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Ectomycorrhizas and tree seedling establishment are strongly influenced by forest edge proximity but not soil inoculum

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Abstract. Reforestation is challenging when timber harvested areas have been degraded, invaded by nonnative species, or are of marginal suitability to begin with. Conifers form mutualistic partnerships with ectomycorrhizal fungi (EMF) to obtain greater access to soil resources, and these partnerships may be especially important in degraded areas. However, timber harvest can impact mycorrhizal fungi by removing or compacting topsoil, removing host plants, and warming and drying the soil. We used a field experiment to evaluate the role of EMF in Douglas-fir reforestation in clearcuts invaded by *Cytisus scoparius* (Scotch broom) where traditional reforestation approaches have repeatedly failed. We tested how planting distance from intact Douglas-fir forest edges influenced reforestation success and whether inoculation with forest soils can be used to restore EMF relationships. We used an Illumina DNA sequencing approach to measure the abundance, richness and composition of ectomycorrhizal fungi on Douglas-fir roots, and assessed differences in Douglas-fir seedling survival and growth near to and far from forest edges with and without forest soil inoculum. Planting Douglas-fir seedlings near forest edges increased seedling survival, growth, and EMF root colonization. Edge proximity had no effect on EMF richness but did change fungal community composition. Inoculations with forest soil did not increase EMF abundance or richness or change community composition, nor did it improve seedling establishment. With Illumina sequencing, we identified two to three times greater species richness than described in previous edge effects studies. Of the 95 EMF species we identified, 40% of the species occurred on less than 5% of the seedlings. The ability to detect fungi at low abundance may explain why we did not detect differences in EMF richness with distance to hosts as previous studies. Our findings suggest that forest edges are suitable for reforestation, even when the interiors of deforested areas are not. We advocate for timber harvest designs that maximize edge habitat where ectomycorrhizal fungi contribute to tree establishment. However, this study does not support the use of inoculation with forest soil as a simple method to enhance EMF and seedling survival.

Key words: Cytisus scoparius; *Douglas-fir;* Pseudotsuga menziesii; *reforestation; Scotch broom; soil transplanting.*

Introduction

Clearcutting and other forms of tree harvest are disturbances that can alter abiotic conditions as well as vegetation and soil community structure in ways that make successful restoration more difficult (Keenan and Kimmins 1993). This is especially true when timber harvests occur on marginal habitat or when harvests are followed by invasion by aggressive nonnative species (Mack et al. 2000, Fischer et al. 2006, Cummings et al. 2007). Nonetheless, reestablishment of forest stands after harvest is often mandated by environmental law in the western United States (Oregon Department of Forestry 1971, Washington Department of Natural Resources 1974). Seedling establishment in clearcuts is often a major bottleneck to successful reforestation (Grossnickle 2012, Jacobs et al. 2015).

There are many factors that contribute to improved conditions for seedling growth at forest edges. Shade provided by the trees at edges results in decreased solar radiation, lower air and soil temperatures, and increased soil moisture relative to the clearcut interiors (Chen et al. 1993, Davies-Colley et al. 2000, Redding et al.

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2003). Hydraulic redistribution by mature trees can bring water within reach of establishing seedlings (Dawson 1993, Schoonmaker et al. 2007). Proximity to mature trees may also allow access to established mycorrhizal networks and inoculum, improving seedling establishment in timber-harvested landscapes (Jones et al. 2003, Cline et al. 2005, Dickie and Reich 2005). Ectomycorrhizal fungi are a ubiquitous and dominant component of soil microbial communities in coniferous forest worldwide (Högberg and Högberg 2002). Ectomycorrhizal fungi form a mantle around fine plant roots and extend hyphae out into the soil to acquire water and nutrients for host plants (Smith and Read 2008), and can be critically important to seedling establishment and growth (Onguene and Kuyper 2002, Cline et al. 2005, Nara 2006, Teste and Simard 2008, Booth and Hoeksema 2010).

Along with their dominance and importance, some EMF are vulnerable to impacts of disturbance (Visser 1995, Twieg et al. 2007). Ectomycorrhizal fungi occur at highest abundance in the top 20 cm of soil (Wallander et al. 2004), so compaction or displacement of topsoil during logging operations can impact EMF communities (Hartmann et al. 2014). Following harvest, the carbon supply from host trees to EMF partners declines or is eliminated altogether (Jones et al. 2003). Canopy removal also increases irradiance, soil temperatures, and evaporation, which can further impact EMF (Fernandez et al. 2017). Because EMF inoculum is present as hyphae emanating from roots for only a short time after host removal (Baath et al. 2004, Drigo et al. 2012), the impacts of deforestation on EMF can be worsened by the invasion of nonnative species that are not suitable EMF host plants.

Declines in EMF diversity associated with timber harvests and invasion may have negative impacts on seedling establishment. Ectomycorrhizal community diversity can affect plant performance (Jonsson et al. 2001). Complementarity among EMF species is expected to lead to a positive relationship between EMF diversity and plant performance because functional diversity of symbionts can satisfy different needs of host plants (e.g., promoting drought tolerance, nutrient acquisition, and disease resistance; Hoeksema et al. 2010, Jones et al. 2010, Wagg et al. 2011). Similarly, changes in EMF community composition can also influence seedling establishment. Ectomycorrhizal species vary in their ability to tolerate disturbance (Koide et al. 2011) and species that persist in the soil following disturbances such as clearcutting may not be the best suited to promote seedling establishment in the degraded environment.

