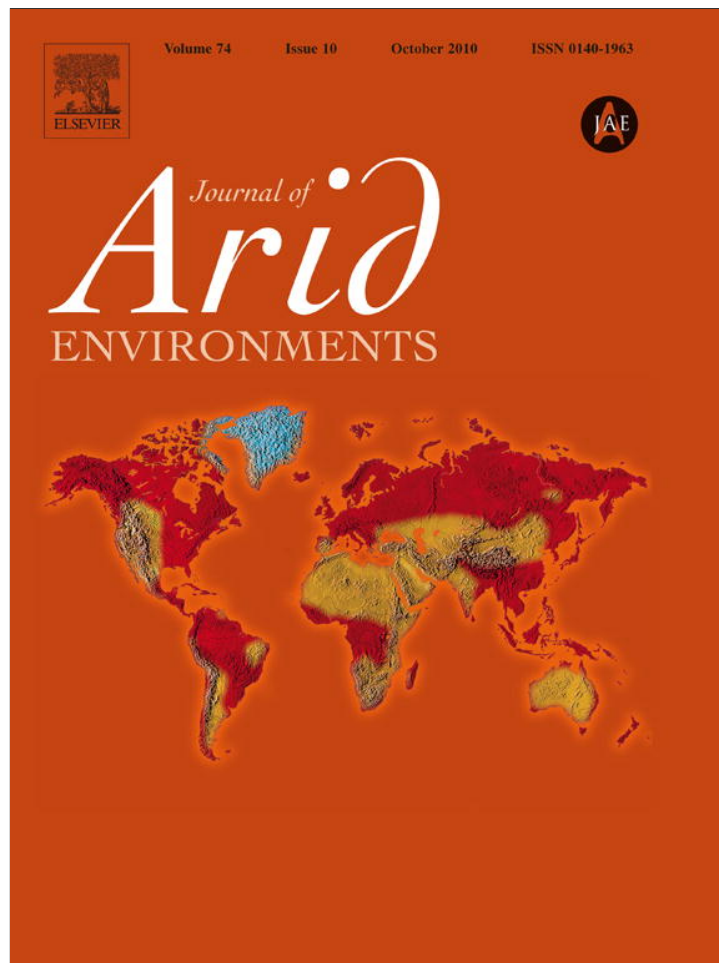


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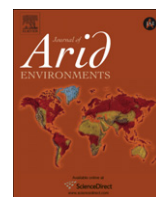
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## Journal of Arid Environments

journal homepage: [www.elsevier.com/locate/jaridenv](http://www.elsevier.com/locate/jaridenv)

## Responses to chronic N fertilization of ectomycorrhizal piñon but not arbuscular mycorrhizal juniper in a piñon-juniper woodland

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## ARTICLE INFO

## Article history:

Received 12 January 2009

Received in revised form

14 April 2010

Accepted 6 May 2010

Available online 12 June 2010

## Keywords:

Drought  
Juniper  
Mortality  
Mycorrhiza  
Nitrogen  
Piñon pine

## ABSTRACT

Responses of mature trees to chronic N additions are poorly understood in ecosystems with high seasonal and spatial variability. To determine the effects of increased N deposition on mature conifers, we fertilized a piñon-juniper woodland in New Mexico at a rate equivalent to the urban interface. Fertilization ( $10 \text{ g m}^{-2} \text{ y}^{-1}$ ) reduced numbers of mycorrhizae and increased leaf production in the ectomycorrhizal (EM) piñon but not in arbuscular mycorrhizal (AM) juniper. Based on N fractionation between EM fungal sporocarps and piñon, EM in piñon utilized 20% of the net primary production in control plots. No sporocarps were produced in fertilized plots. N uptake by piñon could be accounted for by fertilization without mycorrhizae. Leaf N and size increased with fertilization in both species, and positively correlated with leaf  $\delta^{13}\text{C}$ . Leaf N:P increased in piñon but not juniper. Piñon mortality commenced in the N-fertilized plots in 2001, a year before the widespread die-off in western conifers, and continued through 2003. No mortality was observed in control plots or in junipers. The coupling of N enrichment and mycorrhizal decline could affect piñon production and mortality in semi-arid woodlands in the western US.

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### 1. Introduction

Ecological responses to perturbations in long-lived species are complex to study because plants may experience varying resource limitations during their lifetimes. However, evaluating how multiple environmental factors affect long-lived plants, such as trees, provides insights into how species and forested ecosystems are likely to respond to complex environmental change. Nitrogen (N) is a limiting resource for plant growth that interacts with water limitation at the individual and stand level. Elevated N in seedlings and young trees during rainy periods is known to reduce allocation to roots and increase shoot growth (Entry et al., 1998; Gower et al., 1992; Haynes and Gower, 1995; Stober et al., 2000). The resulting increased leaf production (Long et al., 2004) and increased leaf-to-root ratio may cause xylem cavitation and stress under drier conditions (Sperry et al., 1998, 2002). Elevated N also decreases mycorrhizal activity and changes fungal species composition (e.g.,

Egerton-warburton and Allen, 2000; Karen et al., 1997; Porras-Alfaro et al., 2007), thereby altering mycorrhizal-mediated P and water uptake (Allen, 2005; Allen et al., 2003).

