Arbuscular mycorrhizae, glomalin, and soil aggregation in spotted knapweed invaded soils

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ARBUSCULAR MYCORRHIZAE, GLOMALIN, AND SOIL AGGREGATION IN SPOTTED Knapweed Invaded Soils

by

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B.A. Grinnell College, Grinnell, IA, 2000

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Date
Arbuscular mycorrhizae, glomalin, and soil aggregation in spotted knapweed invaded soils

Director: Matthias C. Rillig

This was a three-part study investigating influences of spotted knapweed (*Centaurea maculosa*) on soils in Montana grasslands. In particular, I examined arbuscular mycorrhizae, soil aggregation, and glomalin pools (a fungal glycoprotein important in soil aggregation) in soils from these grasslands. Arbuscular mycorrhizal fungi (AMF) are obligately biotrophic fungi that are closely associated with both host plants and the soil environment. AMF contribute to soil aggregation through the hyphal entanglement process and through the production of extracellular polymeric compounds on hyphal surfaces, including glomalin. Spotted knapweed associates with AMF. However, few studies have examined the relationship between spotted knapweed, AMF, and soil. Influences of spotted knapweed and knapweed management methods on arbuscular mycorrhizae, soil aggregation, and glomalin concentrations were investigated, as well as the variability in spotted knapweed effects on glomalin concentrations through time. I found that soil structure could deteriorate in areas where spotted knapweed infestations are not managed; negative effects on soil parameters were proportional to the density of spotted knapweed. Methods to manage spotted knapweed that were examined did not negatively affect soil structure compared to knapweed-invaded areas. Inter-annual and seasonal variability in effects of spotted knapweed on glomalin in soil were observed through drought and wet years. The observed temporal variability in effects of spotted knapweed on glomalin led to the more fundamental question of how AMF and glomalin vary seasonally in the examined grasslands. In order to more fully comprehend the role of AMF in natural ecosystems, including interactions with invasive plants, it is important to document seasonal changes. Seasonal dynamics over one growing season were observed in some glomalin pools (TG and IREEG) and AMF external hyphal length. This is the first report of seasonal changes in glomalin pools, indicating a sampling regime capturing seasonal variation may have to be employed in future studies measuring glomalin.
Acknowledgments

This work was supported by the U.S. Department of Energy, the National Science Foundation, and the Center for Invasive Plant Management, Montana State University. Thank you to the USFS Lolo National Forest for providing a study site in the Blue Mountain recreation area, Missoula, Montana. Dow AgroSciences LLC and Celestine Duncan (Weed Management Services, Helena, Montana) are gratefully acknowledged for the establishment and maintenance of experimental plots in the Blue Mountain Recreation Area. Celestine Duncan is also acknowledged for providing data and feedback. Dr. Sara F. Wright provided MAb32B11.

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Last, but not least, I would like to express my deepest gratitude toward my family, David, Cathie, Ellen, and Andrew, for all of their support and wise words. Thank you to Ellen for helping me with field work. Thank you to Luke for lending a patient ear and providing support until the finish.
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Introduction

Many invasive exotic plants currently infest and threaten native North American ecosystems. Several invasive exotic weed species have become well established; others are newly introduced. Invasive plant species are highly competitive with native vegetation, threatening native plant biodiversity. Invasive weeds not only have negative effects on native plant communities, but can also alter other biological, hydrological, and physical properties of ecosystems (Gordon 1998). Such invasions are considered a major threat to rare and endangered species as well as to the functioning of ecosystems (Blossey 1999) and can result in large-scale environmental degradation (Macdonald et al. 1989).

The seasonality and growth dynamics of invasive weeds are important in understanding the infestation patterns and effects of the weeds on the native environment. However, there is inconsistent evidence on the establishment and growth patterns of invasive weed infestations, particularly in arid western ecosystems (Smith et al. 1999). In addition, how ecosystem properties and their seasonal dynamics are influenced by the invasion and dominance of invasive weeds has been little studied. Soil properties, for example, are of specific interest because of the resources and nutrient cycling the soil provides for vegetation, native and invasive plant species alike. The characteristics and seasonality of arbuscular mycorrhizae in weed invaded soils are of particular importance because arbuscular mycorrhizal fungi (AMF) provide a direct physical link between vegetation and the soil resource (Miller et al. 1995).
Role of arbuscular mycorrhizal fungi in soil systems and vegetation

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts that colonize the roots of most vascular plants (Smith and Read 1997) and create a network of external hyphae in the soil. AMF can improve plant mineral status (Brundrett 1991) and influence the outcome of competitive interactions of host plant species (Allen and Allen 1990).

AMF are among the most important soil biota in maintaining stable soil aggregation (Jastrow and Miller 1997), a major determinant of soil structure (Schreiner and Bethlenfalvay 1995; Thomas et al. 1993). Soil structure is often utilized in assessing soil quality because it controls many of the biological, physical, and chemical properties of soil. AMF contribute to soil aggregation through the hyphal entanglement process, assisting in soil aggregate formation (Jastrow and Miller 1997), and through the production of extracellular polymeric compounds on hyphal surfaces, which can sorb to inorganic materials, helping to stabilize soil aggregates (Jastrow and Miller 1997). AMF also produce copious amounts of glomalin, a glycoprotein that is important in soil aggregation (Wright and Upadhyaya 1996). Glomalin concentrations in soil aggregates were found to be highly correlated with the percentage of water-stable aggregates (WSA) (Wright and Upadhyahya 1998), a common measure of soil structure (Thomas et al. 1993).

Despite the importance of AMF hyphae in soil ecosystems, studies examining the seasonality of extraradical AMF hyphae are sparse. Miller et al. (1995) quantified external AMF hyphae over a growing season in tallgrass prairie and ungrazed pasture and found that root morphology was strongly associated with external hyphal length. Kabir et al. (1997) examined the seasonal changes of extraradical and intraradical arbuscular
mycorrhizal hyphae affected by tillage and fertilization in an agricultural soil over a
 growing season. Abundance of AM hyphae fluctuated significantly within a growing
 season, with lowest hyphal densities found in the spring. Seasonal dynamics of AMF
 hyphal products, such as glomalin, in the soil are unknown.

**Effects of invasive plants on soil properties**

Plant species within an ecosystem have effects on soil properties such as soil
 carbon and soil aggregation, as well as other properties, including pH and nutrient
cycling. These effects are caused by naturally occurring plant species and invasive plant
species alike. For example, Rillig et al. (2002) examined the effects that co-occurring,
non-invasive plant species have on soil aggregation. They found that different aggregate
water stability values were obtained in monoculture field plots subsequently planted with
different plant species.

Effects of soil properties caused by invasive plant species are especially
interesting because plant invasions have become widespread in many ecosystems.
D’Antonio and Vitousek (1992) examined the effects grass invasions have on ecological
organization. These invasions had effects at multiple levels, from the population to
ecosystem level. Competition by the invasive grasses for water and soil nutrients, as well
as the shallow root systems of these grasses, had effects on soil properties. Kourtev et al.
(1998) and Ehrenfeld et al. (2001) found that areas where two invasive plant species have
established in hardwood forests in the northeast United States, Japanese barberry
(Berberis thunbergii) and Japanese wiregrass (Microstgium vimineum), contain soil with
a significantly lower pH, a thinner litter layer, and higher nitrification rates than soils in
uninvaded areas. Soil and soil food web characteristics were altered, levels of silt increased, and plant material cover was enhanced in areas invaded by the exotic grass *Bromus tectorum* (Belnap and Phillips 2001). These data suggest that a cause-effect relationship between invasive plant species and soil properties exists.

*Invasive plants in Montana; spotted knapweed*

Spotted knapweed, *Centaurea maculosa*, is a prevalent invasive plant species in the middle and western United States, including Montana. Relatively little is known about the relationship between the soil environment and this invasive weed. It has been found, however, that spotted knapweed increases soil erosion in areas where infestations of this plant are well established (Lacey et al. 1989; Cheater 1992). Topsoil is not protected and the sedimentation of surface water increases due to soil erosion (Lacey et al. 1989). No information exists on effects spotted knapweed have on mycorrhizal properties and/or soil structure. A more complete understanding of these processes is key to increasing our mechanistic understanding of the link between plant invasion and soil erosion.

