Soil–Occupancy Effects of Invasive and Native Grassland Plant Species on Composition and Diversity of Mycorrhizal Associations

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Diversified grasslands that contain native plant species can produce biofuels, support sustainable grazing systems, and produce other ecosystem services. However, ecosystem service production can be disrupted by invasion of exotic perennial plants, and these plants can have soil-microbial "legacies" that may interfere with establishment and maintenance of diversified grasslands even after effective management of the invasive species. The nature of such legacies is not well understood, but may involve suppression of mutualisms between native species and soil microbes. In this study, we tested the hypotheses that legacy effects of invasive species change colonization rates, diversity, and composition of arbuscular-mycorrhizal fungi (AMF) associated with seedlings of co-occurring invasive and native grassland species. In a glasshouse, experimental soils were conditioned by cultivating three invasive grassland perennials, three native grassland perennials, and a native perennial mixture. Each was grown separately through three cycles of growth, after which we used T-RFLP analysis to characterize AMF associations of seedlings of six native perennial and six invasive perennial species grown in these soils. Legacy effects of soil conditioning by invasive species did not affect AMF richness in seedling roots, but did affect AMF colonization rates and the taxonomic composition of mycorrhizal associations in seedling roots. Moreover, native species were more heavily colonized by AMF and roots of native species had greater AMF richness (number of AMF operational taxonomic units per seedling) than did invasive species. The invasive species used to condition soil in this experiment have been shown to have legacy effects on biomass of native seedlings, reducing their growth in this and a previous similar experiment. Therefore, our results suggest that successful plant invaders can have legacies that affect soil-microbial associations of native plants and that these effects can inhibit growth of native plant species in invaded communities.

Key words: Arbuscular mycorrhizal fungi, crested wheatgrass, invasion ecology, invasive plants, leafy spurge, plant-soil feedback, smooth brome, soil legacy.

In grassland agroecosystems and other managed grasslands, perennial weed invasions may be strongly affected by plant-soil biota interactions (Hallett 2006; Raizada et al. 2008; Reinhart and Callaway 2006; Wolfe and Klironomos 2005). In particular, site occupancy by invasive plant species may have strong soil-mediated "legacy" effects that influence subsequent interactions between plants and soil microbes. Such effects may change plant community and invasion dynamics, and may thereby interfere with restoration or management of grasslands even after effective control of an invasive species. The mechanistic basis of such legacy effects is not well understood, but may involve changes in soil food webs (Duda et al. 2003), total soil microbial communities (Kourtev et al. 2002), and mutualistic fungi (Allen et al. 2003; Hawkes et al. 2006; Van Der Putten et al. 2007). Such legacy effects can confer a specific advantage to invasive species relative to native species (Allen et al. 2003; Bray et al. 2003; Grman and Suding 2010; Jordan et al. 2008; Ortega and Pearson 2005; Richardson et al. 2000; Stinson et al. 2006; Vogelsang and

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Management Implications

In the midwestern US, regulatory, market and policy pressures could convert large areas from annual agriculture to semi-natural grassland agroecosystems, e.g., as part of a national effort to produce energy crops. Native grassland perennials could be used in these grasslands to reduce production costs, conserve soil quality, conserve native biodiversity, and enhance carbon sequestration in grassland agroecosystems. However, producer interest in semi-natural grassland systems is reduced by current difficulties in reliable and cost-effective establishment of these species, and weed management during establishment is a major concern. Many lines of evidence suggest that weedy exotic species can alter soils physically and/or microbially, creating a "legacy" that persists after control or removal of these species. This legacy effect may contribute significantly to the risk of additional weed invasion and poor performance of desirable species during grassland establishment. In smooth brome, crested wheatgrass and leafy spurge, three exotic perennials that are highly invasive in grasslands, we examined legacy effects on AMF associations of a set of native and invasive species in these grasslands. Smooth brome, crested wheatgrass and leafy spurge had legacy effects that altered AMF colonization rates and community composition in seedling roots of native and invasive species, In a previous analysis of these data (Jordan et al. 2011), we found that these invasive species also had legacy effects that reduced growth of some native species. However, these legacy effects on growth and AMF colonization of natives varied substantially among these invasive species, suggesting that such effects are not uniform. Taken together, these legacy effects on AMF associations and growth suggest that restoration of AMF communities in soil may be important to cost-effective establishment and weed management in semi-natural grassland agroecosystems.

Bever 2009). Invasive species may also have soil-mediated facilitative effects on other invasive species (Jordan et al. 2008, Vogelsang and Bever 2009).

In this report, we examine whether invasive legacies can affect associations between native species and arbuscular mycorrhizal fungi (AMF). If such legacy effects occur and reduce these associations, strong effects on postinvasion plant community and ecosystem dynamics may result (Inderjit and van der Putten 2010). We focused on associations with AMF because of growing evidence that invasive species can have different mycorrhizal associations than co-occurring native species (Batten et al. 2006; Hawkes et al. 2006; Hausmann and Hawkes 2009; Mummey and Rillig 2006; Mummey et al. 2005; Shah et al. 2010; van der Putten et al. 2007). AMF associations are highly important to the functional ecology of many grassland plant species (Hartnett and Wilson 2002; van der Heijden 2004), suggesting that deleterious legacy effects of invasives on natives might occur via differences in hosting behavior between invasives and natives. Additionally, invasives could create a deleterious legacy effect by disrupting AMF associations through other mechanisms. Potential pathways include weak AMF hosting (Seifert et al. 2009) and subsequent reduction in AMF density (Vogelsang and Bever 2009), active antagonism to AMF (Callaway et al. 2008), and formation of low-diversity plant communities (Kivlin and Hawkes 2010).

