

**A Widespread Nitrogen-fixing Invader Experiences Negative Soil Feedbacks Despite
Increased Root Nodulation and Mycorrhizal Colonization**

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Abstract

Invasive species success can be inhibited or intensified by plant-soil feedbacks, in which plants modify the microbial community or abiotic environment of the soil in a way that in turn affects plant growth. Plant-soil feedbacks for introduced species can be either positive (favoring invasion) or negative (limiting invasion impacts). *Cytisus scoparius* (Scotch broom) is a globally invasive species that limits restoration and reforestation success through soil legacies. To better understand the success of *C. scoparius*, we investigated whether the invader exhibits positive or negative plant-soil feedback in its invaded range. We performed a greenhouse experiment using soils collected from a clearcut invaded by *C. scoparius* and nearby uninvaded forest. We compared *C. scoparius* above- and belowground biomass, root nodulation, and the abundance of roots colonized by arbuscular mycorrhizal fungi. *Cytisus scoparius* grown in invaded soils had 67% more root nodules than plants grown in uninvaded forest soil, suggesting that *C. scoparius* invasions into new areas may be limited by their nitrogen-fixing symbionts. *Cytisus scoparius* exhibited 72% greater AMF colonization in plants grown in invaded soils than in uninvaded forest soils, indicating a build up of fungal symbionts in invaded soils. Despite increased root nodulation and arbuscular mycorrhizal fungi, *C. scoparius* grew 21% smaller when grown in invaded soils than in uninvaded soils, indicating negative plant-soil feedback. Taken together, our results suggest that *C. scoparius* has not escaped soil enemies in its introduced range, but increased mutualistic interactions may contribute to *C. scoparius*' persistence. Mutualistic soil biota may counteract negative effects of pathogen build up or nutrient depletion in *C. scoparius* invaded soils, leading to less negative plant-soil feedbacks. This may help explain why *C. scoparius* persists in monospecific stands in invaded regions.

KEYWORDS: *Cytisus scoparius*, plant-soil feedback, invasive, AMF, nodulation

Introduction

The Enemy Release Hypothesis states that invasive species are successful because they escape specialized herbivores and pathogens from their native environment (Elton 1958; Keane and Crawley 2002; Parker and Gilbert 2004; Mitchell and Power 2003; Liu and Stiling 2006). Support for enemy release as a driver for invader success has been mixed. Mitchell and Power (2003) compared virus and certain biotrophic fungal pathogen species richness on 473 plant species naturalized in the U.S. in their native and naturalized ranges and found significantly fewer species in the naturalized range, suggesting a release from natural enemies (Mitchell and Power 2003). While some studies suggest that exotic species may experience success due to a loss of natural enemies (Memmott *et al.* 2000, Keane and Crawley 2002, Klironomos 2002, Reinhart and Callaway 2006), other studies suggest invaders experience equivalent or stronger effects of enemies in introduced regions. This suggests that leaving behind specialized enemies may not drive plant invasiveness (Bever 2002; Beckstead and Parker 2003; Colautti *et al.* 2004). Building and expanding on the Enemy Release Hypothesis, plant-soil feedbacks have been suggested as a mechanism for invader success.

Plant-soil feedbacks occur when plants alter soil abiotic or biotic properties that in turn influence that plant's performance (Parker and Gilbert 2004; Levine *et al.* 2006). Plant-soil feedbacks can result in increased or decreased plant survival, growth, or fitness (Callaway *et al.* 2004; Parker and Gilbert 2004; Reinhart and Callaway 2006). Positive feedbacks occur when plants alter soil biota or chemistry, such as an increase in nitrogen in otherwise nitrogen limited soils, that in turn benefits its own growth. Positive plant-soil can also result from the accumulation of beneficial soil microbes (e.g. mycorrhizal fungi and N-fixing bacteria) whereas negative feedbacks occur when essential nutrients are depleted or soil pathogens accumulate.