Originally from Eurasia, spotted knapweed was likely introduced to the United States around 1900 as a contaminant in hay or alfalfa seed (Lacey et al. 1990; French and Lacey 1983). Since its introduction, spotted knapweed has spread throughout the Northwest. Spotted knapweed is established in every county of Montana, invading at least five million acres of the state (Montana Weed Control Association, August 2002). It is most common in areas disturbed by excessive grazing, logging, rodents, or vehicular traffic.
Several management methods have been developed for spotted knapweed. The most common management method is the use of herbicides. The most widely used herbicide for knapweed management is picloram (French and Lacey 1983). Other herbicides used to manage spotted knapweed include clopyralid and clopyralid plus 2,4-D. Herbicides are applied in the spring or fall in an attempt to maximize the effectiveness of the chemicals. Mechanical means, such as mowing, clipping, hoeing, tilling, and hand pulling, are also implemented to control spotted knapweed by physically removing all or proportions of the plants. Biological control is also used, attempting to control spotted knapweed by exerting stress on the weed to reduce its dominance (DiTomaso 2000). One of the most promising biological controls for spotted knapweed is the larvae of the moth Agapeta zoegana, which are specialist herbivores on the taproots of Centaurea species (Müller-Schärer 1991).

Effects of spotted knapweed on soil properties and AMF seasonality

Non-indigenous plant species, including spotted knapweed, are detrimental to native ecosystems. Several studies have examined the effects of invasive plant species on native vegetation, but fewer studies have examined the invasive plant-soil relationship, and none have investigated the effects of invasive weeds on soil structure, glomalin concentrations, and mycorrhizal seasonal growth. Here I investigate influences spotted knapweed has on the soil ecosystem in Montana grasslands. In particular, I examine arbuscular mycorrhizae and soil aggregation in soils from these grasslands. Influences of spotted knapweed and management methods for spotted knapweed on arbuscular mycorrhizae, soil aggregation, and glomalin concentrations were investigated, as well as
the variability in spotted knapweed effects on glomalin concentrations through time. The seasonality of AMF hyphae (intra- and extra-radical) and glomalin concentrations in spotted knapweed infested areas were also examined over a growing season.
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Chapter 1: Influence of spotted knapweed and spotted knapweed management treatments on arbuscular mycorrhizae and soil aggregation

Abstract

Spotted knapweed is an invasive mycorrhizal weed particularly prevalent in the Pacific Northwest of the United States. Little is known about the effects of spotted knapweed or its management methods on soil quality and soil structure. This study compared soils from spotted knapweed-infested areas to those where spotted knapweed is being managed using several herbicide applications and mechanical treatments. We measured concentrations of glomalin, a glycoprotein produced by arbuscular mycorrhizal fungi (AMF) that is correlated with soil aggregate stability, AMF hyphal length, and percent water stable aggregates (WSA) in soils from managed and unmanaged areas. Areas with high knapweed density (unmanaged areas) generally had lower glomalin concentrations and AMF hyphal lengths compared to areas where chemical and combined mechanical/chemical management treatments were applied. Total glomalin was significantly negatively correlated with percent knapweed cover. However, WSA was high (70-80%) in soils from all management treatments and not affected by knapweed cover. Our results suggest that *Centaurea maculosa* may have negative effects on soil quality, especially in soils with lower aggregate stability.

Introduction

Spotted knapweed, *Centaurea maculosa* Lam. CENMA, is an invasive plant from Eurasia that has invaded much of the Pacific Northwest in the United States. As an
aggressive weed, spotted knapweed competes with native vegetation, often reducing native plant populations and biodiversity (Tyser and Key 1988). Livestock and wildlife forage are reduced and surface water runoff, soil erosion, and stream sediment loads increase in spotted knapweed invaded areas (Jacobs and Sheley 1998; Lacey et al. 1989). The competitiveness of spotted knapweed is likely due to a combination of factors including prolific seed production, high seed viability, and the ability of seeds to germinate during fall and over-winter as rosettes (Lacey et al. 1990; Tyser and Key 1988). The absence of natural enemies and the selective grazing of other desirable forage plants contribute to its success as a competitor (Lacey et al. 1990; Tyser and Key 1988).

Numerous methods have been developed to manage spotted knapweed, including the application of herbicides and mechanical treatments, such as handpulling and mowing. Herbicides such as picloram, clopyralid, dicamba, and 2,4-D effectively control spotted knapweed on rangeland (Sheley et al. 1999). Davis (1990) found that residual grasses in invaded areas increased greatly during the time period of herbicide treatment. Persistent handpulling can also effectively control spotted knapweed, if the entire plant is removed (Sheley et al. 1999). Mowing spotted knapweed at the flowering stage or bud stage is successful in reducing spotted knapweed. However, the long-term effects of mowing on the management of spotted knapweed are unknown (Sheley et al. 1999). Little is known about how these management methods affect the soil environment, including arbuscular mycorrhizae and soil structure.

Several studies have examined the effect of spotted knapweed on native vegetation, but few studies have examined the spotted knapweed-soil relationship. Lacey et al. (1989) conducted a comparative field study and found increased water runoff and
stream sediment loads in areas invaded by spotted knapweed compared to adjacent non-invaded areas.

An experimental approach further investigating spotted knapweed influences on soil properties can help elucidate the underlying mechanisms that explain changes in soil properties in knapweed-invaded soils. Soil structure mediates many biological and physical processes in the soil ecosystem, such as water filtration, soil aeration, biogeochemical cycling, and susceptibility to erosion (Oades 1984; Elliott and Coleman 1988; Hartge and Stewart 1995; Jastrow and Miller 1997). Because soil structure plays such an important role in ecosystem function, monitoring changes in soil structure is important.

The field study described herein examined how spotted knapweed and management methods (mechanical, chemical, and combined mechanical/chemical treatments) for spotted knapweed affect mycorrhizal parameters and soil structure. Although several weedy species are considered to be nonmycorrhizal, particularly in the Chenopodiaceae, Brassicaceae, Polygonaceae, and Amaranthaceae families (Pendleton and Smith 1983), arbuscular mycorrhizal fungi (AMF) associations have been found in spotted knapweed in western Montana (Marler et al. 1999). AMF are among the most important soil biota in maintaining stable soil aggregation (Jastrow and Miller 1997). AMF also produce glomalin, a glycoprotein that is important in soil aggregation. Glomalin concentrations in soil aggregates are highly correlated with the percentage of water-stable aggregates (WSA) (Wright and Upadhyahya 1998) and glomalin has a relatively slow turnover rate in soil (Rillig et al. 2001). Concentrations of glomalin, AMF hyphal length, and percent water stable aggregates were measured in spotted
knapweed invaded areas as parameters crucial to the maintenance of soil structure. Differences in response variables among management methods (mechanical treatments vs. chemical treatments vs. combined mechanical/chemical treatments) were also investigated.

Materials and Methods

Field experiment and sampling

This research was conducted at the Lolo National Forest, in the Blue Mountain Recreation Area, near Missoula, Montana (46° 45' 00" N, 114° 07' 30" W). The site is a grassland area characterized as an upland range with loamy soil dominated by bluebunch wheatgrass (*Agropyron spicatum*), rough fescue (*Festuca scabrella*), and Idaho fescue (*Festuca idahoensis*) (Brown et al. 1999). This area is invaded by spotted knapweed. The experiment of applying different management methods for spotted knapweed in the field consisted of plots (6 x 9 m) arranged in a randomized complete block design containing three blocks based on slope position (n=3). The plots were treated with different management methods for spotted knapweed, including mechanical, chemical, and combined mechanical and chemical treatments. Untreated plots, where no management methods for spotted knapweed were applied, were established as experimental controls.

Chemical treatments included herbicide applications delivered in the spring or fall of 1997. Herbicides were applied using a CO2-pressurized backpack sprayer equipped with a 3.1 m, 6-nozzle spray boom, calibrated to deliver 150 liters per ha. Herbicide applications in this study included picloram at 0.28 kg ai ha⁻¹ (spring 1997) and 0.14 kg ai
ha\textsuperscript{-1} (fall 1997); and clopyralid plus 2,4-D at 0.21 kg ai ha\textsuperscript{-1} plus 1.12 kg ai ha\textsuperscript{-1} (spring 1997) and 0.67 kg ai ha\textsuperscript{-1} plus 1.12 kg ai ha\textsuperscript{-1} (fall 1997). Mechanical treatments included mowing with a standard push-mower twice (early and late bud growth stage) in 1997 only, and twice in 1997, 1998, 1999, and 2000; and handpulling two times per year at four week intervals in 1997, two times per year in 1997 and 1998, and two times per year in 1997, 1998, 1999, and 2000. Combination methods of both mechanical and chemical treatments included mowing during the late bud growth stage and picloram application (0.14 kg ai ha\textsuperscript{-1}) in the fall of 1997, mowing during the late bud growth stage and clopyralid plus 2,4-D application (0.67 kg ai ha\textsuperscript{-1} plus 1.12 kg ai ha\textsuperscript{-1}) in the fall of 1997, and handpulling annually from 1997-2000 and picloram application (0.14 kg ai ha\textsuperscript{-1}) in the fall of 1997. Post-treatment spotted knapweed cover data were collected using the point-frame method (Brown et al. 1999).

