Interactions of arbuscular mycorrhizal fungi, critical loads of nitrogen deposition, and shifts from native to invasive species in a southern California shrubland

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1 **Interactions of arbuscular mycorrhizal fungi, critical loads of**
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3 **southern California shrubland**

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Abstract: Anthropogenic nitrogen (N) deposition and invasive species are causing declines in global biodiversity, and both factors impact the diversity and functioning of arbuscular mycorrhizal fungi (AMF). Shifts in AMF communities also have feedbacks on native plant success, as was observed in California coastal sage scrub, a Mediterranean-type shrubland threatened by invasive grasses. As vegetation-type conversion from native shrubland to exotic annual grassland increased along a gradient of increasing N deposition, the richness of native plant species and of AMF spore morphotypes decreased. Rapid declines in all plant and fungal values occurred at a critical load (CL) of 10-11 kg N ha⁻¹yr⁻¹, indicating that AMF respond to the same environmental signals as the plants and can be used to assess CL. Shrub root colonization also decreased along the N gradient, but colonization of the invasive grass was dominated by a fine AMF endophyte that was unresponsive to elevated N. A greenhouse experiment to assess AMF functioning showed that the native shrub *Artemisia californica* had a negative growth response to inoculum from high-N but not low-N soils, while the invasive grass *Bromus rubens* had a positive response to both inocula. Differential functioning of AMF under N deposition may in part explain vegetation-type conversion and the decline of this native shrubland.

Keywords: fine endophyte, *Glomus tenue*, nitrogen critical load, vegetation-type conversion

Introduction

Anthropogenic nitrogen (N) deposition has recently been documented as a cause of decline of shrub- and grassland ecosystems (Fenn et al. 2010; Bobbink 2010; Rao et al. 2010; Cox et al. 2014). These ecosystems are subject to loss of native plant diversity (Stephens et al. 2010) and vegetation-type conversion to exotic annual grasslands (Fenn et al. 2010; Rao et al. 2010; Cox et al. 2014). Such conversions may be mediated by the direct effects of N fertilization on plants with traits that make some species more productive or competitive than others (Suding et al. 2005; Dimbick et al. 2014), or indirectly through the effects of N on mycorrhizal fungi. Elevated N causes decreases in arbuscular mycorrhizal fungal (AMF) root colonization and spore production (Egerton-Warburton and Allen 2000; Johnson et al. 2003; Egerton-Warburton et al. 2007; van Diepen et al. 2011), and a predominance of poor AMF mutualists that promote reduced growth of host plants (Johnson 1993).

Invasive species increase under N deposition when they are more responsive to N than native species (Rao and Allen 2010). These invasive species have direct impacts on native species composition (Cox et al. 2008, Steers et al. 2011), which may also be mediated by mycorrhizal fungi (Marler et al. 1999; Pringle et al. 2009). Furthermore, invasive species cause feedbacks on soil microbial composition, including shifts in AMF species composition and diversity reductions (Sigüenza and Allen 2006a; Mummey and Rillig 2008; Barto et al. 2011; Busby et al. 2012; Hilbig and Allen 2015). Shifts in AMF species composition have important feedbacks to their hosts by changing the physiological response of their host plants, and plant

species responses to AMF infection vary within a plant community by AMF species identity and plant species dependence on AMF (Allen et al. 2003; Klironomos 2003).

In southern California, which receives high levels of N deposition due to air pollution (Fenn et al. 2010), N deposition has been implicated in the widespread conversion of coastal sage scrub (CSS) to exotic annual grasslands (Allen et al. 1998; Fenn et al. 2010; Cox et al. 2014). Coastal sage scrub is a drought-deciduous Mediterranean-type shrubland with high species richness and endemism that has been reduced to a small fraction of its historic range as a result of land-use change, invasion and increased fire frequency. It is one of the most endangered ecosystems globally with losses in plant species richness across much of its range (Cleland et al. 2016). The role of mycorrhizae in the interactions between exotic annual grasses and native shrubs has been tested (Yoshida and Allen 2004; Sigüenza and Allen 2006b), but the role of AMF in the shift to exotic annual grasslands has not been considered on the landscape scale, and will be considered here.

