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Arbuscular Mycorrhizal Fungi: Factors Affecting Soil Organic Matter Storage

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ARBUSCULAR MYCORRHIZAL FUNGI: FACTORS AFFECTING SOIL ORGANIC
MATTER STORAGE

By

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Dissertation

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Arbuscular mycorrhizal fungi: factors affecting soil organic matter storage

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Arbuscular mycorrhizal fungi (AMF) are soil fungi forming symbiotic associations with majority of land plants. AMF alter soil organic matter (SOM) directly through stabilization of soil aggregates and indirectly providing a path in which plant fixed CO$_2$ is transferred below-ground. Understanding contributions of AMF to SOM via protein production and stabilization of soil aggregates will greatly aid our understanding of soil carbon sequestration, nutrient cycling and mitigation of soil erosion. The work presented in chapter 2 challenges the glomalin extraction process and assesses the accuracy of the Bradford and monoclonal-antibody ELISA detection methods. My results clarify the contribution of glomalin to SOM: suggesting the extraction process is not eliminating all non-glomalin proteins. My results indicate that the Bradford is prone to overestimating the presence of glomalin when soils contain large concentrations of SOM, the ELISA is prone to retention and interference biases depending on the amount and type of organic matter. Chapter 3 determines the contribution of AMF colonized roots to Bradford Reactive Soil Protein (BRSP) and Immunoreactive Soil Protein (IRSP) pools. I test the hypothesis that roots colonized by different species of AMF will produce different quantities of Bradford Root Protein (BRP) and Immunoreactive Root Protein (IRP). These differences could alter BRSP and IRSP pools. I further demonstrate that BRP and IRP concentrations are effective biomarkers in predicting the percentage of AMF root colonization. Chapter 4 assesses the influence of AMF on aggregate dynamics. This work uses Rare Earth Elements (REE) to track aggregate formation and breakdown in the presence of AMF. I confirm that AMF rapidly form and stabilize macroaggregates incorporating intermediate macro- and microaggregates into macroaggregates (> 2000 µm). In chapter 5 I assess the effect of Hieracium invasion and soil fertility on AMF and non-mycorrhizal fungal biomass, carbon mineralization and soil structure. Results from this study provide information essential to understanding how exotic species can alter soil processes. Chapter 6 is the product of my work with the ECOS program; set of investigations aimed at teaching middle school students the ecological importance of soil microbiology. The significance of this body of work is outlined in chapter 7.
ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

Abstract ............................................................................................................................... ii
Acknowledgements ............................................................................................................. iii
Table of Contents ............................................................................................................... iii
List of Tables ....................................................................................................................... vii
List of Figures ...................................................................................................................... vii

Chapter 1 Introduction

Overview .............................................................................................................................. 1
Background and Origin of Research Questions ................................................................... 3
Questions addressed within each chapter ........................................................................... 7
Broader significance of this work ....................................................................................... 10
References .......................................................................................................................... 11

Chapter 2: Glomalin-related soil protein: Assessment of current detection and quantification tools

Abstract ............................................................................................................................... 19
Introduction ........................................................................................................................ 21
Results ................................................................................................................................ 26
Discussion .......................................................................................................................... 28
References .......................................................................................................................... 31

Chapter 3: Intraradical protein and glomalin as a tool for quantifying arbuscular mycorrhizal root colonization

Abstract ............................................................................................................................... 45
Introduction ........................................................................................................................ 47
Results ................................................................................................................................ 53
Discussion .......................................................................................................................... 55
References .......................................................................................................................... 61
Chapter 4: Influence of arbuscular mycorrhizal fungi on soil aggregate dynamics

Abstract .......................................................... 78
Introduction .......................................................... 80
Results .............................................................. 88
Discussion ........................................................... 94
References ........................................................... 103

Chapter 5: Increases in soil fertility alters soil properties in *Hieracium caespitosum*

Abstract .......................................................... 124
Introduction .......................................................... 126
Results .............................................................. 135
Discussion ........................................................... 141
References ........................................................... 151

Chapter 6: Junior Environmental Microbiologist Training

Abstract .......................................................... 178
Introduction .......................................................... 178
Isolating Microorganisms: Inquiry 1 ................................ 180
Plant/microbe symbiosis; Inquiry 2 ................................ 184
Microbial mediated decomposition, Inquiry 3 ................. 188
Assessment .......................................................... 193
References ........................................................... 193

Chapter 7: Synthesis .................................................. 211
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Proposal for new terminology for various fractions of soil proteins and glomalin</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>adapted from Rillig (2004)</td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>Physical description of Nyack Floodplain Soils</td>
<td>40</td>
</tr>
<tr>
<td>2.3</td>
<td>Percentage of BSA remaining after extraction process</td>
<td>41</td>
</tr>
<tr>
<td>2.4</td>
<td>Percentage of ELISA cross reactivity</td>
<td>42</td>
</tr>
<tr>
<td>4.1</td>
<td>Rare-earth element background concentrations and recovery efficiency</td>
<td>113</td>
</tr>
<tr>
<td>4.2</td>
<td>F- and P- values for the aggregate distribution incubation trials</td>
<td>114</td>
</tr>
<tr>
<td>4.3</td>
<td>F- and P- values AMF Treatment Comparison</td>
<td>115</td>
</tr>
<tr>
<td>4.4</td>
<td>F- and P-values Non-AMF treatment comparisons</td>
<td>116</td>
</tr>
<tr>
<td>5.1</td>
<td>Soil characteristics assessed from <em>F. idahoensis</em> and <em>H. caespitosum</em> amended soil</td>
<td>165</td>
</tr>
<tr>
<td>5.2</td>
<td>Total C mineralized from intact and crushed macroaggregates (&lt; 250 µm)</td>
<td>166</td>
</tr>
<tr>
<td>5.3</td>
<td>Plant biomass and root architecture parameters</td>
<td>167</td>
</tr>
<tr>
<td>5.4</td>
<td>Aggregate size class distribution (%) after slaking and rewetting treatments</td>
<td>168</td>
</tr>
<tr>
<td>5.5</td>
<td>Correlation matrix relationships between assessed variables and slaked Mean Weight Diameter (MWD) for <em>F. idahoensis</em> and <em>H. caespitosum</em> soils</td>
<td>169</td>
</tr>
<tr>
<td>5.6</td>
<td>Correlation matrix relationships between assessed variables and slow wetting Mean Weight Diameter (MWD) for <em>F. idahoensis</em> and <em>H. caespitosum</em> soils</td>
<td>170</td>
</tr>
<tr>
<td>6.1</td>
<td>Materials required for selected inquiries</td>
<td>195</td>
</tr>
<tr>
<td>6.2</td>
<td>Internet Resource guide</td>
<td>196</td>
</tr>
<tr>
<td>6.3</td>
<td>Methods of culturing microorganisms inquiry</td>
<td>197</td>
</tr>
<tr>
<td>6.4</td>
<td>Methods of <em>Rhizobia</em> symbiosis investigation</td>
<td>198</td>
</tr>
<tr>
<td>6.5</td>
<td>Methods of decomposition investigation</td>
<td>199</td>
</tr>
<tr>
<td>6.6</td>
<td>Content Standards: National Science Education Standards</td>
<td>200</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

2.1 A. Bradford reactive soil protein (BRSP) for both sites CB and P-22B BSA amended soil treatments. BRSP concentration was calculated as mg g⁻¹ soil.
2.1 B. Immunoreactive soil protein (IRSP) for both sites CB and P-22B BSA amended soil treatments. IRSP concentration was calculated as mg g⁻¹ soil. 43

2.2 A. The amount of leaf litter proteins detected by the Bradford method after GRSP extraction process mg g⁻¹ soil.
2.2 B. The effect of leaf litter proteins on GRSP quantification via ELISA mg g⁻¹ soil 44

3.1 A. Bradford Root Protein (BRP) in roots of Bromus inermis colonized with different AMF isolates
3.1 B. Immunoreactive Root Protein (IRP) in roots of Bromus inermis colonized with different AMF isolates. 72

3.2 A. Bradford Root Protein (BRP) for control and AMF treatments.
3.2 B. Immunoreactive Root Protein (IRP) for control and AMF treatments. 73

3.3 A. Relationship between Bradford Root Protein (BRP) (mg g⁻¹) and percent AMF root colonization in *in vitro* culture
3.3 B. Relationship between Immunoreactive Root Protein (IRP) (mg g⁻¹) and percent AMF root colonization in *in vitro* culture. 74

3.4 A. Relationship between Bradford Root Protein (BRP) (mg g⁻¹) and percent AMF root colonization in Sudan grass grown in the greenhouse
3.4 B. Relationship between Immunoreactive Root Protein (IRP) (mg g⁻¹) and percent AMF root colonization in Sudan grass grown in the greenhouse (n = 18). 75

3.5 A. Relationship between Bradford Root Protein (BRP) (mg g⁻¹) and percent AMF root colonization in narrowleaf plantain grown in the greenhouse
3.5 B. Relationship between Immunoreactive Root Protein (IRP) (mg g⁻¹) and percent AMF root colonization in narrowleaf plantain grown in the greenhouse. 76
3.6 A. Relationship between Bradford Root Protein (BRP) (mg g\(^{-1}\)) and Immunoreactive Root Protein (mg g\(^{-1}\)) from \textit{in vitro} culture

3.6 B. Relationship between Bradford Root Protein (BRP) (mg g\(^{-1}\)) and Immunoreactive Root Protein (mg g\(^{-1}\)) from Sudan grass

3.6 C. Relationship between Bradford Root Protein (BRP) (mg g\(^{-1}\)) and Immunoreactive Root Protein (mg g\(^{-1}\)) from narrowleaf plantain

4.1 Schematic diagram of the growth chamber used in both incubation experiments

4.2 A. Macroaggregate distribution between AMF and Non-AMF treatments during 2 week incubation
4.2 B. Microaggregate distribution between AMF and Non-AMF treatments during 2 week incubation

4.3 A. Macroaggregate distribution between AMF and Non-AMF treatments during 5 week incubation
4.3 B. Microaggregate distribution between AMF and Non-AMF treatments during 5 week incubation

4.4 A. The percentage of 500 µm labeled aggregates isolated from each aggregate size class after 2 weeks of incubation
4.4 B. The percentage of 500 µm labeled aggregates isolated from each aggregate size class after 5 weeks of incubation

4.5 A. The percentage of 250 µm labeled aggregates isolated from each aggregate size class after 2 weeks of incubation
4.5 B. The percentage of 250 µm labeled aggregates isolated from each aggregate size class after 5 weeks of incubation

4.6 A. The percentage of 53 µm labeled aggregates isolated from each aggregate size class after 2 weeks of incubation
4.6 B. The percentage of 53 µm labeled aggregates isolated from each aggregate size class after 5 weeks of incubation
4.7 A. The percentage of labeled silt/clay (< 53 µm) fraction isolated from each aggregate size class after 2 weeks of incubation.

4.7 B. The percentage of labeled silt/clay (< 53 µm) fraction isolated from each aggregate size class after 2 weeks of incubation

5.1 A. Relationship between *F. idahoensis* intact carbon mineralization (mg g⁻¹ soil) and organic matter (%)

5.1 B. Relationship between *H. caespitosum* intact carbon mineralization (mg g⁻¹ soil) and organic matter (%).

5.2 A. AMF hyphal lengths determined for *F. idahoensis* and *H. caespitosum* herbicide and fertilizer treatments.

5.2 B. Non-AMF hyphal lengths determined for *F. idahoensis* and *H. caespitosum* herbicide and fertilizer treatments.

5.3 A. Slaked Mean Weight Diameter (mm) calculated for *F. idahoensis* and *H. caespitosum* herbicide and fertilizer treatments.

5.3 B. Slow wetting Mean Weight Diameter (mm) calculated for *F. idahoensis* and *H. caespitosum* herbicide and fertilizer treatments.

5.4 A. Relationship between *F. idahoensis* AMF hyphal length (m g⁻¹ soil) and slaked Mean Weight Diameter (mm)

5.4 B. Relationship between *F. idahoensis* Saprophytic hyphal length (m g⁻¹ soil) and slaked Mean Weight Diameter (mm)

5.4 C. Relationship between *F. idahoensis* organic matter (%) and slaked Mean Weight Diameter (mm)

5.4 D. Relationship between *F. idahoensis* intact carbon mineralization (mg g⁻¹ soil) and slaked Mean Weight Diameter (mm)

5.5 A. Relationship between *H. caespitosum* intact carbon mineralization (mg g⁻¹ soil) and slaked Mean Weight Diameter (mm)

5.5 B. Relationship between *H. caespitosum* protected carbon mineralization (mg g⁻¹ soil) slaked Mean Weight Diameter (mm)
5.6 A. Relationship between *F.idahoensis* organic matter (%) and slow wetting Mean Weight Diameter (mm)

5.6 B. Relationship between *F.idahoensis* intact carbon mineralization (mg g\(^{-1}\) soil) and slow wetting Mean Weight Diameter (mm)

5.6 C. Relationship between *F.idahoensis* protected carbon mineralization (mg g\(^{-1}\) soil) and slow wetting Mean Weight Diameter (mm)

6.1 Pre and Post inquiry student drawing assessment
Arbuscular mycorrhizal fungi (AMF) alter soil organic matter (SOM) indirectly by providing a path in which plant fixed CO₂ is transferred below ground (Rillig et al., 2001) and directly through the stabilization of soil aggregates (Rillig & Mummey 2006).

Studies quantifying the contribution of AMF biomass to SOM have focused on measuring Glomalin related soil protein (GRSP) fractions: Bradford reactive soil protein (BRSP) and Immunoreactive soil protein (IRSP). Several studies have used GRSP to identify AMF presence in greenhouse sand cultures (Wright et al., 1996), sand cores from tropical forest soils (Lovelock et al., 2004), as well as horticultural mesh traps from field soils (Wright & Upadhyaya, 1999). Additionally, Krivtsov et al., (2004) utilized soil glomalin pools to directly estimate AMF biomass changes in forest soils. While these studies have been important in estimating the significance of GRSP to SOM, no study has assessed the efficiency of the GRSP extraction/detection methods, nor attempted to identify BRSP or IRSP production in source materials such as AMF hyphae, spores or colonized root fragments. If GRSP is to provide an accurate assessment of the influence of AMF on SOM, then a critical evaluation of its detection accuracy is necessary.

AMF directly influence the preservation of SOM through the formation and stabilization of soil aggregates. AMF are hypothesized as being superior “engineers” of soil aggregates when compared to other fungal phyla. This hypothesis is based on the following observations: (i) AMF growth is continuous due to the supply of fixed carbon
from their plant symbiont, (ii) AMF are not consumed by fungal grazers (Klironomos & Kendrick 1996), and (iii) AMF hyphae appear to maintain longer residence times than other fungi. Staddon et al., (2003) suggest that AMF hyphal persistence is measured on the order of weeks. However, these results were obtained from soil lacking clay content, possibly limiting the residence time of AMF hyphae (Zhu & Miller 2003). Studies have suggested that members of the Basidiomycota are equally capable of forming soil aggregates (Caesar-TonThat & Cochran 2000). To date, no study has investigated how specific pools of macro-and microaggregates are influenced in terms of incorporation and decomposition by the presence of AMF.

In order to investigate the impact of AMF on SOM I have undertaken studies to: i) assess the methods used to determine AMF contribution to the SOM via production of GRSP fractions ii) determine if AMF-colonized root fragments contribute to GRSP fractions and thus SOM, (iii) assess the potential mechanisms imposed by AMF to form soil aggregates, and iv) investigate the effect of Hieracium caespitosum invasion and soil fertility on aggregate stability and carbon mineralization. Finally, my dissertation includes a chapter of my work during my ECOS (Ecologists Educators and Schools) fellowship. This summary will describe the development of curricula introducing middle school students to the ecological importance of microorganisms.
Background and Origin of Research Questions

Glomalin-related soil protein

Currently, GRSP is operationally defined since the identification of this protein rests solely on the methods used to extract it from soil (citric acid buffer, autoclaving at a pH of either 7.0 or 8.0) and the assays (Bradford method or ELISA with MAb32B11) utilized to detect and quantify its presence (Rillig, 2004). Several studies suggest that the GRSP pool measured by the Bradford method accounts for all of the GRSP present in the soil and that the ELISA is quantifying the immunoreactive portion of the total GRSP pool (Wright & Upadhyaya, 1996; Wright et al., 1996; Wright & Upadhyaya, 1999, Wright et al., 1999; Rillig & Steinberg, 2002). The current assumption in using the Bradford method is that all, or the vast majority, of non-glomalin proteins are destroyed during the harsh extraction procedure except glomalin. The ELISA assay relies additionally on a monoclonal antibody (MAb32B11) raised against the crushed spores of the AMF Glomus intraradices (Wright et al., 1996). The monoclonal antibody used reacts strongly with all AMF species tested (Wright et al., 1996) and does not significantly cross-react with all non-AMF fungal species examined so far (Wright et al., 1996). Values obtained from the Bradford method and ELISA assay are often well correlated, thus providing further circumstantial evidence that the extraction process is mostly isolating glomalin (Wright & Upadhyaya, 1996; Wright et al., 1996; Wright & Upadhyaya, 1999). Even though the AMF gene product for glomalin has been identified it remains difficult to directly test the validity of the above extraction/detection process applied to soil. Since our knowledge of glomalin is entirely dependent upon these methods, this can result in confusion as to which quantification method (Bradford or ELISA) is more accurately measuring the
glomalin pool. Hence, a new nomenclature has recently been introduced (Rillig, 2004) clearly separating glomalin from soil-derived protein pools (i.e. GRSP). In this context, several questions remain: does the extraction method used destroy all other proteins besides glomalin, or do other proteins survive the extraction process and contribute to the GRSP pool? How specific is the monoclonal antibody used in the MAb3211B-based ELISA process? Is the ELISA a more accurate reflection of glomalin? My research addresses each of these questions.

Colonized root contribution to GRSP pools

The majority of previous studies regarding GRSP have focused on quantifying the concentration of the total GRSP pool in soils, which includes mineral- and aggregate-associated GRSP, AMF hyphae, spores and colonized root fragments (Wright & Anderson, 2000; Rillig et al., 2003; Lutgen et al., 2003; Lovelock et al., 2004). The relative contribution of these fractions to the total GRSP pool is not presently known. Driver et al., (2005) suggested that glomalin is a component of the AMF hyphal wall and that glomalin existence in the soil matrix is primarily a result of hyphal decomposition. As research into identifying mechanisms controlling GRSP production continues (Rillig 2004), it is essential to determine how the root-contained glomalin pools, contributes to the total GRSP.

AMF and soil aggregate formation

Several studies suggest that AMF stabilize macroaggregates against disruptive forces which in turn protects SOM from decomposition (Miller & Jastrow 2000, Jastrow et al., 1998). The ability of soils to sequester organic matter promotes several important
environmental processes which include: sequestration of greenhouse gases (e.g. CO₂), effects on nutrient cycling and retention rates, and mitigation of soil erosion. Since AMF are central organisms within the soil environment, i.e. AMF form mutualistic root/fungi associations with over 80% of all terrestrial plants (Smith & Read 1997), it would be expected that AMF play a significant role in the ability of soil to function as a carbon sink. Several reviews have outlined biochemical and morphological characteristics of AMF that could directly influence macroaggregate formation and stability (Zhu & Miller 2003, Jeffries et al., 2003, Rillig & Mummey 2006). However, the link between fungal abundance and macroaggregate stability has not been directly investigated (Six et al. 2004).

The majority of research to date involving microaggregate formation and stabilization has centered on particulate organic matter acting as a cementing agent of primary particles (Rillig & Mummey 2006). Currently there are two models describing microaggregate formation and incorporation into macroaggregates. Tisdall & Oades (1980) suggest that microaggregates provide the starting material by which macroaggregates are formed. However, Oades (1984) proposed a second model where microaggregates are formed within macroaggregates. Findings from Angers et al., (1997) lend support to the latter model proposed by Oades & Waters (1991). Understanding how AMF influence microaggregate formation would provide essential information on the dynamics that control macroaggregate formation in soils where organic matter is the main binding agent (Oades & Waters 1991). Yet, the contribution of AMF in the formation of
microaggregates and their potential incorporation into larger macroaggregates has not been investigated (Rillig & Mummey 2006).

**Influence on exotic plant invasion and soil fertility on soil structure**

Investigations assessing the impacts of exotic plants suggest that invasion can i) displace native species (Callaway et al., 2003), ii) alter hydrologic cycles (Randall 2000), iii) increase fire intensity and frequency (Melgoza et al., 1990, Mack & D' Antonio 1998, Vitousek et al., 1997) and iv) modify soil structure (Batten et al., 2005). Restoration of soils invaded by exotic species is highly dependent on understanding the effect that management practices have on soil quality and soil-plant relationships (Francis & Cleeg 1990). One particular method used to control rapidly spreading exotic plant populations is the use of N based fertilizers. Several studies have found that alteration of soil fertility via N fertilizers alters AMF communities (Johnson et al., 2003) and carbon mineralization rates (Aoyama et al., 1999a, Graham et al., 2002), as well as soil structural properties (Aoyama et al., 1999b). However, investigations assessing shifts in soil biotic processes and changes in soil properties as a result of *Hieracium caespitosum* (Hawkweed) invasion and soil fertility have not been conducted.

Early research efforts suggest that soils isolated from *Hieracium* infested sites are more acidic, and contain greater quantities of both organic carbon and nutrients (McIntosh & Allen 1993, Boswell & Espie 1998). These results suggest that *Hieracium* is capable of altering soil characteristics as a mechanism of invasion. Recent research further confirms these findings. Knicker et al., (2000) suggest that *Hieracium pratens* infested soils
contain a greater proportion of nitrogen-rich phenolic compounds. Additionally Scott et al., (2001) investigated the litter quality under invading Hawkweed populations and their results indicate that Hawkweed modifies soil resource availability by depleting key nutrients. While these studies are important in understanding Hawkweed invasion, no study to date has determined how fungal communities change as a result of Hawkweed invasion and increases in soil fertility or how these changes influence carbon mineralization or soil structural properties.

**Questions addressed within each chapter**

**Does the glomalin extraction process destroy all other proteins besides glomalin, or do other proteins survive the extraction process and contribute to the GRSP pool?**

Chapter 2 is one of the first studies to effectively challenge the validity of the glomalin extraction process, as well as assess the accuracy of both the Bradford method and the monoclonal antibody-based ELISA detection methods. I test the hypothesis that the glomalin extraction process will successfully disrupt all non-glomalin protein sources added to our test soils. Additionally I hypothesized that if the extraction process fails to denature added protein sources, that the monoclonal antibody-based ELISA would provide a more accurate assessment of GRSP due to the greater specificity of monoclonal antibodies over that of the Bradford method.
Do AMF colonized root fragments influence GRSP pools, and can increasing concentrations of glomalin within colonized roots aid in detecting AMF root colonization?

In chapter 3 I test the hypothesis that AMF colonized roots will contain greater concentrations of glomalin-related protein fractions, Bradford-root protein (BRP) and MAb32B11-immunoreactive-root protein (IRP), when compared to non-AMF colonized controls. This study is the first of its kind to assess the production of BRP and IRP among several isolates of AMF from within the same ecosystem. Furthermore, I hypothesized that AMF colonized roots would significantly increase both BRP and IRP concentrations as AMF colonization increased. This increase could provide a potential pathway for BRP and IRP to enter the soil environment adding to the overall GRSP pool. I further investigated whether changing concentrations of either BRP and/or IRP could be used as a biomarker to assess the percentage of AMF root colonization.

What influence does AMF have on soil aggregate dynamics?

Within chapter 4, I address the influence of AMF on soil aggregate dynamics. This is the first study of its kind to label known aggregate size fractions with Rare Earth Elements, and track their formation and breakdown in the presence of AMF. Previous soil aggregation studies measure aggregation at a single time point, which provides limited information regarding aggregate formation and decomposition. Since AMF are central organisms within the soil environment understanding their contribution to aggregate dynamics is essential in determining soil carbon storage potential. I hypothesized that AMF would rapidly form and stabilize macroaggregates, while incorporating both
intermediate macroaggregates as well as microaggregates. I further hypothesized that the presence of non-mycorrhizal fungi and microorganisms would result in the formation of larger macroaggregates at a much slower rate.

Do increases in soil fertility of *Hieracium* invaded soils alter aggregate stability and carbon mineralization rates?

Chapter 5 is one of the first studies to assess the effect of *Hieracium caespitosum* invasion and increases in soil fertility on AMF and saprophytic fungal biomass, carbon mineralization and soil structure. I test the hypothesis that AMF and non-mycorrhizal fungal biomass will be significantly greater under *H. caespitosum* fertilized treatments (due to low soil pH and large C: N ratios) than native *F. idahoensis* soils. Additionally I hypothesized carbon mineralization and organic matter concentrations would be significantly altered as a result of increased soil fertility and plant species. Furthermore, I expected to find significant differences in fungal mycelium, organic matter, and rate at which carbon is mineralized aspect responsible for aggregate stabilization. I hypothesized that aggregate distribution and Mean Weight Diameter (MWD) would be significantly altered as a result of exotic plant invasion and fertilizer treatments.

**Development of Environmental Microbiology Curriculum**

Chapter 6 is the result of my work through the ECOS program where I served as ecologist in residence at local middle school (grades 6-8). This chapter includes three inquiry based investigations developed in collaboration with undergraduate students and middle school science teachers, intended to teach the importance and significance of soil microbiology to middle school students. Most middle school microbiology curriculum
content is aimed at teaching topics centered on food microbiology, antibiotic resistance of microorganisms, and microorganisms as spoilers of food. I have taken an ecological approach and developed investigations that provide students with an understanding of the environmental significance of microorganisms. These lessons include: i) isolation of microorganisms from environmental samples, ii) assessing microbial/plant symbiosis, and iii) exploration of microbial decomposition and nutrient cycling. I selected these inquiries because they provide a basis for understanding the relevance of soil microbiology from which future question/inquiries could be constructed.

**Broader significance of this work**

Soil is generally considered the most diverse and complex ecosystem on the planet (Young & Crawford 2004). The contribution of organic matter to the soil ecosystem provides the foundation of its structure and the energy that fuels its productivity. SOM provides several essential ecosystem services: nutrient cycling, reducing soil erosion, and potentially providing a reservoir for atmospheric concentrations of CO$_2$. AMF hyphal growth may increase soil organic matter concentrations by providing a conduit in which photosynthetically derived carbon is deposited into the soil. Furthermore, AMF hyphal enmeshment protects organic matter inputs by forming and stabilizing soil aggregates. Several studies have attempted to quantify the contribution of AMF to SOM via the measurement of GRSP fractions BRSP and IRSP, however very few studies have challenged the accuracy of the GRSP extraction/detection process. Accurate accounting of AMF contributions to SOM is essential in order to model fluxes of AMF derived SOM. Additionally, it has been well established that AMF stabilize aggregates against disruptive forces. Yet the influence of AMF on the dynamic nature of soil aggregates has
not been assessed. Understanding how AMF influence aggregate stability over time will greatly improve our knowledge regarding SOM residence times. My work will provide insight into the ability of AMF to contribute to SOM pools, physically protect SOM, and assesses how soil fertility influences sources of SOM. This work will provide substantial information extending our current knowledge, with potential application into the fields of restoration ecology as well as sustainable agriculture.

References


Batten, K.M., Six, J., Scow, K.M., Rillig, M.C., 2005. Plant invasion of native grassland on serpentine soils has no major effects upon selected physical and biological properties. Soil Biology and Biochemistry 37, 2277-2282.