On September 15, 2000, five soil cores (2 cm diameter) were extracted from each plot, physically combined in polyethylene bags, dried in a drying oven at 80\(^\circ\) C overnight, and then stored at room temperature until analysis.

**Hyphal and glomalin measurements**

Hyphae were extracted from soil samples (4 g) using an aqueous extraction and membrane filtration technique according to Rillig et al. (1999). Arbuscular mycorrhizal (AM) hyphae were distinguished at 200x magnification (Miller et al. 1995). Hyphal length was determined using the line intersect method as described in Jakobsen et al. (1992) and Tennant (1975).
Two detection methods are used to quantify glomalin: the Bradford protein assay, yielding the easily extractable glomalin (EEG) and the total glomalin (TG) fractions, and an ELISA assay (employing the monoclonal antibody developed against crushed spores of *Glomus intraradices*; Wright and Upadhyahya 1998), yielding the immunoreactive easily extractable glomalin (IREEG) and immunoreactive total glomalin (IRTG) fractions. These glomalin fractions are operationally defined based on their extractability/solubility and detection methods (much like other soil fractions, such as humic acids). While the ELISA assay is a very specific detection method for glomalin, the more general Bradford protein assay is also utilized. This protein assay may capture glomalin protein that has undergone small (perhaps microbially-mediated) changes, possibly resulting in the destruction or concealment of the epitope for the monoclonal antibody. Because of well-documented and strong correlations with soil aggregate stability (e.g., Wright and Upadhyahya 1998), these glomalin fractions continue to be quantified.

Glomalin fractions were extracted from total soil and also from 1-2 mm aggregates (Wright and Upadhyahya 1996). The protein fractions were extracted using the same method for both size classes of soil. The EEG fraction was extracted with 20 mM sodium citrate, pH 7.0 at 121° C for 30 minutes. Following the EEG extraction, the TG fraction was extracted with 50 mM sodium citrate, pH 8.0 at 121° C for 60 minute cycles until extraction was completed. Both fractions of glomalin were analyzed using the Bradford Protein Assay (Bio-Rad, Melville, NY). The glomalin fractions extracted from the 1-2 mm aggregates were further analyzed using an enzyme-linked immunosorbent assay (ELISA) employing the monoclonal antibody MAb32B11. After
all glomalin analyses were completed, four fractions of glomalin data were obtained: EEG, TG, IREEG, and IRTG.

**Soil aggregate water stability (WSA 1.2 mm)**

The percentage of water stable aggregates in the 1-2 mm size class (WSA 1.2 mm) was determined by a standard wet sieving method (Kemper and Rosenau 1986). Soil particles passing through a 0.25 mm sieve were dried and weighed. The dried aggregates were then dispersed to determine coarse matter. The percentage of water stable aggregates for the 1-2 mm size class was calculated as the mass of aggregated soil remaining after wet sieving, correcting for coarse matter (>0.25 mm).

**Data analysis**

All response variables (glomalin concentrations, AM hyphal length, and WSA 1.2 mm) were first analyzed using multivariate analysis of variance (MANOVA) in the procedure of JMP (JMP version 3.1.6.2, 1996) since these variables cannot reasonably be assumed to be independent (Scheiner 1993). The response variables were then analyzed using analysis of variance (ANOVA). Response variables that contained significant p-values (P≤0.05) from the ANOVA analyses were analyzed further using linear contrasts (JMP version 3.1.6.2, 1996) to test for differences between untreated and treated areas and among the different management methods for spotted knapweed. Management methods that significantly reduced spotted knapweed density compared to the untreated areas were determined by least significant difference (LSD) analysis. Regressions between percent spotted knapweed cover and all response variables were calculated in the
procedure of JMP (JMP version 3.1.6.2, 1996). Due to the low \( n \), we discuss results with \( P<0.10 \).

**Results and Discussion**

Multivariate analysis of response variables measured (EEG, TG, AM hyphal length, and WSA \(_{1-2\text{mm}}\)) on total soil revealed a significant difference (MANOVA: \( F_{14,24}=2.20, P=0.04 \)), justifying further analysis of individual response variables. Multivariate analysis of the 1-2 mm aggregate glomalin data set revealed marginally significant differences (EEG, TG, IREEG, and IRTG; MANOVA: \( F_{13,16}=2.28, P=0.06 \)).

Differences among spotted knapweed management treatments were apparent (Tables 1 and 2). The total soil EEG and TG fractions both exhibited significant differences among treatments (Table 1). AM hyphal length was marginally significant (\( P=0.07 \), Table 1); it was lower in the untreated plots than in plots receiving management treatments (Figure 1). WSA \(_{1-2\text{mm}}\) was not significantly different among management treatments (Table 1). The aggregate water stability for the untreated plots as well as the treatment plots were all high at 70-85%, with little variation between management treatments (Figure 2). Glomalin fractions analyzed from 1-2 mm sized aggregates did not show a clear pattern among treatments. However, the TG fraction did exhibit a significant difference among treatments (Table 2).

Response variables that showed a significant difference among different spotted knapweed management treatments were analyzed further using linear contrasts. Management treatments were separated into groups based on mechanical, chemical, or combined mechanical/chemical means and contrast comparisons were performed.
between these groups of treatments. Untreated plots were significantly different (P≤0.05) from all management treatments for the EEG (total soil) fraction, the TG (total soil) fraction, the TG (1-2 mm) fraction, and AM hyphal length (Table 3). Glomalin concentrations (total soil and 1-2 mm aggregates) and AM hyphal length were lower in untreated plots compared to all management treatments (Figures 1, 3, & 4). Significant differences (P≤0.05) occurred between the mechanical and chemical treatment methods as well as between the chemical and combined mechanical/chemical treatment methods in both the EEG and TG fractions (total soil) (Table 3).

All management methods for spotted knapweed except the mechanical treatments were successful in reducing spotted knapweed cover compared to untreated areas (P≤0.05; Table 4).

Regression analysis showed that percent spotted knapweed cover had a significant negative linear relationship with total soil TG (P≤0.05; Figure 5). Total soil EEG showed a similar negative linear relationship, but was not significant (P=0.13, inset, Figure 5). Other response variables regressed with percent knapweed cover were not significant (P>0.05).

The objectives of this study were to examine the effects of spotted knapweed and spotted knapweed management methods on soil structure and parameters important in determining soil structure. Untreated areas (experimental controls) had the lowest glomalin concentrations compared to areas that received management treatments. Because glomalin is highly correlated with soil aggregate water stability (Wright and Upadhyaya 1998), these results suggest that soil structure can deteriorate in areas where spotted knapweed infestations are not managed. Untreated, dense knapweed areas also
had the lowest hyphal lengths compared to treated areas, further suggesting negative impacts of knapweed invasion. Mycorrhizal hyphae are often directly related to percent aggregate water stability (as they were in this study; \( r=0.98 \)). Hyphae entangle soil particles, assisting in soil aggregate formation (Jastrow and Miller 1997). Also, extracellular polymeric compounds on hyphal surfaces can sorb to inorganic materials, helping to stabilize soil aggregates (Jastrow and Miller 1997). Because hyphae help to form and maintain soil aggregates, they are a key indicator of soil structure.

Despite the established importance of glomalin and hyphae on aggregate formation, soil aggregate water stability, a common measure of soil structure, did not show a similar pattern to glomalin concentrations and AM hyphal length. There were no significant differences in soil aggregate water stability between the untreated areas and those managed for spotted knapweed. The soil aggregate water stability in the soil measured was quite high (70-85\%). The relationship of glomalin and soil aggregate water stability is curvilinear (Wright and Upadhyaya 1998), such that beyond a saturation point, a decrease in glomalin does not correlate to a major decrease in soil aggregate water stability. This may explain why a decrease in soil aggregate water stability in untreated areas was not observed in this study. However, spotted knapweed infestations occur in many areas in the Pacific Northwest where soil aggregate water stability is low. Our results suggest that spotted knapweed may exert a deleterious effect on soil structure in these areas.

Spotted knapweed cover (\%) was significantly negatively correlated with the total soil TG fraction across all treatments. A similar, yet not significant, linear relationship was observed for the total soil EEG fraction. This pattern further suggests that spotted
knapweed has negative effects on soil parameters proportional to the density of this invasive weed.

