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Longterm effects of grazing on arbuscular mycorrhizal fungi

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ABSTRACT

Arbuscular mycorrhizal (AM) fungi are obligate root symbionts and are thought to help plants tolerate grazing. Evidence shows that grazing can stimulate, inhibit, or have no effect on AM fungi. This inconsistency may be due to empirical limitations, specifically: (i) choice of AM fungal response variable, (ii) confounding effects of soil and plant responses to grazing, and (iii) variation in the duration of studies. To test these hypotheses, we compared AM fungi between grazed and ungrazed grassland plots, with grazing exclosures varying in age from 17 to 85 years. Our findings clearly show that grazing does not universally inhibit AM fungi: grazing had no effect on root colonization plots but spore density was higher and soil hyphal length was lower in grazed plots. While soil and plant variables were unrelated to fungal responses, time since grazing cessation was an important factor explaining the difference between grazed and ungrazed AM fungal communities at a site, indicating possible time lags in responses. Understanding of grazing effects on AM fungi can be enhanced by considering multiple fungal responses and increasing the time scale under which they are studied.

1. Introduction

Managed grazing occurs on more than 25% of the global land surface (Asner et al., 2004) and the sustainability of grazed ecosystems is a serious ecological concern as grazing pressure increases worldwide (Dorrough et al., 2004; Kemp and Michalk, 2007). Understanding factors affecting the sustainability of these systems is imperative if we are to maintain current levels of food production. Grazing tolerance in plants is moderated by many factors, but one of the most important is thought to be the symbiosis formed by arbuscular mycorrhizal (AM) fungi.

AM fungi are obligate root endosymbionts which are known to improve host nutrient relations (Smith and Read, 2008), impart protection against pathogens (Sikes et al., 2009), and help hosts tolerate stressful conditions such as drought (reviewed in Augé, 2001) and salinity (Sharifi et al., 2016). AM fungi have also been shown to improve grazing tolerance for their plant hosts (Bennett and Bever, 2007; Hempel et al., 2009; Kempel et al., 2010; Kula et al., 2005; Schausberger et al., 2012) although others have suggested that AM fungi place an added burden on the plant, rather than improving tolerance (Garrido et al., 2010; Pippo et al., 2011).

There has been considerable research on AM fungi and grazing, but fungal responses to grazing are inconsistent and even contradictory among studies (Hokka et al., 2004; Klironomos et al., 2004; Mikola et al., 2005). The *carbon limitation hypothesis* predicts reduced fungal abundance in response to herbivory due to decreased carbon availability (Wallace, 1987). This hypothesis is supported by studies that show decreased AM fungal colonization following herbivory (Gehring and Whitham, 1994; Frank et al., 2003; Wardle et al., 2002; Barber et al., 2012). However, other studies show an increase in fungal colonization following herbivory (Eom et al., 2001; Hokka et al., 2004; Wearn and Gange, 2007; Keitaro et al., 2012; Techau et al., 2004; Nishida et al., 2009). These increases have been attributed to an increase in carbon exudation from the roots following herbivory (Eom et al., 2001; Hokka et al., 2004; Techau et al., 2004; Nishida et al., 2009), which may improve host access to nutrients by stimulating AM fungi and other organisms in the rhizosphere (Techau et al., 2004; Nishida et al., 2009).

The inconsistency in responses to herbivory may be real – suggesting that grazing effects on AM fungi are governed by a suite of contextual factors. Alternatively, such variation may be simply an artifact of differences in study design. We suggest that the following sources of variability may help to elucidate the relationship between AM fungi and grazing:

a *Multiple measures of AM fungi*: Many studies gauge fungal response using only one variable, typically percent root length colonized, a measure of AM fungal abundance that is relative to the total root length of the plant. However, AM fungi may respond to grazing in multiple ways, including changes in propagule availability, and changes in fungal community composition (Frank et al., 2003; Su and Guo, 2007; Ba et al., 2012).

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b *Abiotic and biotic factors*: Many studies do not consider factors which are altered by grazing, and may, in turn, affect AM fungi. For example, grazing often results in increased nutrient availability (Holland and Detling, 1990; Huntly 1991; Hamilton et al., 2008) increased soil compaction (Wallace, 1987), and altered plant community composition (Wikeem et al., 2012). These factors are known to affect mycorrhizal communities directly and indirectly through plant responses (Jansa et al., 2006). Thus herbivory effects may be greatly moderated by soil conditions or plant community – indirectly affecting fungal response.

c *Time scale*: Some fungal responses, such as percent colonization, may fluctuate over time (Nishida et al., 2009). Others, such as changes in community composition may take years (Shelton et al., 2014).

We conducted a field experiment that accounted for differences among fungal response variables along a chronosequence of grazing cessation in the form of grazing exclosures. Specifically, we tested 1) the effect of grazing on multiple measures AM fungal performance, 2) indirect effects of grazing on AM fungi through a variety of abiotic and biotic factors and 3) how grazing response changes over long time scales.

2. Methods

2.1. Study Site

Nine grazing exclosures (established by the Ministry of Forests, Lands, and Natural Resource Operations in BC, Canada) were selected in May 2015 to have similar vegetation (grasslands), soil type (Black Chernozem), elevation (~1100 m), and grazing history (cattle and horses). The exclosures varied in age from 17 to 85 years (See Table 1) and ranged from 0.5–1 ha in size. Adjacent plots with uninterrupted grazing were used for comparison.

