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REGULAR ARTICLE

Nitrogen deposition decreases the benefits of symbiosis in a native legume

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Abstract
Aims Anthropogenic nitrogen deposition can provide legumes with a cheap source of nitrogen relative to symbiotic nitrogen fixation, leading to the potential breakdown of this critical symbiosis. Here, the effects of nitrogen deposition were tested on a native symbiosis between legumes and rhizobia.
Methods Deposition rates, soil nitrogen concentration, and plant nitrogen isotopic composition were quantified along a predicted deposition gradient in California. *Acmispon strigosus* seedlings were exposed to fertilization spanning nitrogen concentrations observed in the plant's California range. Both wild and experimental plants from pristine and nitrogen polluted sites were tested using rhizobial strains that varied in nitrogen fixation.
Results Deposition intensity was tightly correlated with nitrogen concentration in soils. The growth benefits of rhizobial nodulation were dramatically reduced by even modest levels of mineral nitrogen, and all *Acmispon* lines failed to form root nodules at high nitrogen concentrations.
Conclusions Our dataset suggests that anthropogenic deposition has greatly increased soil nitrogen concentrations in Southern California leading to significantly reduced benefits of rhizobial symbiosis. If nitrogen deposition increases continue, plant host mortality and a total collapse of the symbiosis could result.

Keywords Anthropogenic nitrogen deposition · Biological nitrogen fixation · Legume rhizobium symbiosis · Mutualism breakdown

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Introduction

In the legume-rhizobium symbiosis, rhizobia form nodules on the roots of legume hosts and fix dinitrogen (N_2) into ammonium (NH_4^+) and other chemically active forms of nitrogen (N_r; i.e., all N species other than N_2; (Galloway et al. 2013)). Prior to industrialization, biological nitrogen fixation (BNF) from this symbiosis dominated natural inputs of nitrogen into terrestrial ecosystems (Cleveland et al. 1999). Human industrial activity in the past 150 years has more than doubled N_r production globally and the total rate of anthropogenic

N_r production is increasing (Vitousek et al. 1997; Cleveland et al. 1999; Galloway et al. 2004, 2008). Most anthropogenic N_r is emitted into the atmosphere as gaseous NO_x and NH_3 (Galloway et al. 2004) that can be deposited into aquatic and terrestrial ecosystems (Vitousek et al. 1997). As industrialization has spread over the last century, N_r enrichment driven by nitrogen deposition has become global in scale (Galloway et al. 2004; Dentener et al. 2006; Holtgrieve et al. 2011).

Most atmospheric N_r deposition into terrestrial ecosystems likely occurs on historically nitrogen-limited soils (Vitousek et al. 1997; Padgett et al. 1999; Egerton-Warburton et al. 2001). N_r deposition and the resultant fertilization of soils can reduce plant species richness (Roem et al. 2002; Carroll et al. 2003; Maskell et al. 2006; Clark and Tilman 2008; Maskell et al. 2010) by altering outcomes of competitive interactions among plants, and by making the environment unfavorable for nitrogen-sensitive species (Bobbink et al. 2010). N_r deposition can also alter composition of soil fungal communities (Egerton-Warburton et al. 2001) and harm soil bacteria that decompose litter (Janssens et al. 2010; Hobbie et al. 2012; Kamble et al. 2013). Finally, N_r deposition can negate the benefits of plant-microbe symbioses in which root-associated bacteria and fungi provide N_r to plants in exchange for photosynthates. In the case of mycorrhizal fungi, some N_r-enriched soils can render these symbionts superfluous to host plants (Johnson et al. 1997; Egerton-Warburton et al. 2001; Hoeksema et al. 2010; Kivlin et al. 2013). In contrast, less work has examined consequences of N_r deposition for rhizobial symbiosis, despite the central role of rhizobia in terrestrial BNF.