This study has shown that spotted knapweed invasion has a negative effect on parameters associated with soil structure, as shown by a reduction in glomalin concentrations and hyphal length in untreated areas with a high density of this weed compared to managed areas with low spotted knapweed density. However, soil aggregate water stability was not significantly changed by spotted knapweed invasion in this study. Overall, methods to manage spotted knapweed do not negatively affect soil structure compared to invaded, unmanaged areas. Chemical applications tend to result in higher concentrations of glomalin, suggesting that managing spotted knapweed with herbicides may be less deleterious to soil structure than mechanical or combined mechanical/chemical means. These findings provide important information relevant to improving management practices for spotted knapweed, providing evidence that control methods for spotted knapweed are not deleterious to soil structure.

Davis, E. S. 1990. Spotted knapweed (*Centaurea maculosa* Lam.) seed longevity, chemical control and seed morphology. M. S. Thesis. Montana State University, Bozeman, MT.


Table 1. Effect of management treatments for spotted knapweed on different glomalin fractions (total soil; EEG=easily extractable glomalin, TG=total glomalin), arbuscular mycorrhizal (AM) hyphal length, and aggregate water stability (1-2 mm).

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>EEG</th>
<th>TG</th>
<th>AM hyphal length</th>
<th>% water stable aggregates (1-2 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>F</td>
<td>P&gt;F</td>
<td>F</td>
<td>P&gt;F</td>
</tr>
<tr>
<td>Treatments</td>
<td>3.32</td>
<td>0.01</td>
<td>2.14</td>
<td>0.05</td>
</tr>
<tr>
<td>Block</td>
<td>0.97</td>
<td>0.40</td>
<td>1.87</td>
<td>0.18</td>
</tr>
</tbody>
</table>

^a Chi squared non-parametric test performed
Table 2. Effect of management treatments for spotted knapweed on different glomalin fractions (1-2 mm aggregates; TG=total glomalin, IRTG=immunoreactive total glomalin, IREEG=immunoreactive easily extractable glomalin, EEG=easily extractable glomalin).

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>TG</th>
<th>IRTG</th>
<th>IREEG</th>
<th>EEG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P&gt;F</td>
<td>F</td>
<td>P&gt;F</td>
</tr>
<tr>
<td>Treatments</td>
<td>2.71</td>
<td>0.03</td>
<td>0.71</td>
<td>0.71</td>
</tr>
<tr>
<td>Block</td>
<td>5.79</td>
<td>0.01</td>
<td>5.32</td>
<td>0.02</td>
</tr>
</tbody>
</table>

<sup>a</sup> Chi squared non-parametric test performed
Table 3. Contrast comparisons of different management treatments for spotted knapweed from response variables with significant ANOVA p-values (EEG=easily extractable glomalin, TG=total glomalin).

<table>
<thead>
<tr>
<th>Contrast comparison</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>EEG (total soil)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All control methods</td>
<td>14.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mechanical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemical</td>
<td>4.48</td>
<td>0.05</td>
</tr>
<tr>
<td>Mechanical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanical &amp; chemical</td>
<td>0.01</td>
<td>0.92</td>
</tr>
<tr>
<td>Chemical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanical &amp; chemical</td>
<td>3.82</td>
<td>0.06</td>
</tr>
<tr>
<td>TG (total soil)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All control methods</td>
<td>3.83</td>
<td>0.06</td>
</tr>
<tr>
<td>Mechanical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemical</td>
<td>6.20</td>
<td>0.02</td>
</tr>
<tr>
<td>Mechanical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanical &amp; chemical</td>
<td>0.03</td>
<td>0.87</td>
</tr>
<tr>
<td>Chemical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanical &amp; chemical</td>
<td>4.10</td>
<td>0.05</td>
</tr>
<tr>
<td>TG (1-2 mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All control methods</td>
<td>11.2</td>
<td>&lt;0.005</td>
</tr>
<tr>
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<td>Chemical</td>
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<td>0.87</td>
</tr>
<tr>
<td>Mechanical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanical &amp; chemical</td>
<td>1.10</td>
<td>0.31</td>
</tr>
<tr>
<td>Chemical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanical &amp; chemical</td>
<td>0.83</td>
<td>0.38</td>
</tr>
<tr>
<td>Hyphal length</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All control methods</td>
<td>5.09</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Table 4 Percent spotted knapweed cover in plots receiving different management treatments
(average of 3 plots in each block).

<table>
<thead>
<tr>
<th>LSD</th>
<th>Treatment Description</th>
<th>Mean % cover</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated (control)</td>
<td>53.7</td>
<td>1.45</td>
</tr>
<tr>
<td></td>
<td>Handpulled (3 subtreatments combined)</td>
<td>46.7</td>
<td>9.26</td>
</tr>
<tr>
<td></td>
<td>Mowed (2 subtreatments combined)</td>
<td>46.0</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>Picloram (spring '97) and handpulled annually</td>
<td>1.30*</td>
<td>5.29</td>
</tr>
<tr>
<td></td>
<td>Picloram (fall '97) and mowed ('97)</td>
<td>0.70*</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>Clopyralid + 2,4-D (spring '97) and mowed ('97)</td>
<td>9.00*</td>
<td>4.16</td>
</tr>
<tr>
<td></td>
<td>Picloram (spring '97)</td>
<td>0.00*</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Picloram (fall '97)</td>
<td>6.00*</td>
<td>2.52</td>
</tr>
<tr>
<td></td>
<td>Clopyralid + 2,4-D (spring '97)</td>
<td>2.70*</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>Clopyralid + 2,4-D (fall '97)</td>
<td>24.0*</td>
<td>6.56</td>
</tr>
</tbody>
</table>

* indicates significant reduction in percent spotted knapweed cover compared to control (P≤0.05)
Figure 1. Arbuscular mycorrhizal hyphal length measured from soils treated with different management methods for spotted knapweed. Standard errors of the mean are shown (n=3). ANOVA p-values are presented in Table 1; contrast comparison p-values are presented in Table 3.
Figure 2. Aggregate water stability (1-2 mm soil aggregates) measured from soils treated with different management methods for spotted knapweed. Standard errors of the mean are shown (n=3). Chi-squared p-value is presented in Table 1.
Figure 3. Concentrations of different glomalin fractions (total soil) measured from soils treated with different management methods for spotted knapweed. Standard errors of the mean are shown (n=3). ANOVA p-values are presented in Table 1; contrast comparison p-values are presented in Table 3.
Figure 4. Concentrations of different glomalin fractions (1-2 mm aggregates) measured from soils treated with different management methods for spotted knapweed. Standard errors of the mean are shown (n=3). ANOVA p-values are presented in Table 2; contrast comparison p-values are presented in Table 3.
Figure 5. Regression of the concentration of TG (total soil) with percent spotted knapweed cover. $R^2$ value and linear equation are shown. Vertical error bars represent standard errors of mean TG concentrations. Horizontal error bars represent standard errors of mean percent spotted knapweed cover. Inset: Regression of the concentration of EEG (total soil) with percent spotted knapweed cover. $R^2$ value and linear equation are shown.
Chapter 2: Interannual and seasonal variability in effects of spotted knapweed on glomalin concentrations in soil

Abstract

Spotted knapweed is an invasive mycorrhizal weed particularly prevalent in the Pacific Northwest of the United States. Very little is known about how spotted knapweed or management methods for spotted knapweed affect soil properties over time, specifically during drought and wet years. This field study examined changes in the arbuscular mycorrhizal protein glomalin (used as an indicator of soil aggregation) in areas with dense, unmanaged spotted knapweed infestations and knapweed managed areas over a time period that included both drought and wet years. Interannual and seasonal variability in glomalin concentrations due to spotted knapweed and herbicide treatment effects were apparent. These results stress the importance of monitoring invasive weed and management method effects on ecosystem or soil properties over an extended period of time.

Introduction

Spotted knapweed, *Centaurea maculosa* Lam., is an invasive plant from Eurasia that has invaded much of the Pacific Northwest in the United States. As an aggressive weed, spotted knapweed competes with native vegetation, often reducing native plant populations. Livestock and wildlife forage are reduced and surface water runoff, soil erosion, and stream sediments increase in areas where spotted knapweed has invaded (Jacobs and Sheley 1998; Lacey et al. 1989).
Several methods have been developed to manage spotted knapweed, including the application of herbicides. Herbicide applications have been found to be highly effective in reducing spotted knapweed, returning plant communities back to a grass-dominated structure (Rice et al. 1997). Few studies have examined the effects of herbicide treatments for spotted knapweed on the structure and diversity of native plant communities through time (Rice et al. 1997), and possible effects of these herbicide treatments on the soil environment, specifically soil structure, through time have not been examined. Changes in effects of spotted knapweed invasion on soils due to seasonal changes, including those of drought, also have not been thoroughly examined.

