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Direct and indirect effects of allelopathy in the soil legacy of an exotic plant invasion

Sara Grove · Karen A. Haubensak · Ingrid M. Parker

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Abstract Invasive species may leave behind legacies that persist even after removal, inhibiting subsequent restoration efforts. We examined the soil legacy of *Cytisus scoparius*, a nitrogen-fixing, putatively allelopathic shrub invading the western US. We tested the hypothesis that allelopathy plays a critical role in the depressive effect of *Cytisus* on the key native Douglas-fir, both directly on tree growth and indirectly via effects on its ectomycorrhizal fungi (EMF). In a greenhouse factorial experiment, we used activated carbon to inhibit *Cytisus*-produced allelochemicals and sucrose to reduce elevated nitrogen (N). We found that: (1) *Cytisus*-invaded soils depressed Douglas-fir growth compared to uninvaded forest soils. The effect of adding *Cytisus* litter was positive (possibly reflecting an N fertilization effect) only in the presence of activated carbon, providing evidence for a role of allelopathic compounds. Activated carbon did not increase growth in the absence of *Cytisus* litter. Finally, sucrose addition provided weak support for a nitrogen effect of *Cytisus* litter. (2) Seedlings grown in *Cytisus* soils had lower EMF abundance compared to those in uninvaded forest soils. In forest soil from one site, adding *Cytisus* litter also decreased EMF abundance. Douglas-fir growth increased significantly with EMF across sites and soils suggesting that changes in EMF were linked to tree growth. The fungal taxon *Cenococcum geophilum* was significantly depressed in *Cytisus* soils compared to forest soils, while *Rhizopogon rogersii* abundance was similar across soil types. These results together suggest an overall negative effect of *Cytisus* on the growth of a dominant native tree and its fungal symbionts. Our study suggests how the role of allelopathy in ecological restoration may play out on two time scales: through immediate, direct impacts on native plants as well as through long-term, persistent impacts mediated by the collapse or transformation of microbial communities.

Keywords Scotch broom · *Pseudotsuga menziesii* · Ectomycorrhizae · Invasion impact · Soil ecology · Alkaloids · Nitrogen

S. Grove (✉) · I. M. Parker
Department of Ecology and Evolutionary Biology, University of California, Santa Cruz, CA 95064, USA
e-mail: sgrove@ucsc.edu

K. A. Haubensak
Merriam-Powell Center for Environmental Research, Northern Arizona University, Flagstaff, AZ 86011-5018, USA

Introduction

Great effort and resources are spent to control invasive plants usually under the assumption that once the invader is removed, the impact of that species on the community is also eliminated. However, abiotic and biotic impacts during invasions may result in loss of native species, changes in community composition and structure, and changes in resource availability and

nutrient cycling (Vitousek et al. 1987; Maron and Jefferies 2001; Rothstein et al. 2004; Suding et al. 2004; Asner et al. 2008; Rascher et al. 2011). These impacts can persist and hinder native species from reoccupying these habitats even after the invader is removed, known as a "legacy effect."(Maron and Jefferies 2001; Corbin and D'Antonio 2004b). Soil legacies of invasive plants can include changes in nitrogen (Vitousek et al. 1987; Evans et al. 2001; Dougherty and Reichard 2004), carbon storage (Christian and Wilson 1999), soil salinity (Vivrette and Muller 1977; D' Antonio 1993), and soil chemistry via allelopathy (Inderjit et al. 2006; Pollock et al. 2008).

Allelopathy, in which plants produce toxic compounds that give them an advantage over competitors, has received much attention recently as an explanation for the invasion success of certain introduced species. (Bais et al. 2003; Callaway and Ridenour 2004; Vivanco et al. 2004; Pollock et al. 2008; Jarchow and Cook 2009; Lankau et al. 2009). Allelochemicals released by invaders are thought to be especially detrimental to native plants in their introduced range because they lack an evolutionary history with the invader and, therefore, may have fewer mechanisms to counter the toxicity of allelochemicals (Callaway and Aschehoug 2000; Bais et al. 2003; Fitter 2003; Hierro and Callaway 2003). A legacy effect driven by allelopathy can occur if allelochemicals accumulate in the soil and continue to have negative effects on the establishment and growth of native vegetation (Bais et al. 2003; Hierro and Callaway 2003; Lankau 2010). This type of legacy may also indirectly affect plant growth because allelochemicals can disrupt microbial mutualisms such as mycorrhizal associations (Perry and Rose 1983; Rose et al. 1983; Schreiner et al. 1993). For example, abundant evidence suggests that the invader *Alliaria petiolata* (garlic mustard) causes depressive effects on the mycorrhizae of native species through its production of mustard oils (Schreiner et al. 1993; Roberts and Anderson 2001; Kliebenstein 2004). However, to our knowledge, no studies have examined the legacy of indirect impacts on native species via persistent changes in the soil biota.

Cytisus scoparius (hereafter *Cytisus*) is considered one of the most invasive species in western North America with widespread distribution in California, Washington, and Oregon (Peterson and Prasad 1998; Isaacson 2000). Invasion by *Cytisus* into managed forests in the Pacific Northwest has resulted in the loss of more than $40 million annually in timber revenue and control expenses in the state of Oregon alone (Hulting et al. 2008). *Cytisus* is a nitrogen (N)-fixer and enriches soil N pools (Helgerson et al. 1979; Wheeler et al. 1979, 1987; Haubensak and Parker 2004; Caldwell 2006). It also produces high concentrations of alkaloids, primarily sparteine (Wink et al. 1983; Gresser et al. 1996; Wink 2002). Sparteine has been demonstrated to provide defenses against herbivores (Wink et al. 1982) and to inhibit seed germination in some species (Wink 1983). However, there is surprisingly little known about the ecological effects of quinolizidine alkaloids in general and of *Cytisus*-produced compounds specifically.

Throughout much of the Pacific Northwest, Douglas-fir (*Pseudotsuga menziesii*) is both the dominant native and the primary harvested species (Curtis and Carey 1996). In previous work, we showed that Douglas-fir seedlings planted into clearcut sites that had been long invaded by *Cytisus* experienced drastic mortality despite the absence of *Cytisus* (Parker and Haubensak 2011). This, taken with prior work that showed inhibition of target native species grown in *Cytisus* soil (Haubensak and Parker 2004), strongly suggested a negative soil legacy of *Cytisus* invasion.