Long-lived trees, in particular, are currently experiencing simultaneous shifts in soil eutrophication, water availability, stand densification, atmospheric  $\text{CO}_2$ , and invasive species, all of which are shifting ecosystems from the conditions under which they were established (Sala et al., 2000). These shifts are all expected to continue into the future, with dramatic implications for vegetation community structure. Anthropogenic N deposition likely interacts locally with regional environmental perturbations such as drought. Ideally, such N inputs could compensate for the depression in tissue N with elevated  $\text{CO}_2$  (Allen et al., 2005), but elevated N availability could, in turn, also negatively affect mycorrhizal responses, root-shoot allocation, and resilience to chronic drought.

Downwind of urban, industrial and agricultural areas of the arid and semi-arid lands, regional N deposition is increasing dramatically. N deposition in semi-arid regions such as the Los Angeles Basin, Phoenix Basin, the Central Valley of California, Salt Lake City, and Athens, Greece, can exceed  $5 \text{ to } 10 \text{ g m}^{-2} \text{ y}^{-1}$  (e.g., Fenn et al., 2003a; Jones et al., 2004; Schulze, 2000; Tonnesen et al., 2003).

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During dry years, water limits production, a process to which most aridland plants are adapted. But, during wet cycles, or during the rainy season, higher N availability can greatly enhance production. In the southwestern United States, winter precipitation between El Niño and La Niña years varies as much as 4- to 5-fold. La Niña in combination with changes in the Pacific Decadal Oscillation in recent years has led to persistent drought and massive tree mortality (Breshears et al., 2005). In addition to annual variation, even during wet years, pronounced dry seasons affect growth and physiology of individual plants.

Interactions between N and water in mature plants in wildland ecosystems are not well understood. If fertilization or anthropogenic N deposition increases water throughput or decreases mycorrhizae or root:leaf area during wet periods, plants may not be able to adjust to a rapid onset of drought conditions. Deciduous plants can shed leaves, but conifers have perennial needles, generally living 3 to 5 years (Ewers and Schmid, 1981). During drought, it is virtually impossible for a plant to add roots, and without new roots, mycorrhizae to mediate water uptake are not produced (Querejeta et al., 2007). To what extent coniferous plants adjust from a wet year, with high aboveground production, to a low precipitation year, when survival and growth depends on greater root and mycorrhizal mass remains unknown. This may be especially true as drought periods in the future (Seager et al., 2007) exceed those found during the life span of extant plants, or as novel perturbations such as N deposition increasingly interact with more frequent and severe drought periods.

In addition to altering ecosystem dynamics, N deposition may also differentially alter the relative composition of the affected communities, either directly or through an indirect mycorrhizal response to N or to N effects on drought. Swaty et al. (1998) noted that EM were susceptible to drought in soil where water content fluctuated highly. Mueller et al. (2005) found that EM pines were more susceptible to drought than AM junipers, and Gehring et al. (2006) found that in cottonwoods, which form both EM and AM, the AM predominated in the drier end of an experimental gradient, where as EM dominated the wetter end. If N altered relative leaf: root ratio, and C allocation to the fungi, then feedbacks between N, water, and mycorrhizae could affect the composition and functioning of mixed EM/AM communities.

We undertook a study of the impacts of N inputs on leaf, root, and mycorrhiza dynamics in relatively pristine piñon-juniper woodland in south-central New Mexico. Piñon pine has been subjected to large-scale mortality during recent dry years in the western U.S., while interspersed juniper has experienced relatively low mortality (Breshears et al., 2005, 2008). Specifically, we fertilized replicated plots of piñon-juniper woodland at a level equal to N deposition ( $10 \text{ g m}^{-2} \text{ y}^{-1}$ ) measured in seasonally dry pine forests near urban areas in California (Fenn et al., 2003b). This level was also chosen to achieve N saturation, comparable with other fertilization studies in coniferous ecosystems (e.g., Aber et al., 2004; Schulze, 2000). Piñon pine and juniper are especially useful for study because piñon forms ectomycorrhizae (EM) and juniper forms arbuscular mycorrhizae (AM). EM are especially important in transformation and uptake of organic N (Smith and Read, 1997) whereas AM are especially important in uptake of P and  $\text{NH}_4\text{-N}$ . Both play complex roles in water relations (Allen, 2005). We evaluated the response of individual piñon pine and juniper trees to fertilization over a seven-year period beginning in 1997 to better understand the response of piñon and juniper trees to elevated N deposition and regional climate variability.

## 2. Materials and methods

This study was conducted at the Sevilleta National Wildlife Refuge (SNWR) in south-central New Mexico, USA, site of the

Sevilleta Long-Term Ecological Research (LTER) program. The Refuge contains extensive areas of semi-arid grassland, shrubland and piñon-juniper woodland vegetation. Annual precipitation is ca 250 mm, of which 60% falls in episodic monsoon events from July through September (Gosz et al., 1995; Pennington and Collins, 2007). The average annual temperature is  $13.2 \text{ }^\circ\text{C}$  (average low  $1.6 \text{ }^\circ\text{C}$  in January, average high  $33.4 \text{ }^\circ\text{C}$  in July). Aboveground net primary productivity is low, averaging  $51.0\text{--}60.0 \text{ g m}^{-2}$  (Muldavin et al., 2008). Atmospheric N deposition is ca  $0.2 \text{ g m}^{-2} \text{ y}^{-1}$  but increasing (Báez et al., 2007) as Albuquerque expands.