This study investigated how spotted knapweed affects glomalin concentrations over time. Glomalin is a glycoprotein produced by arbuscular mycorrhizal fungi (AMF). Glomalin concentrations in soil aggregates are highly correlated with the percentage of water-stable aggregates (WSA) (e.g., Wright and Upadhyaya 1998), a major determinant of soil structure (Schreiner and Bethlenfalvay 1995, Thomas et al. 1993). In this study, concentrations of glomalin were measured in knapweed invaded and managed soils as a critical indicator of soil structure during a 21 month period.

**Materials and Methods**

**Field experiment and sampling**

The experimental site was located at Lolo National Forest, in the Blue Mountain Recreation Area, near Missoula, Montana (46° 45' 00" N, 114° 07' 30" W). The site is a grassland area characterized as an upland range dominated by bluebunch wheatgrass (*Agropyron spicatum*), rough fescue (*Festuca scabrella*), and Idaho fescue (*Festuca*...
idahoensis) (Brown et al. 1999). The area is invaded by spotted knapweed and occurs in a loamy soil. The experiment consisted of the application of different management methods for spotted knapweed in plots (6 x 9 m) arranged in a randomized complete block design containing three blocks based on slope position (n=3). Untreated plots (experimental controls), where no management methods for spotted knapweed were applied and invasion was dense (Brown et al. 1999), were also established (n=3). Herbicide treatments were focused on in this study because of the considerable reduction in spotted knapweed cover in areas treated with herbicides (C. Duncan, personal communication).

Herbicide treatments included applications delivered in the spring or fall of 1997. Herbicide applications in this study included picloram at 0.28 kg ai ha\(^{-1}\) (spring 1997) and 0.14 kg ai ha\(^{-1}\) (fall 1997); and clopyralid plus 2,4-D at 0.21 kg ai ha\(^{-1}\) plus 1.12 kg ai ha\(^{-1}\) (spring 1997) and 0.67 kg ai ha\(^{-1}\) plus 1.12 kg ai ha\(^{-1}\) (fall 1997). Herbicides were applied using a CO\(_2\)-pressurized backpack sprayer equipped with a 3.1 m, 6-nozzle spray boom, calibrated to deliver 150 liters per ha.

Samples were extracted from plots at the study site five times: September 15, 2000; April 24, 2001; June 15, 2001; September 1, 2001; and June 25, 2002. In September 2000, five soil cores (2 cm diameter) were extracted from each plot. These soil cores from the September 2000 sampling were physically combined and thoroughly mixed in polyethylene bags, dried in a drying oven at 80° C overnight, and then stored at room temperature until analysis. Soil samples from the subsequent sampling times were extracted systematically across the width of each plot, with each core stored in separate polyethylene bags and analyzed separately. The results from the five subsamples from
each plot were averaged prior to applying statistical tests. Soil cores were taken systematically to ensure representation of the entire plot in all analyses.

**Glomalin measurements**

Two fractions of glomalin were extracted from the soil samples: easily extractable glomalin (EEG) and total glomalin (TG) (Wright and Upadhyaya 1996). The EEG fraction was extracted with 20 mM sodium citrate, pH 7.0 at 121° C for 30 minutes. Following the EEG extraction, the TG fraction was extracted with 50 mM sodium citrate, pH 8.0 at 121° C for subsequent 60 minute cycles, until the extracted supernatant showed none of the red-brown color typical of glomalin. Both fractions of glomalin were analyzed using the Bradford Protein Assay (Bio-Rad, Melville, NY).

**Statistical analysis**

Data were analyzed using linear contrasts (JMP 3.1.6.2, SAS Institute, Cary, USA) between areas untreated for spotted knapweed and all herbicide treated areas (P<0.05) to determine differences between untreated, dense spotted knapweed areas and those areas managed with herbicides.

**Results and Discussion**

Seasonal differences in glomalin concentrations due to spotted knapweed were observed. Glomalin concentrations (both EEG and TG fractions) from areas with dense spotted knapweed and areas receiving herbicide treatments varied within a growing season (April 2001 to September 2001) (Figures 6 and 7). Overall, EEG concentrations
decreased from April to September 2001, and TG concentrations increased slightly from April to September 2001.

Spotted knapweed effects on glomalin concentrations measured from untreated and managed soil were apparent in September 2000 in the EEG fraction (P<0.05, Figure 6), with glomalin concentrations being lower in the untreated areas (spotted knapweed cover=53.7%; C. Duncan, personal communication) compared to herbicide treated areas (spotted knapweed cover=8.2%; C. Duncan, personal communication). Although all herbicide treated areas combined had significantly higher glomalin concentrations than untreated areas, no area treated with a particular herbicide treatment had continuously higher or lower glomalin concentrations compared to other treatments (Figure 6). Spotted knapweed effects were not significantly different among treatments in the TG fraction in September 2000 (P=0.27, Figure 7). Knapweed effects on the EEG or TG fractions were not significantly different in April, June, or September 2001 (Figures 6 and 7). Knapweed suppression effects (managed areas) on glomalin concentrations disappear during these months, with glomalin concentrations from each treatment, including untreated areas, becoming almost equivalent by September 2001. In June 2002, spotted knapweed effects were again evident, with untreated areas containing the lowest EEG and TG concentrations (TG, P=0.08 and EEG, P<0.05; Figures 6 and 7). Therefore, it appears that variations in glomalin concentrations due to spotted knapweed vary among years.

Western Montana, including the Missoula area, experienced drought conditions in 2000 and 2001. On a statewide basis, August 2001-April 2002 ranked as the second driest August-April in the 108-year record (National Climatic Data Center 2002).
Precipitation decreased to 0.81 cm by September 2001 (inset, Figure 6). The collapse in spotted knapweed and knapweed suppression effects on glomalin concentrations corresponds to decreases in precipitation (inset, Figure 6; Figures 6 and 7). With the return of more precipitation by June 2002, knapweed and knapweed suppression effects returned in both the EEG and TG fractions.

The time of treatment applications is discontinuous with the sampling period of this study. Treatments were applied three years prior to sampling. However, reduction in spotted knapweed density in herbicide treated areas continued throughout the sampling period (C. Duncan, unpublished), allowing for comparison between dense spotted knapweed areas (untreated) to areas with very little spotted knapweed (herbicide treated).

It is apparent that seasonal and interannual variability of spotted knapweed effects existed during the sampling period. This variability appears to correspond to precipitation data, indicating that knapweed effects vary depending on drought conditions. The largest increase in glomalin concentrations occurred between September 2001 and June 2002. The increase in total glomalin is unexpectedly large. To corroborate the increase in TG concentration between September 2001 and June 2002, arbuscular mycorrhizal extraradical hyphae were measured in soil from untreated areas, since AMF produce glomalin. Hyphal length doubled from September 2001 to June 2002, 23.2 m g⁻¹ soil to 42.6 m g⁻¹ soil, which likely explains the increase in glomalin concentrations between these two time points.

The amount of glomalin in the soil is an indicator of soil structure. Soil structure mediates many biological and physical processes in the soil ecosystem, such as water filtration, soil aeration, biogeochemical cycling, and susceptibility to erosion (Oades
Changes in soil structure are thus important to monitor since soil structure plays such an important role in ecosystem function. Significant interannual and seasonal variability of spotted knapweed effects can be affected by drought or wet years. Our findings stress the importance of determining effects of spotted knapweed and management methods on soil structure over multiple years due to this variability.
Literature Cited


Lacey JR, Marlow CB, Lane JR (1989) Influence of spotted knapweed (Centaurea maculosa) on surface runoff and sediment yield. Weed technology 3: 627 - 631


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Figure 6. Concentrations of easily extractable glomalin (EEG) fraction measured over time from soils treated with herbicides to manage spotted knapweed. Standard errors of means are shown (n=3). Asterisks (*) indicate significant differences between untreated and chemically treated areas (P<0.05, linear contrast). Inset shows change in precipitation and temperature over the sampling period.
Figure 7. Concentrations of total glomalin (TG) fraction measured over time from soils treated with herbicides to manage spotted knapweed. Standard errors of means are shown (n=3).
Chapter 3: Seasonality of arbuscular mycorrhizal hyphae and glomalin concentrations in a spotted knapweed invaded grassland

Abstract

In order to more fully understand the basic biology of arbuscular mycorrhizal fungi (AMF) and their role in natural ecosystems, it is necessary to document seasonal changes of various aspects of the life history of these fungi. Due to their unique position at the root-soil interface, AMF have been described as “keystone mutualists” in ecosystems. Despite the importance of AMF in ecosystems, few studies exist that examine the seasonality of external hyphae and their exuded products (e.g. glomalin), the AMF parameters directly related to ecosystem function through their contributions to soil aggregation. This study examined seasonal dynamics of several soil parameters, with a specific interest in the seasonality of external hyphae and glomalin, a glycoprotein produced by AMF fungi (which is correlated with soil aggregate stability). Here we measured glomalin concentrations and external AMF and non-AMF hyphal length, as well as soil moisture, percent fungal colonization (AMF and non-AMF), and root length in soil in an intermountain grassland in western Montana over one growing season (13 time points). Of the glomalin pools and hyphal lengths measured, significant seasonal changes occurred for total glomalin (TG; 24.5% change), immunoreactive easily extractable glomalin (IREEG; 53.8% change), and AM hyphal length (107% change). Prior studies on glomalin in natural systems have not considered seasonal effects on the measured glomalin. The seasonality of glomalin values observed in this study highlights the importance of implementing a sampling regime that will capture this seasonal
variation. These results also provide valuable information for the development of future studies in this type of natural ecosystem.