Evidence of invasive legacy effects on the AMF symbiosis would support the emerging view that invasive plants can function as "ecosystem engineers," capable of transforming ecosystem attributes such as soil microbes so as to facilitate and maintain invasion (Shah et al. 2009). However, current evidence of such invasive legacy effects on AMF associations of native species is limited. Most previous comparisons of AMF associations of native and nonnative species have not provided robust estimates of legacy effects on AMF associations per se. Rather, most studies have used nonexperimental approaches or methods that do not separate direct legacy effects on AMF associations from indirect effects mediated by interspecific interactions of invasives with natives. We focused directly on legacy effects of invasives on AMF associations via an experimental approach. We worked with plants and soils from a North American grassland system in which many native perennial plant species appear to be highly dependent on AMF for growth and reproduction (Pringle et al. 2009; Wilson and Hartnett 1998). Moreover, this study is part of a larger experiment in which invasive soil-conditioning legacies that reduce native seedling growth have been demonstrated (Jordan et al. 2011). In this report, we assess whether invasive legacy effects on AMF occur and therefore provide a potential explanation for the observed legacy effects at the level of seedling growth.

We conducted a glasshouse experiment to characterize interspecific legacy effects on mycorrhizal associations, in which we examined effects of soil conditioning by nonnative species on AMF associations in a group of native and nonnative perennial species. All of these species occur in mixed-grass prairie grassland communities of North America and are mycorrhizal (Carey et al. 2004; Wilson and Hartnett 1998). We characterized mycorrhizal associations in seedlings of all of these species after three successive periods of separate soil conditioning by three exotic invasive species: smooth brome (*Bromus inermis* Leyss.), crested wheatgrass (*Agropyron cristatum* (L.) Gaertn.), and leafy spurge (*Euphorbia esula* L.; hereafter all species will be referred to by genus). All of these exotic species are "strong invaders" (Ortega and Pearson 2005), able to become community dominants and to form nearly monospecific stands in invaded grasslands. Using terminal restriction fragment length polymorphism (T-RFLP) analysis (Aldrich-Wolfe 2007), we assessed effects of soil conditioning by these invasives on seedling AMF associations, and corresponding effects of three native species and a native species mixture. We used these data to test the hypotheses that legacy effects of invasive species affect the colonization rates, richness, and community composition of arbuscular-mycorrhizal fungi (AMF) colonizing seedlings of co-occurring invasive and native species.

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Materials and Methods

Study Species and Field Sites. Soils were gathered from each of three nature reserves. In each reserve, a particular invasive species was especially problematic, although all reserves are located within the North American range of all three invasive species. Thus, *Bromus* dominated at Lostwood National Wildlife Refuge, ND, USA (48°34′ N, 102°26′ W); *Agropyron* dominated at Medicine Lake National Wildlife Refuge, MT, (48°59′ N′ 104°26′ W); and *Euphorbia* dominated at Theodore Roosevelt National Park, ND (46°59′ N, 103°33′W). These study sites are all located within the northern mixed-grass prairie of western North Dakota and extreme eastern Montana. These prairies are dominated by cool-season grasses, especially western wheatgrass [*Agropyron smithii* (Rydb.) Á. Löve] and needlegrasses (*Stipa viridula, Stipa comata* Trin. & Rupr.) and sedges (*Carex* spp.), although warm-season grasses such as blue grama (*Bouteloua gracilis*) and little bluestem [*Schizachyrium scoparium* (Michx.) Nash.] occur at drier or sandier sites. *S. viridula* Trin., *B. gracilis* (Willd. ex Kunth) Lag. ex Griffiths, and *Aster ericoides* L. were recorded at all native collection sites, but all of the species used in this experiment could be expected to occur in the habitats from which the soils were collected.

At each study site, soil for the glasshouse experiment was collected from areas in which invasive plant species were absent. Three areas of native vegetation at each reserve were selected and roughly 90 L (24 gallons) of soil from the top 15 cm (6 inches) was harvested per area, gathering equal quantities from six separate sampling points chosen randomly in each area. Sampled soils from each reserve were stored under cool (< 25 C (<75 F)) conditions and covered to prevent drying during the 4-d sampling period. Soils were loam, clay loam or sandy loam in texture (details in Jordan et al. 2011).

Glasshouse Experiments. Experiments were conducted in a glasshouse on the University of Minnesota campus, St. Paul, MN from July 2003 to November 2004. At the inception of the experiment, two soil-biotic treatments were imposed: pasteurized (−AMF) and not pasteurized (+AMF) (Jordan et al. 2011). Here we report results only from replicates (detailed below) of the +AMF treatment. Soils were mixed 1:1 with pasteurized sand and placed in 2.5 L pots, keeping soils from each reserve separate.

Effects of soil conditioning by non-native invasive plant species, native plant species and native species mixtures on mycorrhizal associations of seedlings were estimated by creating soil conditioning treatments in three separate soils, collected from nature reserves as above. Each soil was conditioned by the invasive plant species that was dominant at the respective reserve (e.g., *Bromus* was used in soils from Lostwood NWR), and also by each of three native plant species, and a native-species mixture. Therefore, in each soil, five conditioning treatments were established in five replicates: (1) monoculture invasive (with the invasive species specific to each reserve), (2) monoculture *Stipa viridula*, (3) monoculture *Bouteloua gracilis*, (4) monoculture *Linum perenne* L. var. *lewisii* (Pursh) Eat. & Wright, and (5) mixture of six native species (*Stipa*, *Bouteloua*, *Linum*, *Aster ericoides*, *Koeleria pyramidata* (Lam.) Beauv., and *Ratibida columnifera* (Nutt.) Woot. & Standl. (nomenclature according to Flora of the Great Plains (Great Plains Flora Association 1986). Seeds of *Bromus, Agropyron,* and *Euphorbia* were collected at from sites within northern mixed-grass prairie of western North Dakota and extreme eastern Montana; seeds of native species were purchased (Prairie Mountain Roots, Arcola, Saskatchewan, Canada). All native plant species used in the experiment occurred at all of the field sites where we sampled.