This study was designed to examine the soil-based legacy of *Cytisus* on the growth of Douglas-fir. We expected that the net impact of *Cytisus* on Douglas-fir would integrate a positive effect of nitrogen fertilization and a negative effect of allelochemistry, possibly mediated by mycorrhizae. To mediate the effect of nitrogen enrichment, we added sucrose to reduce soil available N (Blumenthal et al. 2003). To mediate the effect of allelopathy, we used activated carbon (AC) to adsorb organic compounds, such as the alkaloids released during the breakdown of *Cytisus* litter (Mahall and Callaway 1992; Wardle et al. 1998; Ridenour and Callaway 2001; Kulmatiski and Beard 2006).

Based on the poor performance of Douglas-fir in *Cytisus*-invaded field sites, we predicted that (1) Douglas-fir seedlings grown in *Cytisus*-invaded soils would be smaller than seedlings grown in uninvaded forest soils, and (2) adding *Cytisus* litter to uninvaded forest soils would decrease Douglas-fir seedling growth. We expected that (3) adding sucrose would reduce the N fertilization effect of both *Cytisus*-invaded soils and soils amended with *Cytisus* litter. We also predicted that adding activated carbon would ameliorate allelopathic effects, and therefore (4) the

effect of adding *Cytisus* litter would be positive when combined with activated carbon. We further expected that the negative effects of *Cytisus* on Douglas-fir growth would be accompanied by the suppression of ectomycorrhizal fungi (EMF), and so (5) colonization by EMF would be lower on Douglas-fir seedlings grown in *Cytisus*-invaded soil or with *Cytisus* litter. If allelochemicals mediate this reduction in EMF, (6) the addition of activated carbon should ameliorate the suppression of EMF by *Cytisus* litter.

Methods

We examined the effects of *Cytisus* on Douglas-fir growth and its associated EMF in a fully randomized factorial greenhouse experiment. In November 2008, we collected soils from two pairs of sites in the south Puget Sound region of western Washington. The two pairs were on opposite sides (hereafter referred to as "East" and "West") of Joint Base Lewis McChord (JBLM). Each pair included a Douglas-fir forest (47°03′12N, 122°40′47W and 47°02′28N, 122°29′30W) and a nearby area that was heavily invaded by *Cytisus* after the removal of trees at least three decades ago (47°02′51N, 122°40′01W and 47°02′01N, 122°31′51W). Organic material was scraped off the surface of the mineral soil; regular spades were used to excavate the top 30 cm of mineral soil, which was passed through a 1.61 cm^2 sieve. Collections were made from at least 10 holes with diameter of approximately 0.5 m, across an area of approximately 1 ha within each site. We transported the soils to the greenhouse at the University of California, Santa Cruz, where collections from each site (east forest, west forest, east *Cytisus*, and west *Cytisus*) were homogenized; we added soil amendments and homogenized again before dividing and distributing into D60 Deepots (6.4 cm × 36 cm, Stuewe and Sons, Tangent, OR).

The three soil amendments were *Cytisus* litter, activated carbon, and sucrose. Each of these amendments was added to the four soil types (west and east forest, and west and east *Cytisus*) in all possible combinations for a total of 32 different soil-treatment combinations. For the *Cytisus* litter addition, we collected fragments of green branches of *Cytisus* from stands of mature plants with a hedge-trimmer. Part of the motivation for this treatment was to provide guidance for resource management on the question of whether mechanical control of *Cytisus* should include the removal of cut and mulched plant material from the site. Therefore, although we call this green material "litter," it is analogous to mulch. *Cytisus* stem and leaf material is comprised of about 4.5 % N (Haubensak 2001). We added 155 cm^3 of plant material to each pot; this volume was calculated based on field data from an invaded site that had undergone mechanical control. The plant material was cut by hand into approximately 2–5 cm segments; diameters of fragments did not exceed 3 mm.

Leaves and stems of *Cytisus* contain high concentrations of sparteine, an N-based quinolizidine alkaloid (Haubensak and Parker, unpublished data). To test for chemical effects of *Cytisus* litter on Douglas-fir seedlings, we added activated carbon ("AC," Marineland Aquarium Products, Cincinnati, OH). We ground the AC into a fine dust with a mortar and pestle and added it at a rate of 1 % w/v, or 10 cm^3 per liter of soil.

Sucrose was added to examine whether soil N enrichment by *Cytisus* increases Douglas-fir growth. Sucrose is a readily available form of carbon and increases N immobilization by microbes, thereby decreasing plant-available N (Blumenthal et al. 2003). We added 1.52 g of sucrose per liter, based on a 200 g C m^{-2} addition rate (Blumenthal et al. 2003). Sucrose was added once at the start of the experiment; the pulse effect of a single sucrose addition on N availability may have been short-lived, and therefore, results should be interpreted cautiously. The magnitude of the effect of carbon addition would likely have been larger with longer term or repeated additions.

Within 2 weeks of soil collection, we planted 480 one-year-old Douglas-fir seedlings into the array of soil amendment treatment combinations described above ($N = 15$ for each treatment). Seedlings were purchased from Silvaseed (Roy, Washington) and were initially grown in potting soil from seed collected from mature trees from two distinct seed zones in western Washington (seed zone 030-05 and 422-15). The (030-05) seed zone is near the town of Hoquiam, which is a coastal zone at a slightly lower elevation than JBLM. The (422-15) seed zone is in Ashford, where environmental conditions and elevation are more similar to those found at JBLM. For each treatment/soil combination, the 15 replicates were split between 8 (422-15) trees and 7 (030-05) trees. Eight seedlings were inspected and found to have no mycorrhizae at the time of planting.

To prevent mycorrhizal cross contamination between soil treatments, we sterilized all containers and potting tools in a 10 % bleach solution immediately prior to planting and the potting tools were treated again between handling a given soil type. The Douglas-fir seedlings were planted into their respective soil treatments and capped off with approximately 2 cm of 30 grit sand to reduce the splash of ectomycorrhizal spores during watering. We watered the trees from above to further reduce cross contamination between individual pots. The seedlings were left to grow outdoors in the natural light and temperature conditions for 19 months.

On April 23, 2010, we harvested the aboveground biomass of all surviving seedlings. We cut each tree at the root crown, placed them into paper envelopes, and dried them at approximately 61° C for up to 24 days after which they were weighed.

During final harvest of Douglas-fir seedlings, we collected subsamples of soils from control (unamended) forest and *Cytisus* soils for nutrient analyses. These subsamples were dried at 105 °C for 24 h, ground with mortar and pestle, then sent to A and L Western Laboratories (Modesto, CA) for the following analyses: soil organic matter, plant-available P (Olsen-Bray), percent N, nitrate pool size, cations, and pH.

