

INTERACTIONS WITH JUNIPER ALTER PINYON PINE ECTOMYCORRHIZAL FUNGAL COMMUNITIES

KRISTIN E. HASKINS¹ AND CATHERINE A. GEHRING

Department of Biological Sciences and Merriam-Powell Center for Environmental Research, Northern Arizona University,
Flagstaff, Arizona 86011-5640 USA

Abstract. Belowground interactions can affect plants either directly or indirectly via their associated mycorrhizal fungi. However, few studies have experimentally examined the consequences of interspecific root interactions for these important mutualists in the field. We used a trenching experiment to examine how belowground interactions between pinyon pine and one-seed juniper affected the ectomycorrhizal (EM) fungal communities of pinyon pine. Three major findings emerged: (1) pinyons responded to the reduction of juniper roots with a near doubling of fine root biomass in just two years, (2) this increase in pinyon roots translated into a potential two-fold increase in EM abundance, and (3) the EM fungal communities of trenched trees differed significantly from controls largely due to a decrease in ascomycete fungi. Because species of EM fungi vary in the benefits they provide, changes in EM communities could have long-term consequences for host-plant establishment, growth, and survival. Belowground interactions with juniper may contribute to the high mortality of pinyons and the reduced diversity of EM fungi associated with recent droughts.

Key words: Arizona (USA) pinyon–juniper woodland; belowground tree interactions and fungal communities; ectomycorrhiza; interspecific competition; *Juniperus monosperma*; *Pinus edulis*; RFLP (restriction fragment-length polymorphism); trenching experiment.

INTRODUCTION

Although recent research demonstrates that the presence and species composition of mycorrhizal fungi can mediate interactions among plants belowground and influence plant community structure (e.g., Perry et al. 1989, Pedersen et al. 1999, Simard et al. 2002, Hart et al. 2003), few studies have examined the effects of belowground interactions on the community structure of these important soil microbes. The presence of the roots or mycorrhizas of a neighboring plant may alter mycorrhizal fungal abundance, diversity, and species composition directly through competition for soil resources or indirectly through effects on the host plant. Because mycorrhizal fungal species vary in their attributes (e.g., Bougher and Malajzuk 1990, Anderson et al. 1999), changes in mycorrhizal fungal communities could influence host-plant establishment (Berliner et al. 1986), growth (Gehring and Whitham 1994), and ecosystem processes (Langley and Hungate 2003). Furthermore, negative effects of belowground interactions on mycorrhizal fungi may be most likely in systems where co-occurring host plants form different types of mycorrhizal associations, as common mycorrhizal networks that may facilitate resource sharing are unlikely to form (Perry et al. 1989).

In this paper we examine the effects of belowground interactions between pinyon pine (*Pinus edulis* En-

gelm.) and one-seed juniper (*Juniperus monosperma* (Engelm.) Sarg.) on the ectomycorrhizal (EM) fungi of pinyons. Pinyon–juniper woodlands provide a model system in which to examine the effects of belowground plant interactions on mycorrhizal fungi for several reasons. First, there is little potential for aboveground interactions because closed canopies rarely form. Second, the soils of many pinyon–juniper woodland sites are nutrient poor and have low water-holding capacity, features that could intensify belowground interactions such as competition (Gehring et al. 1998). Third, these woodlands have experienced severe drought conditions in recent years, potentially exacerbating the effects of belowground interactions. For example, five of the last eight years in northern Arizona woodlands have been moderate to extreme drought years.² Finally, pinyons and junipers form mycorrhizal associations that, at least for pinyons, are positively linked to both seedling and adult plant growth (Gehring and Whitham 1994, Swaty et al. 2004). However, pinyons are the only associate of EM fungi in many areas, while junipers and many understory plant species associate with arbuscular mycorrhizal (AM) fungi. We used a field experiment to address the hypothesis that reduction of juniper fine roots in pinyon rooting zones results in altered EM colonization and community structure and increased pinyon growth. This is the first study to examine the effects of interspecific belowground interactions among plants on EM fungal communities in the field.