Both positive and negative plant-soil feedbacks have been reported for invasive species (Westover and Bever 2001; Bever 2002; Klironomos 2002; Beckstead and Parker 2003; Reinhart *et al.* 2003; Reinhart and Callaway 2006). Release from root pathogens should reduce negative plant-soil feedbacks in invaders (Reinhart and Callaway 2006). However, even when specialist enemies are left behind, other factors may result in a net negative plant soil feedback. For one, invaders may also leave behind specialized beneficial soil biota such as mycorrhizal fungi and nitrogen fixing bacteria (Parker *et al.* 2006; Reinhart and Callaway 2006; Mitchell *et al.* 2006). Another important and often overlooked factor that can result in negative plant-soil feedback is that generalist enemies in the introduced range can also impact invasive plant populations (Keane and Crawley 2002; Beckstead and Parker 2003). Since enemy pressure is generally correlated with host abundance (Janzen 1970; Connell 1971; Gilbert 2002), invasive species that achieve dominance may experience more enemy pressure than native species co-occurring at lower abundance (Parker and Gilbert 2004). This, in theory, could limit invasive species persistence over time (Diez *et al.* 2010). Lastly, negative soil feedbacks could occur if invaders deplete necessary micro or macronutrients (Ehrenfeld 2003).

Arbuscular mycorrhizal fungi (AMF) form symbiotic relationships with roughly 200,000 plant species (Brundrett 2009) and improve nutrient and water uptake (Smith and Read 1997). In addition to AMF mutualistic interactions being incredibly common, a meta-analysis of AMF interactions with native and invasive plants found that AMF is equally likely to form mutualistic relationships with native and invasive plants (Bunn *et al.* 2015). In plant-soil feedback studies in invader systems, positive and neutral feedbacks in invasive species have been attributed to both a loss of pathogens and AMF associations in introduced regions (Richardson *et al.* 2000; Reinhart *et al.* 2003; Callaway *et al.* 2004; Van der Putten *et al.* 2007).

Another beneficial soil microbe that forms associations with some invaders is nitrogen (N) fixing bacteria. Plants in the Fabaceae (legumes) are problematic and successful invasive species worldwide (Daehler 1998) and are known for their ability to form symbiotic relationships with rhizobia. Invasive legume species often increase soil nitrogen availability (Wheeler *et al.* 1987; Ehrenfeld *et al.* 2001; Watt *et al.* 2003; Corbin and D'Antonio 2004; Grove *et al.* 2015). Nitrogen enrichment is often seen as a positive ecosystem service, but the presence of invasive N-fixers can alter plant-plant interactions, resulting in increased dominance of fast growing nitrophylic species and declines in slower growing species (Garcia-Moya and McKell 1970; Maron and Connors 1996; Grove *et al.* 2015). While invasive legumes have the potential to change soil chemistry and plant communities in new environments, their ability to fix nitrogen is dependent upon the presence of their nitrogen-fixing symbionts, which can be host specific or generalists (Wang *et al.* 2012). Thus, plant-rhizobia relationships formed in home and introduced regions can include different microbe species (Horn *et al.* 2014; Traveset and Richardson 2014).

Cytisus scoparius (Scotch broom) is a shrub native to Europe and is invasive on every continent except Antarctica (Peterson and Prasad 1998; Syrett *et al.* 1999). *Cytisus scoparius* was introduced to the Pacific Northwest as an ornamental plant in the 19th century (Gilkey 1957). In the Pacific Northwest (USA), *C. scoparius* invades aggressively after timber harvest in Douglas-fir (*Pseudotsuga menziesii*) forest, and land managers have struggled to both control broom populations and regenerate Douglas-fir populations following *C. scoparius* removal (Waloff 1966; Peterson and Prasad 1998; Grove *et al.* 2012). *Cytisus scoparius* is a legume that forms symbiotic relationships with the nitrogen fixing bacteria, *Bradyrhizobium*, (Sajnaga *et al.* 2001, Lafay and Burden 2006, Horn *et al.* 2014) and generally increases soil nitrogen where it

invades (Watt *et al.* 2003; Grove *et al.* 2012; Grove *et al.* 2015). This N enrichment is associated with an increase in nonnative plant abundance that may be inhibiting native plant recovery following *C. scoparius* removal (Wheeler *et al.* 1987; Watt *et al.* 2003; Grove *et al.* 2015). Alongside changing soil nitrogen, *C. scoparius* produces alkaloids, which have been shown to have negative effects on pathogenic and ectomycorrhizal fungi (Wippich and Wink 1985, Grove *et al.* 2012).