Ectomycorrhizal abundance and identity

We measured the abundance of ectomycorrhizal fungi on a subset of 120 Douglas-fir seedlings grown in both east and west soils and across four of the experimental soil treatments: (1) unamended *Cytisus* soils, (2) unamended forest soils, (3) forest soils with *Cytisus* litter addition, and (4) forest soils with both *Cytisus* litter and AC additions. We measured a minimum of 56 randomly selected root tips on each of 15 replicate trees. To prepare the roots for microscopy, we gently shook off the excess soil and briefly soaked the entire root mass in a bath of DI water. We divided the root mass longitudinally into approximately equal portions, 2–3 depending on the size of the root mass. We randomly selected which of the sections would be analyzed for mycorrhizal colonization. The roots selected for mycorrhizal assessments were further cleaned in a series of water baths where the roots were agitated to remove the soil. Occasionally we very gently used our hands and/or dissecting instruments to physically loosen the remaining persistent large soil aggregates. We took great care not to remove hyphae from the root tips. The roots selected for mycorrhizal assessment were cut with scissors into 3–6 cm fragments and stored in DI water at 3 °C. Within 7 days of the initial processing, the roots were examined for EMF. To measure the abundance of EMF across soil treatments, the Douglas-fir root tips were placed into gridded petri dishes and the roots that terminated nearest to the corner of each of the grid cells were assessed for the presence or absence of EMF and each root tip was assigned a morphotype. To calculate the proportion of root tips colonized by EMF, we divided the number of roots tips with EMF by the total number of root tips observed.

We extracted the fungal DNA from representative samples of each morphotype encountered. DNA was extracted from the fine root tips with a DNeasy plant mini kit (Qiagen). We amplified the ITS region of rDNA with fungal specific primers ITS-1F and ITS-4 (Gardes and Bruns 1993) and Green GoTaq master mix (Promega). The PCR product was cleaned with ExoSAP-It (USB Products) and through a series of ethanol precipitations. The cleaned PCR product was cycle sequenced on an ABI 3100 Sanger Sequencer (applied Biosystems, Foster City, CA, USA). To obtain species identification, we used Geneious (Drummond et al. 2009) to view, manually edit, create contigs, and match our nucleotide sequences with the vouchered specimens in the NCBI GenBank database. Our assessments of the presence/absence of EMF were well supported by our sequence data. However, most morphotypes did not corresponded to a single taxa, while two morphotypes corresponded consistently with a particular fungal taxa. For these two taxa, *Rhizopogon rogersii* and *Cenococcum geophilum*, we used microscopy to quantify individual colonization rates.

Statistical analyses

For soil nutrients, we used 2-way ANOVA to examine the fixed effects of "type" (forest versus *Cytisus*) and "site" (east versus west). Only unamended soils were analyzed, so the effects of *Cytisus* litter, sucrose, and AC were not included here.

For Douglas-fir growth, first, we tested for the significance of differences among our treatments overall by means of a 4 factor (soil type/litter/sucrose/AC) factorial ANOVA model with seed

source as a blocking factor. Because east and west sites showed dramatically different patterns in soil parameters that could influence the effects of various treatments, we analyzed east and west sites separately. The full models were highly significant for both the east ($F_{16,199} = 3.042$, $P = 0.0001$) and west ($F_{16,209} = 6.858$, $P = 0.0001$) sites.

We then tested a series of a priori hypotheses by means of planned contrasts. We compared Douglas-fir seedling growth in *Cytisus*-invaded soil versus forest soil. We tested for an N fertilization effect by comparing soils with sucrose added to those with no sucrose added for (1) *Cytisus*-invaded soil and (2) forest soil to which *Cytisus* litter had been added. To test for allelopathy in *Cytisus*-invaded soils, we compared activated carbon addition to no activated carbon. To test for an allelopathic effect of adding *Cytisus* litter while controlling for other potential positive effects of activated carbon, we used a 2-way factorial mixed model ANOVA with litter and activated carbon as fixed effects and seed source as a random effect.

By means of ANOVA, we compared colonization of EMF across the four treatments for which we assessed EMF abundance: *Cytisus*-invaded soil, forest soil, forest soil with litter, and forest soil with litter and activated carbon. We used subsequent one-tailed *t* tests (assuming unequal variances) to test these specific hypotheses: forest soil > *Cytisus*-invaded soil; forest soils without *Cytisus* litter > with *Cytisus* litter added; and forest soils with activated carbon added along with *Cytisus* litter > *Cytisus* litter added without activated carbon. These comparisons were done for overall EMF abundance, the abundance of *Rhizopogon*, and the abundance of *Cenococcum*. We normalized the data by arcsine square-root transformations, but the *Cenococcum* data could not be normalized and was analyzed by means of a one-tailed, non-parametric Wilcoxon test.

We explored the relationship between Douglas-fir seedling growth and EMF colonization for the subset of trees for which we had assessed EMF, analyzing east and west samples separately. In order to avoid treatment effects, we used only the control (unamended) forest and *Cytisus* soils in this analysis, and a preliminary ANCOVA found no significant effect of soil type and no significant interactions (data not shown). Therefore, the two soil types were combined in a regression of aboveground biomass on EMF colonization. All statistics were performed by means of JMP v. 9.0.0 (SAS Institute 2010).

Results

Characteristics of soil collected from *Cytisus* invasions and uninvaded forests

For virtually all the soil parameters measured (Table 1), there was a significant site x soil interaction term suggesting that the differences between *Cytisus* and forest soils were contingent on site (Table 2). *Cytisus* soils were 60 % lower in organic matter compared to forest soils in the west site, but no different in the east. *Cytisus* soils had higher % N than the forest soils in the east site (0.785 vs. 0.454 %), but lower in the west site (0.365 vs. 0.523 %). Soil NO_3^- followed the same trends as %N (Table 2). That pattern was opposite for available P: *Cytisus* soils had half the available P as the forest soils in the east site, but over three times more in the west. *Cytisus* soils had 90 % greater cation exchange capacity (CEC) than forest soils in the east site, but there was no difference between *Cytisus* and forest CEC values in the west site. There were other such inconsistencies between east and west sites among the other nutrient cations such as Mg and Ca (Table 1). Based on these patterns, we determined that the east and west sites were fundamentally different and chose to analyze the east and west sites separately in all comparisons for seedling growth and ectomycorrhizal colonization.