The experiment was initiated in March of 1997 and continued through the growing season of 2004. This period included El Niño years with above-average precipitation (1999–2000) followed by drought from 2001 to 2004 (Pennington and Collins, 2007). Six  $30 \times 30 \text{ m}$  plots were established in three paired-plot blocks within a piñon-juniper stand in the Upper Goat Draw area of SNWR. The area is dominated by piñon pine (*Pinus edulis* Engelm.) and one-seeded juniper (*Juniperus monosperma* (Engelm.) Sargent) with approximately 50% tree cover, with interspace area occupied by turbin oak (*Quercus turbinella* Greene), and grama grasses (*Bouteloua gracilis* [(Kunth) Lag. ex Griffiths], *B. curtipendula* [(Michx.) Torr], *B. eriopoda* [(Torr.) Torr.]). Additional information concerning the ecology and long-term experiments at these sites can be found at <http://sev.lternet.edu>. Fertilizer ( $10 \text{ g N m}^{-2}$ ) was applied as  $\text{NH}_4\text{NO}_3$  (43:0:0) by hand ( $2.5 \text{ g m}^{-2}$ , four times per year), to fertilized plots. This method of fertilization is appropriate for arid regions in that N deposition results from accumulations on the leaf and soil surfaces during dry periods, followed by flushes of N inputs upon rainfall (Padgett et al., 1999). Measurements to assess response to elevated N were conducted from 1997 to 2004, and included soil and plant tissue N, mycorrhizal abundance, and leaf and sporocarp  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ .

Soil cores (5 cm diameter, 10 cm deep) were taken beneath the canopy of individual piñon pine and juniper trees in October 1997, approximately every other year to evaluate total N, total C, and extractable  $\text{NH}_4$  and  $\text{NO}_3$ .

In March 1997, minirhizotron tubes (1 m in length, 5.6 cm OD) were inserted at a  $22^\circ$  angle to the soil surface beneath eight individuals each, of piñon pine and juniper per plot (48 per species) through the soil profile to the underlying rock layer. This provided an average viewing tube surface extending to 30 cm vertical depth. Along the entire length, each tube was permanently scribed with  $1.4$  (width)  $\times$   $1.2$  (length) cm frames every 1.2 cm along the tube, which was sealed permanently at the bottom. Each tube extended approximately 20 cm out of the soil, where it was painted black and then repainted white, encased in aluminum foil, and capped between measurements.

Beginning in the fall of 1997, we took soil cores and assessed mycorrhizal formation. Direct observations of mycorrhizal infections of first order roots were made annually through 2000. For juniper, the % of root length infected by AM was highly variable among years (20–80% of the root length, from 1997 to 2000) and much of the root was suberized (MF Allen unpublished data; Lansing, 2003). After 3 years, we realized that the soil cores provided openings for invasive species and eventually drastically disturbed the soil being studied. Moreover, fractional colonization of roots has proven unsuitable for assessing mycorrhizal response to environmental change (Alberton et al., 2005; Allen, 2001; Treseder et al., 2003). AM infection units form new infections in the region of elongation (behind the root tip), disappearing as the root ages and suberization and phenolic activity increases (Allen, 2001). The region of elongation behind every new juniper root was found to form arbuscular mycorrhizae (AM). With microscopy of individual tips, or high-resolution minirhizotron observations, we observed AM hyphae radiating from behind each individual root

tip (Allen et al., 2003), and, in stained roots, all newly formed tips were observed to be mycorrhizal. Therefore, we used the numbers of juniper root tips as a proxy for the relative numbers of AM. Subsequent examination of juniper root tips confirmed that all were AM. From 1997 to 2000, we measured ectomycorrhizal (EM) infection of piñon roots by extracting roots from cores (3.5-cm diameter by 12-cm deep), wet-sieving roots using a 0.5-mm sieve, and recovering all EM and nonmycorrhizal root tips. As the roots began to intercept the surface of the minirhizotron tubes (fall of 1997), EM root tips could be distinguished and counted from the videotape images (e.g., Allen et al., 2003; Majdi and Nylund, 1996; Majdi et al., 2001). Minirhizotron images were videotaped three to four times annually during the growing season (May–November) beginning in the fall of 1997 through 2004.

During the first four years of growth (1997–2000), no observable effects of N fertilization on plants were observed. Beginning in November of 2001 (the fourth year of fertilization), leaf samples from trees with minirhizotron tubes were collected to determine physiological activity, and tree mortality was assessed. Physiological activities included total N and P concentration,  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ , and N:P ratios. Trees with minirhizotron tubes were monitored for plant production in November of 2003 after plant growth ended by clipping three individual branches from each tree. Needles of piñon were separated for individual years for the individual growth nodes of 2001, 2002, and 2003. Only the current year growth could be identified and was measured in juniper in 2003.

Soil and leaf C, total soil N, soil-extractable N, and bicarbonate-extractable P were analyzed by the University of California Division of Agriculture and Natural Resources Analytical Laboratory (methods are described at <http://danralab.ucdavis.edu>).  $\delta^{15}\text{N}$  values were measured to determine if N acquisition changed with fertilization.  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  of soils, sporocarps, and plant tissue were analyzed by the Stable Isotope Facility at Michigan Technological University. The standard deviations of repeated measurements of a laboratory standard were 0.4‰ for  $\delta^{15}\text{N}$  and 0.1‰ for  $\delta^{13}\text{C}$ .

We looked for sporocarps in all plots as samples were taken throughout the study. Sporocarps were sampled, along with the individual piñon pine under which they were sporulating. In 2004, sporocarps found on the plots were collected and C/N ratios and  $\delta^{15}\text{N}$  were analyzed at the UC Davis Stable Isotope Facility.