Chapter 2

GLOMALIN-RELATED SOIL PROTEIN; ASSESSMENT OF CURRENT DETECTION AND QUANTIFICATION TOOLS

Abstract

Despite the widely acknowledged importance of arbuscular mycorrhizal fungi (AMF) in soil ecology, quantifying their biomass and presence in field soils is hindered by tedious techniques. Hence biochemical markers may be useful, among which glomalin-related soil protein (GRSP) could show a particular promise. Presently GRSP is operationally defined, its identification resting solely on the methods used to extract it from soil (citric acid buffer and autoclaving) and the assays (Bradford/enzyme-linked immunosorbent assay (ELISA) with a monoclonal antibody) utilized to detect it. The current assumption is that most non-heat stable soil proteins except glomalin are destroyed during the harsh extraction procedure. However, this critical assumption has not been tested. The purpose of this research was to challenge the GRSP extraction process to determine the accuracy of the Bradford method as a measure of glomalin; and to provide some assessment of the specificity of the ELISA monoclonal antibody. In two studies we spiked soil samples either with known quantities of a glycoprotein (BSA: bovine serum albumin) or with leaf litter from specific sources. After extraction 41–84% of the added BSA was detected with the Bradford method. This suggests that the currently used extraction procedure does not eliminate all non-glomalin proteins. Also, ELISA cross-reactivity against BSA was limited, ranging from 3% to 14%. Additions of leaf litter also significantly influenced GRSP extraction and quantification suggesting that plant-derived proteins, as would
occur in the field, had a similar effect as BSA. Litter additions decreased the
immunoreactive protein values, suggesting interference with antibody recognition. We
conclude that the use of GRSP, especially Bradford-based detection, in the assessment of
AMF-derived substances within field soils is problematic, it may be inappropriate in
situations of significant organic matter additions.

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Introduction

Determining arbuscular mycorrhizal fungi (AMF) biomass in field soils is often difficult and tedious (Jakobsen et al., 1992; Miller et al., 1995; Rillig et al., 1999). As a consequence, biochemical markers such as ergosterol, chitin and glomalin have been considered for the study of AMF. Because several organisms produce ergosterol and chitin, their usage as AMF indicators is somewhat limited (Frey et al., 1994). Furthermore, Olsson et al., (2003) found that AMF may not contain ergosterol. Because glomalin related soil protein (GRSP) has been linked with AMF, several studies have used this substance to identify AMF presence in greenhouse sand cultures (Wright et al., 1996). AMF hyphal growth has been related to GRSP production in sand cores from tropical forest soils (Lovelock et al., 2004) and horticultural mesh traps from field soils (Wright and Upadhyaya, 1999). Krivtsov et al., (2004) utilized soil GRSP pools to directly estimate AMF biomass changes in forest soils. Currently GRSP is operationally defined, meaning that the identification of this protein rests on the methods used to extract it (citric acid buffer, autoclaving at pH of either 7.0 or 8.0) and the assays (Bradford method/enzyme-linked immunosorbent assay (ELISA) with MAb32B11) used for quantification (Rillig, 2004). Several studies suggest that the ELISA is quantifying the immunoreactive portion of the total GRSP pool (Wright & Upadhyaya, 1996, 1999; Wright et al., 1996, 1999; Rillig & Steinberg, 2002).

The current assumption in using the Bradford method is that all or the vast majority of proteins are destroyed during the harsh extraction procedure except glomalin. The ELISA assay relies additionally on a monoclonal antibody (MAb32B11) raised against the
crushed spores of the AMF *Glomus intraradices* (Wright et al., 1996). The monoclonal antibody used reacts strongly with all AMF species tested (Wright et al., 1996) and does not significantly cross-react with non-AMF fungal species examined (Wright et al., 1996). Values obtained from the Bradford method and ELISA assay are often well correlated, thus providing further circumstantial evidence that the extraction process is mostly measuring glomalin (Wright & Upadhyaya, 1996; Wright et al., 1996; and Wright & Upadhyaya, 1999).

The gene for the AMF protein glomalin has very recently been sequenced in our laboratory (Gadkar & Rillig, unpublished); yet it remains difficult to assess the relationship between GRSP obtained from soil and glomalin. Hence a new nomenclature has been introduced (Rillig, 2004; Table 2.1), clearly separating glomalin from soil-derived protein pools (GRSP). In this context, several questions remain: does the extraction method used destroy all other proteins besides glomalin, or do other proteins survive the extraction process and contribute to the GRSP pool? How specific is the monoclonal antibody used in the MAb3211B-based ELISA process? Is the ELISA a more accurate reflection of glomalin?

Here we take an indirect approach to addressing some of these questions: spiking soils with non-glomalin protein or non-glomalin containing substrates (containing a mixture of proteins). We wished to test the response of the different GRSP fractions when challenged with extraneous non-glomalin protein additions, and to provide additional tests of the MAb32B11-ELISA. We conducted two studies i) samples from two different
soils were spiked with varying quantities of a thermolabile protein (bovine serum albumin) of similar size to glomalin; and ii) soil samples were amended with leaf litter from specific sources (*Poa annua*, *Populus trichocarpa*, and *Pinus ponderosa*). If the extraction process eliminates all non-heat stable proteins, then the Bradford and ELISA assays will not be strongly influenced by extraneous protein additions of BSA and leaf litter. Furthermore, we hypothesized that the ELISA-based protein values would be influenced by the presence of BSA and leaf litter proteins to a lesser degree.

**Materials and Methods**

**Soils Description**

For experiment 1, we used two different soils (referred to as CB and P22-B) collected from the Nyack Floodplain (western Montana; 48°29’ N, 114°00’ W on the middle fork of the Flathead River) for which soil OM and GRSP (Table 2.2) levels differed as a consequence of soil age (Hamer et al., 2004). Soils were collected to a depth of 20 cm, air dried, and stored at room temperature prior to use. Experiment 2 utilized only the one-year old soils.

**Soil Extraction**

There are currently two detection methods utilized to quantify Glomalinrelated soil protein (GRSP): Bradford protein assay, yielding Bradford reactive soil protein (BRSP), and an enzyme-linked immunosorbent assay (ELISA: using the monoclonal antibody Mab32B11 developed against crushed spores of *Gl. intraradices* (Wright and Upadhyaya,
yielding the immunoreactive soil protein (IRSP). The first step in the extraction process is to recover the EE-BRSP and EE-IRSP soil fraction (EE = easily extractable). This was done by autoclaving 1.0 g of soil with 20 mM sodium citrate, pH 7.0 at 121°C for 30 minutes. Only one autoclave cycle is required to obtain this fraction. Following this extraction process, the BRSP and IRSP fractions were extracted from the same soil sample using 50 mM sodium citrate, pH 8.0 and repeated autoclaving at 121°C for 60 min. After each extraction/autoclaving cycle the sample was centrifuged at 5,000 x g for 15 min. The supernatant was decanted and stored at 4°C until analysis. The extraction process continues until the supernatant is clear/ light yellow in color. Once the extraction process was complete each extract was centrifuged at 10,000 x g. The Bradford assay was first utilized to determine the concentration of EE-BRSP and BRSP using bovine serum albumin as a standard. Immunoreactive protein values were measured using an indirect ELISA with MAb32B11 (Wright & Upadahyaya, 1996).

Protein addition (Experiment 1)

We applied bovine serum albumin (BSA) (Fisher Scientific, Fair Lawn, New Jersey) to each sample at five and ten times the background IRSP level detected in each soil. The CB (one year old soils) soils received 2.8 mg or 5.6 mg of BSA respectively, and P22-B (66 year old soils) soils 11.2 mg or 25.0 mg of BSA. We selected this level of protein additions in order to effectively challenge the extraction process as well as the Bradford and ELISA assay. BSA was thoroughly mixed into 20 g of field soil at the specified rate (Precision Scientific Shaker) for 2 hours. After mixing, 1.0 g of soil was removed from each tube and immediately extracted. This was done in order to decrease the chance of
microbial activity influencing the amount of protein in each sample. All treatments were replicated 8 times.

**Leaf litter addition (Experiment 2)**

The second portion of our experiment set out to determine the influence of plant derived proteins on the GRSP pool. Leaf material was collected from *Poa annua* (annual Bluegrass), *Populus trichocarpa* (Black Cottonwood) and *Pinus ponderosa* (Ponderosa Pine). Samples were air dried at 80 °C for 24 h, and subsequently blended into a fine powder. The blended leaf material (0.15 g) was added to 1.0 g of CB soil and mixed for 2 hours (Precision Scientific Shaker). The soil leaf mixture was extracted immediately and analyzed using the methods described above. All treatments were replicated 5 times.

**Data analysis**

In order to determine the amount of added BSA remaining after the extraction process (%) as detected by the Bradford method we utilized the formula (BRSP - Background BRSP)/ BSA addition x 100. The amount of ELISA cross-reactivity (%) as a result of BSA addition was determined as (IRSP - Background IRSP)/ BSA addition x 100.

ANOVA was used to test for treatment differences. Where F- ratios were significant (P< 0.05) treatment means were compared via Tukey-Kramer (JMP, SAS Institute). Non-parametric analyses (Kruskal-Wallis test) were utilized if data failed to meet parametric assumptions. Where H-values (Kruskal-Wallis test statistic) were significant (P< 0.05) treatment means were compared via Kruskal-Wallis Multiple Comparison Z-Value Test (NCSS, 2000). The CB 5x, 10x and P-22B 5x, 10x treatments contained two statistical
outliers and the *Poa annua* treatment contained one outlier all of which were removed prior to analysis. We defined outliers as data points which are two standard deviations above/below the sample mean. Finally we have restricted comparisons to within soil types.

**Results**

**BSA addition experiment**

Bradford reactive soil protein concentrations (mg g\(^{-1}\)) across all treatments are shown in Figure 2.1A. Significant amounts of BSA survived the extraction process and were detected by the Bradford method for both the easily extractable (\(P_{CB} = 0.002\) [Kruskal-Wallis]; \(P_{F22-B} = 0.0001\) [ANOVA]) and total protein extractions (\(P_{CB} = 0.0001\); \(P_{F22-B} = 0.0001\) [ANOVA]). This pattern of increase occurred regardless of soil type.

The proportion of BSA (%) remaining after the extraction process was determined for each treatment and soil type (Table 2.3). The residual BSA detected by the Bradford method did differ significantly between the CB 5x and CB 10x EE-BRSP extraction. However, we did not detect significant differences in any of the other treatments. We observed that the proportion of BSA remaining after extraction is directly influenced by the amendment amount.

The addition of BSA significantly influenced the amount of EE-IRSP (\(P_{CB} = 0.001\); \(P_{F22-B} = 0.002\) [Kruskal-Wallis]) and IRSP (\(P_{CB} = 0.0001\); \(P_{F22-B} = 0.02\) [ANOVA/ log transformed]) Figure 2.1 B Significant levels of monoclonal antibody (MAb32B11)
cross-reactivity did occur when treatment soils were saturated with BSA (i.e. 10 x treatments).

The percentage of BSA-induced cross reactivity was calculated for each soil type and treatment (Table 2.4). Similar to the trend observed for the Bradford method, as BSA increased in concentration so did the percentage of ELISA cross reactivity. Significant difference in cross-reactivity was observed for the P22-B EE-IRSP treatments. However, no significant differences in percent cross reactivity occurred between any of the other treatments.

**Leaf litter additions**

All of the leaf litter treatments and extractions contained significantly greater proportions of protein than the control ($P_{EE:BRSP}<0.001$ [Kruskal-Wallis]; and $P_{BRSP}<0.001$ [ANOVA/ log transformed]) (Figure 2.2A).

The IRSP values differed significantly ($P<0.001$[ANOVA]) between treatments (Figure 2B). The control and *Poa annua* treatments had greater concentration of IRSP then the other treatments. The EE-IRSP extraction results did not differ significantly ($P<0.06$ [ANOVA]) between treatments, yet they followed the same pattern as the IRSP results.
Discussion

There are several important implications of these results in regards to GRSP detection and quantification. First they challenge the suitability of the Bradford method and the extraction process in measuring glomalin (i.e., the AMF product) pool size. Our results indicate that a significant portion of BSA was not eliminated during the GRSP extraction process and was detected by the Bradford method. BSA is a globular glycoprotein with a molecular weight of ~66 (kDa) (Relkin, 1996, Kanny et al., 1998), denaturing at 65 °C (Ruegg et al., 1977, Kanny et al., 1998, Aparicio et al., 2005). The extraction of GRSP from soil is considered harsh and would be expected to quickly and effectively denature BSA beyond Bradford detection. However, the Bradford assay is capable of detecting peptides as small as 3,000 Da (Sedmak & Grossberg, 1977). It is unlikely that the GRSP extraction process would reduce BSA to less then 3,000 Da. Also, we found that as BSA concentration increased per treatment, the ability of the GRSP extraction process to denature it beyond Bradford method detection was diminished (Table 2.3). BSA when heated forms a gelatin matrix through disulfide and noncovalent bonds (Mastudomi et al., 1993).

Another important finding of this study focuses on the accuracy of the ELISA antibody. We have shown that the extraction process and the use of the AMF monoclonal antibody (MAb32b11) is only slightly cross-reactive when extraneous amounts of BSA were added to our system. This suggests that the ELISA is a more accurate reflection of glomalin pools. Monoclonal antibodies have proven to be highly specific in their ability to differentiate between genera and species of a given fungal isolate (Thornton et al., 2005).
Furthermore, monoclonal antibodies have been successfully utilized to detect saprotrophic fungi in soils (Thornton et al., 1993; Thornton & Gilligan, 1999; Dewey et al., 1996). Before the development of a monoclonal antibody the primary method of detecting AMF involved polyclonal antibodies (Wright et al., 1996). Polyclonal antiserum has mainly been developed against AMF spores (Kough et al., 1983; Hahn et al., 1993; Friese & Allen, 1991) and hyphae (Wilson et al., 1983; Gobel et al., 1995). In all cases the main limitation with these polyclonal antibodies was their high degree of cross reactivity.

Our results further suggest that the GRSP extraction process does not denature all plant derived protein sources. These proteins are of sufficient size and quantity after extraction to be detected by the Bradford method. There are several widespread plant-derived protein classes that may withstand autoclaving, including dehydrins (Robertson et al., 1994; Tabaei-Aghdæi et al., 2000; Volaire, 2002; Pelah et al., 1995; Wisniewski et al., 1996; Caruso et al., 2002; Jarvis et al., 1996; Richard et al., 2000) and heat shock proteins (Wisniewski et al., 1996; Hall, 2002; Burke et al., 1985; Mansfield and Key, 1987; Schöffl et al., 1998). This possibility had not previously been considered in GRSP measurements.

Our results indicate that leaf litter type can significantly reduce the sensitivity of the ELISA assay resulting in an underestimation of the GRSP pool. Otten et al. (1997) suggests that ELISA sensitivity loss can occur as a result of retention or interference bias. Retention bias occurs when the antigen becomes attached to organic compounds and is
not released during the extraction process (Otten et al., 1997). This is especially critical in soils with high organic matter, since humic soil materials are known to bind proteins (Schnitzer, 1982). Interference bias takes place when soluble soil components attach to the wall of the micro titer plate displacing the antigen of interest (Otten et al., 1997).

Based on our Bradford results we know that significant amounts of protein were extracted from each of the leaf litter treatments. It is plausible that some component (i.e. tannins or resins) contained in the *Populus trichocarpa* (Black Cottonwood) and *Pinus ponderosa* (Ponderosa Pine) treatments suppressed the sensitivity of the GRSP antigen.

**Summary**

In conclusion we have shown that the GRSP extraction process does not eliminate all non-glomalin protein sources. Thus the use of the Bradford method can be influenced by soil organic matter and is not an accurate reflection of glomalin (*sensu stricto*, i.e. the gene product; Rillig, [2004]) pool size. Furthermore, the ELISA may be prone to both retention and interference biases depending on the amount of organic matter contained in the soil sample. Based on these findings the Bradford method and ELISA assay may be useful in measuring glomalin pools when organic matter concentrations are low such as washed, autoclaved soil (Lovelock et al., 2004; Wright and Upadhyaya, 1999), or in other controlled experimental conditions. However, when soil organic matter concentrations are high, or when significant extraneous protein additions occur (i.e. manure, sewage, and litter fall) we would caution against the use of the Bradford method to assess glomalin pools in soils. Further research should further include testing methods for the reduction of retention and interference bias of the ELISA as outlined by Otten et al. (1997).
Acknowledgements

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References


Physiologia Plantarum 96, 496-505.


Table 2.1 Proposal for new terminology for various fractions of soil proteins and glomalin adapted from Rillig (2004)

<table>
<thead>
<tr>
<th>Old Usage</th>
<th>Identity</th>
<th>Proposed New Name/Usage</th>
<th>Reason for change</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG (total glomalin)</td>
<td>Bradford-reactive soil protein after extensive extraction</td>
<td>BRSP (Bradford reactive soil protein)</td>
<td>Bradford method measures all protein sources; may be non-specific (see this study)</td>
</tr>
<tr>
<td>EEG (easily extractable glomalin)</td>
<td>Bradford-reactive soil protein after mild extraction</td>
<td>EE-BRSP (easily extractable BRSP)</td>
<td>Bradford method measures all protein sources; may be non-specific (see this study)</td>
</tr>
<tr>
<td>IRTG (immunoreactive total glomalin)</td>
<td>Immunoreactive (MAb32B11) soil protein identified after extensive extractions</td>
<td>IRSP (immunoreactive MAb32B11 soil protein)</td>
<td>Potential for antibody cross reactivity or sensitivity issues (see this study)</td>
</tr>
<tr>
<td>IREEG (immunoreactive easily extractable glomalin)</td>
<td>Immunoreactive (MAb32B11) soil protein identified after mild extraction</td>
<td>EE-IRSP (easily extractable immunoreactive MAb32B11 soil protein)</td>
<td>Potential for antibody cross reactivity or sensitivity issues (see this study)</td>
</tr>
<tr>
<td>Glomalin</td>
<td>Old term used to identify all protein pools measured by Bradford and ELISA (i.e. TG, EEG, IRTG, and IREEG) and the actual protein</td>
<td>GRSP (Glomalin related soil protein)</td>
<td>To clearly separate soil-derived protein from the putative gene product</td>
</tr>
</tbody>
</table>
| Glomalin (sensu stricto)   | Currently unknown identity, theoretically glomalin should be similar to soil glomalin pools (in particular immunoreactive pools) | Glomalin (s) | The name glomalin should be reserved for the gene product.
<table>
<thead>
<tr>
<th>Site</th>
<th>Age</th>
<th>pH</th>
<th>Texture</th>
<th>Sand (%)</th>
<th>Clay (%)</th>
<th>Silt (%)</th>
<th>SOM (%)</th>
<th>IRSP (mg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB</td>
<td>1</td>
<td>7.9</td>
<td>Sandy loam</td>
<td>72</td>
<td>14</td>
<td>14</td>
<td>0.80</td>
<td>0.56</td>
</tr>
<tr>
<td>P22-B</td>
<td>66</td>
<td>7.7</td>
<td>Sandy loam</td>
<td>66</td>
<td>14</td>
<td>20</td>
<td>2.20</td>
<td>2.50</td>
</tr>
</tbody>
</table>
Table 2.3: Percentage of BSA remaining after extraction process

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Soil</th>
<th>BSA addition</th>
<th>F or H (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5x</td>
<td>10x</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
</tr>
<tr>
<td>EE-BRSP</td>
<td>CB</td>
<td>34.0 6.27</td>
<td>61.1 2.73</td>
</tr>
<tr>
<td></td>
<td>P22-B</td>
<td>83.0 8.50</td>
<td>74.0 7.50</td>
</tr>
<tr>
<td>BRSP</td>
<td>CB</td>
<td>52.4 6.30</td>
<td>67.4 7.13</td>
</tr>
<tr>
<td></td>
<td>P22-B</td>
<td>84.7 3.40</td>
<td>83.7 6.62</td>
</tr>
</tbody>
</table>

Treatment comparisons were restricted to within soil type e.g. CB (5) EE-BRSP (%) was analyzed against CB (10) EE-BRSP (%)

*a Kruskal-Wallis test was used
Table 2.4 The percentage of ELISA cross reactivity

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Soil</th>
<th>BSA addition F(P) 5x</th>
<th>Mean</th>
<th>SE</th>
<th>Mean</th>
<th>SE</th>
<th>F(P) 10x</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE-IRSP</td>
<td>CB</td>
<td>3.51</td>
<td>1.40</td>
<td></td>
<td>3.55</td>
<td>1.35</td>
<td>0.001 (0.98)</td>
</tr>
<tr>
<td></td>
<td>P22-B</td>
<td>2.71</td>
<td>1.30</td>
<td></td>
<td>10.4</td>
<td>3.34</td>
<td>5.30 (0.05)</td>
</tr>
<tr>
<td>IRSP</td>
<td>CB</td>
<td>8.50</td>
<td>2.32</td>
<td></td>
<td>14.2</td>
<td>2.42</td>
<td>2.78 (0.13)</td>
</tr>
<tr>
<td></td>
<td>P22-B</td>
<td>10.6</td>
<td>2.88</td>
<td></td>
<td>11.6</td>
<td>2.21</td>
<td>0.07 (0.80)</td>
</tr>
</tbody>
</table>

Treatment comparisons were restricted to within soil type e.g. CB (5) EE-IRSP (%) was analyzed against CB (10) EE-IRSP (%).
Figure 2.1

(A) Bradford reactive soil protein (BRSP) for both sites CB and P-22B BSA amended soil treatments. BRSP concentration was calculated as mg g\(^{-1}\) soil. Means (+) SE were compared with Kruskal–Wallis multiple comparison Z-value test (CB EE-BRSP and BRSP) or Tukey-Kramer (P-22BEE-BRSP and BRSP) significance accepted at P< 0.05.

(B) Immunoreactive soil protein (IRSP) for both sites CB and P-22B BSA amended soil treatments. IRSP concentration was calculated as mg g\(^{-1}\) soil. Means (+) SE were compared with Kruskal–Wallis multiple comparison Z-value test (CB and P-22B EE-IRSP) or Tukey-Kramer (CB and P-22B IRSP), and significance accepted at P < 0.05.
Figure 2.2

(A) The amount of leaf litter proteins detected by the Bradford method after GRSP extraction process mg g⁻¹ soil. Means (±) SE were compared with Kruskal-Wallis Multiple Comparison Z-Value Test (EE-BRSP) or Tukey-Kramer (BRSP), and significance accepted at P<0.05.

(B) The effect of leaf litter proteins on GRSP quantification via ELISA mg g⁻¹ soil. Means (±) SE were compared with Tukey-Kramer, significance was accepted at P<0.05.
Chapter 3

INTRARADICAL PROTEIN AND GLOMALIN AS A TOOL FOR QUANTIFYING ARBUSCULAR MYCORRHIZAL ROOT COLONIZATION

Abstract

Assessment of root colonization by arbuscular mycorrhizal fungi (AMF) is largely dependent upon traditional microscopic techniques as no consistent biochemical marker for AMF is available outside of DNA based methods. Glomalin is an AMF produced protein that has potential to serve as a specific biomarker for rapid detection of AMF. We tested whether AMF-colonized roots contained greater concentrations of two glomalin-related protein fractions, Bradford-root protein (BRP) and MAb32B11-immunoreactive-root protein (IRP), compared to non-colonized controls. Additionally, we tested if these protein fractions were correlated with AMF colonization rate. AMF colonization significantly increased IRP within roots of Bromus inermis colonized by several different AMF isolates. BRP and IRP were also increased in Daucus carota (grown under sterile in vitro conditions), and Plantago lanceolata and Sorghum bicolor (grown in the greenhouse). The relationships between intraradical concentrations of both BRP and IRP and AMF root colonization were approximated by both linear and non-linear models in all plants ($r^2$ from 0.50 to 0.94). Clearly, this method could be useful at least in assessing presence/absence of AMF colonization, for example in large-scale screening situations (e.g., testing for mycorrhizal mutants, verifying colonization in the horticultural/restoration industry). While the MAb32B11-ELISA assay was also useful in detecting AMF colonization, it did not consistently offer greater resolution/precision. This analysis
method is more involved and hence not as practical, and we also could not conclusively attribute the antibody reaction to cross-reactivity or a true glomalin signal in roots.

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Introduction

Assessment of arbuscular mycorrhizal fungi (AMF) root colonization is one of the most elemental and commonly performed procedures in AMF research (Gange et al., 1999; Vierheilig et al., 2005). Typically, colonization is estimated by microscopic observation of fungal structures inside of roots using biological stains such as Trypan blue (Grace & Stribley, 1991). After staining, the extent of AMF colonization can be quantified by several methods, including the grid-line intersects method (Giovannetti & Mosse, 1980), the magnified intersects method (McGonigle et al., 1990) or an intensity of colonization method (e.g., Rillig et al., 1998). The use of biological staining procedures followed by microscopic quantification of AMF colonization is a time-consuming task, often involving toxic chemicals (Coombes & Haveland-Smith, 1982). Many applications in mycorrhizal biology necessitate large-volume screening for root colonization (e.g. horticultural practices, screening of AMF mutants), exacerbating this limitation.

The need for rapid screening of AMF colonization provides motivation to develop a quick and reliable method to determine AMF root colonization, including the development of biochemical markers unique to AMF. Becker & Gerdemann (1977) found that AMF colonization of onion (Allium cepa) produces a yellow pigment that is highly correlated with AMF colonization. Hepper (1976) developed a method by which chitin extracted from AMF colonized roots is converted to glucosamine which is subsequently quantified colorimetrically. Immunological methods using polyclonal antibodies have also been used to detect individual species of AMF (Aldwell et al., 1985; Kough et al., 1983; Wilson et al., 1983). These methods; however, are prone to detection...
limitations such as low specificity and cross reactivity (Wright et al., 1987). Ergosterol has been identified as a potential indicator of AMF presence (Frey et al., 1992; Fujiyoshi et al., 2000). However, Olsson et al. (2003) suggested that AMF may not produce ergosterol. Phospholipid fatty acid (PLFA) 16:1 ω5 and neutral lipid fatty acid (NLFA) 16:1 ω5 have also shown some promise in estimating AMF biomass (Olsson et al., 1995, 1999). For example, Larson et al. (1998) found that PLFA 16:1 ω5 and NLFA 16:1 ω5 correlate strongly with AMF colonization in cucumber (Cucumis sativus). Fontaine et al. (2004) demonstrated that the sterol 24-methyl/methylene may be used in determining AMF colonization in transformed carrot roots (Daucus carota). While these methods can be effective at detecting AMF colonization they have one or several limitations: the detection method is based on a substance that is not exclusive to AMF (i.e. chitin, glucosamine and ergosterol), limiting the use in field collected roots; or they require high pre-sample cost and/or are labor-intensive.

Glomalin is a protein produced by AMF (Gadkar & Rillig, 2006), quantified from soil as glomalin-related soil protein (GRSP; Rillig, 2004). Since we are discussing glomalin fractions from AMF colonized roots, we propose using the following nomenclature: Bradford-root protein (BRP) to describe the protein fraction identified by the Bradford method and Immunoreactive-root protein (IRP) as detected by indirect enzyme-linked immunosorbert assay (ELISA) using monoclonal antibody MAb32B11. BRP and IRP could be a useful set of biomarkers for quantifying AMF root colonization. MAb32B11 was developed by immunizing a BALB/c mouse with spores collected from Glomus intraradices FL208. MAb32B11 has not been used to quantify glomalin extracted from
AMF colonized roots. Wright et al., (1996) microscopically observed immunofluorescence of glomalin-related soil protein on the surface of AMF colonized roots, and first suggested that glomalin may be useful as an indicator of AMF colonization. Driver et al., (2005) showed that AMF extraradical fungal mycelium produce glomalin under sterile conditions, but from this and other studies it is not known whether the fungal mycelium inside the root, which experiences a radically different environment than the soil hyphae, also produces this protein.

