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Neighboring plant influences on arbuscular mycorrhizal fungal community composition as assessed by T-RFLP analysis

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Abstract

Controls on root colonization by arbuscular mycorrhizal fungi (AMF) include host nutrient status, identity of symbionts and soil physico-chemical properties. Here we show, in the field, that the subset of the AMF community colonizing the roots of a common grass species, *Dactylis glomerata*, was strongly controlled by neighboring roots of a different plant species, *Centaurea maculosa*, an invasive forb, thus adding a biological spatial component to controls on root colonization. Using an AMF-specific, 18s rDNA-based terminal restriction fragment length polymorphism (T-RFLP) analysis method, significant differences were found between AMF community fingerprints of samples derived from roots of grasses with (G_{Cm}) and without (G_0) neighboring *C. maculosa*. There were also significant differences between samples derived from *C. maculosa* roots (C_{mac}) and both G_{Cm} and G_0 roots. Sample ordination indicated three generally distinct groups consisting of C_{mac}, G_{Cm} and G_0, with G_{Cm} samples being of intermediate distance between G_0 and C_{mac}. Our results indicate that, with the presence of *C. maculosa*, AMF communities of *D. glomerata* shift to reflect community composition associated with *C. maculosa* roots. These results highlight the importance of complex spatial distributions of AMF communities at the scale of a root system. An additional dimension to our study is that *C. maculosa* is an aggressively invasive plant in the intermountain West. Viewed in this light, these results suggest that pervasive influences of this plant on AMF communities, specifically in roots of its competitors, may represent a mechanism contributing to its invasive success. However, further work is clearly required to determine the extent to which AMF genotypic alteration by neighboring plants influences competitive relationships.

Introduction

Arbuscular mycorrhizal fungi (AMF) are integral components of terrestrial ecosystems (Rillig, 2004), forming arbuscular mycorrhiza (AM) associations with most terrestrial plant families. AMF are obligate biotrophs whose hyphae can act as extensions of plant root systems, typically resulting in increased nutrient uptake and plant growth. In addition to increasing inorganic nutrient uptake, especially phosphorus, benefits of AMF to their hosts include enhanced resistance to pathogens and other environmental stresses, as well as improved water relations (Auge, 2000; Borowicz, 2001; Newsham et al., 1995). AMF typically occur in ecosystems as mixed communities with multiple taxa potentially infecting single roots (e.g., Clapp et al., 1995, 1999). Individual AMF species may have a multiplicity of effects on different hosts, promoting growth in one host while inhibiting growth in another (Klironomos, 2003; Streitwolf-Engel et al., 1997; Talukdar and Germida, 1994; van der Heijden et al., 1998, 2003), thereby altering competitive relationships between plants (Hart et al., 2001). Similarly, individual AMF species may differ in

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their own growth response to different host plants (Bever et al., 1996, 1997; Eom et al., 2000).

Several greenhouse studies have demonstrated that AMF presence can significantly alter resource distributions between competing plant species (Callaway et al., 2001, 2003; Marler et al., 1999a; Zabinski et al., 2002). AMF community composition has also been shown to influence competitive relationships between co-occurring plant species (O'Connor et al., 2002; van der Heijden et al., 1998, 2003). Although the manner and extent to which different AMF species interact with different host species is still poorly understood, van der Heijden et al. (1998) and Stampe and Daehler (2003) demonstrated that the identity of AMF present can influence the outcome of plant competitive relationships as much as whether AMF are present or absent.

Despite the apparent importance of AMF diversity as a determinant of plant community structure and, hence, ecosystem function, little is known about how AMF diversity is controlled. Although AMF are not generally thought to exhibit host specificity, recent studies have demonstrated that co-occurring plant species can have very different AMF communities and that host–plant preferences exist (Helgason et al., 2002; Johnson et al., 2003; Vandenkoornhuyse et al., 2002, 2003). In addition, a recent study (Johnson et al., 2003) strongly suggests that AMF community composition is influenced by the identity of the neighboring plants.

The generally appreciated limitations of spore and hyphal morphological characterization of AMF communities has been an obstacle to the study AMF community dynamics within ecosystems. In recent years, molecular biological tools have significantly increased the ability to characterize microbial communities *in situ*. The development of polymerase chain reaction (PCR) primers specifically targeting the *Glomeromycota* (Di Bonito et al., 1995; Helgason et al., 1988; Simon et al., 1992; Van Tuinen et al., 1998) has allowed for culture-independent characterization of AMF communities within both soils and plant roots. Using such approaches, greater diversity has been revealed than could be recognized with methods based on spore morphology and new AMF species have been identified. The majority of these molecular studies have involved cloning and sequencing of rRNA genes. Recently, terminal restriction fragment length polymorphism (T-RFLP) analysis (Liu et al., 1997) has been shown to be of value for discrimination of AMF communities within plant roots (Johnson et al., 2004; Vandenkoornhuyse et al., 2002, 2003).

