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The invasive plant species *Centaurea maculosa* alters arbuscular mycorrhizal fungal communities in the field

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Abstract While several recent studies have described changes in microbial communities associated with exotic plant invasion, how arbuscular mycorrhizal fungi (AMF) communities respond to exotic plant invasion is not well known, despite the salient role of this group in plant interactions. Here, we use molecular methods (terminal restriction fragment length polymorphism analyses based on the large subunit of the rRNA gene) to examine AMF community structure in sites dominated by the invasive mycorrhizal forb, *Centaurea maculosa* Lam. (spotted knapweed), and in adjacent native grassland sites. Our results indicate that significant AMF community alteration occurs following *C. maculosa* invasion. Moreover, a significant reduction in the number of restriction fragment sizes was found for samples collected in *C. maculosa*-dominated areas, suggesting reduced AMF diversity. Extraradical hyphal lengths exhibited a significant, on average 24%, reduction in *C. maculosa*-versus native grass-dominated sites. As both AMF community composition and abundance were altered by *C. maculosa* invasion, these data are strongly suggestive of potential impacts on AMF-mediated ecosystem processes. Given that the composition of AMF communities has the potential to differentially influence different plant species, our results may have important implications for site restoration after weed invasion.

Keywords *Centaurea maculosa* · Arbuscular mycorrhiza · Terminal restriction fragment length polymorphism · Invasive species · Extraradical hyphae

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Introduction

The large ecological and economic costs associated with invasion of terrestrial ecosystems by exotic plant species (Pimentel et al. 2000) has stimulated great interest in elucidating how invasive plant species influence, and are influenced by, biotic and abiotic interactions.

Many studies have aimed at determining aboveground impacts of exotic plant invasion, such as influences on plant community structure and organisms at higher trophic levels (reviewed by Levine et al. 2003). However, it has become increasingly apparent that a wide range of belowground biotic interactions play important roles in determining plant interactions and ecosystem function (e.g., Bever et al. 1997, 2003; Van der Heijden et al. 1998, 2003; Callaway et al. 2001) and that a much better understanding of plant and

soil microbial interactions will be required to fully understand plant community successional dynamics in the face of invasive alien plant species (Wolfe and Klironomos 2005). Several recent studies indicate that invasive plant species can impact soil microbial community composition and function (e.g., Belnap and Phillips 2001; Kourtev et al. 2002, 2003; Hawkes et al. 2005; Batten et al. 2006). The results of Callaway et al. (2004) indicate that *Centaurea maculosa* Lam. (Asteraceae, spotted knapweed), specifically, can cause shifts in the composition of soil bacterial communities, in part through the release of catechin, an allelochemical exhibiting phytotoxic and antimicrobial activities (Bais et al. 2002; Vivanco et al. 2004). The results of Callaway et al. (2004) also suggest that *C. maculosa* is able to modify the microbial community in invaded soils to its advantage, although the mechanistic basis for the observed positive feedback between the soil microbial community and *C. maculosa* remains unclear.

Plant/mycorrhizal symbioses may be one of the most important biotic interactions regulating plant community structure and successional dynamics (Allen 1991; Hartnett and Wilson 2002). Arbuscular mycorrhizal fungi (AMF) are obligate biotrophs that form symbiotic associations with all but a few terrestrial plant families (e.g., Brassicaceae, Chenopodiaceae). Representing a key interface between plant hosts and soil mineral nutrients, AMF are important to all but a few terrestrial ecosystems (Rillig 2004). In addition to increasing inorganic nutrient availability, benefits of AMF to their hosts include enhanced resistance to pathogens and other environmental stresses, and improved water relations (Allen 1991; Newsham et al. 1995, Borowicz 2001).

The AMF form vast networks that potentially link plants within communities together, allowing for between-plant two-way trafficking of nutrient resources (Simard and Durall 2004). The AMF typically occur in ecosystems as mixed communities with multiple taxa potentially infecting a single host plant. Although AMF are not generally thought to exhibit host specificity, a number of recent studies indicate that host-plant preferences exist (e.g., Hartnett and Wilson 1999; Helgason et al. 2002; Vandenkoornhuyse et al. 2002, 2003; Johnson et al. 2003; Klironomos 2003). Since individual AMF species may have a multiplicity of effects on different hosts (Klironomos 2003), AMF species composition would be expected to differentially influence plant competitive relationships, potentially facilitating plant coexistence in some cases and exclusion or elimination of different plant species in others. The AMF community composition of different sites may make them more or less amenable to establishment of specific plant species (Van der Heijden et al. 1998; Klironomos 2002, 2003; Hart et al. 2003): hence, plant-facilitated alteration of AMF communities may have relevance to ecosystem restoration.

