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Research

Legacy effects of tree mortality mediated by ectomycorrhizal fungal communities

Rebecca C. Mueller[1,2], Crescent M. Scudder[1], Thomas G. Whitham[1] and Catherine A. Gehring[1]

[1]Department of Biological Sciences and Merriam-Powell Center for Environmental Research, Northern Arizona University, 617 S. Beaver Street, Flagstaff, AZ 86011, USA; [2]Chemical and Biological Engineering Department, Montana State University, Bozeman, MT 59717, USA

Author for correspondence:
Catherine A. Gehring
Tel: +1 928 523 9158
Email: Catherine.Gehring@nau.edu

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Summary

- Successive droughts have resulted in extensive tree mortality in the southwestern United States. Recovery of these areas is dependent on the survival and recruitment of young trees. For trees that rely on ectomycorrhizal fungi (EMF) for survival and growth, changes in soil fungal communities following tree mortality could negatively affect seedling establishment.
- We used tree-focused and stand-scale measurements to examine the impact of pinyon pine mortality on the performance of surviving juvenile trees and the potential for mutualism limitation of seedling establishment via altered EMF communities.
- Mature pinyon mortality did not affect the survival of juvenile pinyons, but increased their growth. At both tree and stand scales, high pinyon mortality had no effect on the abundance of EMF inocula, but led to altered EMF community composition including increased abundance of *Geopora* and reduced abundance of *Tuber*. Seedling biomass was strongly positively associated with *Tuber* abundance, suggesting that reductions in this genus with pinyon mortality could have negative consequences for establishing seedlings.
- These findings suggest that whereas mature pinyon mortality led to competitive release for established juvenile pinyons, changes in EMF community composition with mortality could limit successful seedling establishment and growth in high-mortality sites.

Introduction

Changes in climate, including the synergistic effects of increased drought and heat stress, are a major factor driving plant mortality within temperate ecosystems (Allen *et al.*, 2010). The forests of the western United States in particular have shown increased rates of tree mortality (van Mantgem *et al.*, 2009; Allen *et al.*, 2010), leading to dramatic changes in plant community composition (Mueller *et al.*, 2005; Anderegg *et al.*, 2012). Several studies have examined the physiological drivers of mortality in trees experiencing drought (Adams *et al.*, 2009; Allen *et al.*, 2010; Choat *et al.*, 2012) and the consequences of tree mortality for ecosystem processes (Šturšová *et al.*, 2012; Anderegg *et al.*, 2015). However, there has been less emphasis on the biotic legacies of drought that may influence ecosystem recovery (Classen *et al.*, 2013; Peltier *et al.*, 2016). Biotic interactions, such as facilitation and seed dispersal may shape ecosystem trajectories following tree mortality (Simard & Austin, 2010; Redmond *et al.*, 2012, 2015; Redmond & Barger, 2013). For example, recruitment of pines following mortality in the southwestern USA was positively associated with the presence or cover of shrubs and junipers that appeared to function as nurse plants (Redmond & Barger, 2013; Kane *et al.*, 2015; Redmond *et al.*, 2017).

Plant–microbe mutualisms, such as mycorrhizas, also may influence ecosystem recovery following tree mortality (Simard & Austin, 2010). Mycorrhizas are mutually beneficial associations between fungi and the roots of many plant species in which plants exchange fixed carbon for soil resources obtained by fungi (Smith & Read, 2010). Associations with mycorrhizal fungi can improve plant growth and survival, protect plants from herbivory and disease, and improve plant tolerance of abiotic environmental stresses including drought (Mohan *et al.*, 2014). However, mycorrhizal fungi also can be affected by drought stress itself, the pest outbreaks associated with drought and by host tree mortality, resulting in changes in their abundance, diversity and/or species composition (Swaty *et al.*, 2004; Mueller & Gehring, 2006; Karst *et al.*, 2014).

Living host trees can be important sources of ectomycorrhizal fungal (EMF) inoculum for seedlings, but mature trees may be absent or rare following tree mortality, which often falls disproportionately on mature trees (Mueller *et al.*, 2005; van Mantgem *et al.*, 2009). Lack of ectomycorrhizal inoculum in grasslands limited colonization by exotic pines (Nuñez *et al.*, 2009), a phenomenon also observed in other ecosystems (Thiet & Boerner, 2007).

Seedlings closer to congeneric adults had higher EMF colonization than those near non-EMF trees, and the amount of EMF inocula declined with increasing distance from either forest edges (Dickie & Reich, 2005) or living host trees (Teste & Simard, 2008). If living host trees were rare, regenerating

seedlings would instead rely on spores or other propagules as sources of EMF inocula. Despite producing small, wind-dispersed spores, many EMF taxa show strong evidence of dispersal limitation (Peay *et al.*, 2010, 2012). The longevity of EMF spores is poorly understood, although some taxa, such as members of the genus *Rhizopogon*, are long-lived (Bruns *et al.*, 2009; Nguyen *et al.*, 2012). However, EMF spore banks represent a small portion of the EMF community observed on mature trees (Glassman *et al.*, 2015). The consequences of this limited species pool for seedling regeneration following host mortality are largely unknown.

In the present study, we explored the role of living or dead conspecifics and EMF in the potential recovery of pinyon pine (*Pinus edulis*) following drought-induced tree mortality. Aerial surveys conducted by the United States Forest Service revealed substantial *P. edulis* mortality over 12 000 km^2 of the southwestern United States following extreme drought in 2002–2003 (Breshears *et al.*, 2005). Mortality of *P. edulis* exceeded 70% in some stands and was substantially greater for adult than juvenile trees, shifting the age structure of many populations towards dominance by nonreproductive trees (Mueller *et al.*, 2005). Recovery of areas with high pinyon mortality is dependent upon both the continued survival of existing plants and the recruitment of new seedlings (Kane *et al.*, 2015).

Ectomycorrhizal fungi may play a significant role in the re-colonization of high-mortality sites because seedling performance can be dependent upon the abundance and community composition of EMF (Gehring & Whitham, 1994; C. A. Gehring *et al.*, 2017). High levels of pinyon mortality are likely to alter EMF inocula because pinyon is the only ectomycorrhizal host in many pinyon-juniper woodlands; the co-dominant tree species, *Juniperus monosperma*, associates with arbuscular mycorrhizal fungi, as do associated shrub species (Haskins & Gehring, 2005; C. Gehring *et al.*, 2017). Due to limited sporocarp production in this semi-arid environment, mature trees may be the primary source of inocula for establishing pinyon seedlings (Gehring *et al.*, 1998), as evidenced by a positive correlation between the amount of EMF inocula and the density of living pinyons within a site (Haskins & Gehring, 2005). However, sites that have experienced EM host mortality may differ from sites that have historically had few EM hosts such as those dominated by junipers. Previous EMF hosts may leave a legacy of EMF inocula in the form of long-lived propagules (Kjøller *et al.*, 2012).