The first objective of this study was to test roots colonized by several different AMF isolates for the presence of measurable amounts of BRP and IRP. We were further interested in testing whether BRP or IRP concentrations could be used to assess the percentage of AMF colonization. In order to address these questions we conducted a combination of in vitro and greenhouse studies in which we created a range of AMF colonization levels.

**Material and Methods**

*In vitro* culture material

In *vitro* cultures of the AMF *G. intraradices* grown on colonized transformed carrot roots (*D. carota*) were used to obtain soil-free, sterile fungal material; cultures were grown on M medium in phytagel (St-Arnaud et al., 1996). In order to achieve a gradient of AMF root colonization we used a time course harvest approach, harvesting three plates every four weeks for 16 weeks (*n* = 12). Non-colonized carrot roots were obtained by using
plates without AMF (n = 3). Roots were separated from the phytagel medium by suspending samples in 10mM citrate buffer pH 6.0 for 15 min (Doner & Bécard, 1991).

**Greenhouse experiments**

Smooth Brome (*Bromus inermis*) was used during the course of our AMF isolate comparison study. Sudan grass (*Sorghum bicolor*) and narrowleaf Plantain (*Plantago lanceolata*) were used during the correlation study. Seeds for both studies were surface sterilized in 3% H$_2$O$_2$ for five minutes. Pre-germinated seeds were grown in a petri dish for one week. Plants were subsequently grown under greenhouse conditions for 16 weeks (isolate comparison study) or 12 weeks (correlation study). During that time seedlings were exposed to full sunlight for 14 hours, an additional 2 hours of light was administered via (metal halide) growth lights. Plants were fertilized with half strength Hoagland solution every third week of the study. The growth medium was coarse and fine sand (1:1). In order to avoid coextraction of material containing glomalin-related soil protein (GRSP) in our root preparations, we removed GRSP from the sand (Wright, 2000). This was accomplished by an initial water wash for 15 minutes followed by a treatment with 1M NaOH at 80 °C for two hours. This pre-extracted sand mixture was then rinsed with water for 10 minutes, and pH was adjusted to 7.0.

Plants in the AMF isolate comparison study were inoculated with 50 g of AMF material (containing infected roots, hyphae and spores) from one of seven AMF isolates: *Acaulospora* sp., *Entrophospora colombiana*, *Glomus* sp 3., *Gl. aggregatum*, *Gl. mosseae*, and *Gl. intraradices*. All AMF isolates were obtained from the collection...
maintained for the Long-term Mycorrhiza Research Site (LTMRS, Dr. J.N. Klironomos, University of Guelph). To achieve a gradient of AMF root colonization, inoculation for the correlation study consisted of mixing 12, 25 or 50 g of AMF *Glomus etunicatum* into 500 g of mixed sand. Non-AMF control plants for both studies were grown with 50 g of heat killed inoculum (80 °C for 15 minutes). Upon harvest, roots were extracted from the sand and thoroughly rinsed in tap water for 5 minutes to remove adhering material. The AMF isolate comparison study had 6 replicates for each AMF isolate and non-AMF control plant; however, during the course of the study plants either failed to colonize or died, reducing the replicate number in the following treatments: (*Acaulospora* sp. n = 5, *E. colombiana* n = 4, *Glomus* sp 3 n = 6, *Gl. aggregatum* n = 4, *Gl. mosseae* n = 4, *Gl. intra radices* n = 3, and control n = 4). The Sudan grass and narrowleaf plantain correlation study had 3 control replicates and 6 inoculation replicates (n = 21). However, during the course of our study three plants in the Sudan grass experiment died (n =18).

**Glomalin-related protein quantification**

In order to determine the concentration of GRP fractions (BRP and IRP) in root samples we employed the two detection methods used to quantify GRSP: Bradford protein assay, and the ELISA assay (Wright & Upadhyaya, 1998), modified for roots as follows. The first step in the extraction process is to recover the BRP and IRP. This was done by autoclaving 10 mg of dried root sample with 50mM sodium citrate, pH 8.0 at 121 °C for 60 min. After the extraction/autoclaving cycle the sample was centrifuged at 5000 x g for 15 min. The supernatant was decanted and stored at 4°C until analysis. Prior to Bradford and ELISA analysis supernatant was centrifuged at 10,000 x g for 3 min. The Bradford
assay was first used to determine the concentration of BRP using bovine serum albumin as a standard. Immunoreative protein values were measured using an indirect ELISA with MAb32B11 (Wright & Upadahyaya, 1996).

**Mycorrhizal root colonization**

Root samples were cut into approximately 3.0 cm lengths upon harvest from either the *in vitro* cultures or greenhouse material. Roots were cleared and stained as described by Phillips & Hayman (1970). We selected this method based on two criteria: *i)* Trypan blue is a widely used stain for AMF detection (Grace & Stribley 1991), and we thus wished to test BRP and IRP concentrations against this commonly utilized method; *ii)* Trypan blue is considered one of the most effective stains for AMF detection (Gange et al., 1999). AMF colonization was measured by the line intersect method (McConigle et al., 1990) at 200X magnification.

**Data Analysis**

ANOVA was used to test for treatment differences between all colonized vs. non-colonized samples within each plant host (e.g. *in vitro* control vs. *in vitro* AMF) in order to test the null hypothesis that AMF root colonization does not alter protein contents. Where F- ratios were significant (P< 0.05) treatment means were compared via Tukey-Kramer multiple comparison tests (JMP, SAS Institute, Cary, NC, USA). Non parametric analyses (Kruskal-Wallis test) were used if data failed to meet parametric assumptions. Where H –values were significant (P< 0.05) treatment means were compared via Kruskal-Wallis Multiple Comparison Z-Value Test (NCSS, 2000, Kaysville, Utah, USA).
In the correlation study, we used non-linear regression to test for correspondence between BRP or IRP concentrations and percent root colonization. Because we had no a priori basis on which to select a model, we chose the model that best fit the data (SigmaPlot 2001, SYSTAT). The regression equation used was $Y = a \cdot x / (b + x)$. Additionally we used linear equations to assess the relationship between BRP and IRP concentrations and percent colonization in our isolate comparison study as well as BRP and IRP fractions (SigmaPlot 2001, SYSTAT). We confirmed the appropriateness of both models (linear vs. non-linear) by calculating Akaike's information criterion (AIC) values for each models data set. The model with the lowest AIC value (data not shown) was used to assess the relationship between variables.

Results

Colonized roots in our isolate comparison study contained significantly greater amounts of BRP when compared to control treatments (Fig. 3.1A) with the exception of *E. colombiana* and *Gl. intraradices* ($F = 10.8, P = 0.0001$ [ANOV A/log transformed]). However, all AMF isolates tested contained significantly greater concentrations of IRP (Fig. 1B) when compared to control treatments ($F = 6.95, P = 0.0003$ [ANOV A/log transformed]). AMF colonization (Fig. 3.1A) did not significantly differ across AMF isolates ($F = 1.60, P = 0.20$). Additionally the amount of BRP and IRP extracted from colonized roots of *B. inermis* ($n = 29$) significantly increased with the rate of colonization ($r^2_{BRP} = 0.32, P_{BRP} = 0.0006$ and $r^2_{IRP} = 0.30, P_{IRP} = 0.001$) when all treatments were grouped (data not shown).
AMF-colonized roots in our correlation study contained significantly greater concentrations of BRP (Fig. 3.2A) in all host species when compared to control plants ($F_{in\text{-}vitro} = 60.5$, $P < 0.001$ [ANOVA/log transformed], $F_{P.\_lanceolata} = 6.49$, $P = 0.02$ [ANOVA], and $H_{S.\_bicolor} = 6.61$, $P = 0.01$ [Kruskal-Wallis]). Additionally, significant amounts of IRP (Fig 3.1B) were extracted from all AMF-colonized roots when compared to control roots ($H_{in\text{-}vitro} = 6.75$, $P = 0.01$, $H_{P.\_lanceolata} = 7.36$, $P = 0.007$ [Kruskal-Wallis], and $F_{S.\_bicolor} = 7.00$, $P = 0.02$ [ANOVA/log transformed]).

The concentration of BRP and IRP significantly increased with the rate of root colonization in our in vitro study ($F = 25.6$, and $P < 0.0002$ [BRP]; $F = 27.8$, and $P < 0.0002$ [IRP]). We observed 20 times more BRP mg g$^{-1}$ root extracted from heavily colonized roots when compared to non-colonized roots. Additionally, the non-AMF controls had 0.04 mg g$^{-1}$ root of IRP while the highly colonized plant roots contained 1.0 mg of IRP per g$^{-1}$ root (Fig. 3.3A and 3.3B).

The concentration of BRP and IRP extracted from S. bicolor (Fig. 3.3A and 3.3B) significantly increased with increasing root colonization ($F = 105$, $P < 0.0001$ [BRP]; $F = 54.7$, and $P < 0.0001$ [IRP]). Furthermore the P. lanceolata concentrations of BRP and IRP extracted (Fig 3.4A, and 3.4B) also significantly increased with AMF-root colonization ($F = 30.2$, $P < 0.0001$ [BRP]; $F = 73.0$, and $P < 0.0001$ [IRP]). The concentration of BRP ranged from approximately 0.5 mg g$^{-1}$ for the non-colonized AMF controls to 12 mg g$^{-1}$ for heavily colonized plants in both treatments.
Comparison of the ratio of IRP extracted to BRP concentrations quantified by Bradford assay indicates that approximately 9% (isolate comparison study) and 1% (correlation study) of the BRP pool is immunoreactive to the MAb32B11 antibody for both colonized and non-colonized treatments. Regression analysis of the isolate comparison study (data not shown) suggest that BRP and IRP concentrations (n = 29) increase in proportion ($r^2_{BRP/IRP} = 0.11$, $P_{BRP/IRP} = 0.08$). Similar regression results were observed between IRP and BRP for the correlation study (Fig. 3.5). The *in vitro* concentrations of IRP significantly increased with BRP amounts ($F = 7.03$ and $P = 0.02$). This same trend was observed in both the *S. bicolor* ($F = 24.1$ and $P = 0.0001$) and *P. lanceolata* ($F = 5.60$ and $P = 0.03$).

**Discussion**

This study is the first to systematically examine the presence of glomalin-related protein inside plant roots, and to test for a correlation between GRP fractions (BRP and IRP) and AMF root colonization. We provide evidence for the potential general usefulness of using root protein or glomalin to assess AMF root colonization by (i) showing that root protein concentrations were increased for several different AMF isolates, (ii) in different host plant species, and (iii) under sterile *in vitro* and greenhouse conditions.

**Evaluation of Bradford Assay**

Our results suggest that BRP is effective at predicting the presence of AMF colonization in *in vitro* and greenhouse grown cultures (Fig. 3.2A, 3.3A, 3.4A). Several studies have shown an up-regulation of root proteins as a result of AMF colonization (Samra et
al., 1997; Benabdellah et al., 1998; Dumas-Gaudot et al., 2004). Additionally, increased BRP concentrations within AMF colonized roots could be the result of uptake and storage of the amino acid arginine within AMF intraradical hyphae. Jin et al., (2005) indicate that AMF storage of arginine increases as a result of colonization. According to Lucarini & Kilikian (1999) the Bradford assay is sensitive to arginine contained on protein surfaces.

This increased production of proteins within AMF colonized roots provides a detection marker that could be used to assess AMF colonization with the Bradford assay. This method could be very effective where large-scale screening is necessary, including testing for root colonization in mycorrhizal isolate culture, testing for mycorrhiza-defective plant genotypes, and verifying AMF colonization within the horticultural and restoration industry (where screening for AMF colonization is often necessary before plant installation).

In this proof-of-concept study we have not tested the usefulness of this assay for field-collected material. It is possible that root associated organisms may produce proteins that survive the GRP extraction process. Findings from Rosier et al., (2006) indicate that the extraction process used to isolate GRSP does not effectively eliminate all extraneous protein sources. The Bradford assay can detect small proteins and charged compounds (Sedmak & Grossberg, 1977). It is unlikely that the GRSP extraction process would reduce plant root derived proteins or protein sources associated with plant roots below the detection limits of the Bradford method. Until further testing under field condition
occurs, we would therefore recommend the use of this assay primarily for the presence/absence of mycorrhizae (also given the curvilinearity of the relationship in all cases) rather than as a quantitative assay; but this would still be very useful for many screening applications.

**Evaluation of ELISA Assay**

Monoclonal antibodies have been extensively utilized within the field of AMF research (Wright et al., 1987; Hahn et al., 1993) and in detecting saprobic soil fungi (Thornton et al., 1994; Thornton & Dewey, 1996; Dewey et al., 1996). The appeal of the ELISA-based assay is its potential greater specificity compared to the Bradford protein analysis; an advantage that comes with the cost of greater analytical effort and sophistication. Our results suggest that IRP was also effective at predicting AMF colonization (Fig 3.2B, 3.3B, 3.4B), but the relationship was still curvilinear; and the IRP signal was not much better at distinguishing higher levels of root colonization than the Bradford assay.

Our results indicate that approximately 2–12% (isolate comparison study) and 0.2-0.6% (correlation study) of the BRP pool is reacting with the MAb32b11 antibody (Fig. 3.1, 3.2, and 3.5). This observation could reflect that other plant derived proteins increase roughly in proportion with AMF-derived glomalin (i.e. this is a true glomalin signal); alternatively this could be interpreted to mean that a small proportion of root protein is cross-reactive with the MAb32B11 antibody. At this point, a second independent protein detection system would be necessary to conclusively distinguish between these two possibilities; however, at present such a secondary system is unavailable. Using
information on the recently described gene for glomalin (Gadkar & Rillig, 2006), future research could examine AMF colonized roots for glomalin gene expression.

An observation that supports cross-reactivity (instead of a true glomalin signal) is the presence of immunoreactive material in non-mycorrhizal controls (Fig. 3.1) with the exception of Plantago lanceolata. MAb cross reactivity in general is mostly dependent on antigen/foreign compound similarity, based on which cross-reactivity can range from a few % to 100 % (Lee et al., 2001; Wortberg et al., 1996; Giersch, 1993). Several studies suggest that the MAb32B11 antibody is slightly cross-reactive with plant compounds (Nichols, 1999), non-AMF species (Wright et al., 1996), and non-target proteins present in large concentrations, such as BSA (Rosier et al., 2006). Colonization by AMF can significantly influence the biochemistry of the host plant. Studies have shown an increase in protein concentration (Berta et al., 1995; Bago et al., 1997; Blilou et al., 2000) polypeptide content (Dumas-Gaudot et al., 1994; Samra et al., 1996; Benabdellah et al., 1997) and lipid levels (Graham et al., 1995; Pfeffer et al., 1999; Gaspar et al., 1999) as a result of AMF colonization. It is thus possible that the MAb32B11 antibody used is cross reactive with one or several plant produced compounds.

In the isolate comparison study there was a tenfold difference in both BRP and IRP concentrations compared to the correlation study (Fig. 3.1AB and Fig. 3.2AB). This difference in the BRP and IRP quantity could be the result i) longer growth period (25% greater in isolate comparison study), ii) differing host plants, and iii) differences in AMF isolates tested. Several studies have suggested an increase in plant derived compounds as
a result of time since colonization (Maier et al., 1995, Fester et al., 1999), host plant preference (Klingner et al., 1995, Fester et al., 2002) as well as AMF isolates (Vierheilig et al., 2000).

Relationship to GRSP pools in ecosystems

The majority of previous studies regarding GRSP have focused on quantifying the concentration of the total GRSP pool in soils, which includes mineral- and aggregate-associated GRSP, AMF hyphae, spores and colonized root fragments (Wright & Anderson, 2000; Rillig et al., 2003; Lutgen et al., 2003; Lovelock et al., 2004). The relative contribution of these fractions to the total GRSP pool is not presently known. Driver et al. (2005) suggested that glomalin is a component of the AMF hyphal wall and that glomalin arrival in the soil is primarily a result of hyphal decomposition. Our results were not able to conclusively demonstrate the presence of glomalin (sensu stricto) in roots. If this is the case, colonized roots and their decomposition products will not be a major contributor to GRSP, except in the case of Bradford-reactive soil protein pool. As research into identifying mechanisms which control GRSP production and function continues (Rillig 2004), it will be important to determine how all sources of GRSP production, for example how the root-contained pool, contributes to the total GRSP pool. Nevertheless, our study is one of the first to show that contributions to GRSP of co-occurring AMF isolates (i.e. as opposed to fungi that were not isolated from the same ecosystem) can differ significantly.
Conclusion

We have shown that GRP fractions extracted from plant roots can be employed to at least assess AMF presence/absence in roots. Given its comparative ease of use, the Bradford assay would be the best method for this application. Considering the time necessary for conducting the Bradford assay (and assuming processing in 96-well plate format) we have estimated that using this assay could result in a ~40% labor cost savings compared to Trypan blue staining/microscopy. Expenses for supplies should be roughly equivalent, but this does not take into account instrumentation costs (e.g., microplate reader, multi-channel pipettors). Further research should include testing the use of GRP fractions especially BRP, to estimate AMF colonization in field collected samples.

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Figure 3.1

(A) Bradford Root Protein (BRP) in roots of *Bromus inermis* colonized with different AMF isolates. Means (±) SE were compared with ANOVA/log transformed with significance accepted at *P* < 0.05. Numbers inside of bars are percentage AMF root colonization (with standard errors).

(B) Immunoreactive Root Protein (IRP) in roots of *Bromus inermis* colonized with different AMF isolates. Means (±) SE were compared with ANOVA/log transformed, with significance accepted at *P* < 0.05.
Figure 3.2

(A) Bradford Root Protein (BRP) for control and AMF treatments. Means (±) SE were compared with ANOVA or Kruskal-Wallis Multiple Comparison Z-Value Test, with significance accepted at P < 0.05.

(B) Immunoreactive Root Protein (IRP) for control and AMF treatments. Means (±) SE were compared with ANOVA or Kruskal-Wallis Multiple Comparison Z-Value Test, with significance accepted at P < 0.05.
Figure 3.3

(A) Relationship between Bradford Root Protein (BRP) (mg g⁻¹) and percent AMF root colonization in *in vitro* culture (n = 15).

(B) Relationship between Immunoreactive Root Protein (IRP) (mg g⁻¹) and percent AMF root colonization in *in vitro* culture (n = 15).
Figure 3.4

(A) Relationship between Bradford Root Protein (BRP) (mg g⁻¹) and percent AMF root colonization in Sudan grass grown in the greenhouse (n = 18).

(B) Relationship between Immunoreactive Root Protein (IRP) (mg g⁻¹) and percent AMF root colonization in Sudan grass grown in the greenhouse (n = 18).
Figure 3.5

(A) Relationship between Bradford Root Protein (BRP) (mg g\(^{-1}\)) and percent AMF root colonization in narrowleaf plantain grown in the greenhouse (n = 21).

(B) Relationship between Immunoreactive Root Protein (IRP) (mg g\(^{-1}\)) and percent AMF root colonization in narrowleaf plantain grown in the greenhouse (n = 21).
Figure 3.6

(A) Relationship between Bradford Root Protein (BRP) (mg g\(^{-1}\)) and Immunoreactive Root Protein (mg g\(^{-1}\)) from \textit{in vitro} culture (n = 15).

(B) Relationship between Bradford Root Protein (BRP) (mg g\(^{-1}\)) and Immunoreactive Root Protein (mg g\(^{-1}\)) from Sudan grass (n = 18).

(C) Relationship between Bradford Root Protein (BRP) (mg g\(^{-1}\)) and Immunoreactive Root Protein (mg g\(^{-1}\)) from narrowleaf plantain (n = 21).
INFLUENCE OF ARBUSCULAR MYCORRHIZAL FUNGI ON SOIL AGGREGATE DYNAMICS

Abstract

The assembly of mineral soil particles and soil organic components into aggregates and their ensuing decomposition is a dynamic process. Understanding how biotic factors (plant roots, fungal hyphae, and microorganisms) influence aggregate dynamics would provide greater insight into several important soil ecology processes: nutrient cycling and retention rates, mitigation of soil erosion, development of microbial habitats, as well as the potential of soils to serve as a carbon sink. Typically soil aggregation is measured at a single time point; this provides limited information regarding aggregate formation and decomposition. Various methods to access aggregate dynamics have been developed: i) macroaggregate removal via crushing and measuring macroaggregate reformation and ii) incorporation of labeled tracer spheres into macroaggregates. The primary limitation with these methods is only macroaggregate assembly can be measured. An additional method demonstrating promise in measuring aggregate dynamics is the use of Rare Earth Element (REE) oxides. Briefly, an aggregate size class is labeled with a particular REE, and the potential incorporation, stabilization and/or decomposition of the labeled aggregate can be determined by inductively coupled plasma mass spectroscopy (ICP-MS). The purpose of this research was to assess the influence of arbuscular mycorrhizal fungi (AMF) on aggregate dynamics using REE oxides. We selected AMF because they play a prominent role in soil development; their influence on aggregate dynamics is
almost completely unknown. In two studies REE labeled aggregates representing macroaggregate and microaggregate size classes were incubated either in the presence or absence of AMF hyphae for 2 or 5 weeks. After two weeks of incubation our results indicate that AMF treatments significantly slowed macroaggregate turnover. Additionally REE labeled microaggregate and silt/clay fractions (< 53 µm) within the AMF treatment were largely incorporated into macroaggregates. In contrast the Non-AMF treatment exhibited significant decomposition of macroaggregates. Furthermore, we observed slower incorporation of the REE labeled microaggregate into larger aggregates size. This suggests that AMF hyphae influence on aggregate dynamics is rapid, while contributing factors within the Non-AMF treatment (saprophytic fungi, microorganisms) are operating at slower rate and/or smaller spatial scales. Analysis of aggregate distribution after 5 weeks of incubation depicted similar patterns as observed in the 2 week trial. However, when we compared like treatments across incubation times we observed different trends. In the AMF treatment we observed continued incorporation of intermediate macroaggregates into larger macroaggregates as well as potential decomposition of macroaggregates. In terms of Non-AMF treatment we observed steady incorporation of both intermediate macroaggregates and microaggregates into larger aggregates, and limited decomposition. We conclude that: i) REE oxides are an effective tool at assessing aggregate dynamics in response to AMF, ii) AMF promoted rapid formation of macroaggregates while equally incorporating both intermediate macroaggregates as well as microaggregates, iii) AMF stabilize aggregates against decomposition during short incubations, and iv) the presence of saprophytic fungi and microorganisms causes the formation of larger macroaggregates at gradual rates.
Introduction

Soil structure and aggregation affect several important processes including effects on nutrient cycling and retention rates, mitigation of soil erosion, and promotion of favorable water relations (Lynch & Bragg 1985, Bossuyt et al. 2001, DeGryze et al., 2005). Furthermore, soil structure decreases organic matter (OM) decomposition rates, thereby increasing sequestration of greenhouse gases (i.e. CO₂). The ability of soil to protect OM from decomposition is attributed to the physical separation of OM from the microbial community as well as soil fauna (Hattori 1988). An understanding of factors affecting aggregate formation and degradation will significantly improve ecosystem models used to predict soils’ ability to store carbon (Six et al., 2002) and could greatly aid management decisions that promote good soil structure (Bossuyt et al., 2001).

Soil aggregates are not static structures; the assembly of mineral soil particles and soil organic components into aggregates and their subsequent decomposition is an active process (Lei et al., 2002, Plante et al., 2002). This understanding has brought about the idea that aggregates are dynamic and their formation, stability and ensuing turnover is in a constant state of flux (Blanco-Canqui & Lal 2004). Several studies have investigated the effect of organic matter (DeGryze et al., 2006), soil texture (Golchin et al., 1997, DeGryze et al., 2005), and management strategies (Six et al., 1999, Six et al., 2000 and Paustian et al., 2000) on aggregate dynamics; however, no research efforts to date have investigated the effects of soil organisms (i.e. fungi, microorganisms, and microarthropods) on aggregate dynamics.
Qualitative analysis of the influence of arbuscular mycorrhizal fungi (AMF) on the dynamic nature of soil structure is severely lacking. Since AMF are central organisms within the soil environment, i.e. AMF form mutualistic root/fungi associations with over 80% of all terrestrial plants (Smith & Read 1997), it is expected that AMF play a significant role in both aggregate formation as well as prevention of aggregate degradation (Tisdall et al., 1997, Miller & Jastrow 2000). Several studies suggest that AMF stabilize macroaggregates against disruptive forces which in turn protect OM from decomposition, aid water infiltration rates, and create spatially defined areas within the soil environment (Miller & Jastrow 1990, Jastrow et al., 1998). Additionally, several reviews have outlined possible biochemical and morphological characteristics of AMF that could directly influence aggregate dynamics (Zhu & Miller 2003, Jeffries et al., 2003, Rillig & Mummey 2006) yet direct evidence linking AMF to aggregate dynamics is absent. This is most likely due to the difficulty in separating plant contributions from AMF influences, thus a direct relationship between fungal abundance and aggregate dynamics has not been established (Six et al., 2004).

Currently there are two models describing aggregate dynamics. Tisdall & Oades (1980) suggest that microaggregates provide the starting material from which macroaggregates are formed. However, Oades (1984) proposed a second model where microaggregates are formed within macroaggregates. Findings from Angers et al., (1997) lend support to the model proposed by Oades. Understanding how AMF influence microaggregate formation would provide essential information on the dynamics that control macroaggregate formation in soils where organic matter is the main binding agent (Oades & Waters 1981).
Yet, the contribution of AMF in the formation of microaggregates and their potential incorporation into larger macroaggregates has not been investigated (Rillig & Mummey 2006).

Studies of soil aggregate formation and decomposition have used several methods in an attempt to quantify aggregate dynamics. One commonly utilized method involves removing soil macroaggregates via crushing (< 250 µm). The amended soil is then added back to the experimental system and macroaggregate reformation is assessed (Denef et al., 2001, DeGryze et al., 2005). A more sophisticated method of adding labeled tracer spheres has also been used under both field and laboratory conditions; a suite of tracer spheres is applied to the experimental treatment and tracked as they are incorporated into the aggregate size classes of interest (Plante et al., 1999, Plante & McGill 2002a, Plante & McGill 2002b). The primary limitation associated with these methods is only macroaggregate formation can be measured; macroaggregate decomposition and microaggregate dynamics can not be determined (DeGryze et al., 2006). A third method was proposed by Zhang et al., (2001) where Rare Earth Element (REE) oxides are integrated into known aggregate size classes and the redistribution of these labeled aggregates can be traced. Their results indicate that REE oxides are: (i) uniformly incorporated into both macroaggregates and microaggregates, (ii) firmly attached to soil particles reducing the potential for leaching, (iii) easily removed from soil via acid extraction, and (iv) a rarity within the soil environment resolving potential issues with background interference. DeGryze et al., (2006) improved upon the use of REE oxides in aggregate dynamic studies by demonstrating that both aggregate formation and
decomposition can be traced and that the presence of REE oxides did not interfere with the microbial communities associated with aggregates.

The objectives of this study were to: (i) develop an experimental system that targets the influence of only AMF hyphae on aggregate dynamics and (ii) assess the effect of AMF on macroaggregate and microaggregate dynamics during short incubation timescales through the use of rare earth element tracers (REE).