In this study, we used a greenhouse experiment to investigate plant-soil feedback in *C. scoparius* by planting the invader in field soils from *C. scoparius*-invaded and nearby uninvaded forest soils. We tested the hypothesis that *C. scoparius* cultivates positive soil feedbacks through accumulation of beneficial soil microbes, namely N-fixing rhizobia and AM fungi.

Methods

Experiment Setup

We performed a greenhouse experiment using soil collected on January 17, 2017, on two sites in Western Washington (47° 28' 35" N, 122° 50' 15" W). One site was a forest clearcut heavily invaded by *C. scoparius*, hereafter referred to as *C. scoparius*-invaded soil, and the other site was an adjacent uninvaded Douglas-fir forest, hereby referred to as forest soil. The soil was shipped in a cooler to the University of California, Santa Cruz where it was stored in a 4°C refrigerator for 15 days. The soils were then potted in 107 mL cone-tainers (Stuewe and Sons, Tangent, Oregon) with 20 mL of perlite at the bottom to improve drainage, 90 replicates of each soil type. Each replicate was planted with two scarified *C. scoparius* seeds. Seed germination rates were low, so at 24 days after the original seeds were planted, more seeds were germinated on wet filter paper and then added to cone-tainers without a germinated seed. At 92 days after the original planting date more scarified seeds were germinated on wet filter paper and added to

remaining cone-tainers without a germinated seed. Replicates were thinned to one *C. scoparius* seedling per replicate when more than one seed germinated. The experiment was weeded weekly for the duration of the study. After germination, pots were arranged in a randomized blocked design of 6 racks with 45 plants at 10 cm spacing. Plants grew in the UCSC Thimann Greenhouse for 34 weeks.

Aboveground Biomass

We measured dry aboveground biomass of each *C. scoparius* to measure plant-soil feedback responses. Beginning on October 3, 2017, *C. scoparius* seedlings in each block were cut where the stem meets the soil and placed in an envelope. Seedlings were harvested by November 10, 2017, at the time when plants began to show signs of dropping leaves and going into winter dormancy. Plants were dried at 61°C for a minimum of 48 hours and biomass weighed to 0.0001g on an analytical balance.

From October 28 to December 22, 2017 roots were removed from the cone-tainers and rinsed in deionized water and the soil was gently massaged off of the roots. Roots were harvested one rack at a time in random order. A subset of *C. scoparius* plants were selected and further prepped for root nodulation (n=171) and AMF (n= 69) quantification. Total root biomass was measured on a subset of 86 plants.

Root Nodulation

To assess how relationships between *C. scoparius* and nitrogen-fixing bacteria differ in uninvaded and *C. scoparius*-invaded soil types, we counted the number of root nodules on each plant. We also removed, dried, and weighed root nodules from a subsample of 16 plants to assess the relationship between root nodule number and root nodule size. In one rack, we separated out the root nodules and placed them in a labeled coin envelope and the remaining roots in another

labeled coin envelope. Roots and root nodules were then dried at 61°C for a minimum of 48 hours. For the remaining 5 racks, nodules were counted and roots were dried; we could not weigh root nodules on all plants due to time constraints. Once dried, samples were weighed on an analytical balance with precision to 0.0001g.