**Introduction**

AMF are obligately biotrophic fungi that are closely associated with both host plants and the soil environment, functioning as a quasi-extension of the root system, and having numerous effects on plant physiology and plant communities (e.g. Allen 1991, Smith and Read 1997, van der Heijden et al. 1998). AMF play an integral role in the translocation of carbon to soil, having direct access to root carbon (Smith and Read 1997); due to their unique position at the root-soil interface, AMF have been described as “keystone mutualists” in ecosystems (O’Neill et al. 1991).

In order to more fully comprehend the role of AMF in natural ecosystems, as well as their basic biology, it is important to document seasonal changes of various aspects of the life history of these fungi. While numerous studies have examined seasonality of AMF spore production and root colonization (e.g., Gay et al. 1982; Anderson et al. 1984; Johnson et al. 1991; Sanders and Fitter 1992; and Mullen and Schmidt 1993), studies examining the seasonality of AMF extraradical hyphae and their exuded products are sparse. Although fungal spore production and root colonization are important for elucidating fungal life histories (Hart and Reader 2002), neither of these characteristics directly relate to ecosystem function. Conversely, extraradical hyphae and their products, such as glomalin, can be directly related to ecosystem processes, e.g. by virtue of their contributions to soil aggregate stability (Jastrow and Miller 1997; Wright and Upadhyahya 1998). More specifically, AMF contribute to soil aggregation through the
hyphal entanglement process, assisting in soil aggregate formation (Jastrow and Miller 1997), and through the production of extracellular polymeric compounds on hyphal surfaces, which can sorb to inorganic materials, helping to stabilize soil aggregates (Jastrow and Miller 1997). As an example, extraradical hyphae of AMF produce glomalin, a glycoprotein that is highly correlated with the percentage of water-stable aggregates (WSA) in soil (Wright and Upadhyahya 1998; Rillig et al. 2001).

To our knowledge, only two studies have examined the seasonality of extraradical hyphae in the field, and only one of these studies examined hyphae from a natural system. Kabir et al. (1997) examined the seasonal changes of extraradical and intraradical arbuscular mycorrhizal hyphae affected by tillage and fertilization in an agricultural soil over a growing season (n=4). Abundance of AM hyphae fluctuated significantly within a growing season, with lowest hyphal densities found in the spring. Seasonal variation in mycorrhizal root colonization followed corn plant development, increasing up to silking and decreasing thereafter. Miller et al. (1995) examined external hyphal production and its relation to gross root morphology (specific root length: SRL) over a season in two temperate grassland communities (n=4). SRL was strongly associated with external hyphal lengths, where root systems with low SRL had greater lengths of external hyphae. From these studies it is clear that important seasonal patterns in extraradical hyphal length may exist, but the data base is far too small to draw firm conclusions. Seasonal dynamics of AMF hyphal products, such as glomalin, in the soil are unknown.

The field study described herein examines the seasonality of AMF extraradical hyphae and glomalin in a spotted knapweed invaded grassland. To relate these
parameters to intraradical colonization, we also measured AMF root colonization. Total root length and the root length of two root diameter size classes were measured to examine the relationship of hyphae and their products to plant root morphology.

Materials and Methods

Site description

This research site was located in a grassland with an initial invasion of spotted knapweed approximately 1 km north of Missoula, Montana, in the North Hills area. The plant community of this area is an Idaho fescue/bluebunch wheatgrass community type (Festuca idahoensis/Agropyron spicatum) (Mueggler and Stuart 1980). The soil at this site is a sandy loam with pH 6.6 (Table 5).

Field experiment and sampling

Soil cores (2 cm diameter) were extracted systematically along a 5-meter transect, with 3-4 soil samples taken within a 15 x 15 cm area and pooled every meter (n=5). Samples were taken beginning on May 13, 2001, and then approximately bi-weekly until the last sampling date, November 30, 2001, for a total of 13 time points. Samples were repeatedly taken within the same 15 x 15 cm area and 5-m transect through time. Gravimetric soil moisture was determined on a subsample of soil (5 g) from each sample at each time point. All soil samples were then dried overnight at 70 °C. Soil samples were sealed in polyethylene bags and stored at −20 °C until analysis.
Monthly precipitation and temperature data were obtained from the Missoula International Airport weather station, approximately 11 km southwest of the study site (Western Regional Climate Center and National Climatic Data Center).

Extraradical hyphal and glomalin measurements

Extraradical hyphae were extracted from soil samples (4 g) using an aqueous extraction and membrane filtration method (Rillig et al. 1999). Arbuscular mycorrhizal (AM) hyphae were distinguished from non-mycorrhizal hyphae at 200x magnification using similar criteria to Miller et al. (1995). Hyphal length was determined using the line intersect method as described in Jakobsen et al. (1992) and Tennant (1975).

Two detection methods are used to quantify glomalin: the Bradford protein assay, yielding the easily extractable glomalin (EEG) and the total glomalin (TG) fractions, and an ELISA assay (employing the monoclonal antibody developed against crushed spores of Glomus intraradices; Wright and Upadhyahya 1998), yielding the immunoreactive easily extractable glomalin (IREEG) and immunoreactive total glomalin (IRTG) fractions. These glomalin fractions are operationally defined based on their extractability/solubility and detection methods (much like other soil fractions, such as humic acids). While the ELISA assay is a very specific detection method for glomalin, the more general Bradford protein assay is also utilized. This protein assay may capture glomalin protein that has undergone small (perhaps microbially-mediated) changes, possibly resulting in the destruction or concealment of the epitope for the monoclonal antibody. Because of well-documented and strong correlations with soil aggregate
stability (e.g. Wright and Upadhyahya 1998), these glomalin fractions continue to be quantified.

Glomalin extractions from soil (1 gram) were carried out as described by Wright and Upadhyahya (1998). The EEG fraction was extracted with 20 mM sodium citrate, pH 7.0 at 121 °C for 30 minutes. Following the EEG extraction, the TG fraction was extracted with 50 mM sodium citrate, pH 8.0 at 121 °C for 60 minute cycles until the supernatant showed none of the red-brown color typical of glomalin. Both fractions of glomalin were analyzed using the Bradford Protein Assay (Bio-Rad, Melville, NY). The glomalin fractions were further analyzed using an enzyme-linked immunosorbent assay (ELISA) using the monoclonal antibody MAb32B11. After all glomalin analyses were completed, four fractions of glomalin values were obtained: EEG, TG, IREEG, and IRTG.

**Root extraction and quantification**

Roots were removed from the soil samples by a hand flotation and sieving method modified from Cook et al. (1988) and Miller et al. (1995). A 10 g subsample from each soil sample was soaked in 100 ml of tap water and 20 ml sodium hexametaphosphate (35 g L⁻¹) for 30 minutes. The soil suspension was then added to 880 ml tap water (total volume: 1000 ml), manually agitated to suspend roots, and poured through 212 μm and 0.5 mm sieves to retain roots. This process was repeated five times with all soil samples to maximize retrieval of roots. Separated roots were washed with tap water several times to remove any attached soil. Obvious organic material and other debris were removed. Extracted roots were dried in a drying oven at 70 °C overnight and stored at room
temperature until analysis. Total root length and root lengths of two root diameter size classes, fine roots (>0.25 mm diameter) and very fine roots (<0.25 mm diameter), were measured using the Win-Rhizo V. 3.10B root image analysis system (Régent Instruments Inc, Québec, Canada). Total root length colonized by AM hyphae was calculated by multiplying root length by percent AM hyphal colonization for each sample. The efficiency of the root extraction procedure was determined by re-extracting soils (n=3). Any visible roots were removed from the dried soil and measured using the Win-Rhizo root image analysis system. Extraction efficiency was determined to be 98%.

**Percent AM root colonization**

Roots were cleared in 10% KOH for one hour, acidified with 1% HCl for 15 minutes, and then stained with trypan blue. Roots were left in lactoglycerol overnight, and then 1 cm pieces were placed on microscope slides for analysis. Percent colonization was measured by the gridline intersect method as described by Rillig et al. (1999).