In addition to the direct effects of timber harvest on mycorrhizal resources for seedling reestablishment, invasive nonnative plant species may contribute further to the loss of native conifers and their EMF. Invasive species can influence EMF abundance, diversity, and composition by (1) competing with host plants, (2) changing soil properties such as nutrient availability, and (3) chemical inhibition (reviewed in Grove et al. 2017*a*). In the Pacific Northwest, invasive plants contribute to the challenges of forest regeneration and are known to influence EMF associated with native conifers (Grove et al. 2017*b*).

Native to Europe, *Cytisus scoparius* (hereafter *Cytisus*) is a globally problematic woody invader in grasslands, woodlands, and shrublands, and can impact reforestation following timber harvests. In the Pacific Northwest region of the United States, *Cytisus* can form dense stands that prevent forest regeneration (Washington Department of Agriculture, 2017). In Washington State, it is estimated that the economic impact of *Cytisus* on the timber industry is $42,907,000 per year (Washington Department of Agriculture 2017). *Cytisus* is a legume shrub (Fabaceae) that increases soil N availability (Wheeler et al. 1987, Haubensak and Parker 2004, Caldwell 2006) and produces high amounts of the defense compound sparteine (Wink et al. 1982, Gresser et al. 1996, Wink 2002). Our earlier work has shown that Douglas-fir seedlings grown in *Cytisus*-invaded soils had less ectomycorrhizal colonization and were smaller relative to seedlings grown in uninvaded forest soil and that both N enrichment and sparteine can have adverse effects on EMF (Grove et al. 2012, 2017*a*; J. Thompson, S. Grove and I. M. Parker, *unpublished data*).

Where traditional reforestation approaches fail to establish trees in degraded or marginal areas, foresters should consider alternative approaches. One possible approach to reforesting heavily invaded clearcuts is to plant trees in close proximity to forest edges, where EMF communities are presumably intact and readily available to colonize restoration plants (Kranabetter and Wylie 1998, Dickie et al. 2002, Jones et al. 2003). Beyond edges, a second approach would be to transplant soil from nearby intact forests into degraded clearcuts. These soil additions may compensate for the loss of vulnerable but important EMF mutualists and improve seedling survival. Adding soil collected from undisturbed areas as a source of mycorrhizal inoculum has been employed in other restorations with mixed success (Rowe et al. 2007, Carbajo et al. 2011, Emam 2016, Wubs et al. 2016). Most of these studies focused on plant performance and community composition, leaving open the question of the role of the soil microbiome.

We implemented a field experiment to explore the role of EMF in reforestation of invaded clearcuts. We tested two restoration approaches in deforested areas that are heavily invaded by *Cytisus*: planting distance from intact Douglas-fir forest edges and inoculation with forest soils. We measured seedling success and, using Illumina sequencing, we compared EMF species richness, community structure, and relative abundance on seedlings planted close to and far from forest edges. We hypothesized that (1) the abundance and diversity of EMF would be higher nearer to clearcut edges, and (2) seedlings survival and growth would also be higher. We further hypothesized that the addition of forest soil

to seedlings planted farther from edge would increase EMF diversity and abundance, thus improving seedling establishment.

Methods

The sites used in this study were clearcuts at Joint Base Lewis-McChord (JBLM) in the Puget Sound region of Washington State. JBLM is over 35,000 ha and is managed by the U.S. Department of Defense; it is primarily used for military training. The soils throughout the region are shallow and rocky with poor moisture-holding capacity and characterized as glacial outwash (Appendix S1: Table S3; United States Department of Agriculture 2012). More than half of JBLM is forest habitat and managed for timber production. In the 1960s and 1970s, a policy of creating large clearcuts led to *Cytisus* invasion. Reforestation of these invaded clearcuts over the last 40 yr have had limited success.

We implemented an experiment in five invaded clearcuts, all separated by more than 5 km, surrounded by intact Douglas-fir forest stands (Beal Hill, Johnson Marsh, Tank Table, Nisqually, and Rumble Hill; Appendix S1: Table S1). The sites have been extensively invaded for 19–40 yr prior to the onset of this study (Appendix S1: Table S2), and at the time of this study had greater than 60% cover of *Cytisus* (Parker and Haubensak 2011).

Proximity to edge

To test the effect of forest edge on Douglas-fir survival and growth, we planted trees in close proximity to the intact forest edges and further into the *Cytisus*-invaded clearcuts. We installed 34 pairs of transects around the perimeters of the sites, with one transect ~5 m beyond the drip line of the forest edge and a second parallel an additional 15–25 m out into the clearcuts. The distance of the transects far from the forest edge was chosen after a pilot experiment that showed strong survival differences across that spatial scale. Transects varied in length from (25 to 60 m), depending upon the size and shape of the site perimeter. The bare root seedlings used in this experiment were grown from seed collected at JBLM (seed zone 422) at the Silvaseed tree nursery (Roy, Washington, USA) and were two years old at the time of planting. On 16–26 March 2011, we planted Douglas-fir seedlings (N = 958) every 3 m along each transect. Prior to planting, we used hedge trimmers or chain saws to remove *Cytisus* from 5 m wide swaths along each transect. For the duration of the study we cut *Cytisus* off at the base and used hand pruners to remove any shoots that resprouted from cut stumps.

Soil transplant

We isolated the effect of soil from other edge effects and evaluated the inoculation potential of EMF from nearby forests into invaded clearcuts with a soil transplant treatment. We planted the bare root seedlings ~10–20 cm deep into 3 L of transplanted soil collected from either nearby uninvaded forests or from the immediate vicinity of the transect. The volume of soil transplanted was sufficient to completely cover the seedling's roots and was likely sufficient to accommodate most of the root mass produced over the duration of the study. Soil was collected at the time of planting from the top 15 cm of many dispersed locations within the forest. All vegetation, bryophytes, and litter were scraped off the surface and only mineral soils were collected. Soil treatments (forest soil vs. invaded clearcut soil) were applied in an alternating pattern along each transect.