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¹ E-mail: Kristin.Haskins@nau.edu

² (<http://www.noaa.gov/climate.html>)

METHODS

Site description

This study was conducted ~5 km north of Sunset Crater National Monument in northern Arizona, USA, in a pinyon–juniper woodland dominated by pinyon pine and one-seed juniper. The soils were composed of basaltic ash, cinders, and lava flows and belong in the U.S. Department of Agriculture Soil Taxonomic Sub-Group of Typic Ustorthents. These soils are relatively low in nutrients and water storage capacity (Gehring et al. 1998). The mean annual precipitation in Arizona for the period 1998–2000 and 2001–2003 was 31.7 cm and 25.0 cm, respectively.

Trenching experiment

To establish if juniper roots affected pinyon roots and their associated mycorrhizal fungi, we conducted a trenching experiment in which juniper roots were severed outside of the pinyon rooting zone. We chose to use the trenching method because it enabled us to examine pinyon release effects, which would provide evidence of competition, in the field. Although trenching can cause the release of water and nutrients from severed roots (Coomes and Grubb 2000), we expected these effects to be minimal in this system due to slow decomposition (82% of initial root mass remained after one relatively moist year [A. Classen, *unpublished data*]), which was likely exacerbated by drought in the region from 2000–2003.² Furthermore, trenching did not affect the nutrient concentrations of either the bulk soil or pinyon needles (2003 bulk soil total N = 0.76 ± 0.24 mg/g [mean \pm 1 SE] for trenched trees and 0.56 ± 0.11 mg/g, for control trees, $t_{18} = 0.75$, $P = 0.46$; 2003 bulk soil total P concentration = 0.38 ± 0.07 mg/g for trenched trees and 0.49 ± 0.09 mg/g for control trees, $t_{18} = -1.05$, $P = 0.31$; 2001 pinyon needle total N = 12.1 ± 0.28 mg/g for trenched trees and 11.6 ± 0.26 mg/g for control trees, $t_{51} = 1.41$, $P = 0.17$; pinyon needle total P concentration = 1.9 ± 0.08 mg/g for trenched trees and 1.7 ± 0.09 mg/g for control trees, $t_{51} = 1.61$, $P = 0.11$).

In August of 2000 we selected 30 pinyons for trenching and paired those, based on proximity and size (average basal trunk diameter = 5.8 cm), with 30 control trees that were not trenched. Junipers and their roots surrounded trenched and control pinyons. Trenching was accomplished by inserting a spade 30 cm deep into the soil repeatedly around a predetermined trench line. We trenched to a depth of 30 cm because root core data indicated 49% more juniper fine roots (< 2-mm diameter) occurred at 0–30 cm depth than at 30–60 cm depth (biomass [mean \pm 1 SE]: 0–30 cm, 100.8 ± 26.7 g/m²; 30–60 cm, 67.6 ± 43.8 g/m²). Trench lines were determined by measuring the longest radius of the selected-tree's crown, multiplying that distance by 2, and extending out that distance from the drip line of the selected tree. This distance has been observed to in-

corporate nearly all of the fine roots of focal pinyons of this size based on the exposure of the entire root system of 10 similarly sized trees at this site (C. A. Gehring, *unpublished data*). We also periodically verified that pinyon roots were not severed during trench establishment. Trenches were reestablished every 3–4 months.

Nearly two years later (July 2002) we collected soil cores inside the trench lines of trenched trees and within similar distances of control trees to (1) establish if trenching had been successful in reducing the amount of juniper fine roots in the rooting zone of pinyons, and (2) determine if pinyon root growth had increased in response to the trenching of juniper roots. We randomly selected a subset of 20 trenched and 20 control trees to examine live-root densities. Two soil cores (30 cm depth, 6 cm diameter [each 848-cm³ volume]) were collected halfway between the drip line and the trench line on the north and south or east and west aspects of treatment and control trees. Fine roots were separated as living or dead and as pinyon, juniper, or other roots, dried in an oven for 48 h at 60°C, and weighed. Dead roots were brown or black in color and the cortex separated easily from the stele, thus making them distinguishable from living roots. Differences in the mean living juniper and pinyon fine-root mass between trenched and control trees were compared using *t* tests. Pinyon root mass was natural-log transformed to achieve equality of variance. All analyses were performed using SPSS for Windows version 10.0.5 (SPSS 1999).