A few studies (Marler et al. 1999; Callaway et al. 2001, 2003; Zabinski et al. 2002; Carey et al. 2004) indicate that the presence of AMF is central to the invasiveness of *C. maculosa*. Although the mechanisms by which AMF facilitate *C. maculosa* invasiveness have yet to be fully elucidated, a number of different greenhouse-based studies have demonstrated that strong negative responses to the combined presence of AMF and *C. maculosa* are plant species specific and may be at least partially due to AMF-facilitated ''luxury P consumption'' by *C. maculosa* at the expense of mycorrhizal native plant neighbors (Zabinski et al. 2002; Walling and Zabinski 2004). The specificity of AMF-facilitated *C. maculosa* competitiveness could be due to a number of potentially interacting factors, including alteration of AMF functionality resulting from differential host responses of AMF species and/or alteration of AMF community composition comprising mycelial networks.

In *Pseudoroegneria spicata* Pursh (Bluebunch wheatgrass)/*Festuca idahoensis* Elmer (Idaho fescue) communities of western Montana, *C. maculosa* represents an aggressive mycorrhizal invader which typically displaces native grass species. In a previous study (Mummey et al. 2005) we obtained evidence suggesting that the presence of *C. maculosa* can alter AMF communities inhabiting roots of neighboring plants. Thus, *C. maculosa* may alter AMF community functionality at an early stage in the invasion process. In the present study, we examine AMF communities of soils

associated with plant communities dominated by *C. maculosa* in which *P. spicata* and *F. idahoensis* have been almost completely displaced and compare them with adjacent native plant communities lacking *C. maculosa*. Our goal was to determine if *C. maculosa* invasion alters overall site AMF community composition in soil (potentially available to colonize roots) and overall AMF abundance. We hypothesized that, due to AMF host preferences and differential use of hyphal networks by different plants and plant communities, displacement of native grasses by *C. maculosa* results in significant alteration of both AMF species composition and hyphal abundance.

Methods

Field site and sampling design

The study site is situated on a gently sloping grassland 10 km north of Missoula, MT (Lutgen et al. 2003; Mummey et al. 2006a, b) (46°45′00N, 114°07′30W). The area is highly susceptible to invasion by *C. maculosa* and, with the exception of a 18 × 24 m fenced area (Fig. 1), was treated with the herbicide picloram (1.17×10^{-4} L m^{-2}) 3 years prior to sampling. Inside the fenced, herbicide-free area *C. maculosa* dominates the plant community and susceptible native plant species, such as *P. spicata* and *F. idahoensis*, have been almost completely eliminated. Since application of herbicide, the non-treated area has served as a seed reservoir for *C. maculosa*, which has expanded into surrounding, predominantly native plant communities (Fig. 1).

Eight sample locations were selected from areas dominated by native grasses having no *C. maculosa* presence within 1 m. Four meters distant from each native grassland sample point, an additional sample point was located in the *C. maculosa* dominated area. From each of these paired sample points, surface soil (1–10 cm depth; cobbly loam Argixeroll) was collected using a 2 cm diameter soil core extractor and placed in sterile plastic bags. Within 2 h of soil collection, sample bags were frozen (–20°C) prior to subsequent analyses.

Even though herbicide treatment was excluded from only one site in this grassland, the subsequent spread of *C. maculosa* from this remnant population into the surrounding native vegetation could be captured by replicate sample pairs. We based our study design on the assertion that these sample pairs, separated by known distances but differing in direction from one another, would allow for evaluation of affects due to plant community differences even if spatial variability due