Effects of *Cytisus scoparius* on Douglas-fir seedling growth

Cytisus had an overall negative effect on Douglas-fir seedling growth. Despite large differences in soil nutrient status between the east and west sites, the suppressive effect of *Cytisus* soil was clear in both sites. In the east site, Douglas-fir seedlings grown in *Cytisus*-invaded soil were 17 % smaller than seedlings grown in soils collected from uninvaded forest soil ($F_{1,199} = 13.6$, $P = 0.0003$, Fig. 1). In the west site, Douglas-fir seedlings were 13 % smaller in *Cytisus*-invaded soil ($F_{1,209} = 11.2$, $P = 0.0009$; Fig. 1).

Activated carbon treatments produced mixed support for allelopathy. The addition of activated carbon to *Cytisus*-invaded soil had no effect on seedling growth in

Table 1 Soil parameters measured in *Cytisus*-invaded (*Cytisus*) and uninvaded (Forest) mineral soils (0–15 cm)

	Cytisus		Forest	
	East	West	East	West
C:N	16.29 (0.42)	21.57 (1.66)	29.57 (0.78)	24.42 (2.85)
Total N (%)	0.785 (0.02)	0.365 (1.66)	0.454 (0.78)	0.523 (2.85)
SOM	21.99 (0.45)	13.54 (0.99)	23.03 (0.72)	21.71 (2.46)
Available P	16.34 (1.47)	70.51 (3.60)	53.0 (7.97)	33.49 (5.31)
pH	5.49 (0.04)	5.70 (0.05)	5.61 (0.03)	5.14 (0.03)
K	92.68 (25.5)	85.30 (17.3)	98.77 (35.5)	90.73 (47.5)
Mg	40.29 (1.69)	74.96 (5.53)	73.87 (2.96)	55.63 (3.42)
Ca	316.57 (18.9)	609.81 (46.2)	702.91 (29.8)	396.53 (26.5)
Na	29.31 (1.23)	33.81 (3.18)	29.70 (1.73)	29.96 (2.55)
CEC (meq/100 g)	3.11 (0.21)	5.10 (0.31)	5.86 (0.28)	4.41 (0.38)
NO_3^-–N (ppm)	10.29 (1.34)	7.03 (2.28)	7.14 (1.60)	11.07 (1.61)

West and east soils were from two sites on JBLM property. Values are means ($N = 14$) with one standard error in parentheses

SOM soil organic matter, *CEC* cation exchange capacity

the east site ($F_{1,199} = 0.089$, $P = 0.76$; Fig. 2), and had an unexpected negative effect in the west site ($F_{1,209} = 4.55$, $P = 0.03$; Fig. 2). However, with fresh *Cytisus* litter, the effect of activated carbon was positive. That is, in uninvaded forest soils, the effect of adding *Cytisus* litter depended on whether activated carbon was added to the mixture, with activated carbon boosting the impact of litter from neutral to positive in one case and from negative to positive in the other (Fig. 3). In the west, the interaction between litter and activated carbon was significant ($F_{1,106} = 5.37$, $P = 0.02$) and the interaction was marginally significant in the east ($F_{1,108} = 3.192$, $P = 0.07$). The main effect of adding litter was significant in the west ($F_{1,106} = 16.7$, $P = 0.0001$), but not significant in the east ($F_{1,108} = 0.03$, $P = 0.86$). The main effect of adding AC was marginally significant in both the west ($F_{1,106} = 2.86$, $P = 0.094$) and the east ($F_{1,108} = 3.52$, $P = 0.063$).

Our sucrose addition treatment did not consistently reduce plant growth. Adding sucrose to *Cytisus*-invaded soil resulted in a marginally significant 11 % decrease in Douglas-fir seedling growth in the east ($F_{1,199} = 3.6$, $P = 0.059$), but had no effect in the west ($F_{1,209} = 0.11$, $P = 0.10$). In uninvaded forest soils, adding sucrose along with *Cytisus* litter was not different from adding litter by itself in either the east ($F_{1,199} = 0.193$, $P = 0.66$) or west ($F_{1,209} = 1.022$, $P = 0.31$) (Fig. 4).

Effects of *Cytisus scoparius* on ectomycorrhizal fungi

We found substantial evidence that *Cytisus* had a negative impact on fungal mutualists of Douglas-fir. In the east site, *Cytisus*-invaded soil had 22 % lower EMF colonization than uninvaded forest soil, a significant difference (one-tailed $T = 2.37$, DF = 22.31, $P = 0.01$; Fig. 5). In the west site, *Cytisus* soil had 13.8 % less EMF colonization, a marginally significant difference ($T = 1.47$, DF = 20.74, $P = 0.08$; Fig. 5). In forest soils, *Cytisus* litter addition decreased EMF colonization in the east site by 16 % ($T = 2.19$, DF = 25.01, $P = 0.018$) and had no effect on EMF in the west site ($T = 0.41$, DF = 21.19, $P = 0.65$; Fig. 5). The effect of adding activated carbon to forest soil along with *Cytisus* litter counteracted the negative effects of the litter in the east site, but not in the west (Fig. 5). In the east site, EMF were 15 % more abundant on Douglas-fir roots when activated carbon was added with *Cytisus* litter compared to the litter addition alone ($T = 1.94$, DF = 19.32, $P = 0.03$). In the west site, activated carbon did not have a positive effect on ectomycorrhizal fungi abundance ($T = 1.03$, DF = 22.06, $P = 0.84$). Degree of colonization by EMF positively predicted final Douglas-fir biomass and explained a substantial proportion of the variance in biomass in soils from both sites (east: $R^2 = 0.42$, $N = 27$, $P = 0.0002$, west: $R^2 = 0.32$, $N = 29$, $P = 0.001$; Fig. 6).

Fungi identified by ITS included the following genera: *Amanita*, *Cadophora*, *Cenococcum*, *Hygrophorus*, *Laccaria*, *Phialocephala*, *Rhizopogon*, *Rhizoscyphus*, *Suillus*, *Thelephora*, and *Wilcoxina*. Because most of these taxa did not clearly correspond to a morphotype, we could not evaluate all the individual responses to *Cytisus* soils and litter. However, two taxa, *R. rogersii* and *C. geophilum*, were

Table 2 Results for 2-way ANOVAs for all soil variables with "Site" (East versus West) and "Soil" (*Cytisus*-invaded versus uninvaded forest)

