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Inoculum potential of Pinus edulis-associated ectomycorrhizal fungi across a forest extirpation chronosequence

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by

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Inoculum potential of *Pinus edulis*-associated ectomycorrhizal fungi across a forest extirpation chronosequence

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ABSTRACT

Populations of *Pinus edulis* are declining with ongoing climate change. Previous studies have demonstrated the importance of *P. edulis*-associated ectomycorrhizal fungi (EMF) for seedling establishment and resistance to drought. There have been few studies that have examined inoculum potential of EMF in the absence of a plant host, yet the persistence of these fungi may be paramount to the resilience of *P. edulis* and other mycorrhizal plant species. Seven sites were selected in northwestern New Mexico with known dates of *P. edulis* extirpation and a lack of regeneration. Age classes included: two sites extirpated 10-20 years ago, two extirpated 55-65 years ago, two extirpated 500+ years ago, and one extirpated 11,000+ years ago. At each site, two plots were paired: an extirpated plot and the nearest live adult *P. edulis* stand. Soil samples were collected from each plot at 0-5 cm and 20-25 cm depths from four locations. *Pinus edulis* seedlings were inoculated with field soils in a greenhouse bioassay to measure inoculum potential of EMF. It was hypothesized that inoculum potential would decrease with time since extirpation. Additionally, it was predicted that seedlings with higher colonization by EMF would have significantly greater biomass and that EMF communities would differ between sites and plot types. Ectomycorrhizal fungal

diversity and inoculum potential would likely be significantly greater at the 20-25 cm depth. The shallower depth from 0-5 cm would have lower inoculum potential and diversity because of erosion, deposition, and exposure to variable temperatures. Colonization by EMF occurred across sites and inoculum potential decreased significantly from zero to 16+ years post extirpation. Tree biomass was unaffected by colonization by EMF. Communities of EMF in extirpated and live plots differed significantly and were dominated by *Geopora*, *Rhizopogon*, and *Tomentella*. *Geopora* was the only genus found in sites older than 65 years and possibly arrived by dispersion. Trees grown in soils collected from 0-5 cm had significantly greater inoculum potential and diversity. Sites where *P. edulis* was extirpated via drought had less diverse EMF communities that were dominated by *Geopora.* The results of this study will help guide restoration efforts for *P. edulis* and other ectomycorrhizal (ECM) tree species.

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Chapter 1

Introduction

The Earth is undergoing the most rapid extinction of species and populations in its history (Ceballos et al., 2010). Many woody foundational tree populations are declining globally due to numerous factors, including drought stress, regional warming, and increasing water deficits (Anderegg et al., 2013; Van Mantgem et al., 2009). Foundational tree species, such as *Pinus edulis*, play a significant role in structuring communities and ecosystems. With the loss of foundation trees, ecosystem dynamics are dramatically altered (Ellison et al. 2005; Royer et al., 2011). Climate variability and ongoing change are predicted to continue to cause large-scale mortality of woody species and rapid vegetation shifts (Allen and Breshears, 1998; Watson et al., 1998b). Due to their dependence on symbiotic microbes, both resilience and regeneration of foundation trees after major disturbance likely depends on the availability of symbionts, though this remains untested in semi-arid forests.

Semi-arid ecosystems like piñon-juniper (PJ) woodlands are likely to be among the most sensitive to climate change because of their reliance on precipitation patterns for ecosystem functions such as primary production (Pennington and Collins, 2007; Huxman et al. 2004). Piñon-juniper woodlands make up the third largest biome in the United States, covering over 19 million hectares of land (Evans, 1988) and over a million hectares of *P. edulis* mortality in the Southwest has already occurred as a result of climate change (Breshears et al., 2005). This regional die-off has been associated with unusually high temperatures and prolonged drought. Furthermore, the combination of adult mortality and decreased seed production has reduced seedling recruitment (Betancourt et al., 1993).

The elimination of foundation trees can have profound impacts on other biota, especially obligate symbionts. Ectomycorrhizal fungi (EMF) are obligately dependent on their hosts and cannot complete their lifecycle without their host trees (Kuikka, et al., 2003 Hoègberg et al., 2001). Not surprisingly, forest disturbance and host mortality consistently result in lower levels of EMF inoculum and decreased EMF diversity (Boerner et al., 1996; Vogelsang and Bever, 2009; Hagerman et al., 1999). Conversely, ectomycorrhizal (ECM) tree species, including conifers, depend on their fungal partners for successful establishment (Bruns et al., 2009; Terwilliger and Pastor, 1999). Similarly, studies have suggested that EMF species composition and diversity may have important influences on *P. edulis* seedling establishment (Haskins and Gehring, 2005). Unlike most coniferous forests, *P. edulis* is often the sole ectomycorrhizal host plant in PJ ecosystems (Andrews and Gehring, 2016). The lack of an alternative host makes *P. edulis*-associated EMF particularly vulnerable to host extirpation. Thus, it is likely that the success of PJ woodlands depends on the survival and/or re-establishment of *Pinus edulis*-associated EMF (Bruns et al., 2009; Haskins and Gehring, 2005).

Longevity, or inoculum potential of EMF in soil over time, may be a key component to resilience and restoration of these increasingly drought-prone ecosystems. A study in Arizona revealed that host genetics significantly influence *P. edulis* EMF community composition (Gehring et al., 2017). Drought intolerant *P. edulis* trees had associations dominated by the basidiomycete *Rhizopogon roseolus,* whereas drought tolerant *P. edulis* trees associated preferentially with ascomycete *Geopora* species. Moreover, the EMF associated with drought intolerant trees were more beneficial during wetter periods, while the EMF associated with drought tolerant trees were more beneficial during drought. Mueller et

al. (2019) found that fungal inoculum levels of soil under dead *P. edulis* trees remained similar to those of soil under live trees for nine years following host mortality. Longer periods of time and completely extirpated sites have yet to be studied.

Ectomycorrhizal fungi can utilize a large array of adaptations for long-term survival after host death. Some EMF genera, such as *Rhizopogon*, have demonstrated spore heat resistance in fire disturbed soils and increased community dominance post-fire (Peay et al., 2009; Izzo et al., 2006). A bioassay with *Pinus muricata* revealed that *Rhizopogon* inoculum potential in soil increased over four years in the absence of a compatible host tree (Bruns et al., 2009). In fact, *Rhizopogon* has basidiospores that may survive decades or longer (Kjøller and Bruns, 2003). Other EMF form sclerotia to persist in extreme environments. For example, the EM ascomycete fungus *Cenoccocum* forms sclerotia that remain abundant in both burned and unburned forests (Miller et al., 1994). Additionally, many EMF form hypogeous fruiting bodies that depend on mycophagy for dispersal (Frank et al., 2006). Mycophagy can decrease spore encounters with unfavorable weather and provides a nutrient rich fecal pellet that is attractive to plant roots (Luoma et al., 2004).

The lack of information on EMF inoculum potential over time in sites where foundation host species have disappeared represents a critical gap in our knowledge and understanding of forest regeneration, especially in semi-arid ecosystems. We therefore assessed EMF inoculum potential in a chronosequence of *P. edulis* soils using seedling bioassays from sites where *P. edulis* had been extirpated at known dates. We tested the following hypotheses:

1. Inoculum potential:

A. Inoculum potential decreases over time at sites where *Pinus edulis* has been extirpated.

B. Inoculum potential will be significantly greater at 20-25 cm than 0-5 cm because deeper soils are not impacted as severely by fluctuating moisture, erosion, deposition and irradiation.

C. Due to known variation in spore durability and dispersal capacity, we predict that inoculum potential will vary among EMF species (Miller et al., 1994; Darbro and Thomas, 2009; Van Der Heijden et al., 1998; Jonsson et al., 2001).

2. Host growth:

A. *Pinus edulis* seedlings with higher colonization rates by ectomycorrhizae will have significantly greater biomass.

B. Greater EMF diversity will be significantly positively correlated with greater growth (Van Der Heijden et al., 1998; Jonsson et al., 2001).

3. Community structure:

A. There will be higher diversity (Bois et al., 2005) and more basidiomycete fungi (Fernandez et al, 2017; Gordon and Gehring, 2011; Allison and Treseder, 2008) present in positive control soils and lower diversity as extirpation age increases.

Methods

Site descriptions

To identify appropriate sites, several criteria were developed. Each site required a historic record indicating a known *P. edulis* extirpation date, less than a 50% slope, soils loose enough to dig down to at least 25 centimeters, and a distance of 0.5 miles or less from an accessible road. To prevent contaminant inoculum from runoff, sites downhill from healthy *P. edulis* stands were excluded. Each plot within a site had a radius of 120 meters so that sampling occurred a minimum of 100 meters from the nearest live *P. edulis* stand. Seven sites were selected with 10-20 years, 55-65 years, 500+ years, and 11,000 years since extirpation. Sites were located on the Sevilleta National Wildlife Refuge, the Bureau of Land Management (BLM) Rio Puerco Field Office (RPFO), and the BLM Farmington Field Office (FFO) (Table 1).

Table 1. Summary of sites sampled in 2019 in New Mexico for *Pinus edulis*-associated ectomycorrhizal fungi. Paired positive plots are designated by a "+" symbol. All soil profiles were obtained from the United States Department of Agriculture Natural Resources Conservation Service using GPS coordinates.

Location[z]	Age (years)	GPS (plot center)	Elevation (feet)	Collection date	Soil profile	Soil description
AS	11,000	35.0317, -107.0587	5532.5	3/15/2019	Penistaja-fine sandy loam.	fine sandy loam 0-13 cm, sandy clay loam 13-104 cm
FF1	500+	36.0676313, -108.0485282	6,022	5/7/2019	Blanclot	loam 0-5 cm, sandy clay loam 5-38 cm
FF2	500+	36.0823373, -108.0670163	5,990	5/7/2019	Blanclot	loam 0-5 cm, sandy clay loam 5-38 cm
FF+	0	36.044510, -107.316532	7,069	5/8/2019	Blancot-Councelor-Tsosie complex	loam 0-18 cm, clay loam 18-66 cm
SevA	64	34.2203, -106.7094	5,532.01	3/1/2019	Lozier	very flaggy fine sandy loam 0-5 cm, very flaggy loam 5-30.5 cm, bedrock 30.5+ cm
SevA+	0	34.2203, -106.7097	5,492.67	3/4/2019	Lozier	very flaggy fine sandy loam 0-5 cm, very flaggy loam 5-30.5 cm, bedrock 30.5+ cm
Eag	56	35.89360, -107.10083	6,998.07	5/15/2019	Penistaja-Sandstone outcrop	fine sandy loam 0-5 cm, loam 5-50 cm
Eag+	0	35.89305, -107.10017	7,022.36	5/15/2019	Penistaja-Sandstone outcrop	fine sandy loam 0-5 cm, loam 5-50 cm
ICG	16	35.5676475, -107.269633	7,976	5/31/2019	Cabezon	stony loam 0-7.5 cm, gravelly clay loam 7.5-30.5 cm
ICG+	0	35.567435, -107.270595	7,972	5/31/2019	Cabezon	stony loam 0-7.5 cm, gravelly clay loam 7.5-30.5 cm
SevV	16	34.3128, -106.5727	6,224.62	4/12/2019	Harvey	fine sandy loam 0-5 cm, loam 5-150 cm
SevV+	0	34.3112, -106.5732	6,259.88	4/15/2019	Puertecito	very stony loam 0-5 cm, very channery clay loam 5-35 cm

[z]where AS is Ancient Site (bordering Laguna Pueblo), FF is Farmington Field Office (Chaco Canyon), SevA is Sevilleta Arroyo Milagro, Eag is Eagle Mesa, ICG is Ignacio Chavez Wilderness Study Area, SevV is Sevilleta Valley, and + represents paired positive plots

Sevilleta Refuge- Valley (SevV)

Pinus edulis died from the early 2000s drought at this site on the Sevilleta National Wildlife Refuge (Breshears et al., 2005). There was a shift from piñon-juniper woodland to juniper savannah at this site. The site was located approximately 5 miles south of the Goat Draw study area. Current vegetation in the extirpated plot included *Juniperus monosperma, Cylindropuntia imbricata, Bouteloua gracilis, Atriplex canescens,* and *Krascheninnikovia lanata*. Dead, downed, and rotting *P. edulis* was present on the south slope bordering the plot. At the base of the slope was a small arroyo preventing inoculum movement into the extirpated plot. The extirpated plot had a slope of 0-5%.

The live plot had a slope of 0-10% and was 180 meters from the nearest extirpated sampling pit. Sedimentary rocks were abundant and the soil was very rocky. The dominant species were *Pinus edulis* and *J. monosperma*. *Opuntia* spp. were abundant throughout the plot and the dominant grass was *B. gracilis*. *Nolina microcarpa, Escobaria vivipara,* and *Echinocereus* sp. were also present.

Ignacio Chavez (ICG)

This site was burned and cut in 2003 by the Bureau of Land Management (BLM) Rio Puerco Field Office (RPFO). Vegetation within the treated area included *Ericameria nauseosa, Juniperus monosperma, Salsola tragus, Yucca baccata, Gutierrezia sarothrae, Bouteloua gracilis, Bromus tectorum, Sphaeralcea angustifolia, Bahia dissecta, Grindelia squarrosa,* and *Quercus gambelii.* The extirpated plot also had the remnants of downed trees including *Pinus ponderosa*, *Juniperus monosperma*, *Juniperus scopulorum*, *Pinus edulis*, and *Quercus gambelii*. There was also some regeneration of *Pinus edulis* within the treated area.

Three saplings fell within the extirpated plot downhill from sampling. The closest sapling was 29.5 meters northeast of the north pit.

The live plot was 157 meters from the nearest sampling pit in the extirpated plot. The dominant species were *P. edulis* and *J. monosperma* followed by *B. gracilis*. *Ericameria nauseosa*, *B. dissecta*, and *Chenopodium* sp. were also present. Both plots had a slope of 0-5% and clay-loam soils with scoria lava rock sparsely dispersed on the soil surface. All plots were sampled from 1-5 cm and 20-25 cm.

Eagle Mesa (Eag)

Pinus edulis was chained in this area to open up grazing land in 1963. This site was located on BLM land in the RPFO. A few downed *P. edulis* stumps remained at the time of sampling. The identity of these skeletons was confirmed when a small amount of intact bark was found at the base of a stump. The dominant vegetation in the extirpated plot was *Artemisia tridentata*. Grasses were scarce but *Bouteloua gracilis* was present. A few *Juniperus monosperma* trees were found in the plot. A single *Pinus edulis* sapling was also present in the plot but was well out of the sampling radius (25+ meters from plot center). *Gutierrezia sarothrae* was also present.

The paired live stand of *P. edulis* was dominated by *P. edulis* and *J. monosperma*. The dominant grass species were *B. gracilis* and *Sporobolus airoides*. *Artemisia tridentata* and *G. sarothrae* were also present. The live plot was 105 meters from the nearest sampling location in the extirpated plot.

Both plots had a slope of 0-5% and evidence of disturbance by cattle, including fresh and old dung, and footprints. Cattle were spotted about 0.5 miles from the plot.

Sevilleta Refuge- Arroyo Milagro (SevA)

*P*inus *edulis* succumbed to drought in the 1950s and 60s at this site (Betancourt et al., 1993). Mortality occurred between 5,200 and 5,594 feet. What once was a piñon-juniper woodland shifted to a desert shrubland. Vegetation within the extirpated plot included *Teucrium canadense, Parthenium incanum, Ephedra spp., Juniperus monosperma, Aristida purpurea, Cylindropuntia imbricata, Dalea formosa, Nolina microcarpa, Yucca baccata, Thymophylla acerosa, Nerisyrenia camporum, Dasyochloa pulchella, Larrea tridentata, Fouquieria splendens, Bouteloua eriopoda, Rhus trilobata,* and *Rhus microphylla.* The dominant species were *B. eriopoda* followed by *J. monosperma* and *L. tridentata*. The presence of flagstone (shale) made it difficult to dig and sample at both plots.

The nearest live *P. edulis* was about 150 meters downhill from the extirpated plot center. The plot had a 0-10% slope and a sandy-loam soil with shale and sandstone on the soil surface. The soils were compact at 0% slope at the top of the hill but loose on the shoulders. Soils were very rocky and made it difficult to sample precisely at the desired depths.

The live plot was dominated by *Pinus edulis, J. monosperma,* and *N. microcarpa.* The dominant grass was an *Aristida* species. Other vegetation included *Sporobolus airoides, R. trilobata, D. formosa,* and *L. tridentata*.

Farmington Field Office 1 (FF1)

Historical records documented *P. edulis* extirpation from Chaco Canyon at 500-1200 years ago (Betancourt et al., 1993; Betancourt and Devender, 1981). Explanations for the

extirpation of *P. edulis* include fuelwood harvesting by the Anasazi peoples and climate change (Betancourt and Devender, 1981). This site was located on a land parcel bordering Chaco Culture National Historical Park and is managed by the BLM Farmington Field Office.

Vegetation in the extirpated plot was dominated by grasses. These included *Pleuraphis jamesii* and *Sporobolus airoides*. There was also *Achnatherum hymenoide*s. The dominant shrubs were *Atriplex confertifolia* and *Atriplex canescens*. *Gutierrezia sarothrae, Sarcobatus vermiculatus,* and *Abronia fragrans* were also present. No *Pinus edulis* was in sight. This plot was near the meandering channels of the Chaco River. The plot was about 25 meters from a two-track dirt road. Some evidence of cattle and horses was present (old dung).

The live plot was located approximately 82,000 meters east of the extirpated sites in Chaco Canyon. This was the closest live stand covered by our sampling permits and located on public lands. The actual nearest live stand was approximately 5,500 and 8,000 meters away from the FF1 and FF2 extirpated plots respectively. This live stand served as the positive control for both Chaco Canyon extirpated plots. Old cattle prints and dung were present in the plot. Grasses were abundant. The dominant grass was *Bouteloua gracilis*. The dominant tree was *P. edulis*. Other vegetation included *Juniperus monosperma, Artemisia tridentata, Gutierrezia sarothrae, Eriogonum racemosum,* and *Hymenoxys richardsonii*. To ensure that samples were taken under an adult *P. edulis,* the east and west lines were adjusted. The west line was 6.5 meters from plot center while the east line was 13.5 meters from plot center.

Farmington Field Office 2 (FF2)

This site was also located in Chaco Canyon and approximately 1.5 miles from FF1. In the extirpated plot the dominant species was *Sarcobatus vermiculatus*. There were no *Juniperus monosperma* nor *Pinus edulis* in sight. Other vegetation included *Atriplex confertifolia, Gutierrezia sarothrae,* and *Opuntia* spp. Grasses were scarce and grazed. *Sporobolus airoides* was present. This plot was near the seasonal meandering channels of the Chaco River. Evidence of cattle activity was present including footprints, dung, and live animals. Additionally, old horse dung was present.

