenue insurance was provided for durum wheat in North Dakota, which corresponded to a 25% increase in durum wheat planted in the area (Glauber et al. 2002).

Unlike many crops, cellulosic biofuel feedstocks are not covered by crop insurance (Figure 3.2.γ). In 2011 the USDA's Risk Management Agency solicited

input from biofuel researchers on the feasibility of crop insurance programs for biofuel crops (Haugan 2011). In January 2016, the USDA released a statement announcing the beginning of a new crop insurance program for Carinata, also known as Ethiopian mustard, which is an inedible oil seed that can be used to produce biofuels (USDA 2016). The USDA's Risk Management Agency reviews the eligibility of new crops for insurance benefits (Haugan 2011). Historically, the existence of insurance programs has increased the production of subsidized crops (Glauber et al. 2002, USDA 2017). As of 2019, it is too soon to estimate the impact of the new Carinata insurance program on biofuel production.

Uncertainty and communication

Market participants consider their acceptable level of risk before coming to transaction decisions (Awudu and Zang 2012; Klein et al. 2010). Institutions are vital to facilitating the distribution of knowledge among market actors, and to helping actors predict others' behavior (Dequech 2006). When there is a lack of institutional support for markets, such as product standards and disclosure requirements, it is difficult to calculate risk. Actors' decisions under these conditions are based in part off of each actor's perception of the situation in the context of institutions and cultural and social networks (Fernández-Huerga 2008). Instead of being able to rely on probabilistic outcomes connected to experience with existing rules and experience, actors instead have to creatively imagine future scenarios of how regulations will unfold (Bekert 2014; Dequech 2013).

Efforts to reduce market uncertainty drive changes to existing institutions. Market actors can lobby for regulatory changes, otherwise attempt to bypass institutional rules, or exit the market completely (Bylund and McCaffrey 2017). In the event that the institutional status quo is strong, as is the case with agricultural markets, it becomes less likely that actors will be able to effect major reforms (Bylund and McCaffrey 2017).

The current level of farmer participation in the cellulosic biofuel industry suggests that the contract terms offered to farmers or possibly refiners/generators do not compensate for the perceived risk (Weitzman 1980). Interview data suggests that both farmers and extension agents believe that increased communication about best management practices would make growing switchgrass more profitable (Jensen et al. 2007; Mitchell et al. 2012). Awudu and Zang (2012) identify points of uncertainty in the switchgrass-to-biofuel supply chain, including selecting the number of acres farmers devote to biofuel crops and establishing transportation agreements with biofuel refinery operators. These conditions make it more difficult to determine the risk associated with cellulosic biofuel crop production (Awudu and Zang 2012). Due to the extreme uncertainty and internal pressures from social networks, farmers may not challenge the institutional status quo (Bylund and McCaffrey 2017), choosing instead to simply not enter the biofuel market. Informational mimetism also contributes to the preservation of the low-biofuel status quo since actors may decide to copy the behavior of other actors that are believed to have more information (Dequch 2013).

A specific opportunity cost associated with growing switchgrass compared to corn, soy, and other common crops (Dumortier 2016) occurs during the first growing

season (Figure 3.2.α). The one-year stand establishment period required for switchgrass production means that no revenue from production is possible until year two (Wu 2017). This delay in revenues specific to switchgrass compounds the uncertainties already associated with an unestablished market. A related risk concerns the long-term commitment when one plants switchgrass. Switchgrass is a perennial crop and once established lasts upwards of ten years (Wu 2017). A ten-year harvesting time-horizon requires a stronger belief in the permanence of demand compared to annual planting. One survey noted that while some farmers would consider a 7-year contract to supply switchgrass, almost none would consider a 16-year contract (Fewell et al. 2016). A long-term contract could become uncompetitive if the regulatory landscape changes during the contract period.

Improving communication between refinery operators and farming communities may have a positive impact on the rate of farmer adoption of biofuel-friendly crop types and harvesting/transportation practices (Figure 3.2.A). Refinery operators and farmers may be experiencing inefficiencies in information communication (Jensen et al. 2007). When investors and owners are geographically distant from the production site, communication failures can occur between agricultural decision makers and refineries (Gârleanu et al. 2015). Navigating the complex social and economic dynamics present in farming communities could improve the prospects of long-term biofuel production in areas that have the potential to produce large amounts of cellulosic biomass products (White et al. 2013).

Effective communication is especially important when considering crops that are not already commonly grown. Commonly grown crops have an agreed upon set of

standards that to which buyers and farmers adhere (Goodhue and Hoffmann 2006). Agricultural markets trend towards the vertical integration of growers and processors as the number of standards increase (Goodhue and Hoffmann 2006). Specialty crops in particular, such as strawberries, are more likely to be vertically integrated compared to commodity crops (Lajili et al. 1997). With the emergence of new markets, new standards and expectations must be established and captured in contracts (Ripplinger et al. 2012).

Some of the most important metrics for determining the quality of switchgrass are heat values (kJ/kg), ash content after processing, sulfur concentration in the leaves, and the moisture content of the baled product (Figure 3.2.1; USDA 2010). Another area ripe for standardization is pellet size, weight, and composition. The lack of clear standards and guidelines for cellulosic biomass has contributed to farmers' unwillingness to participate in the industry (Jensen et al. 2007; White et al. 2013). Farmers are reluctant to rely on a crop with an uncertain market value regardless of government subsidies (Lajili et al. 1997), although surveyed farmers indicate a moderate willingness to increase production as subsidies increase (Altman and Johnson 2008).