Statistical analyses of treatment differences were analyzed using analysis of variance (Zar, 1974) using Statview 5.0 (SAS Institute, 1998). Root count data were analyzed using a Wilcoxon Two-Sample Test (Sokal and Rohlf, 1995). This test is a non-parametric equivalent to a one-way ANOVA and approximates Student's *t*. Because it is a paired non-parametric test, it serves as a non-parametric repeated measures test, the null hypothesis being that the paired differences equal 0. Based on minirhizotron images, we calculated mean counts of the number of observed roots (number  $\text{cm}^{-2}$  minirhizotron tube $^{-1}$ ).

### 3. Results

Fertilization had no overall effect on total soil N beneath either piñon or juniper, although both extractable  $\text{NO}_3^-$ -N and  $\text{NH}_4^+$ -N increased dramatically with fertilization and remained elevated throughout the study period (Table 1). Fertilization did not alter the  $\delta^{15}\text{N}$  signature of the total soil N pool ( $\delta^{15}\text{N} = -0.6\text{‰}$ ,  $p = 0.27$ , between treatment and control plots). When measured in 2001 and 2003, total soil C (in the top 20 cm) had decreased by approximately 36% in fertilized plots under both piñon and juniper relative to control plots, although  $\delta^{13}\text{C}$  did not change ( $-20.5\text{‰}$ ,  $p = 0.50$ ).

Prior to fertilization, there were no differences in the numbers of mycorrhizae between treatments and control plots (in piñon, 119 + / - 46 EM tips  $\text{core}^{-1}$ , with 78% of the tips mycorrhizal, in

**Table 1**

Soil N concentrations of samples collected near randomly-selected root tubes under the canopy of individual trees. Two samples per tree species per plot (six samples per treatment) were analyzed. Differences between fertilized and control trees for each species were determined by analysis of variance.

Year	Plant	Treatment	Total N <sup>a</sup> (g kg <sup>-1</sup> )	NH <sub>4</sub> -N <sup>b</sup> (mg kg <sup>-1</sup> )	NO <sub>3</sub> -N <sup>c</sup> (mg kg <sup>-1</sup> )	Avail P <sup>d</sup> (mg kg <sup>-1</sup> )	Soil C <sup>e</sup> (g kg <sup>-1</sup> )
1998	Pine	Control	1.52	20.3	6.6	20.3	
		N fert	1.76	31.2	25.7	31.2	
	Juniper	Control	1.41	12.0	9.4	12.0	
		N fert	1.69	24.0	16.2	24.0	
1999	Pine	Control	1.76	6.4	3.8	17.2	
		N fert	1.38	16.7	30.6	9.6	
	Juniper	Control	1.51	7.9	4.2	19.4	
		N fert	1.52	16.3	53.3	12.7	
2001	pine	Control	1.18	3.6	1.6	13.8	12.9
		N fert	1.35	193.1	211.5	12.8	10.2
	Juniper	control	1.35	3.4	1.5	10.8	13.4
		N fert	0.82	70.8	75.2	8.9	7
2003	pine	Control	1.77	11.8	1.8	nd	15.4
		N fert	1.62	99.2	37.0	nd	10.2
	juniper	Control	2.08	11.4	1.7	nd	16.5
		N fert	1.38	45.2	9.5	nd	8

<sup>a</sup> total N: year  $p = 0.003$ , plant  $p = 0.39$ , trt  $p = 0.11$ .

<sup>b</sup> NH<sub>4</sub>-N: plant  $p = 0.046$ , trt  $p = 0.0001$ , year  $p = 0.0058$ , year  $\times$  trt  $p = 0.0013$ .

<sup>c</sup> NO<sub>3</sub>-N: plant  $p = 0.20$ , trt  $p = 0.0010$ , year  $p = 0.0018$ , year  $\times$  trt  $p = 0.0009$ .

<sup>d</sup> Avail P: plant  $p = 0.22$ , trt  $p = 0.68$ , year  $p = 0.0008$ , year  $\times$  trt  $p = 0.0025$ .

<sup>e</sup> soil C: plant  $p = 0.26$ , trt  $p < 0.0001$ , plant  $\times$  trt  $p = 0.053$ .

juniper 70% of the root length was AM). During the first growing season (1997), fertilization actually increased the percentage of tips that formed EM (74% in control to 85% in fertilized,  $p = 0.03$ ), but not the total number of EM tips (125 EM tips  $\text{core}^{-1}$ ,  $p = 0.14$ ). There was no treatment effect in %AM or total AM of juniper. From 1998 to 2000, no changes in percentage mycorrhizae with fertilization were observed for either species. In 1997, no changes in the standing crop of roots were found among plots (Lansing, 2003). No significant treatment differences were detected for either species in 1997.

After 1997, the number of mycorrhizal root tips quantified using minirhizotron images, varied as a function of both year and fertilization (Fig. 1). There was a marginal decline in the number of juniper mycorrhizal tips ( $p = 0.08$ ; Kruskal-Wallis non-parametric test) in fertilized plots during 2000, a year of average precipitation. But in piñon, fertilization significantly reduced mycorrhizal root tips in both 2000 ( $p = 0.048$ ) and in 2004 ( $p = 0.028$ ).

We measured leaf nutrient characteristics during fall of 2001. When fertilized, N concentrations increased 20% in pine and 40% in juniper ( $p < 0.0001$ ), and  $\delta^{15}\text{N}$  significantly increased ( $p = 0.045$ ) in both species (Table 2). P concentration declined with fertilization in piñon, but not juniper ( $p = 0.0002$ ). Needle N:P ratio dramatically increased in both plants. With N fertilization N:P ratios in piñon needles increased 91% and 69% in juniper. N fertilization increased leaf production of both tree species (both  $p < 0.001$ ). In piñon, there was also an effect of year ( $p < 0.0001$ ), in that the dry years had significantly lower relative leaf production than wet years (Fig. 2).