Prior to trenching (July 2000) and again two years after trenching (August 2002), we collected 50–80 root tips, at a depth of 5–20 cm on the west aspect of the drip line of each of the trenched ($n = 30$ trees) and control ($n = 30$) trees at least 1 m from the trench line. We standardized sampling because aspect and crown location can affect ectomycorrhizal (EM) community composition (Gehring et al. 1998). Roots were taken back to the laboratory and scored for percentage EM colonization using the methods of Gehring and Whitham (1991). Living EM tips were classified based on morphology as described by Horton and Bruns (1998) and frozen for molecular analyses. EM colonization data were arcsine-square-root transformed prior to analysis and compared using a *t* test.

Measures of percentage EM colonization categorize root tips as colonized or not, but do not incorporate changes in the abundance of root tips. To account for variation in abundance, we combined estimates of root biomass from cores with data on EM distribution on fine roots using the equation below, where the first term is obtained from the roots we collected from the study trees for EM assessments. We counted the number of living EM tips and measured the length of root (in centimeters). The second term was derived by drying and weighing roots of known length to obtain an estimate of the relationship between root length and root

mass (in grams). The last term came from separately collected soil cores (30 cm depth, 6 cm diameter) where the living pinyon fine roots were separated, dried, and weighed from a known volume of soil.

$$\begin{aligned} \text{EM tips/m}^2 &= (\text{no. EM tips/root length}) \\ &\quad \times (\text{root length/oven-dried root mass}) \\ &\quad \times (\text{oven-dried root mass/soil area}). \end{aligned}$$

These data were natural-log transformed to obtain equal variances prior to analysis using a *t* test.

Fourteen randomly selected trenched trees and 14 control trees were selected for analysis of EM communities using molecular approaches. The DNA from two to three of the saved root tips of each morphotype per tree was extracted and the internal transcribed spacer (ITS) region of the fungal genome, located between the 18S and 28S rRNA, was amplified using PCR (polymerase chain reaction) with the ITS1F and ITS4 primer pair (Gardes and Bruns 1993). Restriction-fragment-length polymorphism (RFLP) data were obtained following the methods of Gehring et al. (1998). The amplified ITS region was characterized using restriction enzyme digestion with *Hinf*I and *Mbo*I, which have been used successfully to discriminate among fungal species in *P. edulis* (Gehring et al. 1998, Swaty et al. 2004). Digital images of agarose gels were recorded and analyzed using a Kodak EDAS 290 gel documentation system and accompanying software (Eastman Kodak Company, Rochester, New York, USA). RFLP patterns were compared to those generated from fungal sporocarps collected over the last 12 years. The dominant unidentified RFLP types were sequenced using an ABI 3100 (Applied Biosystems, Foster City, California, USA) in the laboratory of Dr. Tom Bruns at the University of California, Berkeley (California, USA). Sequence data were analyzed using DNA Star software (DNASTAR, Madison, Wisconsin, USA) and subjected to a GenBank (U.S. National Institutes of Health genetic sequence database) blast search and those with 99% similarity to those in GenBank were considered a match for species identification.

Community analyses of RFLP types were performed using nonmetric multidimensional scaling (NMDS), a nonparametric analytical technique that is applied to the dissimilarity matrix calculated among RFLP types using the Bray-Curtis dissimilarity coefficient (Faith et al. 1987). Comparisons between trenched and control communities were made using an analysis of similarity (ANOSIM) statistical test. Both of these analyses were conducted using DECODA software (Minchin 1999). Simpson's diversity index was computed using PCOrd version 4.02 (McCune and Mefferd 1999). The proportion of ascomycete fungi present within a community, based on total root tips, was compared with a Mann-Whitney test.