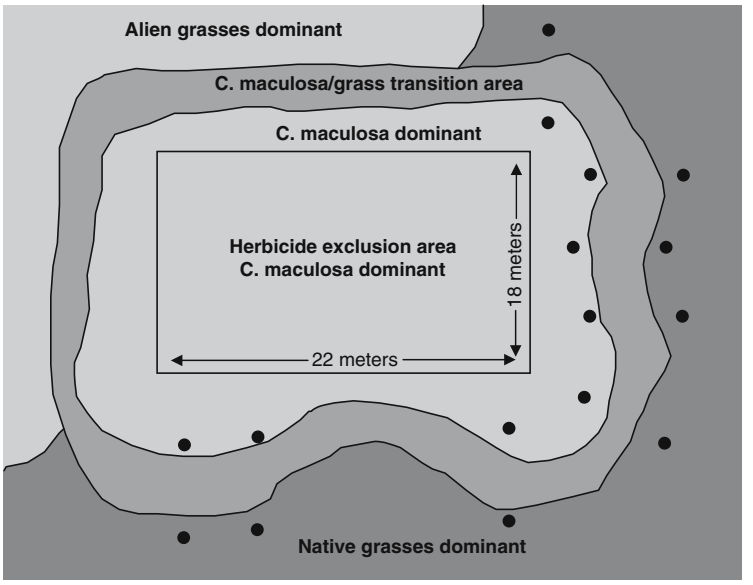

Fig. 1 Diagram of experimental site depicting relationships between the herbicide exclusion area, dominant vegetation types and sample locations (•). Spatial scales are not exact and are intended as an aid for visualization of the experimental design

to other factors, such slope position, was present on the site; an assertion supported by our results (see below).

Since our goal was to examine overall differences in AMF community composition between sites, no attempt was made to specifically examine roots (as in our previous study; Mummey et al. 2005) or the rhizosphere of the different plants present. Instead, our analysis of bulk soils should allow for comparison of AMF communities potentially available to infect plants of each site.

Laboratory analyses

Hyphae were extracted from each soil core and standing total hyphal lengths measured according to Rillig et al. (1999) employing aqueous membrane-filtration with subsequent microscopic examination at 200 ×. Hyphal length was estimated using the line intersect method as described in Jakobsen et al. (1992) and Tennant (1975). The AMF hyphae were distinguished from hyphae of other soil fungi following the morphological criteria described in Rillig et al. (1999).

Plant-available soil phosphorus concentrations were measured using the sodium bicarbonate extraction method of Olsen et al. (1954).

To obtain genomic DNA templates for molecular analyses, we homogenized soils by kneading in plastic bags and extracted genomic DNA from approximately 0.25 g field wet soil using the PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA).

The composition of AMF communities were characterized using large subunit (LSU) rRNA gene-based terminal restriction fragment length polymorphism (T-RFLP) analysis. The method involves end-labeling polymerase chain reaction (PCR) amplicons through the use of fluorescent molecules attached to the 5′-end of each PCR primer. The products of these reactions are digested with select restriction enzymes having specific recognition sequences. Since sequence composition varies between LSU rRNA genes of different AMF species or phylogenetic groups, restriction enzyme recognition sites are also variable. Terminal restriction fragments (T-RFs) are separated by electrophoresis on polyacrylamide gel or capillary DNA sequencers and visualized by excitation of the fluor. The method provides information about T-RF size distributions in each sample, which can be compared between samples to yield measures of community similarity.

The PCR amplification of these genomic templates was based on the AMF LSU rDNA-specific 5′-labeled primer pair FLR3-FAM and FLR4-HEX (Gollotte et al. 2004). We recently demonstrated the specificity of this primer pair by sequencing 139 clones containing FLR3/FLR4 amplicons derived from roots (*C. maculosa*, *Bromus tectorum*, *P. spicata*, *F. idahoensis* and *E. esula*) and soils collected within ((100 m of the study site (Mummey and Rillig, in preparation). While phylogenetic analyses of these cloned sequences indicated that the Glomeromycota were widely represented (Genbank accession numbers DQ468685 to DQ468824), only one sequence could be identified as non-AMF (Basidiomycetes), indicating that these primers and reaction conditions (see below) are highly specific to Glomeromycota of our study sites.

We first determined which restriction enzymes would optimally discriminate between the AMF phylogenetic groups present in our study site. This was achieved by conducting simulated digestion of our cloned sequences with a broad selection of different restriction enzymes, including all common tetrameric restriction enzymes. The results of these analyses indicated that of all restriction enzymes examined, both *Alu I* and *Taq I* both allowed for optimal discrimination between the phylogenetic groups represented in our clone libraries, while predominantly yielding T-RF sizes within the optimal size calling range (between 45 and 500 bp) (Mummey and Rillig, in preparation).