A variety of sporocarp species were collected in 1997, an El Niño year. These were mostly EM fungi (*Lactarius deliciosus* (L. exFr.) S.F. Gray, *Russula alutacea* (Fr.)Fr., *Boletus zelleri* Murrill, and *Rhizopogon pinyonensis* Harrison and Smith), with a few saprobes (including *Clitocybe clavipes* (Fr.) P. Kumm.). However, after initiation of the experiment, between 1999 and 2004, we were unable to find sporocarps on the fertilized plots to undertake comparisons. In 2004, during a weak El Niño year, additional sporocarps of the EM fungi *R. alutacea* and *L. deliciosus*, and the saprobe *Geastrum fornicatum* (Huds.) Fr., were collected in the control plots. No fruiting was observed in the N-fertilized plots. Each of the fungal taxa collected had different isotopic signatures (Table 3). The EM fungi had markedly enriched  $\delta^{15}\text{N}$  signatures, relative to the saprobe *G. fornicatum*, which was intermediate. In contrast, piñon had

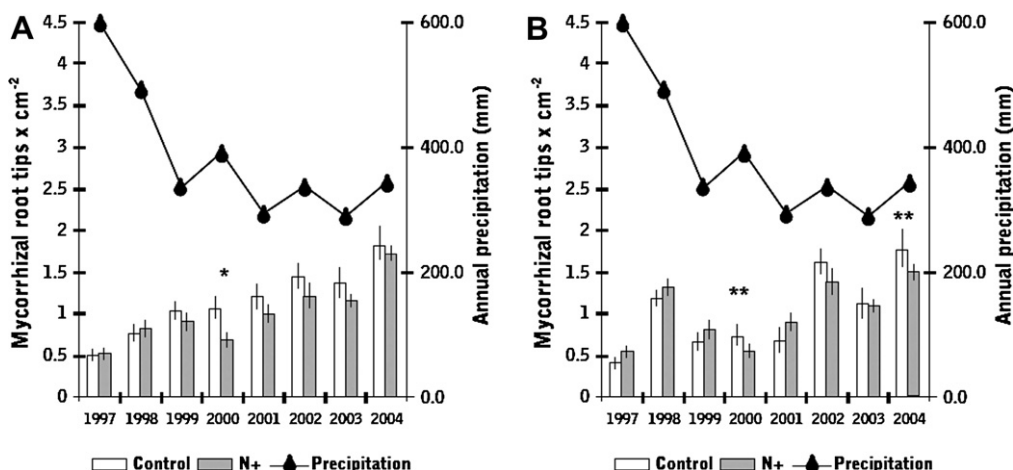


Fig. 1. Numbers of mycorrhizal root tips of juniper (A) and piñon pine (B) contrasting trees in control and N-fertilized plots, and annual precipitation.

a depleted signature relative to its EM fungi. Both EM fungi had slightly less negative  $\delta^{13}\text{C}$  than either the saprobe or the piñon. The C:N ratios were lower in the EM fungi compared with *G. formicatum*, which was exceptionally high for a fungal sporocarp. The piñon C:N ratio was high as expected.

In November of 2001, we began to observe tree mortality in the fertilized plots, but not in the controls. This was notable in that 2000 was an average precipitation year, followed by four dry years. Of the 59 mature piñon pines  $\geq 15$  cm dbh in the three fertilized plots, 11 trees exhibited signs of severe moisture stress. Four trees were dead with needles still attached and seven had numerous dead branches. No mortality of trees or noticeable branch mortality was observed in the control plots. Juniper showed no mortality, but with fertilization, there was a dramatic increase in berry production. By 2003, five additional (for a total of nine) mature piñon trees died in the fertilized plots, compared with only one dead tree in the control plots. An additional 16 mature piñons in the fertilized plots showed signs of bark-beetle damage, compared with 10 trees in the control plots. Saplings ( $< 15$  cm in diameter) appeared unaffected by drought or bark beetles.

Needle  $\delta^{13}\text{C}$  was positively correlated with both leaf %N content ( $r^2 = 0.34, p = 0.0026$ ) and leaf  $\delta^{15}\text{N}$  ( $r^2 = 0.69, p < 0.0001$ ). Multiple regression for needle  $\delta^{15}\text{N}$  and N concentration explained 71% of the variation in  $\delta^{13}\text{C}$ ; adding P to the model further increased the  $r^2$  to 0.80. The  $\delta^{13}\text{C}$  of needles in the fertilized plots (mean difference = 0.245) were marginally lower ( $p = 0.17$ ) than controls. But when  $\delta^{15}\text{N}$  was added to the model as a covariate,  $\delta^{13}\text{C}$  values in fertilized plots were less negative than those from control plots

Table 2

Leaf nutrient status in 2001, the beginning of the drought, when twig and tree mortality was first observed. Two samples were analyzed for each tree species for each plot (six samples per treatment). Values are means (SEM).