**Data analysis**

All response variables were analyzed first using repeated measures multivariate analysis of variance by GLM procedures of SPSS statistical software (SPSS Inc., version 11.0.1, 2001) since these variables cannot reasonably be assumed to be independent (Scheiner and Gurevitch 1993). The effect of time on each response variable was then determined using univariate repeated measures analysis of variance by GLM procedures of SPSS statistical software (SPSS Inc., version 11.0.1, 2001). All response variables were tested with the adjusted Huynh and Feldt (1976) F-test (P< 0.05). Pearson product-
moment correlations ($r$) on the means ($n=13$) were determined in the procedure of JMP (version 3.1.6.2, 1996). The coefficient of variation (cov) was calculated by dividing the standard deviation of means by the grand mean for each response variable. The percent change for response variables was calculated as
\[
\frac{(\text{mean } X_{\text{max}} - \text{mean } X_{\text{min}})}{\text{mean } X_{\text{min}}} \times 100.
\]

Results

Repeated measures multivariate analysis of the response variables measured revealed a significant difference (RM-MANOVA: $F_{130.118} = 2.413$, $P<0.001$), justifying further analysis of individual response variables.

Monthly mean temperature and precipitation data for Missoula, MT in the year 2001 and long-term averages are presented in Table 6. In 2001, Montana experienced severe drought conditions (Montana Drought Monitoring 2001). The mean percent soil moisture for the study site at each sampling time is presented in Figure 8. Variation in the mean percent soil moisture through time was significant (Table 7).

The concentration of TG fluctuated significantly through time (Table 7), with a 24.5% change between the lowest and highest average concentrations. The low coefficient of variation indicates that individual samples did not vary greatly from the overall mean. There was an increase in TG concentration from late May to June (Julian day 145 to 162), followed by a general downward trend until early November (Julian day 307; Figure 9). In November, TG concentrations increased until the end of the sampling period (Figure 9). Conversely, there were no significant differences in the EEG fraction (Table 7).
Variation in the IREEG fraction, but not the IRTG fraction, was significant through time (Table 7), and also had a low coefficient of variation (Figure 10). There was a 53.8% change between the lowest and highest average concentrations of IREEG. An initial decrease in the concentration of IREEG occurred from May to June (Julian day 133 to 162), with IREEG concentrations generally rising thereafter until October (Julian day 286). After October, the concentration of IREEG again decreased (Figure 10). The IRTG pattern through time was similar to that of the IREEG fraction (Figure 10). However, due to large error bars of some IRTG data points, there was not enough power to observe a significant variance of the IRTG concentrations through time.

External arbuscular mycorrhizal (AM) hyphal length varied significantly through time (Table 7), with a large increase from late September to early October (Julian day 265 to 286), followed by a decrease in hyphal length through November (Julian day 286 to 334; Figure 11). The decrease in AM hyphal length at the end of the sampling period was similar to the decrease observed in the IREEG fraction. The percent change between the highest and lowest AM hyphal lengths measured was 107%. Non-AM hyphal length did not vary significantly through time (Table 7).

Percent AM hyphal colonization decreased in November (Julian days 307 to 334), similar to the decrease in external AM hyphal length (Figures 11 and 12; Table 7). Percent vesicle colonization did not vary significantly, while percent arbuscule colonization varied significantly through time (Table 7), with great fluctuation from May to the beginning of August (Julian days 133 to 215), followed by a considerable decrease (Julian day 230; Figure 12) until the end of the sampling period, with colonization leveling out at 2% (Figure 12).
Fine root length (>0.25 mm diameter) changed significantly through time, while very fine root length (<0.25 mm diameter) and total root length did not (Table 7). A decrease in fine root length occurred mid to late May (Julian day 133 to 145), followed by a general increase in root length until late September (Julian day 265; Figure 13). Fine root length then decreased from September to the end of November (Julian day 265 to Julian day 334; Figure 13). This decrease was similar to the decrease observed in AM and glomalin parameters (Table 7; Figures 10, 11, and 12).

AM colonized root length remained constant until July, when AM colonized root length increased and then decreased by August (Julian day 182 to 215; Figure 14). Thereafter, there was an increase until the end of September (Julian day 251), followed by a decrease through the end of the sampling period (Figure 14). Variation in root length colonized by AM hyphae through time was significant (Table 7).

Pearson product-moment correlations ($r$) for glomalin fractions were strongest between IREEG and root length parameters. IREEG was positively correlated with total root length ($r=0.82$, $P<0.01$), fine root length ($r=0.79$, $P<0.01$), and very fine root length ($r=0.80$, $P<0.01$).

**Discussion**

The main objective of this study was to test whether seasonal changes in glomalin fractions occur over a growing season. In a previous study, glomalin had a long turnover time (up to 40 years), much longer than the turnover time expected for AM hyphae (Rillig et al. 2001; Friese and Allen 1991). In a lab incubation comparing glomalin and AMF extraradical hyphae decomposition, glomalin concentrations only decreased by
25% over 150 days, while AMF extraradical hyphal length declined 60% (Steinberg and Rillig 2003). Currently there is no information on the seasonality of glomalin.

Concentrations of the TG and IREEG glomalin fractions changed significantly through time, while the EEG and IRTG fractions did not. This is the first report of seasonal changes of glomalin pools. At this time, it is unclear how these operationally defined glomalin pools differ from each other in terms of biochemistry, function in soil, and age, or how the production of glomalin is controlled (Rillig et al. 2001). The patterns of change in the TG and IREEG concentrations through time are generally dissimilar.

Several fungal parameters fluctuated significantly through time in the soil studied. External AM hyphal length changed significantly over the growing season, with a decrease in November. This decrease is similar to the decrease noted in the IREEG fraction. Hyphal length values measured in this study were fairly high, with the highest hyphal length measured to be 82 m g⁻¹ soil in October, and an average hyphal length throughout the season of 50 m g⁻¹ soil. Our hyphal length values were higher than hyphal lengths from several studies reported in Smith and Gianinazi-Pearson (1988). It is not uncommon for hyphal lengths to vary considerably from study to study, due to varying fungal species and different geographic areas (Smith and Gianinazi-Pearson 1988). However, external hyphal lengths from this study are consistent with hyphal lengths measured in a different intermountain grassland in western Montana (Lutgen and Rillig submitted), where hyphal lengths reached 45 m g⁻¹ soil.

Percent AM and non-AM hyphal colonization in roots decreased in November, similar to the decrease in AM hyphal length. However, the decrease in fungal hyphal colonization begins earlier in the sampling period, during August. A dramatic decline in
percent AM arbuscule colonization also occurs at this time. It is likely that plants were stressed from the drought conditions during the sampling period, and the carbon allocation from plant to fungus may have been diminished as a consequence. Because AM arbuscules are the structures involved in nutrient exchange with plants, the drought response in plants could be reflected quite dramatically in percent arbuscule colonization.

Root length parameters measured did not exhibit much significant change through time, with only the fine root length (>0.25 mm diameter) parameter changing through time. The majority of root length measured was found in the very fine root length (<0.25 mm diameter) parameter.

Fine root length dynamics somewhat follow the dynamics of AM fungal parameters over the growing season. Both root and fungal parameters experience a decrease in late summer (August/September). However, fine root length begins a decline in September, approximately two weeks later than the decline in AM fungal parameters. Again, this decline is similar to that observed in the IREEG protein fraction.

Interestingly, the trade-off experienced by plant root systems between the production of fine roots and external hyphal associations (Miller et al. 1995) was not observed. For example, an increase in external AM hyphal length did not occur when fine root length decreased. Instead, both parameters seemed to decline together. Although mycorrhizal infections are generally considered beneficial, mycorrhizal fungi can represent a carbon drain on plants (Allen 1991). In fact, it has been found that some species of arbuscular mycorrhizae can actually reduce plant tolerance to drought (Allen and Boosalis 1983). By late summer during the sampling period, both plant and fungus could have suffered
from a carbon deficit due to the severe drought conditions experienced during the sampling period.

Correlations between glomalin fractions and root parameters were only observed between the IREEG fraction and all root length parameters. As previously mentioned, it is unclear how these operationally defined glomalin pools differ from each other. It has been suggested that the EEG and IREEG pools are more readily available in the soils, either by being the most recently deposited fractions (Wright and Upadhyahya 1998) or by being the most recently decomposed fractions (Steinberg and Rillig 2003). It has been suggested that degradation of glomalin in the soil also decreases its adsorption to soil particles (Steinberg and Rillig 2003).