Materials and Methods

Experimental Set-up

Narrowleaf Plantain (*Plantago lanceolata*) was selected as our test plant due to its AMF colonization potential and large root diameter. Prior to planting, seeds were surface sterilized in 5% H$_2$O$_2$ for five minutes and grown in a petri dish for one week. Seedlings were subsequently planted into the split chamber system (i.e. one seedling per side) as outlined in Fig. 4.1. Chambers were maintained at a constant water potential via capillary action, this was done in order to prevent wet/dry cycles which could alter aggregate dynamics. Plants were grown under growth chamber conditions (25°C with 14 hr. light cycle) for 2 or 5 weeks. AMF treatments consisted of field soil collected from local grassland that contains abundant AMF (Lutgen et al., 2003). Non-AMF treatments were composed of the same field soil steamed at 80°C for 8 hrs. Both soils were mixed with sand at a 1:1 ratio. The characteristics of our test soil was: sandy loam, pH 6.6, organic matter content 5.72%, texture: 63.5% sand, 21.5% silt, 14.5% clay, CEC (meq 100/g)
13.5 (Lutgen et al., 2003). Additionally non-AMF treatments received a microbial inoculation consisting of 100g soil/ 900 ml of distilled water filtered at 20 µm.

**Rare Earth Element Oxide Characteristics**

Four REE oxides, Gadolinium oxide (Gd₂O₃), Lanthanum Oxide (La₂O₃), Neodymium oxide (Nd₂O₃), and Samarium Oxide (Sm₂O₃) were purchased from the Tianjiao International Trading Co. Inc. U.S.A. The chemical and physical properties of the REE oxides used in our study are reported by Zang et al., (2001 and 2003). The background concentrations of REE oxides in our test soil are reported in Table 4.1.

**Rare Earth Element Oxide Pre-Incubation**

Field soil was initially pasteurized at 80°C for 1 hr. then crushed on a roller table and forced through a 53 µm sieve. This process (i) eliminated the AMF community, (ii) destroyed all previous soil structure (> 53 µm), and (iii) removed both sand and coarse organic matter fractions. Each aggregate size class of interest was labeled by serially diluting REE oxide into separate soil batches of crushed soil. Briefly ten 1.0 g sets of soil were hand mixed with 30.0 mg of REE oxide. Once this step was complete all 1.0 g sets were combined and mixed for 15 min via soil tumbler. Additional blank soil sets were added to the labeled soil mixture at a rate of 10.0 g and mixed for 15 min. This process continued for each REE oxide until a final soil/REE oxide dilution of 300 mg kg⁻¹ soil was achieved. Labeled aggregates were constructed by (i) adding 20 g of organic matter (Medicago sativa [alfalfa]) to 1 kg of soil, (ii) adjusting water content to field capacity via di-water and (iii) incubating soil mixture for 3 weeks at 25°C. Prior to addition,
organic matter was ground to a fine powder and passed through a 500 µm sieve.

Additionally, mixing efficiency was tested by measuring the REE oxide concentration of three subsamples taken from each of the four aggregate incubations.

**REE Oxides Treatment Installation**

The split chamber system used in this experiment is equipped with a center compartment constructed of 38 µm mesh (Fig. 4.1) which restricts plant roots but allows AMF hyphal growth. Initially, we inserted a sterile soil “plug” into the compartment and allowed the AMF hyphae to colonize this area for approximately 2 weeks. This step allowed us to install the REE oxide labeled soil aggregates at a juncture when AMF hyphae would be primed to enter the center compartment; this would decrease aggregate turnover as a result of abiotic forces. Labeled soil aggregates were added at the following rate: 5.0 g of 500 µm (SM₂O₃), 6.5 g of 250 µm (La₂O₃), 5.0 g of 53 µm (Ad₂O₃), and 3.5 g of silt/clay (< 53 µm) (Gd₂O₃). This ratio of macroaggregates and microaggregates are typical of the ratio found within field soils. The addition of known quantities of aggregate also enabled us to track their distribution over time.

**Aggregate Separation**

At harvest soil from the incubation process and split chamber center compartment was separated into several aggregate size classes via the modified wet sieving method. This modification used separated sieves as oppose to the nested sieve method originally described by Yoder (1936). Preliminary studies suggest that the nested sieve method provides an overestimation of macroaggregate stability (data not shown). With the
separate sieve method each individual sieve is slowly moved in and out of deionized water over a period of 5 min (100 cycles). Upon completion of the sieving cycle, the next smaller sieve class is lifted into place and the separation cycle is repeated. The material remaining after the separation process is backwashed off the sieve, collected and oven dried at 80°C. The fraction is then weighed and the percentage of water stable aggregates calculated. We used a series of four sieves in order to obtain the following five fractions from a 10 g sample: > 1000 µm (large macroaggregates), 500-1000 µm (intermediate macroaggregates) 250-500 µm (small macroaggregates), 53-250 µm (microaggregates) and < 53 µm (silt and clay).

Mycorrhizal measurements

AMF hyphal length (m hyphae/soil g⁻¹) from the hyphal compartment was measured using an aqueous extraction and filtration method (Rillig et al., 1999). AMF hyphae were distinguished from other soil fungi at 200 times magnification (Miller et al., 1995). The following criteria were used to distinguish AM hyphae: dichotomous branching (as oppose to right angles), non-regular septa, and irregular growth (seldom in straight-line with elbow-like protrusions). Hyphal length was determined using the line intersect method as described by Jakobsen (1992) and Tenant (1975).

Extraction and Quantification of REE oxides

We used the acid extraction protocol proposed by Zang et al., (2001) in order to remove REE oxides from aggregate samples. Initially, 500 mg of each aggregate size class was placed into an acid washed Erlenmeyer flask. Next 10 ml of concentrated HNO₃ was
added to each sample and heated to 85° C via water bath for 1 hour. Samples were then removed and allowed to cool for 30 min. (< 70° C). Then 10 ml of 30% H_{2}O_{2} was added and reheated for approximately 2-3 min; this step removes organic material within the sample and consequently releases bound REE oxides. 5 ml of HCL is then added to each sample and heated to 85° C. After 1 hr of heating, samples were cooled, eluted with 5 ml of di-water and filtered through a 0.45 µm filter.

The prepared samples were analyzed using an Agilent 7500CE ICP-MS (Agilent Technologies, Palo Alto, CA) by the Interdisciplinary Center for Plasma Mass Spectrometry at the University of California at Davis (ICPMS.UCDavis.edu). The samples were introduced using a MicroMist Nebulizer (Glass Expansion 4 Barlow’s Landing Rd., Unit 2A Pocasset, MA 02559) into a temperature controlled spray chamber. Instrument standards were diluted from Certiprep ME 2A and ME 4 (SPEX CertiPrep, 203 Norcross Avenue, Metuchen, NJ 08840) to .5ppb, 2ppb, 10ppb, 100ppb, 500ppb, and 1000ppb respectively in 3% Trace Element HNO_{3} (Fisher Scientific) in 18.2 mohm water. A NIST 1643E Standard (National Institute of Standards and Technology, 100 Bureau Drive, Stop 2300, Gaithersburg, MD 20899-2300) was analyzed every 12th sample as a quality control. Sc, Y, and Ge Certiprep standards (SPEX CertiPrep) were diluted to 100ppb in 3% HNO_{3} and introduced by peripump as an internal standard.

Data Analysis

In order to determine the amount of REE within an aggregate size class (%) we used the following formula: 

\[
\frac{\text{((ICPREE - Background REE) x dilution rate))}}{\text{initial REE}}
\]
concentration x 100. ANOVA was used to test for treatment differences between AMF and non-AMF treatments in order to test our null hypotheses. Where F-ratios were significant (P < 0.05) treatment means were compared via Student-t test (JMP, SAS Institute, Cary, NC, USA). AMF and Non-AMF treatments were replicated six times (n = 6) however, in the Non-AMF treatment two plants died (n = 4) during our 2 week incubation trial.

Results

Verification of REE Oxide Incorporation

The use of REE oxides in aggregate dynamic studies requires that the REE oxides of interest be thoroughly mixed into the soil and that recovery of the label is sufficiently efficient (i.e. > 95%). Our results suggest that the REE oxide concentration within each aggregate subsample was homogeneously incorporated as the standard error was not greater then 1.5% (Table 4.1). Furthermore, the recovery rate for each of the REE oxides tested was relatively high (> 97%), with the exception of Gd2O3, which was smaller at 95% (Table 4.1). These findings are similar to the results reported by DeGryze et al., (2006).

Fungal hyphal lengths

To determine the potential binding capabilities of fungal hyphae in aggregate dynamics we measured the hyphal lengths of both AMF and saprophytic fungi. Significant differences in AMF hyphal lengths in the root exclusion chamber were measured between the AMF and Non-AMF treatments after 2 weeks of incubation (F = 32.7 and P = 0.001
AMF treatments contained 14.63 (4.40) meters of AMF hyphae while the Non-AMF treatments contained 1.54 (0.11) meters of AMF hyphae. AMF hyphal lengths also significantly differed \((F = 13.4, \text{ and } P = 0.006 \text{ [ANOVA/log transformed]})\) between AMF and Non-AMF treatments after 5 weeks of incubation. AMF treatments contained 49.14 (10.42) meters of hyphae while non-AMF treatments contained 1.42 (0.29) meters of AMF hyphae.

The lengths of Non-AMF hyphae did not significantly differ between treatments or incubation times. After two weeks of incubation, AMF treatment contained 0.22 (0.14) meters of hyphae while the Non-AMF treatment contained 0.51 (0.11) meters of hyphae \((F = 1.94 \text{ and } P = 0.21 \text{ [ANOVA]})\). The hyphal lengths of Non-mycorrhizal fungi continued to increase after 5 weeks of incubation. The AMF treatment has 0.84 (0.22) meters of hyphae and Non-AMF treatment had 0.78 (0.12) meters of hyphae \((F = 0.04 \text{ and } P = 0.84 \text{ [ANOVA]})\).

**Aggregate Size Distribution**

Aggregate size distribution was assessed for the purposes of tracking the treatment effects (Fig 4.2, 4.3 and Table 4.2). After two weeks of incubation 76.7% (3.9) of the AMF treatment was composed of the large macroaggregate size class (> 1000 µm). This was significantly greater than the amount measured in the Non-AMF treatment 2.8% (1.3) Table 4.2. This observation was reversed in the smaller macroaggregate size classes: the 500 µm aggregates in the AMF treatment contained 1.7% (0.52) which was significantly lower than the amount measured in the Non-AMF treatment 8.5% (2.3) Table 4.2.
Results from the 250µm aggregate size class display a similar trend: AMF treatment contained 4.4% (1.2) which was significantly lower than the Non-AMF 28.6% (1.4) Table 2. Additionally, the Non-AMF treatment in the 250 µm size class contained approximately the same amount of aggregates as initially placed into the incubation chamber (Fig 4.2). The allocation of microaggregates (53 µm) and silt/clay (<53 µm) after two weeks of incubation was significantly lower in the AMF treatment when compared to the Non-AMF treatment (Fig 4.2 and Table 4.2). The Non-AMF treatment contained a greater portion of 53 µm aggregates when compared to the initial amount of aggregates added to the incubation chamber; a similar trend was observed in the labeled silt/clay fraction (<53 µm).

A similar aggregate distribution pattern was observed for the 5 week incubation study (Fig 4.3). The formation of large macroaggregates (>1000 µm) was significantly greater in the AMF treatment when compared to the Non-AMF treatment (Fig 4.3 and Table 4.2). The proportion of small macroaggregates (500 µm and 250 µm) in the 5 week incubation was significantly greater in the Non-AMF treatment when compared to the AMF treatment (Fig 4.3 and Table 4.2). In the 53 µm size class the Non-AMF treatment contained a significantly greater percentage of aggregates than the AMF treatment (Fig 4.3 Table 4.2). Additionally, the Non-AMF treatment contained a greater fraction of 53 µm than was originally placed in the center compartment. In the silt/clay fraction (<53 µm) both treatments were statistically equivalent (Fig 4.3).
A comparison of the AMF treatment between the 2 and 5 week incubations suggest that no significant differences occurred between the distribution of aggregate size classes measured (Fig 4.2 and 4.3). In the Non-AMF treatment the 5 week incubation contained a significantly greater fraction of 1000 µm aggregates than the 2 week incubation (F = 8.34 and P = 0.03 [ANOVA]). The 500 µm size class significantly increased over the incubation time as well: 8.5% (2.3) at 2 weeks to 15% (1.4) at 5 weeks (F = 5.51 and P = 0.07 [ANOVA]). A significant decrease (F = 16.2 and P = 0.02 [ANOVA]) in the percentage of 250 µm aggregates was observed during the incubation time; 28.6% (1.4) to 21% (2.5). Decreases in the microaggregate size classes were also observed over the incubation period. The 53 µm size class significantly decreased (F = 10.0 P = 0.02 [ANOVA]) from 43.7% (1.6) to 33.0% (4.2). The 5 week incubation contained significantly less (F = 9.0 P = 0.03 [ANOVA]) of the silt/clay fraction (< 53 µm) size, 4.7% (2.4), when compared to the 2 week incubation, 16.2% (0.3).

**REE Oxide Transfers**

The potential incorporation and decomposition of both macroaggregate sizes classes (500 and 250 µm) were assessed to determine the distribution of the REE label. The contribution of 500 µm aggregates towards the formation of larger macroaggregates (1000 µm) during the 2 and 5 week incubations was significantly greater in the AMF treatment (Fig 4.4A and 4.4B). As a result the Non-AMF treatment retained a significantly greater proportion of 500 µm aggregates during both incubation trials. Additionally, a significantly greater fraction of the labeled 500 µm aggregate size class was measured within the Non-AMF 250 µm treatment (Fig 4.4A), yet this trend was not
observed during the 5 week incubation. The distribution of the 250 µm aggregate size class followed a similar trend with regard to macroaggregate formation as the 500 µm size class (Fig 4.5A and Fig 4.5B). Results from the Non-AMF treatment demonstrate a greater incorporation of the 250 µm aggregates into 500 µm aggregates for both incubation trials (Fig 5A and Fig 5B). Decomposition of the 250 µm aggregates into microaggregates (53 and <53 µm) was slightly greater in the Non-AMF treatments (Fig 5A) however, this trend was not observed in the 5 week incubation (Fig 4.5B).

Additionally the contribution of microaggregates to aggregate dynamics was assessed via REE oxide labels. The incorporation of 53 µm aggregate size class into 1000 µm aggregates was significantly greater in the AMF treatment after two weeks of incubation (Fig 4.6A). The Non-AMF treatment maintained greater incorporation of the 53 µm size class into the intermediate macroaggregates (500 and 250 µm) during both incubation trials (Fig 4.6A and Fig 4.6B). Decomposition of the 53 µm aggregates was significantly greater in the Non-AMF treatments; this trend was not observed in the 5 week incubation. The transfer of the silt/clay fractions (<53 µm) into larger aggregates followed the same trend as the 53 µm size class (Fig 4.7A and Fig 4.7B). The Non-AMF 500 µm and 250 µm size class incorporated a significantly greater fraction of the <53 µm after 2 weeks of incubation. Similar results were measured after 5 weeks of incubation with the exception of <53 µm incorporation into 250 µm aggregates (Fig 4.7B). A significantly greater fraction of the labeled silt/clay (<53 µm) remained within the non-AMF treatment after 2 weeks of incubation, yet a similar trend was not measured in the 5 week incubation. The
AMF treatment incorporated a greater fraction of silt/clay into the 53 µm size class after 5 weeks of incubation.

**REE Oxide Treatment Transfers**

Aggregate distribution data observed from the 2 and 5 week incubation trials (Fig 4.2 and 4.3) for the AMF treatment suggest that aggregate dynamics are static. Yet, further analysis of the labeled aggregate distribution within each size class suggests this is not the case. The 1000 µm aggregates in the AMF 5 week incubation continued incorporating 500 µm aggregates when compared to the AMF 2 week incubation (Fig 4.4A, 4.4B and Table 4.3). We observed a similar trend in the 250 µm labeled aggregate size class, incorporation into 1000 µm aggregates was significantly greater in the 5 week AMF treatment (Fig 4.5A, 4.5B, and Table 4.3). In terms of labeled microaggregate (53 µm) dynamics we measured significantly less incorporation into the 250 µm aggregate size class (Fig 4.6A, 4.6B and Table 4.3). We measured a significantly greater fraction of labeled silt/clay material (< 53 µm) within the 250 µm aggregate size class after 5 weeks of incubation.

Aggregate distribution data from the Non-AMF 2 and 5 week trials suggest that after five weeks of incubation significant gains occurred in the 1000 and 500 µm aggregate size classes; while the 250, 53 aggregates and silt/clay fraction (<53 µm) underwent significant losses (Fig 4.2 and 4.3). Comparison of each of the labeled aggregate size classes enabled us to determine where these significant gains and losses occurred. After the 5 weeks of incubation, the 1000 µm aggregates incorporated a greater proportion of
500 µm labeled aggregates than the 2 week trial (Fig 4.4A, 4.4B and Table 4.4). Furthermore, decomposition of the 500 µm aggregates into 250 µm aggregates was significantly less when compared to the 2 week trial. In addition the Non-AMF treatment after 5 weeks of incubation incorporated a significantly greater fraction of 250 µm aggregates into the 1000 µm size class (Fig 4.5A, 4.5B and Table 4.4). The labeled 53 µm aggregate size class was extremely active and we measured significant incorporation into both the 1000 and 500 µm aggregate size classes after 5 weeks of incubation (Fig 4.6A, 4.6B and Table 4.4). Lastly significantly less of the labeled silt/clay (<53 µm) material was recovered from the 53 µm aggregate size class after 5 weeks of incubation.

**Discussion**

This study is the first to systemically investigate the influence of AMF on aggregate dynamics through the use of REE oxides and a modified growth chamber which isolates AMF hyphae from plant roots. We provide evidence that the presence of AMF i) increased large macroaggregate formation (> 1000 µm) via equal incorporation of macroaggregates and microaggregates and ii) slowed intermediate macroaggregate and microaggregate decomposition. Additionally we found that in the absence of AMF macroaggregate formation continues at a gradual rate possibly as a result of saprophytic fungi.

**Macroaggregate formation via AMF hyphal enmeshment**

Fungal hyphae adhere to soil particles as they expand through the soil environment which indirectly results in the physical entanglement of primary soil particles into soil.
aggregates (Beare et al., 1997, Rillig & Mummey 2006). Oades & Waters (1991) suggest that fungal hyphae act as “sticky-string bag” enmeshing large quantities of soil particles thus preventing their disruption. We tested aggregate stability via the fast wetting method which provides a greater degree of disruption when compared to other methods (Abiven et al., 2007). Our results suggest that the presence of AMF hyphae greatly improved macroaggregate (> 1000 µm) formation and stability (Fig 4.2 and 4.3). Degens et al., (1996) calculated the potential of growing hyphae to enmesh several aggregate size classes into an aggregate greater than 1000 µm. When we apply the same formula and assumptions to our soil we find that the enmeshment potential of AMF hyphae tripled over the incubation period. Since the distribution of 1000 µm macroaggregates within the AMF treatment was similar during both incubation trials (Fig 4.2 and 4.3), this suggests that AMF hyphal growth beyond a given density did not improve macroaggregate formation. However, REE labeled aggregate analysis suggests that this extensive enmeshment of aggregates via AMF hyphae provided greater long term macroaggregate stabilization as well as continued incorporation of intermediate macroaggregates (Fig 4.5A, 4.5B, 4.6A, and 4.6B). Future studies should investigate how AMF influence aggregate dynamics in response to disintegrating forces (i.e. wet/dry, freeze/thaw or fungal grazers). To our knowledge no investigations have attempted to address this question.

**Macroaggregate formation via saprophytic hyphal enmeshment**

Several studies suggest that saprophytic fungal growth contributes to the formation of stable soil aggregates (Kinsbursky et al., 1989, Toyota et al., 1996, Tisdall et al., 1997,
Caesar-TonThat & Cochran 2000). Results from our study suggest that increased saprophytic fungal expansion contributed to increases in macroaggregate formation within the Non-AMF treatment during the incubation period (Fig 2 and 3). Additionally, the presence of saprophytic hyphae may have contributed to the continued incorporation of intermediate macroaggregates and microaggregates into 1000 µm aggregates (Fig 4.4A, 4.4B, 4.5A, 4.5B, 4.6A, and 4.6B). The spread of saprophytic hyphae is highly dependent on organic matter additions (Boswell et al., 2002, Boswell et al., 2007, and Falconer et al., 2007), however, the concentration of saprophytic hyphae in our experiment was significantly less when compared to AMF treatment. Since our test soil received limited organic matter additions it would be expected that saprophytic fungal growth would be restricted, limiting their potential to form aggregates. Investigations examining saprophytic hyphal growth on aggregate dynamics as a function of organic matter quality and quantity remain an interesting area of future research.

**AMF hyphal influence on aggregate dynamics**

AMF hyphae greatly affect macroaggregate stabilization (Jastrow et al., 1998, Tisdall et al., 1997, Rillig & Mummey 2006). Our results indicate that 500 and 250 µm macroaggregates were rapidly incorporated into 1000 µm aggregates within the AMF treatment (Fig 4.4A, 4.4B and 4.5A, 4.5B). The presence of AMF also slowed the decomposition of 500 and 250 µm macroaggregates into smaller aggregate size classes. Thomas et al., (1993) found that the presence of AMF hyphae significantly halts macroaggregate disintegration. Similar to our results Andrade et al., (1998) found that the presence of AMF hyphae greatly increased aggregate stability over a short timeframe.
Additionally their results suggest that both hyphae and plant roots improved aggregate stability individually, yet root/hyphae effects were additive when acting together. Piotrowski et al., (2004) suggests that hyphal spread may be of greater significance to aggregate stability than the total length of AMF hyphae. This observation is consistent with our results; as AMF hyphae continued to spread throughout the incubation chamber the presence of the hyphae slowed macroaggregate decomposition while promoting aggregate incorporation (Table 4.3).

**GRSP influence on aggregate dynamics**

In addition to AMF hyphal influence on aggregate dynamics the production of proteinaceous compounds (i.e. glomalin) may also serve as an important component affecting aggregate formation and stabilization (Wright & Upadhyaya 1998, Rillig & Mummey 2006). Several studies indicate that glomalin-related soil protein (GRSP) released from AMF hyphae promote water stable macroaggregates (Wright & Upadhyaya 1998, Wright & Anderson 2000, Rillig et al., 2001). Recent experimental evidence however, suggests that glomalin is not secreted and is bound within the living mycelium of AMF (Driver et al., 2005). Gadkar & Rillig (2006) identified glomalin as a cellular heat shock protein, approximately 63.1 kDa, with unknown function. Purin & Rillig (2008) confirmed the presence of glomalin in the cytoplasm matrix and cell walls of mycelium as well as within spores via electron microscopy. One hypothesis that lends support to the correlative evidence linking glomalin to aggregate stability suggests that as the mycelium originally forming the aggregate begins to decay, cellular glomalin is then released thus forming a nucleation center bridging primary particles. The persistence of AMF hyphae
is a widely debated topic with estimates ranging from days (Staddon et al., 2003) to months (Tisdall & Oades 1990, Olsson & Johnson 2005).

**Saprophytic fungi influence on aggregate dynamics via production of extracellular materials**

Several studies suggest that saprophytic fungi are equally important to aggregate formation and stabilization as are members of the phylum Glomeromycota (Cesar-TonThat & Cochran 2000, Toyota et al., 1996). Results from this study suggest that incorporation as well as destabilization of macroaggregates (500 and 250 µm) occurred within the Non-AMF treatment (Fig 4.4A, 4.4B and 4.5A), yet decomposition of 500 µm and 250 µm aggregates did not increase after 5 weeks of incubation (Fig 4.4B and 4.5B). AMF and saprophytic fungi both influence aggregate stability via hyphal enmeshment and/or the production of extracellular materials. Since we observed a gradual increase in aggregate incorporation in the Non-AMF treatment this suggests that the binding of aggregates by saprophytic fungi may not be as strong as the aggregate bond formed by AMF hyphae or the growth of saprophytic fungi may be significantly slower than AMF. Additionally, extracellular polysaccharides secreted by saprophytic fungi would have to reach a set concentration in order to support aggregate stability. The current hypothesis indicates that saprophytic fungal production of extracellular materials is more important to aggregate formation and stabilization than hyphal enmeshment (Chenu 1989, Hu et al., 1995, Caesar-TonThat & Cochran 2000). This hypothesis is consistent with the known biology of saprophytic fungi; the majority of energy resources are allocated towards acquisition of carbon (i.e. enzyme). Since enzymes maintain a high metabolic cost,
resource distribution for the construction of “durable” hyphae would be limited. This idea also fits with the growth strategy employed by saprophytic fungi. Several models of fungal growth suggest that the ability of saprophytic fungi to cover large spatial scales is dependent on biomass recycling capacities (Boswell et al., 2002, Boswell et al., 2007, Ritz & Young 2004, Falconer et al., 2007). If the hyphae of saprophytic fungi were constructed of recalcitrant materials, biomass recycling would be rather difficult. In order for the formation of stable soil aggregates to occur via saprophytic fungal growth carbon addition rates must meet the energy demands of the rapidly expanding mycelium.

Research efforts aimed at identifying extracellular products produced by the microbial community that improve aggregate stability will need to overcome several technological issues such as: quantifying specific (i.e. fungal vs. plant) sources of polysaccharide production.

**AMF influence on microaggregate dynamics**

The majority of research to date involving microaggregate formation and stabilization has centered on particulate organic matter acting as a bridging agent of primary particles, and the influence of AMF has been completely unexplored (Rillig & Mummey 2006). Our results suggest that 53 µm aggregates and silt/clay fraction (< 53 µm) exposed to AMF hyphae were quickly incorporated into large macroaggregates (> 1000 µm). Our results fit the hypotheses of Tisdall & Oades (1982) and Miller & Jastrow (1992) who suggest that AMF hyphae are the main contributor to microaggregate incorporation into larger macroaggregates. Additionally, Jastrow et al., (1998) suggest that AMF hyphae provide the initial mechanism by which microaggregates are incorporated into macroaggregates.
Further stabilization of microaggregates occurs as a result of prokaryotic activities. Rillig & Mummey (2006) proposed that microbial communities at the microaggregate level are influenced by AMF: i) production of carbon sources that promote bacterial growth, ii) modification of rhizodeposition providing the additional resources of carbon for microbial growth, and iii) alteration of the soil environment providing protective niche for microbial growth.