Tree	Treatment	N (g/kg) <sup>a</sup>	$\delta^{15}\text{N}_{\text{‰}}$ <sup>b</sup>	P (g/kg) <sup>c</sup>	N:P <sup>d</sup>
Piñon	Control	11.1 (0.40)	-0.37 (0.14)	1.73 (0.07)	6.5 (0.36)
	Fertilized	13.6 (0.40)	0.30 (0.18)	1.12 (0.07)	12.4 (0.77)
Juniper	Control	8.0 (0.26)	-1.43 (0.27)	1.02 (0.06)	8.1 (0.53)
	Fertilized	12.9 (0.54)	-1.16 (0.23)	0.98 (0.06)	13.3 (0.63)

N = 6 for each tree species and treatment. Fertilizer levels were  $100 \text{ kg ha}^{-1} \text{ y}^{-1}$

<sup>a</sup> N concentration: tree species  $p = 0.0002$ , fertilizer treatment  $p < 0.0001$ , tree species  $\times$  treatment  $p = 0.0074$ .

<sup>b</sup>  $\delta^{15}\text{N}$ : tree species  $p < 0.0001$ , fertilizer treatment  $p = 0.045$ , tree species  $\times$  treatment  $p = 0.41$ .

<sup>c</sup> P concentration: tree species  $p < 0.0001$ , fertilizer treatment  $p = 0.0002$ , tree species  $\times$  treatment  $p = 0.0005$ .

<sup>d</sup> N:P: tree species  $p = 0.05$ , fertilizer treatment  $p < 0.0001$ , tree species  $\times$  treatment  $p = 0.578$ .

( $p = 0.07$ ). This trend was more pronounced in piñon than in juniper (plant species by fertilization treatment;  $p < 0.01$ ).

#### 4. Discussion

A major difficulty in understanding long-lived species is that individuals may persist through changing environmental conditions. Based on responses from more mesic ecosystems, we postulated that N fertilization would increase water throughput and C fixation, and reduce allocation to roots and mycorrhizae in piñon-juniper woodlands. We hypothesized that in these semi-arid environments an AM plant, juniper, and an EM plant, piñon, would respond similarly to fertilization. In addition, we asked whether or not responses were short-term allowing these mature trees to adjust physiologically, or if the changes in resource availability that occurred over multiple years would affect the survival of these long-lived individual trees.

Mycorrhizal infection rates for both EM and AM were high and well within the range expected for this ecosystem. Although sporocarps were present during wet years in the control plots, and surrounding trees, no sporocarps have been observed on treatment

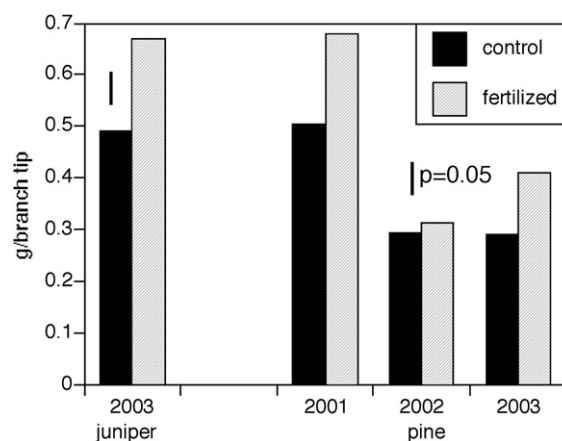


Fig. 2. Relative leaf production of individual branch tips of piñon pine in control and fertilized plots from 2001 through 2003. Leaf production in piñon control and piñon N-fertilized was significantly different in 2001, 2002, and 2003. For piñon, repeated measures ANOVA showed that fertilization ( $p = 0.0013$ ) and year ( $p < 0.0001$ ) were highly significant, but there was no interaction between fertilization and year ( $p = 0.51$ ). Leaf production was also significantly different ( $p = 0.0005$ ) between control and N-fertilized junipers in 2003, the only year available for analysis.

**Table 3**

$\delta^{15}\text{N}$  and C/N ratios of sporocarps and leaf tissue from the associated piñon. After 1997, sporocarps in the plots were only found in the fall of 2004 and only in control plots. No sporocarps were observed in the fertilized plots.

Material	Sample size	$\delta^{15}\text{N}$	C/N
<sup>a</sup> <i>Lactarius deliciosus</i>	7	8.95 (0.32) <sup>a</sup>	18.7 (0.64) <sup>a</sup>
<sup>a</sup> <i>Russula alutacea</i>	6	4.22 (0.83) <sup>b</sup>	16.0 (0.38) <sup>a</sup>
<sup>a</sup> <i>Boletus zelleri</i>	2	9.5 (0.9)	11.6 (0.05)
<sup>a</sup> <i>Rhizopogon pinyonensis</i>	3	9.1 (0.5)	9.1 (2.1)
<sup>b</sup> <i>Clitocybe clavipes</i>	1	3.7	8.7
<sup>b</sup> <i>Geastrum fornicatum</i>	3	-0.13 (0.3) <sup>c</sup>	49.9 (10.3) <sup>b</sup>
<i>Pinus edulis</i>	4	-3.28 (0.23) <sup>d</sup>	57.8 (0.38) <sup>b</sup>

The sporocarps included all found under four individual piñon trees. Values are mean  $\pm$  (SEM). Different letters denote significantly different at  $p > 0.05$ .

<sup>a</sup> ectomycorrhizal fungus.

<sup>b</sup> saprotrophic fungus.

plots since the start of fertilization. This lack of sporulation coupled with the lowered total numbers of EM tips indicates a loss in mycorrhizal functioning. This pattern has been observed in Europe in areas with high levels of N deposition (Karen et al., 1997; Wallenda et al., 2000). There were no differences in the richness of morphotypes or RFLP-types between the fertilized and control plots through 2000 (Lansing, 2003). In August 2000, there was an average of 18.8 morphotypes per plot regardless of the fertilization treatment. There were no consistent differences in RFLP-types between treatments (Treseder et al., 2004). In the AM juniper, no drop in mycorrhizal activity was observed.