Once guidelines and standards are in place, both farmers and processors will have clearer understandings and expectations. This will reduce both miscommunication risks as well as risks associated with negotiating new, unfamiliar contracts (Cusumano et al. 2015). In response to inconsistencies in switchgrass storage, processing, and pretreatment, as of 2017 the U.S. DOE was working on a preprocessing system that produces one consistent fuel out of multiple feedstocks (USDOE 2017). This is an

example of creating a standardized technology. Meanwhile, the biofuel refining company POET (http://poet-dsm.com/) has developed standardized contracts and fuel inputs, and employs a team of crop buyers using standardized approaches. This is an example of the private sector generating uniform transaction methods. The emergence of professional consultants on contracting risk reduction for ethanol producers, including United Bio Energy (AgMotion Inc.), is bringing further uniformity to the emerging ethanol market.

Conclusions

This chapter has identified three areas for improvement that could increase the production of biofuel crops and the refining of second generation biofuels. They include addressing the gap in knowledge transfer between government/academic extension resources, which report extensive knowledge of biofuel crop production, and farmers, who do not report having received this information. Another area for improvement involves extending crop insurance opportunities to more biofuel source crops, which would reduce the risk to farmers of growing crops that only have markets in biofuel refining. Related to crop insurance would be subsidies that provide incentives to plant switchgrass to compensate for the lack of first-year revenue and the added risk of a multi-year production commitment. Providing payments for ecosystems services to farmers who plant switchgrass may also increase farmer willingness to grow switchgrass for biofuel.

The third area for improvement involves developing guidelines and standards for biofuel crop production, storage, and transportation. These reforms are independent

of and complementary to the often-noted need for improvements in the technology of refining. The drivers of these reforms will include private-sector experience with the biofuel industry as well as government action. Federal policy changes with continued subsidy support are an important short-term support for the industry.

In the absence of more extensive reforms, but with biofuel mandates left in place, a possible future is an increase in corn stover sold to cellulosic biofuel refineries compared to farmer investment in devoted energy crops. Corn stover is a secondary product, meaning farmers will have primary income from corn production in case they cannot sell the stover. Farmers are also familiar with corn production compared to switchgrass production for biofuel. And crop insurance is available for corn, making corn stover a less risky option compared to switchgrass. Possible yield reductions in corn crops in stover-harvested fields would need to be considered in comparison to the revenue increase from stover sales to refiners. Through policy choices, except for the income from the primary product, these same advantages could be conferred on other biofuel sources, which would lead to increased production over time.

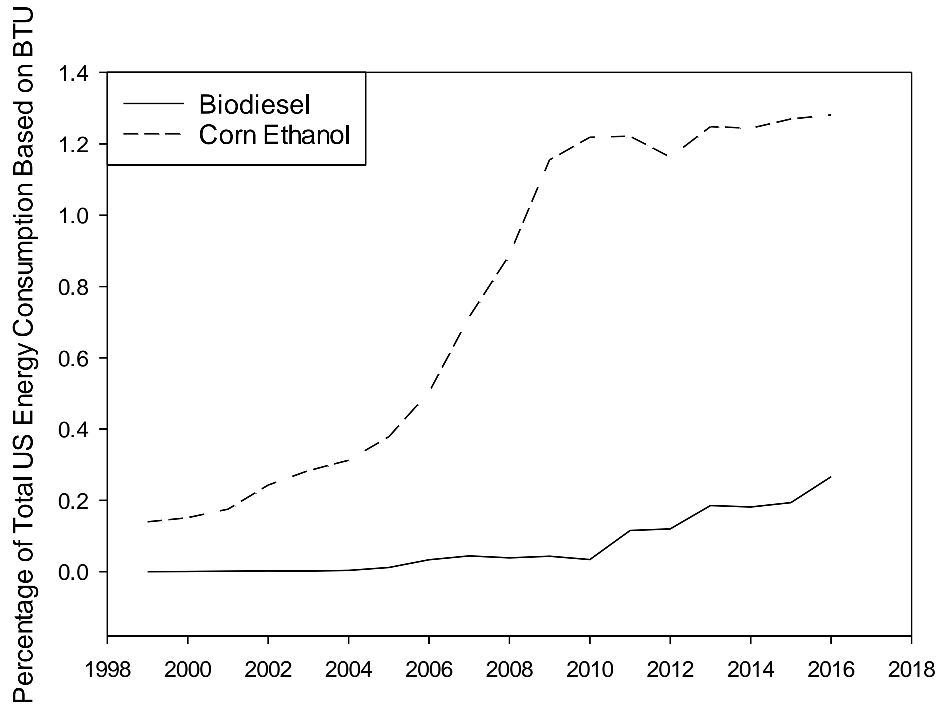


Figure 3.1.

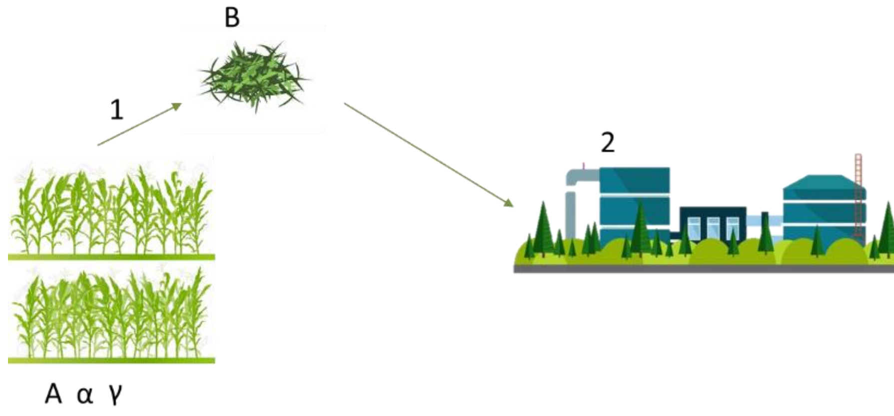


Figure 3.2.

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