AMF Colonization

To study how *C. scoparius* invasion influences interactions between this species and arbuscular mycorrhizal fungi, we stained *C. scoparius* roots grown in each soil type using methods based on Phillips and Hayman (1970). Once root nodules were counted, we cut the roots to 0.5-1.5 cm sized segments prior to clearing and staining. We ran a pilot study using *Genista monspessulana*, a close relative of *C. scoparius*, to optimize root clearing and staining times and temperature. Roots were submerged in a 10% KOH solutions and kept in a 55°C hot water bath for 12 hours. The cleared roots were then rinsed with deionized water and neutralized with 1% HCl for 5 minutes at ambient room temperature. We soaked the roots in a 0.05% trypan blue w/v in lactoglycerol staining solution (1:1:1 lactic acid: glycerol: DI water) in a hot water bath at 55°C. After staining for 30 minutes the roots were strained, and covered them with lactoglycerol (1:1:1 lactic acid: glycerol: DI Water).

To quantify AMF abundance, we evaluated 72 subsampled stained root segments. All roots were placed on a 1 cm grid 10 mL square petri dish, and we sampled the root on top of each line intersect and noted the presence or absence of hyphae, arbuscules, and vesicles observed on each root cutting under a stereoscope microscope at 60X magnification. Arbuscules and vesicles are indistinguishable at 60X magnification so their presence was marked if either structure was present.

Data Analysis

We used JMP-Pro version 13 to perform the statistical analyses. We compared above and belowground biomass, number of root nodules, and mycorrhizal colonization in *C. scoparius* grown in the two soil types using t-tests. We used regression to see how well the number of root nodules could predict total nodule weights.

Results

Root Nodulation

The number of root nodules was an estimate of plant-rhizobia interactions. There were about three times more root nodules on *C. scoparius* grown in *C. scoparius*-invaded soils than uninvaded forest soils ($t=5.5$, $df=94$, $P<0.0001$; Figure 1). On the subsample of root nodules, root nodule number significantly predicted total root nodule weight, explaining a high amount of the variance (Nodule Weight (g) $=0.013+0.00011*\text{Number of Nodules}$, $n=16$, $R^2=0.79$, $P<0.0001$; Figure 2). Using the same subsample of roots, we compared the dry weight of all the root nodules between *C. scoparius* invaded and uninvaded soils ($t=2.5$, $df=14$, $P=0.026$; Figure 3).

AMF Colonization

AMF colonization of *C. scoparius* roots was greater in roots grown in *C. scoparius*-invaded soils than uninvaded forest soils ($t=12$, $df=56$, $P<0.0001$, Figure 4; $t=14$, $df=64$, $P<0.0001$, Figure 5).

Aboveground and Belowground Biomass

Aboveground biomass in *C. scoparius* was significantly greater when grown in uninvaded forest soils than in invaded soils ($t=-7.0$, $df=167$, $P<0.0001$; Figure 6). There was also greater root biomass in *C. scoparius* grown in forest soil than in *C. scoparius* soil ($t=-3.6$, $df=79$,

$P < 0.001$; Figure 7). The root to shoot ratio in *C. scoparius* grown in *C. scoparius*-invaded soil was greater than in uninvaded soil ($t=2.3$, $df=62$, $P=0.026$; Figure 8).

Discussion

Greater root nodulation in invaded soils suggests that *C. scoparius* may be rhizobia limited as it invades new territory. Nitrogen fixation by *C. scoparius* and other invasive legumes can be symbiont limited (Parker 2001; Parker *et al.* 2006; Lau and Suwa 2016), and *C. scoparius* has been found to be specifically *Bradyrhizobium* limited, in Parker *et al.*'s (2006) study (Parker 2001; Parker *et al.* 2006). Buildup of rhizobia in soil over time can lead to down-regulation of N-fixation over time due to lower nitrogen demands (Schulze 2004). The high levels of nodulation seen here do not suggest down-regulation of N-fixation, despite that our site of *C. scoparius* invasion is more than a decade old and *C. scoparius* has been found to increase soil nitrogen levels (Watt *et al.* 2003; Grove *et al.* 2012; Grove *et al.* 2015).