Not surprisingly, leaf production increased in both species with fertilization. But in piñon, that production also came with a cost. Initially, N fertilization in this relatively low deposition area (Báez et al., 2007) increased mycorrhizal formation in piñon, but not juniper. Majdi and Andersson (2005) also observed increased EM formation over a two-year period with fertilization in a mesic European forest. However, after the first year, N fertilization resulted in reduced mycorrhizal activity in EM piñon, but, again, not the AM juniper. These lowered mycorrhizal numbers, especially in piñon, could affect tree survival in these semi-arid ecosystems.

Two possible explanations could account for the increased  $\delta^{15}\text{N}$  in fertilized trees. The first hypothesis (Koba et al., 2003) postulated that, although the  $\delta^{15}\text{N}$  of fertilizer is approximately 0‰, the depleted-fraction ammonia may have been volatilized and nitrified, resulting in more enriched  $\delta^{15}\text{N}$ -NO<sub>3</sub><sup>-</sup> (e.g., Högborg et al., 1995). This enriched NO<sub>3</sub><sup>-</sup>-N would be readily available to the tree bypassing mycorrhizal fungi.

Second, with a reduction in EM, more of the N uptake likely came directly from the fertilizer and the expected fractionation between EM fungi and the tree may have been reduced (e.g., Hobbie et al., 1999; Högborg, 1997). EM fungi utilize and transport primarily NH<sub>4</sub><sup>+</sup>-N and organic N from soil (e.g., Read and Perez-Moreno, 2003). In the control plants, the production of new needles required 0.55 g N m<sup>-2</sup>, or 150 nmol kg<sup>-1</sup> per year. But the available nitrate was 0.4 g m<sup>-2</sup>. As soils moistened, soil microbes, and plants (such as grasses) with fine root systems would have rapidly acquired most of the exchangeable N (Collins et al., 2008), which was also subjected to leaching and gaseous loss. The amount actually acquired by the tree was probably far less than the required amount. The increased acquisition of N likely occurred through the extended mycorrhizal hyphae network accessing bound NH<sub>4</sub><sup>+</sup>-N and amino acids in these soils.

To understand the allocation of N and C between piñon and its EM fungi, we estimated net transport of N from fungus to host and the retention of N by the fungus using the equations of Hobbie and Hobbie (2006) based on isotopic fractionation of <sup>15</sup>N from soil to fungus to plant in the control plots. This model assumes that the EM fungi take up NH<sub>4</sub><sup>+</sup> and organic N, and transport <sup>15</sup>N-depleted

amino acids to plants, retaining <sup>15</sup>N-enriched amino acids within fungal tissue. Using this model, 42% (*L. deliciosus*) to 36% (*R. alutacea*) of N in piñon was derived from EM fungi indicating that 58% (*L. deliciosus*) to 64% (*R. alutacea*) of the N extracted from the soil was retained by the fungi. These values were representative of the range of <sup>15</sup>N fractionation of all EM fungal sporocarps collected. Based on the estimated aboveground net primary production (ANPP) of 55 g C m<sup>-2</sup> y<sup>-1</sup> for piñon-juniper woodland (D. Moore, Sevilleta LTER unpublished preliminary estimate), and doubling for total net primary production, we further estimated the allocation of N to EM fungi. Under this scenario, at a C:N ratio of 58 in piñon, 0.95 g N were needed to support current annual needle production. This means that EM fungi transported 0.35–0.40 g N to the host plant for needles. By retaining 1.3 g m<sup>-2</sup> y<sup>-1</sup> of N, at a C:N ratio of 18 (for the fungi), 23.6 g C will be needed for the fungi, or approximately 20% of the total NPP. These calculations could be done only for pine, as arbuscular mycorrhizae do not fractionate N (Hobbie and Hobbie, 2006).

With approximately 5 g m<sup>-2</sup> y<sup>-1</sup> of NO<sub>3</sub><sup>-</sup>-N added by fertilization, during the experiment, 8–40 g m<sup>-2</sup> y<sup>-1</sup> of NO<sub>3</sub><sup>-</sup>-N was potentially available (from Table 1). Production in the fertilized piñon trees was estimated at 83 g C m<sup>-2</sup> y<sup>-1</sup> (AGPP X 1.51, Fig. 2) requiring 1.6 g N. This amounts to only 6.5% of the added fertilizer NO<sub>3</sub><sup>-</sup>-N. Thus, the amount added by fertilization far exceeded the required N for leaf growth along with the NPP of other species and microbial biomass. As there was no production of EM sporocarps in the fertilized plots, we were unable to calculate the amount of NPP allocated to the EM fungi, but the added NO<sub>3</sub><sup>-</sup>-N would certainly provide adequate N without the presence of the mycorrhiza. NH<sub>4</sub><sup>+</sup>-N and any organic N transport would have been surplus. Thus, mycorrhizal fungi likely became superfluous in the N balance of piñon pines and, because the N was not required, any exchange of P or water for C might have been lessened or eliminated.