Soil conditioning treatments were repeated through three successive growth cycles (I: July 8, 2003 to November 10, 2003; II: February 5, 25, 2004 to May 17, 2004; III: June 21, 2004 to August 20, 2004); all experimental units were held in a dark cold treatment at ~ 4 C after each growth cycle). These growth periods were only a small fraction of the life cycle of these species, which are all perennial. All conditioning treatments began with 15 to 20 established seedlings per pot and produced abundant above-ground biomass. The conditioning treatments were imposed in a glasshouse with 400 watt high-pressure sodium lamps (Ruud Lighting, Racine WI) used for supplemental lighting (14 to16 h daylength) from September to May. Average summer glasshouse temperatures were 27/23 C (day/night); average spring and fall temperatures were 21/19 C. PAR values from March to July peaked at 1433 μM m^{-2} (mean 440 μM m^{-2}); PAR values from July to September peaked at 1235 μM m^{-2} (mean 400 μM m^{-2}). Pots were watered as needed. Osmocote 14–14–14 (The Scotts Company LLC, Marysville, Ohio), a slow release fertilizer, was added at the start of the second growth cycle (1.25 g pot^{-1} [0.04 oz; ¼ teaspoon]); soil nutrients were below field levels for N, P, K (Jordan et al. 2011; Jordan, unpublished data) by the end of the experiment but plants did not show visible signs of nutrient stress. Pots containing soil from a given collection site were held on adjacent glasshouse benches with no intermingling during the first two growth cycles. In the third cycle, all pots were combined in a randomized complete-block design. A final growth cycle (September 15, 2004 to November 19, 2004) was used to assess host and soil conditioning on seedling growth. Prior to this cycle, all pots were frozen at −4 C to minimize regrowth of conditioning species.

In the final cycle, half of each pot was planted with seeds of six native species; the other half was planted with seeds of six invasive species in a fixed pattern (Figure 1);

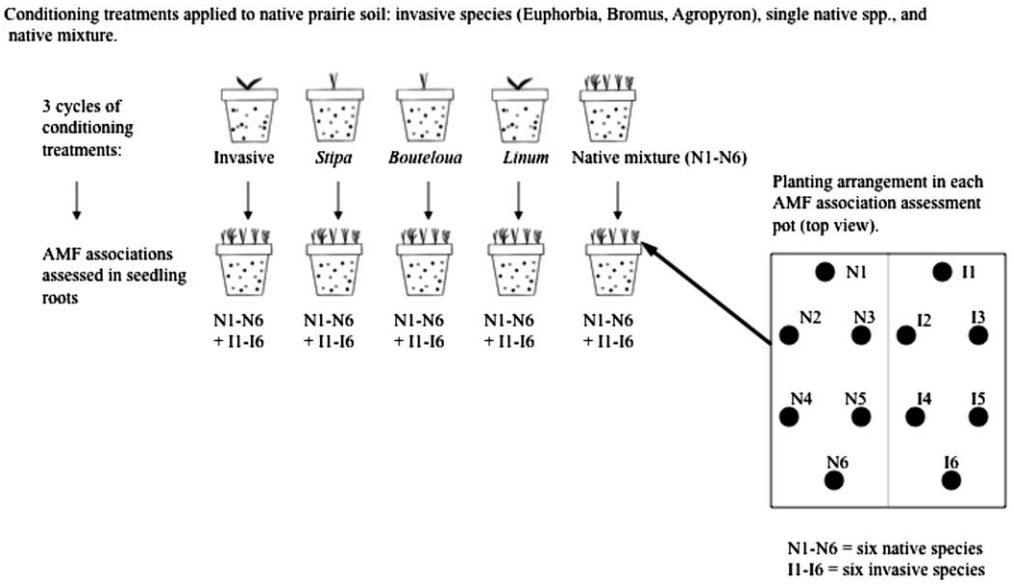

Figure 1. Protocol for estimating effects on AMF associations of soil conditioning by individual invasive species (*Agropyron* (I1), *Bromus* (I2) and *Euphorbia* (I3)), individual native species (*Stipa* (N1), *Linum* (N2) and *Bouteloua* (N3)), and native mixture (*Stipa*, *Bouteloua*, *Linum*, *Aster ericoides* (N4) *Koeleria macrantha* (N5), and *Ratibida columnifera* (N6)). Root samples were collected from six native species (N1–N6) and six invasive species (I1–I3 and *Cirsium arvense* (I4), *Centaurea stoebe* (I4), and *Poa pratensis* (I6). Soil conditioning treatments were applied to each of three soils collected from separate prairie nature reserves as noted in text; each soil was conditioned by natives alone and in mixture, and by the single invasive species that was most dominant at the respective soil collection site. Diagram depicts conditioning treatments applied to one of the three soils.

consequently, the experimental unit for assessment of AMF associations is a sowing position within a pot conditioned by a given species. Seedlings were thinned to one plant/species/pot at 10 d after planting. Invasives were *Bromus, Agropyron, Euphorbia, Cirsium arvense* (L.) Scop., *Centaurea stoebe* L., and *Poa pratensis* L.; natives were *Stipa, Bouteloua, Linum, Aster, Koeleria,* and *Ratibida*. *Centaurea* seed was obtained from Montana (R. Callaway, University of Montana); *Cirsium* seed was collected in Ramsey Co., MN, and *Poa* was obtained from Roseau Co., MN. Pots were monitored for regrowth from perennating structures remaining in the soil. Little regrowth occurred; it was clearly distinguishable from emerged seedlings and was immediately removed by clipping. After 12 wk, whole plant biomass was harvested from a subsample of five randomly-chosen replicates of each treatment. At this time, seedlings of each species were small and well separated from other species in each pot. Plants were dried at 60 C for 3 to 7 days and weighed. Dried root samples were stored at room temperature until processed for DNA extraction.

All native species and the invasive species used for conditioning (*Agropyron, Bromus* and *Euphorbia*) are capable of forming mycorrhizae in the growth stage examined in this experiment (data not presented). In a separate experiment concurrent with the first growth cycle of this experiment, roots of 10-wk-old seedlings were examined microscopically to assess mycorrhizal colonization via an adaptation of the magnified intersections method (McGonigle et al. 1990); further details in (Jordan et al. 2011). Roots of *Poa*, *Centaurea*, and *Cirsium* were not examined, but all three species have been found to be well-colonized at comparable ages (Hart and Reader 2002; Vatovec et al. 2005; Walling and Zabinski 2004).

Molecular Assessment. To characterize AMF associations of seedlings grown after three rounds of soil conditioning, T-RFLP analysis was used. In total, 671 root samples were analyzed using a nested polymerase chain reaction (PCR; modified from Renker et al. 2003), followed by restriction digests of the second PCR product to identify AMF operational taxonomic units (OTUs) in root samples, as detailed below.