PCR amplification of soil DNA extracts consisted of two PCR rounds, the first employing the primer pair LR1 and FLR2 (van Tuinen et al. 1998; Trouvelot et al. 1999) and the second using primers FLR3-FAM and FLR4-HEX. The 25 µl reaction mixtures included 1 µl template DNA (soil extract or PCR products from the initial PCR), 100 pmol of each deoxynucleoside triphosphate, 10 pmol of each primer and 2 U HotMaster™ Taq DNA polymerase (Eppendorf, Hamburg, Germany). Thermal cycling for all

reactions included an initial denaturing step of 95° C for 5 min, 25 cycles (primer pair LR1/FLR2) or 30 cycles (primer pair FLR3-FAM/FLR4-HEX) consisting of 1 min at 95°C, 1 min at 58°C and 1 min at 65°C, followed by a final extension step of 65°C for 10 min. Products of these reactions were quantified using agarose-gel electrophoresis with Low DNA Mass Ladder (Invitrogen, Carlsbad, CA) as the size standard. PCR products were purified using the GenCatch™ PCR cleanup kit (Epoch Biolabs, Inc. Sugar Land, TX) and subsequently digested with the restriction enzyme *Taq I* (Fermentas Life Sciences, Hanover, MD). Each digestion, containing 16 µl purified PCR product and 5 U *Taq I* in the manufacturer's recommended buffer, was incubated for 4h at 37°C.

The T-RF sizes in each sample were determined using an ABI 3100 automated capillary DNA sequencer (Applied Biosystems, Foster City, CA, USA) with ROX-500 (Applied Biosystems) as the size standard. T-RF size determination and quantification was performed using Genemapper software (Applied Biosystems). We used the Microsoft-Excel macro Treeflap (Rees et al. 2004; http://www.wsc.monash.edu.au/~cwalsh/treeflap.xls) to convert each fragment size present to the nearest integer value and to subsequently align peaks against rounded sizes of the fragments. Relative fluorescence of T-RFLP profiles derived from different samples were standardized to 7,000 relative fluorescence units with a minimum peak height threshold of 50 fluorescence units.

Using binary presence or absence data for each sample, Jaccard distance matrixes were constructed (SPSS, Version 13.0). An additional matrix was constructed consisting of dummy variables identifying sample pairs having within-group members or members from different groups (*C. maculosa* or native plant site samples). Using these matrixes, the computer program zt (Bonnet and Van de Peer 2002) was employed to examine differences between sample sites using a modification of the Mantel test (Mantel 1967; Kropf et al. 2004).

The AMF hyphal lengths, phosphorus concentrations and numbers of T-RF sizes in T-RFLP profiles derived from each sample were compared using Pairwise *t*-tests (SPSS).

Results

Extraradical hyphal lengths averaged 3.24 (SD 0.79) and 2.50 (SD 0.84) m g^{-1} for native grass and *C. maculosa* sites, respectively (Fig. 2). Pairwise analysis indicated that hyphal lengths were significantly greater in samples collected in the native grass-dominated area compared to the *C. maculosa* dominated area ($P < 0.001$).

Plant available phosphorus (Olsen P) concentrations were found to be highly variable, averaging 20.75 (SD 9.83) and 17.62 (SD 4.87) mg kg^{-1} for native grass and *C. maculosa* site samples, respectively. No significant differences ($P = 0.44$) among locations were found (data not shown).

Total number of T-RF sizes (both forward and reverse) averaged 24.2 (SD = 5.8) and 20.8 (SD = 3.3) in T-RFLP profiles derived from samples collected in native grassland and *C. maculosa*-dominated sites, respectively. Pairwise analysis of all sample pairs indicated no significant differences in T-RF numbers associated with the two plant community types. However, after removal of an anomalous sample pair (sample pair no.3, which also had the lowest *P*-content of all native grassland samples; 1.4 SD below the mean), *C. maculosa*-associated samples were found to have significantly fewer (on average 5.3) T-RF numbers than native grassland samples ($P = 0.041$).