Douglas-fir survival and growth

During 6–12 May 2011, we measured the initial height and diameter of the Douglas-fir seedlings. Height was measured from the base of the plant to the tallest leaf bud (usually terminal, but sometimes lateral). Stem diameter was measured at 5 cm above the root crown. Initial stem heights and widths were not different across treatments (heights, $F_{3,944} = 2.10$, $P = 0.10$; widths, $F_{3,944} = 0.70$, $P = 0.55$). Toward the end of the first growing season, 12–16 September 2011, we measured seedling survival, height, and diameter. After a second growing season, 7–12 November 2012, we collected final survival, height, and diameter measurements. To obtain aboveground biomass values, we clipped the seedling at the root crown and collected the aboveground portion of the plants for all surviving individuals (N = 243). All individuals were oven dried for a minimum of 7 d at 65° C and then weighed.

Ectomycorrhizae

We collected the entire root system from a subset of surviving Douglas-fir seedlings. We assessed differences in EMF abundance, diversity, and community composition across treatments for three sites. We examined EMF on seedlings from the three sites that had high enough survivorship to provide a reasonable sample from all four treatments (Tank Table N = 58, Nisqually N = 19, and Rumble N = 22; Appendix S1: Table S2). Ectomycorrhizal abundance and diversity measurements were only made on seedlings that survived through the second year of the study. Entire root masses along with the surrounding soil were carefully excavated from the ground and put into 1-gallon freezer bags (1 gallon = 3.79 L). The root samples were stored on ice and transported to the University of California, Santa Cruz. The intact root masses were washed by placing the entire root mass into a tub of deionized water, the roots were gently agitated in the water bath to loosen and remove soil particles. The total root mass of each seedling was then divided in half longitudinally and we randomly selected which half to use for EMF colonization measurements. The other

half of the root mass from each tree was kept frozen at −20° and later used for molecular characterization of the EMF community.

We quantified the proportion of Douglas-fir seedling roots colonized by EMF across treatments for one site, Tank Table (N = 53 seedlings). We cut one-half of the roots up into 0.5 cm segments and placed them into gridded petri plates. We randomly selected 72 root tips per tree and assessed presence or absence of EMF on each tip under a stereoscope. A root segment was considered ectomycorrhizal if root hairs were absent and a sheathing mantle of hyphae was present, or the root tips had morphology that results from EMF infection, such as swelling and secondary tuberculate or coralloid branching.

Ectomycorrhizal communities

We characterized EMF community composition and diversity on the same Douglas-fir seedlings, but not the same roots, that were assessed for EMF abundance. We cut the roots into ~0.5-cm segments and stored them in 2× CTAB lysis buffer. We used a Qiagen DNeasy mini plant kit to extract DNA from 0.5 g of Douglas-fir roots from 102 individual seedlings. We added 10 2.3 mm chrome beads to each sample along with 400 μL of AP1 buffer (Qiagen Dneasy; Qiagen, Hilden, Germany) and agitated the samples on a bead beater for 5 min. We then added 4 μL of RNase from the extraction kit and incubated the samples in a 65°C heat bath for 10 min and then followed the manufacturer's instructions. To increase capture of present diversity and minimize stochastic biases that occur in DNA amplifying, we performed two independent DNA extractions and PCRs per Douglas-fir seedling and combined the data into one species list per tree following sequencing. For each individual extraction, we used 250 mg of prepared roots. To obtain the 250 mg root samples, we randomly selected one-half of the root mass and cut it into 0.5-cm segments. Root segments were pooled and 250 mg were haphazardly selected. The percentage of the total root mass used for molecular characterization ranged from 25% to 100%, depending on seedling size. The DNA extracts were quantified with a Qubit fluorometer (Invitrogen; Thermo Fisher Scientific, Waltham, Massachusetts, USA). Samples were then diluted to equimolar concentration with 5 ng DNA/μL.

With PCR, we amplified the internal transcriber spacer (ITS 2) region of rDNA using the ITS 3 (forward) with ITS 4 (reverse) fungal specific primer pairs (White et al. 1990). We created fusion primers for ITS 2 by incorporating partial Illumina-specific adapters and indexes to the ITS3 (5′-GCATCGATGAAGAACGC AGC-3′; White et al. 1990) and ITS4 (5′-TCCTCCG CTTATTGATATGC-3′; White et al. 1990) primer sequences. Both primers included internal Levenshtein distance indexes (eight nucleotides) and partial sequences identical to the Illumina Nextera adapters. The internal indexes were used in combination to identify each well of a 96-well PCR plate, and partial Nextera sequences were used in a second PCR reaction to complete the Illumina Nextera adapter while an additional set of eight nucleotide Levenshtein distance indexes identified each plate of PCR reactions during sequencing.