Source	F	P
C:N		
Site	0.0017	0.962
Soil	22.31	<0.0001
Site*Soil	9.31	0.055
Total N (%)		
Site	87.09	<0.0001
Soil	21.22	0.001
Site*Soil	168.5	<0.0001
SOM		
Site	12.34	0.002
Soil	10.99	0.003
Site*Soil	6.57	0.017
Available P		
Site	11.25	0.003
Soil	0.001	0.974
Site*Soil	50.84	<0.0001
pH		
Site	11.85	0.002
Soil	32.92	<0.0001
Site*Soil	84.29	<0.0001
K		
Site	0.05	0.822
Soil	0.02	0.864
Site*Soil	0.001	0.991
Mg		
Site	5.01	0.031
Soil	3.77	0.061
Site*Soil	51.95	<0.0001
Ca		
Site	0.042	0.832
Soil	7.33	0.012
Site*Soil	88.06	<0.0001
Na		
Site	1.07	0.311
Soil	0.57	0.453
Site*Soil	0.85	0.362
CEC		
Site	0.82	0.373
Soil	11.82	0.002
Site*Soil	32.84	<0.0001
NO_3^-–N		
Site	0.037	0.854
Soil	0.066	0.792
Site*Soil	4.248	0.051

DF = 1 for each main effect and interaction term

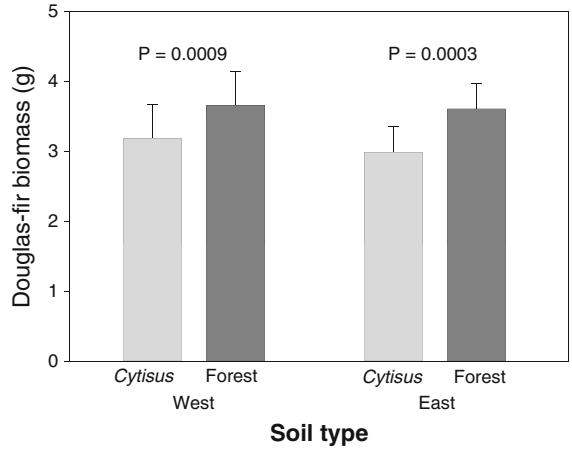

Fig. 1 Above ground dry biomass (g) of Douglas-fir seedlings after 19 months of growth in either *Cytisus*-invaded soils or uninvaded forest soils, collected from two sites (east and west) at Joint Base Lewis McChord in southwestern Washington. *Error bars* represent ±1 standard error. Significance levels are derived from planned contrasts for individual comparisons (see text)

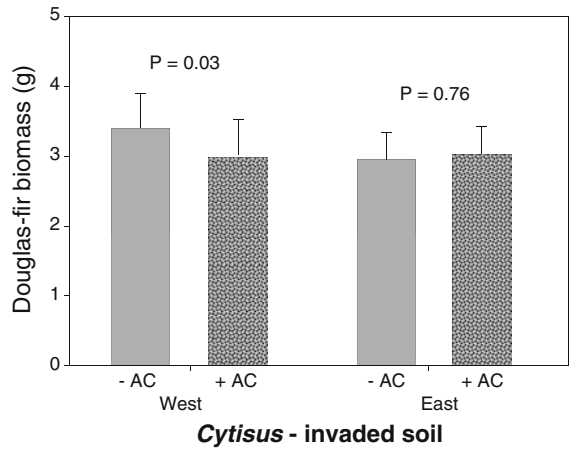

Fig. 2 The effect of activated carbon (AC) addition on aboveground biomass (g) of Douglas-fir seedlings after 19 months of growth in *Cytisus*-invaded soils collected from two sites (east and west) at Joint Base Lewis McChord. *Error bars* represent +1 standard error. Significance levels are derived from planned contrasts for individual comparisons (see text)

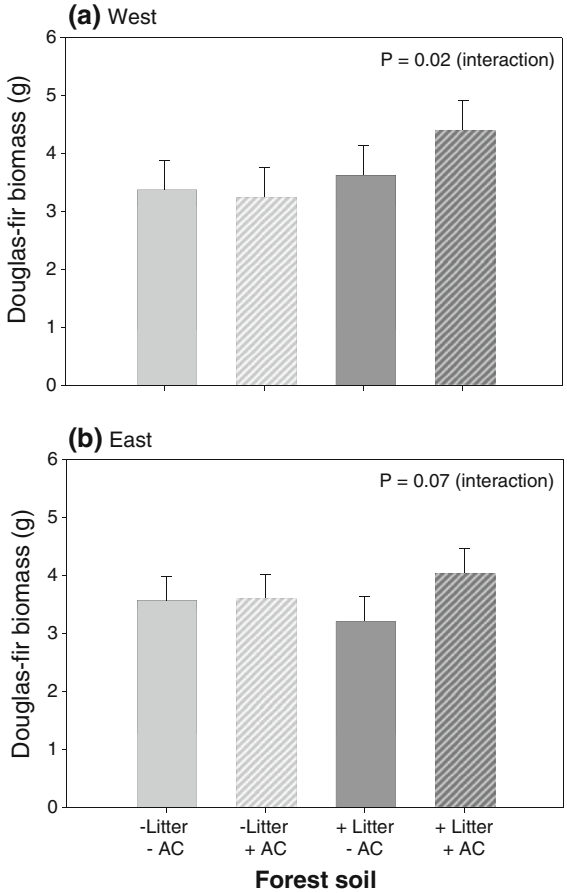

Fig. 3 The joint effects of adding Cytisus litter (Litter) and activated carbon (AC) on aboveground biomass (g) of Douglas-fir seedlings after 19 months of growth in *Cytisus*-invaded soils collected from two sites (east and west) at Joint Base Lewis McChord. *Error bars* represent +1 standard error. Significance corresponds to the interaction term in a two-way ANOVA

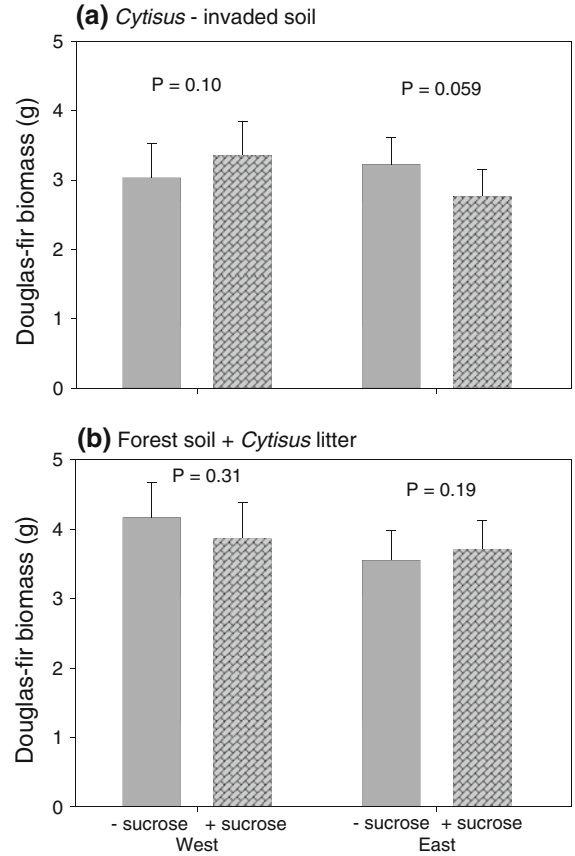

Fig. 4 The effect of adding sucrose on aboveground biomass (g) of Douglas-fir seedlings grown in two types of soils: **a** *Cytisus*-invaded soils, and **b** uninvaded soils to which *Cytisus* litter was added. Soils were collected from two sites (east and west) at Joint Base Lewis McChord. *Error bars* represent +1 standard error. Significance levels are derived from planned contrasts for individual comparisons (see text)