We examined the impact of *P. edulis* mortality on the growth and survivorship of established juvenile pinyons and quantified the potential for mutualism limitation of seedling establishment via altered EMF communities. We compared the performance of seedlings and the EMF communities of the soil associated with living vs dead conspecifics at two scales: the tree scale, which in our focus was the understory of trees within a stand, and the stand scale in which we compared stands in the same area that differed in degree of mature *P. edulis* mortality. We also inoculated *P. edulis* seedlings with the EMF communities of stands that varied in living *P. edulis* density to determine if changes in EMF influenced seedling performance. We hypothesized that: (H1) Juvenile pinyons that established before drought would perform better beneath a dead conspecific than a living conspecific as a result of competitive release (Redmond *et al.*, 2017); (H2) The soil associated with living pinyons would have higher EMF abundance and species richness and altered species composition relative to the soil of dead pinyons at both the tree and stand scale because inoculum beneath dead trees will be limited to long-lived EMF propagules; (H3) The EMF community of stands with high pinyon mortality would be less beneficial for *P. edulis* growth than the EMF community of stands with high numbers of living *P. edulis*. Because the altered EMF communities associated with dead pinyons would lead to reduced EMF function. Given the high likelihood of increased drought in the intercontinental US (Seager *et al.*, 2007; Garfin *et al.*, 2013), and the predicted impacts of drought on forests world-wide (Allen *et al.*, 2010), understanding the forces that drive tree re-establishment in high-mortality sites could suggest management strategies that promote ecosystem recovery.

Materials and Methods

Study sites

This study was conducted within pinyon–juniper woodlands north of Flagstaff, AZ, USA from 2002 to 2007 using sites established to measure the impact of two extreme drought years (1996 and 2002) on tree mortality (Mueller *et al.*, 2005). Cumulative pinyon mortality following both droughts was 72.2% across the region (Mueller *et al.*, 2005). The Palmer drought severity index, a measure of dryness based on precipitation and temperature, averaged −4.98 for 2002, −3.65 for 2003, −2.67 for 2004, 3.77 in 2005, −3.40 in 2006 and −3.94 in 2007 (www.noaa.gov). Negative values indicate drought, with values between −3 and −4 considered severe drought. Positive values indicate wetter than normal conditions.

Previous studies indicate that seedling establishment is dependent upon association with established vegetation, often a mature conspecific, and access to EMF inoculum. To determine if conspecific nurse plant mortality affected juveniles, we compared stem growth of juveniles associated with living and dead conspecifics before and after drought. To quantify the effects of overstory mortality on EMF inoculum and seedling performance, we conducted several bioassays, two focused on individual trees, and two focused on stands that differed in pinyon mortality (Table 1). Ideally, our study would also include measures of EMF on established juveniles; however, we did not sample juveniles because collecting the necessary roots may have resulted in juvenile mortality.

The effect of overstory mortality on established juvenile pinyons: Hypothesis 1

We documented juvenile *Pinus edulis* survival following mature *P. edulis* mortality by using data on juvenile pinyons associated with living or dead mature pinyons collected in 2002 from six high-mortality sites covering 85 km^2 of northern Arizona (Mueller *et al.*, 2005). Across these sites, all juvenile pinyons

Table 1 Overview of studies used to test Hypotheses 1, 2 and 3 (H1–H3).

	Hypothesis	Scale	Type	Duration	EMF measures
Field study	H1	High-mortality sites	Survey	6 yr	None
Experiment 1	H2	Tree: living and dead; Site 1	Inoculum potential	6 months	Colonization, composition
Experiment 2	H2	Tree: living; Site 1	Inoculum potential	6 months	Colonization
Experiment 3	H2	Stand: sites 1 and 2	Inoculum potential	5 months	Colonization, composition[1]
Experiment 4	H2, H3	Stand: Site 1	Growth response	9 months	Colonization, composition, function

[1]Ectomycorrhizal fungal (EMF) composition and species richness data were collected only at Site 2.

(basal trunk diameter (BTD) < 3.0 cm) were noted as living or dead and associated with a living pinyon or a pinyon that died during the 1996 drought. The percentage mortality of juveniles associated with living and dead pinyons was compared using a Pearson's chi-squared goodness-of-fit test. All statistical tests were performed using the statistical platform R (R Core Team, 2016) primarily using the package VEGAN (Oksanen *et al.*, 2016).

The remainder of the study took place primarily at one of the sites described by Mueller *et al.* (2005) located at 35.47194°N, −111.62916°W (Site 1), with soils classified as Typic Argiustolls (Miller *et al.*, 1995). This site experienced two successive mortality events allowing us to examine both the immediate and longer-term impacts of overstory mortality on the microclimate experienced by associated pinyon juveniles, as well as their growth. Drought-induced mortality was higher in larger pinyons, leading to a patchwork of living and dead pinyons, including juvenile trees associated with living and dead mature trees (Mueller *et al.*, 2005). We selected 10 mature pinyons in the following three groups: living (live), died during the drought of 2002 (2002 dead) and died during the drought of 1996 (1996 dead). We were able to differentiate the two classes of dead pinyons based on tree characteristics and information from mortality surveys conducted in 2002. Each study tree had an associated juvenile that was at least 8 yr old (quantified using bud scars) to ensure that it had established before the 1996 drought. The height of juveniles at the onset of the study averaged 44 cm and did not differ significantly among treatments ($F_{2,27} = 0.10$, $P = 0.90$), indicating similar ages.

We characterized the microclimate of the understory of the three treatment groups by measuring photosynthetically active radiation (PAR; $\mu mol\ m^{-2}\ s^{-1}$), soil moisture, soil temperature, and litter depth. We measured PAR at the four cardinal directions using a Li-Cor LI-250 light meter (Li-Cor Biosciences, Lincoln, NE, USA), soil temperature to a depth of 13 cm using a Barnant type K thermometer (Barnant Co., Barrington, IL, USA), and soil moisture to a depth of 15 cm using time domain reflectometry with a Soilmoisture Equipment Corp. (Goleta, CA, USA). We measured litter depth to the nearest cm using a ruler. We measured soil moisture, temperature and litter depth on the east and west aspect of focal mature trees midway between the dripline and trunk of the tree. Measurements were made in August 2004, April 2005 and August 2005 and compared using repeated measures ANOVA. Due to variable cloud cover in August 2004, PAR was measured only in 2005.