We initially amplified DNA extracts in a PCR reaction combining 5.0 μL buffer, 0.75 μL dNTPs, 2.0 μL fusion primer mix (5 μmol/L each), 0.5 μL Kapa Biosystems HiFi polymerase, 14.75 μL ddH_2O, and 2.0 μL DNA template. We cycled this reaction mixture using a touchdown thermal profile that included an initial denaturation step at 95°C for 3 min, followed by 20 cycles of 98°C for 20 s, 70°C for 15 s (dropping 0.5°C every cycle), and 72°C for 15 s followed by 15 cycles of 98°C for 20 s, 60°C for 15 s, 72°C for 15 s, and a final extension of 72°C for 10 min. We included a negative, sterile water control in each plate of PCR amplicons, and we treated these samples identically throughout subsequent library preparation and sequence stages. Following PCR, we validated amplification success by running 8 μL of each PCR product on 1.5% (w/v) agarose for 1.5 h at 100 V and visualizing the results. Each PCR reaction was cleaned and normalized by adding 9 μL of PCR product to a Sequal-Prep Normalization Plate (Invitrogen) and following the manufacturer's protocol. After normalization, we pooled 10 μL of normalized amplicons into 1.5 μL microtubes, and dried them down overnight in a vacuum concentrator (Thermo Fisher Scientific). We resuspended the dried PCR products in sterile, deionized water, and purified the combined pool of amplicons with Agencourt Ampure XP magnetic beads (Beckman Coulter, Brea, California, USA). Prior to adding the outer portion of the Illumina-Nextera adapters, we quantified DNA concentrations of the pooled and cleaned PCR reactions with a Qubit 2.0 Fluorometer (Life Technologies, Grand Island, New York, USA). We then used PCR to extend the partial-length Nextera-adapters to full length while incorporating two additional indexes that we used to identify each plate of PCR reactions. We prepared a reaction mix containing 10 μL HiFi HotStart Buffer, 1.5 μL NTPs, 5.0 μL Nextera primer mix (5 μmol/L each), 1.0 μL HiFi HotStart polymerase, 30 μL ddH_2O, and 20 ng of pooled PCR product. We amplified the reaction mix using a thermal profile that included an initial denaturation step at 95°C for 3 min, followed by 20 cycles of 98°C for 20 s, 60°C for 15 s, 72°C for 15 seconds, and 72°C for 10 min. Following PCR, we purified reactions with 1.8× Agencourt Ampure XP magnetic beads (two times) and quantified purified reactions with the Qubit. We estimated fragment size distributions for each plate of amplicons using a Bioanalyzer (Agilent, Santa Clara, California, USA), and we performed a final quantification of each pool (plate) of amplicons using qPCR (Kapa Biosystems qPCR Library Quantification Kit; Kapa Biosystems, Wilmington, Massachusetts, USA). Before sequencing, we combined multiple pools of amplicons at

equimolar ratios, and we sequenced the pool of pooled libraries using PE300 sequencing on an Illumina MiSeq at the Genotype and Sequencing Facility at University of California Los Angeles.

Raw sequences were demultiplexed by plate using the Illumina BaseSpace platform. Raw sequences were demultiplexed by well using a publicly available python script, Splitaake (*available online*).[7] We employed a strict filter that excluded indexes with a Levenshtein distance (mismatch with possible barcodes) > 1. All subsequent sequence handling was done with the open source software MacQIIME pipeline (Caporaso et al. 2010), unless otherwise noted. Paired-end sequences were concatenated wherever possible. When there was no overlap in paired sequence, the forward read was retained and the reverse read was removed from downstream analysis. We trimmed low-quality base pairs (Phred score <20) and removed sequences shorter than 100 bp. We also removed sequences that matched adaptor sequences after adapter trimming. We then standardized the number of reads per sample by randomly subsampling 9,292 reads for ITS2 with seqtk, a publicly available python script (*available online*).[8] This represented a minimum of 2.5% quantile of the average read number per sample for each ITS fragment separately.

Assignment of operational taxonomical units (OTUs)

Reads were assigned to OTUs using the MacQIIME toolkit (Caporaso et al. 2010), where USEARCH v5.2.236 (Edgar 2010) was applied to create clusters of two or more sequences with a maximum of one mismatch. Taxonomy was assigned to a representative of each cluster with BLAST v2.2.22 (Altschul et al. 1990, 1997), which searched sequences against the UNITE database (Koljalg et al. 2005), filtered at 97% identity match. Finally, we selected OTUs assigned to ectomycorrhizal lineages compiled by Tedersoo et al. (2010) and Tedersoo and Smith (2013) with a python script provided by Branco et al. (2013).

To resolve the identity of OTUs not assigned to the species level, we generated a maximum likelihood phylogenetic tree with PhYML (Guindon et al. 2010) and included the following settings: substitution model HKY85, estimated transition/transversion ratio, fixed proportion of invariable sites, four substitution rate categories, estimated gamma distribution, optimized for topology/length/rate, topology search algorithm = nearest neighbor exchange (NNI).

We excluded certain taxa from the ectomycorrhizal community comparisons: all of the fungi in the order Helotiales, including *Acephala* sp., *Cadophora* spp., *Rhizoscyphus* sp., *Hymenoscyphus* sp., and *Meliniomyces* spp., are dark septate fungi and are most frequently considered root endophytes rather than ectomycorrhizas, per se (Newsham 2011). It is worth noting, however, that these taxa represented 58% of the total sequence reads, and *Cadophora finlandica* was the most abundant fungus on all Douglas-fir seedlings sequenced, irrespective of edge proximity or soil inoculation.

Low numbers of reads (<0.01%) were assumed to be contaminants, as confirmed by our negative controls (Appendix S2). For each EMF OUT, we subtracted the number of reads in the negative control from all samples as described by Nguyen et al. (2015). Because it is possible that some of these fungi were actually present, although at very low abundance, this approach may underestimate true fungal diversity. This reduced the average number of EMF species per sample by 5.75.

Statistical analyses

We modeled Douglas-fir seedling dry biomass and ectomycorrhizal root colonization with two-factor ANOVA models that included proximity to forest edge, inoculation type, and their interaction as fixed factors; field site was included as a random factor.