N_r fertilization can reduce or eliminate the immediate growth benefits of rhizobial nodulation for legumes (Regus et al. 2014, 2015) in part because soil N_r can be less costly for legumes to use than biologically fixed nitrogen (Voisin et al. 2002). In the short term, some legumes have been shown to reduce nodule formation when exposed to high concentrations of nitrate (Streeter 1988), but it is unknown whether plants reduce nodule formation in response to a loss of benefit from rhizobial nodulation or other factors such as nitrogen toxicity. Moreover, the nodulation response to nitrogen addition can depend upon both the plant and the rhizobial genotype (Heath et al. 2010). Over longer time scales, exposure to N_r deposition and enrichment could favor plants that adapt to better utilize mineral N_r for growth or tolerate high soil N_r concentrations, as can occur in agricultural systems (Herridge and Danso 1995). Moreover, increased N_r concentrations in soil is predicted to lead to plants that depend less on BNF and thus evolve relaxed control over rhizobia (Kiers et al. 2007; Akcay and Simms 2011; Regus et al. 2014; Weese et al. 2015).

Here we examined both the immediate and potential evolutionary effects of nitrogen deposition on legume-rhizobium interactions. Populations of the native annual legume *Acmispon strigosus* (formerly *Lotus strigosus*) are found throughout much of California, including sites that are predicted by simulation models to receive little N_r deposition (coasts and high deserts) and regions with predicted intense N_r deposition (Los Angeles and Santa Ana River basins; (Fenn et al. 2010)). To infer the relationship between nitrogen deposition and soil fertility at our field sites, we quantified atmospheric deposition rates and soil nitrogen across the predicted deposition gradient. To quantify the relative contributions of symbiotic versus mineral nitrogen fixation at different sites, we conducted nitrogen isotopic analyses on wild collected host seeds and also on host plants inoculated with soil rinsates. Finally, we generated four plant lines sourced from two *Acmispon* populations at opposite extremes of predicted deposition and exposed them to an experimental gradient of mineral N_r concentrations in the greenhouse. Plants were grown axenically or were exposed to one of two single-strain rhizobial inoculation treatments that represent the most and least effective strains we have tested (Sachs et al. 2010a, 2011). Previous work on *A. strigosus* showed that host differential investment to effective versus ineffective rhizobia was not affected within a range of nitrogen fertilization (Regus et al. 2014), but this range is greatly expanded upon here and multiple plant genotypes are tested. We examined how plants responded to the simulated N_r deposition gradient and whether the response depended on the plant's past history of N_r deposition.

Materials and methods

Atmospheric sampling and deposition estimates

We measured daily ambient atmospheric concentrations of gaseous nitrogen species (NH_3, NO_2, HNO_3) at eleven *A. strigosus* populations in California (Table 1) using passive samplers and following published methods (Bytnerowicz et al. 2002). We also measured deposition

of aerosol nitrogen species (particulate fraction of NH_4^+ and NO_3^-) which is calculated as a fraction of ambient gas concentrations following Zhang and colleagues (Zhang et al. 2003). The fractions of NH_3 and HNO_3 were based on mean concentrations of HNO_3, NO_3^-, NH_3, and NH_4^+ measured in the San Bernardino Mountains in southern California (Bytnerowicz and Fenn 1996). Measurement periods included July 2012, September 2012, February 2013, and August 2013.

Deposition of gaseous nitrogen species into soils was calculated as the product of daily ambient gas concentrations and gas deposition velocity (Hanson and Lindberg 1991), for which we used published average values for land use categories (LUC) that best correspond to each sampled site (Table 1; (Zhang et al. 2003)). Annual dry nitrogen deposition was calculated as the sum of deposition of atmospheric gaseous nitrogen species (NH_3, NO_2, HNO_3) and deposition of aerosol nitrogen species (NH_4^+, NO_3^-). Total annual nitrogen deposition was calculated as the sum of annual dry nitrogen deposition and estimated wet deposition. Based on historical precipitation records, wet nitrogen deposition was estimated as 1.0 kg N ha^{-1} $yr.^{-1}$ for inland sites and 1.5 kg N ha^{-1} $yr.^{-1}$ for the coastal sites (Table 1; (EPA 2012).