In the current study, we use T-RFLP analysis to test the hypothesis that the Eurasian forb, *Centaurea maculosa* L. (spotted knapweed), influences the composition of AMF communities infecting roots of a common naturalized forage grass, *Dactylis glomerata* L. Our study can be viewed in light of neighbor-controls on AMF communities: can adjacent plant roots of one species exert a strong influence on the subset of the AMF community colonizing roots of a different species? This phenomenon has been examined at the level of root colonization (Jastrow and Miller, 1993), but not at the higher level of resolution of the plant-associated AMF community.

An additional aspect of our study is that *C. maculosa* is one of the most economically and environmentally destructive weeds in western North America (LeJeune and Seastedt, 2001; Pimentel et al., 2000). Although a number of studies indicate that the presence of AMF strongly influences competitive relationships between *C. maculosa* and Northwestern American grasses (Callaway et al., 2003; Marler et al., 1999a, 1999b; Zabinski et al., 2002), the question of whether *C. maculosa* influences AMF community composition in the roots of grass species it displaces has not been addressed. Such information is a critical step towards understanding the potential mechanistic role of AMF community composition as part of the strategy by which *C. maculosa* attains dominance.

Materials and methods

Field site and sample collection

Root samples were collected from depths between 5 and 15 cm in a gently sloping meadow approximately 10 km north of Missoula, MT. *D. glomerata* is the predominant plant species on the site, although the grasses *Poa pratensis* and *Avena fatua* are represented as minority species. The Eurasian forb, *C. maculosa,* is invading the site and, at this stage, is present primarily in a

number of small patches dispersed across the field. Although both *C. maculosa* and *D. glomerata* are not native to the Pacific N.W., *D. glomerata* is a desirable forage grass intentionally introduced to eastern Montana many times beginning in the 1800s. *C. maculosa* is a noxious weed first observed in Montana in 1920. Since that time it has become widely distributed across the region (INVADERS Database System; http://invader.dbs.umt.edu).

Sampling was conducted in August, 2003 when *C. maculosa* was flowering. Eight *D. glomerata* individuals separated from *C. maculosa* by at least 5 m (G_0) and eight *D. glomerata* individuals in close proximity (<10 cm) to *C. maculosa* (G_{Cm}) were randomly selected. Fine roots from each plant were carefully excavated from the soil for further processing. In the case of G_{Cm}, entwined *D. glomerata* and *C. maculosa* roots were carefully separated and samples of each collected. All samples were placed in sterile plastic bags and transferred to cold storage (−20 °C) within 2 h of sample collection.

DNA extraction from root material

To recover DNA from AMF associated with the roots, each root sample was rinsed with distilled water (diH_2O), surface cleaned ultrasonically for 1 min using the low-energy FS60 ultrasonic system (Fisher Scientific, Pittsburg, PA) to remove attached soil and mycelium, then rinsed again with diH_20 and blotted dry using paper towels. The root DNA extraction method of Bahnweg et al. (1998) was modified for DNA recovery. Between 150–200 mg of fine root material from each sample was immersed in liquid N_2 and ground to powder using a mortar and pestle. To 100 mg of ground root material, 1 mL of 50% (v/v) methanol, 50 μL of 20% (w/v) $CaCl_2$ and 10 μL β-mercaptoethanol were added. This mixture was agitated on ice for 10 min, then concentrated by centrifugation at 4 °C. The supernatant was discarded and 1 ml 50% methanol and 10 μL β-mercaptoethanol were added to the pellet, followed by agitation on ice and centrifugation as before. Supernatants were discarded and 400 μL benzyl chloride, 500 μL extraction buffer (100 mM Tris-HCl, 20 mM EDTA, 1.4 mM NaCl, 2% (w/v) CTAB, pH 8.0), 10 μL β-mercaptoethanol and 20 mg PVP (polyvinyl pyrollidone) were added to the pellet, followed by vortexing for 10 min at 4 °C. Chloroform (300 μL) was then added and the mixture agitated on ice for 5 min, followed by centrifugation as above. The aqueous phase was then precipitated in isopropanol for 2 h to collect DNA. After centrifugation and removal of supernatants, the pellets were washed sequentially with 70% ethanol/0.1 M Na-acetate and then 95% ethanol for 5 min each. After air drying for 30 min, each DNA pellet was resuspended in 100 μL TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA).