**Saprophytic fungi influence of microaggregate dynamics**

Microaggregates begin as small pieces of decomposing organic matter which become saturated with plant, fungal and bacterial polysaccharides (Beare et al 1994). Their ensuing incorporation into larger aggregates is often facilitated by plant roots as well as fungal hyphae (Oades 1984). Based on electron microscopy Foster (1981) identified a greater proportion of bacterial colonies within microaggregates, suggesting the importance of the role of bacteria over fungi with regard to microaggregate formation and stabilization. Results from our study suggest that the Non-AMF 53 µm aggregates underwent incorporated and destabilized after two weeks of incubation (Fig 4.6A and Fig 4.6B). We also observed a similar trend in the silt/clay (< 53 µm) fraction with significant incorporation into intermediate macroaggregates (Fig 4.7A and Fig 4.7B). This gradual incorporation and subsequent stabilization of microaggregates into larger aggregates suggests that saprophytic fungal hyphae may have provided the initial mechanism for incorporation. Further stabilization between micro and macroaggregate could be the result of microbial activity. In order for microbial activity to support incorporation/ stabilization of microaggregates into macroaggregates two criteria must be
met: i) environmental conditions at the bridging site must support microbial growth, and ii) microorganisms growing at that site must produce extracellular polysaccharides capable of bridging microaggregates to macroaggregates. Several studies suggest that the interior regions of microaggregates are characterized by low predation, limited oxygen diffusion, low nutrient concentrations as well as stable moisture (Chenu et al. 2001, Ranjard and Richaume 2001). It is plausible that as microaggregates are pulled into close proximity of macroaggregates an environment which favors microbial growth could be established. Additionally, investigations identifying specific groups of microorganisms within microaggregates are ongoing. Mummey & Stahl (2004) found that Actinobacteria were highly abundant within the interior of microaggregates while members of Proteobacteria were located at microaggregate surfaces. Assessing the influence of microorganisms on microaggregate incorporation is hampered by several technological difficulties such as isolating specific organisms from soil, quantifying their population, and characterizing the extracellular polysaccharides they produce. As a result of these limitations investigations involving the identification of specific species of bacteria and the extracellular byproducts production potential to bind microaggregates have not been thoroughly completed. In spite of these difficulties Caesar-TonThat et al., (2007) utilized a series of methods (spiral plating, fatty ester methyl profiles, and DNA analysis) in order to identify two species of microorganisms (Stenotrophomonas and Sphingobacterium) capable of microaggregate formation through polysaccharide production. Future studies that address the communities of microorganisms and mechanisms capable of stabilizing microaggregates will have to employ a suite of methods.
Experimental considerations

Prior to our study two potential limitations regarding this method remained unanswered:
i) would the characteristics of our field soil promote REE oxide desorption from labeled aggregates, and ii) would the concentration of REE oxides used prove to be toxic, limiting AMF and/or saprophytic fungal growth. According to Jones (1997) REE oxide absorption to soil surfaces is a function of soil pH, cation exchange capacity, and organic matter content. As a cautionary note, use of soils with low pH may promote REE oxide desorption and possible leaching; Cao et al., (2001) found that REE oxide desorption increased with decreasing pH. Our test soil maintained a slightly acidic pH, and high CEC value which would suggest that the soil used would promote REE oxide binding. In order for REE oxides to be an effective tracer in aggregate dynamic studies they must not hinder the growth of microorganisms. The toxicity of REE oxides on soil microorganisms has not been thoroughly investigated; DeGryze et al., (2006) found that microbial respiration was not affected by the presence of REE oxides. While our study did not directly test for toxic effects of REE oxides on AMF or saprophytic fungi, the continued growth of both AMF and saprophytic hyphae over the incubation period indirectly suggests that REE oxides at these concentrations do not limit fungal growth. Results from this study, suggest that studies using REE oxides are feasible with regard to plant/fungal aggregate dynamics.
Acknowledgements

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References


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## Tables

### Table 4.1 Rare-earth element (REE) background concentrations and recovery efficiency

<table>
<thead>
<tr>
<th>Element</th>
<th>Background concentration (mg kg(^{-1}) soil)</th>
<th>REE Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>La</td>
<td>22.6 (1.4)</td>
<td>98.2 (0.03)</td>
</tr>
<tr>
<td>Nd</td>
<td>23.4 (1.5)</td>
<td>97.1 (0.01)</td>
</tr>
<tr>
<td>Gd</td>
<td>4.40 (0.171)</td>
<td>95.0 (0.0001)</td>
</tr>
<tr>
<td>Sm</td>
<td>4.61 (0.237)</td>
<td>99.3 (0.002)</td>
</tr>
</tbody>
</table>

Values are presented as Means (+) standard error (n = 4)
Table 4.2 F- and P-values for the relative abundance of each aggregate size class for both incubation trials

<table>
<thead>
<tr>
<th>Size Class</th>
<th>2 week</th>
<th>5 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
</tr>
<tr>
<td>&gt;1000 µm</td>
<td>111.2</td>
<td>0.0001</td>
</tr>
<tr>
<td>1000-500 µm</td>
<td>13.8</td>
<td>0.001</td>
</tr>
<tr>
<td>500-250 µm</td>
<td>163.8</td>
<td>0.0001</td>
</tr>
<tr>
<td>250-53 µm</td>
<td>148.7</td>
<td>0.001</td>
</tr>
<tr>
<td>&lt; 53 µm</td>
<td>52.1</td>
<td>0.004</td>
</tr>
<tr>
<td>Label</td>
<td>Size (µm)</td>
<td>% Δ</td>
</tr>
<tr>
<td>-------</td>
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</tr>
<tr>
<td>Sm</td>
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</tr>
<tr>
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<td>-13.9</td>
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<tr>
<td></td>
<td>250</td>
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<tr>
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<td>500</td>
<td>-9.4</td>
</tr>
<tr>
<td>Nd</td>
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<td>-15.4</td>
</tr>
<tr>
<td></td>
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<td>-12.0</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>Gd</td>
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</tr>
<tr>
<td></td>
<td>250</td>
<td>+6.0</td>
</tr>
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Percentage change (%Δ) was calculated as the difference of 2 week treatment average subtracted from the 5 week treatment average. * indicates significance accepted at P < 0.1.
Table 4.4 F and P values Non-AMF treatment comparisons

<table>
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<th>Label</th>
<th>Size (µm)</th>
<th>% Δ</th>
<th>F(P)</th>
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<td>+ 4.7</td>
<td>5.02 (0.08)*</td>
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<tr>
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<td>500</td>
<td>- 11.0</td>
<td>8.00 (0.04)</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>- 5.0</td>
<td>9.58 (0.03)</td>
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<tr>
<td></td>
<td>&lt;53</td>
<td>- 6.8</td>
<td>4.3 (0.09)*</td>
</tr>
<tr>
<td>La</td>
<td>1000</td>
<td>+ 9.6</td>
<td>24.4 (0.004)</td>
</tr>
<tr>
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<td>250</td>
<td>-10.0</td>
<td>29.5 (0.003)</td>
</tr>
<tr>
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<td>1000</td>
<td>+13.5</td>
<td>264 (0.0001)</td>
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<td>44.6 (0.001)</td>
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<td>31.1 (0.003)</td>
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<td>136 (0.0001)</td>
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<tr>
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<td>&lt;53</td>
<td>- 8.1</td>
<td>50.1 (0.001)</td>
</tr>
<tr>
<td>Gd</td>
<td>1000</td>
<td>+ 3.2</td>
<td>4.71 (0.08)*</td>
</tr>
<tr>
<td></td>
<td>&lt;53</td>
<td>- 9.4</td>
<td>102 (0.0002)</td>
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Percentage change (%Δ) was calculated as the difference of 2 week treatment average subtracted from the 5 week treatment average. * indicates significance accepted at P < 0.10
Figure 4.1

Schematic diagram of the growth chamber used in both incubation experiments. Black arrows indicate mesh screens restricting root growth, while white arrow indicates root restriction towards labeled soil.
(A) Macroaggregate distribution between AMF and Non-AMF treatments during 2 week incubation. Significant differences between treatments are indicated by different upper case letters. Lower case letters indicates significant differences detected between the 2 and 5 week studies within Non-AMF treatments. Means (±) SE were compared with ANOVA significance accepted at $P < 0.05$. The predicted bar is the amount of REE labeled aggregate size class added to the incubation chamber. Additionally, the $> 1000 \, \mu m$ size class was not labeled with REE oxide therefore no predicted bar is included in this figure.

(B) Microaggregate distribution between AMF and Non-AMF treatments during 2 week incubation. Significant differences between treatments are indicated by different upper case latters. Lower case letters indicates significant differences detected between the 2 and 5 week incubation studies within NonAMF treatments. Means (±) SE were compared with ANOVA significance accepted at $P < 0.05$. 

Figure 4.2
Figure 4.3

(A) Macroaggregate distribution between AMF and Non-AMF treatments during 5 week incubation. Significant differences between treatments are indicated by different upper case letters. Lower case letters indicates significant differences detected between the 2 and 5 week studies within Non-AMF treatments. Italicized lower case letters indicates significance detected at $P < 0.10$. The predicted bar is the amount of REE labeled aggregate size class originally added to the incubation chamber. Additionally, the > 1000 µm size class was not labeled with REE oxide therefore no predicted bar is included in this figure.

(B) Microaggregate distribution between AMF and Non-AMF treatments during 5 week incubation. Lower case letters indicates significant differences detected between the 2 and 5 week studies within Non-AMF treatments. Means (±) SE were compared with ANOVA significance accepted at $P < 0.05$. Italicized lower case letters indicates significance detected at $P < 0.10$. 
Figure 4.4

(A) The percentage of 500 µm labeled aggregates isolated from each aggregate size class after 2 weeks of incubation. * indicates significant difference detected at $P < 0.05$ between Non-AMF 2 week incubation and Non-AMF 5 week incubation.

(B) The percentage of 500 µm labeled aggregates isolated from each aggregate size class after 5 weeks of incubation. † indicates significant difference detected at $P < 0.05$ between AMF 2 week incubation and AMF 5 week incubation.
Figure 4.5

(A) The percentage of 250 µm labeled aggregates isolated from each aggregate size class after 2 weeks of incubation. * indicates significant difference detected at P < 0.05 between Non-AMF 2 week incubation and Non-AMF 5 week incubation.

(B) The percentage of 250 µm labeled aggregates isolated from each aggregate size class after 5 weeks of incubation. † indicates significant difference detected at P < 0.05 between AMF 2 week incubation and AMF 5 week incubation.
Figure 4.6

(A) The percentage of 53 µm labeled aggregates isolated from each aggregate size class after 2 weeks of incubation. * indicates significant difference detected at P < 0.05 between Non-AMF 2 week incubation and Non-AMF 5 week incubation.

(B) The percentage of 53 µm labeled aggregates isolated from each aggregate size class after 5 weeks of incubation. † indicates significant difference detected at P < 0.05 between AMF 2 week incubation and AMF 5 week incubation.
(A) The percentage of labeled slit/clay (< 53 µm) fraction isolated from each aggregate size class after 2 weeks of incubation.* indicates significant difference detected at P < 0.05 between Non-AMF 2 week incubation and Non-AMF 5 week incubation.

(B) The percentage of labeled slit/clay (< 53 µm) fraction isolated from each aggregate size class after 2 weeks of incubation. † indicates significant difference detected at P < 0.05 between AMF 2 week incubation and AMF 5 week incubation.
Hawkweed, a perennial native of Europe, is a serious ecological threat to northwestern states including Washington, Idaho, and Montana. Studies suggest *Hieracium caespitosum* is well adapted to low nutrient soils and can alter several soil processes; potentially promoting a soil environment that favors or stabilizes the species after establishment. Several studies suggest that increases in soil fertility slow Hawkweed growth and spread. However, no study has investigated changes in soil process under Hawkweed as a result of increased soil fertility. The purpose of this study was to compare fertilizer influences on the soil environment under *H. caespitosum* to that of neighboring weed-free *F. idahoensis* in order to gain an understanding of the potential mechanisms by which *H. caespitosum* invasion modifies the soil environment. We analyzed plant shoot/root biomass, soil pH, organic matter (OM) concentrations, arbuscular mycorrhizal fungi (AMF) and non-AMF biomass as these are key soil properties that promote plant establishment. We further hypothesized that soil aggregate size distribution and stability as well as the mineralization rates of available and macroaggregate protected soil carbon would shift as a result of fertilization treatments. Our results suggest OM significantly increased under *F. idahoensis* soils in response to fertilizer addition; this trend was not observed in *H. caespitosum* soils. We attribute the increase of OM concentrations in *F. idahoensis* soils to increased plant productivity; this finding is further supported by
increased shoot and root biomass production. The presence of OM is important as it provides a nutrient reservoir and increases soil water holding capacity. AMF hyphal lengths significantly decreased as a result of fertilization in *F. idahoensis* soils, this trend was not observed in *H. caespitosum*. AMF hyphal lengths decreased in the *F. idahoensis* soils possibly as a result of improved soil nutrient status. In contrast AMF hyphal length remained consistent across all *H. caespitosum* treatments; this suggests that even with improved soil nutrient status Hawkweed is still dependent on AMF. Additionally several studies suggest that Hawkweed species are highly mycorrhizal dependent. Available and macroaggregate protected carbon mineralization rates significantly increased as a result of fertilization in *F. idahoensis* treatments, yet in the *H. caespitosum* soils available as well as macroaggregate protected carbon mineralization significantly decreased. Studies suggest that fertilization alters mineralization rates by shifting the C: N ratios of the labile carbon pool. In the *F. idahoensis* treatments the addition of fertilizer possibly modified the C: N ratios increasing mineralization and nutrient availability. The addition of fertilizer in the *H. caespitosum* soils possibly increased the concentration of recalcitrant compounds in Hawkweed biomass slowing mineralization. We conclude *H. caespitosum* potentially alters several soil processes that modify the soil environment thereby promoting its establishment. Results from this study provide pertinent information which greatly improves our knowledge of Hawkweed invasion mechanisms at the soil level; this understanding could aid Hawkweed invasion models as well as management efforts in preventing the spread of Hawkweed.
**Introduction**

*Hieracium caespitosum* (Hawkweed) a member of the Asteraceae family is a weedy stoloniferous plant, with milky sap and a shallow fibrous root system (Panebianco & Willemsen 1976, Wilson et al., 1997). Hawkweed, a perennial plant native of Europe, has become a serious ecological threat in both the United States as well as New Zealand (Wilson et al., 1997, Makepeace et al., 1985). Typically Hawkweed is found on well drained, coarse textured soils containing low organic matter (Wilson et al., 1997). These general growth requirements have allowed Hawkweed populations to invade several habitat types: mountain meadows, forest clearings, pastures, and abandoned farmland (Wilson et al., 1997). Until recently Hawkweed populations have been mainly restricted to the eastern United States (Wilson et al., 1997). However, rapidly invading populations in both Washington and Idaho have prompted its listing as a noxious weed, with potential designation in Montana, if invading populations continue to gain ground (Toney et al., 1997). Invasion of exotic weeds results in changes to several ecological processes: i) displacement of native species (Callaway et al., 2003), ii) alteration of hydrologic cycles (Randall 2000), iii) increases in fire intensity and frequency (Melgoza et al., 1990, Mack & D' Antonio 1998, Vitousek et al., 1997) iv) modification of nutrient cycling (Ashton et al., 2005, Ehrenfeld et al., 2003) vi) shifts in microbial communities (Bradford et al., 2002, Holly et al., 2008) as well as vii) a reduction in soil structure (Batten et al., 2005). According to Pimentel et al., (2005) economic losses and management control expenses associated with exotic weed invasion of range and crop lands costs $33 billion annually. An understanding of how invasives influence soil ecosystems is essential to potential
management strategy. This concept is likely a more effective approach than attempting to restore damaged ecosystems to their pre-invaded state (Belnap & Phillips 2001).

Early research efforts utilizing fertilizer application have identified the potential alteration of soil nutrient cycling as a mechanism of Hawkweed invasion. Scott et al., (1990) found that Hawkweed populations were reduced on moderately productive soils as a result of direct seeding and fertilizer application. In contrast, their results indicate that fertilizer application and direct seeding on less productive soils failed to halt Hawkweed spread. Results from Scott (1993) suggest that large application rates of phosphate and sulfur fertilizer together with seeding controlled Hawkweed, while lower application rates were not as successful. These results provide important insight into the role of soil fertility on Hawkweed establishment, yet below ground biotic processes were entirely ignored. Increased soil fertility can significantly alter soil microbial communities (Marschner et al., 2003, Sessitsch et al., 2001), carbon mineralization rates (Aoyama et al., 1999a, Graham et al., 2002) as well as soil structural properties (Aoyama et al., 1999b, Marinari et al., 2000, Munkholm et al., 2002, Celik et al., 2004). In regards to microbial community changes several studies suggest that large fertilizer additions reduce the diversity of Arbuscular Mycorrhizal Fungi (AMF) (Corkidi et al., 2002, Johnson et al., 2003, Sigüenza et al., 2006), an important plant symbiont, which may aid in exotic plant invasion. Investigations assessing shifts in soil biotic processes as a result of Hawkweed invasion could provide essential information that prevents and/or controls invading populations.
Building upon previous research efforts, several studies have attempted to develop a model that explains Hawkweed invasion by quantifying ecosystem factors that enhance invasion potential including: management, geographical conditions, resident plant community structure, browse intensity, and general soil characteristics (Rose et al., 1995, Fan & Harris 1995, Duncan et al., 1997, Rose & Frampton 1999). Currently three models have been proposed as a result of these studies: grassland decline, site suitability/propagule rain, and niche creation (Fan & Harris 1995, Duncan et al., 1997, and Rose et al., 1995). The predictive power of these models was assessed by Rose et al., (1998); their results suggest that the grassland decline model provides the best fit in predicting Hawkweed invasion due to its ability to assess temporal and spatial variation of Hawkweed abundance. However, one of the primary limitations associated with Hawkweed models of invasion is that they are exclusive to New Zealand systems which maintain different soil properties, native plant communities, as well as climatic conditions when compared to North America Hawkweed populations. Additionally, these models only consider the most basic of soil characteristics (i.e. soil moisture and nutrient status) which are not representative of the complex nature of soils. Our investigation will explore changes in key soil characteristics as a result of Hawkweed invasion as well as increasing soil fertility within North American soils. Additionally, these results will provide pertinent information greatly enhancing the predictive power of current Hawkweed invasion models.
Investigations assessing changes in critical soil properties as a result of Hawkweed presence are beginning to provide important insight into possible invasion mechanisms. Studies assessing soil pH, organic carbon and nutrient concentrations suggest that Hawkweed soils are acidic, and contain greater quantities of both organic carbon and nutrients (McIntosh & Allen 1993, Boswell & Espie 1998). Saggar et al., (1999) found that, as Hawkweed populations expand, carbon and nitrogen concentrations increase in comparison to pasture soils. They further found that microbial carbon, nitrogen, phosphorus and CO₂ respiration rates were greater under Hawkweed. They concluded that Hawkweed is able to modify the soil environment to its own advantage. Knicker et al., (2000) assessed the chemical composition of soil organic matter induced by Hawkweed invasion. Their results suggest that Hawkweed soils contain a greater proportion of phenolic compounds which are hypothesized as being influential in soil nitrogen sequestration. Building on this idea Scott et al., (2001) investigated the litter quality under invading Hawkweed populations; their results indicate that Hawkweed modifies resource availability by depleting key nutrients. Their results further suggest that Hawkweed biomass contains larger quantities of aromatic compounds (i.e. phenolics and hybrid carbon) which are less decomposable. The large C: N ratio, low soil pH, and recalcitrant plant biomass within Hawkweed soils suggest that a greater proportion of fungal biomass should exist in Hawkweed soils (Saggar et al., 1999). These studies assess soil changes within New Zealand systems and do not consider fungal community changes as a result of Hawkweed invasion or how these shifts may influence soil structural properties.
The purpose of this study was to compare fertilizer influences on the soil environment under *H. caespitosum* to that of neighboring weed-free *F. idahoensis* within North American system. We analyzed plant shoot/root biomass, soil pH, organic matter (OM) concentrations, arbuscular mycorrhizal fungi (AMF) and non-AMF biomass as these are key soil factors that promote plant establishment. We further hypothesized that soil aggregate Mean Weight Diameter (MWD), distribution, as well as the mineralization rates of available and macroaggregate protected soil carbon would shift as a result of fertilization treatment.

**Materials and Methods**

**Experimental design description and sampling**

The soils for our study were taken from an established field experiment located near Santa, Idaho (49° 9' 1' N; 116° 26' 57" W). Wallace et al., (in press) provides a complete plot design as well as site description. The experimental design was composed of an initial herbicide treatment (May 2003) applied to native grassland plots consisting of Clopyralid administered at a rate of 0.6 kg ae ha⁻¹. *H. caespitosum* plots received no herbicide treatment. Gold Medal fertilizer (23-5-5, 1% Fe, 14% S) was hand broadcast into both native and invaded plots approximately 2 weeks after herbicide application at a rate 44 and 88 kg N ha⁻¹ an additional set of invaded and native grassland plots received no fertilizer application. The treatments for this study are thus defined as: *H. caespitosum* control (no herbicide/fertilizer), *H. caespitosum* low (no/herbicide/44 kg N ha⁻¹) and *H. caespitosum* high (no herbicide/88 kg N ha⁻¹); *F. idahoensis* control
(herbicide/no fertilizer), *F. idahoensis* low (herbicide/44 kg N ha\(^{-1}\)), *F. idahoensis* high (herbicide/88 kg N ha\(^{-1}\))

In the center of each treatment plot (10 x 30 meter) plant biomass was collected by clipping all above ground vegetation at the root collar within a 0.30 m\(^2\) quadrant. Upon removal of above ground plant biomass two 0-15 cm soil cores were collected from the center of the quadrant. The first core was used to assess aggregate stability, respiration, and root biomass. The second core was used to determine soil chemical characteristics. All soil samples were stored at 4 °C prior to analysis. We sampled four plots within each treatment: *H. caespitosum* (n = 4) and *F. idahoensis* (n = 4).

**Plant Biomass**

Total above ground biomass was determined by oven drying shoot samples for approximately 24 h at 60 °C. Plant roots were extracted by suspending 50 g of soil in 500 ml of 5.0 % sodium hexametaphosphate solution. Suspended roots were initially agitated for 3 minutes, left for 1-2 hrs, then re-agitated for an additional 3 minutes: this method promotes aggregate disruption. Roots were then collected over a 250 μm sieve and rinsed with tap water. Prior to biomass assessment (oven drying at 60 °C for 24 hr.) total root length was determined; this step was conducted first in order to prevent possible alteration of root widths via drying. Total root length, fibrous root length (> 0.20 mm) as well as very fine root length (< 0.20 mm) was measured using the WinRhizo V 3.10B root image analysis system (Régent Instruments Inc, Québec, Canada).
Carbon Mineralization

Soil carbon mineralization was measured using closed chamber incubation system with an alkali CO₂ trap as described by Hopkins (2008). Our carbon mineralization analysis had two treatments: i) undisturbed field soil and ii) field soil crushed to less than 250 µm. The first treatment was used to assess potential carbon mineralization within whole soil (defined; intact); the second treatment was done in order to liberate potential labile carbon stored within macroaggregates (defined; protected). Initially field wet soil was passed through a 2.0 mm sieve to remove large OM residues and stones. Setup of the incubation chamber involved placing 25 g of soil into a plastic specimen cup which was then inserted into a 1 liter Ball Jar containing 25 ml of CO₂ free water. Next a scintillation vial containing 10 ml of 1 M NaOH solution was placed into the middle of the soil sample. NaOH traps were removed after 4, 8, 12, 20, 32 days. During harvest, incubation chambers were vented for 30 minutes; water lost from both the chamber and soil sample was determined by weighing, and was replaced with CO₂ free water. Traps were immediately sealed with screw caps and wrapped with parafilm tape; traps were analyzed within 24 hrs of harvest. Incubation chambers were maintained at room temperature (25°C) for the duration of the study. Total carbon mineralization during the incubation period was determined by back titrating the NaOH trap with 0.5 M HCL, using phenolphthalein as an indicator. Carbonates within the trap were removed by precipitation via 2 ml of M BaCl₂. The amount of carbon mineralized was determined by the equation presented by Hopkins (2008).
Aggregate fractionation

Disruption of soil aggregates from the *H. caespitosum* and *F. idahoensis* plots was assessed via slaking as a result of fast wetting and micro cracking due to slow wetting (Le Bissonnais 1996). During the slaking treatment, 10 g of soil was immersed in de-ionized water for ten minutes; the slow wetting method involves capillary slow wetting 10 g of soil for 30 minutes prior to aggregate fractionation. Soil from both disruption methods was then separated into several aggregate size classes via the modified wet sieving method. This modification used separated sieves as oppose to the nested sieve method originally described by Yoder (1936). Results from Rosier and Mummey (unpublished data) suggest that the nested sieve method provides an overestimation of macroaggregate stability. With the separate sieve method each individual sieve is slowly moved in and out of deionized water over a period of 5 min (100 cycles). Upon completion of the sieving cycle, the next smaller sieve class is lifted into place and the separation cycle is repeated. The material remaining after the separation process is backwashed off the sieve, collected and oven dried at 50 C. The fraction is then weighed and the percentage of Water Stable Aggregates (WSA) is calculated. We used a series of three sieves in order to obtain the following four aggregate fractions: > 2000 µm (large macroaggregates), 250-2000 µm (intermediate macroaggregates), 53-250 µm (microaggregates) and < 53 µm (silt and clay). We calculated the Mean Weight Diameter (MWD) of both slaked and slow wetted aggregates using the equation presented by Batten et al., (2005), the two largest size fractions (> 2000 and 250-2000 µm) into one macroaggregate size class. Additionally Larney (2008) provides a detailed description of the formula and the method to determine MWD.
MWD = ((>250 μm % WSA mass X 5.125) + (53-250 μm % WSA mass X 0.1515) + (< 53 μm % WSA mass X 0.00275))/100

Mycorrhizal measurements

Root samples were cut into approximately 3.0 cm lengths upon harvest. Roots were cleared with 10% KOH and stained with Trypan blue as described by Phillips & Hayman (1970). AMF and saprophytic root colonization was measured by the line intersect method (McGonigle et al., 1990) at 200X magnification. AMF and saprophytic hyphal length (m hyphae/soil g⁻¹) from H. caespitosum and F. idahoensis treatment plots was measured using an aqueous extraction and filtration method (Rillig et al., 1999). AMF hyphae were distinguished from other soil fungi at 200 times magnification Miller et al., (1995), the following criteria were used to distinguish AM hyphae: dichotomous branching (as oppose to right angles), non-regular septa, and irregular growth (seldom in straight-line with elbow-like protrusions). Hyphal length was determined using the line intersect method as described by Jakobsen (1992) and Tenant (1975).

Data Analysis

Two-way ANOVA was used to evaluate statistical differences by herbicide treatment and fertilization rate. Additionally a one-way ANOVA was used to test for significant differences between available and macroaggregate protected carbon mineralization rates in both H. caespitosum and F. idahoensis treatments. Where F- ratios were significant (P< 0.05) treatment means were compared via Tukey-Kramer analysis (JMP, SAS Institute, Cary, NC, USA). Non parametric analyses (Kruskal-Wallis test) were utilized if
data failed to meet parametric assumptions. Where H-values were significant (P< 0.05) treatment means were compared via Wilcoxon Signed Rank test (NCSS, 2000, Kaysville, Utah, USA). Pearson-product moment correlation and regression analysis was used to assess relationships between MWD and organic matter, AMF/non-mycorrhizal hyphal lengths, and plant parameters (JMP, SAS Institute, Cary, NC, USA). Non-parametric Spearman rank correlation analysis was used to assess relationships between available and macroaggregate protected carbon mineralization and MWD as well as organic matter concentrations. In the *F. idahoensis* low fertilizer one replicate treatment was excluded from statistical analysis due to the presence of an outlier identified in the organic matter concentration and AMF hyphal length. We define outliers as data points which are two standard deviations above/below the sample mean.