In order to examine the impact of mature tree mortality on juvenile growth, we measured stem growth from 1999 to 2005 of established juvenile pinyons located in the understory of the living, 2002 dead and 1996 dead mature pinyons using bud scars to delineate years. Differences in stem growth were compared before (1999–2002) and after (2003–2005) tree mortality from the 2002 drought using repeated measures ANOVA.

Effects of overstory mortality on EMF inoculum – glasshouse Experiment 1: Hypothesis 2

We measured the amount of EMF inocula in the understory of living, 2002 dead and 1996 dead *P. edulis* using a Mycorrhizal Inoculum Potential (MIP) bioassay as in Haskins & Gehring (2005). In January 2005, soil cores (15 cm depth × 6 cm width) were taken on the east side of the above study trees midway between the dripline and trunk and transferred into 600-ml pots. At the time of soil collection, the 1996 dead pinyons had been dead for 9 yr and the 2002 dead pinyons had been dead for 3 yr. Soil cores were taken ≥ 1 m from any associated understory pinyon seedlings to limit the likelihood of collecting inoculum associated with a living juvenile. Pinyon seeds from a general collection from northern Arizona were surface-sterilized in 10% bleach for 20 min and rinsed with distilled water. Viable seeds of similar weight (0.24–0.29 g) were planted into pots in a glasshouse under 16 h : 8 h, light : dark cycles and watered daily until germination and weekly following seedling emergence. To determine the level of airborne EMF contaminants in the glasshouse, seeds also were planted into steam-sterilized soil (two successive nights at 95°C). Seedlings were grown for 6 months and then harvested to quantify EMF colonization as in Gehring & Whitham (1991). Levels of EMF colonization on seedlings planted in sterile soils were low with most seedlings having no colonization and one having a colonization of 5%, indicating minimal airborne contaminants. Due to seedling mortality, final sample sizes were seven, four and five for living, 2002 dead and 1996 dead, respectively. To determine EMF community composition, all living EMF tips were categorized into morphological groups (morphotypes) as described by Horton & Bruns (1998). DNA was extracted from representatives of each morphotype from each seedling using a DNEasy 96 well plant kit (Qiagen) according to the manufacturer's instructions. The internal transcribed spacer (ITS1-5.8S-ITS2) region of the fungal genome was amplified with PCR using the ITS1F and ITS4 primer pair (Gardes & Bruns, 1993). Preliminary analyses were done with restriction fragment length polymorphism (RFLP) using restriction enzyme digestion with *Hin*fI and *Mbo*I (Gehring *et al.*, 1998). Representative RFLP types were sequenced from the forward and reverse primer using an Applied Biosystems Inc. (Foster

City, CA, USA) 3730xl in the Environmental Genetics and Genomics laboratory at Northern Arizona University. Consensus sequences were constructed using CAP3 (Huang & Madan, 1999) and compared to sequences in the GenBank database using BLASTN (Altschul *et al.*, 1990). Sequences not submitted previously were deposited in GenBank under accession nos. MK053653–MK053655. The community similarity of seedlings growing in soil from living, 2002 dead and 1996 dead trees was calculated using Bray–Curtis based on relative abundance and visualized using a nonmetric multidimensional scaling (NMS) ordination. Differences among groups were determined using PERMANOVA (Anderson, 2001). Taxonomic richness among groups was compared using one-way ANOVA.

Spatial distribution of EMF inoculum – glasshouse Experiment 2: Hypothesis 2

Experiment 1 compared EMF inoculum beneath living and dead pinyons but did not assess the spatial distribution of inoculum associated with living trees. We examined the spatial extent of EMF inoculum by quantifying the MIP inside and outside the rooting zone of five mature living pinyons. We estimated the rooting zone based on root system exposure studies as the distance from the trunk to the canopy extended out from the dripline (C. A. Gehring, pers. obs.). We took four soil cores (15 cm depth × 6 cm width) inside and outside the rooting zone for each tree. Target trees were located ≥ 200 m from other pinyons to limit collection of inocula from nontarget trees. Seedlings were grown and EM colonization measured as in Experiment 1 and compared between canopy locations using a Student's *t*-test. We were unable to perform molecular analyses so no data on EMF community composition are presented.

EMF inoculum potential, community composition and seedling growth at the stand scale – glasshouse Experiments 3 and 4: Hypotheses 2 and 3

Studies of individual trees provide information about EMF dynamics following tree mortality, but measurements at larger scales are necessary given the variability in *P. edulis* mortality across the landscape. We examined the effect of pinyon mortality on EMF inoculum potential, EMF community composition and pinyon seedling growth at the stand scale by conducting two experiments using soil from four stands of *P. edulis* that differed in mature pinyon mortality and thus living pinyon density (Exact values given in Table 3). The four stands were located on flat terrain within a 2 km^2 area near the site used for Experiments 1 and 2 (Site 1). We established 50 × 50 m plots within each stand and measured the basal trunk diameter of each living and dead *P. edulis* to calculate pre-mortality and post-mortality pinyon basal area. Because ecosystem recovery depends upon the reproductive potential of surviving trees, we counted the number of mature female cones on all living *P. edulis* in each plot. We then estimated the total cone production in each plot by multiplying the number of living mature pinyons (BTD > 2 cm) by the mean number of cones per tree. The number of cones was square-root transformed and analyzed among stands using a one-way ANOVA. Stand measurements and glasshouse Experiment 3 also were conducted at a site located on poorly developed, 55 000-yr-old cinder soils, classified as Typic Ustorthents (Miller *et al.*, 1995) adjacent to Strawberry Crater in northern Arizona (Site 2) (Supporting Information Table S1).

The first experiment that used soil from these stands, Experiment 3, was an MIP bioassay similar to the ones described above except that it compared the EMF colonization of pinyon seedlings grown in soil cores collected haphazardly from the four stands of *P. edulis* described above ($n = 12$ per stand) at Sites 1 and 2 in October 2006. We followed the methods for Experiment 1 for soil collection, seed planting, seedling growth, seedling harvest and measurements of EM colonization. We used seeds of similar size collected from trees in the area; seeds from all maternal trees were included in all treatments. One seedling died in soils from Site 1 and five seedlings did not germinate in soils from Site 2, resulting in sample sizes of 47 and 43 seedlings, respectively. Percentage EM colonization was compared among seedlings grown in soil from the four stands per site using a one-way ANOVA. EMF community composition data were collected in association with this experiment from Site 2 but not from Site 1. Instead, EMF community data were collected from Site 1 in conjunction with Experiment 4 described below.