Soil nitrogen sampling

We estimated concentrations of extractable NO_3^- and NH_3 in soil at the same eleven *A. strigosus* populations using published methods (Regus et al. 2014). For each plant population we sampled three soil cores (10 cm depth) along a 3 m transect where *A. strigosus* plants had been collected previously. Soil samples were collected in February and August 2013. Soil was sieved, dried, and analyzed using published methods (Santiago et al. 2005). Nitrogen analysis was performed at the FIRM Isotope Facility at UC Riverside. We used regression analysis to determine the relationship between annual rates of atmospheric nitrogen deposition and the local concentrations of extractable NO_3^- and NH_3 (collectively, mineral N) in soils.

A. strigosus plant lines

We developed lines of *A. strigosus* from seeds collected at the Bodega Marine Reserve (BMR) in Northern California and from a natural site at the University of California, Riverside (UCR) in Southern California. Simulation models predict that BMR experiences negligible N_r deposition (e.g. < 5 kg N_r ha^{-1} $yr.^{-1}$) and that UCR has high levels of deposition (e.g. > 20 kg N_r

Table 1 Field Sites and measures of atmospheric and soil nitrogen

Site	Land Use Category[a]	Total Soil N ± (%)[b]	Mineral N (ppm)[b]	Modeled N Deposition (kg ha^{-1} $yr.^{-1}$)[c]	Dry N Deposition (kg ha^{-1} $yr.^{-1}$)	Total N Deposition (kg ha^{-1} $yr.^{-1}$)[d]
Anza-Borrego	Deserts (24)	0.01 ± 0.01 c	2.02 (0.20)d	5.0–7.0	1.68	2.68
Bernard Field Station	Urban (21)	0.11 ± 0.01 a	10.81 (1.78)b,c	>25.0	7.42	8.42
Bodega Marine Reserve	Deserts (24)	0.01 ± 0.01 c	4.08 (2.38)c,d	3.0–5.0	0.34	1.84
Burns Pinon Ridge Reserve	Deserts (24)	0.03 ± 0.01 c	7.04 (0.30)b,c,d	3.0–5.0	1.61	2.61
Griffith Park	Broadleaf shrubs (10)	0.04 ± 0.02 b,c	6.70 (2.58)b,c,d	19.0–25.0	4.32	5.32
Guadalupe-Nipomo Dunes	Deserts (24)	0.03 ± 0.01 c	1.69 (0.09)d	3.0–5.0	1.35	2.85
Madrona Marsh Preserve	Urban (21)	0.03 ± 0.01 c	3.94 (0.88)c,d	15.0–19.0	3.42	4.92
Sweeney Granite Mountains	Deserts (24)	0.01 ± 0.01 c	1.65 (0.07)d	3.0–5.0	4.08	5.08
Motte-Rimrock Preserve	Broadleaf shrubs (10)	0.05 ± 0.01 b,c	12.65 (1.88)b	9.0–15.0	12.57	13.57
UC Riverside	Broadleaf shrubs (10)	0.07 ± 0.02 b	20.47 (1.54)a	11.0–15.0	7.67	8.67
Whitewater Preserve	Deserts (24)	0.01 ± 0.01 c	5.47 (0.64)b,c,d	11.0–15.0	3.73	4.73

[a] Numbers in parenthesis refer to LUC from Zhang et al. 2003

[b] Letters show significant differences in pairwise t-test corrected for multiple comparisons ($p < 0.05$)

[c] Estimates of N deposition taken from model of Fenn et al. 2010

[d] Total N deposition is the sum of dry and wet deposition. Wet deposition data was taken based on published estimates based on land use categories above

ha^{-1} $yr.^{-1}$; (Fenn et al. 2010)). Consistent with these deposition models, BMR soils have low N concentrations (0.01% total N, ~ 4.00 ppm mineral N) and UCR has high N concentrations (0.1% total N_r, ~20.00 ppm mineral N; (Regus et al. 2014)). For context, the UCR soil N concentrations are comparable to tilled agricultural soils (Bremner 1965).