For seedling survival, which was treated as a binomial variable, we used a GLMM with a binomial logit link function approach to assess the importance of forest edge proximity and soil inoculation type one- and two-years post planting. The full GLMM models included proximity to edge, inoculation type and their interaction as fixed factors and site as a random factor. We compared AIC scores of nested models to evaluate which predictive factors contributed to Douglas-fir survival. The survival, growth (as measured as aboveground dry biomass), and EMF abundance analyses were performed in R version 3.5.0 (R Core Team 2018) with the lme4 (Bates et al. 2015) and MuMIn (Barton 2012) packages.

We analyzed the structure and composition of the fungal community using the Vegan package in R (Oksanen et al. 2016). Species rarefaction curves (specaccum) were used to evaluate the adequacy of our sampling scheme. In each treatment, 20 samples captured >90% of the diversity (Appendix S3: Fig. S1). To evaluate the effects of forest edge proximity and soil inoculations on EMF community composition we performed a permutational analysis of variance (PERMANOVA), and NMDS (metaMDS) using Bray-Curtis dissimilarity. We evaluated the heterogeneity of the EMF communities with a homogeneity of variance (betadisper) analysis. We used species accumulation curves (accumcomp) to depict EMF species richness as a function of sampling effort and Log scale rank abundance curves (rankabuncomp) to evaluate species evenness for each of the four edge proximity and soil inoculation experimental treatments.

We used logistic regression models with Bonferroni corrections ($\alpha = 0.05/20$), to compare the relative abundances of the dominant 20 EMF species found on Douglas-fir seedlings among treatments (Appendix S4), with edge proximity and soil inoculation as fixed effects and site as a random effect. The relative abundance of each fungal species was based on presence-absence data

[7] https://github.com/faircloth-lab/splitaake
[8] https://github.com/lh3/seqtk

rather than sequence read abundance. We calculated the relative abundance of each fungal species as the proportion of trees colonized by that species. We determined the 20 most frequently occurring fungal species, then compared these occurrences across treatments. We compared relative abundance across treatments for the 20 most abundant fungal species.

Results

Douglas-fir seedlings planted near the forest edge survived better than seedlings planted an additional 20–25 m from the edge into the invaded clearcuts. After one growing season, we found that seedlings planted near the forest edge had 30% higher survival than seedlings planted away from the edge (Fig. 1A). After the second growing season, the positive effect of being planted near the forest edge was even more pronounced, with seedling survival more than two-fold greater near the forest edge compared to away (Fig. 1B). In both years, the model with the lowest AIC_c score included edge proximity but not soil transplant or their interaction (Table 1).

After the second growing season, seedlings growing near the edge had 6% more aboveground biomass than seedlings growing further out into the invaded clearcuts ($F_{1,238.9} = 5.30$, $P = 0.02$; Fig. 1c). There was no effect of soil inoculation on seedling aboveground dry biomass ($F_{1,235.5} = 0.06$, P = 0.81), and there was no interaction of proximity to forest and soil inoculation type ($F_{1,235.5} = 0.02$, $P = 0.89$).

Ectomycorrhizal colonization was substantially greater on the seedlings near the forest edge than seedlings far from the edge, irrespective of soil type ($F_{1,52} = 18.65$, $P = 0.001$; Fig. 1d). Seedlings grown in soil transplanted from the forest into invaded clearcuts far from forest edges did not have greater EMF abundance than seedlings planted into the *Cytisus*-invaded clearcut soils two growing seasons later ($F_{1,52} = 0.001$, $P = 0.97$), and there was no interaction between inoculation type and proximity to forest edge ($F_{1,52} = 0.058$, $P = 0.81$).

We identified 95 unique ectomycorrhizal OTUs (hereafter referred to as taxa) on Douglas-fir seedlings. The number of EMF taxa found on an individual Douglas-fir seedling ranged from 3 to 29. Of the 95 total EMF taxa observed, 75 were found on seedlings planted into forest soil near to forest edges, 77 taxa were found on seedlings planted into *Cytisus* soil near forest edges, 68 taxa were on seedlings planted into forest soil far from forest edges, and 76 taxa were found on seedlings planted into *Cytisus* soil far from forest edges. Total EMF taxa richness was not different among treatments ($F_{3,98} = 0.53$, $P = 0.67$). The mean number of taxa identified per seedling varied between 14 and 16 across the four treatments. Inspection of the species accumulation curves revealed that species richness saturated more quickly for seedlings planted near forest edges without added forest soil inoculum than for seedling planted far from forest edges with forest soil addition (Appendix S3: Fig. S1). Sequence read abundance per seedling ranged