Although MIP studies examine the EMF inoculum present in soils, they are too short to examine the effects of varying inocula on seedling performance. To determine if stand-level pinyon mortality affected EMF community structure and seedling performance, we conducted a longer-term study (9 months), in which pinyon growth and EMF communities were measured on seedlings grown in sterile soil inoculated with soil from the four stands of *P. edulis* from Site 1 and compared to sterile inoculated controls (Experiment 4). This longer study allowed us to examine the effects of the EMF community on seedling performance, as opposed to inoculum bioassays, that aim to quantify the EMF community present during seedling establishment. Background soil for the experiment consisted of mixed soil from all four stands that was steam-sterilized twice. We collected soil cores from each stand as described previously and a portion of a core (*c.* 25 g) was added as inoculum *c.* 7 cm below the surface of 1-l pots at the time of planting. Soil was collected in July 2007, 5 yr after tree mortality. We used seeds collected from the local area as in Experiment 3. An additional group of seedlings served as sterile-inoculated controls. Seeds were planted directly in the steam-sterilized field soil mix. We used a small amount of soil inoculum in pooled soil for this experiment to reduce the possible effect of differences in soil chemical and physical properties among the four stands, and to more clearly determine the influence of the EMF community on seedling growth. Replication was 12 seedlings per inoculum source except for the sterile-inoculated treatment, which had five seedlings. We watered seeds and seedlings daily until establishment and then every 3 d. We harvested seedlings after 9 months of growth under a 16 h : 8 h light regime and

measured their EM colonization and community composition as in Experiment 1. Seedlings grown in the sterile-inoculum treatment had either no EMF colonization ($n = 4$) or 2% colonization by EMF ($n = 1$). Data on total seedling biomass were obtained by drying seedlings at 70°C for 48 h and weighing them to the nearest 0.01 g. We expressed seedling growth as microbial growth response (MGR), calculated as the natural log of the biomass of seedlings inoculated with live soil divided by the average biomass of seedlings grown in sterile soil (Johnson *et al.*, 2015). Values of MGR > zero indicate that inoculum positively affected seedling growth. One seedling died during the experiment, leaving $n = 47$ for the growth study, excluding sterile controls. EMF measurements were made on 10 seedlings per group, but insufficient molecular data were obtained from four seedlings, resulting in a sample size of 8–10 per group. Data on percentage colonization by EMF, EMF species richness and MGR among sites were analyzed using one-way ANOVA. As above, EMF community similarity was measured using Bray–Curtis on relative abundance data, visualized with NMS and compared with PERMANOVA. Analysis of EMF community data indicated a decline in the abundance of *Tuber* in stands with higher mortality so we tested for an association between *Tuber* relative abundance and seedling biomass using linear regression.

Results

Effect of overstory mortality on established juvenile pinyons – Hypothesis 1

Across the six sites, the mortality of juvenile pinyons associated with mature pinyons that died during the 1996 drought did not change significantly during the subsequent drought of 2002. Mortality of juvenile pinyons associated with dead pinyons was 22.2% compared to 13.0% for those associated with living pinyons ($\chi^2 = 2.39$, $P > 0.05$).

Stem growth of juvenile pinyons in the understory of dead pinyons was significantly higher than in juveniles associated with living conspecifics (Fig. 1). Before the mortality event of 2002 (1999–2002), stem growth of juveniles located in the understory of mature trees that died during the 1996 mortality event was greater than growth of juveniles associated with mature trees that survived. Similar results were found after the 2002 mortality event (association: $F_{2,27} = 13.7$, $P < 0.001$, Year: $F_{1,27} = 3.8$, $P = 0.02$, association by year: $F_{2,27} = 0.88$, $P = 0.52$). Growth did not differ significantly between juveniles associated with living and 2002 dead trees before 2002, but significant differences were observed following the 2002 drought (2003–2005; association: $F_{2,27} = 6.76$, $P = 0.004$, Year: $F_{1,27} = 0.43$, $P = 0.66$, association by year: $F_{2,27} = 0.43$, $P = 0.78$), with the growth of juveniles beneath 2002 dead trees intermediate between live and 1996 dead.

Litter depth was significantly lower under 1996 dead trees than live trees with 2002 dead trees intermediate (Table 2). There were no significant differences in soil moisture or soil temperature among live, 2002 dead and 1996 dead pinyons (Table 2), but light intensity was significantly greater under 1996 dead pinyons than under live or 2002 dead pinyons. The latter two groups were not different from one another.

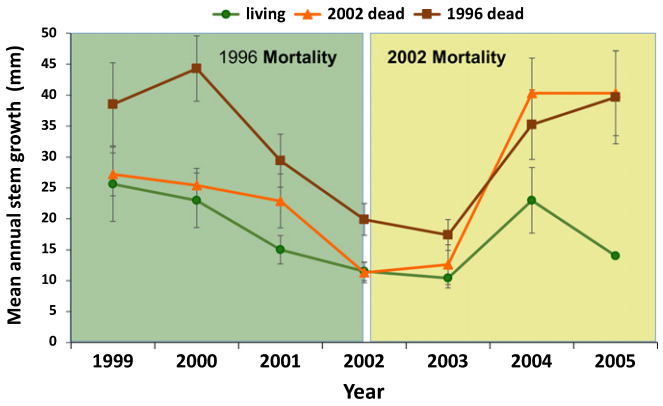

Fig. 1 Mean annual stem growth of juvenile pinyons (*Pinus edulis*) located in the understory of living (green lines), 2002 dead (orange lines) and 1996 dead (brown lines) pinyons. From 1999 to 2002, growth of juveniles associated with 1996 dead trees was significantly greater than seedlings associated with living or 2002 dead pinyons. Following the 2002 drought (2003–2005) growth of juveniles associated with 2002 dead pinyons was significantly greater than that of juveniles associated with live pinyons. Standard errors not visible are within the bounds of the symbols used.

Table 2 Microclimate and ectomycorrhizal fungal (EMF) data for living, 2002 dead and 1996 dead pinyons (*Pinus edulis*) collected in 2004–2005.