2.2. Soil sampling

In 2015 we took soil samples both inside and outside each exclosure, with all samples 10 m apart and 10 m from the fence. At each point we sampled two soil cores (2.5 cm diameter), one at the surface (0–15 cm) and one at the subsurface (15–30 cm). In total, we collected 180 samples: 9 sites × 2 grazing treatments × 2 depths × 5 replicates. Samples were kept on ice until reaching storage at −20 °C. Samples were dried for two days at 60 °C, homogenized with a 500 µm sieve, and subsampled for DNA extraction and sequencing. Samples were then pooled at the plot level for measurements of soil chemistry and mycorrhizal abundance resulting in 36 soil samples: 9 sites × 2 grazing treatments × 2 depths.

In June 2015 sampling was undertaken to determine soil bulk density. Five undisturbed cores (5 cm diameter) were taken in each plot near the original samples at all sites except Muscrat, which was too rocky. Again, cores were taken at the surface (0–15 cm) and subsurface (15–30 cm). The samples were dried for five days at 40 °C and weighed. The average bulk density was calculated per plot.

Table 1
Description of the nine exclosures used in this study.

Site	Latitude (N)	Longitude (W)	Elevation (m)	Slope	Age*
Muscrat Lk	50° 8′2.15”	120°26′30.45”	1025	30	17
Tunkwa New	50°35′53.45”	120°51′55.15”	1150	4	21
Bluegrass	50° 4′45.27”	120°28′2.70”	1000	0	21
Aspen	50° 4′54.14”	120°25′55.74”	1200	7	37
Stipa rich	50° 3′58.90”	120°26′51.29”	1125	8	42
Stipa nel	50° 4′43.39”	120°26′58.88”	1000	5	48
Tunkwa Old	50°35′58.03”	120°51′57.96”	1150	0	55
Repeater	50° 4′32.08”	120°25′30.02”	1306	0	65
Goose Lk	50° 6′11.59”	120°27′55.19”	1160	5	85

*Age indicates age of exclosure.

2.3. AM fungal responses

2.3.1. Percent root colonization

Only surface samples contained enough root segments to accurately quantify percent colonization. Root segments in dried soil samples were arbitrarily selected for staining, comprising multiple plant species. Washed root segments were cut into 1 cm pieces and stored in 50% ethanol before being cleared then stained with Chlorazol Black E. Percent colonization was determined using the magnified intersection method (McGonigle et al., 1990).

2.3.2. Spore density

Spores were extracted using a wet-sieving technique and counted (Klironomos et al., 1993)

2.3.3. Soil hyphal length (SHL)

was determined using 200 mL of soil suspension combined with 1 mL of a mixture of europium (III) thenoyl-trifluoroacetonate and a fluorescent brightener (Anderson and Westmoreland, 1971). The suspensions were then stained for 1 h and filtered through nitrocellulose filter papers using a 50% ethanol wash. The filters were mounted on microscope slides and computer-imaging software (NIS Elements-Nikon) was used to estimate hyphal length. While soil hyphal length is not a discrete measure of AM fungal abundance, it is considered a robust relative measure of AM fungi in the soil (Miller et al., 1995; Hart and Reader, 2002a).

2.3.4. AM fungal community

DNA was extracted from 125 mg of soil from all 180 soil samples following the protocol of the FastDNA-96™ Soil Microbe DNA (MP Biomedicals) after first drying, sieving and homogenizing the soil. Nested PCR was performed on the samples, first to amplify, then to attach barcodes for Illumina sequencing. In the first PCR, Glomeromycota specific primers WANDA (Dumbrell et al., 2010) and AML2 (Lee et al., 2007) were used to amplify a 500 bp fragment of the small subunit of rDNA. Samples were amplified in a 20 µL reaction consisting of 11.75 µL ddH20, 5 µL 5x PCR buffer (Promega), 1 µL MgCl2 (BioLabs), 0.5 µL dNPTs (Amresco), 0.25 µL BSA (BioLabs), 0.5 µL GoTaq (Promega), and 0.5 µL of each primer. This mixture was heated for 2 min at 95 °C then 34 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C 1 min, finishing with 72 °C for 5 min. Barcodes were added to PCR products in a 20 µL reaction of 9.2 µL ddH20, 4 µL 5 x PCR buffer (Promega), 3.6 µL MgCl2(BioLabs), 0.4 µL dNPTs(Amresco), 0.6 µL BSA(BioLabs), 0.2 µL GoTaq(Promega), and 0.5 µL of each barcode. Cycling conditions were: 1 min at 95 °C, 10 cycles of 30 s at 95 °C, 30 s at 60 °C, 1 min at 68 °C, and ending with 5 min at 68 °C. Samples were frozen and sent to the IBEST Genomics Resources Core at the University of Idaho for sequencing using Illumina Sequencing Technology (Bennett, 2004).

2.4. Sequence analysis

Raw sequences were processed using QIIME (Caporaso et al., 2010). The raw sequence files contained approximately 4.2 million sequences, 3.2 million of which were retained after low quality (Phred quality score of less than Q3), and reads with more than 3 unknown characters were removed. We used USEARCH (Edgar, 2010) and a 97% identity threshold to pick Operational Taxonomic Units (OTUs) using maarjAM database (Opik et al., 2010) as a reference database. This resulted in 28927 OTUs, which was reduced to 351 OTUs after filtering out those comprising less than 0.01% of the data set. Representative sequences of these OTUs were aligned using MUSCLE (Edgar, 2004), then BLASTed (Altschul et al., 1990) against both the maarjAM database (Opik et al.,