To determine if reduced juniper-root biomass altered pinyon growth aboveground, we measured the stem and

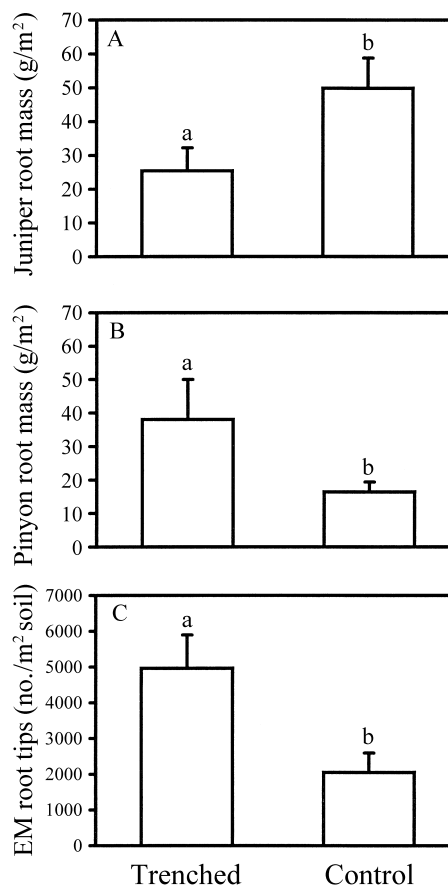


FIG. 1. Trenching significantly reduced juniper live root mass (A) and increased pinyon live root mass (B). The increase in live pinyon fine roots can be translated into a significant increase in ectomycorrhizal (EM) root tips (C). The bars represent means and 1 SE for trenched trees and control trees. Bars with different lowercase letters are significantly different at $P = 0.05$.

needle lengths of 18 trenched and 18 control trees in October of 2003. Annual stem lengths from 1998–2003 were measured on one nonterminal shoot on the east and on the west aspect of each tree at mid-crown. We also measured the lengths of three randomly selected 2003 needles to the nearest millimeter using calipers. Mean annual stem length data were analyzed using repeated-measures ANOVA and needle length data were analyzed with a *t* test.

RESULTS

Trenching successfully reduced the amount of juniper fine roots in pinyon rooting zones and pinyons responded by increasing fine-root biomass. There was nearly twice as much juniper root associated with control than trenched trees (Fig. 1A: $t_{38} = -2.20$, $P = 0.03$), and more than double the pinyon root mass in trenched than control trees (Fig. 1B: $t_{38} = 1.99$, $P = 0.05$).

Although pinyons responded belowground to the reduction of juniper roots, there was no aboveground response in shoot or needle length. Pretreatment (1998–2000) annual stem length was 31.6 ± 4.8 mm (mean ± 1 SE) for trenched trees and 35.0 ± 4.2 mm for control trees, whereas the post-treatment (2001–2003) annual stem length was 11.3 ± 0.3 mm and 13.2 ± 0.6 mm for trenched and control trees, respectively. Mean annual stem lengths did not significantly differ between trenched and control trees in any year (repeated-measures ANOVA: $F_{5,30} = 0.56$, $P = 0.46$). There also were no differences between trenched and control trees in needle length in 2003 (24.5 ± 2.1 mm for trenched trees and 26.6 ± 1.6 mm for control trees, $t_{34} = -0.82$, $P = 0.42$).

*EM colonization, root tip production,
and community structure*

Percentage EM colonization did not differ between trenched trees and control trees prior to trenching in 2000 (trenched, $21.9 \pm 3.1\%$ [mean ± 1 SE], control, $20.8 \pm 2.9\%$; $t_{57} = 0.27$, $P = 0.79$) or following trenching in 2002 (trenched, $20.6 \pm 1.9\%$, control, $23.0 \pm 2.6\%$; $t_{55} = -0.29$, $P = 0.77$). However, when the near doubling of pinyon fine roots associated with trenching is considered, trenched trees had twofold more EM tips than control trees (Fig. 1C: $t_{31} = -2.52$, $P = 0.017$).

We collected RFLP data from 1–3 tips per morphotype per tree for 14 trees per group for a total of 648 EM tips and 9 unique RFLP types. Within a tree, each morphotype represented a single RFLP type with two exceptions where one morphotype represented two RFLP types. There were two rare RFLP types, representing 8.4% of all EM examined, that were unidentified due to poor sequence data. Nearly 76% of all tips, representing four RFLP types, could be identified only to Order (Pezizales) using sequence data, 9.6% were identified to genus (*Rhizopogon* sp., two species), and 6% to species (*Tricholoma terreum* (Schaeff. ex Fr.) P. Kumm.).