	Live	2002 dead	1996 dead	*P*-value
% soil moisture	7.8 (0.48)	7.7 (0.47)	7.3 (1.0)	0.62
Soil temperature (°C)	13.9 (0.43)	15.5 (1.9)	14.1 (0.49)	0.08
PAR ($\mu mol\ m^{-2}\ s^{-1}$)[1]	237 (40.4)[a]	407 (61.1)[a]	714 (99.1)[b]	**< 0.001**
Litter depth (cm)	43.9 (4.3)[a]	41.1 (1.1)[ab]	26.2 (6.5)[b]	**0.026**
% EMF colonization[2]	57.8 (7.1)	44.6 (5.4)	50.7 (3.1)	0.35
EMF richness[1]	1.86 (0.26)	1.60 (0.40)	1.67 (0.29)	0.72

Data presented are the means across groups (1 SE). Different letters indicate significance at $\alpha = 0.05$ and *P*-values represent the statistic for among group comparisons. *P*-values in bold are statistically significant at $\alpha < 0.05$.
[1]PAR, photosynthetically active radiation.
[2]EMF data are from glasshouse Experiment 1.

Effects of overstory mortality on EMF inoculum – glasshouse Experiment 1; Hypothesis 2

Pinus edulis seedlings grown in soils collected beneath living and dead pinyons had similar EM colonization (Table 2). Eight EMF species were observed on the bioassay seedlings and richness of the EMF communities on seedlings grown in soil from live, 2002 dead and 1996 dead pinyons was similar ($F_{2,14} = 0.06$, $P = 0.89$) (Table 2). EMF community composition differed among living, 2002 dead and 1996 dead trees ($F_{2,14} = 18.6$, $P < 0.001$) (Fig. 2a). These differences were most pronounced between living

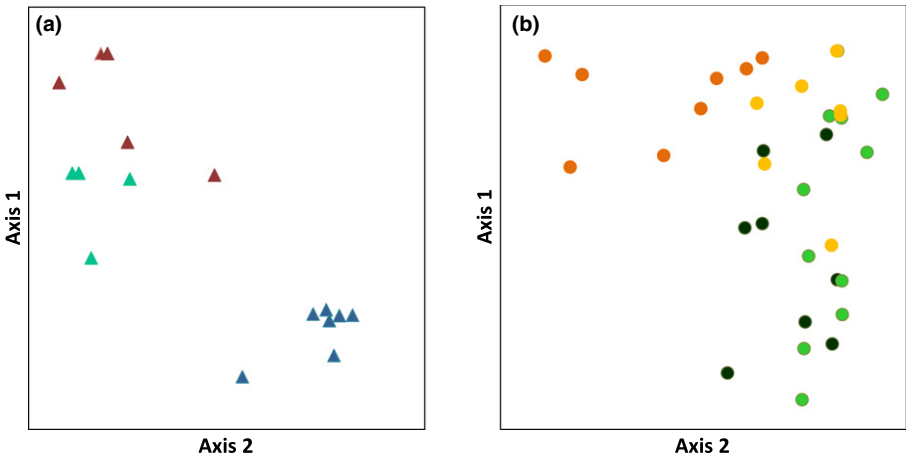

Fig. 2 Nonmetric multidimensional scaling (NMDS) ordination of the ectomycorrhizal fungal (EMF) communities associated with *Pinus edulis* seedlings grown in Experiments 1 and 4. (a) Communities of seedlings grown in soil from beneath living (blue triangles), 2002 dead (aqua triangles) and 1996 dead (maroon triangles) pinyons (Experiment 1). (b) Communities of seedlings grown in soil from stands that varied in living pinyon basal area: 3.84 m^2 ha^{-1} (dark green circles), 1.80 m^2 ha^{-1} (light green circles), 1.09 m^2 ha^{-1} (yellow circles) and 0.10 m^2 ha^{-1} (orange circles) (Experiment 4).

and both classes of dead trees ($F_{1,15} = 22.7$, $P < 0.001$), but the EMF communities of 1996 dead and 2002 dead pinyons also differed ($F_{1,8} = 4.2$, $P < 0.049$) suggesting that time since mortality may influence the EMF species beneath dead pinyons.

All communities were dominated by ascomycete fungi from the order Pezizales, and the single basidiomycete genus observed, *Rhizopogon*, was only found on three seedlings (Fig. 3). The majority of these taxa have been observed in other studies of pinyon pine (Swaty *et al.*, 2004; McHugh & Gehring, 2006; C. A. Gehring *et al.*, 2017). The dominant species of EMF in the inoculum from living trees was a member of the genus *Tuber* (*Tuber* sp. 1), whereas the inocula of dead pinyons was dominated by *Geopora* species (Fig. 3). Both dominant *Geopora* species have been studied previously and consisted of *Geopora pinyonensis* for the 1996 dead and morph J (*Geopora* sp.) for the 2002 dead (Gordon & Gehring, 2011; Flores-Rentería *et al.*, 2014).

Spatial distribution of EMF inoculum – glasshouse Experiment 2; Hypothesis 2

In agreement with other studies (Dickie *et al.*, 2002; Dickie & Reich, 2005), the amount of EMF inocula found inside the rooting zone of mature pinyons was nearly double that found outside the rooting zone, with percentage EMF colonization of seedlings averaging 19.04 ± (1 SE) 3.1 and 10.30 ± 2.9, respectively ($t = 6.43$, $P = 0.017$). Levels of colonization were lower than