Furthermore, the decline in AMF, similar to the decline in native plant species richness, may be used as an indicator of critical load (CL) for N deposition. A CL for N deposition is that amount of N below which there are no negative impacts on an ecosystem (Pardo et al. 2011). These can be measured as changes in structure (e.g., species richness, vegetation-type conversion) or function (e.g., mycorrhizal functioning, increased fire due to invasion). Cox et al. (2014) calculated a CL of 11 kg N ha⁻¹yr⁻¹ for the shift from native CSS to exotic annual grassland dominated by Mediterranean invasive grasses, but this study was focussed on vegetation rather than AMF.

During the last 20+ years, we have undertaken a series of studies to understand how anthropogenic N deposition affects plants and AMF of CSS. The objectives of this review are to synthesize previous studies on the relationships between N deposition, native and invasive species, and AMF activity, especially pertaining to CSS, and to compare this information with a more recent analysis of CL for N deposition in CSS (Cox et al. 2014). The specific objectives are to review (1) impacts of N deposition on abundance of native and invasive species of CSS in California; (2) impacts of N deposition on arbuscular mycorrhizal diversity and activity; (3) critical loads for N deposition impacts on AMF and relate these to CL for vegetation-type conversion; and (4) functional responses of native and invasive species to shifts in AMF diversity and to elevated N.

Nitrogen deposition gradient in coastal sage scrub

Before proceeding with N deposition impacts on AMF and associated CSS vegetation, we review the N deposition values that occur in this vegetation type. After the EPA Community Multiscale Air Quality (CMAQ) model of N deposition for California became available (Fenn et al. 2010), we were able to calculate critical loads of N deposition for impacts on plants and AMF. The California version of CMAQ was generated at a 4×4 km scale for 2002, which provides finer resolution than the 12×12 km national CMAQ model. The N deposition values in southern California CSS range from 2 to 20 kg N ha⁻¹yr⁻¹ (Allen et al. 2014), with 2 being the background level in unpolluted areas and 20 the highest value in wildland areas downwind of urban Los Angeles. The range of high values across the U.S. is also >20

kg N ha⁻¹yr⁻¹ (NADP 2013) so values for N deposition in CSS are as high as any found in the U.S. By contrast, the highest values in a European grassland N deposition gradient were near 40 kg N ha⁻¹yr⁻¹ (Stephens et al. 2010), i.e. higher than recorded U.S. values. Both oxidized and reduced forms of N are deposited, as nitrate/nitric acid from internal combustion, and ammonia/ammonium from agriculture and catalytic converters (Fenn et al. 2010). About 90% is deposited in the dry form during the long summer dry season in this Mediterranean-type climate and accumulates on soil surfaces (Padgett et al. 1999, Bytnerowicz et al. 2016). The N deposition gradient we studied was in CSS in western Riverside County, and ranged from 8.7 to 19.6 kg N ha⁻¹yr⁻¹ (Fig. 1).

Growth of individual CSS plants under elevated N

Elevated N preferentially increases the growth and rate of N uptake of the dominant invasive grass *Bromus rubens* (= *B. madritensis* subsp. *rubens*) over the dominant native shrub it is displacing *Artemisia californica* (Yoshida and Allen 2001; 2004). When individuals of *B. rubens* and *A. californica* were grown individually in pots in sterile soil, growth rates of both species were similar, although mortality of *A. californica* was high at the highest level of N fertilization (Padgett and Allen 1999). However, when individuals were grown in non-sterile soil, AMF-colonized roots of *B. rubens* acquired labeled ¹⁵NH₄⁺ (but not ¹⁵NO₃⁻) at a higher rate than *A. californica* (Yoshida and Allen 2004). This suggests that *B. rubens* can take up N from NH₄⁺-dominated field soils (most of the soils in the region are slightly acidic in pH and NH₄⁺-dominated; Padgett et al. 1999). However, these growth results of individual

plants in pots are insufficient to explain the role of N deposition and AMF in the shift from shrubland to grassland, and further studies in the field plus greenhouse are presented that are more revealing.