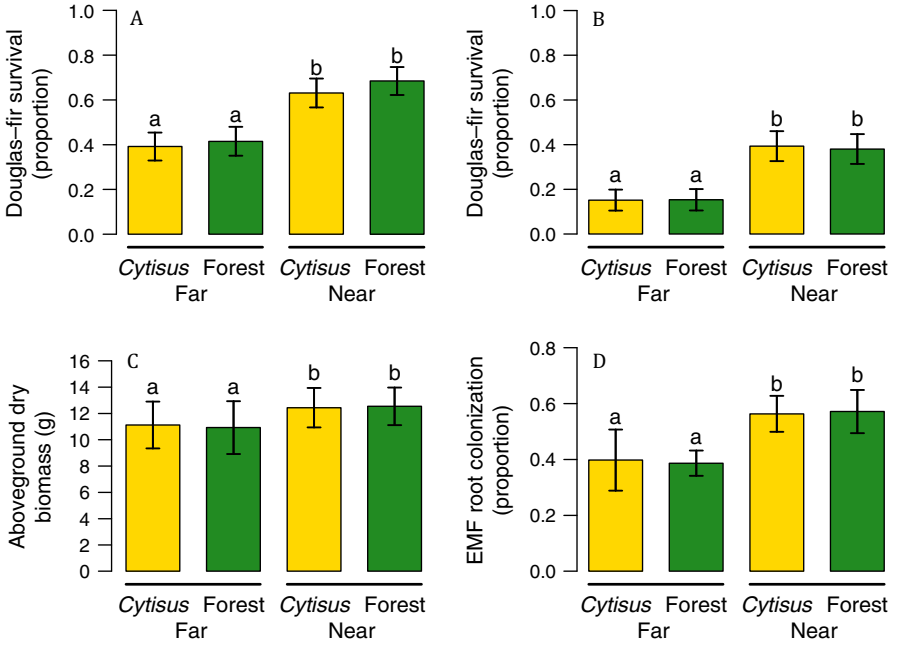

Fig. 1. (A) Proportion of Douglas-fir seedlings that survived through the first growing season, (B) proportion of Douglas-fir seedlings that survived through the second growing season, (C) Douglas-fir aboveground biomass, and (D) the proportion of roots colonized by ectomycorrhizal fungi on seedlings planted near to and far from forest edges in *Cytisus*-invaded clearcut soils (yellow bars) and in soils transplanted from nearby forests (green bars). Bars are mean ± SE.

TABLE 1. Douglas-fir seedling survival GLMM model selection tables ranked by ΔAIC_c results after (A) one and (B) two growing seasons after planting.

Intercept	Proximity	Soil	Proximity × soil	df†	logLik‡	AIC_c§	ΔAIC_c	Weight
(A) Model selection table 2011								
−0.52720	+			3	−557.69	1121.4	0.00	0.46
−0.62020	+	+		4	−556.86	1121.8	0.38	0.38
−0.58170	+	+	+	5	−556.70	1123.5	2.06	0.16
−0.05090				2	−592.88	1189.8	68.39	0.0
−0.03665		+		3	−592.09	1190.2	68.80	0.0
(B) Model selection table 2012								
−2.039	+			3	−443.3	892.6	0.00	0.660
−2.021	+	+		4	−443.2	894.6	1.97	0.247
−2.050	+	+	+	5	−443.2	896.5	3.91	0.094
−1.259				2	−478.3	960.5	67.89	0.000
−1.247		+		3	−478.2	962.5	69.88	0.000

Notes: The + signs indicate the factors included in the model.
† Degrees of freedom.
‡ Log likelihood.
§ Akaike Information Criterion corrected for sample size.

from 66 to 15,242. There was a total of 128,272 reads on seedlings in forest soil transplanted near the forest edge; 101,599 reads on seedlings planted into *Cytisus* soil near the forest edge; 73,931 reads on seedlings from *Cytisus* soil planted far from the forest edge; and 60,537 reads on seedlings in forest soil transplanted far from the forest edge.

Ectomycorrhizal communities differed when near to or far from the forest edge (adonis, $F_{1,98} = 1.8$, $R^2 = 0.02$, $P = 0.03$, Fig. 2A), were not affected by forest soil inoculations (adonis, $F_{1,98} = 0.95$, $R^2 = 0.01$, $P = 0.35$), and there was no edge proximity and forest soil inoculation interaction ($F_{1,98} = 1.49$, $R^2 = 0.01$, $P = 0.13$). Ectomycorrhizal fungal communities exhibited greater heterogeneity near the forest edge than the EMF communities farther out in the invaded clearcut (betadisper, *Cytisus* soils near vs. far, $P = 0.01$; forest soils near vs. far, $P = 0.04$; Fig. 2B; Appendix S4).

Only 2 of the 20 most common taxa showed a frequency of occurrence that was affected by soil inoculation or edge proximity (Appendix S4). *Cenococcum* occurred more frequently on seedlings planted with forest soil inoculations. This effect depended on edge proximity because, near the forest edge, *Cenococcum* was as abundant in forest and *Cytisus* inoculated soils but, far from the edge, it was 5.5-fold more frequent on seedlings planted with forest soil inoculum (Appendix S4). *Tomentella* sp. 2 was found 50% more frequently on seedlings planted far from forest edges, irrespective of soil inoculation type (Appendix S5).

DISCUSSION

We found that Douglas-fir seedling survival was drastically improved in edge environments compared to the interior of clearcuts that had been invaded by *Cytisus*. When planted near the forest edge, Douglas-fir seedling survival was nearly threefold higher two years after planting compared to seedlings planted an additional 20–25 m into the invaded clearcuts. Improved seedling establishment near forest edges could be influenced by a number of factors. For example, shade provided by the forest can reduce drought stress (Gray and Spies 1996, Hughes and Bechtel 1997, Dovciak and Brown 2014). Reduced herbivory at forest edges has also been shown to improve seedling establishment (Ruzicka et al. 2010).

Increased availability of mycorrhizal fungi is also an important feature of forest edges. Several studies have shown a positive relationship between EMF abundance and plant performance (Smith and Read 2008, Hoeksema et al. 2010) as well decreased survival and growth of ectomycorrhizal plants where their fungal symbionts are absent (Nuñez et al. 2009, Hoeksema et al. 2010, Hynson et al. 2013). We found that Douglas-fir trees experienced a 45% increase in root colonization by EMF when planted near forest edges (within 5 m) compared to 20–25 m away from forest edges, suggesting less EMF availability away from the edge. There are several, nonmutually exclusive factors that could explain the EMF limitation away from edges. The lateral roots of Douglas-fir generally extend less than 1 m past the canopy edge (Mauer and Palátová 2012), and because EMF require carbon from a host plant to be metabolically active, seedlings planted beyond the canopy may be unable to access mycorrhizal networks associated with the mature edge trees. Additionally, mycorrhizal fungi cannot reproduce without host plants, and 95% of basidiospores disperse less than 1 m from the fruiting body (Galante et al. 2011). Spore dispersal limitation is likely an important factor contributing to decreased EMF colonization on seedlings far from the forest edge. Deploying spore traps across a mosaic landscape, Peay et al. (2012) found that abundance and richness of EMF spores declined with increasing distance from hosts,