Ancient Site (AS)

This site is managed by the BLM RPFO. Historical records indicated extirpation of *P. edulis* at approximately 11,000 years ago (Betancourt, et al., 1993). The present-day range of *Pinus edulis* does not overlap with its range during the last ice age (11,000 to 40,000 years before present). During the last ice age, *P. edulis* occurred at elevations between 300 and 1700 meters. Site location was determined using this information on ArcGis including elevation and current *P. edulis* range. The site slope was between 0 and 5%.

The dominant species was *Atriplex canescens* followed by *Juniperus monosperma*. *Artemisia filifolia, Lycium pallidum, Bouteloua gracili*s, *Pleuraphis jamesii, Dimorphocarpa wislenzi*i, and *Eriogonum* sp. were also present. There was no live plot paired with this site although the nearest live stand was approximately 10,500 meters away. The AS extirpated plot was intended to serve as an over-arching negative control for the greenhouse bioassay. It was not anticipated that colonization would occur at this site given than *P. edulis* was extirpated 11,000 years ago.

Seed germination

Pinus edulis seeds for bioassays were obtained from Plants of the Southwest in Santa Fe, New Mexico. All seeds were guaranteed to species and were collected from central and northwestern New Mexico.

Five hundred seeds were placed within 8x8 inch cheesecloth bags. Each cheesecloth bag was two fabric layers thick. Bags were stapled shut to prevent seeds from floating away during rinsing. Cheesecloth bags were placed in a shallow plastic container (about two inches deep) in a single layer. The shallow container was tilted to ensure continuous water flow over the seeds. Seeds were rinsed in deionized water for 48 hours. Cheesecloth bags were rotated approximately every twelve hours to ensure equal rinsing of all seeds.

Post rinsing, seeds were spread out in a single layer on a cookie sheet disinfected with 70% ethanol. Seeds were dried in an Enviralab Sterility Module laminar flow hood for 48 hours. Seeds were then transferred to 5 1/8" x 3 1/8" x 10 5/8" paper bags. Approximately 500 seeds were added to each paper bag. Each paper bag was placed inside a second bag of equal size.

Paper bags were stored at 2-4°C for a minimum of sixty days prior to germination. Seeds were checked for mold weekly for thirty days and then monthly.

Seeds were surface sterilized in a 10% bleach solution (one part 5.25% hypochlorite solution to nine parts deionized water) for one minute and 30 seconds with continuous agitation. The seeds were then rinsed twice in sterile deionized water.

Following surface sterilization, seeds were placed in 21"x 13"x 3" heat-sterilized vermiculite trays. Sterile water was added to each vermiculite tray until the substrate was moist enough to form a ball, but water did not drip out of the ball nor did water pool at the

bottom of the tray. Each tray contained 50-100 seeds covered with 1-3 cm of vermiculite. Trays were covered with plastic wrap and watered every other day with a deionized water mist within a laminar flow hood. Germinating seeds were incubated at 18-24 °C in a greenhouse.

Approximately 3,200 seeds were germinated to ensure the availability of the 1,520 seedlings needed for the bioassays.

Collecting and storing soil

Soil collection

As described above, soil samples were collected from sites in northwestern New Mexico with varying *Pinus edulis* extirpation dates (10-20 years, 55-65 years, 500+ years, and 11,000 years).

The exact locations of sites were recorded using GIS and GPS. All sampling in extirpated sites occurred a minimum of 100 meters from the nearest live *P. edulis* stand. Studies have demonstrated that the dissemination of fungal inoculum decreases as distance from host increases and that most spores land a few meters from their source (Bahram et al., 2013; Malloch and

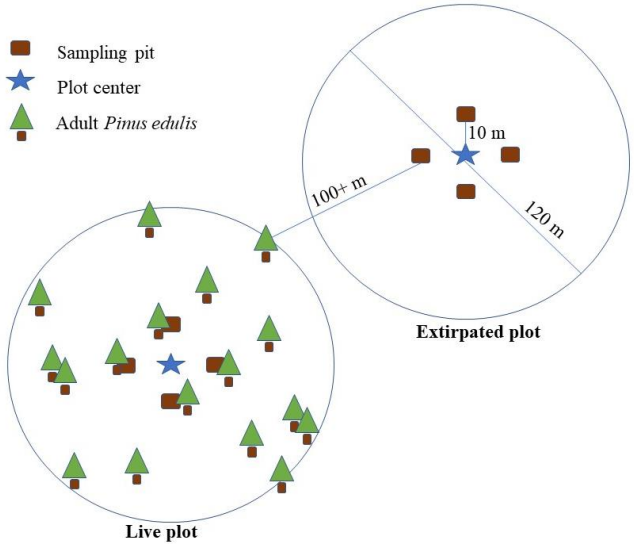

Figure 1. Site layout: circles indicate 120m diameter plots. Sampling pits at north, south, east, and west 10m from plot center, and extirpated pits are a minimum of 100m from the nearest live *Pinus edulis*.

Blackwell, 1992; Peay et al., 2010; Galante et al., 2011). Additionally, spores that disperse further distances are subject to degradation by the external environment (Norros et al., 2015). Soil samples were collected ten meters from plot center at 0°N, 90°E, 180°S and 270°W (Fig. 1). Some pit locations in live plots were moved to guarantee sampling under *P. edulis* canopy. In the Eag live plot, the north, east, and south pits were dug 7.5 meters from plot center and the west pit was sampled 9 meters from plot center. Additionally, at the SevA site the live plot the east and west pits were moved 10 degrees (80 and 280 degrees instead of 90 and 270 degrees). Finally, in the SevV live plot the north, east, south, and west pits were 14, 22, 31 and 14.5 meters from plot center respectively. Sampling was conducted at four locations within a plot to more thoroughly document fungal composition and diversity, since fungal communities in soil are extremely patchy (Lilleskov et al., 2004; Genney et al., 2006).

A shallow 30 x 100 cm trench was dug at the ten-meter mark (the nearest trench was ~14 meters distant). Soil samples were dug out of the side of each trench using a trowel sterilized with 70% ethanol. The trowel was sterilized between trenches and depths. From each trench, 0.44 L of soil was collected from 0-5 cm and 20-25 cm. Samples were taken from the first 25 cm of soil where 65% of soil microbes reside (Paul, 2014). All pits were sampled from 1-5 cm and 20-25 cm except the extirpated plot in SevA. In this plot the north, east, and south pits were sampled from 4-8 cm and 23-28 cm. The west pit hit bedrock at 23 cm, so sampling occurred from 4-8 cm and 20-23 cm. We chose to sample two depths because we expected deeper soil to be isolated from airborne inoculum but also more relevant to root colonization potential. Sampling terminated at the 25 cm level because the majority of microbial biomass occurs in the first 25 cm of soil (Fierer et al., 2003). An additional 0.44 L of soil was collected from each trench and depth to serve as a sterile

negative control. The negative controls were sterilized in Primus Steam Sterilizer. Positive control soils were collected in a similar manner beneath the nearest live *P. edulis* canopy. Control soils were collected from one live stand for every site for the 10-20 and 55-65-year-old sites. The 500-year sites shared positive controls and the 11,000-year site did not have a positive control. It was anticipated that the 11,000-year site would in itself serve as a negative control for the entire study.

All samples were transported to the University of New Mexico within 48 hours of collection. Samples were stored at room temperature in paper envelopes until the greenhouse bioassay began. Soils were stored for a maximum of four months.

Using sterile technique, 0.5 grams of soil from each replicate envelope was mixed together. Five grams of this mixture was stored at -80°C for molecular analysis in a 15 mL falcon tube. These samples will be analyzed in a follow-up study. An additional 45 grams from pooled replicates was used for soil chemistry analysis. Analyses included pH, soil organic matter, cation exchange capacity, and plant available nutrients (N and P). Soil pH was measured at UNM using the Standard Soil Methods for Long-Term Ecological Research (Robertson et al., 1999). Other tests were conducted at the Forest Soils Laboratory at the University of Alaska, Fairbanks. Cation exchange capacity was determined using perchloric acid digestion. Soil organic matter was measured using the loss of ignition or ashing protocol. Plant available nitrogen and phosphorus was determined using the KCL extraction solution and dilute acid fluoride methods respectively. These tests allowed for the analysis of potential effects of soil chemistry. Several studies have demonstrated the effects of soil type, soil organic matter and pH on rate of colonization, mycorrhizal community composition, and mycorrhizal abundance (Oehl, 2010; Lauber et al, 2008; Kluber et al, 2012).

Greenhouse trials

Seeds were germinated in sterile vermiculite trays at 18-24°C in the greenhouse. Soil samples were not mixed for the bioassay. Each soil sample collected was used to inoculate a single tree. Seedlings were transplanted to 164 mL conetainer pots (Stuewe and Sons) with the following treatments:

A) Extirpated plot

1. Negative control 0-5 cm: Sterile extirpated soil
2. Negative control 20-25 cm: Sterile extirpated soil
3. Extirpated soil 0-5 cm: Not sterile
4. Extirpated soil 20-25 cm: Not sterile

B) Live plot (*P. edulis* stand)

1. Positive control 0-5 cm: Not sterile
2. Positive control 20-25 cm: Not sterile

Figure 2. Greenhouse bioassay with a randomized block design and overhead irrigation.

Each treatment consisted of ten trees resulting in a total of 160 or 240 trees/site (10 replicates x 6 treatments x 4 pits). Conetainer pots were placed in 1x2 ft raised trays. Each pot was filled with a mix of three parts sterilized sand and one part native soil. The sand was sterilized in a Primus Steam Sterilizer. Sand was chosen as a primary substrate to make visualization and washing of roots more feasible. Native soil was placed between two layers of sterile sand to minimize contamination between pots. To statistically remove the effects of environmental variability within the greenhouse, a

complete randomized block design was used where one replicate from each pit was located in a randomized position within each block, for a total of ten blocks (Fig. 2). Seedlings were checked weekly for mortality and grown for five months. Overhead irrigation with UV treated water was turned on every other day and temperatures were maintained at 18-24°C in the greenhouse. Irrigation turned on for one hour so that each plant received approximately 100 mL of water every other day.

Toward the end of the greenhouse bioassay, replicates 4-10 from the ICG site were killed due to a greenhouse temperature malfunction. Presence/absence and shoot biomass data was collected for ICG replicates 1-10 while extent of colonization, root biomass, and community data were collected for replicates 1-3.

Processing seedlings

Above-ground biomass

After five months, seedlings were pulled put up to measure inoculum potential and effect of colonization on above-ground biomass. Each seedling was removed from the greenhouse and above-ground biomass was cut off at the crown of the plant using clippers. Above-ground parts were placed in pre-labeled paper bags and dried at 60°C for 48 hours in a SHEL LAB 120 volt drying oven. Weights were then recorded to the nearest 0.01 grams.

Morphotype procedure

Root balls were removed from their pots and placed in a 35-micrometer mesh sieve for rinsing. The soil aggregates were gently broken apart by hand with running water. Clean

roots were then picked from the sieve with forceps and placed in a quart Ziploc bag. Each root system was then observed under an Olympus SZ-CTV dissecting microscope for colonization. All fine root tips were then removed using forceps and placed into a 1x1 cm gridded petri dish filled with deionized water. The petri dish was swirled, and fifty root tips were counted randomly as colonized or uncolonized. Colonized root tips were then described based on physical characteristics and assigned a morphotype number (Fig. 3). Uncolonized roots were given the morphotype identity "M0001" (Fig. 4). Morphotypes were assigned separately to each seedling. Morphotypes were then placed in individual pre-weighed 1.5 mL centrifuge tubes and stored in the -80°C freezer until DNA extraction. If a tree had entirely uncolonized roots, no tips were stored in the -80°C freezer. All roots were processed within a week of the five month pull date. Remaining roots were placed into a prelabeled paper bag and dried at 60°C in the drying oven for 48 hours.

Figure 3. Example morphotypes sorted by physical characteristics. Each morphotype represents a species. Photos taken at 10-20x magnification.

All centrifuge tubes were freeze dried for 48 hours using a Labconco benchtop lyophilizer. After freeze drying, tubes were weighed to 0.0001

Figure 4. Pinus edulis root cluster on the left is colonized. The cluster on the right is uncolonized. 10x magnification.

grams to get the dry weight of root tips and associated morphotypes.

DNA extraction, electrophoresis, PCR

To identify morphotypes, a single root tip or cluster was used. Samples were ground with 2.3 mm metal beads and incubated in an AP1 buffer for 1.5 minutes twice at 30 Hz. Total genomic DNA from each sample was extracted via a DNeasy 96 Plant Kit (QIAGEN Incorporated, Germantown, Maryland) following the manufacturer's instructions. The DNA was then amplified using the universal fungal primers, ITS1-F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) using a Bio-Rad C1000 Touch Thermal Cycler with a lid temperature of 95°C, 40 cycles of denaturation, annealing, and extension and final extension at 72°C for ten minutes. Twenty-five of 192 samples extracted were randomly selected to confirm amplification via electrophoresis. A 100 bp DNA ladder was used and the gels were visualized using a Gel Logic 200 Imaging System. The raw PCR products were then shipped to Functional Biosciences (Madison, WI) to process samples via Sanger sequencing.

The sequences received from Functional Biosciences were trimmed and assembled using Phred and Phrap in the CodonCode Aligner program. Sequences were uploaded into USEARCH-UPARSE and clustered into operational taxonomic units (OTUs) using a 97% identity threshold. To identify OTUs, representative sequences were submitted to Protax for comparison against the UNITE database on the PlutoF platform. Poorly identified OTUs were subsequently analyzed using BLASTN searches against all sequences in NCBI GenBank. Operational taxonomic units that were not EMF including saprotrophs and plant pathogens were removed for downstream analysis. To check whether any OTUs belonging to

the same genus had been oversplit, we also carried out genus-level phylogenetic analyses. First sequences were aligned using Muscle (Edgar, 2004) in Aliview (Larsson, 2014). Phylogenetic trees generated for *Rhizopogon*, *Geopora,* and *Tomentella* were created using RAxML (Stamatakis, 2014). Ectomycorrhizal OTUs were combined if they resolved to the same terminal clade with minimal branch length (see Supplemental Figures 3, 4, 5).

Statistical analysis

Analysis was performed using the R statistical program and PRIMER-e (RStudio Team, 2015; Clarke and Gorley, 2006). Prior to analysis, data was normalized, and a principal component analysis was performed on soil chemistry data in R using the "tidyverse" package. After a principal component analysis, it was determined that soil organic matter (SOM) and cation exchange capacity (CEC) were correlated and, therefore, these variables were collapsed into "component two" to simplify analysis. Plant available nitrogen, plant available phosphorus, and soil pH were also correlated and collapsed into "component one" (Fig. S-1). If either component had a significant effect on the response variables, it was broken up into its original variables for further analysis.

During all analyses, plot age was combined with plot type into a categorical variable where age "0" represented positive controls and age "infinity" represented negative controls. Additionally, the Eag and SevA extirpated plots were lumped into a 55-65-year age bin and SevV was lumped with ICG into a 10-20 year age bin.

Multi-level linear mixed effects (lmer) models were developed using the "lme4" R package to analyze the response variables: tree aboveground biomass, tree belowground biomass, total tree biomass, and colonization rate of ECM. Each model analyzed a single

response and included the fixed effects of plot age, depth, and soil chemistry properties and interactions as well as the random effect of tree replicate nested in pit and pit nested in site. Additionally, colonization rate and EMF diversity were treated as predictors in the total biomass model. Ectomycorrhizal presence/absence was analyzed using a generalized mixed effects model that included the same fixed and random effects and interactions as the lmer models.

Alpha diversity (species richness, Shannon index, Simpson index) were calculated using the "vegan" package in R. Fixed effects were site, depth, and plot type and replicates were nested in pit while pit was nested in site. Interactions between fixed effects were included in the models. The R packages "tidyverse" and "ggplot2" were used to construct stacked taxonomic bar plots. A Bray-Curtis analysis of community similarity was performed in PRIMER-e using PERMANOVA with a Euclidean distance matrix and 9999 permutations. The model included fixed effects of site, plot age, plot type and depth with all the interactions. To analyze dispersion between plot types and sites in the Bray-Curtis NMDS, PERMDISP was used in PRIMER-e. Ordination figures were constructed in R using the Bray-Curtis dissimilarity index. Additional plots were constructed using "ggplot2" on R. An indicator species analyses was performed at the plot and site level using the "indicspecies" package in R and EMF species nestedness between extirpated and paired positive plots was assessed using the "betapart" package.

Results

Inoculum potential

Colonization using soil inoculum occurred at all plots, but inoculum potential was strongly related to depth and plot history, especially host extirpation. The positive plots and shallower depths had the greatest inoculum potentials. All negative control trees had zero colonization by EMF. In contrast, colonization was recorded in trees grown in extirpated soils of all ages, from 16 to >11,000 years. Plot age and sampling depth had significant effects ($p < 0.0001$) on presence/absence of ECM on roots. Overall, trees grown in positive control soils had significantly greater ECM presence ($p < 0.0001$) than those grown in *Pinus edulis* extirpated soils. The shallower depth produced significantly more trees ($p < 0.0001$) with ECM present than the deeper soil. There was a significant interaction between age and depth ($p = 0.0013$). This interaction could be driven by the low number of trees with ECM from the 500-year-old plots (just five plants were colonized) as well as the absence of ECM trees grown in the deeper soils in these plots (Fig. S-8).

Patterns in the percent of colonized roots (colonization rate) of individual seedlings followed those seen for presence/absence of colonization. Plot age and depth had a significant effect on root colonization rate by EMF ($p < 0.0001$). Colonization did tend to decrease over time in extirpated plots (Fig. 1), though this trend was not significant. Trees grown in soils from the shallower depth had significantly ($p < 0.0001$) greater colonization rates by EMF and in a pairwise comparison, the positive plots had significantly greater colonization rates than the extirpated plots ($p < 0.0001$) in all age categories (Fig. 1). There was a significant interaction ($p < 0.0001$) between depth and age. This interaction could be driven by the low number of colonized plants in the 11,000-year plot (Fig. S-9).