DNA Extraction. Three to five 1-cm pieces of dried root of each surviving replicate (a single plant) from the glasshouse experiment were placed in a 2.0 ml (0.07 fl oz) tube with one 3-mm-(diam (0.12 inch) steel bead (#4 steel shot, Ballistic Products Inc., Midway USA) and shaken on a plant shaker (TissueLyser, Qiagen, Germany) for 2 min to pulverize (median dry mass of root samples was 44 mg (.002 oz)). Modest amounts of root material were used in order to facilitate identification of AMF species by T-RFLP. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. DNA extracts were stored at −20 C until used.

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Nested PCR. The ITS region was amplified via nested PCR, using primers SSU-Glom1 and LSU-Glom1 (Renker et al. 2003) in the first PCR (PCR1) and fluorescently labeled universal fungal primers ITS4 and ITS5 (White et al. 1990) in the second (PCR2). This technique has been used successfully by several researchers (Aldrich-Wolfe 2007; Lekberg et al. 2007; Renker et al. 2003) to amplify all currently described genera of AMF with the exception of *Paraglomus* (see below). While SSU-Glom1 is a general primer compatible with many eukaryotic sequences, LSU-Glom1 appears to be specific to the Glomeromycota and some basal groups in the Basidiomycota (Aldrich-Wolfe 2007; Renker et al. 2003). Consequently PCR1 was designed to amplify the ITS region of all arbuscular mycorrhizal fungi, as well as that of some nontarget fungi in the Basidiomycota, while excluding amplification of DNA from other root fungi and plants.

PCR1 was carried out in a total volume of 40 µL, containing 4 µL 10× CoralLoad buffer, 4 µL SSU-Glom1, 4 µL LSU-Glom1, 4 µL dNTPs (Fisher Scientific), 0.4 µL *Taq* polymerase (Qiagen), and 2 µL genomic DNA extract. All primers and dNTP concentrations were 2.5 mM. Sterile water was used as a negative control. Hot start PCR was carried out for 35 cycles (94 C for 2 min; 35 cycles of 92 C for 30 s; 57 C for 15 s; 72 C for 30 s; and a final extension at 72 C for 5 min).

To limit further amplification of DNA from nonAMF species, five microliters of PCR1 product from each reaction were incubated at 37 C for a minimum of 4 h or overnight with 4 units of *Alu*I (New England Biolabs (NEB)) following the manufacturer's instructions. The ITS region of most AMF lacks an *Alu*I cut site, while the ITS regions of other fungi likely to have been amplified during PCR1 typically contain multiple *Alu*I cut sites (Aldrich-Wolfe 2007; Renker et al. 2003). The only AMF likely to be consistently eliminated by this restriction digest are the Paraglomaceae and some members of the Archaeosporaceae (Renker et al. 2003; Aldrich-Wolfe 2007), groups against which other primers commonly used for amplification of AMF are biased as well (Krüger et al. 2009).

PCR2 followed the same recipe and thermocycling as PCR1, using the *Alu*I digested product as template and fluorescently labeled primers: ITS4 labeled with HEX and ITS5 labeled with 6-FAM (Invitrogen). PCR2 products were checked on a 0.8% agarose gel. Samples showing a PCR product size consistent with those expected for AMF (500 to 650 bp) were purified using the UltraClean PCR Clean-Up Kit (MoBio Laboratories) following the manufacturer's protocol. Of 671 possible root DNA samples, 121 had no band (i.e. no amplification); thus, 550 root samples amplified for AMF, nonAM fungi, or both types of fungi. Of these, 155 samples had an AMF band and 395 samples had only nonAM fungi. Purified PCR2 products were digested with *Mbo*I (NEB) and *Hinf* I (NEB) in a 10 µl reaction following the manufacturer's instructions.

PCR2 and digested products for each sample were sent to the University of Minnesota BioMedical Genomics Center DNA Sequencing and Analysis Facility (BMGC), St. Paul, MN to determine PCR2 product and terminal restriction fragment lengths using an ABI 3130xl automated capillary DNA sequencer with MapMarker1000 as the size standard. Data were analyzed with Peak Scanner Software v1.0 (Applied Biosystems) to acquire fragment size and strength.

Identification of AMF OTUs. To define operational taxonomic units (OTUs), fragment size and strength data from each sample were used to construct a 4-point profile (Aldrich-Wolfe 2007, Lekberg et al. 2007); profiles were comprised of PCR2 product length, and 5′-*Hinf* I, 3′-*Hinf* I, and 3′-*Mbo*I T-RFLP fragment lengths. Samples that contained products of equivalent length (± 3 bp) for PCR2, 5′-*Hinf* I, 3′-*Hinf* I, and 3′-*Mbo*I were considered to share an AMF OTU. Fragments of equal strength were considered as additional bands (Aldrich-Wolfe 2007; Lekberg et al. 2007), and were considered to be different OTUs. Peaks that were less than 10% of the strongest peak were discarded, because strong peaks in all four fragments were needed for OTU identification. To confirm that OTUs represented AMF taxa, 23 of the PCR products of the most frequently detected lengths were cloned with either pGEM-T® Easy Vectors (Promega) or PCR Cloning Kit (Qiagen) following the manufacturer's instructions. Five to ten colonies were selected from each sample and clones were sequenced using an ABI 3730xl automated capillary DNA sequencer at BMGC. Out of 195 clones from this study, 84% were AM species (163 were AM fungi, 32 were nonAM fungi). Sequences were analyzed with Sequence Scanner v1.0 (Applied Biosystems). In each sequence, both primer and restriction sites were identified and virtual code was produced to match the T-RFLP fragments. These sequences plus additional sequences from companion studies on similar soils were compared via ClustalW (Goujon et al. 2010; Larkin et al. 2007). Eighty-nine representative sequences were distilled into 40 distinct taxa based on the virtual T-RFLP patterns created from the sequences. Sequence data highlighted similar sequences that differed at a single base pair within the enzyme cut sites, suggesting that several T-RFLP patterns indicated closely related species. Again, T-RFLP patterns were considered to represent a single OTU if all 4 regions were ± 3bp, or if sequence data produced multiple digest sites for the same T-RFLP pattern. If no sequence was representative of a T-RFLP pattern, it was considered a single OTU. Across the range of root samples examined, we observed 28 AMF taxa; all were consistent with Glomus groups A and B (Schwarzott et al. 2001). AMF community composition in each root sample was described by the number and identity of observed AMF OTUs in the sample.