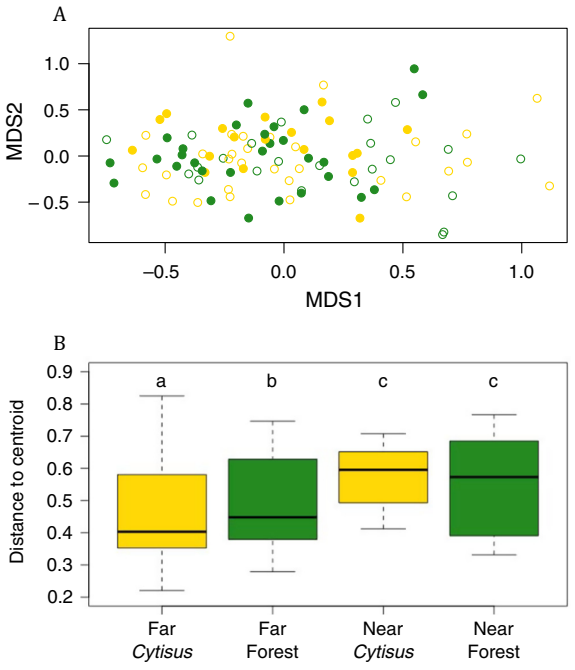

FIG. 2. (A) Nonmetric multidimensional scaling (NMDS) plot of ectomycorrhizal fungal communities near (open circles) and far (closed circles) from forest edges in *Cytisus*-invaded soils with (green) and without (yellow) an addition of forest soil inoculum. Ectomycorrhizal communities near the forest edge are more variable than communities an additional 20–25 m farther into the *Cytisus*-invaded clearcut ($N = 102$ seedlings). (B) Variance in ectomycorrhizal community composition near to and far from forest edges, with and without forest soil inoculation. The band inside each box is the median Euclidean distance in principal coordinate space between the samples and their respective group centroid. Pairwise Tukey HSD comparisons are shown; different letters denote significant differences between groups at $P < 0.05$, details in Appendix S5.

providing important evidence that EMF are dispersal limited. The abiotic conditions that result from greater light influx away from forest edges may also contribute to declines in EMF abundance, because EMF can be sensitive to desiccation (Querejeta et al. 2007), and increased sun exposure may result in increased EMF loss. Finally, Douglas-fir productivity is sensitive to water stress (Simard 2009), and the ability of seedlings to provide necessary carbon to their fungal symbionts may have been limited away from forest edges.

We predicted that seedlings near the forest edge would show greater overall EMF diversity because they would have access to the fungi actively forming mycorrhizal networks in addition to those persisting in the clearcut. We expected seedlings planted far from the edge would primarily be colonized by EMF that produce long-lived spores. Early successional communities are generally associated with ectomycorrhizal fungi with this life history strategy (Horton et al. 1998, Baar et al. 1999, Ishida et al. 2008). Mature forests generally have greater mycorrhizal richness than open stands following disturbance (Visser 1995, Twieg et al. 2007). We also expected EMF dispersal limitation to result in decreased EMF diversity far from the forest edge (Galante et al. 2011, Peay et al. 2012). Contrary to our prediction, we did not find increased EMF richness on seedlings growing near forest edges. This surprising finding is in contrast with previous studies of Douglas-fir edge effects, which all report a negative relationship between EMF richness and distance to host plants (Kranabetter and Wylie 1998, Durall et al. 1999, Outerbridge and Trofymow 2004, Cline et al. 2005).

Our study is distinctive in that we used a high throughput Illumina DNA amplicon sequencing approach, which provides a different kind of data from studies that characterized diversity with traditional Sanger sequencing, restriction fragment length polymorphism (RFLP), and morphotyping approaches. Illumina gave us a more complete picture of the true diversity of EMF because we were able to analyze a larger proportion of the total root mass rather than a collection of individual root tips, and we circumvent technical challenges assigning species identity (Agerer 1997). We identified 89 unique EMF OTUs on seedlings near forest edges and 85 OTUs away from edges. With the Illumina approach, we identified two to three times more species than described in the previous Douglas-fir edge effects studies (Kranabetter and Wylie 1998, Durall et al. 1999, Hagerman et al. 1999, Outerbridge and Trofymow 2004, Cline et al. 2005). We were able to detect rare species that would otherwise be undetectable with former approaches that analyzed much smaller subsets of seedling root tips. Of the 95 unique EMF taxa we identified, 40 (~40%) of these taxa occurred on <5% of the seedlings (Appendix S6) and are therefore considered rare (McCune et al. 2002). Capturing these rare species revealed the EMF communities near and far from forest edges, and irrespective of soil inoculation type, have low evenness, and that a few species within the community are numerically dominant (Appendix S3: Fig. S2). The ability to detect more rare species may explain why we did not detect differences in EMF richness with distance to hosts, as previous studies have using the morphotyping approach (e.g. Hagerman et al. 1999, Cline et al. 2005, and Dickie and Reich 2005). Nickel et al. (2018) also found treatment effects with morphotyping and Sanger sequencing but not with Illumina; in their study, which focused on soil cores rather than live roots, Illumina sequencing of non-viable EMF may explain the difference. We suspect patterns of richness in previous studies may primarily reflect shifts in abundance. A standard method for determining EMF richness is to score a random subset of plant roots for the presence or absence of EMF, then identify the fungal taxa on the roots where EMF is present. A problem with this approach is that EMF richness will increase with root sample number and therefore treatment groups with greater EMF colonization will also have greater EMF richness. Previous studies that report increased richness with increased

edge proximity all used this common approach. It is unclear whether patterns of species fungal richness would persist in the other studies if a larger proportion of the total root mass were included.