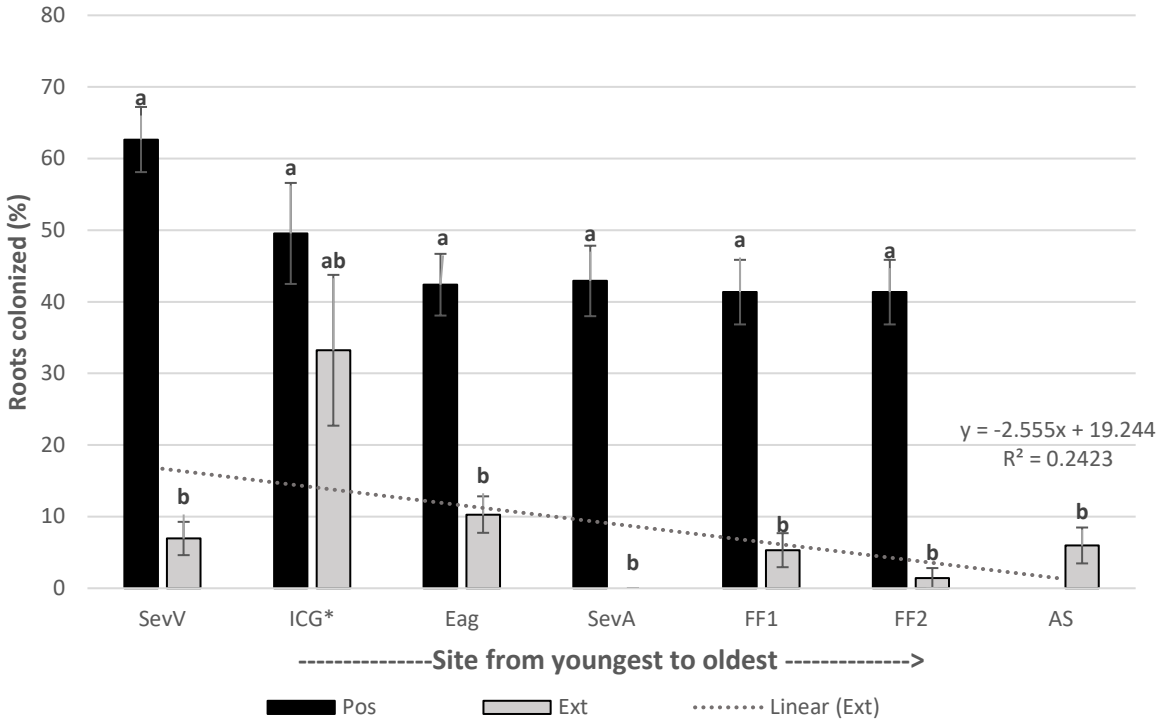

Figure 1. Roots colonized by EMF across sites in chronological order, separated by plot type. There is a non-significant trend toward decreasing inoculum potential over time following the extirpation of *Pinus edulis.* Positive plots had significantly greater colonization across sites ($p < 0.0001$). Significant differences indicated by lower case a, ab, b.

Host growth

Aboveground and belowground biomass

Sterile negative control soils and live soils from the shallower depths resulted in greater root and shoot biomass overall. There was a significant effect of plot age on both shoot and root biomass ($p < 0.0001$) as well as a significant effect ($p = 0.0014$) of depth on shoot biomass wherein seedlings in the shallower soil had significantly greater shoot biomass. There were no significant interactions between variables. On average, negative control trees had the greatest shoot biomass followed by plots in the 55-65 and 11,000-year age bins. The 10-20 and 500-year-old plots had the smallest shoot biomass on average.

Results for belowground biomass mirrored those for aboveground biomass. The only difference was that depth did not have a significant effect on root biomass. A pairwise comparison of the different plot ages is summarized in Table S-2.

Total biomass

Total biomass was related to plot type (sterile controls vs live soils) and soil organic matter. Pine seedlings grown in the negative control soils (age = "infinity") had the greatest total biomass on average. Additionally, as soil organic matter increased, tree biomass decreased. Plot age had a significant effect ($p < 0.0001$) on total biomass as did SOM ($p = 0.0067$). Trees with the greatest biomass were associated with no EMF or *G. pinyonensis* (Fig. 4). Trees with the smallest biomass were associated with other *Geopora* spp. and *Rhizopogon milleri* or exclusively *Geopora* spp. (including *G. pinyonensis*). The negative control trees differed from the positive, 10-20-year-old plots, and 500-year-old plots (Fig. S-12). Colonization rate ($p = 0.2034$) and fungal diversity per seedling ($p = 0.1784$) were not correlated with total biomass. Biomass did not increase as fungal diversity increased, although there was a non-significant positive relationship between rate of root colonization and biomass (Fig. 2, Fig. S-11). There was a significant interaction between plot age and colonization ($p = 0.01426$). The interaction may be explained by the low number of colonized trees in extirpated plots compared to positive plots. There were 195 and 39 colonized trees in the positive and extirpated plot types, respectively.

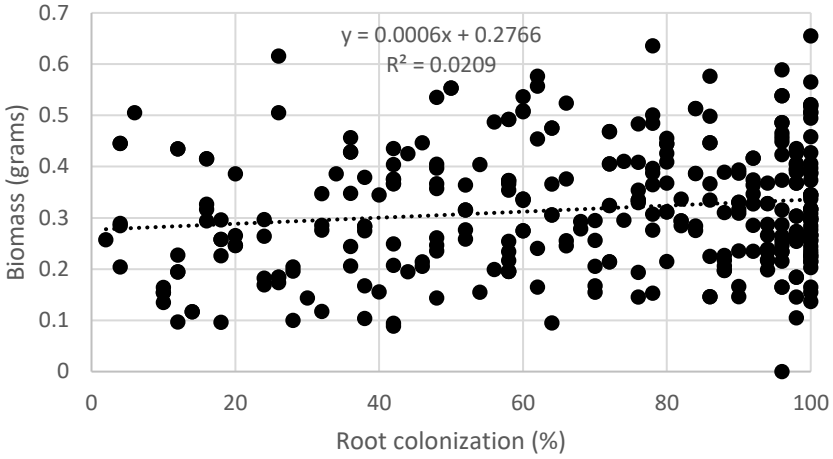

Figure 2. Total tree biomass versus root colonization. There was no significant relationship between root colonization rate and total biomass (p = 0. 2034), although there was a positive trend.

On average as CEC (R^2 = 0.0464), nitrogen (R^2 = 0.1272), phosphorus (R^2 = 0.2094), and SOM (R^2 = 0.0732), increased, total tree biomass decreased. Total tree biomass and pH were positively correlated (R^2 = 0.0099). The ICG site had the highest levels of nitrogen and the lowest total tree biomass on average. There were no other significant effects of soil chemistry revealed by our analyses.

Community analysis

Taxonomic IDs

After sequence analysis, thirty-seven individual operational taxonomic units (OTUs) were identified using 97% sequence similarity for OTU thresholds, along with slight OTU revisions based on phylogenetic analyses. Twenty-four putative EMF OTUs were identified. Operational taxonomic units that were removed from analysis were plant pathogens or saprotrophs. These were identified as *Fusarium*, *Phellinus gilvus*, *Monosporascus eutypoides*, *Dactylonectria estremocensis*, and *Nectria*. Most of these were recovered as

singletons on a single cluster because they yielded EMF taxa when the same morphotype was re-sequenced.

The dominant phylum, Ascomycota (54.2% of OTUs), occurred across sites, depths, and plot types (Fig. 3) as did Basidiomycota (45.8% of OTUs). The most common genera were *Geopora* (Ascomycota), *Rhizopogon* (Basidiomycota), and *Tomentella* (Basidiomycota) (Fig. 4 and Fig. S-7). As age increased, communities shifted from *Rhizopogon-Geopora* dominated to *Geopora* dominated (Fig. 4). Trees that were colonized in the 11,000 and 500-year-old plots were exclusively colonized by *Geopora* spp. including *G. pinyonensis*. Finally, the deeper soil from the positive and younger sites tended to be dominated by *Rhizopogon* while the shallower soils tended to be dominated by *Geopora*.

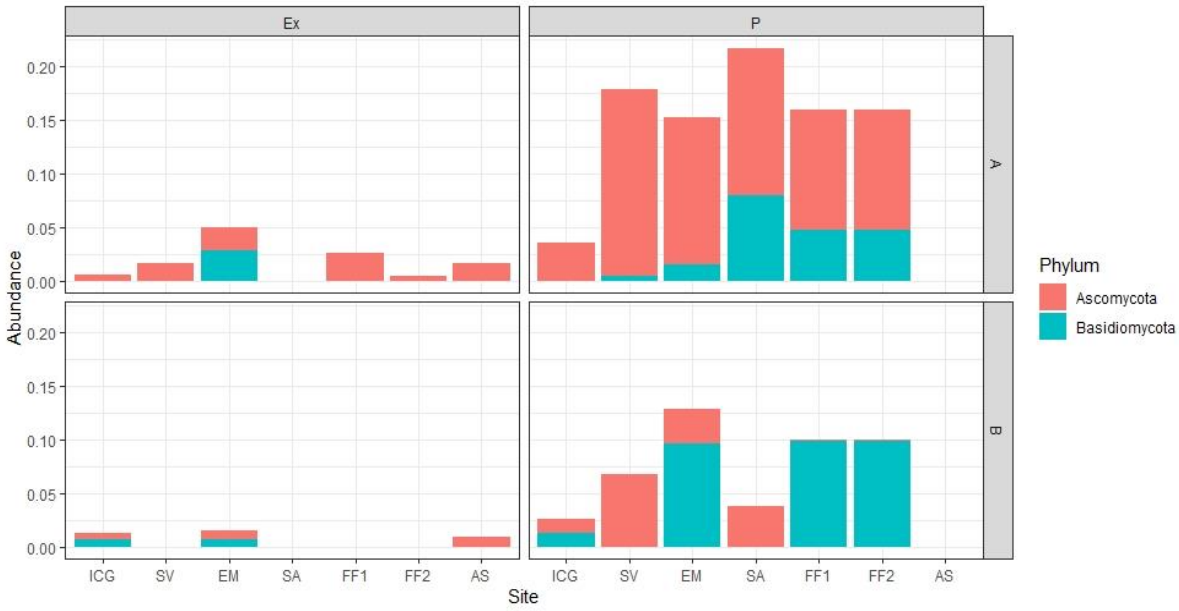

Figure 3. Absolute abundance of fungal phyla across sites and depths. The positive and extirpated plots are "P" and "Ex" respectively. Positive control soils had a greater occurrence of basidiomycete fungi as did deeper samples (B). Depths are 0-5 cm (A) and 20-25 cm (B).

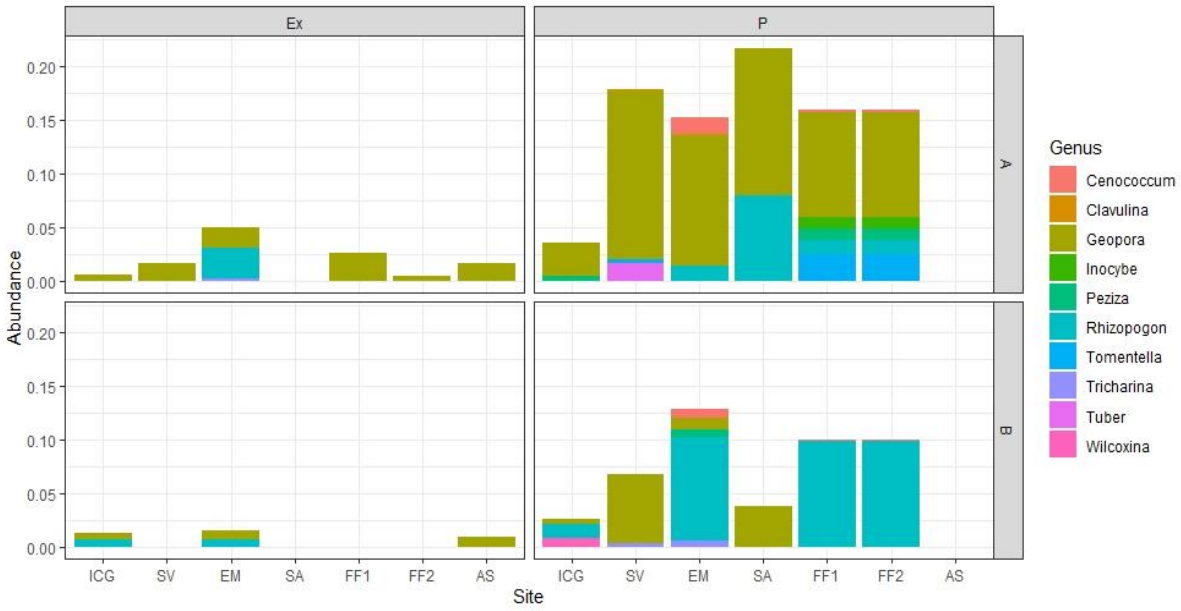

Figure 4. Genus level absolute abundance by site from youngest to oldest. Plot types are extirpated (Ex) and positive (P). Depths are 0-5 cm (A) and 20-25 cm (B). The most common genera were *Geopora*, *Rhizopogon*, and *Tomentella*. Depth B had higher incidence of *Rhizopogon* while Depth A had higher incidence of *Geopora*.

Richness

The positive control soils (p=0.0074) and shallower depth (p = 0.0826) had a greater number of EMF species associated with *P. edulis* seedlings (Fig. 5). All colonized trees grown in extirpated soils were associated with a single species of EMF. In the positive controls 88.7%, 9.7%, and 1.5%, of colonized trees had one, two, and three EMF species while the positive-shallow, positive-deep, extirpated-shallow, and extirpated-deep had 6.6, 4, 2.14, 0.86 species respectively. There was a significant effect of site (p = 0.0483) and plot type (p = 0.0074) on richness. Additionally, depth was nearly significant (p = 0.0826).

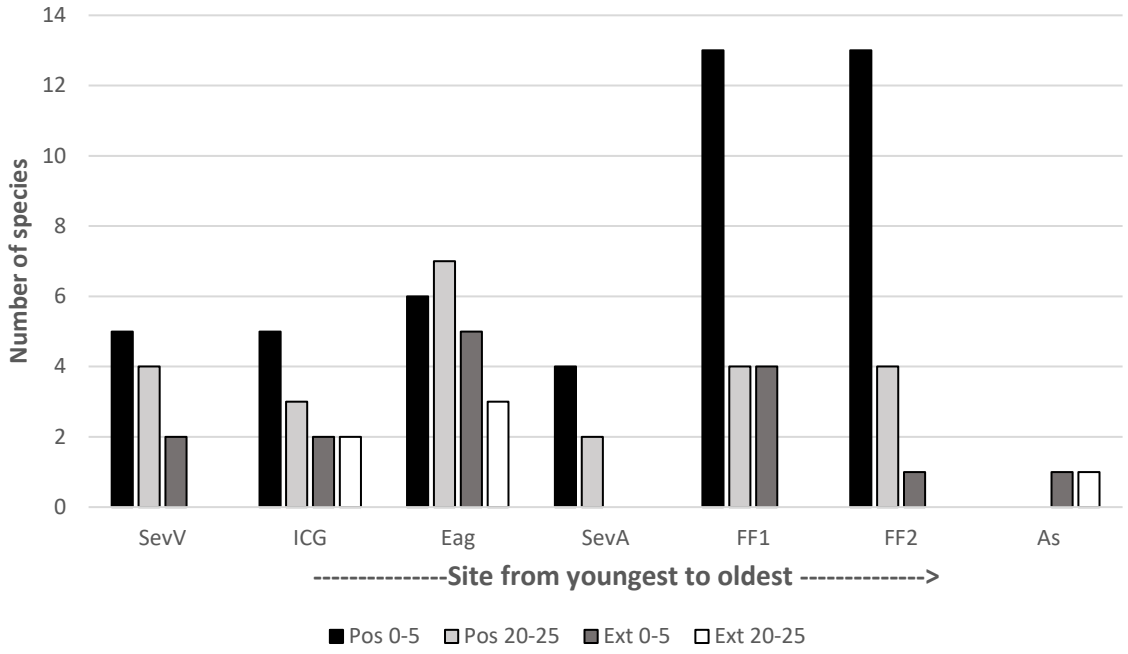

Figure 5. Richness by site, depth, and plot type. Site and plot type had significant effects on richness. The positive plots had significantly greater richness than the extirpated plots (p = 0.0074) as did the shallower depth. The FF positive plot had the greatest richness overall.

Shannon and Simpson diversity indices

Shannon diversity was also low across sites and plot types and the positive plots and shallower depth had the greatest EMF diversity. There was a significant effect of plot type (p = 0.01807) wherein the positive control had significantly greater diversity, but there was no relationship with site or depth. There was no effect of soil chemistry nor were there significant interactions between site, depth, and plot type. Trees grown in positive control soils had the greatest diversity in the FF2 and FF1 sites at the deeper depth, while Eag had the greatest diversity at the shallower depth (Fig. 6, Table S-1).

The Simpson diversity analyses yielded similar results. Site, plot type, and depth had significant effects on EMF diversity. The shallower depth (p = 0.0343) and positive plots (p

= 0.0080) had greater diversity overall and the Eag site had greater diversity than the SevV site (p = 0.0197).

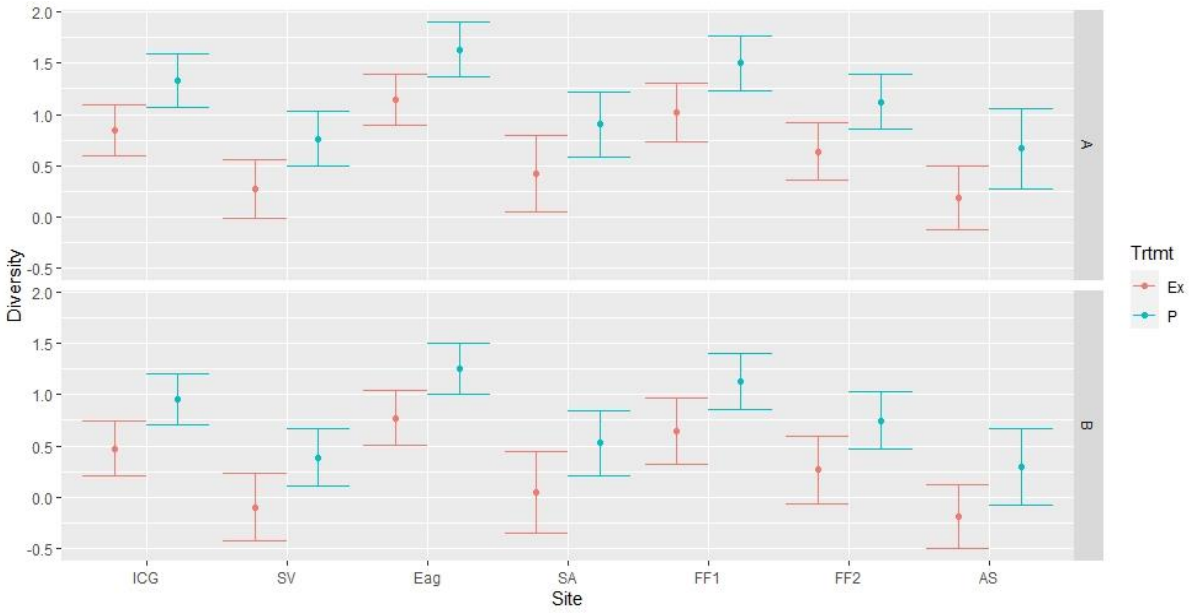

Figure 6. Shannon diversity by site from youngest to oldest. Plot age did not have an effect on diversity (p = 0.7351). Plot types are extirpated (Ex) and positive (P). Depths are 0-5 cm (A) and 20-25 cm (B).