**Results**

**Soil characteristics**

Available soil nutrient levels across all sites in our study were statistically equivalent (Table 5.1). Our data suggest significant differences (H = 11.1, P = < 0.001[Kruskal-Wallis]) in organic matter (OM) concentration with high fertilizer treatments maintaining the greatest OM (Table 5.1). The addition of herbicide significantly influenced soil pH (F = 7.94, P = 0.01[two-way ANOVA]) the lowest soil pH was identified in the *H. caespitosum* high fertilizer treatment (Table 5.1). Our data further suggest that within *F. idahoensis* treatments a negative relationship exists between slaked MWD and OM (r = -0.755 [Table 5.5 and Fig. 5.4 C]), we also observed a negative relationship between *F. idahoensis* slow wetting MWD and OM (r = -0.71 [Table 5.6 and Fig 5.6A]),
**Fungal variables**

Invading exotic weed species as well as fertilizer additions can alter soil fungi. Furthermore, the mycelium of fungi can serve as a binding agent controlling aggregate distribution as well as MWD. We assessed fungal biomass of AMF and Non-AMF in *F. idahoensis* and *H. caespitosum* amended plots in order to: i) assess the influence of fertilizer additions on fungal abundance and ii) identify a potential fungal impact on MWD. Significant differences in AMF hyphal lengths were observed as a result of herbicide treatment ($F = 19.2$, $P = 0.001$ [two-way ANOVA]) as well as the interaction of herbicide and fertilization ($F = 4.00$, $P = 0.04$ [two-way ANOVA]) the greatest AMF hyphal lengths were measured under *H. caespitosum* high fertilizer amended soils (Fig 5.2 A). The lengths of Non-AMF hyphae significantly differed as a result of fertilization ($F = 4.10$ and $P = 0.04$ [two-way ANOVA]) *H. caespitosum* control treatment maintaining significantly greater Non-AMF hyphae than *F. idahoensis* low fertilizer treatment (Fig 5.2 B). Assessment of AMF root colonization (Fig 5.2 A) did not significantly differ in response to herbicide ($F = 0.10$, $P = 0.76$ [two-way ANOVA]), fertilization ($F = 0.21$, $P = 0.81$ [two-way ANOVA]), or the interaction of both treatments ($F = 2.48$, $P = 0.12$ [two-way ANOVA]) between *H. caespitosum* and *F. idahoensis*. Significant differences in Non-AMF hyphal root colonization was not observed as a result of herbicide application ($F = 1.21$, $P = 0.29$ [two-way ANOVA]) or the interaction of herbicide and fertilizer ($F = 0.10$, and $P = 0.76$ [two-way ANOVA]) (Fig 5.2 B). However, a trend towards greater Non-AMF root colonization was observed in response to fertilizer addition ($F = 2.85$, $P = 0.09$ [two-way ANOVA]), suggesting that increases in fertilizer resulted in greater root colonization (Fig 5.2 B). Slaked *F. idahoensis* MWD had
a positive relationship with AMF hyphae ($r = 0.53$ [Table 5.5 and Fig. 5.4 A]), however a negative relationship ($r = -0.82$ [Table 5.5 and Fig. 5.4 B]) was observed between Non-AMF hyphae and slaked MWD. Additionally, no significant correlation was observed between any of the fungal parameters measured and the slaked or slow wetting MWD of $H. caespitosum$ treatments.

**Carbon mineralization**

The labile component of SOM plays a significant role in nutrient turnover as well as substantially influencing soil structural stability. In light of these effects we assessed the labile factor of SOM via carbon mineralization within whole soil fractions in $H. caespitosum$ and $F. idahoensis$ treatments. Our results suggest that both $H. caespitosum$ (control) and $F. idahoensis$ (high fertilizer/herbicide) treatments (Table 5.2) contained the highest rates of available carbon mineralization in response to herbicide application ($F = 20.9$ and $P = < 0.001$ [two-way ANOVA]), as well as the interaction of both treatments ($F = 38.8$ and $P = < 0.001$ [two-way ANOVA]). However, fertilization did not have a significant influence on available carbon mineralization ($F = 0.88$ and $P = < 0.43$ [two-way ANOVA]). Correlation assessment of the $F. idahoensis$ treatments suggest that the rate of available carbon mineralization significantly increases in response to greater OM concentrations ($r_S = 0.68, P = 0.02$ [Fig. 5.1 A]). Additionally the $F. idahoensis$ high fertilizer/herbicide treatment maintained the greatest mineralization rate as well as OM concentration. An opposite trend was observed in $H. caespitosum$ treatments; OM concentration significantly decreased ($r_S = -0.85, P = 0.002$) in response to higher carbon mineralization rates (Fig. 5.1 B). Furthermore, the $H. caespitosum$ high fertilizer/no herbicide treatment maintained the highest mineralization rate yet lowest OM
concentration. Correlation analysis further suggests that available carbon mineralization rates also influenced MWD. Our results suggest that a negative relationship exists between available carbon mineralization rates and slaked MWD of *F. idahoensis* plots ($r_s = -0.51$ [Table 5.5 and Fig. 5.4D]), and we observed a similar negative relationship between of available carbon mineralization rates and slow wetting MWD ($r_s = -0.78$ [Table 5.6 and Fig. 5.6 B]). In contrast the *H. caespitosum* treatments maintained a positive relationship between slaked MWD and available carbon mineralization rates ($r_s = 0.43$ [Table 5.5 and Fig. 5.5A]).

Labile carbon maintained within macroaggregates represents an organic carbon pool that

1) physically protected from microbial decomposition, 2) influential in stabilizing macroaggregates against destabilizing forces, and 3) dependent on standing plant biomass production. As a result of these characteristics we assessed the amount of protected labile carbon within macroaggregates (> 250 μm) in both *H. caespitosum* and *F. idahoensis* plots. Our results suggest that herbicide had no influence on macroaggregate protected mineralization rates ($F = 0.01$, $P = 0.90$ [two-way ANOVA]), however, fertilizer addition ($F = 8.63$, $P = 0.003$[two-way ANOVA] and the interaction of both treatments did significantly ($F = 23.0$, $P = 0.001$ [two-way ANOVA]) influence the amounts of macroaggregate protected mineralization rates (Table 5.2). We further compared available and macroaggregate protected soil mineralization rates within plant treatments. Our results indicate that fertilization of *H. caespitosum* soils significantly increased ($H = 22.8$, $P = 0.003$ [Kruskal-Wallis]) the amount of labile carbon within macroaggregates when compared to whole soils (Table 5.2). We observed a similar trend within the *F. idahoensis* plots; all macroaggregate protected treatments maintained
significantly greater \( F = 640.0, P = 0.0001 \) [ANOVA] amounts of labile carbon than whole soil (Table 5.2). Correlation assessment suggest a negative relationship between slaked MWD and macroaggregate protected carbon mineralization rates \( r_s = -0.32 \) (Table 5.5 and Fig. 5.5B) in the \textit{H. caespitosum} soils. Additionally, a positive relationship was observed between macroaggregate protected carbon mineralization rates and slow wetting \textit{F. idahoensis} MWD \( r_s = 0.55 \) (Table 5.6 and Fig 5.6 C).

**Plant parameters**

Above and belowground biomass directly contributes to soil organic carbon which in turn influences aggregate distribution and stability. In order to determine the effects of fertilizer and herbicide amendments on the biomass of invaded and native plots we assessed both above and below ground biomass. The addition of herbicide significantly influenced shoot biomass \( F = 10.6, P = 0.005 \) [two-way ANOVA], however, this trend was not observed in the fertilizer or interaction of both treatments (Table 5.3). Significant differences were also measured in terms of root biomass: herbicide, fertilizer, as well as the interaction of both treatments (Table 5.3); \textit{F. idahoensis} low fertilizer treatment produced seven times the root mass when compared to all \textit{H. caespitosum} treatments. Results from our correlation assessment suggest that neither shoot nor root biomass significantly influenced the slaked or slow wetting MWD (Table 5.5 and 5.6).

Fertilizer additions can significantly alter total root length as well as the distribution of fine roots and very fine roots; additionally plant roots can serve as binding agents controlling aggregate stability as well as MWD. We measured the effect of fertilizer amendments on root development within \textit{H. caespitosum} and \textit{F. idahoensis} plots in order
to determine fertigation effects on MWD. Our results indicate that the addition of herbicide significantly affected total root length \((F = 33.7, P = < 0.001 \text{ [two-way ANOVA]})\), a similar trend was observed in fertilizer treatment \((F = 7.24, P = 0.001 \text{ [two-way ANOVA]})\). Additionally we observed a significant difference in fine root and very fine root production with respect to herbicide, fertilizer and the interaction between both treatments (Table 5.3). Results from our correlation assessment suggest that none of the root parameters measured within \textit{F. idahoensis} or \textit{H. caespitosum} treatments had an effect on slaked or slow wetting MWD (Table 5.5 and 5.6).

**Aggregate size distribution**

Aggregate size distribution was assessed via slaking and slow wetting in order to determine the effect of an invasive exotic weed as well as fertilizer and herbicide amendments on soil structure. The addition of herbicide as well as fertilizer significantly influenced the distribution of larger macroaggregates (> 2000 µm) within slaked treatments (Table 5.4). In contrast no significant differences were observed in the remaining aggregate size class as a result of slaking. Upon competition of the slow wetting analysis significant differences were observed in larger macroaggregates (> 2000 µm) as a result of herbicide addition \((F = 22.1, P = 0.002 \text{ [two-way ANOVA]})\) additionally a trend was also observed in the fertilizer treatment \((F = 1.62, P = 0.007 \text{ [two-way ANOVA]})\). The distribution of 250 µm and 53 µm aggregates did not significantly differ between any of the \textit{H. caespitosum} or \textit{F. idahoensis} plots (Table 5.4). However, significant difference \((F = 6.34 \text{ and } P = 0.02 \text{ [two-way ANOVA]})\) in the silt/clay size class (< 53 µm) was observed as a result of the herbicide treatment.
Mean weight diameter

Previous research suggests that the Mean Weight Diameter (MWD) within a given soil can be significantly influenced by several biotic factors (e.g. plant roots, fungal hyphae, and OM). As a result of this understanding we assessed the MWD in slaked and slow wetting soils in both *H. caespitosum* and *F. idahoensis* treatments. Our results indicate that no significant differences in slaked MWD occurred as a result of herbicide, fertilizer, or the interaction of both treatments (Fig. 5.3 A). In contrast significant differences in slow wetting MWD were observed as a result of herbicide application (Fig. 5.3 B).

Discussion

This is the first study to thoroughly investigate alterations of several key soil characteristics as a result of *H. caespitosum* invasion. We further utilized modifications of soil fertility status via fertilizer addition in order to assess shifts in critical soil process possibly affecting Hawkweed invasion potential. We provide evidence that *H. caespitosum* invasion significantly alters several soil properties. However, in the presence of increasing soil fertility each of these factors were reversed under *H. caespitosum*; and accelerated within *F. idahoensis* soils. As a result of this investigation we were further able to identify several mechanisms affecting soil structure changes.

Soil properties

Soil pH

Our results indicate that soil pH significantly decreased under *H. caespitosum* soils as the growth of Hawkweed increased (Table 5.1). Several New Zealand based studies suggest
that soil pH declines under Hawkweed species (McIntosh & Allen 1993, McIntosh et al., 1995, Scott et al., 2001), however, response of North American soils to invasion and how increases in soil fertility influences Hawkweed soil pH have not been thoroughly investigated. Several studies suggest that plant litter may act as an acidifying agent releasing organic acids and lowering soil pH (Ponge et al., 1998, Jobbagy & Jackson 2003). Additionally Delhaize & Ryan (1995) reviewed the effects of aluminum on plant growth/development, suggesting that the solubility of aluminum significantly increases in acid soils at a pH below 5.5 resulting in toxic effects. According to Scott et al., (2001) suggest that aluminum phytotoxicity may be an invasion mechanism employed by Hawkweed. In contrast, we observed a marked increase in soil pH under *F. idahoensis* soils (Table 5.1). *F. idahoensis* biomass may be capable of reducing soil acidity. Similarly the grass specie *Calamagrostis villosa* is capable of reducing soil acidity by actively acquiring ammonium and various cations (Fiala et al., 2005). Another potential hypothesis would suggest that our test soils initially maintained low pH due to previous land use (i.e. conifer forest converted to pasture). The growth of *F. idahoensis* could be increasing soil pH, while Hawkweed biomass may be reversing this trend.

**Organic matter concentration and mineralization rates**

Another important finding of this study centers on the development of soil OM as a result of *H. caespitosum* invasion and increases in soil fertility. Our results suggest that OM significantly increased under *F. idahoensis* soils reaching levels equivalent to *H. caespitosum* control treatment soils (Table 5.1). Several agricultural based studies have shown that soil OM significantly increases as a result of increased soil fertility via
fertilizer additions (Haynes & Naidu 1998, Goyal et al., 1999, Šimek et al., 1999, Kanchikerimath & Singh 2001, Graham et al., 2002), attributing this increase in soil OM to increased plant productivity. Our results suggest a similar mechanism since fertilizer additions resulted in a significant increase in \textit{F. idahoensis} biomass (Table 5.3) and this may have contributed to increased soil OM. Additionally our results indicate that available carbon mineralization rates significantly increased as a result of herbicide application, and the interaction of both herbicide and fertilizer in \textit{F. idahoensis} treatments, yet an opposite trend was observed in \textit{H. caespitosum} soils (Table 5.2).

According to Goyal et al., (1999), and Kanchikerimath & Singh (2001) the rate of carbon mineralization increases with fertilization. Their results further suggest that the C: N ratio of the labile carbon pool directly affects mineralization. Several studies have shown that organic carbon concentrations and carbon mineralization rates are greater under Hawkweed invaded soils leading to the hypothesis that Hawkweed alters resource availability as a mechanism of invasion (Saggar et al., 1999, Knicker et al., 2000, Scott et al., 2001). However, our results show that the increases in soil fertility changes this relationship (Fig. 5.1 A, B). Fertilizer addition in the \textit{F. idahoensis} treatments accelerated mineralization (Fig 5.1A) possibly due to the low C: N ratio of \textit{F. idahoensis} litter. Plant litter composed of low C: N ratios decompose quicker releasing nutrients and improving soil fertility (Ehrenfeld et al., 2005). In contrast addition of fertilizer in the \textit{H. caespitosum} soils slowed mineralization resulting in an accumulation of OM (Fig 5.1B). This result suggests that the chemical constituents (i.e. C: N or C: P ratios) of \textit{H. caespitosum} litter under increased soil fertility may not favor decomposition. Several
studies have shown leaf litter with high C: N or C: P ratios maintain a slow decomposition rate (Hobbie 1992, Sinsabaugh & Moorhead 1994, Aerts 1997).

**Macroaggregate protected carbon mineralization rates**

Macroaggregate-protected organic matter is identified as the increased mineralization that results when macroaggregate structure is reduced to < 250 µm via crushing (Beare et al., 1994). This method has been extensively utilized within agricultural systems, however, results reporting macroaggregate-protected organic matter mineralization within native grasslands or invasive weed monocultures are severely lacking. Our results indicate that macroaggregate-protected organic matter mineralization did not significantly change as a result of soil fertility increases within the *F. idahoensis* treatments (Table 5.2). It is possible that the increased mineral N added to *F. idahoensis* soils favored rapid labile carbon mineralization, resulting in reduced amounts of labile carbon being protected within macroaggregates. Aoyama et al., (1999) found that macroaggregates contained smaller amounts of labile carbon as a result of fertilization. They attributed this observation to increased labile carbon mineralization resulting from fertilizer additions. In contrast, our results suggest that macroaggregate-protected organic matter mineralization significantly increases as a result of increased soil fertility within the *H. caespitosum* soils (Table 5.2). A potential hypothesis that explains this observation is that the addition of fertilizer slows available carbon mineralization within *H. caespitosum* treatments, and results in an increase in labile carbon reserves, which were then incorporated into macroaggregates. The *H. caespitosum* control treatments provide further evidence which supports this observation. The similarity between mineralization
rates in both available and macroaggregate-protected treatments (Table 5.2) suggests that labile carbon pools were mineralized prior to macroaggregate incorporation. Results from Saggar et al., (1999) lend support to this observation as they suggest that *H. caespitosum* increases carbon mineralization, increasing nutrient cycling as a mechanism of invasion.

**AMF colonization and hyphal lengths**

Our results further suggest significant changes in AMF biomass occurred as a result of exotic plant invasion and increased soil fertility. We found that AMF extraradical hyphal lengths significantly decreased as a result of herbicide application and the interaction of herbicide and fertilizer in *F. idahoensis* soils, yet we observed an opposite trend in *H. caespitosum* treatments. The existence of invasive plant species (Mummey & Rillig 2006) as well as soil N: P ratios (Johnson et al., 2003), can alter AMF hyphal presence. In the *F. idahoensis* treatments the functional equilibrium model provides the most parsimonious explanation for the reduction of AMF. Fertilizer addition improved the soil nutrient status in *F. idahoensis* treatments. Since nutrients were no longer limiting, less carbon was allocated below ground essentially reducing root biomass, length, (Table 5.3) and AMF hyphae (Fig 5.2 A). However, in the *H. caespitosum* treatments we observed a steady state in AMF hyphal lengths as fertilizer addition increased (Fig 5.2 A). One potential hypothesis would suggest that *H. caespitosum* host specific AMF species that produce greater lengths of hyphae and are not influenced by nitrogen additions. Scheublin et al., (2004) found that *Hieracium pilosella* roots maintain low AMF diversity, hosting communities mostly composed of the genus *Glomus*. *Glomus intraradices* abundance was found to increases after N and P fertilization (Emo et al.,
Furthermore, *Glomus intraradices* are fungi maintaining a generalist life history resulting in ecological versatility (Börstler et al., 2008). We observed no significant differences in hyphal (Fig. 5.2 A), vesicle or arbuscular colonization (data not shown) between herbicide and fertilizer treatments. Since AM fungal structures are dynamic fluctuating as a result of seasonal and annual changes (Johnson et al., 2003) and we sampled at one time-point, we did not capture the potential variability of these fungal structures.

**Non-mycorrhizal fungi**

Belnap & Phillips (2001) suggest that exotic plant invasion increases the presence of saprophytic fungi coupled with a decrease in specialized fungi such as AMF. Our results indicate that the addition of fertilizer significantly increased non-AMF hyphal lengths in both *H. caespitosum* and *F. idahoensis* high fertilizer treatments (Fig 5.2 B). This result is supported by the trend towards increased root colonization also observed in high fertilizer treatments (Fig 5.2 B). Our findings are contrary to the results of several studies which have applied multiple sampling strategies in order to assess the fungal community in grassland soils after exotic plant invasion (Bittman et al., 2005, Klein et al., 2006). In order to gain greater understanding of the dynamic nature of the non-mycorrhizal fungal community in Hawkweed soils, future studies should employ multiple sampling efforts, assessment of total and active hyphal lengths, and biochemical makers such as phospholipids and ergosterol.
Fertilizer effects on invasion mechanisms

Scott et al., (1990) found that additions of fertilizer to moderately productive soils limited Hawkweed invasion, however, the mechanisms responsible for this observation have not been identified. Results from our investigation provide evidence that supports two potential mechanisms. First addition of fertilizer may be altering the activity of certain soil enzymes. Alteration of soil enzymes as a result of fertilizer addition has been reported in several studies (Ajaw et al., 1999, Kandeler et al., 1999, Marschner et al., 2003). Our data indicate that high fertilizer additions increased carbon mineralization in F. idahoensis treatments, yet reduced mineralization in the H. caespitosum soils (Table 5.2 and Fig. 5.1 A, B). It is possible that the application of fertilizer altered the quality of soil organic matter inputs in the F. idahoensis treatments; this in turn increased enzyme activity and resulted in greater nutrient turnover. Secondly our results support a potential AMF invasion mechanism. According to van der Heijden et al., (1998) H. pratense is highly dependent on AMF, and maintains a mycorrhizal dependency of 0.98. Our data indicate that high fertilizer additions reduced AMF lengths under F. idahoensis. It is possible that the application of fertilizer to plants with low mycorrhizal dependency (i.e. F. idahoensis) may reduce AMF abundance to a level insufficient to support H. caespitosum growth/invasion.

Soil structure
Mechanisms influencing mean weight diameter

Several biotic factors such as plant roots and fungal mycelium influence aggregate stability by providing the binding force that stabilizes aggregates. The balance between
organic matter inputs and the rate at which organic matter is mineralized significantly influences aggregate formation and stability (Trujillo et al., 1998). We constructed a correlation matrix in order to evaluate how increased soil fertility influences the potential relationships between measured biotic parameters and slaked and slow wetting MWD with respect to *H. caespitosa*um and *F. idahoensis* soils. Root parameters and fungal hyphal lengths did not significantly influence slaked MWD within *H. caespitosa*um treatments (Table 5.5). In contrast our results suggest a positive correlation between AMF hyphae and slaked MWD within *F. idahoensis* treatments (Fig. 5.4 A). Several previous studies have shown positive correlations between macroaggregate stability and AMF hyphae (Miller & Jastrow 2000, Rillig et al., 2001, 2002). Additionally our results suggest that a reduction of AMF hyphae as a result of fertilization reduced MWD (Fig. 5.5 A). Wilson et al., (2009) reported a similar finding suggesting that AMF hyphal loss due to fungicide application resulted in a reduction of macroaggregate stability. We also observed a negative correlation between saprophytic hyphal lengths and slaked MWD (Fig. 5.4 B). This result is contrary to the findings of several studies that indicate that saprophytic fungi are influential in aggregate stability (Tisdall et al., 1997, Caesar-TonThat & Cochran 2000). A possible explanation for this result is that the addition of fertilizer reduced AMF hyphae lengths (Fig 5.2 A) and increased labile organic matter production (Table 5.2); the resulting conditions favored saprophytic fungal growth that is not capable of supporting greater MWD capable of resisting slaking forces. This explanation is consistent with the path analysis conducted by Rillig et al., (2002); their results demonstrate that AMF are highly influential in regards to aggregate stability.
Mechanical disruption of aggregates via slaking occurs when water rapidly enters an aggregate causing entrapped air to explosively release; this action results in aggregate swelling and disintegration (Kay & Angers 1999, Lado et al., 2004). Le Bissonnais & Arrouays (1997) suggest that soils with high organic matter concentrations will resist slaking forces. We observed that increases in both soil organic matter as well as intact carbon mineralization were negatively correlated with slaked MWD within the *F. idahoensis* treatments (Fig 5.4 C, D). Additionally in the *H. caespitosum* treatments we observed a positive correlation between slaked MWD and intact carbon mineralization (Fig 5.5 A), yet a negative correlation was observed between macroaggregate protected carbon mineralization (Fig 5.6 B). The correlations we observed in both the *F. idahoensis* and *H. caespitosum* soils were driven by high fertilizer addition treatments; our results suggest that fertilizer addition influence the rate of mineralization impacting the quality of soil organic matter which in turn affects aggregate stability. Amézketa (1999) reviewed several studies suggesting that the quality of the soil organic matter pool has a greater impact on stabilizing aggregates than total soil organic matter concentrations. Martens (2000) investigated the biochemistry of several organic residues and assessed their rate of decomposition in relation to aggregate stability. His results indicate that organic matter composed of carbohydrates and amino acids resulted in rapid aggregate formation with limited long term stability, however recalcitrant organic matter such as phenolic acids resulted in slower aggregate formation with greater long term stability.
Slow wetting is a method of aggregate disruption that weakens cementing forces (i.e. clay and organic matter) between particles inside the aggregate (Ghezzehei & Or 2000). According to Lado et al., (2004) slow wetting is an effective method for determining interior aggregate stability factors that are not often identified via slaking. We observed no significant binding or cementing mechanism in the *H. caespitosum* treatments, which could explain slow wetting MWD (Table 5.6). In the *F. idahoensis* we observed a negative correlation between organic matter as well as available carbon mineralization in response to slow wetting MWD (Table 5.6 and Fig 5.6 A, B), yet a positive correlation was observed between protected organic matter mineralization and slow wetting MWD (Table 5.6 and Fig 5.6 C). These findings are similar to the results reported for the slaked MWD treatments, suggesting that high fertilizer treatments are influencing the correlation values. This observation lends further support to the hypothesis that fertilization is affecting carbon quality and MWD resistance to slaking and slow wetting forces in *F. idahoensis* soils.

**Conclusion**

We have shown that *H. caespitosum* invasion alters several soil characteristics (i.e. SOM, carbon mineralization, and AMF hyphal lengths) in comparison to native *F. idahoensis* soils. Application of herbicide and fertilizer to *F. idahoensis* soil increased SOM and carbon mineralization rates as well as decreasing AMF hyphal lengths, alteration of these factors possibly limited the further spread of Hawkweed. Since the abundance of AMF hyphae, organic matter, and the rate of mineralization were altered as a result of *H. caespitosum* invasion and soil fertility, we observed further changes in aggregate
distribution as well as decreases in MWD. This shift in aggregate distribution could change the potential of native soils to cycle and sustain nutrients, maintain water holding capacity, as well as altering microbial habitats. Additionally our results suggest that Hawkweed invasion does not deplete soil nutrient reserves which is typically the case observed with other invasive plants. Future research efforts should include investigations assessing the nutrient status of H. caespitosum biomass, potential differences in nutrient cycling, as well as the quality of SOM. Additionally alteration of symbiotic/pathogenic fungi could offer interesting insight into potential invasion mechanisms of H. caespitosum.

Acknowledgements

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### Table 5.1 Soil characteristics assessed from *F. idahoensis* and *H. caespitosum* amended soils

<table>
<thead>
<tr>
<th></th>
<th><em>F. idahoensis</em></th>
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</tr>
</thead>
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<tr>
<td></td>
<td>No Low High</td>
<td>No Low High</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>pH</td>
<td>5.07 (0.05) 5.13 (0.02) 5.10 (0.07)</td>
<td>5.00 (0.04) 4.95 (0.09) 4.97 (0.03)</td>
<td></td>
<td></td>
<td>7.94 (0.011)</td>
<td></td>
<td>0.01 (0.91)</td>
<td></td>
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<tr>
<td>CECa</td>
<td>10.5 (0.33) 10.6 (0.33) 10.7 (0.44)</td>
<td>9.88 (0.19) 10.0 (0.46) 10.3 (0.21)</td>
<td></td>
<td></td>
<td>3.33 (0.08)</td>
<td></td>
<td>0.39 (0.68)</td>
<td></td>
</tr>
<tr>
<td>OMb</td>
<td>3.0 (0.04)a 3.3 (0.09)b 3.9 (0.06)d</td>
<td>3.70 (0.07)c 3.90 (0.12)d 4.00 (0.13)d</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001 (0.001)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pc</td>
<td>43.9 (10.4) 38.4 (5.32) 39.8 (2.84)</td>
<td>38.6 (3.95) 36.1 (3.97) 42.3 (9.01)</td>
<td></td>
<td></td>
<td>0.10 (0.76)</td>
<td></td>
<td>0.24 (0.79)</td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td>1898 (64.4) 1895 (173) 1919 (125)</td>
<td>1815 (90.4) 1918 (90.1) 1934 (102)</td>
<td></td>
<td></td>
<td>0.03 (0.87)</td>
<td></td>
<td>0.21 (0.82)</td>
<td></td>
</tr>
<tr>
<td>Mg</td>
<td>244 (7.10) 239 (23.8) 241 (8.71)</td>
<td>220 (10.8) 248 (9.21) 243 (13.7)</td>
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<td></td>
<td>0.20 (0.66)</td>
<td></td>
<td>0.43 (0.67)</td>
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</tr>
<tr>
<td>Kc</td>
<td>254 (31.1) 234 (15.8) 242 (14.3)</td>
<td>217 (15.5) 270 (25.4) 252 (27.7)</td>
<td></td>
<td></td>
<td>0.03 (0.87)</td>
<td></td>
<td>0.29 (0.75)</td>
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</tr>
<tr>
<td>NO3-d</td>
<td>0.40 (0.08) 0.25 (0.12) 0.45 (0.18)</td>
<td>0.28 (0.08) 0.35 (0.12) 0.30 (0.07)</td>
<td></td>
<td></td>
<td>0.40 (0.53)</td>
<td></td>
<td>0.40 (0.80)</td>
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<tr>
<td>NH4+d</td>
<td>17.0 (6.9) 9.80 (1.1) 13.1 (5.3)</td>
<td>9.13 (0.95) 13.9 (4.98) 12.3 (4.87)</td>
<td></td>
<td></td>
<td>0.15 (0.70)</td>
<td></td>
<td>0.04 (0.96)</td>
<td></td>
</tr>
</tbody>
</table>

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*meq/100g*

*%*

*kg ha⁻¹*

*µg g⁻¹*
Table 5.2 Total C mineralized from available and crushed macroaggregates (<250 µm)

<table>
<thead>
<tr>
<th></th>
<th>Available</th>
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<th>Protected</th>
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<tbody>
<tr>
<td></td>
<td>F. idahoensis</td>
<td>H. caespitosum</td>
<td>F. idahoensis</td>
<td>H. caespitosum</td>
</tr>
<tr>
<td>No</td>
<td>1.24 (0.01) bb</td>
<td>1.27 (0.02) ab</td>
<td>1.33 (0.01) aa</td>
<td>1.27 (0.01) bb</td>
</tr>
<tr>
<td>Low</td>
<td>1.26 (0.03) abb</td>
<td>1.25 (0.01) abb</td>
<td>1.31 (0.02) abaa</td>
<td>1.30 (0.01) ba</td>
</tr>
<tr>
<td>High</td>
<td>1.28 (0.02) ab</td>
<td>1.23 (0.01) bb</td>
<td>1.32 (0.01) aa</td>
<td>1.35 (0.02) aa</td>
</tr>
<tr>
<td>Herbicide</td>
<td>20.9 (&lt;0.001)</td>
<td>0.01 (0.904)</td>
<td></td>
<td></td>
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<tr>
<td>Fertilizer</td>
<td>0.880 (0.432)</td>
<td>8.63 (0.003)</td>
<td></td>
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<tr>
<td>Interaction</td>
<td>38.8 (&lt;0.001)</td>
<td>23.0 (0.001)</td>
<td></td>
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</tbody>
</table>

Bold letters indicate comparison of intact soil carbon mineralization rates between plant species and fertilization treatments.