Seeds were collected from wild plants at BMR in June 2005 (BMR05) and 2007 (BMR07) and from UCR in April 2008 (UCR08) and April 2009 (UCR09) from separated locations within each field site. To generate seed sets for the experiment, plant lines were developed that only included descendants from a single wild-collected seed. Plants for seed production were grown in one gallon pots in sterile soil (UC Mix-3) from January to June, 2011. Plants were only allowed to self-pollinate (greenhouses were sprayed weekly with the insecticide Mavrik). We refer to these four descendent seed sets as lines BMR05, BMR07, UCR08, and UCR09.

Isotopic analysis of wild-collected *A. strigosus* seeds and hosts inoculated with soil rinsates

Wild *A. strigosus* seeds were collected from the BMR and UCR field sites between 2005 and 2014. Mature pods were collected from 3 to 12 plants at each of 9 GPS locations per field site. Approximately 30 seeds per GPS location were dried 2–3 days at 60° C, weighed, and pulverized in a bead beater (using a 5 mm stainless steel bead) ≥ 4 times at 4 m/s for 10 s. Samples were analyzed for %N, C:N ratio, and $\delta^{15}N$ (UC Santa Cruz Stable Isotope Laboratory).

Soil cores were collected from the BMR and UCR field sites in March 2015. Twenty soil cores of ~13 cm depth were collected from each field site within a radius of ~10 m, always sampling nearby but not directly over *A. strigosus* plants. Soil cores were homogenized and sieved under sterile conditions to <2 mm, combined with sterile water to form a 1 g soil / mL H_2O slurry, and allowed to settle overnight. The resultant supernatants were used as inoculants for axenic *A. strigosus* seedlings, either using 5 mL of the supernatant directly (live soil treatment) or after autoclaving (dead soil treatment). Each soil rinsate was inoculated onto the *A. strigosus* plant lines derived from the same field site (2 plant lines per field site × 2 field sites × 2 soil treatments [live, dead] (Zhang et al. 2003) × 10 plant replicates =80 plants total). Inoculation took place 9 March, 2015, and plants were raised in a greenhouse and fertilized weekly with N-free Jensen's solution. Plants were harvested at 8 weeks post inoculation, checked for nodulation, and plant shoot tissue was dried in a 60 °C oven for 2–3 days. Dry leaves were removed from stems and powdered with a 5 mm stainless steel bead for 10 s at 4 m/s. Four out of 10 plant replicates per treatment were analyzed for %N, C:N ratio, and $\delta^{15}N$ (UC Santa Cruz Stable Isotope Laboratory).

We calculated %Ndfa (%N derived from atmospheric N_2) for both the wild-collected *A. strigosus* seeds and for the plants inoculated with soil rinsates. We used the method of Wanek and Arndt (Wanek and Arndt 2002), which requires estimation of $\delta^{15}N$ for non-nitrogen fixing reference plants ('$\delta^{15}N_{refplant}$') and for legumes with N_2 as the sole source of N_r ('*B*'). The $\delta^{15}N_{refplant}$ was estimated based on *A. strigosus* inoculated with autoclaved soils from each soil sample and *B* was estimated based *A. strigosus* inoculated with our most effective strain (#49) and no access to mineral nitrogen ($B = -2.75$).