Community Composition

Ectomycorrhizal community similarity differed between depths and plot type. Although plot age had a significant effect between age zero and 16+, there were no differences in community composition among extirpated plots. A PERMANOVA analysis of community similarity yielded a significant effect of age (p = 0.0144) and depth (p = 0.0010). Positive plots (age = 0) were significantly different from the 10-20 and 55-65 age bins (p < 0.05). A secondary analysis excluding the positive controls indicated that age did not have a significant effect on community similarity for the extirpated plots (p = 0.1454). A tertiary analysis with only age and plot type yielded a significant effect of plot type (p = 0.0024) but not age. Positive and extirpated plots did not have significantly different dispersion (p = 0.8145) (Fig. 7).

There were no indicator species by site or by plot type and all sites had overlapping taxa between positive and extirpated plots. The SevV and ICG extirpated taxa were perfect subsets of their positive pairs and were therefore highly nested (Table 1).

Table 1. Nestedness between extirpated and paired positive plots.

Site	Turnover	Nestedness	Total Sorensen
Eag	0.2000	0.2667	0.4667
FF1	0.5000	0.2778	0.7778
FF2	0.0000	0.8667	0.8667
ICG	0.3333	0.2667	0.6000
SevV	0.0000	0.6000	0.6000

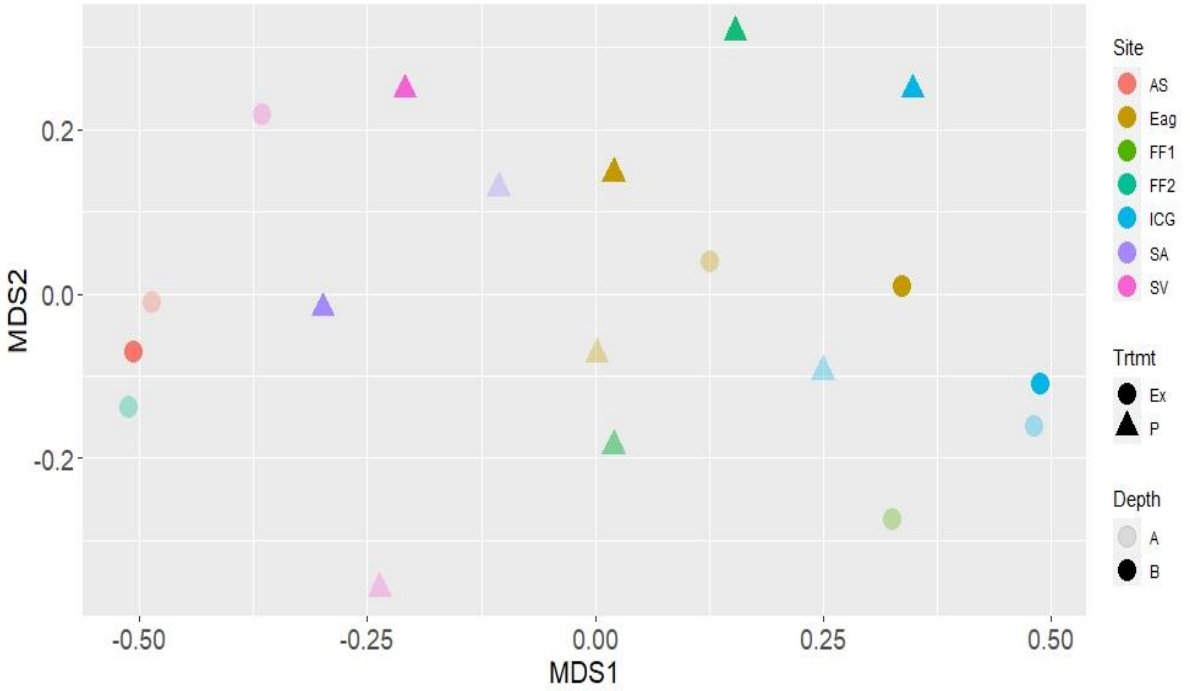

Figure 7. A Bray-curtis similarity NMDS ordination by plot X depth. Sites are various colors and the positive (P) and extirpated (Ex) plots are triangles and circles respectively. Depth is indicated by a hollow (shallow) and filled (deep) shape. Dispersion did not differ among plot types (p = 0.8242).

Discussion

Inoculum potential

Our study is the first to measure inoculum potential of EMF in piñon-juniper forests in which trees have been extirpated for over ten years. Seedlings were colonized via soil from every extirpated plot except SevA. Our finding of inoculum in extirpated sites could be due to long-term persistence of EMF propagules in soil. Alternatively, dispersal from outside the sites might explain the presence of EMF inoculum at older plots. The greater inoculum potential found at the 0-5 cm depth ($p < 0.0001$) is contrary to our predictions. However, it supports the idea that fungal dispersal has contributed to the small but non-zero inoculum potential that was found in the extirpated plots. If inoculum has arrived recently to a site, we would expect to find it on soil surfaces rather than 20 cm or more below the surface, particularly in these semi-arid sites that have almost no organic layer and hard, densely packed soils. In the oldest extirpated plots (FF1, FF2, and AS), *Geopora* was the only genus present on colonized trees. The fruiting bodies of *Geopora* are often hypogeous like many truffle fungi and likely depend on mycophagy to disperse spores in fecal pellets (Fogel and Trappe, 1978; Frank et al., 2006). A dispersal event from mycophagous migratory birds, ungulates, or small mammals could explain the presence of *Geopora* in these old plots and why inoculum potential was greater at the shallower depth (Alsheikh and Trappe 1983; Launchbaugh and Urness, 1992). In the oldest sites, the nearest live stands were 5.5, 8, and 10.5 km away, distances that could easily be covered by migratory ungulates (Sawyer and Kauffman, 2011). Additionally, the high winds and dust storms that occur throughout the Southwest could disperse spores that are resistant to desiccation, heat, and exposure to UV light.

In prior studies, inoculum potential of *P. edulis*-associated EMF has only been measured up to nine years after extirpation (Mueller et al., 2019). These studies found either no difference after five years or increased inoculum potential (Mueller et al., 2019; Bruns et al., 2009). As we hypothesized, inoculum potential in our study, measured by root colonization rate, decreased as time from *P. edulis* extirpation increased. There were also significant differences in colonization between age zero (positive controls) and the FF1, FF2, SevA, Eag, and SevV extirpated soils. These results demonstrate a sharp decrease in EMF inoculum only 16 years after *P. edulis* extirpation (Fig. 1). However, it must be noted that in both our study and Mueller et al. dispersal could have contributed to colonization in extirpated plots. Furthermore, the ability to generalize from our results is limited due to the small number of sampling sites and restricted extirpation age range. If future studies narrow sampling age to 0-50 years since extirpation with more sampling sites, a more robust relationship between inoculum potential and time might be observed.

Host growth (biomass)

We found no relationship between seedling biomass, colonization rates, EMF diversity, and associated EMF species and genera. The trees with the greatest biomass were grown in negative and 11,000-year-old extirpated soils, with very low rates of colonization. These trees associated with no EMF or *Geopora pinyonensis,* while trees with the lowest biomass (10-20 and 500-year age bins) associated with *Geopora* (including *G. pinyonensis*) and *Rhizopogon milleri* or exclusively *Geopora* (including *G. pinyonensis*). In one study, *Geopora* was associated with greater growth in *P. edulis* under drought conditions (Gehring

et al., 2017). Seedlings in our study were grown under ideal temperature and moisture, so the effects of EMF species on biomass could be masked.

Although EMF diversity was low and greater diversity and colonization did not result in greater biomass, EMF diversity was similar to previous studies. Greater EMF diversity and colonization rates were not positively correlated with tree biomass thus, our hypotheses were not supported. Increasing diversity of sampling sites and extirpation dates could reveal clearer results in future studies. Additionally, growing seedlings in larger amounts of soil and for a longer period would have likely resulted in higher EMF diversity. Nonetheless, low EMF diversity is not uncommon in PJ woodlands. In Arizona, 15-19 EMF species have been reported in association with *P. edulis* (Gehring et al., 1998). Another study recorded eight EMF species in six genera associated with *P. edulis* (Patterson et al., 2019). In contrast, another low elevation pine, *P. contorta*, was associated with 26 genera of EMF (Cullings and Makhija, 2001). Arid environments may limit fungal diversity due to low host diversity, high temperatures, low moisture, and high solar radiation (Gallo et al., 2006).

Previous studies have reported effects of EMF colonization on pine biomass as positive, negative, or neutral depending on plant developmental stage (Gehring et al., 2017; Corrêa et al., 2006; Colpaert et al., 1992). In the early stages of tree development, mycorrhizal relationships can slow seedling growth because the cost of carbon outweighs the benefit of nutrient acquisition by the mycorrhizae (Colpaert et al., 1992; Bidartondo and Bruns, 2005). This could explain why our hypothesis was not supported (i.e. in our study higher colonization by EMF was not significantly correlated with greater biomass) and why the negative control trees had greater biomass on average. However, in a very similar soil bioassay in which *P. edulis* seedlings were grown for over a year, seedlings in live soils

(most of which formed ECM) were twice as large, on average, as the sterile controls (Erik Olivas and D. Lee Taylor, unpublished data). Thus, had we grown the seedlings longer in the current study, the results would likely have been dramatically different.

Finally, soils did not vary greatly in abiotic properties and did not have a strong effect on growth. Trees grown in deep and shallow soils did not have differing biomass. The lack of depth effect could be explained by the low organic matter levels in arid land soils; in other words, the shallow and deep samples had very similar soil structure and chemistry. Many of the soils in this study were sandy, and sand holds fewer nutrients than other soil types because of its smaller surface area to volume ratio (Brady et al., 2008). The combination of these factors could homogenize the distribution of nutrients between the depths, resulting in similar nutritional makeups.

Community analysis

As we hypothesized, diversity did decrease significantly from age zero to sixteen years post extirpation, suggesting a pattern of decreased diversity as time since extirpation increases. The greater EMF diversity found in the shallower depth and positive control trees is likely explained by the recent arrival of inoculum and presence of live *P. edulis*. Because diversity was so low across extirpated plots, it is difficult to draw conclusions about how communities changed beyond the zero to 16-year window.

As we hypothesized, the positive plots had a greater incidence of basidiomycete EMF and communities shifted towards ascomycete dominance over time, with *Geopora* as the primary genus at older plots. Although *Geopora* occurred across sites and plot types, its increase in dominance over time suggests persistence and/or superior dispersal capacities.

Not only was *Geopora* associated with the oldest extirpated plots, but extirpated soils from the driest sites (Sev sites) were almost entirely occupied by *Geopora* species. We predict a shift to *Geopora*-dominated communities as temperatures increase, drought intensifies, and *P. edulis* continues to suffer large-scale mortality events. Although this implies a major loss of biodiversity as PJ woodlands become drier, there is some evidence that *Geopora* affords greater drought tolerance (Gehring et al., 2017). Further research is necessary to understand the functional and ecological impacts of shifting EMF communities and loss of biodiversity, especially in ecosystems such as semi-arid PJ forests where alternate hosts are unavailable, and conditions are harsh.

Our results suggest that mode of extirpation may be an important predictor of future fungal communities. Though the number of sites we could study was limited, *P. edulis* extirpation driven by drought appeared to lead to the most rapid and dramatic decline of inoculum potential. Drier sites also had lower diversity overall. *Pinus edulis* trees in the ICG extirpated plot were cut and burned while trees in the SevV plot died of drought. The drier SevV site had lower diversity in both the extirpated and positive plots compared to ICG. Additionally, both paired live stands were under 200 meters away from the nearest extirpated sampling pit, yet the paired communities showed strong differences 16 years after extirpation. This trend exists when comparing the Eag and SevA sites as well. The drought extirpated SevA site had lower diversity in both plots compared to the Eag site. Although differences in community similarity were not significant, this trend suggests an effect caused by mode of extirpation. In future studies, it would be informative to measure moisture levels at sampling sites or to experimentally manipulate methods of tree removal and soil conditions.

The EMF genera in extirpated plots tended to be a subset of their positive counterparts which demonstrated both species turnover and a decrease in EMF diversity over time (Results Table 1). Both the SevV and ICG extirpated communities were completely nested within paired positive plots and, dominant genera persisted in both plot types suggesting both a decrease in biodiversity and an increase in relative abundance of dominant genera (Fig. 4, S-7). For example, The SevV extirpated plot community was exclusively *Geopora* while its live counterpart had a community of *Geopora*, *Tuber*, *Tricharina*, and *Tomentella*.

Finally, EMF communities did not seem to vary predictably with elevation, distance, latitude or longitude. The FF and Sev sites were the furthest northwest and southeast, respectively, yet both FF and Sev extirpated plots were entirely dominated by *Geopora* spp. including *G. pinyonensis*. Similarly, the positive FF and Sev plots were both dominated by *Geopora* spp. and *Rhizopogon* spp. On the other hand, our finding of strong nestedness in extirpated plots compared to neighboring positive control plots does suggest that there are location-specific communities of EMF. In contrast, if there were widespread dispersal across our entire study region, we would not expect these similarities among adjacent paired plots.

Conclusions

Low levels of EMF inoculum were found in soil where *P. edulis* had been extirpated as little as 16 and up to 11,000 years ago which suggests that *P. edulis*-compatible EMF may be present in areas where mass mortality events have occurred. This could promote successful reintroduction of *P. edulis* in these sites although it seems unlikely that EMF inoculum persisted for 11,000 years; long-distance dispersal of *Geopora* most likely occurred

in these older plots. To measure inoculum coming into extirpated sites, long-term monitoring of arriving fungi via spore traps or fecal collections could be useful. The limited, patchy inoculum recovered from extirpated sites suggest that favorable sites for *P. edulis* establishment may occur somewhat unpredictably across the landscape (Lilleskov et al., 2004; Genney et al., 2006).

Based on our results, PJ woodland land managers could consider inoculating new plantings of *P. edulis* in areas extirpated for over 20 years with soils from healthy nearby stands. Sites that have been extirpated beyond 20 years will have little or no EMF inoculum based on our results, which would hinder establishment and continued survival of seedlings in recovering forests. Land managers should also consider the environmental conditions of extirpated sites. For example, in drier sites seedling plantings may not be feasible without irrigation and therefore water availability would be a greater concern than lack of EMF inoculum. Understanding the persistence and dispersal of obligate EMF inoculum and the benefits of field inoculations could be crucial in restoration efforts as foundational woody species continue to decline worldwide.

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Supplemental materials

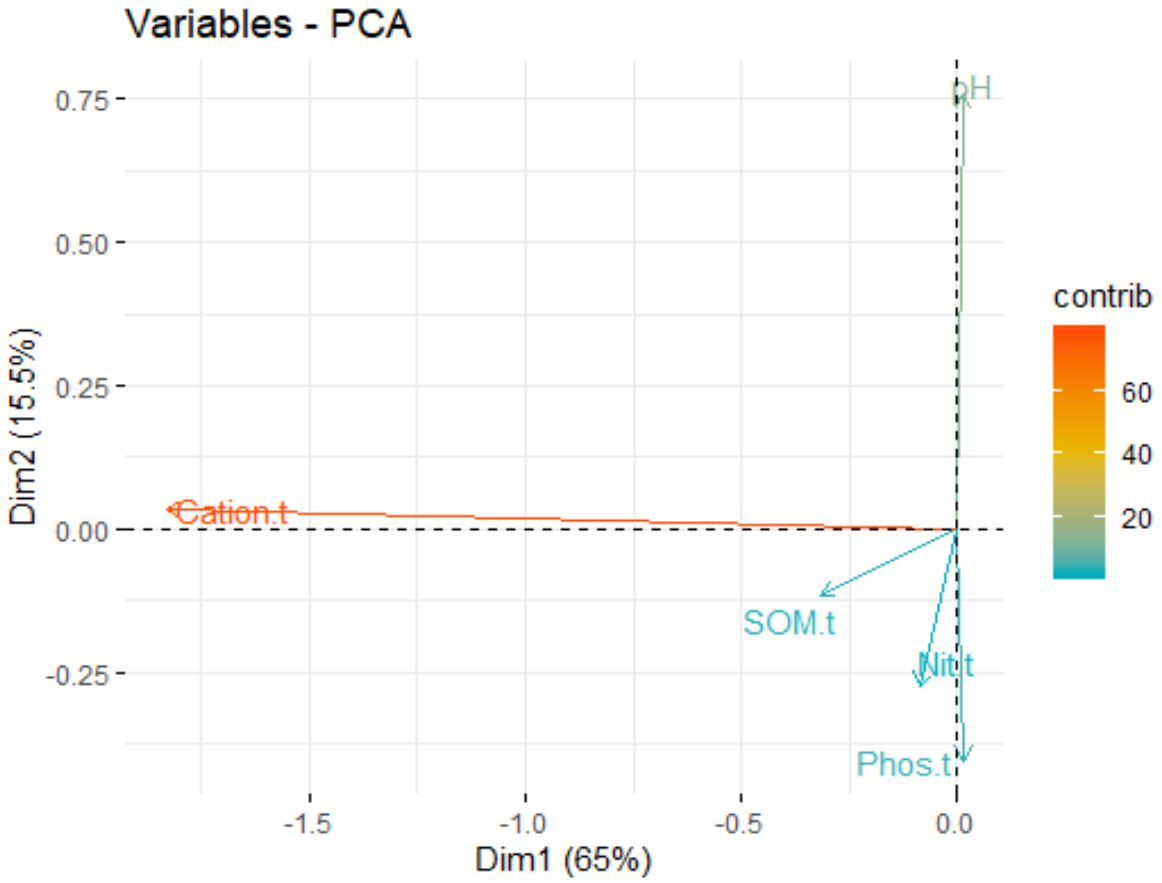

Figure S-1. In this figure correlations among SOM, P, N, and CEC are represented by vectors. Vectors that point in the same direction are positively correlated. Vectors that point in opposite directions are negatively correlated. Axes represent the components that explain the most variance. The longer the vector the more contribution to the component.