In addition to greater nodulation, we observed greater AMF colonization in *C. scoparius*-invaded soils, once again suggesting symbiont limitations in uninvaded forest soils. AMF limitation in forest soils is not surprising, as Douglas-fir forms symbiotic relationships with ectomycorrhizal fungi (EMF) (Grove *et al.* 2012; Grove *et al.* 2017); we therefore suspect the surrounding uninvaded forest soils are dominated by EMF and are AMF limited. Although AMF associations function along a continuum from antagonistic to mutualistic (Johnson *et al.* 1997; Hoeksema *et al.* 2010), AMF associations are more frequently mutualistic (Bunn *et al.* 2015), suggesting that the increased AMF colonization is likely beneficial for *C. scoparius*.

Despite evidence of increased mutualistic interactions in invaded soils, *C. scoparius* grew smaller, suggesting a negative plant-soil feedback. Our results are similar to other studies on

invaders that experienced negative plant-soil feedbacks in their introduced ranges (Bever 2002; Reinhart and Callaway 2006; Suding *et al.* 2013). *Cytisus scoparius* has also been in northwestern North America for more than a century (Gilkey 1957), potentially enough time for some of its native pathogens to be introduced to the region or to gain other pathogens which can lead to negative plant-soil feedbacks (Hallett 2006; Diez *et al.* 2010). Known soil pathogens have been observed on other *Cytisus* species in North America, such as *Pythium* sp. and *Rhizoctonia* sp. (Farr *et al.* 1989). Additionally, the hyphae we observed may have been pathogenic not just AMF, and increased hyphal abundance reflects the negative response in *C. scoparius* growth.

Two other factors could explain the lower performance of *C. scoparius* in invaded soil. First, the observed negative plant-soil feedback may be driven by nutrient dynamics, such as phosphorus (P) limitation in *C. scoparius*-invaded soils (Vance 2001; Bever *et al.* 2010). Increased allocation to root biomass in *C. scoparius*-invaded soils is consistent with this possibility. N-fixation is a process that requires high amounts of P, and the greater nodulation in invaded soils could have the effect of depleting P. Increased AMF colonization, as we found in invaded soils, has been found in legumes grown P-limited soils (Ossler and Heath 2018).

Alternatively, the soils from this study were collected at sites with underlying differences which may contribute to plant-soil feedback dynamics. The *C. scoparius*-invaded site was clearcut and therefore disturbed, possibly leading to soil compaction and/or leaching. To rule out these site differences as a driver of the observed plant-soil feedbacks would require an additional feedback experiment in the greenhouse. We will be performing a follow up experiment investigating plant-soil feedbacks by taking forest soils in which Douglas-fir and *C. scoparius* have grown for 8 months, then testing the performance of *C. scoparius* in those soils. Our future

results will help clarify the mechanisms behind reduced performance in the invaded soils of this experiment.

Our results suggest that positive plant-soil feedbacks are not likely the driver of *C. scoparius* invasion success. However, soil biota interactions may help explain how *C. scoparius* persists despite its negative plant-soil feedbacks in invaded soils. AMF colonization of legumes in P-limited soils is more frequently beneficial for the plant (Hoeksema *et al.* 2010). Therefore increased AMF colonization and nodulation may counteract the negative effects of pathogen buildup or nutrient limitation leading to less negative plant-soil feedbacks (Morris *et al.* 2007). Less negative plant-soil feedbacks in their own soil may be enough for an invader to outcompete native plants, which experience more negative plant-soil feedbacks in invader soils (Suding *et al.* 2013). In previous studies Douglas-fir, the dominant native species at our sites, does grow worse in *C. scoparius* invaded soils (Grove *et al.* 2015; Grove *et al.* 2017). The negative growth response of Douglas-fir in *C. scoparius* soils has been suggested to be caused by allelopathy in *C. scoparius*, which harms ectomycorrhizal fungi associated with Douglas-fir (Grove *et al.* 2012). Suppression of native EMF by AMF associating invaders aligns with the degraded mutualist hypothesis, in which exotic species decrease symbiotic relationships in native plants and fungi (Stinson *et al.* 2006; Vogelsang and Bever 2009; Suding *et al.* 2013). If either *C. scoparius* AMF associations or allelopathic chemicals prevent EMF associations, the Douglas-fir-EMF system may experience mutualism disruption preventing Douglas-firs from moving in and reestablishing in *C. scoparius*-invaded areas (Grove *et al.* 2017).