Nitrogen gradient inoculation experiment

We inoculated experimental plants with two genetically diverged *Bradyrhizobium* strains (referred to as #s 2 and 49), which were originally collected from *A. strigosus* at BMR (Sachs et al. 2009). Strain #49 is highly effective on *A. strigosus* from BMR, providing ~500% increase in *A. strigosus* shoot biomass when hosts are grown in soil without soil nitrogen, and #2 is ineffective, not significantly affecting shoot biomass (Sachs et al. 2010a). These strains bracket the natural variation of *Bradyrhizobium* symbiotic quality on *A. strigosus* (Sachs et al. 2010a). Both strains readily nodulate this host in single strain inoculations and attain high population density within nodules, both in the absence of mineral nitrogen (Sachs et al. 2010a) and when fertilized with mineral nitrogen (Regus et al. 2014). *Bradyrhizobium* strains were grown on agar plates with modified arabinose gluconate medium (MAG), and cultures were scraped and resuspended in sterile ddH_2O to generate inocula of $1 \times 10_8$ cells ml^{-1}, with 5.0 ml inoculated per plant (Sachs et al. 2009).

Seedlings were prepared under axenic conditions and grown in sterilized quartzite sand, which is inert and provides negligible nutrients (Sachs et al. 2009). Seedlings were moved to the greenhouse one week prior to

inoculation, and after four days in the greenhouse, plants were fertilized with 10.0 mL nitrogen-free Jensen's solution with dissolved KNO_3 for nitrogen treatments (Somasegaran and Hoben 1994). Three days after initial fertilization, plants were inoculated with 5.0 ml of either strain #2, #49, or sterile ddH_2O. Four days after inoculation, plants were fertilized per treatment as above and then once per week until harvest. For each plant line, 126 size-matched sterile-grown seedlings were randomly assigned to inoculum/fertilizer treatment groups. Fertilizer treatments consisted of a range of N_r concentrations that bracket and exceed the N_r levels observed at the two sites (0.00, 0.25, 0.50, 1.00, 3.00 and 5.00 g L^{-1} KNO_3). For comparison, the third fertilizer concentration (0.50 g L^{-1} KNO_3) provides plants with approximately 15 ppm NO_3 or 75% of mineral nitrogen content at UCR. We used KNO_3 because plants most readily take up NO_3^- in nature and soil processes convert most mineral nitrogen to NO_3^- (Streeter 1988). The experiment ran for eight weeks, from inoculation to harvest (12 March to 7 May, 2012). At harvest, plants were carefully depotted, and all nodules were dissected, counted and photographed. Roots, shoots and nodules were separated and dried in an oven (60 °C, > 4 days) before weighing dry biomass. The experiment included 504 plants in total (7 replicate plants per treatment, 4 plant lines, 3 inoculation treatments, 6 N_r treatments).

Host plant mortality was analyzed using multiple logistic regressions (Fit Model Platform, JMP 10.0; SAS Institute Inc. 2012). Host growth response to nodulation was calculated as the percent difference in dry shoot biomass between inoculated plants and size-matched uninoculated control plants (Sachs et al. 2010a). We tested whether growth response differed significantly from zero (i.e., no growth response to nodulation) using a one sample t-test (JMP 10.0; SAS Institute Inc. 2012). Mean individual nodule mass was calculated as total per-plant nodule mass divided by nodule number. Differences in host growth response, nodule number, mean nodule mass, and shoot weight of uninoculated plants among plant lines or fertilizer treatments were assessed with general linear models (GLM; Fit Model Platform in JMP 10.0) to test main effects (rhizobial genotype, fertilizer, host line) and interactions among effects within each experiment. We also used pairwise analyses correcting for multiple comparisons using Tukey's Honestly Significant Difference test (HSD).

Results

Nitrogen deposition estimates

Atmospheric sampling took place over time periods without precipitation. Estimates of N deposition varied >35× among the tested sites, ranging from total annual dry deposition of 0.34 kg ha^{-1} $yr.^{-1}$ at BMR to 12.57 kg ha^{-1} $yr.^{-1}$ at Motte Rimrock Reserve (Table 1; Supplemental Table 1). Our empirical measures of N deposition paralleled but were lower than the published simulation data for these same locations (Table 1; Supplemental Table 1).

Soil nitrogen concentrations

Measures of mineral N in soils varied >12× among the sampled sites, ranging from 1.65 ppm in the Granite Mountain Preserve to 20.47 ppm at UCR. Measures of mineral N and total N percentage roughly paralleled each other among sites (Table 1; Supplemental Table 1).