T-RFLP analyses

The T-RFLP analysis method involves end-labeling PCR amplicons by attachment of fluorescent molecules to PCR primers. The products of these reactions are digested with select restriction enzymes having specific recognition sequences. Since sequence composition varies between 18S rRNA genes of different AMF species, restriction digestion of mixed community PCR products results in terminal restriction fragments (T-RFs) of variable length. End-labeled T-RFs are separated by electrophoresis on polyacrylamide gel or capillary DNA sequencers and visualized by excitation of the fluor, providing quantitative data about each T-RF detected, including size in base pairs and intensity of fluorescence (peak height).

In this study, PCR products were amplified from genomic templates using the *Glomales*-specific primer AM1 (Helgason et al., 1998), labeled with 5′-FAM, in conjunction with the nearly 'universal' eukaryotic primer NS31 (Simon et al., 1992). Although the AM1 primer site is not well conserved in certain divergent lineages of AMF, such as the *Archaeosporaceae* and the *Paraglomaceae* (Redecker, 2000), AM1 reliably amplifies the three traditional AMF families (*Glomaceae, Acaulosporaceae,* and *Gigasporaceae*), whilst excluding known non-target plant and fungal DNA. The specificity of this primer set has previously been demonstrated in a number of studies (e.g., Daniell et al., 2001; Helgason et al., 1998; Husband et al., 2002; Kowalchuk et al., 2002; Vandenkoornhuyse et al., 2002).

Reaction mixtures (25 μL) included 10 pmol each of primers AM1 and NS31, and 12.5 μL iTaq supermix (BioRad Laboratories, Hercules,

CA). Thermal cycling included an initial denaturing step of 95 °C for 5 min, 30 cycles consisting of 30 s at 95 °C, 1 min at 58 °C and 1 min at 72 °C, followed by a final extension step of 72 °C for 10 min. Amplification reactions were conducted utilizing a PTC-100 thermocycler (MJ Research, Inc., Waltham, MA). PCR products were purified using UltraClean PCR cleanup kits (MoBio Laboratories, Solana Beach, CA). Product quantification and size verification was accomplished using agarose-gel electrophoresis with Low DNA Mass Ladder (Invitrogen, Carlsbad, CA) as the standard. Additional PCR mixtures, including only AM1 or NS31 were analyzed to verify PCR specificity. To further verify product specificity, PCR products generated from root templates were cloned into the pT7Blue-3 plasmid using the Perfect Blunt cloning kit (Novagen, Madison, Wis.). Plasmids were purified from overnight cultures using Qiagen mini-prep kits (Qiagen, Valencia, Calif.) according to the manufacturer's specifications. The insert size of individual clones was confirmed by restriction fragment analysis using *EcoRI*. Clones having inserts of correct size were used as templates in DNA sequencing reactions using vector-specific primers T7 and U19 (Novagen) and subsequently sequenced on a Prism 3100 capillary DNA sequencer (Applied Biosystems, Foster City, CA). Sequences from these analyses were deposited in GenBank under accession numbers AY702061 to AY702066.

Simulated digestion of all available database AMF sequences, as well as AMF sequences cloned in our laboratory, with all available restriction enzymes indicated that restriction enzymes Hinf I, Mbo I and Nla III yielded the most unique T-RFs. We tested both Hinf I and Mbo I on a subset of the samples used in this study. Mbo I yielded greater numbers of T-RF sizes and was used for all subsequent analyses.

Each restriction digestion reaction, containing 8 μL purified PCR product and 2 U Mbo I in the manufacturer's recommended buffer (New England Biolabs, Beverly, MA), was incubated for 4 h at 37 °C. Digestion products were purified using Performa® DTR gel filtration cartridges (Edge Biosystems, Gaithersburg, MD).

T-RF sizes in each sample were determined using an ABI 3100 automated capillary DNA sequencer (Applied Biosystems) with ROX-500 (Applied Biosystems) as the size standard. T-RF size determination and quantification was performed using Genemapper software (Applied Biosystems).