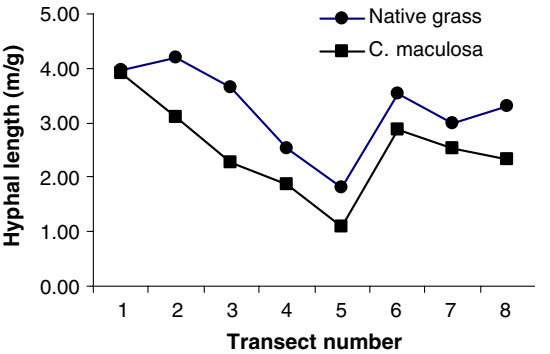

Fig. 2 Hyphal lengths calculated for samples collected in native grass-dominated and *Centaurea maculosa*-dominated areas. Data points corresponding to native grass and *Centaurea maculosa*-dominated areas are connected for clarity. Pairwise *t*-tests indicated significantly reduced hyphal length in samples obtained from *Centaurea maculosa*-dominated areas ($P < 0.001$)

Ordination analysis of T-RFLP profiles derived from all soil samples indicated separation of samples from *C. maculosa* and native grass dominated areas along both PCA 1 and PCA 2 (Fig. 3). Comparison of samples collected in *C. maculosa* versus native grass-dominated areas using the Mantel test indicated that the differences suggested by sample ordination were significant ($r = 0.22$, $P = 0.02$).

A total of 46 T-RF sizes were found to be common to T-RFLP profiles derived from at least one native grassland and one *C. maculosa*-dominated site sample. A total of 34 and 9 T-RF sizes were unique to native grassland and *C. maculosa*-dominated samples, respectively (Fig. 4).

Discussion

Our results clearly indicate that the soil AMF community, and therefore the diversity of AMF available to infect plant roots, was significantly altered upon *C. maculosa* invasion. The data also suggest that a gradient of change exists with some overlap between groups; this could be indicative of neighboring plants affecting AMF communities (Mummey et al. 2005; Hawkes et al. 2006) or legacy effects of past presence of invaders (see below). Moreover, the large number of T-RF sizes found to be unique to the native grassland relative to the *C. maculosa*-dominated site samples (34 and 9 T-RF sizes, respectively) suggest that *C. maculosa* facilitates an overall decrease in AMF diversity. Decreased AMF diversity could potentially have implications for the ecosystem since greater AMF species richness can facilitate plant species diversity and ecosystem productivity (Van der Heijden et al. 1998). Although further analyses will be required to determine whether decreased or altered AMF diversity represents an important mechanism facilitating *C. maculosa* invasiveness, this could be important to restoration (e.g., seedling establishment; Van der Heijden 2004) of *C. maculosa* invaded sites.

While our results indicated significant differences in AMF community composition between sites, they also showed that 46 different T-RF sizes were shared in T-RFLP profiles derived from both *C. maculosa*-dominated and native grassland samples. Although this does suggest that a similar subset of the AMF community was present in both native grassland and *C. maculosa*-dominated areas, it is important to keep in mind that our analyses were conducted on soil that may contain genetic materials (spores) of remnant AMF communities established prior to *C. maculosa* invasion. Additionally, *C. maculosa* was present across the hillside, including our native grassland sample points, just three years prior to our study. Thus, our results likely underestimated differences in the AMF communities between the invaded and non-invaded areas.

Even though our results indicate that alteration of AMF community composition with

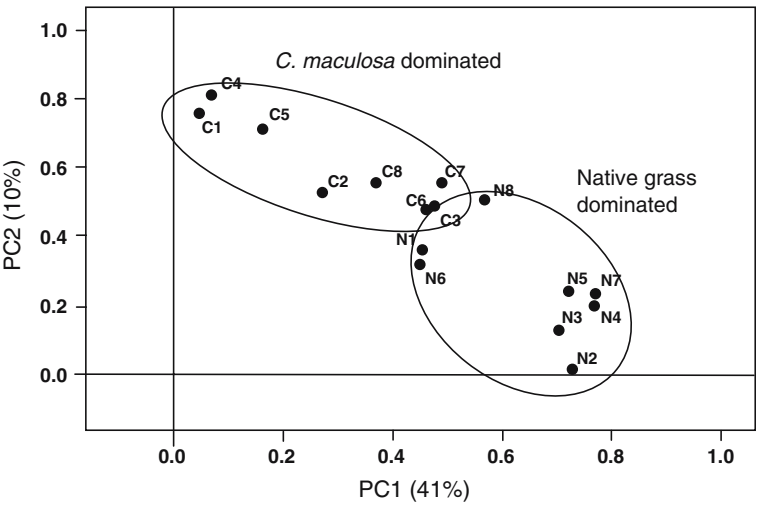

Fig. 3 Ordination diagram based on T-RFLP profiles derived from samples obtained from *Centaurea maculosa* (*C*) and native grass (*N*) dominated sites. Numbers in parentheses indicate the amount of variance accounted for by each principal component axis