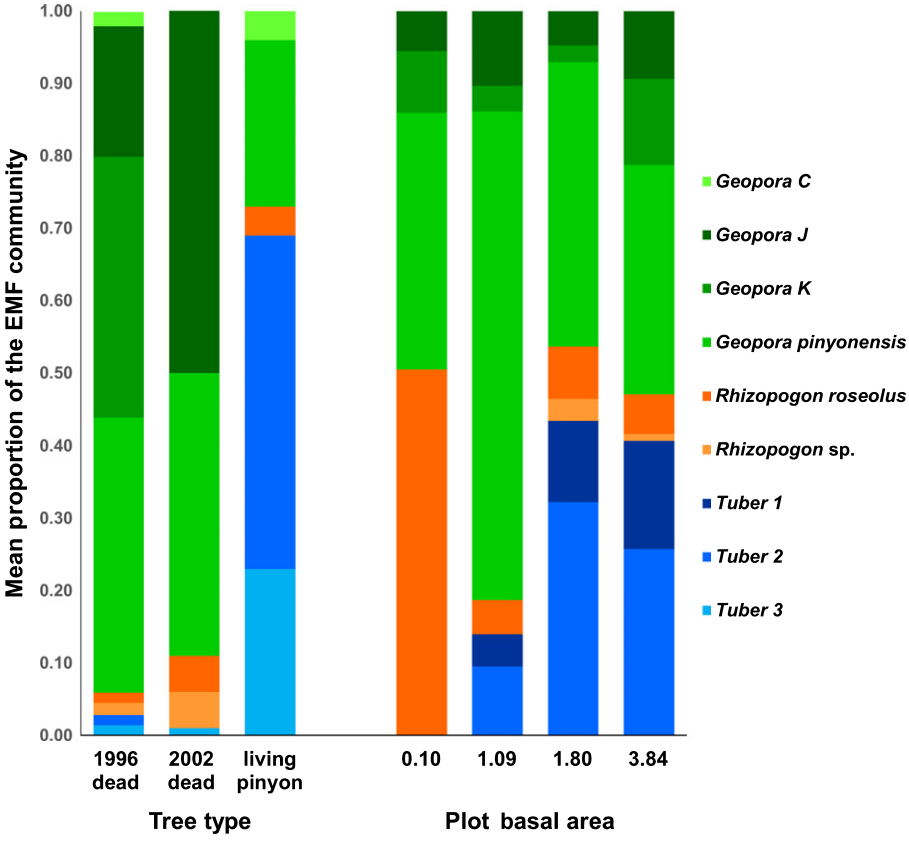

Fig. 3 Mean relative abundance of ectomycorrhizal fungal (EMF) communities of seedlings grown in soil collected beneath living, 2002 dead and 1996 dead pinyons (three bars on left) and in association with soil inoculum collected from stands of varying living pinyon (*Pinus edulis*) density as measured by basal area (m^2 ha^{-1}) (four bars on right). Bars are color-coded by fungal genus: green, *Geopora*; blue, *Tuber*; orange, *Rhizopogon*.

observed in Experiment 1, possibly due to differences in drought stress between years.

EMF inoculum potential, community composition and seedling growth at the stand scale – glasshouse Experiments 3 and 4; Hypotheses 2 and 3

Pre-mortality living pinyon basal area varied among the stands, but by a smaller amount than post-mortality living pinyon basal area at both sites (Tables 3, S1). Living pinyon basal area was nearly 40-fold greater in the highest density stand of *P. edulis* than in the lowest density stand after the 2002 drought and associated mortality at Site 1 and three-fold greater at Site 2 (Tables 3, S1).

On average, living trees produced similar numbers of cones regardless of stand mortality ($F_{3,36} = 0.40$, $P = 0.75$ for Site 1; $F_{3,36} = 0.09$, $P = 0.96$ for Site 2) suggesting that surviving trees performed well in higher mortality stands following drought. However, estimated cone production differed by 2.5–5-fold between the lowest and highest mortality stands due to the low numbers of living mature pinyons in the higher mortality stands (Tables 3, S1).

Results of Experiment 3 indicated that ectomycorrhizal inoculum potential did not differ among stands, with similar mean % EMF colonization regardless of living tree density ($F_{3,43} = 0.717$, $P = 0.55$ for Site 1 and $F_{3,39} = 1.283$, $P = 0.29$ for Site 2) (Tables 3, S1). For Site 2, mean EMF species richness per seedling differed significantly among stands ($F_{3,35} = 8.621$, $P = 0.001$); seedlings grown in soil from the stand with the highest living pinyon basal area had more species than seedlings grown in soil from the other stands (Table S1). EMF community composition also varied among stands within Site 2 ($F_{3,35} = 2.235$, $P = 0.027$), with seedlings grown in soil from the stand with the highest living pinyon basal area having distinct communities relative to other seedlings (Fig. S1), with *Geopora* less common, and *Rhizopogon* and *Hebeloma* more common (Fig. S2).

In Experiment 4, EMF species richness and EMF community composition differed among seedlings inoculated with soils from the four stands within Site 1, whereas EMF colonization was similar. Levels of EMF colonization ranged from 33% to 45% comparable to Experiment 3 ($F_{3,35} = 0.451$, $P = 0.79$). Average EMF species richness per seedling was significantly higher in seedlings inoculated with soil from the stand with highest living pinyon basal area than in seedlings inoculated with soil from the stand with the lowest living pinyon basal area, with seedlings inoculated with soil from the other two stands intermediate ($F_{3,31} = 4.58$, $P = 0.009$) (Table 3).

The EMF community composition varied significantly among seedlings inoculated with soil from the four stands of Site 1 ($F_{3,31} = 5.2$, $P = 0.001$), with pairwise comparisons indicating that the EMF communities of seedlings inoculated with soil from the stand with the lowest pinyon basal area differed significantly from all other stands (Figs 2b, 3). The EMF community composition of seedlings grown with soil inoculum from the 1.80 basal area site was not significantly different from seedlings grown with soil inoculum from the lowest basal area site ($P = 0.08$). Consistent with Experiment 1, differences among stands were due to shifts in members of the genus *Tuber* with tree mortality (Fig. 3). Taken together, the results of Experiments 1–4 showed no significant effect of tree mortalityon EMF abundance, variable effects on EMF species richness and consistent changes in EMF community composition.

Pinyon MGR was positive in all cases, indicating that even inoculum from the stand with the lowest living pinyon density promoted pinyon growth relative to sterile controls. However, MGR was significantly lower (33–39%) in seedlings inoculated with soil from the stand with the lowest living pinyon basal area (0.10) than in seedlings inoculated with soil from the stands with the highest living pinyon basal area (1.80, 3.84), whereas the 1.09 basal area site was intermediate ($F_{3,43} = 4.66$, $P = 0.007$) (Fig. 4). Seedling biomass was significantly positively associated with the relative abundance of *Tuber* ectomycorrhizas ($R^2 = 0.585$, $F_{1,33} = 45.12$, $P = 00001$) (Fig. 5) These results support our third hypothesis that changes in EMF communities with mature tree mortality would negatively affect *P. edulis* seedlings.

Discussion

Positive effects of overstory mortality on established juveniles

Although we expected that the death of an overstory pinyon would alter juvenile mortality during subsequent drought, we found that mortality was similar among juveniles associated with living and dead conspecifics. Mortality of juveniles associated

Table 3 Characteristics of the four *Pinus edulis* stands used in the stand-scale analysis for Site 1.

Stand	Pre-mortalityPinyon Basal Area (m^2 ha^{-1})[1]	% Pinyon mortality	Post-mortality Pinyon Basal Area (m^2 ha^{-1})	Mean cones per tree (SE)[2]	Estimated cones per plot[1]	% EM colonization (SE)[2]	EMF species richness
1	3.03	64.4	0.10	19.4 (17.6)	155	36.7 (5.4)[a]	2.0 (0.27)[a]
2	5.27	22.4	1.09	14.5 (10.8)	290	28.7 (6.6)[a]	2.1 (0.22)[ab]
3	6.69	43.9	1.79	26.0 (14.4)	676	39.1 (6.5)[a]	3.1 (0.33)[ab]
4	3.84	0.0	3.84	11.2 (3.7)	762	38.4 (3.4)[a]	3.2 (0.36)[b]

EMF, ectomycorrhizal fungal.
[1]Pinyon basal area, percentage pinyon mortality and estimated cones per plot were measured at the stand level, and so have no variance measures. Estimated cones per plot was calculated using mean number of cones per tree and the total number of mature pinyons in each plot and also has no estimate of variation.
[2]Values represent the mean (1, SE). Different letters indicate significance at $\alpha = 0.05$.