Beyond species richness, we did find differences in community structure near and far from edges. We also found that the communities were more heterogeneous near the edge. Our bare root seedlings were not intentionally inoculated with EMF, but they did grow for a year in an outdoor nursery plot near our experimental sites. They would have been exposed to EMF and likely brought fungal associates with them. Although we did not assess which fungal species were associated with the seedlings at planting time, the EMF communities associated with tree nurseries can be different from forest communities (Cline et al. 2005). Our observed differences in fungal composition on the seedlings after growing in the field for 20 months may be conservative because all seedlings likely started with some nursery fungi. In this study, differences in composition were driven by rare species; when rare species were excluded from the analyses, leaving the 55 most common taxa the differences between near and far from edge EMF communities disappeared. Though the abundance of EMF was strongly affected by isolation, these data suggest that most of the common, and presumably important, EMF species were not differentially affected by isolation from host or forest soil inoculation. Rare EMF species with unknown importance may have been affected by stressors associated with *Cytisus* invasion or by increasing distance from the forest edge. However, it is also possible that variation in rare species is a result of spatial variation and stochastic sampling (Lynch and Neufeld 2015). Differences in fungal community composition near and far from the edge may have influenced the ability of seedlings to obtain soil resources from varied sources and had consequences on seedling survival. Future research is needed to evaluate the importance of EMF richness, community composition, and rare taxa on plant performance.

We transplanted local forest soil as a source of EMF inoculum for Douglas-fir seedlings in the hopes of improving reforestation success. We predicted that forest soil would contain EMF species similar to those that occur at forest edges, and that by moving these forest soils into the long invaded clearcuts, we would increase EMF colonization and EMF species richness. In the forestry industry, it is common practice to ensure that seedlings are exposed to beneficial soil microbes either by adding commercial inoculum or by planting seedlings into an outdoor nursery bed that contains EMF prior to transplantation for reforestation (Cram and Dumroese 2012). In a meta-analysis, Maltz and Treseder (2015) concluded that applying live soil collected from reference sites consistently results in increased mycorrhizal colonization and improved seedling establishment. They further determined that mycorrhizal inoculation with field soil additions were more effective than commercial sources. However, the vast majority of the studies reviewed were arbuscular mycorrhizal plants and fungi rather than the ectomycorrhizal system studied here. Inoculation with EMF has had mixed success. While it has been successful in some scenarios (Baez-Perez et al. 2017, Cortese and Bunn 2017), other studies have shown a lack of effect (Sykorova et al. 2016, St-Denis et al. 2017). In our study, forest soil inoculation did not change EMF abundance or species richness, and it did not improve Douglas-fir survival or growth. There may have been soil mixing by macroinvertebrates and earthworms, an explanation that is also consistent with the lack of differences in EMF community composition. Alternatively, conditions in the invaded clearcut may have been too harsh to support the forest fungi. Our previous work has shown that *Cytisus* invasion into forest soils reduced EMF abundance and resulted in decreased Douglas-fir performance (Grove et al. 2012, 2017*a*), lending some support to the idea that altered conditions in the *Cytisus*-invaded clearcuts may not be suitable for forest EMF.

Our study design is unable to discern the relative importance of EMF networks vs. spores or other soil propagules in facilitating seedling establishment. If our inoculation treatment near the forest edge had significantly increased EMF diversity or abundance, and had improved seedling establishment, that would have provided some evidence that EMF networks at the edge were not as important as spore availability. Because inoculations did not have an effect on seedling performance, however, we cannot rule out the possibility that access to EMF networks contributed to increased seedling survival at the forest edge. This remains speculation because we did not collect data on seedling root growth beyond the initial transplanted soil volume or hyphal growth from networked EMF into the transplanted soils.

We tested two possible restoration approaches to improve seedling establishment: planting along edges and reintroduction of EMF into invaded clearcuts with forest soil additions. Our results suggest that forest edges are suitable for reforestation, even when the interiors of these degraded landscapes are not. In areas where reforestation is challenging due to invasive species dominance or suboptimal soil or climate conditions, we advocate for timber harvest patterns that maximize forest edge habitat. It may be possible that patches of adult EMF-associated trees retained during timber harvest may also improve EMF colonization and seedling establishment in areas that are otherwise difficult to reforest. It should be noted that 70–95% survival of planted Douglas-fir seedlings is expected in this region (S. Loy, *personal communication*); the 40% survival rate we observed in seedlings planted near edges is therefore less than desirable. Although seedlings were EMF limited, we found that inoculation with forest soil was not an effective strategy to increase abundance or richness of EMF, or seedling survival. We do not know if a larger volume of soil inoculum might have been more effective, but our forestry collaborators considered even the 3 L we used per

tree to be unrealistic for implementation on a commercial scale. A message from our study is that to support Douglas-fir seedling establishment by maximizing EMF colonization, we will need to be strategic in the design of timber harvests and take advantage of natural mycorrhizal hyphal networks, rather than attempting to recreate fungal communities. We suggest that the findings from these *Cytisus*-invaded Douglas-fir clearcuts can inform reforestation of other ectomycorrhizal tree species in areas with suboptimal abiotic and biotic conditions.

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Supporting Information

Additional supporting information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/eap.1867/full

Data Availability

Data are available on the Dryad Digital Repository: https://doi.org/10.5061/dryad.43b7j27