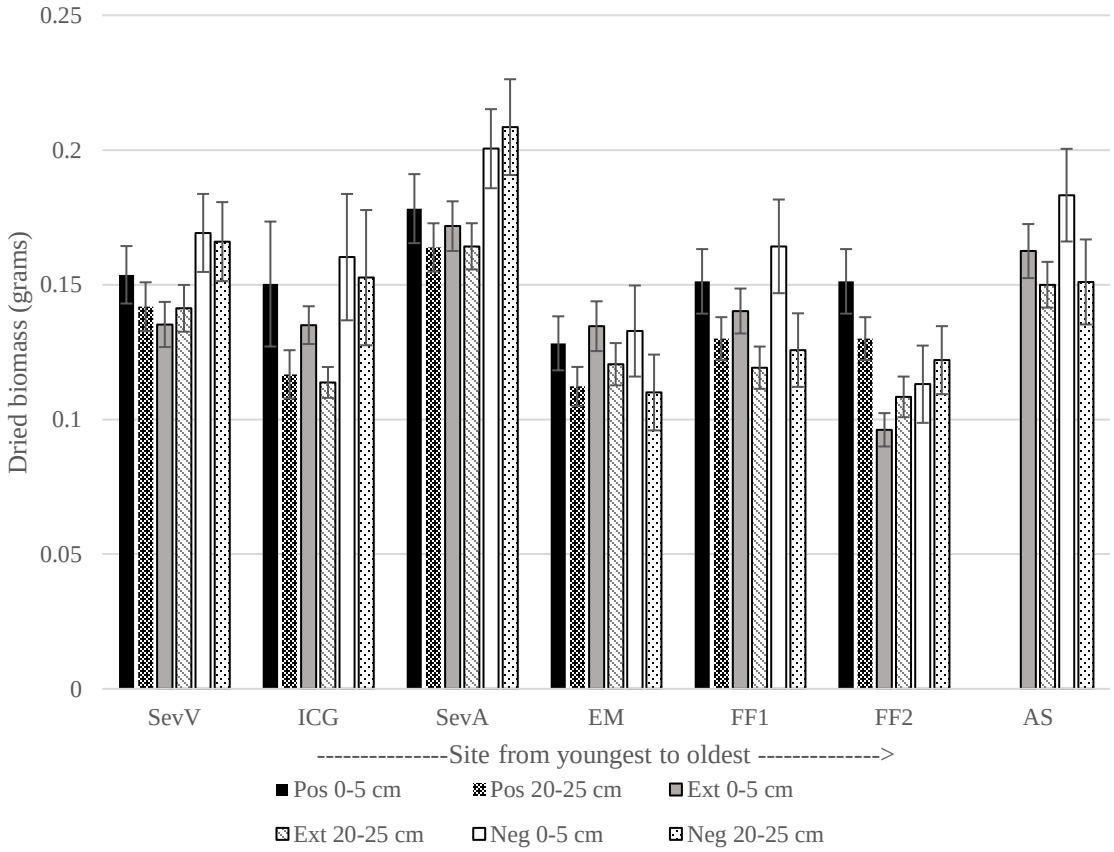

Figure S-2. Aboveground biomass separated by depths and plot type. The 0-5 depth has greater shoot biomass than the 20-25 cm depth on average.

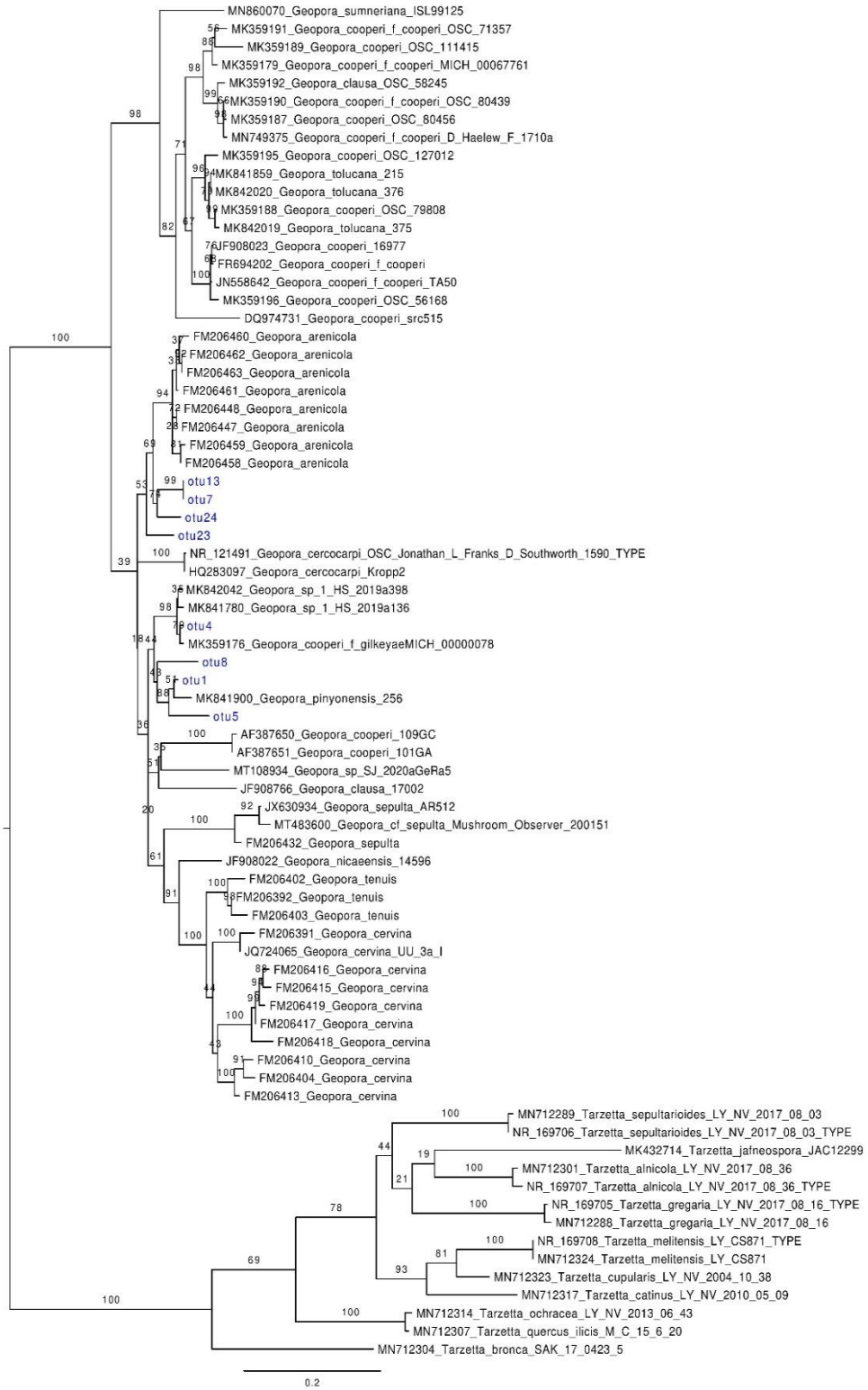

Figure S-3. Phylogenetic tree of the genus *Geopora* with OTUs from this study in blue. *Tarzetta* was used as the outgroup. Maximum-likelihood bootstrap support values from RAxML are provided above branches.

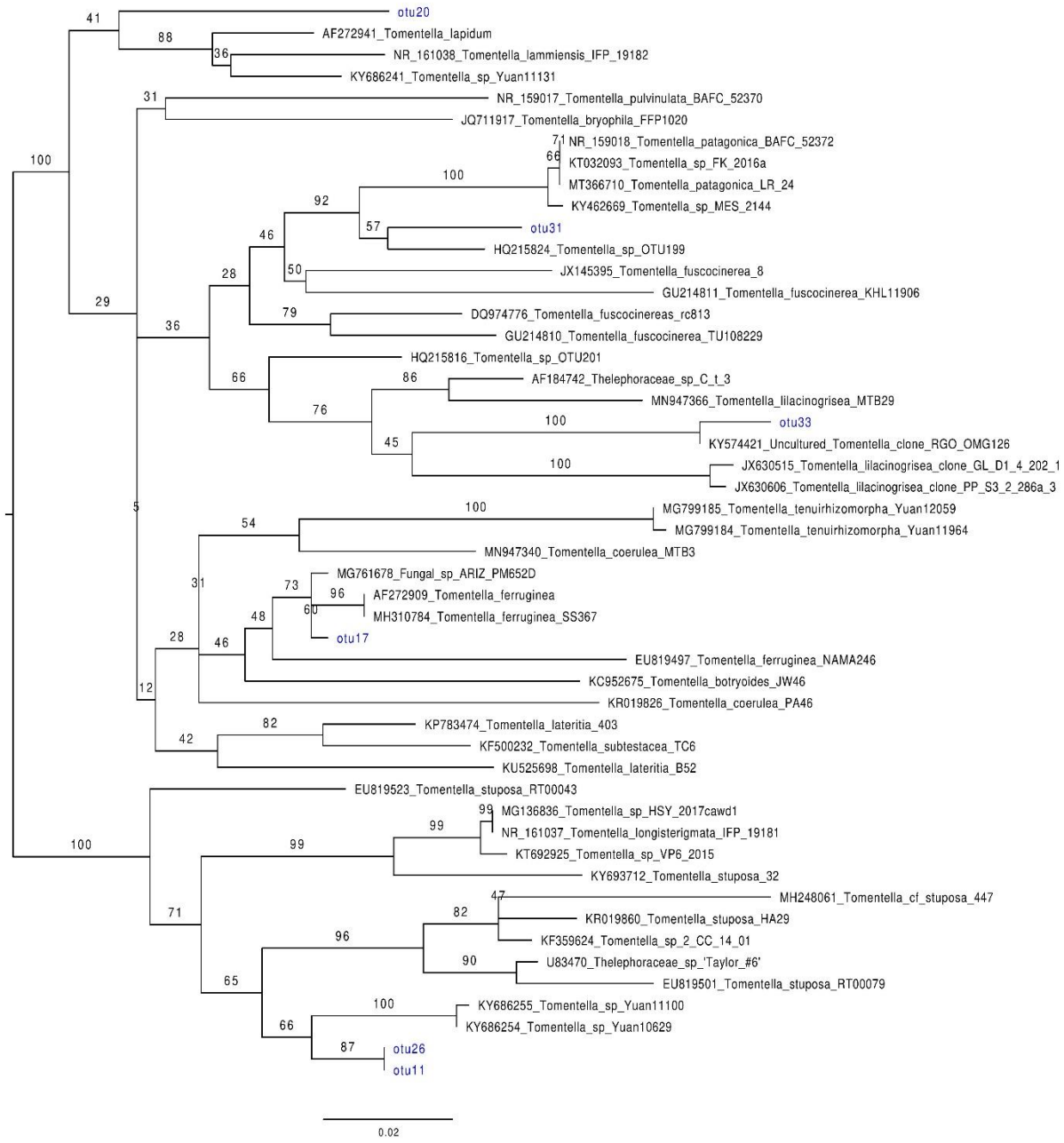

Figure S-4. Phylogenetic tree of the genus *Tomentella* with OTUs from this study in blue. The tree is midpoint rooted. Maximum-likelihood bootstrap support values from RAxML are provided above branches.

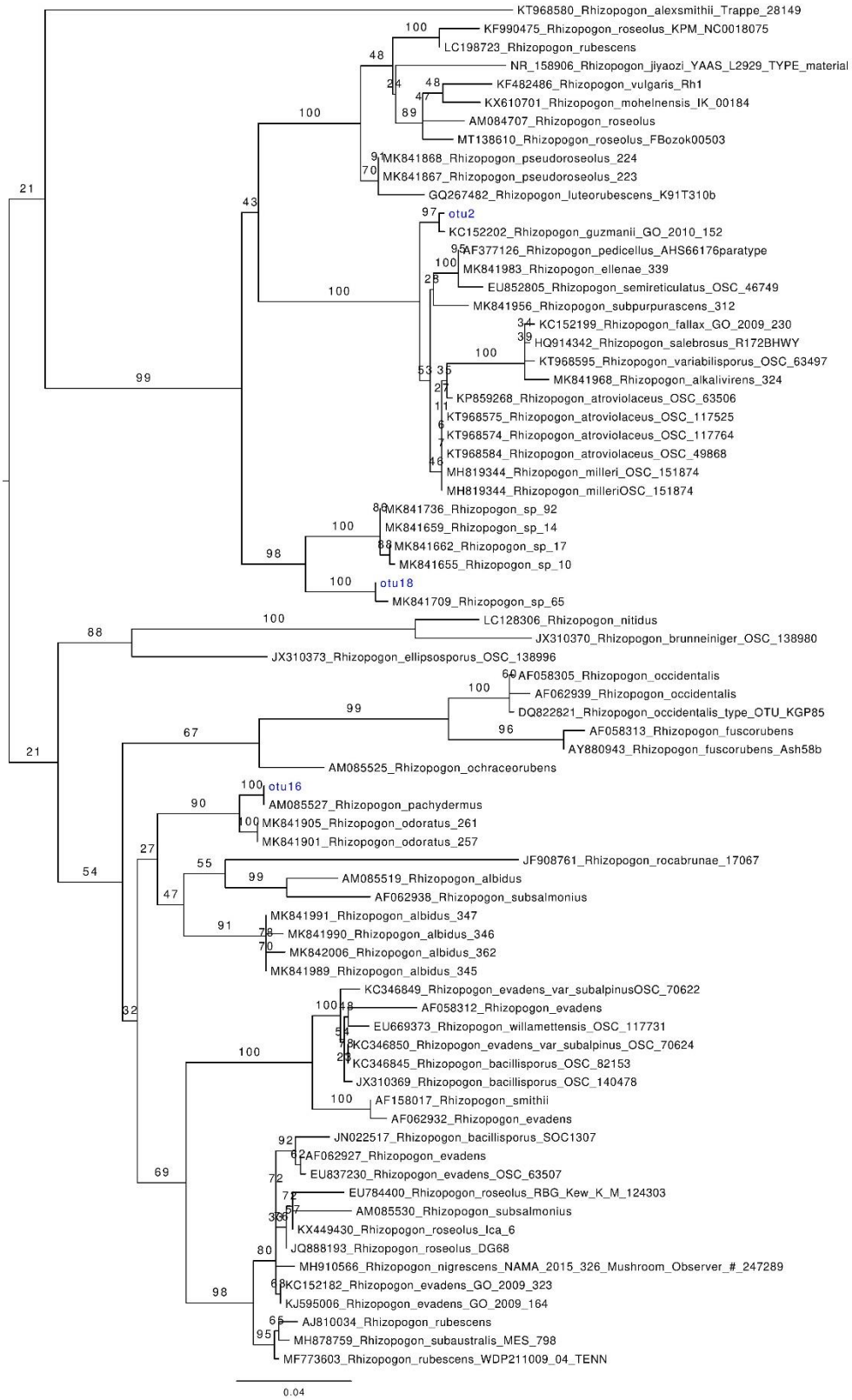

Figure S-5. Phylogenetic tree of the genus *Rhizopogon* with OTUs from this study in blue. The tree is midpoint rooted. Maximum-likelihood bootstrap support values from RAxML are provided above branches.

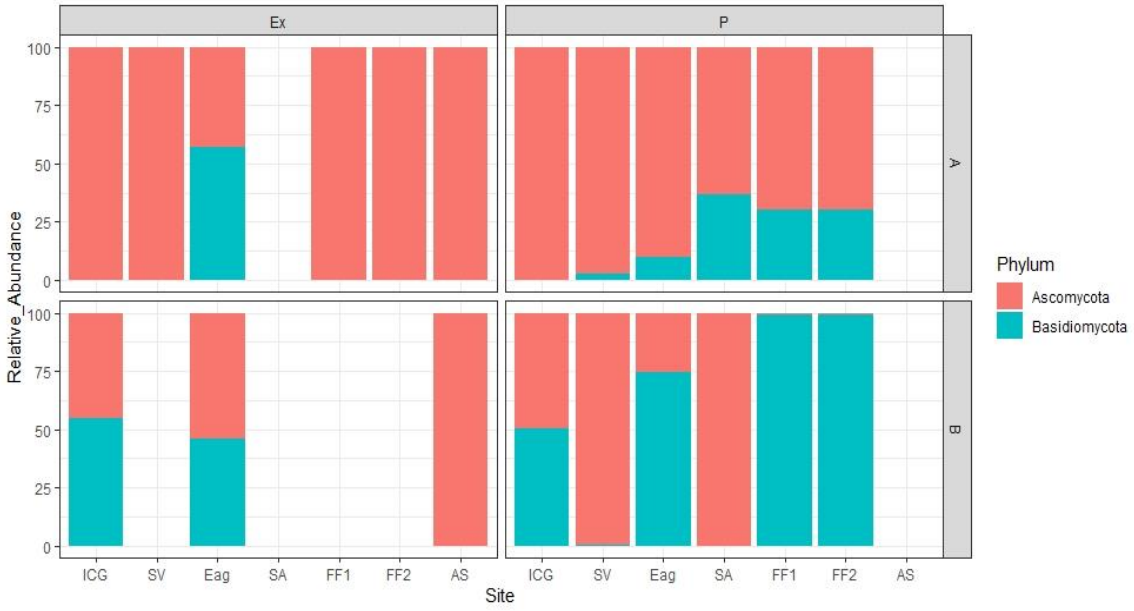

Figure S-6. Relative abundance of fungal phyla across sites and depths. The positive and extirpated plots are "P" and "Ex" respectively. Positive control soils had a greater occurrence of basidiomycete fungi as did deeper samples (B). Depths are 0-5 cm (A) and 20-25 cm (B).