Plant community shifts in response to N fertilization and AMF implications

Studies in California grasslands have shown that N fertilization or N deposition increases exotic annual grasses preferentially over native grasses and forbs (Huenneke et al. 1990, Weiss 1999). Similarly, invasive grasses increased in abundance in southern California CSS with increased N availability (Fig. 2a, b). CSS vegetation was fertilized beginning 1994 with 60 kg N ha⁻¹yr⁻¹ as NH₄NO₃ following a 1993 wildfire in an area of relatively low N deposition (Fig. 2a, b). The biomass of exotic annual grasses (predominantly *B. rubens*) increased in N-fertilized plots, while the cover of native shrubs, dominated by *A. californica*, decreased. As this community reassembled from bare ground following the fire, competition from productive, N-fertilized *B. rubens* likely reduced shrub seedling establishment, as was previously demonstrated in a grass removal experiment (Eliason and Allen 1997). However, AMF may also be implicated, as AMF colonization of native shrub roots was lower in plots with elevated N (Egerton-Warburton and Allen 2000; Sigüenza et al. 2006b), but there was no significant difference in AMF colonization between N-fertilized and unfertilized *B. rubens* roots in these plots (Sigüenza et al. 2006b). Loss of AMF may negatively impact the hyphal network that promotes water transport to shrubs during the summer dry season (Allen et al. 2010, Querejeta et al. 2012), further reducing their growth.

Critical load for loss of plant richness on a N deposition gradient

The N fertilization experiments reported above confirmed the patterns in native and invasive species shifts on the N deposition gradient, where native species richness declined from 67 to 15 species between 8.7 to 19.6 kg N ha⁻¹yr⁻¹ (Allen et al. 2014; Table 1). Based on the steep drop in species richness between 9.6 and 11 kg N ha⁻¹yr⁻¹, we estimated that the CL for species richness loss is ~10 kg N ha⁻¹yr⁻¹. Percent cover of native forbs and shrubs also declined steeply along this gradient, while cover of invasive annual grasses increased (Table 1). The low level of N deposition we observed in this CSS gradient is not the lowest level of N found in southern California, and precludes testing the response at lower levels at this site. For instance, impacts of N deposition on plant species richness in European grasslands were reported by Stephens et al. (2010), who observed species losses over an N deposition range of 2 to 40 kg N ha⁻¹yr⁻¹. However, lower levels of N deposition occur in the Venturan (coastal) association of CSS, and this N gradient is the topic of current research in the Santa Monica Mountains. Studying a gradient with lower N deposition may result in a lower CL for loss of species richness.

This observation of CL for loss of plant species richness is based on relatively few sites in which a steep drop in richness occurs above 9.6 kg N ha⁻¹yr⁻¹. To confirm this value, a more rigorous approach to estimate CL was taken by quantifying vegetation-type conversion from native shrubland to exotic annual grassland (Cox et al. 2014). A vegetation survey from 1930 was compared with a modern resurvey from 2002. The analysis of 150 sites showed that 35% of CSS

converted to exotic grassland within 72 years, and mostly in areas with high N deposition. The probability of type-conversion to exotic grassland increased steeply at 11 kg N ha⁻¹yr⁻¹ (Cox et al. 2014). We took this to be the CL for vegetation-type conversion, similar to the CL for richness loss (Table 1).

AMF activity across N gradients as indicators of critical load

A similar loss of AMF spore morphospecies, reduced percent root colonization and spore density in rhizospheres of native CSS shrubs (*A. californica*, *Encelia farinosa*, *Eriogonum fasciculatum*) occurred on this N gradient (Egerton-Warburton and Allen 2000; Table 2, Table 3). Root colonization declined from 45 to 19% and spore density declined from 105 to 23 spores/g soil with elevated N (Table 2). A total of 24 species of AMF were observed, with 19 being the highest number at any site (Table 3; Egerton-Warburton and Allen 2000). Spore species richness declined from 19 to 11 per site across the N gradient. The greatest decline was in the 8 species of large-spored genera (*Scutellospora* [3 species], *Acaulospora* [2 species], *Gigaspora*, *Entrophospora*, and *Sclerocystis* [1 species each]), which declined from a total of 37% to 7% of the total species richness with elevated N (Table 3). The remaining 16 species were all *Glomus* spp. *Glomus aggregatum* was the most abundant species in density at all sites, and increased further with elevated N. Other *Glomus* species either declined in density with elevated N or showed no discernible pattern in response to N (Egerton-Warburton and Allen 2000).