clearly identifiable by means of microscopy. Root colonization by *R. rogersii* was not significantly lower in *Cytisus*-invaded soil than forest soil in the east ($T = 1.19$, DF $= 24.97$, $P = 0.12$) or west ($T = 1.14$, DF $= 27$ $P = 0.13$) (Fig. 7). *Cytisus* litter addition did not decrease *Rhizopogon* colonization in forest soils (east: $T = 0.86$, DF $= 25.60$, $P = 0.80$, west: $T = 0.43$, DF $= 23.77$, $P = 0.66$). Additionally, activated carbon did not increase *Rhizopogon* colonization in soils amended with *Cytisus* litter (east: $T = 0.06$, DF $= 23.16$, $P = 0.52$, west: $T = 0.52$, DF $= 21.87$, $P = 0.69$). In contrast, the abundance of *C. geophilum* on Douglas-fir roots was 55 % lower in long-invaded *Cytisus* soil compared to the uninvaded forest soil in the east (one-tailed Wilcoxon, $\chi^2 = 5.06$, $P = 0.02$; Fig. 7). In the west, *Cenococcum* abundance decreased by 81 % in *Cytisus* soils ($\chi^2 = 8.05$, $P = 0.004$) compared to forest soils (Fig. 7). The addition of litter to forest soils marginally significantly decreased the abundance of *Cenococcum in the east* ($\chi^2 = 2.38$, $P = 0.12$), but not in the west ($\chi^2 = 0.29$, $P = 0.58$). Activated carbon did not increase *Cenococcum* abundance in either site (east: $\chi^2 = 0.65$, $P = 0.42$; west: $\chi^2 = 0.35$, $P = 0.55$).

Discussion

The soil legacy of *Cytisus*, a putative allelopathic N-fixer, may be comprised of both positive and

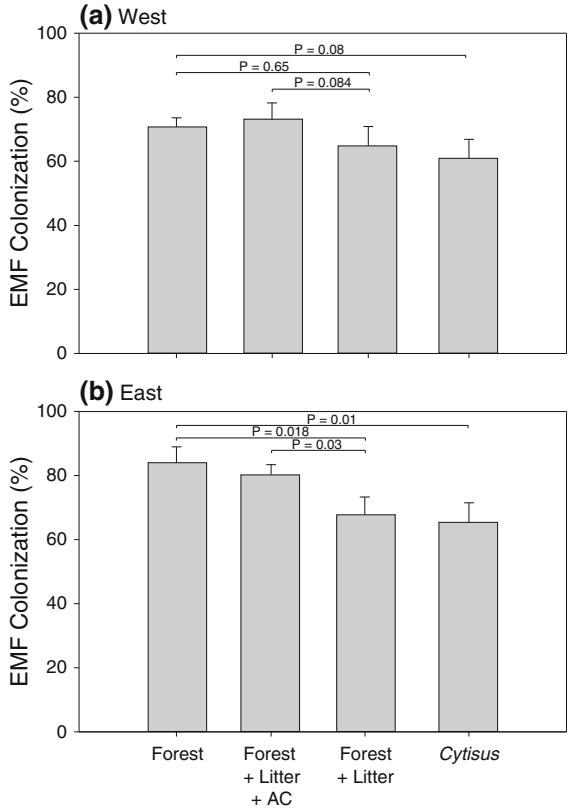

Fig. 5 Colonization of roots by ectomycorrhizal fungi (EMF) for Douglas-fir seedlings grown in four types of soils: uninvaded forest soils with no amendments, forest soils to which both *Cytisus* litter and activated carbon were added (+Litter +AC), forest soils to which only *Cytisus* litter was added, and soils long invaded by *Cytisus*. Soils were collected from two sites (east and west) at Joint Base Lewis McChord. *Error bars* represent +1 standard error. *Brackets* above *bars* denote significance of *t* tests for specific comparisons

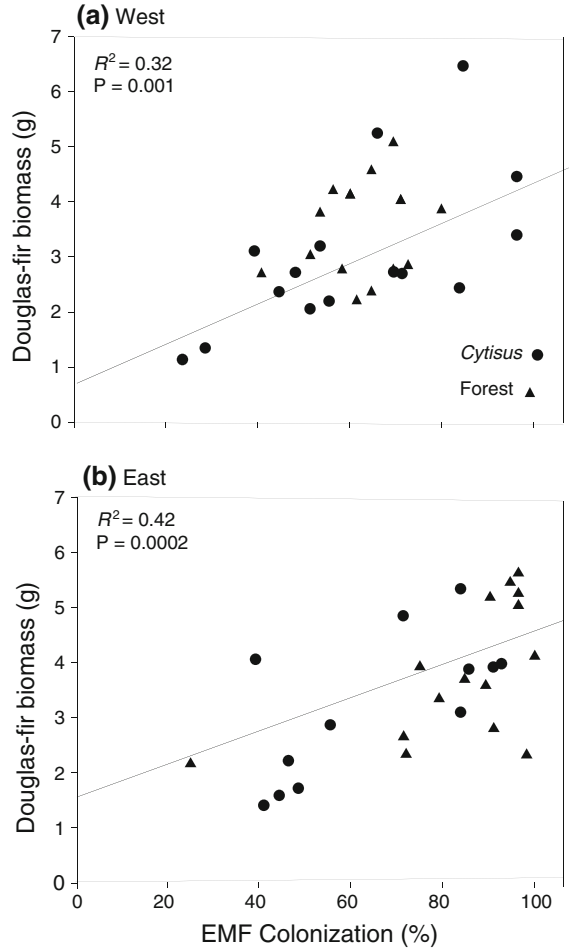

Fig. 6 Above ground biomass (g) of Douglas-fir seedlings is significantly predicted by the percentage of roots colonized by ectomycorrhizal colonization (EMF). Data from *Cytisus*-invaded and uninvaded forest soils (with no amendments) were combined for this analysis; soils were collected from two sites (east and west) at Joint Base Lewis McChord

negative effects: *Cytisus* elevates soil N, while also producing leaves with high concentrations of sparteine, a toxic alkaloid (Gresser et al. 1996). Our previous results suggested negative effects of *Cytisus* have the potential to outweigh positive effects on the growth of native herbaceous species (Haubensak and Parker 2004). Here, we found that the growth of a dominant native tree and its fungal symbionts also demonstrated a net negative impact of the invader. However, we found complex and variable patterns in how nitrogen fertilization and allelopathy interact to produce a legacy effect through both direct and indirect mechanisms.