Statistical Methods. Amplification of AMF DNA from a root sample was used to indicate colonization of that root sample by AMF. Only roots for which fungal DNA (AMF, nonAMF fungal, or both) was successfully amplified were included in the analysis, since reactions that fail to amplify fungal DNA could not be distinguished from reactions for which no AMF fungal DNA was present. Differences in likelihood of amplification between invasive and native hosts, between soils conditioned by invasive and native plant species, and between soil types were assessed by logistic regression in JMP v.8 (SAS Institute 2009).

Legacy effects of soil conditioning by invasive species on richness of AMF in roots of native and invasive plants were tested using analysis of variance (ANOVA) in JMP v.8 (SAS Institute 2009) with host type, soil conditioning treatment, and soil origin as main effects and with all possible interactions, and number of AMF taxa per root as the dependent variable. Too few individuals of *Euphorbia* and *Ratibida* survived to be included as hosts in the analysis. All remaining 150 origin/host/conditioning combinations included 4 to 5 replicate root samples each, except six with only three replicate root samples (*Linum* hosts in soil conditioned by *Linum* at Lostwood NWR; *Koeleria* hosts in soil conditioned by *Bouteloua* and *Stipa* hosts in soil conditioned by *Agropyron* at Medicine Lake NWR; and *Bouteloua* hosts in soil conditioned by *Stipa*, *Linum* hosts in soil conditioned by *Euphorbia*, and *Stipa* hosts in soil conditioned by *Bouteloua* at Theodore Roosevelt National Park). Because each invasive species was only used to condition the soil from one site, soil conditioning by invasives was treated as a single category for this analysis. Consequently the soil-origin effects used in ANOVA (above) accounts for differences in both soil and invasive type; this confounding prevents us from comparing the legacy effects of different invasive species, but does not affect the focus of our analysis, which was comparison of legacy effects of invasive and native species on AMF associations of seedlings. Differences in richness of AMF among host species were assessed using Tukey's HSD in JMP.

Effects of soil conditioning treatment (invasive vs. native), host type (invasive vs. native), and soil origin on AMF community composition in roots of exotic invasive and native perennial plant species were assessed by nonmetric multidimensional scaling (NMS) in PC-ORD v.5 (McCune and Mefford 2006). Because abundance of AMF cannot be reliably determined from PCR samples and variable numbers of samples were colonized by AMF in each treatment, Bray-Curtis pairwise distances were calculated using AMF OTU presence–absence data for each soil origin-conditioning species–host species combination. The optimal number of dimensions was determined via Monte Carlo simulation comparing 50 runs with empirical data against 50 randomized runs with a step-down in dimensionality from 6 to 1 and a random starting configuration; very similar results (not shown) were obtained from a 100-run simulation. To test whether AMF community composition differed between invasive and native hosts and invasive and native soil conditioning effects, axis scores for each of the three NMS axes were compared using a two-way ANOVA with host type and soil conditioning type as main effects and host type by soil conditioning as the interaction term in JMP. Axis scores were normally distributed with equal variance, meeting the assumptions for ANOVA.

Results

AMF Colonization and Richness. From the 671 root samples from which DNA was extracted, we successfully amplified fungal DNA from 550. From this, 28% of these roots were found to contain AMF as determined by T-RFLP analysis. We detected 28 taxa of AMF, with a maximum of 5 taxa detected in a single root sample. We observed consistent differences in mycorrhizal associations in seedling roots of the native and invasive species examined by T-RFLP analysis. AMF colonization of invasive species occurred at low rates relative to native species (Figure 2; logistic regression $\chi^2 = 64.8$; $df = 1$; $p < 0.0001$). There was no general legacy effect on AMF colonization rates of soil conditioning by invasive species, when compared to native species.

However, we observed contingent legacy effects involving certain invasive species–soil combinations, as manifested by a significant interaction (logistic regression $\chi^2 = 7.16$; $df = 2$; $p = 0.028$) among conditioning treatment (native or invasive), soil origin (soil–invasive conditioning species) and host type (native or invasive), and a significant interaction between soil conditioning treatment and soil origin (logistic regression $\chi^2 = 9.08$; $df = 1$; $p < 0.011$). These interactions indicate invasive legacy effects on colonization rates that were contingent on other factors. In native host species, AMF colonization rates were increased by *Bromus* conditioning of Lostwood soils, (Figure 2a) but not affected by other invasive–soil origin combinations. Invasive conditioning species also decreased AMF colonization rates in some cases; among invasive host species grown in Medicine Lake soil conditioned by *Agropyron*, no AMF colonization was observed, while moderate colonization was observed in these species when grown in Medicine Lake soil conditioned by native species (Figure 2b). Finally, AMF colonization was increased in invasive hosts by *Euphorbia* conditioning in Theodore Roosevelt soils, relative to invasive host colonization in Theodore Roosevelt soil conditioned by natives (Figure 2c). Therefore, each invasive species produced a legacy effect on AMF colonization in seedling roots. However, these effects were not consistent in direction or magnitude across the