The declines in AMF richness and percent colonization were primarily a function of elevated N rather than host species (shrub vs. grass). We believe this to

be the case because soil and root samples were collected under shrubs that occurred in remnant patches even in regions with high N deposition. Rhizosphere soils of grasses were not collected for this study (Egerton-Warburton and Allen 2000). Furthermore, exotic grasses occur throughout the gradient, although more extensively across the landscape under elevated N deposition. A reduction in shrub root colonization with elevated N may be triggered by molecular signaling and reduced provisioning of photosynthate to AMF (reviewed in Egerton-Warburton et al. 2000; Johnson et al. 2003). Soils of this region are relatively high in phosphorus with 30 $\mu\text{g/g}$ P (Padgett et al. 1999). Reduced mycorrhizal activity following N eutrophication is especially pronounced in soils with initially low N:P ratios, such that elevated N causes both N and P to be in high supply (Johnson et al. 2003). Preferential loss of large-spored species with elevated N may be related to their greater carbohydrate demand for reproduction (reviewed in Egerton-Warburton and Allen 2000, Egerton-Warburton et al. 2007).

The patterns in reduction of AMF activity are largely similar to loss of plant species richness under N deposition. The CL for decline of AMF activity is above 9.6 $\text{kg N ha}^{-1}\text{yr}^{-1}$ (Tables 2, 3). This corresponds to plant species richness decline above 9.6 $\text{kg N ha}^{-1}\text{yr}^{-1}$ (Table 1) and vegetation type conversion at $\sim 11 \text{ kg N ha}^{-1}\text{yr}^{-1}$ from native shrubland to exotic annual grassland (Cox et al. 2014). Both decreased shrub AMF activity and loss of plant species richness are related to increased soil N, but native plant richness decline is also related to increased invasive grass productivity. This suggests AMF spore richness and percent infection of shrub roots can be used as indicators of elevated N, as they declined under sparse, remaining shrubs

sampled in the high deposition sites (Egerton-Warburton and Allen 2000). Alternatively, the grasses tend to form associations with fine AMF (Sigüenza et al. 2006a; 2006b; Hilbig and Allen 2015), which might cause a decline in spore richness even in the absence of N deposition. However, spore richness was not quantified for grass rhizospheres. In support of our shrub rhizosphere analysis, a retrospective study using archived soil samples from a higher elevation chaparral site in the Los Angeles air basin showed a decline in density of all spore species and a loss of large spored genera between 1937 and 1961, with further declines through 1999 (Egerton-Warburton et al. 2001). In this site, soil $\delta^{15}\text{N}$ values (an indicator of N emissions from internal combustion) were elevated since at least 1958. As a result, N deposition has affected AMF activity for a sufficient time to cause feedbacks to the plant community.

Feedbacks of N-fertilized AMF inoculum on invasive and native plant species

Another analysis of AMF activity was done along the same N deposition gradient (Egerton-Warburton and Allen 2000), that included the invasive grass *B. rubens* as well as the native shrub *A. californica* (Sigüenza et al. 2006b). Spore counts (richness was not assessed) in the rhizosphere of the invasive grass did not decline significantly with elevated soil N, while spore counts of the native shrub decreased, the latter also observed by Egerton-Warburton and Allen (2000). Furthermore, approximately one-half of *B. rubens* root colonization was by a fine AMF endophyte, *Glomus tenue* (with up to 80% total root infection, sum of coarse plus fine endophyte). In contrast, *A. californica* roots showed only traces of fine

AMF colonization (Sigüenza et al. 2006b), while *G. tenue* spores and root infection were not observed in shrub rhizospheres by Egerton-Warburton and Allen (2000). The next step was to determine whether there is a functional response of mycorrhizal fungi in the success of *B. rubens* and the poor performance of *A. californica* under N deposition.

We report on a greenhouse study to demonstrate feedbacks of N-deposition altered AMF communities on growth of *B. rubens* and *A. californica* (Sigüenza et al. 2006a). Three inoculum treatments from this study were: A) soil inoculum from low N deposition site (8 kg N ha⁻¹yr⁻¹); B) soil inoculum from high N deposition site (20 kg N ha⁻¹yr⁻¹); and C) sterile soil (sterilized inoculum from high deposition and low deposition sites to balance the nutrients). Each of the inoculum treatments was given a high or low N fertilizer treatment. The potting mix was soil from the low N deposition site, and the N fertilization treatment had added NH₄NO₃ to increase N to levels found in soils of the high N deposition site.