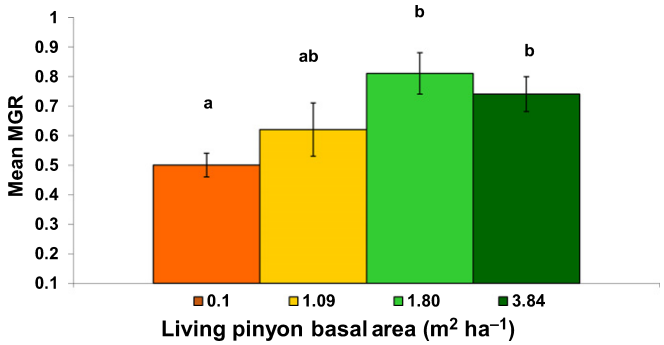

Fig. 4 Mean microbial growth response (MGR) of seedlings grown for 9 months in steam-sterilized field soil inoculated with live soil from stands varying in living pinyon (*Pinus edulis*) density as measured by basal area (m^2 ha^{-1}): 3.84 m^2 ha^{-1} (dark green), 1.80 m^2 ha^{-1} (light green), 1.09 m^2 ha^{-1} (yellow) and 0.10 m^2 ha^{-1} (orange). Error bars show ± 1 SE and different letters above bars indicate significant differences at $P < 0.05$.

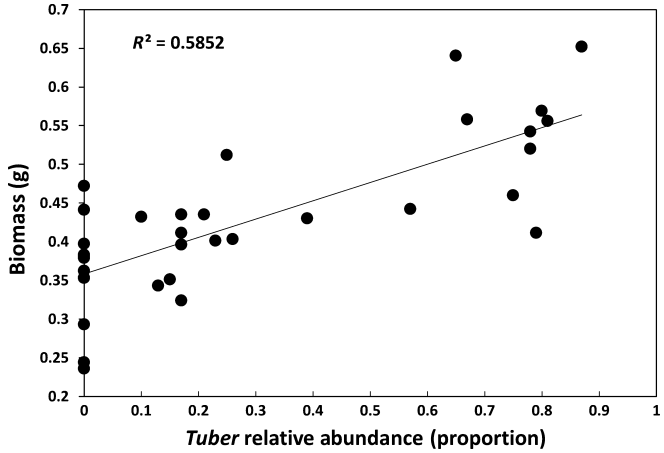

Fig. 5 Total *Pinus edulis* seedling dry biomass was significantly positively associated with the relative abundance of *Tuber* ectomycorrhizas in Experiment 4.

with dead pinyons also was lower than of juveniles in vegetation interspaces, which was > 40% (Mueller *et al.*, 2005). These findings suggest that the positive effects of dead pinyons on associated juveniles persisted during the 6 yr period between the two droughts (1996–2002). Similarly, Redmond *et al.* (2015) found that *Pinus edulis* juveniles had lower mortality beneath dead pinyons than canopy interspaces; however, they did not document mortality under living pinyons.

Juvenile pinyons associated with dead mature pinyons had significantly higher stem growth than juveniles found with living pinyons, a pattern observed following the 1996 and 2002 mortality events. Importantly, before the drought and associated mortality, no significant differences in stem growth were observed (Fig. 1). This result suggests that death of the overstory tree led to competitive release for understory juveniles. This competitive release was likely the result of reduced competition for light, as soil moisture and temperature were similar under live and dead pinyons (Table 2). However, increased nutrient availability from decomposition of dead roots may have contributed to increased growth, although rates of decomposition are exceedingly low in these semi-arid sites (Classen *et al.*, 2007).

Similar trends in juvenile growth following overstory mortality were observed by Kane *et al.* (2015), although differences between the growth of seedlings associated with living and dead pinyons was not statistically significant in their study. Previous studies indicate that mature pinyons facilitate conspecific seedling establishment (Chambers, 2001), but our findings suggest that this interaction shifts to competition as juvenile pinyons grow, as predicted by Holmgren *et al.* (1997). Similar shifts have been observed between pinyon and shrubs in high stress sites, where the interaction is facilitation when pinyons are seedlings (Sthultz *et al.*, 2006), but shifts to competition as pinyons age (McHugh & Gehring, 2006) or where abiotic stress is reduced (Sthultz *et al.*, 2006). Regardless, our findings suggest that established juvenile pinyons benefited from the death of the overstory pinyon, with limited negative effects.

Legacy effects of dead pinyons on ectomycorrhizal fungi (EMF) inocula

With the exception of research on forest clear cuts (Teste & Simard, 2008; Barker *et al.*, 2013), few studies have examined the legacy effects of large-scale tree mortality on belowground microbial communities (but see Karst *et al.*, 2015). Our study shows that even 9 yr following the death of the tree, soil in the understory of pinyons that died in 1996 had levels of EMF inocula similar to that of living trees (Table 2). Likewise, levels of EMF inocula did not differ among stands that varied substantially in post-drought living pinyon density. Proximity to living EMF hosts increased the abundance of EMF inoculum in other studies (Dickie & Reich, 2005; Haskins & Gehring, 2005; Teste & Simard, 2008), similar to our observation that the abundance of EMF propagules was nearly double inside the rooting zone of living pinyons compared to adjacent areas that were bare soil or colonized by non-EMF host plants. This finding, combined with the results of Experiment 1 that found persistence of EMF propagules following mature tree mortality, suggests that soil communities in sites that have experienced mortality differ from sites that have not been colonized by EMF hosts, such as juniper-dominated ecosystems (Haskins & Gehring, 2005).