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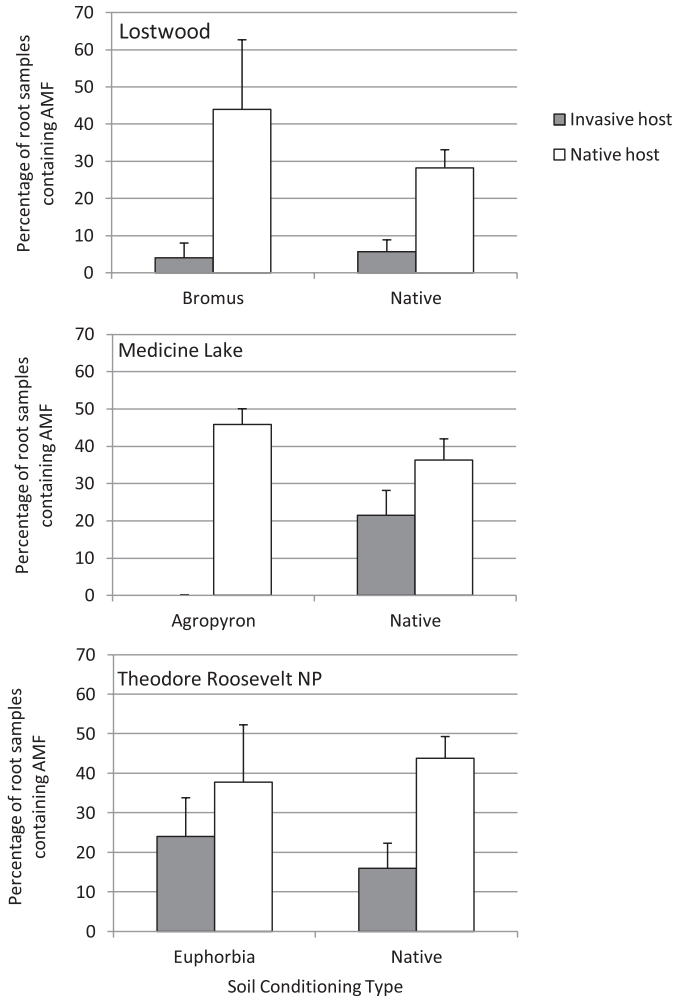

Figure 2. Frequency of colonization as assessed by PCR of invasive and native seedlings by arbuscular mycorrhizal fungi in soils conditioned by invasive plant species and native species in soils collected from three sites. Each soil was conditioned by one invasive species, 3 native species and a mixture of natives. (a) Lostwood soils conditioned by Bromus and natives; (b) Medicine Lake soils conditioned by Agropyron and natives; (c) Theodore Roosevelt National Park soils conditioned by Euphorbia and natives. Error bars indicate standard error of mean colonization frequencies across all relevant combinations of host species and conditioning species. $N = 4$ to 6 for invasive-conditioned soils and $N = 21$ to 24 for native-conditioned soils.

combinations of invasive conditioning species and soils that we examined. In particular, soil conditioning by certain combinations of invasive species and soil type increased AMF colonization, while others had the opposite effect.

Mean AMF richness per root sample differed among the 10 host species (Figure 3; $F = 18.9$; $df = 9{,}554$; $p < 0.001$). The invasive species *Centaurea, Cirsium, Bromus,* and *Agropyron* had significantly lower levels of AMF OTU richness than the native species *Bouteloua,*

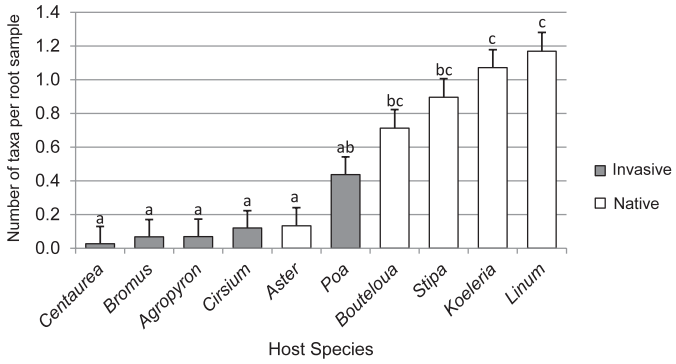

Figure 3. Effect of host species on species richness of arbuscular mycorrhizal fungi in roots of greenhouse-grown seedlings. Values are means ± SE; $n = 66$ to 75. Means that share a letter do not differ by Tukey HSD at $\alpha = 0.05$.

Stipa, Linum, and *Koeleria* (Figure 3). However, we did not observe a general invasive legacy effect on AMF richness in seedling roots; soil conditioning treatment effects on AMF richness did not differ significantly between invasive and native conditioning species.

Effects of Invasive Soil Conditioning on AMF Community Composition. Soil conditioning by invasive species altered the composition of AMF associations in seedling roots (Figure 4; $F_{1,88} = 6.05$; $p = 0.0159$). Seedlings from soils conditioned by invasive soils exhibited a more variable AMF community than seedlings from soils conditioned by natives, as evidenced by the larger error bars for invasive species in the ordination (Figure 4). Fewer OTUs were common (i.e., were found in five or more root samples) in the invasive hosts than in native hosts in both soil conditioning treatments and the lowest numbers were found in invasive hosts growing in soils conditioned by invasives (Figure 5).

Discussion

We found that legacy effects of soil occupancy by three invasive species had little effect on AMF richness in seedling roots, but did affect AMF colonization rates in roots and the taxonomic composition of mycorrhizal associations of a set of native and invasive perennial grassland species. Also, we found categorical differences in hosting behavior between native and invasive members of this group of grassland species: seedling roots of invasive species were less likely to be colonized, and were likely to have fewer AMF taxa per root. Our findings are consistent with relevant previous studies in a range of plant communities, which show that mycorrhizal associations of invasive species can differ from those of co-occurring native species (Batten et al. 2006; Hawkes et al. 2006; Mummey and Rillig 2006; Mummey et al. 2005; Shah

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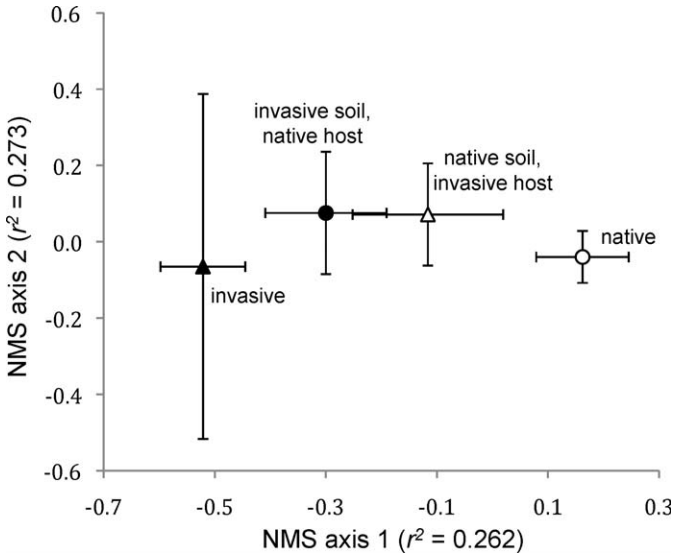

Figure 4. Ordination by nonmetric multidimensional scaling (NMS) of arbuscular mycorrhizal fungal communities in roots of native (circles) and invasive (triangles) hosts grown in soils conditioned by native (open symbols) and invasive (filled symbols) perennial plant species. Values are mean NMS scores along each axis ± SE. Final stress for the three-dimensional optimal solution was 17.42 with 139 iterations and an instability value of zero. Samples were pooled across soils from different sites and across plant species within conditioning and host types.