Bromus rubens responded positively to both inoculum sources in comparison to sterile controls in low N soil, but there were no significant effects of inoculum in high N soil (Fig. 3a). Conversely, *A. californica* seedlings had a negative response to inoculum in high and low N soils, and inoculum from high N deposition soil was especially detrimental to its growth. The greenhouse study of Sigüenza et al. (2006a) was done under a moderate water regime, i.e., pots were watered to field capacity and surface soils allowed to dry without wilting seedlings. Recently, we performed a variation on this study by watering pots to field capacity or limiting soil moisture to simulate drought stress in *A. californica* seedlings, and found that

inoculum from high N deposition soil was especially detrimental under drought stress (Valliere and Allen 2016).

In a greenhouse study by Sigüenza et al. (2006a), as in the field (Sigüenza et al. 2006b), *B. rubens* was colonized by fine AMF hyphae, likely *Glomus tenue*, while *A. californica* was colonized by coarse hyphae. The negative response of *A. californica* seedlings to AMF colonization may be related to the high P (30 $\mu\text{g g}^{-1}$; Padgett et al. 1999) of these soils, as high P may cause growth reductions in mycorrhizal plants, especially at the seedling stage (e.g., Johnson et al. 1997; Allen et al. 2003).

Alternatively, *Glomus tenue* may have promoted a positive growth response in *B. rubens* in these high P soils under low N relative to sterile controls. Under elevated N, there was no significant difference in biomass response to inoculation in the greenhouse experiment, even though there was a reduction in percent mycorrhizal colonization in high N deposition soil (Sigüenza et al. 2006a). Nonmycorrhizal, potentially pathogenic fungi were also assessed microscopically in this study, and their colonization was greater for both species only in one treatment, high N deposition inoculum under high N fertilizer (Sigüenza et al. 2006a), so incidence of pathogens does not explain the growth responses in this study. These findings suggest that *G. tenue* is an effective mutualist even at low levels of root colonization and under high soil N availability, (Hilbig and Allen 2015). *Glomus tenue* (syn. *Rhizophagus tenuis*) is commonly found in soils of harsh and disturbed environments (Olsson et al. 2004), and is considered to be more frequent in pioneer plants (Blaschke 1991). A positive feedback from *G. tenue* was also found to contribute to the overall success of the exotic annual *Bromus diandrus* by

compensating for negative impacts of putative pathogens in an abandoned agricultural field (Hilbig and Allen 2015).

Conclusions

By synthesizing a series of studies on AMF and plant response with recently available data on N deposition (Fenn et al. 2010), we have shown that AMF may be an important indicator of CL for N deposition, and may also have a central role in the vegetation-type conversion of coastal sage scrub to exotic annual grassland. Threshold losses in AMF morphospecies richness, native plant species richness, and vegetation-type conversion from native shrubland to exotic annual grassland all occurred at 10-11 kg N ha⁻¹yr⁻¹. These losses in AMF diversity co-occurred with a decrease in AMF root colonization in *A. californica* but not *B. rubens* in field-collected samples (Sigüenza and Allen 2006a). Furthermore, AMF of *A. californica* are sensitive to elevated N, producing small seedlings that are likely unable to compete with fast-growing *B. rubens* with fine AMF that are insensitive to elevated N (Sigüenza and Allen 2006b). By combining these studies, we suggest that processes occurring on very small scales of individual plant-mycorrhizal interactions can have ramifications on the landscape scale for vegetation-type conversion under the impact of N deposition.

Further evidence for AMF-induced vegetation changes may come by studying additional invasive and native species of CSS, and by expanding these studies to other ecosystem types. For instance, a closely related invasive grass, *B. tectorum*, is causing vegetation type conversion throughout *Artemisia tridentata* shrublands of

the western U.S. even in the absence of N deposition, but most of the invasion appears to be in soils that are naturally high in N and P (Belnap et al. 2016). Losses of richness in European grasslands and shrublands is not related to invasive species, but rather to increases in native grass species under N deposition (Bobbink et al. 2010; Stephens et al. 2010). The role of AMF under changing nutrients has been little explored in these situations of species loss, and deserves further study.