Regression analysis that only compared simultaneously gathered data from atmospheric and soil sources indicated that local annual dry nitrogen deposition was an excellent predictor of mineral soil N (R^2 = 0.754; $p < 0.0011$; Fig. 1). A regression of all the atmospheric and soil data gathered without respect to sampling date was also significant (R^2 = 0.250; $p < 0.0018$).

Isotopic analysis of wild-collected *A. strigosus* seeds

Seeds collected from wild plants at BMR had significantly lower mean %N (BMR, 2.68%; UCR, 3.45%; $F_{1,17}$ = 24.106, p = 0.0002) and significantly higher C:N ratio compared to seeds from UCR (BMR, 16.7; UCR, 13.2; $F_{1,17}$ = 24.827, p = 0.0001), suggesting that UCR plants at the N_r polluted site are incorporating more nitrogen on average. However, seeds from BMR had significantly lower $\delta^{15}N$ values than seeds from UCR (BMR, −1.56; UCR, 0.47; $F_{1,17}$ = 6.366, p = 0.0226) and higher %Ndfa (BMR, 85.00%; UCR, 66.63%; $F_{1,17}$ = 8.440, p = 0.0103), suggesting that UCR plants at the N_r polluted site are receiving a lower percentage of their nitrogen from BNF (Supplemental Tables 2 and 3).

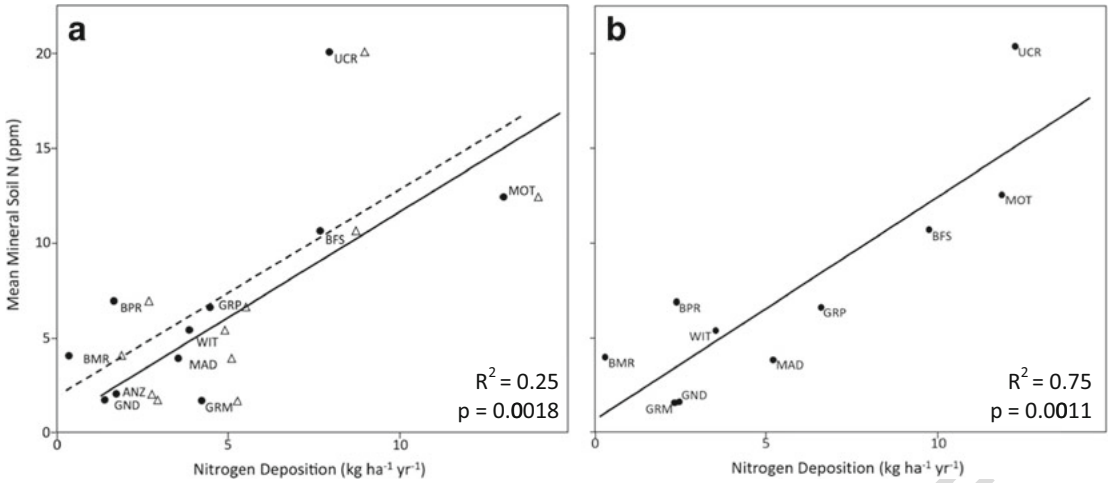

Fig. 1 Correlation of mean N_r dry deposition rates and mineral soil N across the 11 field sites. The left panel shows mean measures from all three sampling periods. The dashed line is dry deposition (circles); solid line is the sum of dry deposition and estimated wet deposition (triangles). The right panel shows single measures taken in Feb. 2013, the only time point where soil and atmospheric measures were able to be taken for all sites simultaneously (ANZ, Anza Borrego; BFS, Bernard Field Station; BMR, Bodega Marine Reserve; BPR, Burns Pinon Ridge; GRP, Griffith Park; GND, Guadalupe-Nipomo Dunes; MAD, Madrona Marsh; GRM, Granite Mountains Preserve; MOT, Mott-Rimrock Preserve; UCR, UC Riverside; WIT, Whitewater Preserve)