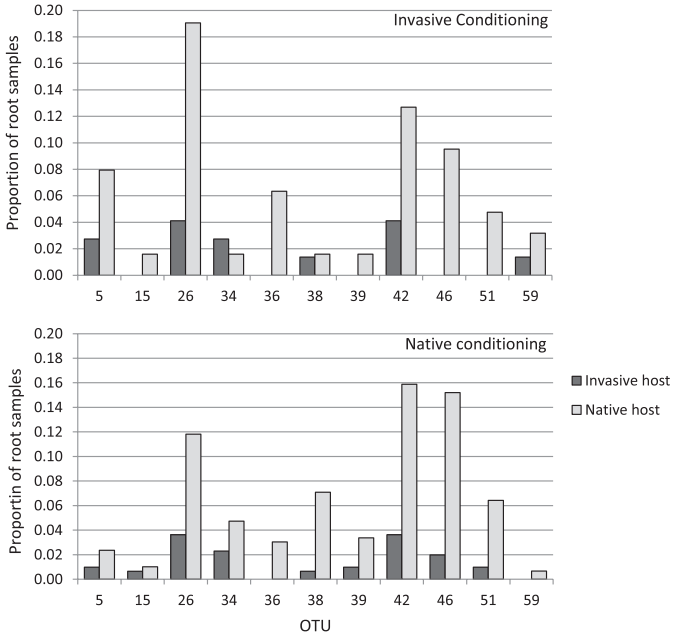

Figure 5. The proportion of root samples containing particular AMF taxa as a function of type of soil conditioning and plant host (invasive soil, invasive host (n = 73); invasive soil, native host (n = 63); native soil, invasive host (n = 304); native soil, native host (n = 296). Only the 11 OTUs occurring in at least five root samples are shown.

et al. 2010; van der Putten et al. 2007). Moreover, certain mycorrhizal invasives have been shown to alter AMF associations in natives growing in close spatial proximity to these invasive species (Mummey et al. 2005, Hawkes et al. 2006, Hausmann and Hawkes 2009). For example, (Mummey et al. 2005) found that AMF community composition in roots of a native grass was altered by the presence of a highly invasive forb. Other studies have shown that less invasive exotics can also strongly influence AMF associations in native species (Hawkes et al. 2006), and that invasive species can differ qualitatively in their effects on AMF associations of natives (Hausmann and Hawkes 2009).

However, our study differs from most previous work by explicit focus on a particular pathway by which invasive species might affect AMF communities in native species: legacy effects from a period of invasive soil occupancy by mycorrhizal invasive species. Most previous studies have examined effects of mycorrhizal invasives on AMF associations of natives that were exerted during concurrent growth of the invasive and native species, or used experimental designs or nonexperimental approaches that confounded legacy effects with other pathways, e.g., above-ground interactions that may affect below-ground mutualisms. Moreover, most previous assessments of invasive effects on mycorrhizal associations of natives have focused on effects of a single invasive species, and have examined responses to invasive soil occupancy in only a few native and/or invasive species. By comparing individual legacy effects of multiple invasive and native species on AMF associations of a range of co-occurring invasive and native species, our results provide a more comprehensive and community-scaled assessment of these effects on AMF associations of natives and invasives.

Additionally, we found a consistent difference in AMF relations between seedlings of these native and invasive species, all of which are considered to be mycorrhizal on the basis of previous studies. The consistently lower rate of AMF colonization and richness observed in a group of co-occurring invasive seedlings provides new evidence of commonalities in AMF relations among mycorrhizal invasive perennial species (Hausmann and Hawkes 2009, Seifert et al. 2009, van der Putten et al. 2007; Vogelsang and Bever 2009). Such commonalities suggest the possibility that qualitative differences may frequently occur between invasives and natives in the functional ecology of associations with AMF. Presently, it is not clear how differences in AMF associations between invasives and natives may affect community and ecosystem processes and dynamics in invaded communities, but rapidly-growing evidence of major functional differences among AMF taxa in a wide range of ecologically significant traits (Verbruggen and Kiers 2010) suggests strongly that invasives may be

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capable of significant impact on plant communities and ecosystems via alteration of AMF communities.

Together, these considerations suggest a possible scenario of cofacilitation among invasives by reduction in abundance and/or diversity of AMF that are mutualists of native species (Jordan et al. 2008; Seifert et al. 2009). In this scenario, multiple co-occurring invasives contribute to disruption of native mutualisms with AMF by any of a range of nonexclusive mechanisms (e.g., reducing AMF density and abundance, or forming strong mutualisms with AMF taxa that are relatively ineffective mutualists of natives; Zhang et al. 2010). In effect, such functional similarities among co-occurring invasives may create a ''cooperative guild'' (Perry 1995), i.e., a set of species that have mutually facilitative effects. Such joint effects of invasive species on AMF relations with native species could support self-reinforcing invasion processes—a so-called ''invasional meltdown''—(Best and Arcese 2009) that reduce the ability of native species to establish and persist in modified soils (Standish et al. 2008) even when management actions are taken to aid establishment (Lombardo et al. 2007). Such processes may establish a stable degraded state in a grassland agroecosystem (Kulmatiski 2006; Suding et al. 2004), enforced by a legacy of extensive invasive modification of soil that facilitates reinvasion even after effective control or removal of invasives (Peltzer et al. 2009, Rout and Callaway 2009).