We had two different approaches to measuring the effects of *Cytisus* on soils: (1) comparing uninvaded forests to nearby, long-invaded clearcuts and (2) adding fresh *Cytisus* litter directly to uninvaded forest soils. The *Cytisus*-invaded soils used in our study had been invaded for several decades and so could be considered a "worst-case scenario" in terms of the legacy effects of *Cytisus* on the soils. On the other hand, other site differences between the two intact forests and the two invaded clearcuts could strongly influence our results. Certainly, the four locations varied substantially in their nutrient status and stoichiometry, which probably reflects underlying site history and soil features as well as (and interacting with) the role of *Cytisus* invasion. For example, trees

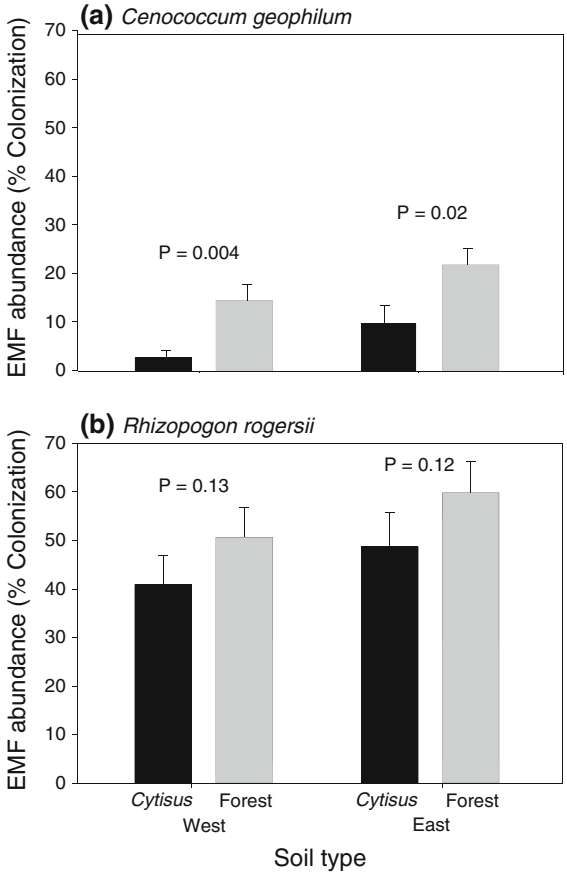

Fig. 7 Colonization of Douglas-fir roots by two species of ectomycorrhizal fungi (EMF), *Cenococcum geophilum* and *Rhizopogon rogersii*, for seedlings grown in uninvaded forest soils and soils long invaded by *Cytisus*. Soils were collected from two sites (east and west) at Joint Base Lewis McChord

were probably removed earlier at the east than the west invaded site, and the west invaded site may have burned more frequently and more intensively than the east site.

Our experimental addition of *Cytisus* litter circumvents the problem of underlying variability among the sites, and it can suggest how a recent *Cytisus* invasion might influence abiotic and biotic features of an intact forest soil environment. For example, if soil microbial communities change slowly over time in response to *Cytisus* invasion, long-invaded soils would represent an endpoint; addition of activated carbon to these soils would not show a benefit to microbial communities because the sensitive species would be absent and, therefore, unable to respond. In contrast, activated carbon would have the potential to ameliorate the effects of *Cytisus* litter on an intact forest microbial community, thereby allowing for the indirect effects of allelopathy to be seen.

Direct effects of *Cytisus* on Douglas-fir

As we predicted, the net effect of soil long invaded by *Cytisus* was to suppress the growth of Douglas-fir seedlings. Several others have demonstrated similar suppression effects of invasive species' soil legacies on target native species. For example, Batten et al. (2008) showed that the soil microbial community was altered in soils invaded by the exotic grass *Aegilops triuncialis* resulting in delayed flowering, decreased biomass, and increased root allocation in some native species. However, we found more examples in the literature of studies that demonstrated a legacy effect without a specific mechanism tested (Grman and Suding 2010). Several mechanisms have been suggested for legacies, including altered resources (Corbin and D'Antonio 2004a) or accumulation of pathogens (Bever 1994; Klironomos 2002). However, comparative studies that use invader-conditioned soils and uninvaded soils, and that demonstrate reduced growth of target native species in the former relative to the latter, are surprisingly uncommon.

Allelopathy is another possible mechanism by which invasive plants may alter the soil and affect growth of subsequent colonizers. For example, catechin root exudates produced by *Centaurea maculosa* contribute both to success in its introduced range and depressed growth of native grasses (Ridenour and Callaway 2001). The production of mustard oils by the invader *A. petiolata* results in depressed tree seedling recruitment (Stinson et al. 2006, Wolfe et al. 2008). In our study, the addition of activated carbon to *Cytisus*-invaded soils did not increase Douglas-fir growth. The effectiveness of activated carbon in field and lab studies is mixed suggesting an indiscriminate nature of activated carbon's affinity for organic compounds. For example, Kulmatiski and Beard (2006) showed that some native species responded positively and others negatively to activated carbon addition following non-native removal. Other studies have shown no effect of activated carbon on target species (Barto and Cipollini 2009). In this study, the lack of effect of activated carbon on Douglas-fir growth may reflect ineffectiveness at binding sparteine, the primary alkaloid produced by *Cytisus*. An alternative explanation is that allelopathic compounds produced by *Cytisus* do not

directly affect Douglas-fir growth, but instead have an indirect effect via its mycorrhizal partners. As explained above, adding activated carbon to long-invaded soils would not reveal such an indirect effect because those mutualistic partners would presumably be absent from those soils.

In support of this hypothesis, we did find a strong interaction between the effect of adding *Cytisus* litter and the effect of activated carbon to forest soils. We will explore the potential role of ectomycorrhizae in this pattern below. In both sites, activated carbon transformed the effect of litter into a strong positive effect. Interestingly, without activated carbon, litter addition depressed tree growth in soils from the east site, whereas litter appears to have a slight positive net effect in the soils from the west site. This may suggest that the net sum of positive and negative factors can vary from site to site. We expected that the net effect of litter addition would be more positive in the site with greater nitrogen limitation (i.e., lower nitrogen availability), but our nutrient analysis did not support our hypothesis.