Trenched and control trees differed significantly in EM community composition (Fig. 2; ANOSIM: $R = 0.21$, $P = 0.006$). This difference was partially due to a significantly higher proportion of ascomycete fungi associated with control trees (Mann-Whitney $U = 46$, $P = 0.014$). However, trenched and control communities had similar RFLP type richness (six and five, respectively) and there was no significant difference in Simpson's diversity index between the two groups (trenched, 0.67 ± 0.17 [mean ± 1 SE], control, 0.39 ± 0.12 ; t test: $t_{26} = 1.31$, $P = 0.20$). Based on the total number of EM tips examined and identified, trenched trees had 57.2% ascomycetes and 42.8% basidiomycetes, whereas control communities had 94.9% ascomycetes and 5.1% basidiomycetes. Three species of basidiomycetes, two *Rhizopogon* species and *Tricholoma terreum* were present in trenched communities

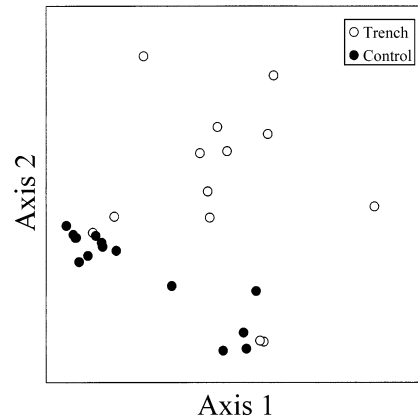


FIG. 2. NMDS (nonmetric multidimensional scaling) ordination of EM (ectomycorrhizal) fungal RFLP (restriction-fragment-length polymorphism) types demonstrates that trenched and control trees differed significantly in community composition (ANOSIM: $R = 0.21$, $P = 0.006$). Each point represents the RFLP type community for a given tree ($n = 14$ trees per group).

while only one unidentified basidiomycete was present in controls.

DISCUSSION

*Pinyon roots respond positively to reduction
of juniper roots*

The reduction of juniper roots with trenching resulted in a doubling of fine-root production by pinyons and an estimated two-fold increase in ectomycorrhizas on trenched trees. These findings are consistent with definitions of competitive release (niche expansion in absence of a potential competitor; Begon et al. 1990). Furthermore, the increase in fine-root biomass of the target species in response to trenching has been confirmed in other studies examining belowground interactions of woody species (e.g., Holl 1998). Thus, competition with juniper likely reduced pinyon access to water and nutrients directly through suppression of fine-root production and indirectly through reduction in soil exploration by ectomycorrhizal (EM) fungi. Junipers are thought to be more drought tolerant than pinyons because they are better able to access shallow, intercanopy water (Breshears et al. 1997). The competitive release we observed provides an additional mechanism for the lower tolerance of pinyons than junipers to water stress (Linton et al. 1998) and the greater susceptibility of pinyon to drought-induced mortality (R. C. Mueller, C. M. Scudder, M. E. Porter, R. T. Trotter, C. A. Gehring, and T. G. Whitham, *unpublished manuscript*).

Pinyons did not exhibit an aboveground response to trenching. Root trenching in nutrient-poor soils generally has a positive effect on aboveground plant growth (reviewed by Coomes and Grubb [2000]), yet we did not observe a growth response in either shoot or needle length. These results may be due to the 2001–

2002 extreme drought that resulted in a 70% reduction in shoot growth compared to 2000, a year of moderate drought. Pinyons likely invested in fine-root production at the expense of shoot growth, a strategy that could maximize uptake of the materials most limited during drought.

Reduction of juniper roots alters pinyon ectomycorrhizal communities

Differences between trenched and control EM communities were driven by a shift in composition, including the proportion of ascomycete fungi, rather than by differences in diversity. Basidiomycete fungi were rare in control communities and belonged to a single RFLP type. In contrast, basidiomycetes made up more than one third of the abundance of the EM community of trenched trees where they also exhibited higher species richness. Many members of the subdivision Ascomycotina are hypogeous and thought to be drought tolerant (States 1990). The decrease in ascomycete fungi with reduced belowground interaction with juniper is consistent with previous studies demonstrating that ascomycete fungi increase as host-plant stress increases. For example, increases in herbivory and water stress were associated with higher proportions of ascomycete fungi in pinyon pine (Gehring et al. 1998, Whitham et al. 2003). Greater prevalence of ascomycete fungi also has been observed in other semi-arid environments (e.g., Danielson and Pruden 1989) and in the early stages of fungal succession on Mount Saint Helens (Carpenter et al. 1987). The increase in basidiomycete fungi we observed following trenching is consistent with these patterns and provides the first experimental demonstration that stress relief, via competitive release from juniper, results in increased basidiomycete abundance and diversity.