The EMF community shifts observed in high-mortality sites may be associated with the production of resistant propagules found in a subset of EMF species. EMF colonization can occur through contact with hyphae emanating from EMF that have colonized the roots of adjacent EMF hosts, or through propagules such as spores. In our system, given the length of time between sampling of soils and death of the host tree, we suspect that the EMF detected persisted beneath dead trees in the form of spores or other resistant propagules (Bruns *et al.*, 2009). These propagules also likely occured beneath living pinyons, but may have been less abundant or outcompeted by other EMF taxa (Peay, 2016). A synthesis of studies that examined EMF spore longevity found that spores of EMF species in the Basidiomycota often were unable to persist for long enough to accumulate in spore banks in soils (Nara, 2008). In stands of *Pinus muricata,* EMF species with resistant propagules were primarily members of the phylum Ascomycota, along with species in the basidiomycete

genus, *Rhizopogon* (Taylor & Bruns, 1999). In our study the EMF that persisted beneath dead trees or in sites with high host mortality were ascomycetes or species of *Rhizopogon*, which can retain viability in the absence of a host through the production of long-lived spores (Tedersoo *et al.*, 2006; Bruns *et al.*, 2009).

Our study may not fully represent the effect of overstory mortality on the EMF community because we used glasshouse MIP experiments which may have underestimated the inoculum available beneath living trees by eliminating the potential for colonization by hyphae attached to living trees. In boreal forest, root connections between seedlings and mature trees influenced the EMF communities of establishing seedlings (Tedersoo *et al.*, 2008). Similarly, willow seedlings obtained most of their EMF community from established willows in an early successional environment (Nara & Hogetsu, 2004). Because the potential to obtain EMF from living mature pinyons is much greater in low-mortality stands, seedlings establishing in the field beneath dead pinyons or in high-mortality sites may experience more dramatic changes in community composition and species richness than observed in our bioassays. The juvenile trees that established before severe mature tree mortality described above may have been buffered from these changes because they established EMF associations before severe drought.

Altered EMF community composition and consequences for seedling growth

The EMF communities associated with living and dead trees were significantly different, patterns also observed when comparing stands that differed in living pinyon density. The EMF communities associated with *P. edulis* mortality were composed primarily of ascomycete fungi within the order Pezizales (Gordon & Gehring, 2011). Ectomycorrhizal fungal communities dominated by ascomycete fungi are common in *P. edulis* and increased dominance by ascomycetes has been observed in response to herbivory (C. A. Gehring *et al.*, 2014), parasitism (Mueller & Gehring, 2006), drought (C. Gehring *et al.*, 2014) and interspecific competition (Haskins & Gehring, 2004; McHugh & Gehring, 2006). Furthermore, studies of *P. edulis* showed that ascomycetes within the genus *Geopora* contributed significantly to drought tolerance (C. A. Gehring *et al.*, 2017).

Although ascomycetes in the genus *Geopora* persisted following mature *P. edulis* mortality, members of the genus *Tuber* were absent or reduced in association with dead trees or high-mortality stands. Perhaps most importantly, this loss of *Tuber* from the EMF community was associated with a significant decline in seedling growth (Fig. 5). Although members of the genus *Tuber* are among the most well-studied ascomycete EMF, functions such as nitrogen uptake vary among species (Pena & Polle, 2013). Concomitant with the loss of *Tuber* following *P. edulis* mortality was lower species richness, which could have contributed to reduced seedling growth. *Pinus sylvestris* and *Betula pendula* seedling biomass was affected by both EMF diversity and community composition (Jonsson *et al.*, 2001). Mortality of lodgepole pine (*Pinus contorta*) due to mountain pine beetle (*Dendroctonus ponderosae*) herbivory altered EMF communities, seedling growth and defensive chemistry (Karst *et al.*, 2015). Our inoculation experiment supports these findings and suggests that shifts in an EMF community dominated by closely related species also can alter host plant performance.

Indirect species interactions and ecosystem recovery

Although some climate models predict that pinyon pine may be extirpated from Arizona this century (Rehfeldt *et al.*, 2006), our study provides two encouraging signs for this species. First, the death of an overstory conspecific led to dramatically higher growth among established juvenile pinyons. As pinyon size is correlated with reproductive maturity in this slow-growing species (Mueller *et al.*, 2005), this increased growth could reduce the time required for surviving juveniles to reproduce. Second, trees that had been dead for 9 yr provided similar amounts of EMF inoculum as living trees, patterns that also were observed 5 yr after mortality at the stand scale. Although other factors, such as abiotic environmental conditions, affect the establishment of pinyon seedlings, access to EMF inocula can have a strong positive impact on the establishment and survival of pine seedlings (Peay *et al.*, 2009), including pinyon pine (Gehring *et al.*, 1998).

Despite these positive effects, widespread adult pinyon mortality may ultimately have negative consequences for pinyons for three reasons. First, the microclimate modifications afforded by dead pinyons that were associated with enhanced juvenile growth are temporary, and over time will resemble the microclimates of vegetation interspaces, where pinyon seedling establishment is virtually nonexistent (Chambers, 2001; Redmond & Barger, 2013; Redmond *et al.*, 2015). Mortality rates during drought also are much higher in the absence of a nurse plant (Mueller *et al.*, 2005). Second, although the number of cones produced per tree did not differ between high- and low-mortality stands, estimated stand-level cone production was markedly reduced in low density stands. Third, the amount of EMF inoculum was unchanged in the understory soil of dead pinyons, yet the composition of the EMF community was significantly different. Very few EMF species form spores that can survive in the absence of a host (Bruns *et al.*, 2009), suggesting that differences between living and dead pinyons, and high- and low-mortality stands, will increase over time.

Climate models predict that many forest ecosystems will experience more frequent and severe droughts (Seager *et al.*, 2007; Garfin *et al.*, 2013). Most temperate and boreal forest ecosystems have dominant plants that associate with EMF, and our findings suggest that large-scale mortality events will have consequences for EMF communities. Further, because EMF in the phylum Ascomycota appear to be more resilient to droughts and disturbances, changes in communities dominated by EMF in the Basidiomycota may be more pronounced. Greater understanding of the functional consequences of changes in EMF communities may help us predict the trajectories of forest ecosystems.

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Author contributions

RCM, CMS, CAG and TGW designed the study; RCM, CMS, CAG collected and analyzed field, glasshouse and/or laboratory data; RCM and CAG wrote the manuscript; all authors contributed to revisions.

ORCID

Catherine A. Gehring https://orcid.org/0000-0002-9393-9556
Rebecca C. Mueller https://orcid.org/0000-0003-2254-399X

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 NMDS Ordination of Ectomycorrhizal fungal (EMF) community data for *Pinus edulis* at Site 2, Experiment 3.

Fig. S2 Stacked bar graph illustrating Ectomycorrhizal fungal (EMF) species differences with *Pinus edulis* stand density for Site 2, Experiment 3.

Table S1 *Pinus edulis* stand characteristics, Ectomycorrhizal fungal (EMF) colonization and EMF species richness data for Site 2, Experiment 3.

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