Procedures for analysis of T-RFLP profiles were based on the methods of Dunbar et al. (2001). Briefly, three replicate TRF profiles derived from each root sample were aligned and each TRF size differing by less than 0.5 bp considered of identical size. Since total DNA quantity, and hence total relative fluorescence, can vary between samples or sample replicates, relative fluorescence of each replicate profile was standardized to the smallest quantity by iterative proportional reduction of peaks heights in larger profiles (Dunbar et al., 2001). After proportional reduction of larger profiles, peaks with fluorescence values less than the threshold value (75 relative fluorescence units, RFUs), and peaks not occurring in all three replicates, were eliminated from subsequent analyses. A single standardized T-RFLP profile for each sample was produced by taking the average peak height for each T-RF of all replicates (average deviation for each peak from the replicate mean = 5%, SD = 0.46).

To compare different samples, standardized profiles for all samples were aligned and total relative fluorescence standardized by iterative proportional reduction of peak heights as above.

Data analyses

T-RF sizes and peak heights of each sample were compared using multivariate statistical methods. Initial detrended correspondence analysis indicated that the data exhibited a linear, rather than a unimodal, response to sample origin, justifying the use of linear ordination methods (Leps and Šmilauer, 2003). Therefore, relationships between T-RFLP profiles of samples were evaluated by principal components analysis (PCA), using Canoco software (Microcomputer Power, Ithaca, NY).

A modification of the Mantel test (Kropf et al., 2004; Mantel, 1967), employing the computer program zt (Bonnet and Van de Peer, 2002), was then utilized to determine differences between sample groups. In this analysis, two

matrices were constructed, one consisting of Pearson correlation coefficients for all sample pairs, with the second consisting of 1 or 0, depending upon whether two samples belong to the same group or not. Test statistics were calculated based on Monte Carlo sampling (1000 permutations).

Results

PCR specificity was verified by product absence in single primer PCR reactions, PCR products yielding single, discrete bands of the anticipated size when analyzed by agarose gel electrophoresis, and through cloning and sequencing of PCR products. Comparison with database sequences indicated the seven sequenced clones represented three *Glomus* species and that no non-AMF sequences were detected. Simulated digestion with *Mbo I* resulted in three different anticipated T-RF sizes (281, 442, 443) that correspond to T-RF sizes found in the T-RFLP profiles derived from the same sample.

Overall, a total of 25 different T-RF sizes were detected. The percentage of samples in which each T-RF was detected within the different sample groups is presented in Table 1. Although the number of different T-RF sizes detected was similar between T-RFLP profiles for individual samples, a total of 23, 22 and 18 different T-RF sizes were detected within all T-RFLP profiles derived from C_{mac} roots, G_{Cm}, and G_0, respectively. Five different T-RF size classes were found to be shared by C_{mac} and G_{Cm}, but were not detected in G_0 samples. Conversely, only two T-RF classes that were found in grass derived samples were not detected in C_{mac} samples.

Principal components 1 and 2 accounted for 61% of the total sample variance (39 and 22% for PC1 and PC2, respectively) (Figure 1). Three distinct clusters, comprised of C_{mac}, G_{Cm} and G_0 samples, are apparent, with G_{Cm} samples being of intermediate distance between C_m and G_0 samples. The modified Mantel test indicated significant differences between all three sample groups (G_0 vs. C_{mac}, $r = 0.41$, $P < 0.001$; G_0 vs. G_{Cm}, $r = 0.41$, $P < 0.01$; C_{mac} vs. G_{Cm}, $r = 0.37$, $P < 0.01$).

Table 1. T-RF sizes and the percentage of C_{mac}, G_{Cm} and G_0 samples in which each T-RF class was found

T-RF Size	*C. maculosa*	Grass + *C. maculosa*	Grass
94	100*	100	100
443	87.5	75	100
71	100	12.5	100
302	37.5	37.5	87.5
279	25	50	75
93	100	37.5	75
441	25	37.5	62.5
146	0	50	50
301	12.5	62.5	37.5
303	62.5	50	37.5
281	75	37.5	37.5
442	75	62.5	25
158	12.5	12.5	25
67	50	100	12.5
343	0	87.5	12.5
90	37.5	37.5	12.5
304	12.5	25	12.5
135	25	0	12.5
145	25	50	0
98	37.5	25	0
66	12.5	25	0
114	87.5	12.5	0
81	12.5	12.5	0
95	37.5	0	0
97	37.5	0	0

*Percentage of samples ($n = 8$) in which each respective T-RF size was found.