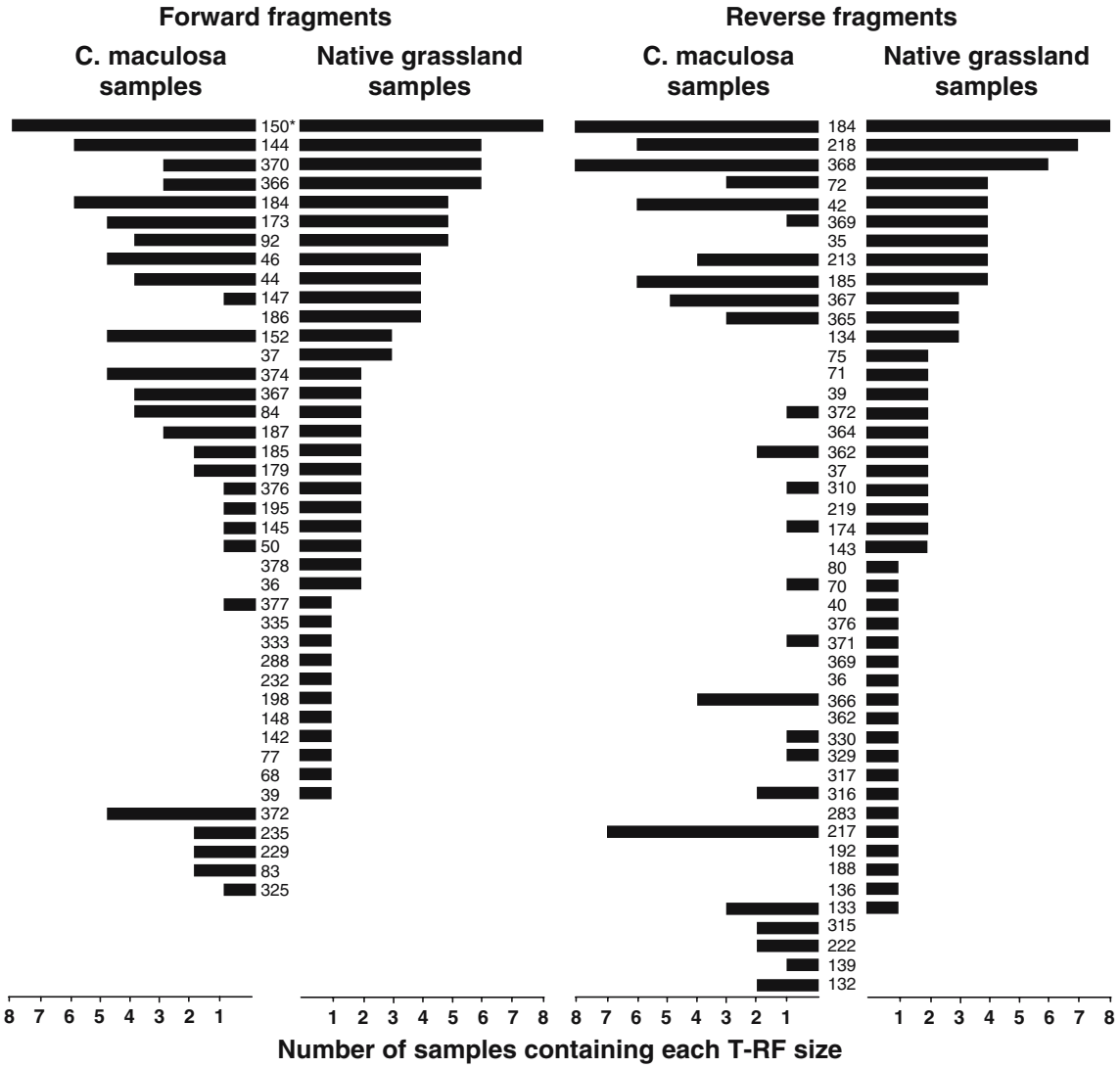

Fig. 4 Graphical presentation of the number of occurrences of each forward and reverse T-RF size found in *Centaurea maculosa* and native grass dominated sites. *Indicates T-RF size in base-pairs