Isotopic analysis of *A. strigosus* hosts inoculated with soil rinsates

A. strigosus hosts inoculated with live soil rinsates were nodulated in every case, whereas none of the hosts inoculated with autoclaved rinsates had nodules. Comparing effects of live and dead soil rinsates, we found that plants inoculated with live soil rinsates exhibited higher %N, lower C:N ratios, and lower $\delta^{15}N$ values at both sites, indicating that compatible nitrogen-fixing rhizobia exist in the sampled soils (BMR: %N, $F_{1,15} = 1735$, $p < 0.0001$; C:N, $F_{1,15} = 205.9$, $p < 0001$; $\delta^{15}N$, $F_{1,15} = 412.8$, $p < 0.0001$; UCR: %N, $F_{1,15} = 46.54$, $p < 0.0001$; C:N, $F_{1,15} = 34.38$, $p < 0001$; $\delta^{15}N$, $F_{1,15} = 48.38$, $p < 0.0001$; Supplemental Tables 2 and 3).

Plants inoculated with the live BMR soil rinsates had significantly higher %N than plants inoculated with the live UCR soil rinsates ($F_{1,15} = 4.809$, $p = 0.0457$). However, plants inoculated with the autoclaved rinsates did not show a difference in %N between field sites ($F_{1,15} = 0.598$, $p = 0.4521$), suggesting that the differences in plant nitrogen content is caused by the rhizobia and possibly other microbes in the soils, rather than abiotic differences. No significant differences were found in C:N ratio, $\delta^{15}N$, or %Ndfa in the live soils between BMR and UCR, although the trends were the same as in the seed samples, with evidence of (C:N, $F_{1,15} = 1.4181$, $p = 0.2535$; $\delta^{15}N$, $F_{1,15} = 1.1886$, $p = 0.2940$; %Ndfa, $F_{1,14} = 2.0788$, $p = 0.1730$; Supplemental Tables 2 and 3).

Response of uninoculated *A. strigosus* to N_r gradient

For shoot biomass of uninoculated *A. strigosus*, the GLM uncovered significant effects of nitrogen treatment ($F_{5,148} = 40.29$, $p < 0.0001$) but no effect of plant line ($F_{3,148} = 2.360$, $p = 0.0747$) or their interaction ($F_{15,148} = 1.292$, $p = 0.2129$). Shoot mass of uninoculated plants increased over the span of the four lower N fertilizer concentrations (0.00–1.00 g L^{-1} KNO_3) and then leveled off or decreased in the highest concentrations (3.00–5.00 g L^{-1} KNO_3; Fig. 2; Supplemental Table 3). Three uninoculated plants became contaminated by rhizobia in the greenhouse, each exhibiting <5 nodules (compared to inoculated plants which averaged ~46 and ~78 nodules for strains #2 and #49, respectively). The contaminated plants were removed from analysis (BMR05, 0.25 g L^{-1} KNO_3; BMR07, 5.0 g L^{-1} KNO_3; UCR08, 0.5 g L^{-1} KNO_3).

Twenty of the 168 uninoculated plants died during the experiment. A multiple logistic regression of mortality found significant main effects of both fertilizer ($p < 0.0001$) and plant line ($p < 0.001$). Mortality of uninoculated plants increased with increased nitrogen for all plant lines ($\chi^2 = 20.80$, $p < 0.0001$), although mortality was greater for BMR05 than other plant lines ($\chi^2 = 17.11$, $p < 0.0007$; Table 2).