Plants produce more roots during certain times in a growing season due to environmental factors. During this active root production, plant roots release exudates that are a labile source of carbon for the soil microbial community, enhancing the environment for decomposers (saprobes and bacteria). Priha et al. (1998/1999) found that two species of trees had a stimulative effect on soil microbes, possibly due to the tree species having more roots and releasing more root exudates in soil. Perhaps root production in the current study (represented here by root length) served as a priming effect for the microbial community by producing abundant exudates and enhancing the environment for decomposers. As a result, these abundant decomposers degraded the more readily available EEG and IREEG fractions, decreasing their adsorption to soil particles. Hence, an increase in root length may have resulted in an increase in either of these fractions, as observed with the IREEG fraction.
The sampling schedule for this study was quite intense (soil cores extracted bi-weekly from May through November 2001; 13 time points) compared to other field studies on AM hyphal dynamics, particularly those that measured external hyphal length (four time points; Jastrow et al. 1995, Kabir et al. 1997). Our sampling frequency allowed for a more detailed resolution of the seasonal patterns of AM hyphal dynamics and glomalin pools.

Although glomalin has a slow turnover time in soil, it appears that some glomalin fractions can fluctuate through a growing season. Changes in AM fungal and root parameters did not exactly match changes observed in glomalin concentrations. However, the decline in IREEG concentration in November is comparable to the general decrease in AM fungal and root parameters in late summer through the end of the sampling period. The overall lack of correlation between AM fungal parameters and glomalin is important, as it points out that glomalin fractions, as currently defined, may not be useful as indicators of AMF hyphal length and fungal activity in general.

Although this study was conducted only over one growing season, it is the first study to examine glomalin concentrations (an AM hyphal product) over a growing season in an intermountain grassland community. Every study on glomalin in natural ecosystems thus far has not considered the importance of seasonal fluctuations in glomalin (since only one time point was examined). Our study indicates that, while fluctuations in this seemingly stable soil pool are relatively small, they were nevertheless significant in our study. Hence a sampling regime capturing seasonal variation may have to be employed in future studies measuring glomalin.
Literature Cited


Steinberg P D and Rillig M C 2003 Differential decomposition of arbuscular mycorrhizal
fungal hyphae and glomalin. Soil biology and biochemistry, in press.


Table 5. Physical and chemical properties of soils* at the North Hills field site, Missoula, MT.

<table>
<thead>
<tr>
<th></th>
<th>Percent</th>
<th>ppm</th>
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<tbody>
<tr>
<td>Organic matter</td>
<td>5.72</td>
<td></td>
</tr>
<tr>
<td>Sand</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Silt</td>
<td>21.50</td>
<td></td>
</tr>
<tr>
<td>Clay</td>
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<tr>
<td>Kjeldahl N</td>
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<tr>
<td>Nitrate-N</td>
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<tr>
<td>Olsen P</td>
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</tr>
<tr>
<td>K</td>
<td>358.2</td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td>39.1</td>
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</tr>
<tr>
<td>CEC (meq/100 g)</td>
<td>13.50</td>
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* determined by South Dakota State University Soil Testing Laboratory
Table 6. Monthly mean temperature and precipitation data for Missoula, MT for 2001 and long-term (LT) averages.

<table>
<thead>
<tr>
<th>Month</th>
<th>Temperature (°C)</th>
<th>Precipitation (cm)</th>
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<tr>
<td></td>
<td>2001</td>
<td>LT average</td>
</tr>
<tr>
<td>January</td>
<td>-5.5</td>
<td>-5.2</td>
</tr>
<tr>
<td>February</td>
<td>-5.1</td>
<td>-1.6</td>
</tr>
<tr>
<td>March</td>
<td>2.9</td>
<td>2.1</td>
</tr>
<tr>
<td>April</td>
<td>6.0</td>
<td>6.8</td>
</tr>
<tr>
<td>May</td>
<td>12.6</td>
<td>11.0</td>
</tr>
<tr>
<td>June</td>
<td>14.6</td>
<td>15.6</td>
</tr>
<tr>
<td>July</td>
<td>19.2</td>
<td>19.3</td>
</tr>
<tr>
<td>August</td>
<td>21.2</td>
<td>18.8</td>
</tr>
<tr>
<td>September</td>
<td>16.3</td>
<td>13.2</td>
</tr>
<tr>
<td>October</td>
<td>6.8</td>
<td>6.8</td>
</tr>
<tr>
<td>November</td>
<td>2.2</td>
<td>0.2</td>
</tr>
<tr>
<td>December</td>
<td>-3.4</td>
<td>-4.8</td>
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Table 7. F- and P-values from univariate repeated measures analysis of variance for glomalin, arbuscular mycorrhizal (AM), and root parameters. P-values <0.05 are bolded. All response variables were tested with the adjusted Huynh and Feldt F-test.

<table>
<thead>
<tr>
<th>Variable</th>
<th>F</th>
<th>P</th>
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<tr>
<td>Percent soil moisture</td>
<td>109.2</td>
<td>&lt;0.001</td>
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<tr>
<td>Easily extractable glomalin (EEG)</td>
<td>0.942</td>
<td>0.515</td>
</tr>
<tr>
<td>Total glomalin (TG)</td>
<td>2.405</td>
<td>0.016</td>
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<tr>
<td>Immunoreactive easily extractable glomalin (IREEG)</td>
<td>2.097</td>
<td>0.047</td>
</tr>
<tr>
<td>Immunoreactive total glomalin (IRTG)</td>
<td>0.494</td>
<td>0.259</td>
</tr>
<tr>
<td>AM hyphal length</td>
<td>2.416</td>
<td>0.033</td>
</tr>
<tr>
<td>Non-AM hyphal length</td>
<td>1.697</td>
<td>0.097</td>
</tr>
<tr>
<td>Percent AM hyphal colonization</td>
<td>4.678</td>
<td>0.001</td>
</tr>
<tr>
<td>Percent AM vesicle colonization</td>
<td>1.459</td>
<td>0.227</td>
</tr>
<tr>
<td>Percent AM arbuscule colonization</td>
<td>3.414</td>
<td>0.009</td>
</tr>
<tr>
<td>Percent non-AM hyphal colonization</td>
<td>3.361</td>
<td>0.003</td>
</tr>
<tr>
<td>Total root length</td>
<td>1.398</td>
<td>0.200</td>
</tr>
<tr>
<td>Fine root length (&gt;0.25 mm diameter)</td>
<td>3.037</td>
<td>0.003</td>
</tr>
<tr>
<td>Very fine root length (&lt;0.25 mm diameter)</td>
<td>1.299</td>
<td>0.250</td>
</tr>
<tr>
<td>AM colonized root length</td>
<td>2.576</td>
<td>0.028</td>
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</table>
Figure 8. Average percent soil moisture through time (Julian days). Standard errors of the mean (\(n=5\)) and coefficient of variation (cov) are shown. Univariate repeated measures ANOVA p-value is presented in Table 7. \(cov=\)variability of the means (\(n=13\))
Figure 9. Mean concentrations of glomalin fractions through time (Julian days). Note scale and axis break. Standard errors of the mean (n=5) and coefficient of variation (cov) are shown. Univariate repeated measures ANOVA p-values are presented in Table 7. cov=variability of the means (n=13)
Figure 10. Mean concentrations of immunoreactive glomalin fractions through time (Julian days). Note scale and axis break. Standard errors of the mean (n=5) and coefficient of variation (cov) are shown. Univariate repeated measures ANOVA p-values are presented in Table 7. cov=variability of the means (n=13)
Figure 11. Mean external hyphal length of arbuscular and non-arbuscular mycorrhizae through time (Julian days). Standard errors of the mean (n=5) and coefficient of variation are shown. Univariate repeated measures ANOVA p-values are presented in Table 7. cov=variability of the means (n=13)
Figure 12. Average percent colonization of arbuscular mycorrhizal (AM) hyphae, vesicles, and non-AM hyphae through time (Julian days). Standard errors of the mean ($n=5$) and coefficient of variation (cov) are shown. Univariate repeated measures ANOVA p-values are presented in Table 7. cov=variability of the means ($n=13$) Inset: Mean arbuscular colonization through time (Julian days). Standard errors of the mean ($n=5$) and coefficient of variation (cov) are shown. Univariate repeated measures ANOVA p-value is presented in Table 7. cov=variability of the means ($n=13$)
Figure 13. Mean total root length, fine root length (>0.25 mm diameter), and very fine root length (<0.25 mm diameter) through time (Julian days). Note scale and axis break. Standard errors of the mean (n=5) and coefficient of variation (cov) are shown. Univariate repeated measures ANOVA p-values are presented in Table 7. cov=variability of the means (n=13)
Figure 14. Mean arbuscular mycorrhizal fungal (AMF) root length through time (Julian days). Standard errors of the mean (n=5) and coefficient of variation (cov) are shown. Univariate repeated measures ANOVA p-value is presented in Table 7. cov = variability of the means (n=13).