Although our findings regarding ascomycete vs. basidiomycete fungi are consistent with other studies, we found remarkably lower levels of fungal species richness than a previous study of pinyon pine (Gehring et al. 1998). This discrepancy with Gehring et al. (1998) is likely due to the much greater geographic scale of sampling both within and among sites and to the greater numbers of trees sampled by Gehring et al. (1998). Gehring and Whitham (2002) sampled a comparable number of trees (20 vs. 28 trees in this study) in a nearby study site and observed similar levels of species richness (11 vs. 9 species reported here).

Other studies that have used trenching as a tool to investigate EM communities have focused on juvenile trees found in association with adult trees also colonized by EM fungi (e.g., Fleming 1984, Simard et al. 1997). Changes in EM community composition with trenching were attributed to destruction of hyphal connections between juvenile and overstory trees. The latter were hypothesized to provide carbon to species of EM fungi with high energy requirements that were unlikely to be met by seedlings alone (Fleming 1984,

Simard et al. 1997). The EM shifts we documented, however, were unlikely to be due to loss of hyphal connections because focal pinyons were surrounded by juniper (an AM [arbuscular-mycorrhizal] species) and occurred at large distances from other pinyons (the only EM species at the study site). Likewise, we feel that it is unlikely that the changes in community composition we observed were induced by the physical disturbance of trenching because samples for EM community analysis were collected at least 1 m away from the trench lines, where disturbance was minimal.

Long-term implications of stress for ectomycorrhizal communities and pinyons

Although several studies have shown that mycorrhizal fungi can alter competitive relationships among plants (e.g., Perry et al. 1989, Pedersen et al. 1999), this study is the first to demonstrate that interspecific root interactions alter EM fungal abundance and community composition in the field. Changes in the EM fungal community may have long-term consequences for both the fungi and their host plants. Some basidiomycete fungi may be vulnerable to local extinction due to increasing competition with juniper with continued drought. While the increase in basidiomycetes in our trenching experiment argues that these fungi persist in the soil, the 16-fold increase in mortality rates of pinyon compared to juniper during recent droughts (R. C. Mueller, C. M. Scudder, M. E. Porter, R. T. Trotter, C. A. Gehring, and T. G. Whitham, *unpublished manuscript*) may result in greater occupation of pinyon rooting zones by juniper and fewer pinyons to act as hosts for these fungi.

Changes in EM abundance and species composition may affect pinyon performance as well. Species of EM fungi are known to vary in both environmental tolerance and mutualistic abilities and EM fungal diversity has been positively correlated with nutrient uptake (Baxter and Dighton 2001). EM population and community changes with competition may alter the probability of pinyon reestablishment following drought-related mortality. Adult plants can be important sources of EM inoculum for establishing seedlings (Dickie et al. 2002), and adult pinyons frequently act as pinyon nurse plants (R. C. Mueller, C. M. Scudder, M. E. Porter, R. T. Trotter, C. A. Gehring, and T. G. Whitham, *unpublished manuscript*). The reductions in EM abundance and alteration in EM community composition with interspecific competition may reduce the likelihood that pinyon seedlings become colonized with EM fungi and establish successfully. High rates of pinyon mortality led to reductions in EM abundance and changes in EM communities including a significant decline in EM species richness (Swaty et al. 2004). Haskins (2003) observed that only 9% of pinyon seedlings were colonized by EM fungi in juniper-dominated pinyon-juniper woodlands. Resource competition, particularly when accompanied by drought, may be the initial

steps in a cascade of events that ultimately lead to shifts in the ecotonal boundaries of juniper–grasslands and pinyon–juniper woodlands.

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