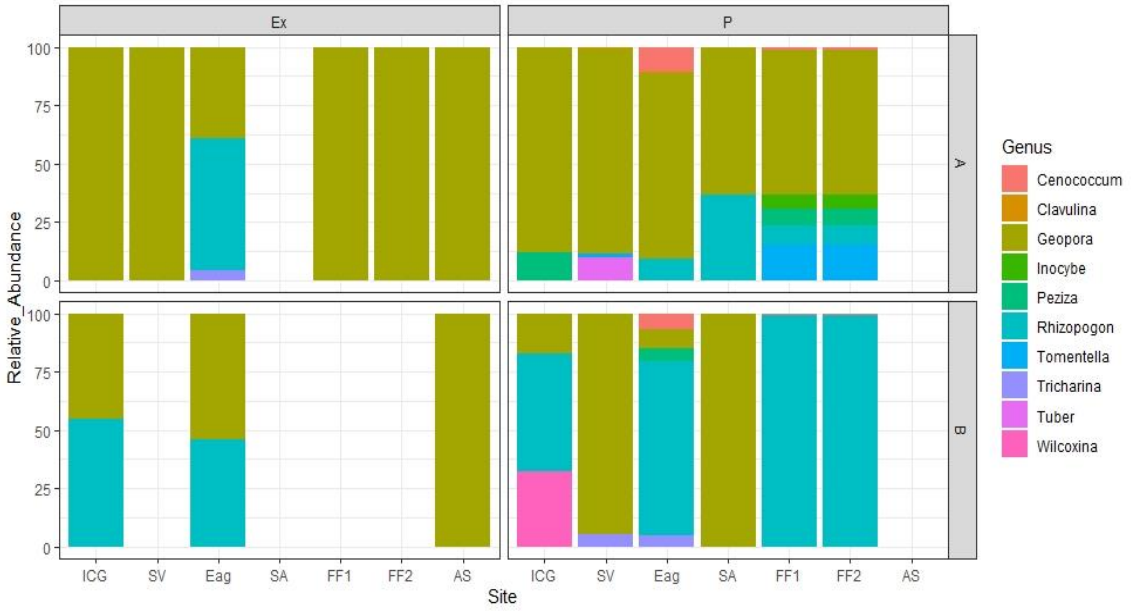

Figure S-7. Genus level relative abundance by site from youngest to oldest. Plots are extirpated (Ex) and positive (P). Depths are 0-5 cm (A) and 20-25 cm (B). Depth B had higher incidence of *Rhizopogon* while Depth A had higher incidence of *Geopora*.

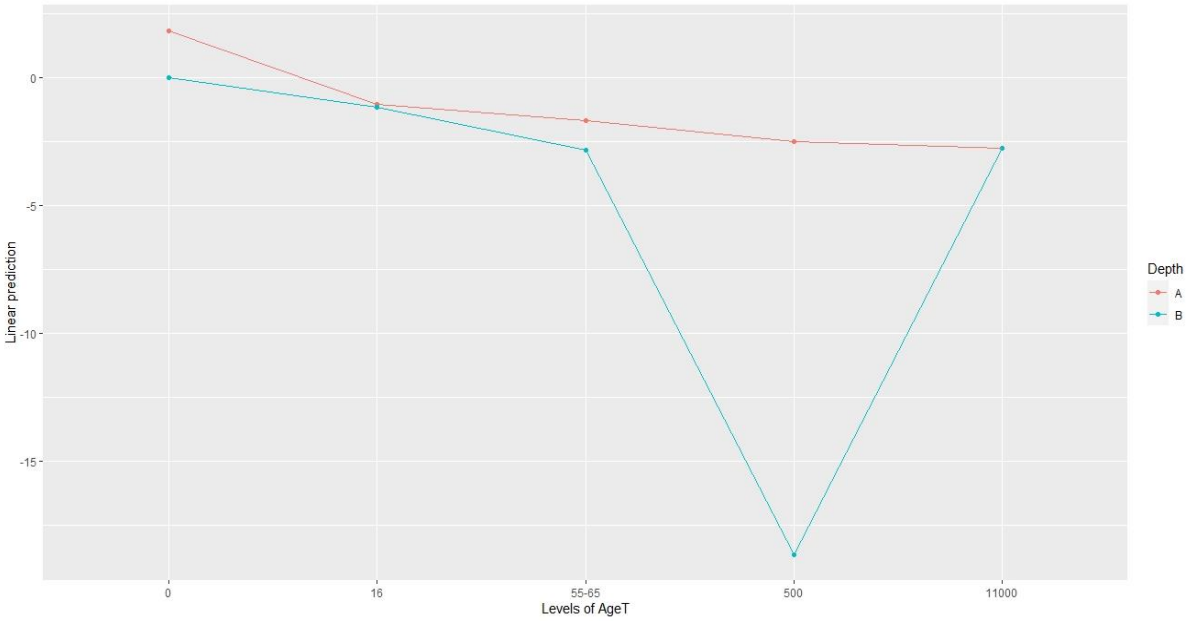

Figure S-8. A visualization of differences in intercepts for the 0-5 cm depth (A) and the 20-25 cm depth (B) in the presence/absence model. Plot age and depth had a significant interaction effect ($p = 0.0013$). Depth B in the 500-year-old plot had zero colonization which could be driving this interaction. Additionally, the low number of colonized plants from the 500 year-old plots (just five plants were colonized) could be a driver of this interaction.

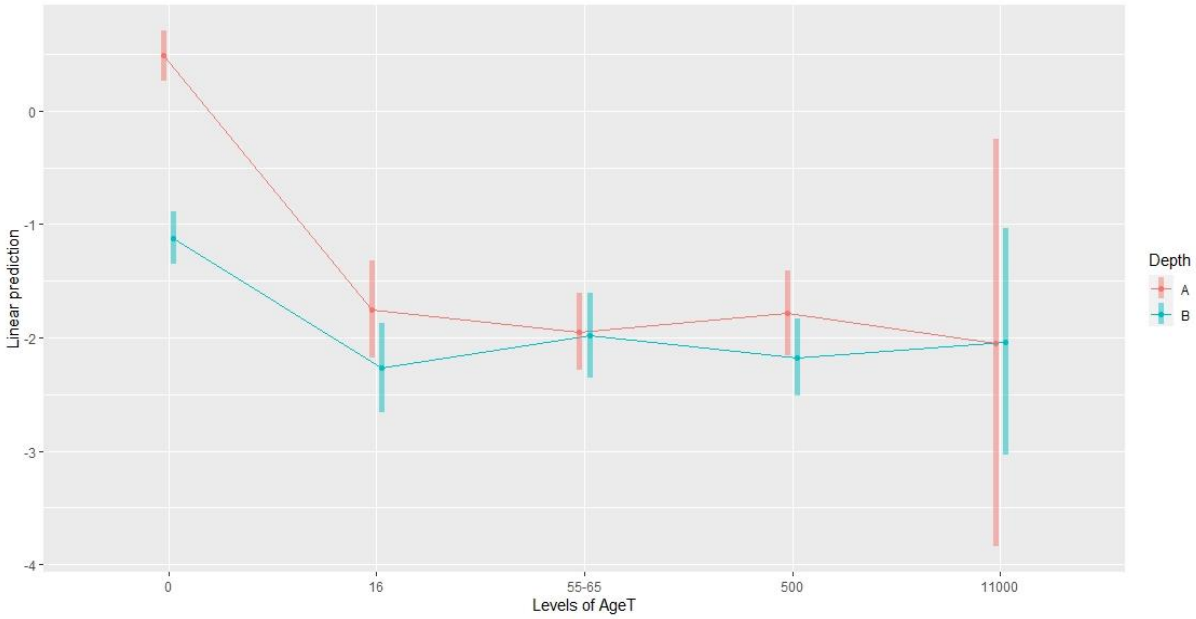

Figure S-9. A visualization of differences in intercepts for the 0-5 cm depth (A) and the 20-25 cm depth (B) in the colonization model. There was a significant interaction ($p < 0.0001$) between depth and plot age. The confidence interval for the 11,000-year plot is much larger than the other age categories. This is likely driven by the low number of colonized plants in the 11,000-year-old plot. No effect of site could also be driving this.

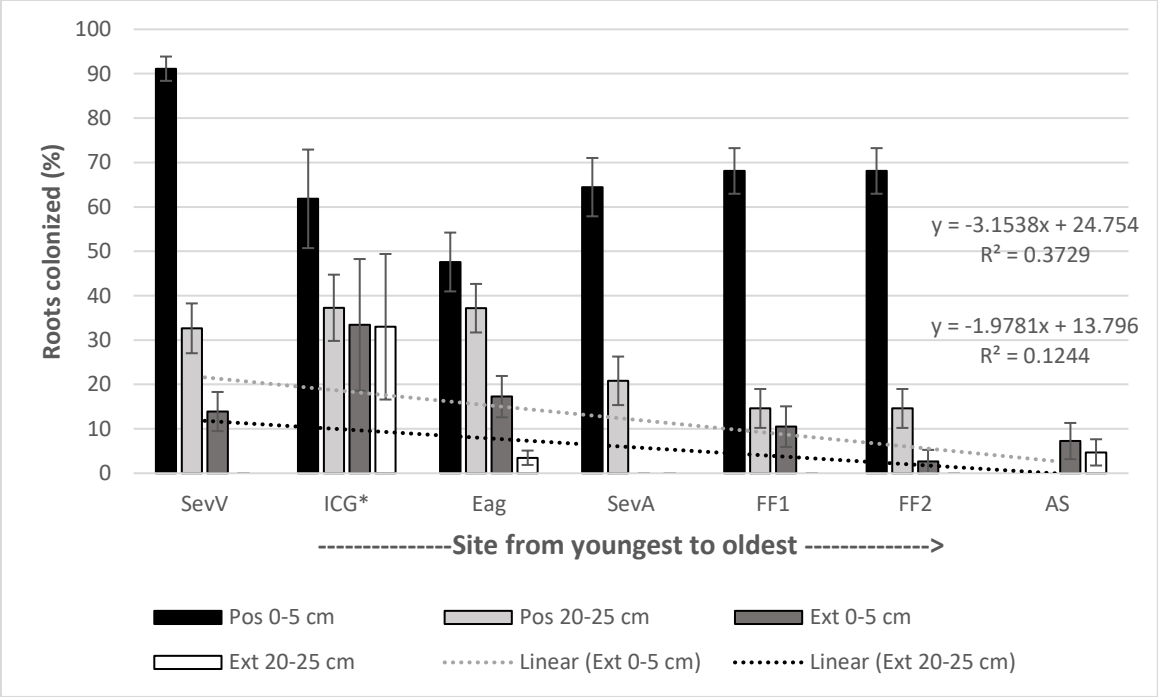

Figure S-10. Roots colonized by EMF across sites in chronological order, separated by plot type and depth. There is a non-significant trend toward decreasing inoculum potential over time following the extirpation of *Pinus edulis.* Positive plots have significantly greater colonization across sites ($p < 0.0001$) as does the shallower depth ($p < 0.0001$).

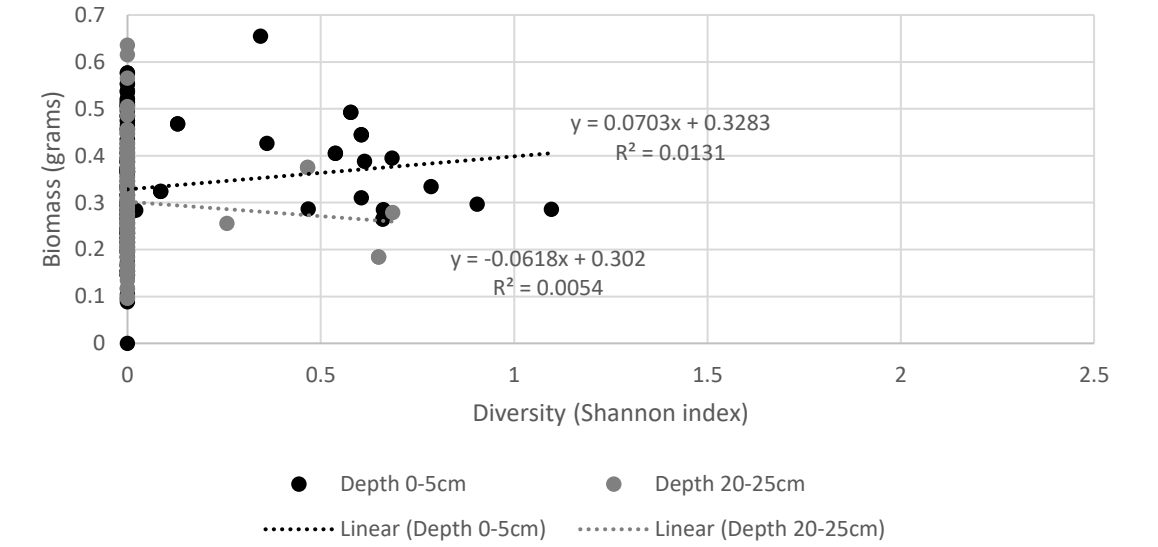

Figure S-11. Total tree biomass versus EMF diversity by depth. Black circles indicate the 0-5 cm depth and gray circles indicate the 20-25 cm depth. Diversity did not affect biomass ($p = 0.1784$).

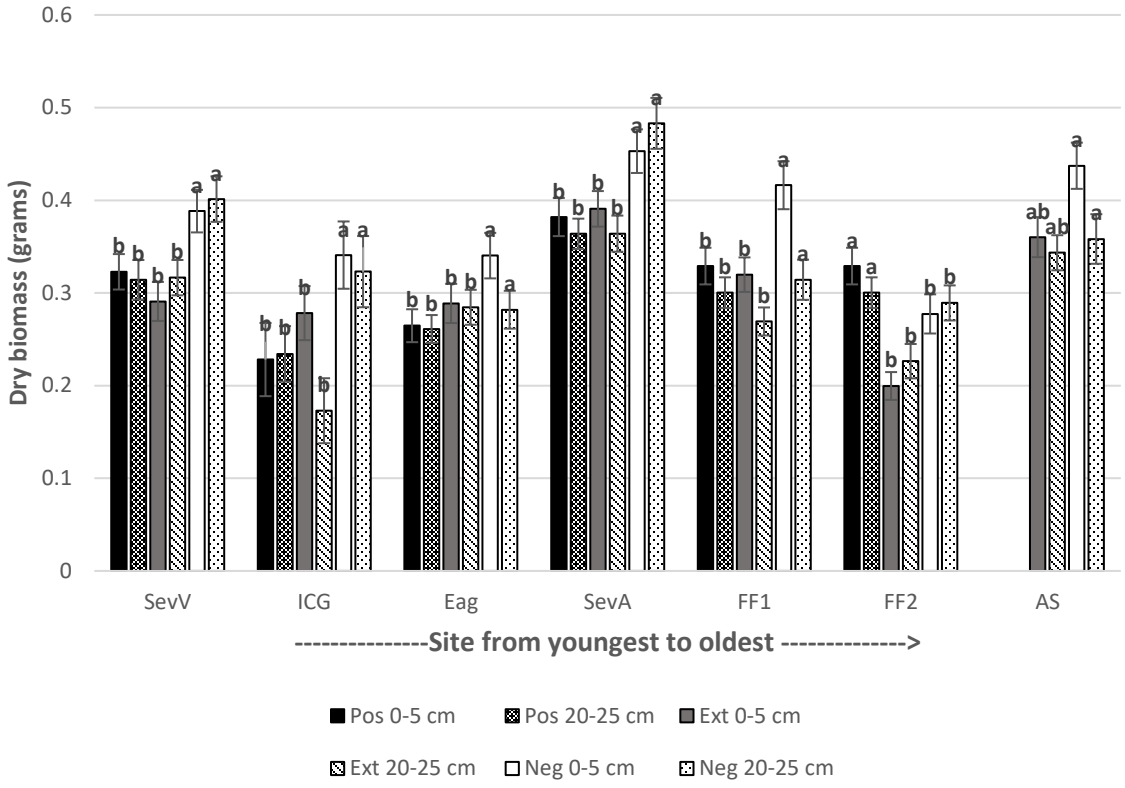

Figure S-12. Total tree biomass by site separated by depth and plot type. Age had a significant effect ($p < 0.0001$) on tree biomass. There was no significant effect of depth on biomass. Significant differences indicated by lower case letters.

Table S-1. Shannon diversity separated by site, depth, and plot type. Plot type had a significant effect on diversity. The positive plots had significantly greater richness than the extirpated plots (p = 0. 01807).

Site	Depth	Plot Type	Diversity (Shannon)
AS	A	Extirpated	0.0000
AS	B	Extirpated	0.0000
EM	A	Extirpated	1.2200
EM	A	Positive	1.2500
EM	B	Extirpated	0.9240
EM	B	Positive	1.4000
FF1	A	Extirpated	1.1400
FF1	A	Positive	1.9300
FF1	B	Positive	0.5720
FF2	A	Extirpated	0.0000
ICG	A	Extirpated	0.6620
ICG	A	Positive	1.2400
ICG	B	Extirpated	0.6890
ICG	B	Positive	1.0100
SevA	A	Positive	0.9780
SevA	B	Positive	0.4510
SevV	A	Extirpated	0.5420
SevV	A	Positive	0.4500
SevV	B	Positive	0.4340

Table S-2. Pairwise comparisons of biomass by plot age. Negative (age = infinity) control plants tended to have the greatest biomass compared to live soils. The 10-20 year and 500 year plots tended to have the smallest biomass on average. An "*" indicates a significant difference in biomass between pairs according to the Bonferroni correction.

Response variable	Pairs	P value
Aboveground biomass	Neg, Pos controls	0.0063
	Neg, 10-20 year plots	0.0003*
	Neg, 55-65 year plots	0.3035
	Neg, 500 year plots	0.0012*
	Neg, 11k plot	0.9478
	Pos controls, 10-20 year plots	0.5001
	Pos controls, 55-65 year plots	0.9992
	Pos controls, 500 year plots	0.7232
	Pos controls, 11k plot	0.9883
	10-20, 55-65 year plots	0.5920
	10-20, 500 year plots	1.0000
	10-20, 11k plot	0.6181
	55-65, 500 year plots	0.7293
	55-65, 11k plot	0.9989
	500, 11k plot	0.7090
Belowground biomass	Neg, Pos controls	<0.0001*
	Neg, 10-20 year plots	<0.0001*
	Neg, 55-65 year plots	0.0012*
	Neg, 500 year plots	<0.0001*
	Neg, 11k plot	0.2766
	Pos controls, 10-20 year plots	0.9231
	Pos controls, 55-65 year plots	0.8950
	Pos controls, 500 year plots	0.0693
	Pos controls, 11k plot	0.8817
	10-20, 55-65 year plots	0.6495
	10-20, 500 year plots	0.8610
	10-20, 11k plot	0.6016
	55-65, 500 year plots	0.0307
	55-65, 11k plot	0.9982
	500, 11k plot	0.0749
Total biomass	Neg, Pos controls	<0.0001*
	Neg, 10-20 year plots	0.0001*
	Neg, 55-65 year plots	0.0091
	Neg, 500 year plots	<0.0001*
	Neg, 11k plot	0.5077
	Pos controls, 10-20 year plots	0.8480
	Pos controls, 55-65 year plots	0.9864
	Pos controls, 500 year plots	0.2579
	Pos controls, 11k plot	0.9526

10-20, 55-65 year plots	0.7148
10-20, 500 year plots	0.9929
10-20, 11k plot	0.6436
55-65, 500 year plots	0.2153
55-65, 11k plot	0.9978
500, 11k plot	0.2666

Chapter 2

Introduction

The species *Populus fremontii*, *Populus deltoides*, *Populus angustifolia*, and hybrid *Populus* spp., commonly known as cottonwood trees, occur throughout the Southwest and Mexico as foundational trees with many ecosystem functions in riparian habitats (USDA Forest Service, 2019). The introduction of invasive species (Zavaleta, 2000), anthropogenic hydrological alterations (Rood and Mahoney, 1990; Rood and Mahoney, 1995; Friedman et al., 1998), and climate variability (Perry, et al., 2012; Rood et al., 2003; Braatne et al., 1996) is threatening the health of these riparian *Populus* forests. These factors decrease *Populus* seedling recruitment and large trees die without replacement (Braatne et al., 1996). *Populus* occurs along streams, channels, and canyons including the Rio Grande in New Mexico. Many *Populus* stands along the Rio Grande are around 75 years old and may disappear within decades because of their roughly 100-year lifespans (Loehle, 1988; USDA Forest Service, 2019). Although older trees are more vulnerable to drought and increasing temperatures, all *Populus* genotypes are drought intolerant and are at risk under the current climate regime (Monclus et al., 2006). There are several current restoration strategies that can be implemented to restore these forests, including altering water flow patterns, increasing water table levels, and removing invasive species. It is imperative to continue restoration and management research as these trees become increasingly threatened.