Nitrogen fertilization likely explains the positive effect of *Cytisus* litter on Douglas-fir growth. *Cytisus*-invaded soils have elevated rates of plant-available nitrogen in California grasslands (Haubensak 2001), and glacial outwash prairies (Haubensak and Parker 2004). However, our sucrose addition results only weakly support the hypothesis that Douglas-fir growth is responding to nitrogen enrichment by *Cytisus* litter in forest soils. When added to the *Cytisus*-invaded soil, sucrose significantly reduced growth in one of the two sites, but when combined with the litter addition treatment, sucrose did not significantly reduce growth. We added a single dose of sucrose at the onset of the experiment, and it is likely that the effect of sucrose on N availability was intensive, but short-lived (Torok et al. 2000). Further, slower-growing species from infertile sites, like Douglas-fir, may not respond strongly to short-term changes in N dynamics (Chapin et al. 1990).

Indirect effects of *Cytisus* on Douglas-fir: ectomycorrhizal fungi

The abundance of ectomycorrhizal fungi was lower on seedling grown in *Cytisus* soils compared to uninvaded forest soils. Adding *Cytisus* litter to forest soil also suppressed EMF colonization, at least in soil from one site. These effects on mycorrhizal fungi are likely to have important implications for the growth and establishment of Douglas-fir seedlings. Others have found that EMF can be critical to conifer establishment because of their role in nutrient acquisition (Read et al. 2004; Nuñez et al. 2009; Teste et al. 2009). For example, some Pinaceae in nutrient-limited environments show higher leaf nutrient content and faster growth when colonized by EMF (Parke et al. 1983; Abuzinadah and Read 1986; Gehring and Whitham 1994; Van Tichelen and Colpaert 2000). Consistent with those studies, we also found that Douglas-fir growth was positively and significantly related to mycorrhizal colonization across sites and soil treatments.

One possible explanation for the negative effects of *Cytisus* on ectomycorrhizae is that mycorrhizal fungi may be suppressed by allelopathic compounds. We tested this with addition of activated carbon to forest soils with *Cytisus* litter; EMF colonization increased in the presence of activated carbon relative to soils with *Cytisus* litter alone, but only in one site. Few studies have experimentally shown that invasive species inhibit the mycorrhizal colonization of native species via the mechanism of allelochemicals. An exception is Zhang et al. (2007), who examined interactions between invasive and native species grown in soil containing constructed arbuscular mycorrhizal fungi communities. Using activated carbon and extracts from the rhizome of the invader, they showed that the depressive effect of the invader on the native was via allelochemicals contained in those extracts.

Until recently, allelopathy mediated by mycorrhizae has been an unexplored mechanism by which invasive plants may impact native plant communities. The invader *A. petiolata*, an entirely non-mycorrhizal plant, suppresses both arbuscular (Stinson et al. 2006; Callaway et al. 2008; Vogelsang and Bever 2009) and ectomycorrhizal fungi (Wolfe et al. 2008) in invaded soils. *Alliaria* and other plants in the Brassicaceae suppress mycorrhizal fungi through root exudates of anti-fungal allelopathic mustard oils (glucosinolates) (Kliebenstein 2004). The end result is that establishment, growth and competitive ability of native plants are negatively impacted. It is still unclear whether this type of impact is a common and important feature of plant invasions.

Two other mechanisms could also contribute to the reduction we saw in EMF colonization of Douglas-fir seedlings in *Cytisus* soils and with *Cytisus* litter: nitrogen enrichment and absence of suitable plant

hosts. In the presence of elevated nitrogen, some plants will suppress mycorrhizal associations (Treseder and Allen 2002; van Diepen et al. 2007; Hoeksema et al. 2010). This could occur in our system in response to nitrogen enrichment of the soil by *Cytisus*. Finally, *Cytisus* itself does not associate with ectomycorrhizal fungi, and EMF generally require a plant host in order to persist in a site over the long term (Molina et al. 1992; Outerbridge and Trofymow 2004). Therefore, soils that have been invaded for several decades are likely to be depauperate of ectomycorrhizal fungi suitable for Douglas-fir. All considered, the long-invaded *Cytisus* soils supported what are perhaps surprisingly high rates of EMF colonization on Douglas-fir. This could be explained either by long-distance dispersal of fungal spores or by extended spore dormancy, although little is known about the longevity of EMF spores in the absence of suitable plant hosts (Nara 2009).

In addition to overall EMF colonization, we quantified the abundance of two specific fungal taxa, *C. geophilum* and *R. rogersii*, which are broadly distributed and commonly associated with Douglas-fir. The two taxa responded differently to *Cytisus* invasion. *Rhizopogon* was not significantly reduced in the *Cytisus*-invaded soils, which may reflect the ability of *Rhizopogon* spores to remain dormant in the absence of a host for at least a decade (Bruns et al. 2009). In contrast, we found that *Cenococcum* was strongly reduced in the *Cytisus*-invaded soil compared to uninvaded forest soil. The apparent differential sensitivity of different fungal species to *Cytisus* invasion would be expected to result in shifts in overall community structure and composition. Ectomycorrhizal fungi vary in their effects on their plant host, providing different degrees of benefit and even being parasitic under certain conditions (Johnson et al. 1997; Jones and Smith 2004). Thus, differential sensitivity of mycorrhizal fungi to the soil changes that accompany invasions may have important consequences for reforestation success.

In conclusion, *Cytisus* invasion has a net negative impact on Douglas-fir growth through its effects on soil, consistent with an earlier bioassay and with the hypothesis that *Cytisus* is an allelopathic invader. Ectomycorrhizal fungi are also suppressed in *Cytisus* soils, and the strong relationship we found between plant size and mycorrhizal colonization could reflect the potential for the suppression of mycorrhizae to feed back to tree growth and establishment. The legacy of *Cytisus* invasion integrates both positive and negative factors; and, as we found here, their relative importance is likely to be highly context-specific.

Allelopathy may play an important role in biological invasions and in ecological restoration (also see Cummings et al. this issue). Our study suggests how this role may play out on two time scales. Allelopathic invaders may have immediate, direct impacts on native plant communities. At the same time, there may be long-term, persistent impacts mediated by the collapse or transformation of microbial communities. Ultimately, the chronic, indirect effects could be at least as important as the direct effects, even though they are rarely quantified or even considered.

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