Underscored letters indicate comparison of protected soil carbon mineralization rates between plant species and fertilization treatments.

Italics letters indicate comparison intact and protected carbon mineralization rates within plant treatments.
<table>
<thead>
<tr>
<th></th>
<th>Shoot&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Root&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Total Root&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Fine Root&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Very Fine Root&lt;sup&gt;c&lt;/sup&gt;</th>
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<td><strong>F. idahoensis</strong></td>
<td></td>
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</tr>
<tr>
<td>No</td>
<td>400 (70) a</td>
<td>4.1 (1.2) b</td>
<td>2.65 (0.80) c</td>
<td>1.30 (0.45) bc</td>
<td>1.11 (0.33) bc</td>
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<td>Low</td>
<td>390 (44) a</td>
<td>14.7 (1.2) a</td>
<td>7.50 (0.25) a</td>
<td>3.75 (0.13) a</td>
<td>3.75 (0.21) a</td>
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<tr>
<td>High</td>
<td>460 (76) a</td>
<td>7.8 (2.0) b</td>
<td>4.50 (0.90) b</td>
<td>2.48 (0.60) b</td>
<td>2.06 (0.46) b</td>
</tr>
<tr>
<td><strong>H. caespitosum</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>270 (13) bc</td>
<td>2.4 (0.6) c</td>
<td>0.90 (0.17) d</td>
<td>0.45 (0.10) d</td>
<td>0.44 (0.10) d</td>
</tr>
<tr>
<td>Low</td>
<td>340 (40) ab</td>
<td>2.2 (0.3) b</td>
<td>1.32 (0.35) cd</td>
<td>0.72 (0.21) cd</td>
<td>0.60 (0.15) cd</td>
</tr>
<tr>
<td>High</td>
<td>260 (11) c</td>
<td>3.6 (0.7) b</td>
<td>1.46 (0.16) cd</td>
<td>0.70 (0.10) cd</td>
<td>0.73 (0.08) cd</td>
</tr>
<tr>
<td>Herbicide</td>
<td><strong>10.6 (0.005)</strong></td>
<td><strong>49.1 (&lt;0.001)</strong></td>
<td><strong>33.7 (&lt;0.001)</strong></td>
<td><strong>39.1 (&lt;0.001)</strong></td>
<td><strong>20.2 (&lt;0.001)</strong></td>
</tr>
<tr>
<td>Fertilizer</td>
<td>0.29 (0.75)</td>
<td><strong>11.6 (0.001)</strong></td>
<td><strong>7.24 (0.001)</strong></td>
<td><strong>10.5 (&lt;0.001)</strong></td>
<td><strong>7.10 (0.006)</strong></td>
</tr>
<tr>
<td>Interaction</td>
<td>1.38 (0.28)</td>
<td><strong>13.6 (&lt;0.001)</strong></td>
<td>1.80 (0.20)</td>
<td><strong>6.00 (0.01)</strong></td>
<td><strong>4.72 (0.02)</strong></td>
</tr>
</tbody>
</table>

<sup>a</sup> Shoot Biomass mg m<sup>-2</sup>

<sup>b</sup> Root Biomass mg g<sup>-1</sup> soil

<sup>c</sup> Root length m g<sup>-1</sup> soil
Table 5.4 Aggregate size class distribution (%) after slaking and rewetting treatments

<table>
<thead>
<tr>
<th>Slaked/rewet</th>
<th>Slaked - F. idahoensis</th>
<th>Slaked - H. caespitosum</th>
<th>F(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>2000 - 250</td>
<td>6.6 (0.3)c</td>
<td>5.5 (0.3)c</td>
<td>6.5 (0.5)c</td>
</tr>
<tr>
<td>250 - 53</td>
<td>46.0 (3.0)</td>
<td>48.0 (3.0)</td>
<td>43.3 (1.9)</td>
</tr>
<tr>
<td>53</td>
<td>24.0 (1.2)</td>
<td>22.1 (1.0)</td>
<td>22.1 (1.4)</td>
</tr>
<tr>
<td>&lt; 53</td>
<td>22.0 (1.7)</td>
<td>21.5 (3.4)</td>
<td>26.0 (2.7)</td>
</tr>
</tbody>
</table>

Rewet

<table>
<thead>
<tr>
<th></th>
<th>No</th>
<th>Low</th>
<th>High</th>
<th>No</th>
<th>Low</th>
<th>High</th>
<th>Herbicide</th>
<th>Fertilizer</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000 - 250</td>
<td>5.8 (0.9)c</td>
<td>4.3 (0.1)d</td>
<td>6.7 (0.6)c</td>
<td>12.0 (2.0)a</td>
<td>9.5 (1.5)b</td>
<td>12.0 (1.7)ab</td>
<td>22.1 (0.002)</td>
<td>1.62 (0.07)</td>
<td>0.03 (0.97)</td>
</tr>
<tr>
<td>250 - 53</td>
<td>39.3 (1.9)</td>
<td>37.1 (1.0)</td>
<td>33.7 (1.6)</td>
<td>40.0 (1.0)</td>
<td>37.4 (2.0)</td>
<td>37.6 (1.3)</td>
<td>1.54 (0.23)</td>
<td>3.22 (0.07)</td>
<td>0.84 (0.45)</td>
</tr>
<tr>
<td>53</td>
<td>16.3 (0.7)</td>
<td>16.5 (0.7)</td>
<td>17.6 (0.9)</td>
<td>16.4 (1.6)</td>
<td>16.3 (0.9)</td>
<td>17.0 (1.4)</td>
<td>0.04 (0.84)</td>
<td>0.35 (0.71)</td>
<td>0.09 (0.93)</td>
</tr>
<tr>
<td>&lt; 53</td>
<td>33.4 (3.0)</td>
<td>31.7 (2.9)</td>
<td>38.0 (1.6)</td>
<td>27.1 (1.6)</td>
<td>33.6 (1.5)</td>
<td>28.0 (3.0)</td>
<td>6.34 (0.02)</td>
<td>0.79 (0.50)</td>
<td>3.42 (0.06)</td>
</tr>
</tbody>
</table>
Table 5.5 Correlation matrix displaying relationships between assessed variables and Slaked Mean Weight Diameter (MWD) *F. idahoensis* and *H. caespitosum* soils

<table>
<thead>
<tr>
<th></th>
<th><strong>F. idahoensis</strong></th>
<th></th>
<th><strong>H. caespitosum</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>r</em></td>
<td><em>F (P)</em></td>
<td><em>r</em></td>
</tr>
<tr>
<td>OM&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.755</td>
<td>11.9 (0.007)</td>
<td>-0.206</td>
</tr>
<tr>
<td>Shoot Biomass&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.250</td>
<td>0.09 (0.76)</td>
<td>-0.311</td>
</tr>
<tr>
<td>Root Biomass&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-0.102</td>
<td>0.598 (0.45)</td>
<td>0.004</td>
</tr>
<tr>
<td>Total Root&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.324</td>
<td>1.03 (0.34)</td>
<td>-0.323</td>
</tr>
<tr>
<td>Fine Root&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.314</td>
<td>0.98 (0.35)</td>
<td>-0.350</td>
</tr>
<tr>
<td>V-Fine Root&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.317</td>
<td>1.01 (0.34)</td>
<td>-0.391</td>
</tr>
<tr>
<td>AMF&lt;sup&gt;e&lt;/sup&gt;</td>
<td><strong>0.538</strong></td>
<td><strong>3.68 (0.08)</strong></td>
<td>0.057</td>
</tr>
<tr>
<td>Non-AMF&lt;sup&gt;e&lt;/sup&gt;</td>
<td><strong>-0.821</strong></td>
<td><strong>18.6 (0.002)</strong></td>
<td>0.250</td>
</tr>
<tr>
<td>Intact min&lt;sup&gt;f&lt;/sup&gt;</td>
<td><strong>-0.518</strong></td>
<td><strong>1.65 (0.05)</strong></td>
<td><strong>0.434</strong></td>
</tr>
<tr>
<td>Protected min&lt;sup&gt;f&lt;/sup&gt;</td>
<td>-0.164</td>
<td>0.51 (0.30)</td>
<td><strong>-0.328</strong></td>
</tr>
</tbody>
</table>

<sup>a</sup> (%)  
<sup>b</sup> Shoot Biomass mg m⁻²  
<sup>c</sup> Root Biomass mg g⁻¹ soil  
<sup>d</sup> Root Lengths (m g⁻¹ soil)  
<sup>e</sup> Fungal hyphae (m g⁻¹ soil)  
<sup>f</sup> Soil Carbon Mineralization (mg g⁻¹ soil) after 35 days of incubation  

* Spearmans Correlation  
Bold indicates significance accepted at P < 0.05  
Italics indicates significance accepted at P < 0.1
Table 5.6 Correlation matrix displaying relationships between assessed variables and slow wet Mean Weight Diameter (MWD) *F. idahoensis* and *H. caespitosum* soils

<table>
<thead>
<tr>
<th></th>
<th><em>F. idahoensis</em></th>
<th></th>
<th></th>
<th><em>H. caespitosum</em></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>r</em></td>
<td><em>F (P)</em></td>
<td></td>
<td><em>r</em></td>
<td><em>F (P)</em></td>
<td></td>
</tr>
<tr>
<td>OM&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.715</td>
<td>9.10 (0.01)</td>
<td></td>
<td>-0.240</td>
<td>0.60 (0.46)</td>
<td></td>
</tr>
<tr>
<td>Shoot Biomass&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.392</td>
<td>1.09 (0.32)</td>
<td></td>
<td>-0.084</td>
<td>0.07 (0.79)</td>
<td></td>
</tr>
<tr>
<td>Root Biomass&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-0.402</td>
<td>1.74 (0.21)</td>
<td></td>
<td>-0.310</td>
<td>1.03 (0.33)</td>
<td></td>
</tr>
<tr>
<td>Total Root&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-0.156</td>
<td>0.22 (0.64)</td>
<td></td>
<td>-0.170</td>
<td>0.291 (0.56)</td>
<td></td>
</tr>
<tr>
<td>Fine Root&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-0.093</td>
<td>0.07 (0.79)</td>
<td></td>
<td>-0.213</td>
<td>0.47 (0.51)</td>
<td></td>
</tr>
<tr>
<td>V-Fine Root&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-0.211</td>
<td>0.419 (0.53)</td>
<td></td>
<td>-0.092</td>
<td>0.08 (0.77)</td>
<td></td>
</tr>
<tr>
<td>AMF&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.341</td>
<td>1.13 (0.312)</td>
<td></td>
<td>-0.103</td>
<td>0.102 (0.75)</td>
<td></td>
</tr>
<tr>
<td>Non-AMF&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.382</td>
<td>1.42 (0.28)</td>
<td></td>
<td>0.483</td>
<td>3.19 (0.15)</td>
<td></td>
</tr>
<tr>
<td>Available min&lt;sup&gt;f&lt;/sup&gt;</td>
<td>-0.778</td>
<td>2.44 (0.007)</td>
<td></td>
<td>0.241</td>
<td>0.81 (0.21)</td>
<td></td>
</tr>
<tr>
<td>protected min&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.557</td>
<td>1.75 (0.04)</td>
<td></td>
<td>0.295</td>
<td>0.97 (0.16)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> (%)  
<sup>b</sup> Shoot Biomass mg m<sup>-2</sup>  
<sup>c</sup> Root Biomass mg g<sup>-1</sup> soil  
<sup>d</sup> Root Lengths (m g<sup>-1</sup> soil)  
<sup>e</sup>Fungal hyphae (m g<sup>-1</sup> soil)  
<sup>f</sup> Soil Carbon Mineralization (mg g<sup>-1</sup> soil) after 35 days of incubation  
*Spearmans Correlation  
Bold indicates significance accepted at *P* < 0.05  
Italics indicates significance accepted at *P* < 0.10
Figure 5.1

(A) Relationship between *F. idahoensis* available carbon mineralization and organic matter. Treatments included: No fertilizer (triangles), Low fertilizer (squares), High Fertilizer (circles).

(B) Relationship between *H. caespitosum* available carbon mineralization and organic matter. Treatments included: No fertilizer (triangles), Low fertilizer (squares), High Fertilizer (circles).
Herbicide: $F = 19.2, P = 0.001$
Fertilizer: $F = 0.03, P = 0.98$

Interaction: $F = 4.00, P = 0.04$

Figure 5.2
(A) AMF hyphal lengths determined for *F. idahoensis* and *H. caespitosum* herbicide and fertilizer treatments. Means (±) SE were compared with two-way ANOVA with significance accepted at $P < 0.05$. Numbers inside of bars are percentage AMF root colonization (SE).

(B) Non-AMF hyphal lengths determined for *F. idahoensis* and *H. caespitosum* herbicide and fertilizer treatments. Means (±) SE were compared with two-way ANOVA with significance accepted at $P < 0.05$. Numbers inside of bars are percentage non-AMF root colonization (SE).
Figure 5.3

(A) Slaked Mean Weight Diameter (mm) calculated for *F. idahoensis* and *H. caespitosum* herbicide and fertilizer treatments. Means (+) SE were compared with two-way ANOVA significance accepted at $P < 0.05$.

(B) Slow wetting Mean Weight Diameter (mm) calculated for *F. idahoensis* and *H. caespitosum* herbicide and fertilizer treatments. Means (+) SE were compared with two-way ANOVA significance accepted at $P < 0.05$. 
Figure 5.4

(A) Relationship between *F. idahoensis* AMF hyphal length and slaked MWD.
Treatments included: No fertilizer (triangles), Low fertilizer (squares), High Fertilizer (circles).

(B) Relationship between *F. idahoensis* Non-AMF hyphal length and slaked MWD.
Treatments included: No fertilizer (triangles), Low fertilizer (squares), High Fertilizer (circles).
(C) Relationship between *F. idahoensis* organic matter and slaked MWD. Treatments included: No fertilizer (triangles), Low fertilizer (squares), High Fertilizer (circles).

(D) Relationship between *F. idahoensis* available carbon mineralization and slaked MWD. Treatments included: No fertilizer (triangles), Low fertilizer (squares), High Fertilizer (circles).
Figure 5.5

(A) Relationship between *H. caespitosum* available carbon mineralization and slaked MWD. Treatments included: No fertilizer (triangles), Low fertilizer (squares), High Fertilizer (circles).

(B) Relationship between *H. caespitosum* macroaggregate protected carbon mineralization and slaked MWD. Treatments included: No fertilizer (triangles), Low fertilizer (squares), High Fertilizer (circles).
Figure 5.6

(A) Relationship between *F.idahoensis* organic matter and slow wetting MWD. Treatments included: No fertilizer (triangles), Low fertilizer (squares), High Fertilizer (circles).

(B) Relationship between *F.idahoensis* available carbon mineralization and slow wetting MWD. Treatments included: No fertilizer (triangles), Low fertilizer (squares), High Fertilizer (circles).

(C) Relationship between *F.idahoensis* macroaggregate protected carbon mineralization and slow wetting MWD. Treatments included: No fertilizer (triangles), Low fertilizer (squares), High Fertilizer (circles).
Chapter 6

JUNIOR ENVIRONMENTAL MICROBIOLOGIST TRAINING

Abstract

Investigations involving soil microorganisms provide the perfect venue in which to teach students about several biological processes as well as dispelling the misconception that microbes are only agents of disease. We have designed three investigations aimed at instructing students about microbial habitat, symbiotic relationships with plants and microbial mediated decomposition.

Introduction

What types of microorganisms live within a particular soil? Do these microorganisms increase the growth of certain plants by establishing beneficial relationships providing the plant with a competitive edge? Can the presence of certain microbes speed up litter decomposition (i.e. "rotting") in turn releasing nutrients aiding faster growing plants? These are just some of the many questions that Soil Microbiologists think about as they ponder a gram of soil. Typically soil microbiology focuses on an assortment of very small soil organisms including viruses, bacteria, fungi, and protozoa. However, soil microbiology investigations have also included larger soil organisms such as the nematodes, mites, and other microarthropods.

Soil microbiologists strive to understand several important environmental processes which begin at the soil level such as: nutrient cycling, organic matter decomposition, and soil structure. Why is it important to understand these processes? Knowledge of soil
microbiology helps organic farmers plan crops, by establishing the necessary plant/microbe relationships in order to avoid the need for chemical fertilizers. Awareness of the importance of soil microbiology also aids restoration planners in applying the correct soil amendment which could ultimately restore a damaged ecosystem to its original state. In addition plant ecologists use information regarding soil microorganisms to predict the potential spread of invasive plants. On a global scale, knowledge of how microorganisms influence soil structure may help scientists better understand the potential carbon storage capacity of soil, possibly alleviating global warming. Soil microorganisms even hold the possibility of helping us determine the origins of a planet; for example the mission goal of the Mars Lander Phoenix was to look for life on Mars at the soil level.

Typical curriculum pieces aimed at teaching microbiology topics to middle school students are often centered on food microbiology, antibiotic resistance of microorganisms, and microorganisms as spoilers of food. While understanding these concepts is important, concentrating only on these areas could lead students to the misconception that all microorganisms are agents of disease (Blair & Bowen 1996; Simonneaux 2000; Byrne 2003). Environmental microbiology curriculum pieces designed to expound on the global benefits of microorganisms are becoming available. However these educational units tend to focus primarily on certain aspects of microbiology: isolation (Wagner & Stewart 2000, Farone & Farone 2005) nutrient cycling (Ambler et al. 2001, Rogan et al. 2005) and decomposition (Byington 2001, Brunell 2003).
Our activity “Investigations into Soil Microbiology” includes three inquiry based studies designed to teach the importance and significance of soil microbiology to middle school students. In this set of investigations students will: i) isolate microorganisms from environmental samples, ii) assess microbial/plant symbiosis, and iii) explore microbial decomposition and nutrient cycling. We selected these three inquiries because they provide an essential base for understanding the relevance of soil microbiology from which future question/inquires could be built: isolating microbes that could “eat” oil or determining the physiological characteristic that a bacteria would need to survive on the surface of Mars.

Isolating Microorganisms: Inquiry 1

Teacher Preparation

In advance of this inquiry, prepare the materials listed in Table 6.1. Sterilization of all necessary equipment can be easily completed by wrapping materials in aluminum foil and heating to 121° C or 260° F in a conventional oven. Agar plates can be purchased from Carolina Biological Supply (catalogue item: LB agar plates), or for an inexpensive alternative Blair & Bowen (1996) developed a recipe for agar plates using common household items. Several web sites offer excellent tutorials on isolation techniques as well as facts about microorganisms (Table 6.2 internet resources).

Inquiry 1: where do microorganisms live?

In order to get students excited about microorganisms, discuss what students know about bacteria and fungi. Most likely students will suggest that bacteria cause diseases, and
fungi are spoilers of food, (as most of them will have come across the fungal ridden remnants of lunches gone by). Listed are a few questions that might help get the conservation started:

- **How big is a bacterial cell?** ("very small; typically a single cell is not visible by the naked eye; approximately 0.1 µm in size")

- **Are bacteria and fungi the same?** ("No, bacteria are simple, single celled organisms and typically fungi have multiple cells and grow as long strands called mycelium, also fungi are much larger than bacteria")

- **Where are bacteria and fungi found?** ("On almost every surface imaginable, from the highest mountain to the deepest ocean")

- **What do bacteria and fungi need in order to survive?** ("Water, nutrients, and oxygen [Not Always! that is a topic we will cover later]")

- **How are bacteria and fungi eliminated from an area?** ("Through the use of cleansers such as bleach or alcohol; hospitals use a device called an autoclave which produces high heat and pressure to rid surgical instruments of microorganisms")

- **How do microbiologists isolate microorganisms?** ("Microbiologists often culture microorganisms via agar, which for all practical purposes is clear jello")

- **When you view an inculcated agar plate are you seeing one bacterial cell?** ("No you are seeing thousands of bacterial cells growing in a colony")

**Key concepts for students to gain from this introductory lesson and discussion:** i) bacteria and fungi are everywhere, ii) general ideas about the differences between fungi and bacteria, and iii) growth requirements of bacteria and fungi. Additionally, students should
begin to realize that not all microorganisms are agents of disease. Another key concept that students may also begin to understand is that microorganisms are critical to several ecosystem process.

After completing the discussion about microorganisms show students the equipment they will use to isolate microorganisms and begin their Junior Microbiologist training. We found it helpful to pass the equipment around the room as we described how to take an environmental sample. Additionally, we had cultured plates on display which helped the students get an idea of what the inquiry would look like when complete.

After the students had heard the discussion on isolating microorganisms, understood the investigation, and asked all pertinent questions, they were then instructed to explore the school yard in search of a good sampling site. This took approximately 10-15 minutes. Upon completion of this task, students then began the assignment of sampling and preparing their plate for incubation, following the procedure outlined in Table: 6.3. Next the students were asked to descied where their samples were taken and answer the following predictions: i) how many colonies would grow on their plate, ii) would they have more fungal than bacterial colonies, and iii) what criteria would they use to differentiate specific colonies of microorganisms. Additionally students were asked to develop one original question they could answer based on data collection and observations. During the incubation period students can monitor their plates for several days while keeping notes of how their plates have changed. Students can count the number of different colonies formed on their plates and compare their results with colony
counts from other plates. This information was recorded in students' "microbial investigations notebook".

At the end of the microbial isolation inquiry setup, students reconvened and were asked to describe where they took their sample and why. Also students were asked to answer each of the preselected questions, which enhanced the general conversation regarding the inquiry. The majority of our students felt that samples taken from either plants or soil would produce plates with more bacterial and fungal colonies than samples from other locations, because these areas maintained more of the necessary requirements for microbial growth. Some of our students felt that samples taken from building doorknobs, benches, and paved surfaces would have a greater number of bacterial and fungal colonies due to constant human contact.

Upon completion of our investigation students discovered that plates with the most diversity and largest colony number were samples taken from outdoors. These observations lead to a discussion as to why certain plates contained more colonies of microorganisms. This conversation was supplemented with a general discussion of factors controlling microbial biodiversity including water, oxygen, temperature and nutrient availability (see internet resources). This inquiry illustrates microbes are a diverse groups of organisms which can be found in unexpected areas. As a safety precaution plates should remain closed during the course of the investigation as there is the slight possibility of culturing a pathogenic microorganisms ($\leq 1\%$). We found that
taking digital pictures of the plates and requiring students to post them in their notebooks was a well received option.

**Plant/microbe symbiosis: Inquiry 2**

**Teacher Preparation**

From the previous inquiry students should have a good foundational understanding of microbiology. This inquiry i) builds on the knowledge from the first inquiry that microorganisms are everywhere and ii) introduces students to *Rhizobium* legume symbiosis. Students explore symbioses by observing *Rhizobium* nodule formation on legumes. Students will determine if commercial *Rhizobium* inoculants are more successful at root colonization than non-treated garden soil. Most of the materials necessary for this inquiry are common household items (Table 6.1): growth containers-plastic cups, grow lights-sunny window sill. Legume seed can be purchased at any garden store, as can the *Rhizobium* inoculum (manufactured by Nitragin Inc). In advance of this inquiry we found it useful to pre-sterilize the legume seed coats (submerge the seeds in 10% bleach solution for 2 min. then rinse with tap water) as well as to pre-germinate the legume seeds within a damp paper towel wrapped in aluminum foil. The motivation behind these steps is to eliminate potential microbial colonies from the seed coat which could influence the results of the experiment; pre-germinating the seeds allows students to pick actively growing plants and also provides greater accuracy in biomass measurements. Soil for this inquiry was collected from a common garden area, and plant debris and stones were removed by sieving. Additionally half the soil was heat sterilized at 121° C or 260° F for 1 hour to provide test soil for our *Rhizobium* inoculum treatment.
Inquiry 2: do microorganisms help plants grow?

In order to get students thinking about plant/microbe symbiosis, discuss with students what they know about this relationship. Most students should be aware of some classic symbiotic relationship: clownfish/anemones, sharks/remoras, fungi/algae (i.e. lichens) or quite possibly termites/bacteria. Here are a few questions that should aid the conservation in getting started:

- What is symbiosis? ("A relationship between two different organisms where both benefit")
- Does symbiosis always benefit both partners? ("Not always, in some relationships one member will benefit at the expense of the other; this is referred to a parasitic relationship")
- What is Rhizobium? ("A bacteria that forms a symbiotic relationship with plants known as a legume")
- What is legume? ("Plant that harbors nitrogen fixing bacteria on its roots: soybean, pea and bean")
- How does the plant benefit from a relationship with Rhizobium? ("Rhizobium takes atmospheric nitrogen [N₂] and converts it to a plant accessible form [NH₄⁺], the plant uses this source of nitrogen to construct essential biochemical molecules: DNA, carbohydrates, and enzymes")
- What does the Rhizobium gain from the relationship? ("Oxygen free [anaerobic] environment required for growth, and food in the form of carbon from photosynthates")
• How can you tell a legume root is harboring *Rhizobium*? ("Legume roots harboring *Rhizobium* will have bumpy texture as a result of nodule formation")

• How does the *Rhizobium* find the plant root? ("Scientists believe that both the plant and the *Rhizobium* emit a series of complex biochemical signals which aid in recognition and location of bacterial colony")

• How does the root nodule form? ("Once the bacterial signal is received by the plant the root hair forms a pocket that allows colonization. Bacteria enter the plant and a nodule is formed. The atmosphere within the nodule is anaerobic as the enzymes necessary to convert N\textsubscript{2} to NH\textsubscript{4}\textsuperscript{+} will only function under anaerobic conditions")

Upon completing the discussion about symbiosis students were shown a display of all the materials they would need in order to perform this inquiry. We found it helpful to have one experiment containing an actively growing plant. Also we had pictures of legume colonized roots we had found via internet sources.