Assuming that with fertilization the plant had sufficient N, as indicated by the leaf concentrations and the N:P ratios, then other benefits such as water transport and P uptake provided by mycorrhizae would have also been reduced. In piñon pine, fertilization with N reduced P concentrations in needles. In contrast to the piñon, the numbers of roots and AM did not decline in juniper with N fertilization. AM fungi transport NH<sub>4</sub><sup>+</sup>-N, but have a lesser impact on NO<sub>3</sub><sup>-</sup>-N (Tu et al., 2006; Yoshida and Allen, 2001). Both EM and AM are critical to P uptake because P concentrations are extremely low in soil solution and not acquired by mass flow. Mycorrhizal hyphae both scavenge for and mobilize this resource. In dry soils, mycorrhizae also are able to move to patches of moist soil increasing water uptake during dry periods (e.g., Allen, 2007) and acquiring both N and P in dry soils via hydraulic lift (Egerton-Warburton et al., 2008). With N fertilization, EM abundance declined (and EM fungal sporocarp production stopped) but AM abundance did not. Thus, the additional benefits for water and P provided by mycorrhizae would have declined in piñon but not juniper.

The less negative  $\delta^{13}\text{C}$  indicates greater water throughput in trees with higher N concentrations (Ehleringer, 1993; West et al., 2007). In addition,  $\delta^{13}\text{C}$  covaried with  $\delta^{15}\text{N}$  in response to the N fertilization. What this means is that as the fractionation of N due to mycorrhizal transport of N declined and numbers of mycorrhizae declined with fertilization, more water was used by fertilized than unfertilized plants. Yet, mycorrhizae are also important to water uptake (Allen, 2005; Hobbie and Colpaert, 2004). Additionally, the piñon in the fertilized plots would require greater water uptake to maintain the larger needle mass. While this would have improved production during the wet season, it would also have required greater amounts of water to maintain turgor during the dry period, requiring more roots and/or mycorrhizae, not less. Lower numbers

of mycorrhizal fungal hyphae can reduce the uptake of soil water when drought is imposed, placing an added drought stress on the larger needles.

The death of needles at the tips of branches in 2001 and the high mortality rate of mature trees that we observed were also exhibited in the large-scale piñon die-off throughout the American southwest beginning in 2003. Importantly, the mortality observed in our fertilized plots occurred following the high rainfall years of the late 1990s, two years before the large drought-induced mortality across the region. Also crucial, there was no evidence of beetle damage in the dead trees or those showing drought symptoms at our sites. By 2001, the loss of four piñon trees per plot during an average precipitation year corresponded to a mortality of 44.4 trees ha<sup>-1</sup>, a number considered as high mortality by the USDA Forest Service at the peak of the drought in 2003. The loss of nine trees by 2003 is equivalent to a mortality of nearly 100 trees ha<sup>-1</sup>, an extremely high mortality response to the drought (USDA Forest Service, 2005). These values contrast with the loss of only a single piñon in the control plots, and no loss of juniper in any of our plots.

The decline in total soil C in fertilized plots was likely driven by a priming of soil organic matter decomposition (Mack et al., 2004) and an increase in root turnover (Majdi and Andersson, 2005; Pregitzer et al., 2002), coupled with a decline in root-derived C inputs to the soil. Importantly, the C:N ratio dropped from 11 to less than 8 in piñon, and from 10 to as low as 6 under juniper. This suggests that soil C in these low nutrient soils dropped below values commonly found in wild land ecosystems (Zak et al., 1994). The lack of change in soil  $\delta^{13}\text{C}$  ( $p = 0.50$ ) suggested that there was no change in the source of soil organic matter C.

One factor associated with urbanizing regions has been higher rates of atmospheric N deposition associated with drastic increases in transportation (Fenn et al., 2003a). Increasing N may result in increased invasive plant establishment and increased fire frequency due to greater productivity in western forest ecosystems (Fenn et al., 2003b). Understanding interactions of drought, N deposition, and elevated atmospheric CO<sub>2</sub> will be critical to developing models that identify changing fire regimes and plant dynamics. Long-term records demonstrate that drought is a major factor influencing tree distribution (Allen and Breshears, 1998; Breshears et al., 2005). However, the recent expansion of suburban housing into forested landscapes, coupled with fire suppression, elevated CO<sub>2</sub> and N deposition has triggered interest in the importance of anthropogenic factors driving ecosystem change. Across the southwestern USA, high piñon pine mortality was evident during the recent drought (Breshears et al., 2005; USDA Forest Service, 2005). Increased tree mortality was also observed with elevated N of Jeffrey pine in the San Bernardino Mts. during drought (Fenn et al., 2003b). Other studies showing pine mortality with chronic N fertilization include the Harvard Forest Red Pine plot, which had a 56% mortality rate after 15 years (Magill et al., 2004). Today, all mature pines in this plot are dead (Allen, personal observations).

We have shown that increased soil available N resulted in increased mortality of piñon pines. Elevated soil N lowered mycorrhizal activity and increased leaf production in piñon pine. This decline in the root/mycorrhiza area to leaf area ratio would have reduced the capacity of piñon to take up P and to maintain a positive water balance during drought. Other studies (e.g., Haskins and Gehring, 2004) would suggest that once the EM pines are lost, AM juniper could well restrict the re-establishment of the EM and with these fungi, the pines. Other similar EM/AM conifer mixtures occur elsewhere, such as EM mixed pines and AM cedars in southern California. Thus, chronic environmental changes, such as elevated rates of atmospheric N deposition, are likely to interact with periodic drought to alter ecosystem structure and function in mixed conifer woodlands such as piñon-juniper woodlands in the western US.

## Acknowledgements

We thank William Swenson and Chris Glover for help with the minirhizotron observations and the Sevilleta Long-term Ecological Research project for use of field sites and equipment. This research was funded by the US National Science Foundation numbers DEB 9996211, DEB 9981548, EF 0410408, and DEB 0080529.

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