While *C. scoparius*' invasiveness cannot be explained by positive plant-soil feedbacks in this system, our results suggest that soil biota may contribute to *C. scoparius*' continued dominance. AMF and rhizobia may counteract the negative effects of either nutrient depletion or

pathogen buildup in the soil (Morris *et al.* 2007) and when combined with *C. scoparius*' allelopathic properties and life history traits may allow *C. scoparius* to outcompete Douglas-fir despite negative plant-soil feedbacks.

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Figures

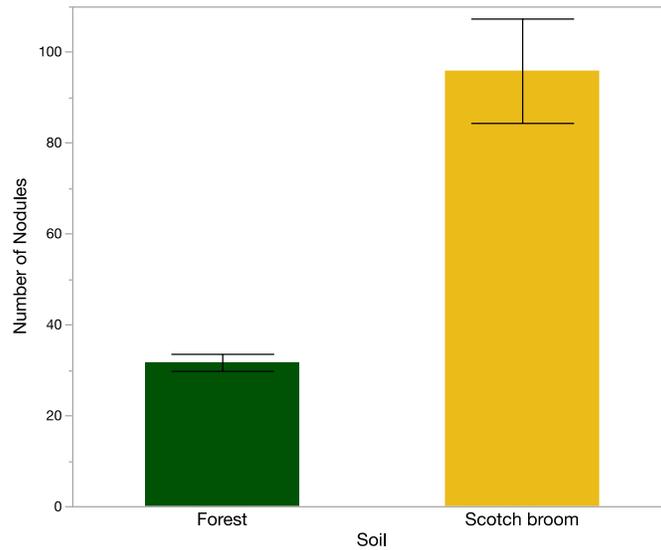


Figure 1. Number of root nodules on *Cytisus scoparius* grown in uninvaded forest soil (green bar) and Scotch Broom invaded soil (yellow bar). The error bars show +/- 1 standard error.

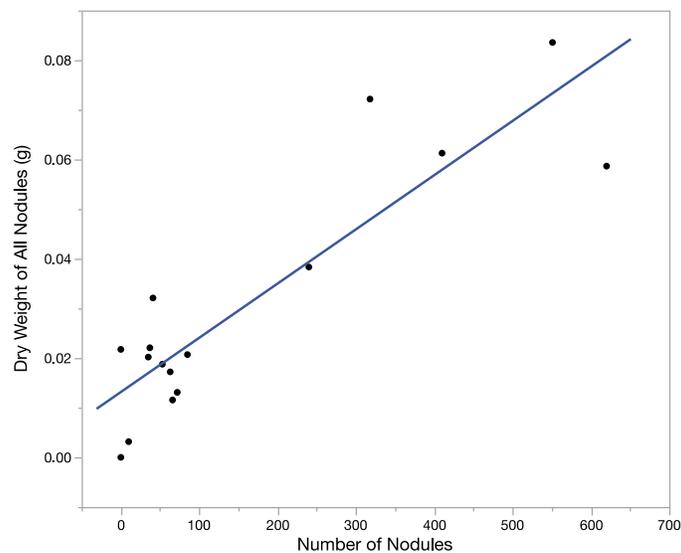


Figure 2. Linear regression of *Cytisus scoparius* root nodule weight (g) on root nodule number. The points represent root nodules of individual *C. scoparius*. The blue line is the line of best fit (Nodule Weight (g)=0.013+0.00011*Number of Nodules).