The likelihood of such a scenario depends on how AMF-related invasive soil legacies affect the functional ecology and population growth rates of native species. At present there is little direct experimental evidence on these points, but Zhang et al. (2010) demonstrated that soil occupancy by an invasive herb causes changes in the composition of AMF communities that favor growth of that invasive over certain natives in both monoculture and mixture. Our findings extend this result to a broader community scale, because the three invasive species used in our soil-occupancy treatments have been shown in this (Jordan et al. 2011) and a similar previous experiment (Jordan et al. 2008) to have deleterious effects on aboveground biomass of native grassland perennial seedlings. In the previous experiment, soil conditioning by *Agropyron* sharply reduced growth of *Aster* and *Ratibida*; *Bromus* had antagonistic conditioning effects on *Linum* and *Ratibida*, while soil conditioning by *Euphorbia* resulted in reduced growth of *Aster*, *Ratibida* and *Linum* (Jordan et al. 2008). In the present experiment (biomass effects reported in (Jordan et al. 2011), soil conditioning by *Agropyron* had significant negative effects on *Bouteloua* and *Stipa* relative to growth in soils conditioned by native species; *Euphorbia* had significant negative effects on *Bouteloua* and *Linum*. *Bromus* also reduced growth of three of the four natives analyzed (Jordan et al. 2011), but these effects were not significant. Taken together, these results indicate an association, in these species, between invasive legacy effects on biomass and AMF associations. Our findings are therefore consistent with the hypothesis (Inderjit and van der Putten 2010; Seifert et al. 2009) that invasive plants can interfere with native species by affecting mutualisms between natives and soil organisms, with potentially major effects on community dynamics and the success of ecological restoration. However, we hasten to emphasize that we have no evidence of a causal link between legacy effects on biomass and AMF community composition. Such a link can be tested by assessment of native seedling growth after experimental manipulations of soil AMF communities that parallel the shifts in AMF community composition observed in this study, thereby isolating AMF effects from other pathways by which invasive legacy effects may occur. Such an approach has been taken to verify that changes in AMF communities resulting from soil occupancy by a single invasive herb reduce growth of a native herb and performance in mixture with the invasive herb (Zhang et al. 2010); this protocol can be extended to examine the joint effects of co-occurring invasives.

Despite indications of certain commonalities among invasive species in relations with AMF, we observed qualitative differences in certain invasive legacy effects of particular species/soil combinations. For example, *Agropyron* conditioning of Medicine Lake soil eliminated AMF colonization of invasive hosts, while *Euphorbia* conditioning in TRNP soil appeared to enhance colonization rates, particularly for invasives. These findings suggest that *Euphorbia* may culture a robust AMF community that differs from the AMF community resulting from other invasives as well as from native conditioning of soils. Finally, *Bromus* conditioning of Lostwood soil resulted in greater colonization of natives than soils conditioned by native species. These differences indicate that generalizations about the direction and magnitude of invasive legacy effects on AMF communities in native and invasive hosts are certainly premature. In particular, our results suggest that soil legacy effects of different invasive species will affect native plant communities differently; moreover, these legacy effects appear to create ''winners'' and ''losers'' in native communities via differential effects on growth of native species (Jordan et al. 2011).

We note that inferences from our findings are limited by a number of aspects of our study. First, the establishment phase of grassland perennial plant species examines only one component of their fitness; however, establishment may be a strongly limiting phase for grassland perennials (van der Heijden 2004), and mycorrhizal associations of grassland perennials appear to have some degree of temporal stability (Hausmann and Hawkes 2010). Also, recent evidence from a grassland system shows that strong deleterious soil legacies can result from brief (5 wk) periods of growth by a conditioning species (Grman and Suding

2010). Second, our T-RFLP analysis technique restricts the detection of certain groups of AMF, particularly species in the genera *Paraglomus* and *Archaeospora*, that share an *AluI* cut site in the ITS region with nonAMF fungi (Renker et al. 2003), so our measures of AMF richness should be regarded as conservative. However, unless representatives of these two genera are much more likely to be encountered in one soil conditioning treatment than the other, this bias is unlikely to alter the overall conclusions from our analysis. Third, our findings are based on colonization of seedlings over a relatively short time frame in a glasshouse setting in roots growing in disturbed soils; despite indications of temporal stability of mycorrhizal associations noted above (Hausmann and Hawkes 2010), our experimental conditions may favor rapidly colonizing AMF taxa and therefore additionally limit the taxonomic and functional range of our analysis. Fourth, our findings are based on an analysis of presence/absence data on AMF richness and community composition and therefore are not able to detect differences in AMF communities based on abundance. Lastly, we treated AMF clades that differed only in a few base pairs as the same taxon, potentially lumping together clades with significantly different biological properties.

Despite these limitations, our results have an important practical implication: if invasive perennials can create depauperate or otherwise altered AMF communities in grasslands or other plant communities, then a cost-effective method for remediating these soil effects will be needed. One possibility is the use of native species that function as "nurse species" that can readily establish on sites after invasive occupancy, and which can by various mechanisms facilitate the establishment and growth of other native species (Lockwood and Samuels 2004). For example, Canada wild rye (*Elymus canadensis* L.) is a native species widely regarded as a nurse species by grassland managers. It is able to establish and produce substantial above-ground biomass in situations where large seed banks of annual weeds are present (Jordan et al., unpubl.); such above-ground growth presumably creates potential for substantial effects on soil microbial communities. Canada wild rye has been shown (Noyd et al. 1995) to develop high levels of AMF colonization in soils where other native perennial grasses were poorly colonized. Our findings revealed a range of AMF richness levels among native grassland perennials (e.g., *Aster* versus *Linum*, Figure 2) suggesting that functional nurse species may occur in these plant communities. Assessments of potential nurse species can now be extended by T-RFLP analyses of mycorrhizal associations of these species and subsequently-establishing natives.

In summary, two main results emerge from this experiment. First, we found that a set of perennial grassland invasive species changed the composition of AMF communities in seedling roots of native perennial grassland species. These same invasive species were previously found to be capable of reducing native perennial grassland seedling growth via a soil-legacy effect, suggesting a possible causal association between invasive legacy impacts on native AMF associations and native seedling growth. However, our experimental design does not permit a test of causal relationships between these two legacy effects. Secondly, we found that a group of perennial grassland invasives was substantially less colonized by AMF, and formed associations with fewer AMF taxa, compared to a set of co-occurring native perennial grassland species. This finding suggests that invasive legacy effects on AMF community composition may result, at least in part, from this apparent difference in the functional ecology of plant–soil interaction between these natives and invasives.

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