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500

1 Table 1. Percent cover and richness (number of species per 3 ha) of plant groups
 2 along an N deposition gradient in western Riverside County. Sites are arranged from
 3 north to south along an urban to rural gradient. Forb species richness in bold shows
 4 a rapid drop in richness, suggesting a critical load of 10 kg N ha⁻¹ y⁻¹ based on the
 5 CMAQ model of N deposition (Fenn et al. 2010).

Site	Exotic grass % cover	Native forb % cover	Shrub % cover	# Native forb species	Soil N μg g ⁻¹	N deposition kg N ha ⁻¹ y ⁻¹
Jurupa Hills	63.5	4.0	2.2	16	37.7	19.6
Box Springs	69.2	18.5	2.4	31	32.6	14.7
Botanic						
Garden	36.0	25.4	0.2	20	28.9	13.4
Lake Perris	0.5	26.1	2.8	30	20.3	11.1
Mott Reserve	6.7	14.3	11.2	37	30.6	11.1
Lopez						
Canyon	11.1	19.6	19.3	67	9.6	9.0
Tucalota						
Hills	1.5	35.7	35.0	50	10.5	8.7

6

7

8 Table 2. Spore counts and percent root colonization of AMF along a N deposition
9 gradient in western Riverside County. Sites are arranged from north to south along
10 an urban to rural gradient (data from Egerton-Warburton and Allen 2000). Values in
11 bold show a drop in spore density and root infection and are interpreted to
12 represent a critical load.

Site	Spores/ g soil	% root colonization	Soil N $\mu\text{g g}^{-1}$	N deposition $\text{kg N ha}^{-1} \text{y}^{-1}$
Jurupa Hills	23	19	86.2	19.6
Waterman Rd.	54	18	15.6	16.9
Box Springs Mt.	66	17	26.4	14.7
Mockingbird Cyn	74	24	56.8	12.5
Lake Mathews	70	23	39.5	11.1
Motte Reserve	80	27	26.1	11.1
Hemet	78	22	12.8	9.6
Lake Skinner	103	39	7.5	8.7
Santa Margarita	105	45	9.7	8.7

13

14 Table 3. Diversity of AMF spores decreased with increasing extractable soil N along the N deposition gradient of Western
 15 Riverside County. Many large-spored species failed to reproduce in high-N soil (Egerton-Warburton and Allen 2000). Modeled
 16 N deposition values from Fenn et al. (2010).

Site	Santa Margarita	Lake Skinner	Hemet	Motte Reserve	Lake Mathews	Mockingbird Cyn	Box Springs Mt.	Jurupa Hills
Soil N ($\mu\text{g g}^{-1}$)	9.7	7.5	12.8	26.1	35.0	39.5	26.4	86.2
N deposition ($\text{kg N ha}^{-1} \text{y}^{-1}$)	8.7	8.7	9.6	11.1	12.5	12.5	14.7	19.6
Diversity (H')	0.766	0.564	0.269	0.422	0.579	0.315	0.163	0.243
No. AMF species	19	19	11	18	15	14	14	12
No. Large-spored taxa	7	4	2	4	4	2	1	1
% Large-spored taxa	36.8	21.1	18.2	22.2	26.7	14.3	7.1	8.3

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Figure Captions

Fig. 1 Nitrogen deposition in western Riverside County, southern California, showing a gradient 60 km long that ranges from 8.7 to 19.6 kg N ha⁻¹yr⁻¹ and lies within coastal sage scrub vegetation. Map created by Robert Johnson, Center for Conservation Biology, UC Riverside.

Fig. 2. A.) Percent cover of native shrubs following a 1993 fire in coastal sage scrub in plots that were fertilized with N and controls. B.) Biomass of exotic grass *Bromus rubens* in control and N fertilized treatment. P indicates significance for repeated measures analysis, * = $P < 0.05$ for individual dates (E. Allen, unpublished data).

Fig. 3. Greenhouse growth responses of the invasive grass *Bromus rubens* and the native shrub *Artemisia californica* to soil inoculum from sites with low and high N deposition compared to sterile soil (NM = nonmycorrhizal) under two levels of levels of soil N fertilizer. Different letters indicate significantly different at $P < 0.05$. Redrawn using data from Sigüenza and Allen (2006a).

Los Angeles

Riverside