C. maculosa invasion occurred on our study site, it is not clear whether this AMF community alteration is exclusive to the habit of an invasive plant, or if similar patterns could have arisen by any plant attaining local dominance in a patchy community. Nevertheless, given the previous studies indicating that the presence of *C. maculosa* can strongly influence soil microbial community structural and functional attributes, a potential mechanism (catechin, which may influence AMF, if not directly, indirectly via phytotoxicity and, potentially, antibacterial activities; Bais et al. 2002), and the resulting unusual dominance of this plant strongly suggest that we were observing an aspect intricately connected with the invasive habit of this plant species.

Our results showed not only altered AMF molecular diversity but also decreased AMF abundance with *C. maculosa* invasion, a finding corroborated by other field-based studies. For example, Lutgen and Rillig (2004) observed a similar pattern in soil AMF hyphal lengths in plots in which *C. maculosa* abundance was experimentally reduced. Klein et al. (2006), in a

field experiment in which nutrient concentrations were manipulated by sucrose addition, found both total and active hyphal lengths to exhibit a significant negative correlation with *C. maculosa* biomass. Additionally, Batten et al. (2006) used PLFA analysis to demonstrate that the presence of *Centaurea solstitialis* (a close relative of *C. maculosa*) significantly reduces biomarkers for AMF in invaded sites.

Since we measured total standing biomass of soil AMF hyphae, which does not permit separation into living and dead hyphal length (Klein and Paschke 2000), our data provide little insight into functionality of AMF in the relation to hyphal nutrient transport processes, which would perhaps be better approximated by knowledge of active hyphal lengths. However, since AMF hyphae and hyphal products are of great importance for aggregate stabilization in grassland ecosystems (Rillig and Mummey 2006), decreased hyphal abundance, whether living or dead, could have a pronounced influence on overall ecosystem function via alteration of soil structure; this may represent one of the mechanisms by which *C. maculosa* increases soil erosive losses (Lacey et al. 1989).

The reasons for the observed decrease in hyphal abundance are unclear but could be due to decreased allocation of resources to AMF by *C. maculosa* relative to native plant species, alteration of hyphal decomposition rates, the changes in AMF community composition, or decreased overall active plant biomass to support mycelial growth. Further research will be required to elucidate these relationships.

In addition to biotic factors that could potentially influence both AMF community composition and abundance at our study site, such as host preferences or differences in plant exudates and decomposition products, a number of abiotic factors may have also played a role. For example, soil moisture can be an important driver of exotic plant invasions (Eliason and Allen 1997; Booth et al. 2003; Wood et al. 2006). Enloe et al. (2004) showed that *C. solstitialis* invasion resulted in significant alteration of soil water dynamics. Although soil moisture was not measured in the present study, *C. solstitialis* and *C. maculosa* have very similar growth habits and may similarly influence soil moisture. Since soil water availability is known to have a strong influence on AMF communities and their function (Augé 2001, 2004) potential changes could have strongly influenced our results.

Similarly, pH is a key soil variable that broadly influences soil abiotic and biotic properties, including nutrient availability and other factors which can directly influence AMF (Clark 1997). Potential *C. maculosa*-facilitated alteration of this important variable (Callaway et al. 2003) could also have influenced AMF characteristics on our study site. Finally, plant species are known to differentially influence nutrient cycling (e.g., Hobbie 1992), which could cause changes in AMF communities. Even though we did not find evidence for changes in soil phosphate this may be due to high variability and relatively low replication masking effects. Additionally, other nutrients which we did not measure may have been altered.

In summary, our results indicate that invasion of a native grassland ecosystem by *C. maculosa* results in alteration of AMF community composition with concurrent decreases in extraradical hyphae in the soil. Many questions remain to be addressed concerning how these changes influence plant competitive relationships and ecosystem function. Further work is clearly needed to determine how alteration of AMF communities influences plant competitive relationships during *C. maculosa* invasion and the role played by AMF community composition in determining seedling establishment and subsequent vigor. The time required for an altered AMF community to revert to its original state, even if a native community is establishing, is unknown. If persisting, altered AMF community composition in invaded areas may represent a limiting factor for restoration efforts even after removal of invasive species, i.e. there may be a soil ecological legacy of invasion.

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