Economic importance

Populus has both industrial and recreational uses. *Populus* species have been utilized worldwide to make lumber, paper, and veneer (Labbé et al., 2014). More recently *Populus* has been the subject of biofuel research because of its fast growth (Sannigrahi, 2010).

Additionally, the *Populus* genus has become a model system for answering many physiological questions about woody species that could not be answered using traditional model systems such as *Arabidopsis* or rice (Jansson and Douglas, 2007). *Populus* is an ideal model system because it is fast growing, reproduces prolifically, can be easily cloned, and several species have complete genomes (Bradshaw et al., 2000). In addition to their industrial uses, *Populus* trees have become an icon of the New Mexican Bosque. City dwellers and naturalists alike enjoy *Populus* forests in recreation areas throughout New Mexico and the Southwest. Without intervention, we will lose these iconic trees.

Ecological importance

Populus forests provide many ecosystem functions for wildlife (Fig. 1). The Bosque offers an oasis for several animal species in arid lands throughout the southwestern United States and Mexico. *Populus* trees provide important food and shelter resources for many animals including beavers, porcupines, bats, and birds. *Castor canadensis*, commonly known as the American beaver, is a keystone species in riparian ecosystems because of its ability to engineer habitats (Bailey and Whitham, 2006). Beaver activity influences both plant and animal dynamics within ecosystems. Beavers prefer *Populus* and willows as a food source over non-native trees like *Tamarix* (Mortenson et al., 2008) and herbivory via beavers results in prolific production of new shoots in *Populus* trees.

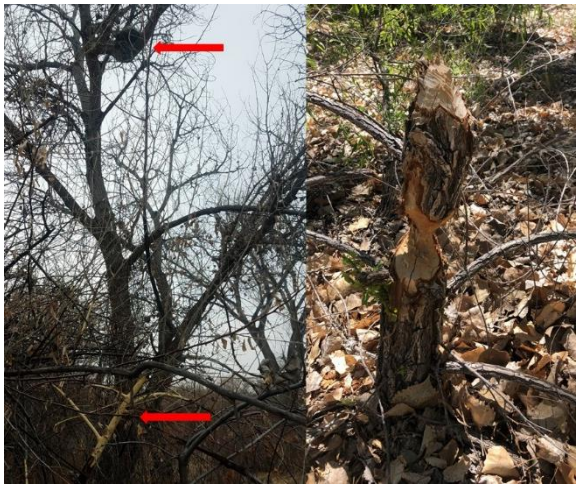

Figure 1. Left: A porcupine in a *Populus deltoides* tree with cambium herbivory below. Photo credit Gregory Reynolds. Right: Beaver herbivory on *P. deltoides* in the Rio Grande Bosque.

New sprouts provide habitat for arthropods such as sawflies, *Phyllocolpa* spp., that in turn serve as a food source to other animals (Bailey and Whitham, 2006).

Other insects depend on *Populus* as well. The cottonwood leaf beetle, *Chrysomela scripta* depends on spring buds as a primary food source after emergence (Andersen and Nelson, 2002). Additionally, abundance and richness of phreatophyte-dependent butterflies decreases with increased populations of *Tamarix* and declining populations of native *Populus* and willows (Nelson and Andersen, 1999; Nelson and Wydoski, 2008)

Numerous other vertebrates also utilize the *Populus* ecosystem. Porcupines, *Erethizon dorsatum*, feed on *Populus* by chewing wood down to the cambium during winter months (Johnson et al., 2013; Taylor, 1935) and *Populus* trees can serve as nesting sites for porcupines (Chapman and Feldhamer, 1982). Bats and birds can use *Populus* trees as their habitats as well (Valdez et al., 1999; Rumble and Gobeille, 2004). The Middle Rio Grande Valley serves as a habitat for seven species of bats (Valdez et al., 1999). Bird diversity is also supported by *Populus* riparian forests. A study documenting bird diversity in riparian and adjacent uplands found that 82% of bird species observed occurred in riparian habitats (Knopf, 1985). About 54% of observed species occurred in uplands indicating that species richness was significantly greater in riparian habitats (Knopf, 1985). A 2004 study concluded that 79% of identified bird species in riparian and surrounding grasslands occurred only in *Populus* forests (Rumble and Gobeille, 2004). Moreover, diversity and abundance of bird species was highest in seral and late seral woodlands indicating that the loss of *Populus* woodlands could negatively affect avian biodiversity (Rumble and Gobeille, 2004). Although riparian habitats make up less than 1% of western ecosystems in the United States, most bird species depend on these systems for breeding habitats (Knopf and Samson, 1994). Nearly

50% of bird species in the New Mexico Gila and San Juan Valley depend on these ecosystems for breeding (Schmitt, 1976; Hubbard, 1971). As foundational trees, *Populus* define their ecosystem and without them riparian forests may be altered for the foreseeable future.

Cottonwood biology

Populus species become sexually mature around 5-10 years of age and can live 100-200 years under ideal environmental conditions (Braatne et al., 1996; Loehle, 1988; USDA Forest Service, 2019). The trees are dioecious and both sexes flower for about 1-2 weeks during peak river flow from February through April (Karrenberg et al., 2002; Braatne et al., 1996) (Fig. 2). Seeds are produced prolifically and disperse via wind and water (Karrenberg et al., 2002; Braatne et al., 1996). Seed dispersal has evolved to take advantage of high flooding events and regeneration is dependent on disturbance via flood cycles (Karrenberg et al., 2002; Braatne et al., 1996). Flooding events clear out other vegetation and deposit alluvium, allowing the successful establishment of seedlings (Karrenberg et al., 2002) (Fig. 3). After dispersal, seeds are viable for only 1-2 weeks, creating a short window for successful germination and establishment (Braatne et al., 1996). In order to germinate, seeds must be exposed to high light and moisture (Braatne et al., 1996). Under these conditions, germination can occur within 24 hours (Karrenberg et al., 2002). The small endosperm of the seed makes it highly dependent on the success of the first cotyledons performing photosynthesis in an open canopy (Braatne et al., 1996). Seedlings are poor competitors in high vegetation areas, but they can survive inundation for 3-4 weeks

Figure 2. Sexually mature *Populus deltoides* female catkin (left) and male catkin (right). Trees are dioecious.

Figure 3. Recruitment of *Populus deltoides* seedlings on an alluvial sand bar on the Rio Grande after flooding.

making them highly adapted to flooded areas lacking vegetation (Braatne et al., 1996). These adaptations have made *Populus* a dominant tree in riparian areas (Braatne et al., 1996).

Shortly after establishment, seedlings invest most of the energy produced by photosynthesis into root growth to anchor them against future flooding events (Karrenberg et al., 2002). Additionally, seedlings and saplings have flexible branches that can withstand flooding and can re-sprout after being uprooted by floods (Karrenberg et al., 2002).

Adult trees can also reproduce asexually. Flooding may knock over older trees or break off large branches. These can then be buried and sprout new clones (Braatne et al., 1996). Exposed roots can also sprout root suckers which can be mistaken for saplings in old growth *Populus* forests (Braatne et al., 1996).

Threats

Previous literature includes comprehensive experiments looking at the effects of invasive species on *Populus* forest health, the effects of modifying natural hydrology, and impacts of climate change. These factors contribute to increased mortality of adult trees and decreased seedling recruitment and establishment.

Invasive species

Tamarix species

Tamarix species, commonly known as salt cedar, were originally introduced as ornamental species on the East Coast in the early 1800s (Brotherson and Field, 1987) (Fig. 4). In the early 1900s, *Tamarix* began to spread throughout the west in major waterways and by 1920, the ecological harm caused by *Tamarix* became clear (Brotherson and Field, 1987). *Tamarix* is now a widespread invasive species of the United States, particularly in the Southwest (Zavaleta, 2000). *Tamarix* decreases the success of native species by inhibiting populations of beneficial microbes, competing for water resources, and increasing the salinity of surface soil (Beauchamp et al., 2005; Sala et al., 1996; Cleverly et al., 2006; Zavaleta, 2000). Additionally, *Tamarix* is fast growing, produces seeds prolifically, and produces seeds longer than *Populus* species (Zavaleta, 2000; Merkel and Hopkins, 1957).

Figure 4. *Tamarix chinensis* in full flower in the Rio Grande Bosque.

Tamarix may inhibit populations of beneficial microbes associated with *Populus*. Beauchamp et al. (2005) performed a study inoculating both *Populus fremontii* and *Tamarix ramosissima* with soils from healthy adult stands of *P. fremontii* known to contain

mycorrhizal fungi. Each pot either contained *Populus*, *Tamarix,* or *Tamarix* and *Populus*. *Tamarix* had insignificant colonization by arbuscular mycorrhizae (AM), zero colonization by ectomycorrhizae (EM), and significantly high colonization by dark septate endophytes while *Populus* had high colonization from both EM and AM fungi (Beauchamp et al., 2005). These results suggest that the presence of *Tamarix* could inhibit native populations of mycorrhizae. Meinhardt and Gehring (2012) report a negative effect of *Tamarix* on *Populus* biomass that could be influenced by mycorrhizal inhibition by *Tamarix*. When grown in the field with *Tamarix*, *Populus* had less associations with EM and AM fungi and greater colonization by potential pathogens (Meinhardt and Gehring, 2012). These results support the potentially antagonistic behavior between *Tamarix* and *Populus*-associated microbial communities.

Populus may also negatively affect *Tamarix*. In pots that were inoculated with mycorrhizae and had both *Tamarix* and *Populus, Tamarix* biomass and height decreased (Beauchamp et al., 2005). However, there were no significant differences in biomass or height between inoculated with uninoculated *Populus*. This indicates that the benefits of mycorrhizae could be greater in the presence of a competitor and that these fungi could indirectly suppress the biomass and growth of *Tamarix* (Beauchamp et al., 2005). Research conducted by Bhattacharjee et al. (2009) in the Bosque del Apache uncovered how tree density affected the growth of *Tamarix* and *Populus*. This study reported that *Populus* density beyond 7-8 seedlings/0.25 m^2 negatively influenced the biomass of *Tamarix*. Furthermore, *Populus* had the highest survival rate at 10 seedlings/0.25 m^2.

Tamarix trees can constrain the success of native vegetation by competing for water resources and taking up more water in monospecific stands (Sala et al., 1996; Cleverly et al.,

2006). Additionally, unlike native phreatophytes which depend on constant contact with the water table, *Tamarix* can switch between the water table and vadose-zone water (Nippert et al., 2009). These characteristics can lower water tables and decrease perennial spring and stream flow. After the removal of *Tamarix* in mixed stands of *Tamarix* and *Populus*, Cleverly et al. (2006) recorded a 21% decrease in water loss via evapotranspiration. Without perennial access to water, *Populus* seedling recruitment decreases because water tables drop too quickly for seedlings to establish (Mahoney and Rood, 1998). Furthermore, *Tamarix* will often develop deeper roots than native species and can survive desiccation for longer periods (Zavaleta, 2000; Braatne et al. 2007). These characteristics give *Tamarix* a competitive advantage over *Populus*.

Finally, *Tamarix* trees salinate surface soils. *Tamarix* draws salts up from deeper soils and secretes them into leaves (Zavaleta, 2000). These leaves drop in the fall and increase salt content on the soil surface. *Tamarix* can survive 36,000 ppm of salt in soils whereas *Salix gooddingii* and *Populus* have thresholds around 1,500 ppm (Zavaleta, 2000). In 1995, Shafroth et al. investigated how increasing salinity affected first year survival of *Populus fremontii* and *Tamarix ramosissima*. Seeds from these species were grown at 0, 1, 3, and 5 times the mean ion concentration of soil collected from a healthy *Populus* stand in the San Marcial Bosque in New Mexico. Trees were grown from seed in outdoor pots. Germination of *Tamarix* was unaffected at all salinity levels while *Populus* germination decreased by 35% on average with increasing salinity. Thus, by making surrounding soils uninhabitable, *Tamarix* can inhibit the establishment of new *Populus* seedlings.

The combination of the above characteristics has allowed *Tamarix* to successfully encroach on native riparian habitats. *Populus* trees are less able to establish and survive without beneficial microbes, in high salinity soils, or in areas with low water tables.

Elaeagnus angustifolia

Elaeagnus angustifolia, also known as Russian olive, is an escaped ornamental tree species that now occurs across most of the western United States (Olson and Knopf, 1986). This species is particularly successful in riparian ecosystems where it is displacing native *Populus* and willows (Fig. 5).

Figure 5. *Elaeagnus angustifolia*, Russian olive, in the Rio Grande Bosque.

A study by Shafroth et al. (1995a) demonstrated the advantages *E. angustifolia* has over *Populus* species. They compared germination and establishment of *Populus deltoides* and *E. angustifolia* under various light and moisture regimes over one year. Treatments included full sun, 89% shade, and five moisture levels ranging from dry to moist. In all treatments *E. angustifolia* germinated at different times with little mortality whereas *P. deltoides* germinated in a large pulse during mid-June with a single mortality event. There were no significant differences under ideal conditions (i.e. full sun and high moisture). Under non-ideal conditions (i.e. low light, dry soil), *E. angustifolia* had higher rates of germination and establishment.

The results of this study reveal three advantages *E. angustifolia* has over native *Populus*. First, the reproductive strategy of *E. angustifolia* has greater germination than *Populus* throughout the growing season. *Elaeagnus angustifolia* seeds are also more durable

than *Populus* seeds and can persist in soil until suitable conditions emerge (Shafroth et al., 1995a). *Elaeagnus angustifolia* is therefore able to establish throughout the growing season with low seedling mortality, unlike *P. deltoides*. Second, *E. angustifolia* is shade tolerant whereas *Populus* species are shade intolerant (van Haverbeke, 1993; Shafroth et al., 1995a). Consequently, *E. angustifolia* can grow in the shade of adult *Populus* and fill the canopy gaps as older native trees fall. Third, *E. angustifolia* is not dependent on flooding and deposition (Currier, 1982), unlike native *Populus*. In fact, *E. angustifolia* can successfully establish in wetland meadows and other ecosystems where *Populus* cannot (Currier, 1982). These advantages have favored the spread of *E. angustifolia* in riparian ecosystems, especially after human alterations to the hydrological cycle.

Although the spread of non-native species is often interpreted negatively, it must be noted that *E. angustifolia* does provide some benefits to ecosystems such as providing structure for avian and arthropod wildlife. However, research has demonstrated that bird diversity is greater in native plant-dominated stands/forests such as *Populus* (Boss, 1984). Arthropod diversity is also higher in native stands than in stands dominated by *E. angustifolia* (Shafroth et al, 2010).

Anthropogenic hydrological alterations

Anthropogenic modification of rivers in the Southwest has occurred for hundreds of years to protect infrastructure, promote agricultural practices, and supply water to human populations (Swanson et al., 2011). For example, in the 1920s and 1930s the Rio Grande was modified with levees and irrigation diversions to prevent flooding and accompany rapid urban development (Swanson et al., 2011). The construction of dams and diversion channels

has greatly influenced the success and health of *Populus* forests. Several studies have observed a decrease in *Populus* populations downstream from dams (Braatne et al., 1996; Rood and Mahoney, 1990; Rood and Mahoney, 1995; Friedman et al., 1998). Dams are reducing volume and changing flow patterns in ways that can lead to drought stress for *Populus* (Rood and Mahoney, 1990). Changes in the frequency and timing of flooding events negatively impact regeneration of seedlings, which require both flooding and alluvial banks for establishment (Rood and Mahoney, 1990; Rood and Mahoney, 1995) (Fig. 6). Additionally, water tables are dropping too quickly for successful seedling establishment due to hydrological alterations (Braatne et al., 1996).

Figure 6. A 2019 flooding event in Belen, NM. Flooding events disperse *Populus* seeds and create alluvial banks for seedlings to establish. Dams and levees prevent natural flooding.

Upstream dams have caused downstream channel narrowing in the southwestern plains and decreased water flow in the north and east United States (Friedman et al., 1998). In their unaltered form, river channels experience a great deal of disturbance that benefits pioneer species like *Salix*, *Populus*, and *Tamarix*. Seedling recruitment has declined as channels narrow because available area to colonize is decreasing. The sides of channels have become more stabilized, therefore lessening the probability of channel migration. Eventually, shade tolerant species, like *Elaeagnus angustifolia*, move into adult *Populus* stands and *Populus* dies off without replacement. A study by Rood and Mahoney (1995) supports this. Their study analyzed the effects of the Tiber Dam on the Marias River in Montana. They found a decrease of *Populus*

recruitment downstream of the dam but previously established *Populus* adults were doing equally well upstream and downstream. This likely occurred because erosion and deposition were reduced by the Tiber Dam which worsened environments for seedling establishment. Additionally, nutrient rich sediment was being caught by the dam and decreasing the occurrence of downstream alluvial sand bars.

Alterations in flooding and disturbance events are also affecting *Populus* success. In 2007 Rood et al. examined the influence of disturbance via flooding, fire and ice on *Populus* ecology. Five species were used in the study: *Populus angustifolia*, *P. balsamifera*, *P. fremontii*, *P. deltoides*, and *P. trichocarpa*. All five species required some form of disturbance for establishment, but each responded differently depending on the type of disturbance. Results from this study suggest that natural disturbance likely favors native *Populus* over invasive woody species.