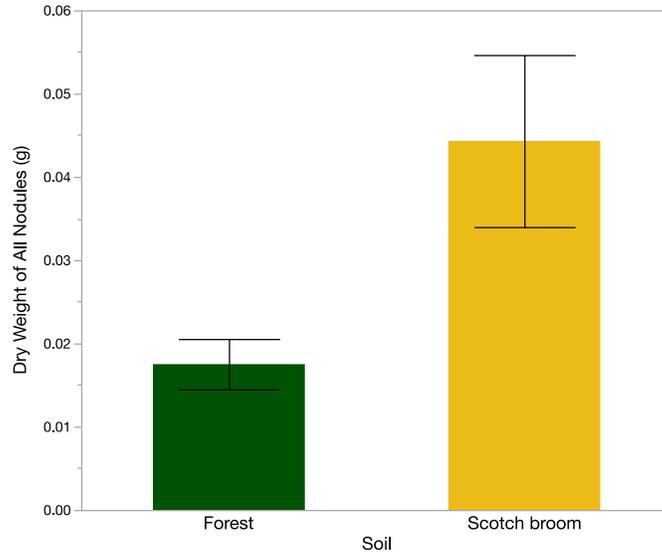


Figure 3. Dry weight of all root nodules (g) on *Cytisus scoparius* grown in uninvaded forest soil (green bar) and Scotch Broom invaded soil (yellow bar). The error bars show +/- 1 standard error.

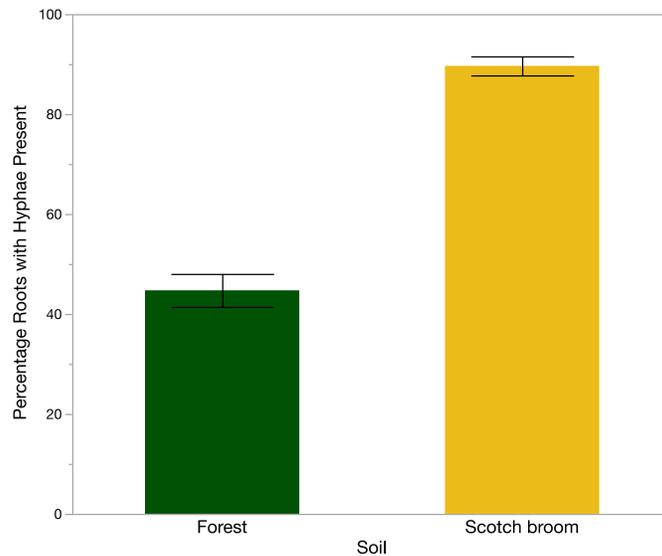


Figure 4. Percentage of roots with AMF hyphae present on *Cytisus scoparius* grown in uninvaded forest soil (green bar) and Scotch broom invaded soil (yellow bar). The error bars show +/- 1 standard error.

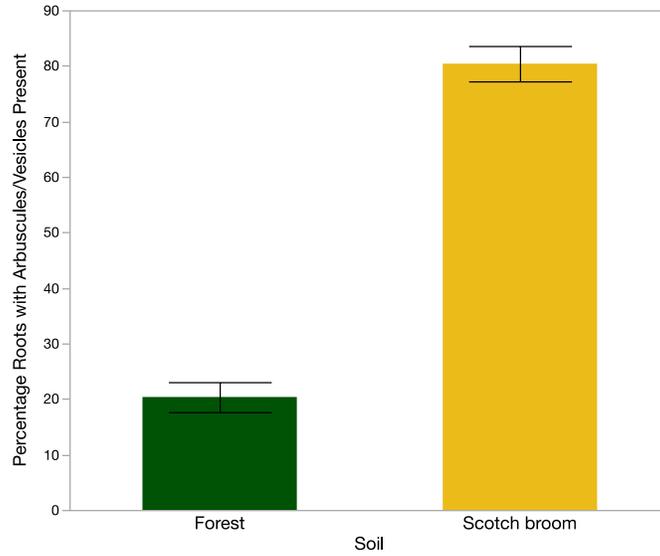


Figure 5. Percentage of roots with AMF arbuscules and/or vesicles present on *Cytisus scoparius* grown in uninvaded forest soil (green bar) and Scotch broom invaded soil (yellow bar). The error bars show +/- 1 standard error.