Once students understood the goal of the investigation and had asked all necessary questions, they were provided the materials to set up the garden soil non-*Rhizobium* treatment following the procedure outlined in Table 6.4. We conducted this treatment first in order to avoid any cross contamination potential with the *Rhizobium* incolum. Next students were provided the necessary materials to setup the *Rhizobium* treatment. After the experiment was established the students were asked to make the following predictions: i) which treatment would contain more *Rhizobium* nodules, ii) which
treatment would contain the larger plants, and iii) what criteria would they use to assess these questions. Students were asked to develop one original question they could answer based on data collection and observations. Students monitored their plants for several weeks, taking measurements of plant height, recording watering amounts, and observing general plant characteristics. This information was recorded in students “microbial investigations notebook”.

At the end of the plant/microbe symbiosis investigation setup, students were asked to discuss the answers they provided to each question. The majority of students felt that plants inoculated with *Rhizobium* would have the greatest number of root nodules, and maintain significantly greater shoot biomass. This theory was based on the idea that the plants were receiving a huge dose of *Rhizobium* bacteria. However, a smaller group of students felt that both treatments would maintain equal number of root nodules as well as biomass. They based this idea on the first inquiry that microorganisms are everywhere. Additionally a group of students felt we should take weekly measurements of shoot growing during the experimental phase.

Upon conclusion of this inquiry students counted the number of nodules on each root and measured shoot biomass, and height as well as root biomass for each treatment. At this point we were able to integrate math related concepts into the inquiry: averages, and use of bar graphs. When we analyzed our data we concluded that no significant differences occurred between our non-treated garden soil and our sterilized soil spiked with *Rhizobium*. Our results suggest that the administration of commercial inoculants did not
provide a significant benefit when compared to garden soils. Results from this inquiry lead to additional investigation ideas such as comparing the use of the Rhizobium inoculated soils to disturbed soils such as those recovered from a walking path. The student felt that soils devoid of vegetation should have reduced microbial populations.

Microbial mediated decomposition, Inquiry 3

Teacher preparation

At this point students should be aware that microorganisms can provide beneficial services to plants but what about global services provided by microorganisms? This inquiry introduces students to the role of microorganisms as decomposers and nutrient recyclers. Students will investigate; i) the efficiency of anaerobic vs. aerobic decomposition and ii) how the chemical complexity of an organic material will determine its potential decomposition via microorganisms. The materials necessary for this study are inexpensive, household items (Table 6.1). The miniature composters were 1 liter canning jars; these could easily be substituted with any type of glass jar and accompanying lid. The soil for this investigation was collected from a common garden plot, and was not heat sterilized as an active microbial community is needed for decomposition. In advance of this inquiry we found it useful to collect organic material from the school grounds (i.e. leaves, and sticks). Additional easily degradable organic material was collected from uneaten lunch leftovers. A small fraction of students readily enjoyed the idea of watching their lunch “rot”.
Inquiry 3: do microorganisms recycle?

In order to get students excited about microbial mediated decomposition ask students what they think about this process. Typically microorganisms are considered spoilers of food rather than ecosystem recyclers. In addition, most students will have the misconception that when an organic compound decays it disappears or is just broken into smaller and smaller pieces (Leach et al. 1992; Hogan and Fisherkeller, 1996; Grotzer and Basca 2003). Here are a few questions that should aid the conservation in getting started:

- What is decomposition? ("Decomposition is the chemical and physical breakdown of organic matter into primary components [e.g. carbon, oxygen (CO₂) and nitrogen], this process is mediated by microorganisms")
- What happens to the material that is degraded by microorganisms? ("Some of the organic matter is released as a gas, CO₂, some of the organic matter is converted to simpler compounds [i.e. proteins become amino acids], and some of the organic matter is incorporated into the body of the bacteria")
- Can other organisms use organic matter degraded by microorganisms? ("YES microbial decomposition release nutrients that are then used by higher organisms such as plants")
- Can microorganisms serve as a food source? ("YES microorganisms serve as the foundation of the soil food web. Microorganisms become a food source of larger soil organisms (e.g. protozoa, nematodes, and arthropods) creating the decomposer food chain")
- Do all materials degrade at equal rates? ("NO the chemical complexity of an organic material will govern how quickly it is degraded by microorganisms")
• Why do certain organic materials degrade faster? (Materials composed of sugars, starch and even cellulose will rapidly decay [days-months]; this is because several populations of microorganisms maintain specific enzymes (proteins) that can degrade this type of organic material.)

• Why do certain organic materials degrade slower? ("Lignin, a component of wood, decomposes very slowly because only certain groups of specialist microorganisms maintain the enzymes required to decompose lignin. These microorganisms tend to grow very slowly because of the large amount of energy required to produce the necessary enzymes for wood decomposition.")

• What does anaerobic mean? ("The ability to live and grow where there is no oxygen. Bacteria are the only known organism capable of this growth form.")

After completing the discussion on microbial decomposition students were shown how to construct their miniature composters. Students were provided with two jars; one would serve as the aerobic (holes placed in the lid) and the second is their anaerobic composter. In order to provide students with an example of composter construction we built one prior to the investigation. At this point we also found it helpful to discuss the use of the digital scale which students would use to record pre-composted and post-composted weights of their organic materials.

Once students understood the purpose of the investigation, they were provided with the necessary materials, and the experimental set-up followed the protocol outlined in Table 6.5. After the investigation was established the students were asked to make the
following predictions: i) would organic materials in the aerobic composter decompose faster then the anaerobic composter [explain your answer]? ii) which materials would degrade the fastest? and iii) what mathematical formula could be used to determine the amount of material decomposed over the incubation period. Students monitored their composters for several weeks, recording observations (presence vs. absence of organic material), smell, and presence of fungal mycelium (i.e. green bread mold). This information was recorded in students “microbial investigations notebook”.

Upon conclusion of the microbe/decomposition inquiry set-up students were asked to discuss their predictions. About half the class felt that material in the anaerobic composter would degrade faster; they felt this treatment was representative of landfill conditions. Further questioning of students’ ideas on this concept revealed the misconception that “materials are buried in a landfill so they degrade quicker”. Approximately half of the remaining class felt that material in the aerobic composter would degrade quicker. Several of these students had backgrounds in both organic farming and composting. This extensive questioning led to an in depth discussion about landfills, recycling, and composting of food waste.

At the conclusion of our inquiry students opened their aerobic composter and began the task of indentifying the remains of their organic material. Once they completed this task they weighed the remaining material and recorded all pertinent information. Next the students opened their anaerobic composter: note their will be a strong odor emanating from this treatment. Again students record weights of remaining material as well as any
interesting observations. Next students calculated the percentage of weight lost from each material using a formula we derived at the beginning of the inquiry \( \frac{\text{initial weight (g) - remaining weight (g)/initial weight (g)}}{\ast \ 100} = \text{amount of material degraded (\%)} \). Also we were able to integrate several of the same math concepts introduced from the second inquiry (averages and bar graphs).

Results from this inquiry indicate that organic materials within the aerobic composter degraded faster than the anaerobic composter. At this point we were able to discuss why materials do not degrade in a landfill since the anaerobic composter is very similar to conditions we would find at a landfill. Basically landfills are built on the principle of excluding water and oxygen to avoid the leakage of waste into ground water. The buried material remains intact with decomposition occurring very slowly. In contrast, most of the easily degradable material (fruit, bread, vegetables, and leaves) in our aerobic composter was reduced rather quickly, while more recalcitrant materials (wood) remained. Based on these results we were then able to speculate on where the material went, and how the decomposition of organic materials is a type of natural recycling.
Assessment

In order to determine the effectiveness of these inquiries in changing students’ misconceptions about microorganisms we performed pre/post assessment (Figure 6.1). The students were asked to either draw or use words to describe what factors (above and below ground) they thought were important for plant growth and survival. Results from the pre-assessment indicate that none of the students thought microorganisms would be important factors in plant growth/survival. However, in 72% of the post assessment drawings students indicated that microorganisms were important in plant growth/survival. Before we began this series of inquiries there was the distinct concern that student misconceptions of microorganisms would significantly lessen their enthusiasm for these investigations. However, this distress was quickly diminished as the students became actively engaged in the first inquiry and discussion, this interest increased throughout the remaining inquiries. Finally the majority of supplementary discussions were actually the result of student questions and independent research.

References


Tables

Table: 6.1 Materials required for selected inquiries.

<table>
<thead>
<tr>
<th>Inquiry</th>
<th>Materials</th>
<th>Instructions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Agar Plates</td>
<td>General Isolation Agar</td>
</tr>
<tr>
<td>1</td>
<td>Sterile Swabs</td>
<td>Wrapped in aluminum foil heated to 120° C or 250° F</td>
</tr>
<tr>
<td>1</td>
<td>Sterile water</td>
<td>Water heated to boiling placed in sterile container</td>
</tr>
<tr>
<td>1</td>
<td>Sharpie</td>
<td>Marking plates</td>
</tr>
<tr>
<td>1</td>
<td>Tape</td>
<td>Sealing plates</td>
</tr>
<tr>
<td>1</td>
<td>Dark warm area</td>
<td>Incubating plates</td>
</tr>
<tr>
<td>2</td>
<td>Soil</td>
<td>Collected from garden or lawn.</td>
</tr>
<tr>
<td>2</td>
<td>Seed</td>
<td>Legume seed (bean, pea or clover).</td>
</tr>
<tr>
<td>2</td>
<td>10% Bleach solution</td>
<td>Seed Sterilization.</td>
</tr>
<tr>
<td>2</td>
<td>Plastic cups</td>
<td>Growing containers.</td>
</tr>
<tr>
<td>2</td>
<td>Growth light</td>
<td>Plants will require 12-14 hours of daylight.</td>
</tr>
<tr>
<td>2</td>
<td>Garden inoculant</td>
<td>Legume inoculant manufactured by Nitragin Inc.</td>
</tr>
<tr>
<td>3</td>
<td>Soil</td>
<td>Collected from garden or lawn.</td>
</tr>
<tr>
<td>3</td>
<td>2 quart Ball jar</td>
<td>Function as a miniature composter.</td>
</tr>
<tr>
<td>3</td>
<td>Organic material</td>
<td>bread, vegetables, fruits, leaves (green and brown), wood, and paper</td>
</tr>
<tr>
<td>3</td>
<td>Aluminum foil</td>
<td>Cover ball jars</td>
</tr>
<tr>
<td>Inquiry</td>
<td>Description</td>
<td>Resource Reference</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------</td>
<td>--------------------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>Agar Plates</td>
<td>www2.carolina.com (LB agar plates)</td>
</tr>
<tr>
<td>1</td>
<td>Isolation Technique</td>
<td>.bioed.org/ECOS/inquiries/Micro_Isolation_inquiry.pdf</td>
</tr>
<tr>
<td>1</td>
<td>Microbe habitats</td>
<td><a href="http://www.pbs.org/opb/intimatestrangers">www.pbs.org/opb/intimatestrangers</a></td>
</tr>
<tr>
<td>1</td>
<td>Review/Terms</td>
<td>helios.bto.ed.ac.uk/bto/microbes/#The</td>
</tr>
<tr>
<td>1</td>
<td>Review</td>
<td><a href="http://www.microbeworld.org">www.microbeworld.org</a></td>
</tr>
<tr>
<td>1</td>
<td>Definitions</td>
<td><a href="http://www.bact.wisc.edu/Bact100/Effects.html">www.bact.wisc.edu/Bact100/Effects.html</a></td>
</tr>
<tr>
<td>1</td>
<td>Nitrogen Fixation</td>
<td>edis.ifas.ufl.edu/SS180</td>
</tr>
<tr>
<td>2</td>
<td>Biological Nitrogen</td>
<td><a href="http://www.soils.wisc.edu/~barak/soilsience326/nitrogen.htm">www.soils.wisc.edu/~barak/soilsience326/nitrogen.htm</a></td>
</tr>
<tr>
<td>2</td>
<td><em>Rhizobia</em> symbiosis</td>
<td>users.rcn.com/jkimball.ma.ultranet/BiologyPages/N/NitrogenFixation.htm</td>
</tr>
<tr>
<td>2</td>
<td>Nodule Picture</td>
<td>forages.oregonstate.edu/nfgc/topics.cfm?ID=187</td>
</tr>
<tr>
<td>3</td>
<td>Aerobic vs anaerobic</td>
<td><a href="http://www.rivenrock.com/composttypes.htm">www.rivenrock.com/composttypes.htm</a></td>
</tr>
<tr>
<td>3</td>
<td>Role of microbes in decomposition</td>
<td><a href="http://www.globalchange.umich.edu/globalchange1/current/lectures/kling/microbes/microbes.html">www.globalchange.umich.edu/globalchange1/current/lectures/kling/microbes/microbes.html</a></td>
</tr>
</tbody>
</table>
Table: 6.3 Methods of culturing microorganisms inquiry

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>All materials should be sterilized at 120°C or 260°F. Isolation kits can be used.</td>
</tr>
<tr>
<td>2</td>
<td>Students make predictions as to areas of high and low microbial diversity.</td>
</tr>
<tr>
<td>3</td>
<td>Students inoculate plates by wetting swab in sterile water.</td>
</tr>
<tr>
<td>4</td>
<td>The wet swab is then gently wiped across the surface of interest.</td>
</tr>
<tr>
<td>5</td>
<td>The swab is then gently wiped across the agar plate.</td>
</tr>
<tr>
<td>6</td>
<td>The student writes their name on the plate and seals the plate edges with tape.</td>
</tr>
<tr>
<td>7</td>
<td>Plates are incubated in a warm (72°F) dry environment.</td>
</tr>
<tr>
<td>8</td>
<td>During the incubation process students monitor plates, recording when new colonies occur.</td>
</tr>
<tr>
<td>9</td>
<td>Plates should remain sealed at all times, since there does exist the possibility of isolating pathogenic microbes.</td>
</tr>
<tr>
<td>10</td>
<td>Dispose of plates by heating to 121°C or 260°F.</td>
</tr>
</tbody>
</table>
Table: 6.4 Methods of *Rhizobia* symbiosis investigation.

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Students make predications.</td>
</tr>
<tr>
<td>2</td>
<td>Add <em>Rhizobia</em> inoculant to sterilized soil treatment.</td>
</tr>
<tr>
<td>3</td>
<td>Place plants into soil treatments.</td>
</tr>
<tr>
<td>4</td>
<td>Plants should receive at least 12-16 hours of light per day.</td>
</tr>
<tr>
<td>5</td>
<td>Do not fertilize plants, water plants as needed.</td>
</tr>
<tr>
<td>6</td>
<td>After 4-5 weeks of growth the students harvest the plants.</td>
</tr>
<tr>
<td>7</td>
<td>Plant shoots are separated from the roots by clipping at root collar.</td>
</tr>
<tr>
<td>8</td>
<td>Plant shoots are dried for 48 hrs and weighed to determine shoot biomass.</td>
</tr>
<tr>
<td>9</td>
<td>Roots are rinsed in water and nodules counted.</td>
</tr>
<tr>
<td>10</td>
<td>Students compare biomass and nodule formation between the different soil treatments.</td>
</tr>
</tbody>
</table>
### Table: 6.5 Methods of decomposition investigation.

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Students make predictions regarding which materials will degrade first and which treatment will be more effective at decomposition.</td>
</tr>
<tr>
<td>2</td>
<td>Wrap aluminum foil around ball (mason) jars. Simulate soil environment and prevents potential water loss.</td>
</tr>
<tr>
<td>3</td>
<td>Create holes in lids of ball jars (aerobic treatments only).</td>
</tr>
<tr>
<td>4</td>
<td>Collect soil (1 Kilogram of soil per ball jar).</td>
</tr>
<tr>
<td>5</td>
<td>Students weigh their organic materials and record the weights.</td>
</tr>
<tr>
<td>6</td>
<td>Weighed organic materials are placed in ball jar with soil.</td>
</tr>
<tr>
<td>7</td>
<td>Add water, soil should be damp.</td>
</tr>
<tr>
<td>8</td>
<td>Place jars in cool dark area for 4-6 weeks, water occasionally.</td>
</tr>
<tr>
<td>9</td>
<td>At the end of the study students identify remaining organic material.</td>
</tr>
<tr>
<td>10</td>
<td>Students determine rate of decomposition.</td>
</tr>
</tbody>
</table>
Table 6.6: Content Standards: National Science Education Standards (NRC 1996)

<table>
<thead>
<tr>
<th>Content Standard</th>
<th>Grades 6-8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Standard A: Science as an inquiry</strong></td>
<td></td>
</tr>
<tr>
<td>Abilities necessary to do scientific inquiry</td>
<td></td>
</tr>
<tr>
<td>Understanding scientific inquiry</td>
<td></td>
</tr>
<tr>
<td><strong>Standard B: Physical Science</strong></td>
<td></td>
</tr>
<tr>
<td>Properties and changes of matter</td>
<td></td>
</tr>
<tr>
<td><strong>Standard C: Life Science</strong></td>
<td></td>
</tr>
<tr>
<td>Structure and function in living systems</td>
<td></td>
</tr>
<tr>
<td>Diversity and adaptations of organisms</td>
<td></td>
</tr>
</tbody>
</table>
Figures

Figure 6.1 Pre and Post student drawing assessment

Pre-assessment

Post assessment

[Diagram showing pre- and post-assessment drawings involving elements like sun, oxygen, soil, and bacteria]
Chapter 7

SYNTHESIS

Approximately 2400 petagrams (Pg-10^{15} g) of carbon are stored in the Earth’s soil as soil organic matter (SOM), representing two times the amount of carbon stored in the Earth’s vegetation and atmosphere combined (Brady & Weil 2002). Several essential ecosystem services are highly dependent upon SOM including nutrient cycling, mitigation of soil erosion, and storage of atmospheric CO₂. Plant biomass is the primary contributor to SOM; however, a host of soil organisms are responsible for incorporating plant tissues into the soil environment (i.e. macro/micro invertebrates, and fungi). A significant fungal contributor to SOM is Arbuscular mycorrhizal fungi (AMF). These fungi form mutualistic symbiotic relationships with approximately 80% of terrestrial plants (Smith & Read 1997); this represents a significant proportion of photosynthetically fixed carbon shuttled below ground. Several studies have attempted to quantify the indirect contribution of AMF to SOM via the measurement of Glomalin related soil protein (GRSP), as well as the direct effects through the stabilization of soil aggregates. Yet several questions remain; i) how accurate are the glomalin extraction and detection methods, ii) do AMF colonized root fragments contribute to GRSP pools, iii) what influence does AMF have on aggregate dynamics and iv) how do soil fertility and exotic plant invasion influence AMF abundance as well SOM concentrations. The work presented addresses each of these questions, and improves our current understanding of AMF influences on SOM. Understanding ecosystem soil carbon budgets is critical as stable ecosystems release SOM via microbial oxidation in balance with organic matter
inputs. Accurate assessment of specific carbon inputs is necessary if this vital balance is to be maintained.

Current estimates suggest that up to 20% of host photosynthetic carbon can be transferred to AMF (Smith & Read 1997); this finding suggests that AMF significantly contribute to SOM. Several studies have relied on GRSP extraction and detection methods to accurately assess and model fluxes of AMF derived carbon, and according to Wright & Upadhyaya (1996) 30-60% of soil carbon can be attributed to GRSP in undisturbed soils. The work presented in chapter 2 challenges the GRSP extraction efficiency and detection methods (Bradford/ELISA assay). Our results are significant as they suggest that previous efforts to quantify the contribution of GRSP fractions to SOM possibly overestimated the true contribution of AMF. Additionally, we found that leaf litter biochemistry can significantly alter the assessment of AMF contribution to GRSP pools. Results from this study and similar research efforts have lead to a refinement of methods to better estimate AMF contributions to SOM. From a soil ecology perspective, these findings are valuable as they provide a critical assessment of the presence of GRSP within the soil environment, greatly aiding our understanding of AMF contribution to SOM pools.

Research efforts to date have focused on quantifying the concentration of the total GRSP pool in soils from various sources including aggregate-associated GRSP, AMF hyphae, spores and colonized root fragments (Wright & Anderson, 2000; Rillig et al., 2003; Lutgen et al., 2003; Lovelock et al., 2004). The relative contribution of each of these
specific sources to the total GRSP pool has yet to be assessed. AMF maintain two different growth forms that experience radically different environments: extraradical mycelium, which explore the soil, and intraradical hyphae, which colonize host root tissue. We expect the contributions of each growth form to GRSP pools to differ. The focus of the work presented in chapter 3 evaluates the potential production of Glomalin-root protein (GRP) fractions BRP and IRP (Bradford-reactive root protein and Immunoreactive-root protein) within AMF colonized root tissue. Additionally, we evaluated whether BRP and IRP could provide a useful biomarker in assessing AMF root colonization. Findings from this study are valuable as they suggest that BRP appears to be present in AMF-colonized root and contributes to GRSP pools, however, we cannot conclusively identify IRP as a source of GRSP due to antibody cross-reactivity. Furthermore, these results demonstrate that the Bradford method offers an effective process for assessing AMF root colonization. This study is the first to measure the potential production of GRP fractions within AMF colonized roots, and these results provide evidence quantifying a source of AMF derived SOM as well as assessing a possible physiological function. Given that AMF colonize a significant proportion of host root tissue we expected root decomposition products to add significantly to GRSP pools, yet our results do not support this hypothesis and SOM models should exclude IRP as a source of AMF derived SOM. Gadkar & Rillig (2006) suggest that glomalin is a heat shock protein (Hsp), which is a class of proteins up-regulated as a result of stress gradients (i.e. heat, cold, dehydration). It is possible that the intraradical hypha of AMF experience a stable environment within host root tissue not necessitating the production of glomalin.
Soil macroaggregates (> 250 µm) physically protect labile SOM from the surrounding decomposer community which in turn promotes greater soil carbon storage. Soil aggregates are dynamic structures and their assembly and subsequent decomposition is an active process (Lei et al., 2002, Plante et al., 2002). Understanding how specific soil organisms influence aggregate dynamics is important in regards to understanding soil carbon storage potential. Due to the ubiquity of AMF within most soil environments it is expected that AMF play a significant role in aggregate assembly/turnover, however, direct evidence linking AMF to aggregate dynamics is completely absent. The work presented in chapter 4 is the first to employ Rare Earth Elements (REE) as a tracer to assess the influence of AMF on aggregate dynamics. Our results are significant as they indicate AMF increased large macroaggregate formation via equal incorporation of macroaggregates and microaggregates, and slowed intermediate macroaggregate as well as microaggregate decomposition. These findings suggest that AMF directly improve SOM storage via the stabilization and formation of macroaggregates. Additionally the long-term stabilization of macroaggregates favors the formation of microaggregates which are essential for sequestration of soil carbon. The majority of SOM storage models assess the turnover of distinct soil carbon pools mostly ignoring the physical process of soil structure. Six et al., (2002) proposed a conceptualized model focusing primarily on the influence of soil structure on SOM storage. The main limitation associated with this model is the lack of evidence depicting how specific soil organisms influence aggregate dynamics. The results from our study greatly improve the predictive power of this newly proposed model.
Several studies suggest that exotic plant species alter soil processes, creating a soil environment that favors establishment and spread of invasive plants. Exotic plant modification of the soil environment could influence several factors that affect SOM storage i.e. AMF abundance, OM concentrations, and aggregate stability. Understanding how invasive plants change soil processes is essential in terms of preventing/restoring invaded ecosystems as well as modeling how invaded landscapes could lose their potential to store carbon. The focus of the work presented in chapter 5 was to compare soil properties of *H. caespitosum* soils to that of neighboring weed-free *F. idahoensis* soil in order to determine how changes in the soil environment affect invasion and possibly alter SOM storage. Our results suggest that *H. caespitosum* invaded soils maintained greater AMF hyphal abundance and OM concentrations; increases in these factors resulted in greater aggregate stability. Our results further indicate that as soil fertility increased under both *H. caespitosum* and *F. idahoensis* soils a loss of aggregate stability occurred and correlation analysis suggests that as OM increased in both invaded and native grassland soils Mean Weight Diameter (MWD) decreased. In contrast to several studies which suggest that losses in OM result in a decrease in MWD, results from our study provide support to the hypothesis that the quality not the quantity of OM drives aggregate stability. From a soil/plant ecology perspective these results are significant as they suggest an alteration of key soil processes as well as potential changes in AMF community structure as a result of exotic plant invasion.

My work with the ECOS program at the University of Montana has led me to the understanding that soil microbiology/ecology education at the primary level (K-12) is
virtually nonexistent. Basic knowledge of soil microbiology/ecology is necessary if an informed voting society is to make critical decisions which could ultimately affect the quality of its soil (i.e. heap leach mining, promotion of sustainable agriculture). Civilizations that understood the necessity of proper soil stewardship prospered; because they realized that soil quality was directly related to crop production. For example during the Yao dynasty a soil classification system recognizing nine distinct soil types was established, this system was used as a taxation base for land usage (Montgomery 2007). Development of a soil classification system early in Chinese culture illustrates a significant understanding of the importance of soil management, and provides evidence suggesting why China did not suffer the same fate as several earlier empires (Coleman et al., 2004). In contrast, history is riddled with accounts of the crippling effects of soil degradation indirectly leading to the demise or reduction of several civilizations. Early examples of soil exhaustion have been documented in Mesopotamia, Greek, Roman and Mayan cultures. Additionally several modern examples of soil degradation also exist from the famine that struck China in the 1920’s, Dust Bowl era within the USA, to the current desertification of North Africa. The underlying factor of all these examples is a lack of understanding of the most fundamental principles of soil ecology. As a result of this understanding I have developed a series of lessons which build upon each other with the primary goal of focusing on the importance and significance of soil microbiology/ecology to middle school students. In this set of investigations students will: i) isolate microorganisms from environmental samples, ii) assess microbial/plant symbiosis, and iii) explore microbial decomposition and nutrient cycling. I selected these three inquires because they provide an essential base for understanding the relevance of
soil microbiology/ecology from which future question/inquires could be built. These lessons will be available to teachers via publication as well as the ECOS website.

In conclusion 80% of terrestrial plants host mycorrhizal symbiosis representing a significant input to SOM. As a result AMF contributions to SOM pools and formation of soil structure need to be better understood. Experimental manipulations measuring glomalin’s existence via exclusion of AMF should cease as they offer limited new information. Glomalin research should continue to focus on evaluating the specificity of the ELISA assay/monoclonal antibody (MAb32B11). Another area of research should consider the physiological function of glomalin. Is there an environmental trigger(s) that causes an overproduction of glomalin (i.e. herbivory, metal toxicity, osmotic stress); no research to date has addressed this question. Until there is a clear understanding of glomalin’s structure and function the ecological significance of this compound remains uncertain. It is apparent that AMF influence aggregate stability/turnover, the next avenue of AMF-aggregate research should consider AMF modification of soil structure. Alteration of aggregate pore distribution can create localized environments which increase microbial activity due to changes in environmental gradients as well as changes in food web structure. Exploring these avenues of research would greatly aid our understanding of soil ecosystem development as well as providing essential information necessary for long-term soil management.
References