Lastly, the rate of decreasing water tables levels can have alarming effects on the establishment of *Populus* seedlings. *Populus* trees are phreatophytes and depend on a constant supply of ground water even as adults (Thibault et al., 2017). *Populus* seedling roots can grow up to a centimeter a day and up to 60-100 cm in their first year under ideal conditions (Mahoney and Rood, 1998). However, if water table drops exceed 2.5 cm a day, seedlings are unable to establish (Mahoney and Rood, 1998). Rates of decreasing water tables are already exceeding the necessary threshold for seedling establishment (Braatne et al., 1996). A 14-year study looking at water table depth along the Rio Grande in Albuquerque, La Joya State Game Refuge, the Sevilleta National Wildlife Refuge, and the Bosque del Apache found drought conditions during most of the study's duration (Thibault et al., 2017). Current and future drought conditions put *Populus* species at a distinct

disadvantage compared to more drought resistant invasive species like *Tamarix* (Braatne et al., 2007).

Modifications of hydrological patterns can also cause shifts in plant distribution. Drought intolerant species like native *Salix* and *Populus* will struggle as water tables drop and hydrological patterns become increasingly irregular (Perry et al., 2012). Overall, the anthropogenic inhibition of flooding and low water tables is obstructing the health of riparian forests.

Variable climate

Riparian ecosystems, particularly those in semi-arid and arid regions, are being altered by increasing CO_2 levels and temperatures caused by human-induced climate change (Perry et al., 2012). These global alterations are drastically changing the physiology of riparian trees, altering hydrological patterns, impacting animal communities, and modifying ecosystem structure and functions (Perry, et al., 2012).

As temperatures rise and droughts become longer and more severe (IPPC CHANGE, O.C., 2007), heat stress increases in native riparian trees (Perry et al., 2012). Increasing heat stress decreases rates of photosynthesis and escalates the occurrence of branch and crown die-off (Rood et al., 2003). Additionally, xylem cavitation increases and overall tree productivity decreases as water no longer can efficiently flow to leaves (Rood et al., 2003). Warming climate can also delay autumnal leaf senescence in upland *Populus* spp. and likely affects riparian species as well due to their physiological similarities (Taylor et al., 2008). Furthermore, a warming climate alters plant phenology because plants depend on environmental signals like temperature and daylight to time flowering (Bertin, 2008).

Changes in plant phenology can make it so soil resources no longer align with plants' physiological processes like reproduction, new leaf emergence, and shoot elongation (Perry et al., 2012). For example, *Populus fremontii*, *Salix exigua*, and *Salix gooddingii* will release seeds earlier with increasing temperatures which could affect dispersal of seeds (Stella et al., 2006). This could also disrupt the synchronized release of seeds with snow melt. All these physiological changes can result in shifting distributions of tree species as they attempt to colonize more suitable habitats. For example, warmer adapted species, such as *P. deltoides*, may move northward or further upstream (Perry et al., 2012). If populations cannot migrate quickly enough, they face extirpation.

Warming climate alters the timing of snow melt, total annual river flow, flooding, and late summer flow volume (Regonda et al., 2005; Barnett et al., 2008). Snow-pack is melting sooner, increasing winter flooding and pushing potential *Populus* establishment earlier in spring (Perry et al., 2012). Although *Populus* seeds are being released earlier with earlier snow melt, they are restricted by photoperiod which will eventually misalign with the timing of snow melt (Rood et al., 2008). Additionally, the Southwest is getting more of its precipitation from rain which can increase the intensity of summer floods (Perry et al., 2012). These floods could then kill seedlings that were established in the spring and new *Populus* seedlings will be unable to establish later in the season. Increased summer flooding will then favor the germination of invasive species, like *Tamarix*, that release seeds throughout the summer.

Climate change will also modify animal communities associated with riparian plant species. Canopy and shrub-nesting birds prefer native foliage whereas other species can benefit from dense *Tamarix* forests (van Riper et al., 2008; Brand et al., 2010). These shifts

in riparian tree species will also affect insect communities. For example, *Populus* leaf beetle (*Chrysomela scripta*) populations could suffer because they use temperature to time emergence (Andersen and Nelson, 2002). *Populus* trees are budding out later in the season causing beetles to emerge before their primary food source does (Andersen and Nelson, 2002). Insects that prefer *Tamarix,* like the tamarisk leafhopper (*Opsius stactogalus*), will do better than those that prefer native foliage (Perry et al., 2012). A decrease in arthropod abundance could decrease bird reproduction because of a lack of food (Bolger et al., 2005). Climate change has also been associated with amplified nest predation of birds as stressed trees provide less foliage for protection (Martin, 2007).

Finally, increasingly variable climate and altered forest structure can have dramatic effects on ecosystem function. Warming climate alters nutrient cycling (Graaf et al., 2006). For example, soil respiration and nitrogen mineralization rates increase with rising temperatures (Rustad et al., 2001). Carbon cycling is modified as litter decomposition slows down when communities shift from *Populus* litter to *Tamarix* litter which is known to be higher in lignin and can take longer to decompose (Pomeroy et al., 2000; Moline and Poff, 2008). Nitrogen cycling is also modified by the litter of *Elaeagnus angustifolia* which decomposes rapidly compared to *Populus* leaf litter and has higher levels of nitrogen (Harner et al., 2009; Moline and Poff, 2008).

The changing climate favors a shift in plant communities where native early successional trees will be replaced by herbaceous drought tolerant plants and late seral woody species (Rumble and Gobeille, 2004). This unfortunately includes non-native trees such as *Tamarix*. This can then decrease suitable habitat for associated riparian wildlife such

as birds and arthropods. The combination of these climatic effects demonstrates the need for future research.

Management and restoration

Mycorrhizal benefits

Figure 7. *Populus deltoides* root tip colonized by the EM fungus *Cenococccum* sp. An AM species would not form a visible outer sheath like this EM fungus.

Most land plants, 80% of species and 92% of families, associate with mycorrhizal fungi (Wang and Qiu, 2006). Mycorrhizae can act as an extension of the root system and more effectively explore soils for valuable nutrients such as nitrogen and water (Allen et al., 2003). *Populus* species have the rare ability to associate with both ectomycorrhizae and endomycorrhizae or arbuscular mycorrhizae (Gehring et al., 2006; Gherghel et al., 2014; Karliński et al., 2010; Lodge, 1989) (Fig. 7). *Populus deltoides*, *P. trichocarpa*, *P. fremontii*, *P. tremuloides*, *P. angustifolia*, and *P. nigra* can form associations with both EM and AM fungi (Beauchamp et al., 2005; Gehring et al., 2006; Gherghel et al., 2014; Karliński et al., 2010; Lodge, 1989; Vozzo and Hacskaylo, 1974; Neville et al., 2002). These fungi benefit *Populus* species by providing nutrients like nitrogen in exchange for photosynthates (Allen et al., 2003), increasing tolerance to heavy metals (Chen et al., 2016), and reducing the effects of pathogens (Pfabel et al., 2012). *Populus*' ability to initiate fungal relationships is influenced by abiotic and biotic conditions.

A study in 2016 examining the effects of cadmium contaminated soils found that AM fungi increase *P. deltoides* tolerance to heavy metals (Chen et al., 2016). To compare sexes, the study inoculated male and female *P. deltoides* trees with the AM fungus *Rhizophagus irregularis*. Male trees that were exposed to cadmium had significantly higher levels of colonization by AM than female trees as well as less severe reactions to toxicity. Soils that

are contaminated with lead and copper have lower mycorrhizal colonization, fungal biomass, and root tip abundance (Karliński et al., 2010). The results from these two studies suggest that plant genetics play an important role in heavy metal tolerance as well as the presence of mycorrhizal fungi (Chen et al., 2016; Karliński et al., 2010).

In addition to increasing tolerance to heavy metals, mycorrhizae may inhibit plant pathogens. Pfabel et al. (2012) investigated the effects of the EM fungus *Hebeloma mesophaeum* on *Populus* infected by the foliar rust *Melampsora*. Both the EM and rust caused an increase of tannins in leaves which demonstrated EM ability to affect secondary metabolism. However, while rust increased the plant defense hormone, salicylic acid, ectomycorrhizal fungi unexpectedly did not. In fact, the EM fungus counteracted the effects of the rust on *Populus* suggesting that EM fungi may provide a layer of defense against pathogens.

In order to benefit from mycorrhizae, successful root colonization must occur. Colonization by EM and AM is dependent on biotic and abiotic factors (Gherghel et al., 2014; Tagu et al., 2001; Karliński et al., 2010; Gehring et al., 2006). *Populus* species and hybrids vary in their ability to form EM (Tagu et al., 2001). For example, *Populus deltoides* has significantly higher colonization rates by the EM fungus *Laccaria bicolor* than *Populus trichocarpa* (Tagu et al., 2001). Furthermore, tree genetics play a role in enzyme excretion by colonized root tips. Root excretions influence rates of colonization and vary among individual *P. deltoides* x *P. trichocarpa* crosses colonized by *L. bicolor* (Courty et al, 2010).

Other biotic factors, such as the presence of fungal endophytes and bacteria, can influence colonization as well (Neville et al., 2002; Labbé et al., 2014). Colonization by non-mycorrhizal fungal endophytes is negatively correlated with EM colonization and positively

correlated with AM colonization in *Populus* (Neville et al., 2002). Other soil microbes can influence ratios of colonization. The mycorrhizal helper bacteria *Pseudomonas* can stimulate EM formation on *Populus deltoides* (Labbé et al., 2014). In a greenhouse study, *Pseudomonas* had a significant positive effect on *Laccaria bicolor* growth and mycelial architecture in 81% of *P. deltoides* clones (Labbé et al., 2014). Clones also made more secondary roots with a *L. bicolor-Pseudomonas* combination than with *L. bicolor* alone. This demonstrates the complex relationship between mycorrhizae and *Populus*.

Many other factors drive variation in AM:EM ratios. Such factors include abiotic influencers like soil depth, soil moisture, site location, history of land use, and natural history (Karliński et al., 2010; Gherghel et al., 2014). Other factors are more biotic such as host genotype and age as well surrounding litter accumulation rates (Karliński et al., 2010; Gherghel et al., 2014). Gehring et al. (2006) examined the effect of elevation on AM to EM ratios in hybrid *Populus fremontii* clones. Ectomycorrhizal colonization was higher at greater elevations (1,400-2,300 meters), whereas AM colonization decreased with elevation. Moreover, soil moisture was a driver of colonization more than host genetics in the study. Ectomycorrhizal fungi tend to do better in moist well-drained soil compared to their AM counterparts (Gehring et al., 2006; Lodge, 1989). However, colonization by AM fungi is more common with flooding or inundated soils (Lodge, 1989; Truszkowska, 1953). Finally, when EM colonization is high, AM colonization is low likely because these symbionts are antagonistic (Lodge, 1989).

In sum, *Populus*-associated mycorrhizae can provide many benefits to their host trees including tolerance to heavy metals and pathogens. The rate of colonization by mycorrhizal fungi is influenced by both biotic and abiotic factors which should be considered when

managing *Populus* forests. These factors determine the AM:EM ratio of fungi that associate with *Populus*. As temperatures and drought severity increase, these mycorrhizal communities may be important in the migration of *Populus.* Further research is required to understand how integral these fungi are to the survival of *Populus* and their use in restoring these ecosystems.

Restoration strategies

It is important to maintain healthy vegetation to improve water quality, fisheries, and aesthetics for recreation areas (Braatne et al., 1996). *Populus* restoration efforts will require a multifaceted approach including creating artificial disturbance, modifying water flow, selecting resilient plant genotypes, and invasive species management. By utilizing the results of previous studies, effective plans for managing *Populus* can be developed.

Experiments have demonstrated the importance of both disturbance and proper moisture for *Populus* regeneration (Friedman et al., 1995, Rood et al., 2007). If adult *Populus* are present, it is unnecessary to add seeds under these ideal conditions (Friedman et al., 1995). This method preserves the genetics of a given area and decreases the cost of importing cuttings. However, if irrigation is not feasible this strategy will not work. It is also important to note that not all sites will have a sufficient seedbank if adult trees are absent. Artificial disturbance such as manually removing litter or using controlled burns is highly effective in combination with irrigation (Friedman et al., 1995; Rood et al., 2007). Additionally, increasing flood and ice disturbance opens new areas for establishment (Rood et al., 2007). If possible, restoration should be synchronized with natural seed drop (Friedman et al., 1995).

For those areas with an insufficient seed bank, it should be noted that optimum germination occurs with fluctuating temperatures between 2-25°C when seeds spend eight hours at the lower temperature and sixteen hours at the highest temperature (Young and Clements, 2003). When mass producing seedlings for restoration, the sex of seedlings should be also be considered. In sites with heavy metal contamination, it would be beneficial to plant male trees inoculated with AM fungi (Chen et al., 2016).

To maintain sufficient moisture levels, it is crucial that land managers mimic historic hydrographs with either simulated or natural flooding (Bhattacharjee et al., 2009; Braatne et al., 1996; Thibault et al., 2017; Rood and Mahoney, 1990). This is especially important on dammed rivers. River flow should be slowly decreased allowing flow throughout the summer (Rood and Mahoney, 1990). By draining the water table slowly, downstream seedlings will be able to establish more effectively (Mahoney and Rood, 1998; Rood and Mahoney, 1990). During drought years instream flow should be implemented (Braatne et al., 1996).

Lastly, monitoring water table depths and removing invasive species can help ensure high enough water tables to support native vegetation (Thibault et al., 2017). This strategy will counteract the effect of invasive species like *Tamarix* and *Elaeagnus angustifolia.* We can target areas with shallower water tables and less variability in water levels for restoration to increase chances of success.

Removal of invasive species requires applying the concepts of integrated pest management in which a combination of biological, physical and chemical control is utilized (Shafroth et al., 2010). Each site will require a unique combination of restoration techniques depending on funding, invasive plant cover, water table dynamics, and the objective of land

managers (e.g. saving water, preserving avian habitat). Management of both *Tamarix* and *E. angustifolia* necessitates a multi-year plan to prevent re-infestation (Carruthers et al., 2008).

Biological control via the tamarisk leaf beetle (TLB) can be effective for managing *Tamarix* and minimizes environmental impacts because of the insect's high host specificity (Carruthers et al., 2008). The TLB causes mortality in *Tamarix* by feeding on foliage until root reserves are depleted (Hudgeons et al., 2007). If feeding does not kill adult trees, growth is still suppressed which gives native plants a competitive advantage (Fig. 8). In Lovelock, Nevada the release of TLB caused 65% mortality of *Tamarix* after five consecutive years of defoliation. However, some adult *Tamarix* trees can go dormant and resprout years later (Hudgeons et al., 2007). In these cases, the utilization of TLB needs to be in conjunction with other management practices. Success using TLB depends on the levels of herbicides being used, disturbance via flooding, abundance of beetle predators, and the initial quality of *Tamarix* foliage (DeLoach et al, 2008; Dudley et al., 2006). For example, if an adult *Tamarix* is already weak then the beetles will be unable to establish without their primary food source (Dudley et al., 2006).

Figure 8. *Tamarix chinensis* refoliation of its upper canopy after TLB herbivory. Refoliated branches appear clumpy compared to healthy branches.

Research studying potential biocontrols for *E. angustifolia* are underway. *Elaeagnus angustifolia* is a common ornamental tree so a biocontrol should target reproductive parts to preserve aesthetics within cities but stop the spread of *E. angustifolia* in surrounding ecosystems (Bean et al., 2008).

Another option for managing *Tamarix* and *E. angustifolia* is the use of mechanical controls. This typically involves first cutting aboveground biomass and then removing roots (Shafroth et al., 2010). This technique requires heavy equipment and the removal of belowground biomass can cause soil disturbance in ecosystems. One of the benefits of this technique is fast results.

Figure 9. The cut-stump method utilized on *Tamarix chinensis*. Trees are cut and painted over with an herbicide (green). Photo credit: Joey Pruitt, BLM RPFO.

Often a combination of aboveground cutting and application of an herbicide on cut stumps (known as the cut-stump method) can be effective (Fig. 9). Although the cut-stump method is very effective, it is labor intensive. Herbicides have been effective in managing both *E. angustifolia* and *Tamarix.* Applications are usually affordable, easy to apply over large areas, and they avoid soil disturbance (Shafroth et al., 2010).

Removal of non-native species can have mixed effects. Removal is beneficial to herpetofauna and has a positive impact on bat activity (Bateman et al., 2008). However, midstory avian nesters can decrease in population after the removal of invasive trees without the addition of native midstory trees (Bateman et al., 2008). With every restoration plan costs and benefits must be considered.

Conclusions

Populus trees make up the foundation of riparian habitats throughout the southwestern United States. A combination of invasive species encroachment, modification of natural water regimes, and climate variability has resulted in a decline of successful seedling recruitment and establishment. Counteracting this will be difficult and require a diverse plan of action. The most important restoration strategies will involve disturbance and perennial water flow. Removing invasive species and selecting appropriate genotypes will also contribute to the restoration of *Populus* in riparian ecosystems.

Further research is required to understand soil communities. For example, a study on soil community variation between areas with invasive species and areas where invasive species have been removed would be beneficial. Soil microbes including mycorrhizae may be helpful in restoration efforts. Additional drought studies with and without compatible mycorrhizae could shed light on their ability to mitigate effects of climate change on *Populus*. Increasing drought studies could also help predict the fate of *Populus* forests. Future research should also investigate potential biological controls for both *Tamarix* and *Elaeagnus angustifolia* to minimize adverse management effects such as soil disturbance. Long-term tamarisk leaf beetle studies throughout the Southwest could reveal more promising results for the management of *Tamarix* as well.

Definitions

Arbuscular mycorrhizae- A symbiotic relationship between plant roots and a mycorrhizal fungus where the fungal partner penetrates cortical cells and form arbuscules for nutrient exchange.

Endomycorrhizae- A symbiotic relationship between plant roots and a mycorrhizal fungus where the fungal partner exchanges nutrients with a plant host and penetrates cortical cells.

Ectomycorrhizae- A symbiotic relationship between plant roots and a mycorrhizal fungus where the fungal partner exchanges nutrients with a plant host without penetrating cortical cells.

Evapotranspiration- the movement of water via plant transpiration from soil and through plant leaves into the atmosphere.

Braided river- A river composed of multiple small channels feeding in and out of each other and separated by braid bars or temporary islands.

Inundation- Flooding caused by an overflowing river.

Meandering river- A river that curves back and forth on a sloping landscape while slowly breaking away sediment.

Phreatophyte- A deep-rooted plant that has roots in constant contact with moisture, primarily the nearest water table.

Seral- An intermediate stage in ecological succession.

Vadose-zone water- water that is between the soil surface and water table that is readily available to the biosphere of plants.

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