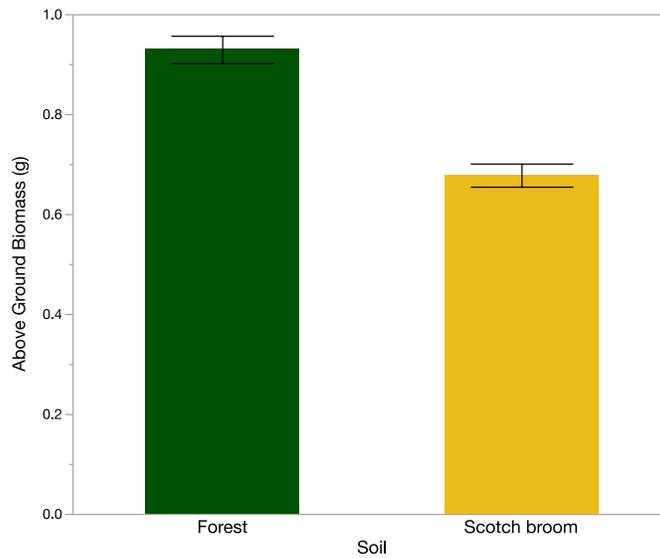


Figure 6. *Cytisus scoparius* dry aboveground biomass in uninvaded forest soil (green bar) and Scotch Broom invaded soil (yellow bar). The error bars show +/- 1 standard error.

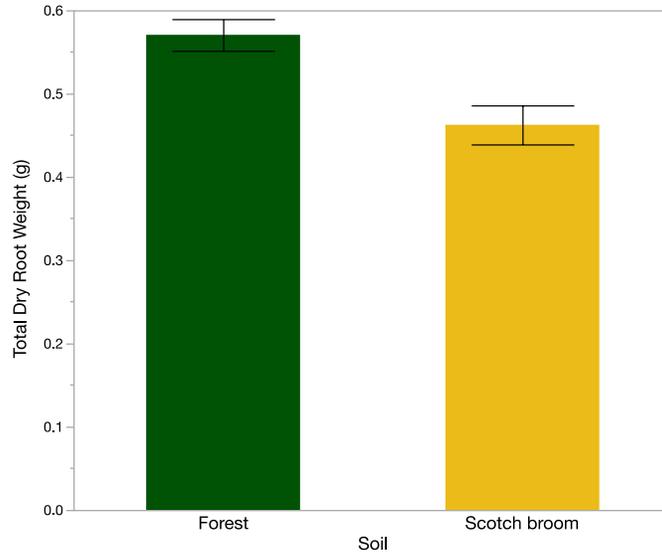


Figure 7. *Cytisus scoparius* root biomass in uninvaded forest soil (green bar) and Scotch Broom invaded soil (yellow bar). The error bars show +/- 1 standard error.

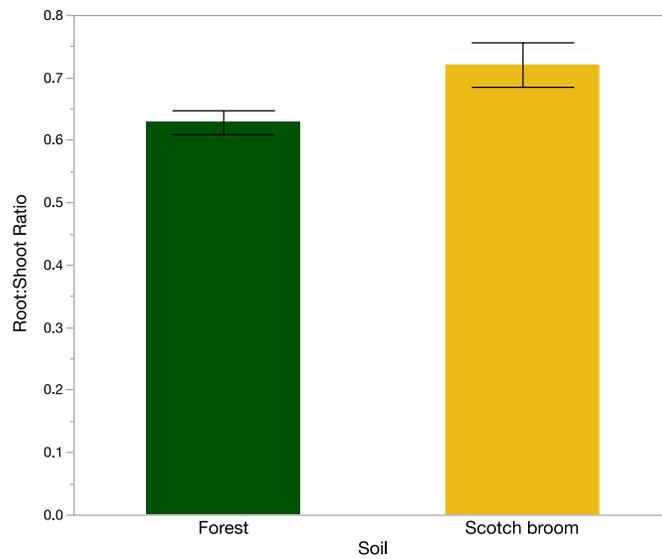


Figure 8. Root to shoot ratio of *Cytisus scoparius* grown in uninvaded forest soil (green bar) and invaded Scotch Broom soil (yellow bar). The error bars show +/- 1 standard error.