Discussion

In the current study, application of the T-RFLP approach demonstrated that both host and neighboring plants exert an influence on AMF community composition of individual plants. Overall, fewer ribotypes were associated with G_0 samples (18) than with either G_{cm} (22) or C_{mac} (23) samples (Table 1). Moreover, only two ribotypes present in G_0 samples were absent in G_{Cm} samples, whereas five ribotypes present in both C_{mac} and G_{Cm} were absent in Go samples. These results, especially when taken in light of the ordination analysis (Figure 1), suggest not only greater AMF diversity in grass roots with neighboring *C. maculosa*, but that the AMF community shifts are reflective of AMF communities infecting roots of *C. maculosa*.

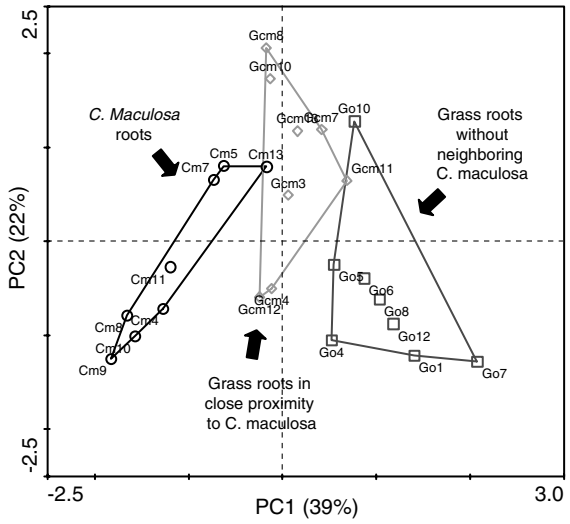

Figure 1. Scatter plot of structure loading for PC 1 and 2. Numbers in parentheses represent the percent of variance explained by the PC. Polygons enclose samples derived from *C. maculosa*, G_{Cm} and G_0 roots and are intended as a visual aid for group discrimination.

While our data indicates that *C. maculosa* influences AMF species composition of neighboring *D. glomerata*, it provides little insight into the mechanisms by which this is accomplished. There are basically three different groups of mechanisms that could be responsible for this observed difference: (1) direct interaction of AMF communities, for example by AMF propagule production in the source root; (2) host effects, such as carbon subsidization of AMF in the source root; or (3) modification of the physico-chemical environment adjacent to the target roots.

A number of prior laboratory and field studies have indicated that sporulation rates for different AMF species are host-dependent (Bever et al., 1996; Johnson et al., 1992; Johnson and Wedin, 1997; Sanders and Fitter, 1992; Struble and Skipper, 1988). Thus, alteration of the rate of infective propagule production for different AMF species might be at least partially responsible for alteration of AMF communities infecting grass roots.

Mycorrhizal propagule concentration has a significant effect on root colonization, at least in the short term; an effect that has been frequently documented in the mycorrhiza literature (for review see Smith and Read, 1997), and that is the basis of the 'mycorrhizal inoculum potential' assay (Johnson et al., 1991). In this sense, production of propagules of AMF species present in the *C. maculosa* roots may have 'overwhelmed' the nearby grass roots. It is particularly interesting that this effect appears to have been stronger than the selectivity of the grass roots for their characteristic subset of the AMF community, in turn suggesting that selectivity of root colonization is strongly determined by the fungal symbiont.

Conversely, primarily 'source' root physiological properties could have been responsible for the changes in AMF communities in the target root. For example, Francis and Read (1984) showed that establishing symbioses in a target root could be carbon-subsidized by the originating root; if this propensity to serve as a carbon source is particularly strong in certain plant species, such as *C. maculosa*, colonization of target roots could be primarily plant (resource) driven, and hinge less on individual fungal species characteristics.

An additional, potentially important, influence on fungal symbiont selection within roots of both *C. maculosa* and neighboring plants may be the chemical nature of *C. maculosa* root exudates. *C. maculosa* is known to secrete catechin, which exhibits both phytotoxic and antimicrobial properties (Bais et al., 2002, 2003), and the presence of catechin has been shown to result in significant alteration of soil bacterial communities (Callaway et al., 2004). Although AMF community responses to catechin are unknown, the presence of *C. maculosa* may alter AMF communities indirectly via phytotoxic mechanisms or alteration of fungal/bacterial relationships, or directly by selection for catechin-resistant AMF species. Other physico-chemical properties could have also been modified in the vicinity of the target grass roots, such as nutrient concentrations or pH, which could have indirectly led to AMF community changes within the target root.

These results highlight the need for further study of mechanisms driving assembly of AMF communities and the resulting functional relationships between plants, fungi and other soil biota. In addition to furthering our understanding of basic AMF biology and ecology, such information may provide important insights into microbial feedback mechanisms influencing plant competitive and successional relationships.

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