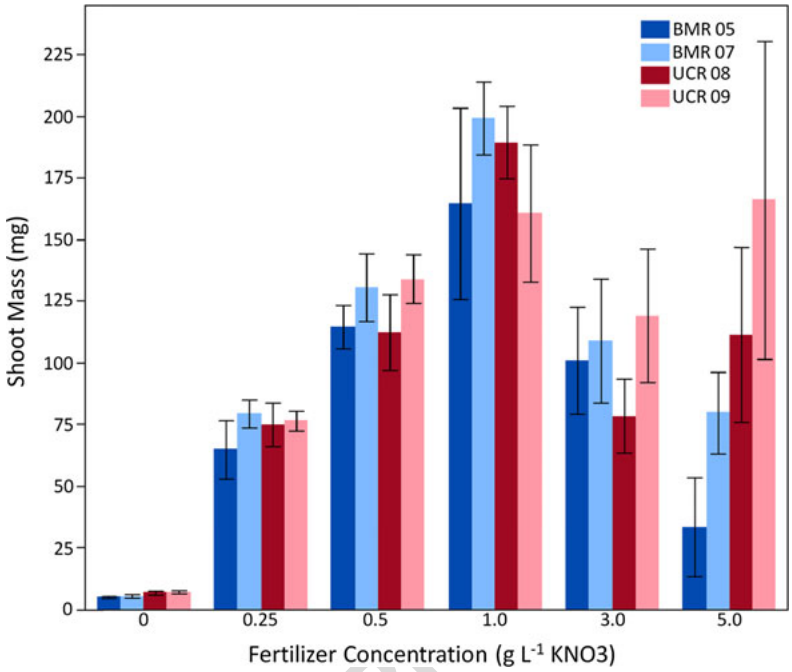

Fig. 2 Shoot mass of axenic *A. strigosus* in a range of mineral nitrogen concentrations. Error bars are ± one standard error

Nodule number of plants in N_r gradient

A GLM analysis of nodule number was performed that included the four lowest fertilizer concentrations. It was not practical to make statistical comparisons of nodule number for the two highest fertilizer treatments because many plants died or did not form nodules (see mortality analysis; Table 2). There were significant effects of inoculation treatment ($F_{1,193} = 25.79$, $p < 0.0001$) and fertilizer concentration ($F_{3,191} = 16.68$, $p < 0.0001$) on nodule number, but plant line was not significant ($F_{3,191} = 0.2277$, $p = 0.8770$), and none of the

Table 2 Plant mortality and nodulation status. Seven total replicates per treatment combination. 'Control' plants are un-inoculated. 'Nodules' columns show the number of plants that formed nodules irrespective of nodule counts. 'Live' column shows the number of plants that were alive at the end of the experiment

			0 g L^{-1} KNO_3		0.25 g L^{-1} KNO_3		0.5 g L^{-1} KNO_3		1.0 g L^{-1} KNO_3		3.0 g L^{-1} KNO_3		5.0 g L^{-1} KNO_3	
			Nodules[a]	Live[b]	Nodules[a]	Live[b]	Nodules[a]	Live[b]	Nodules[a]	Live[b]	Nodules[a]	Live[b]	Nodules[a]	Live[b]
Control	BMR05	n.a.	6	1	6	n.a.	6	n.a.	5	n.a.	4	n.a.	3
	BMR07	n.a.	7	n.a.	7	n.a.	7	n.a.	7	n.a.	7	1	6
	UCR08	n.a.	7	n.a.	7	n.a.	7	n.a.	7	n.a.	6	1	5
	UCR09	n.a.	7	n.a.	7	n.a.	7	n.a.	7	n.a.	6	n.a.	4
Strain 2	BMR05	6	6	5	6	5	6	5	6	0	5	0	4
	BMR07	7	7	7	7	7	7	7	7	2	6	0	6
	UCR08	6	7	7	7	7	7	6	7	1	7	0	5
	UCR09	7	7	7	7	6	7	5	7	1	7	0	7
Strain 49	BMR05	6	6	6	6	6	6	6	6	2	4	0	1
	BMR07	7	7	7	7	7	7	7	7	3	6	2	5
	UCR08	7	7	7	7	6	7	2	7	5	7	3	5
	UCR09	7	7	7	7	7	7	7	7	4	7	1	2

[a] Total number of plants with nodules

[b] Total number of plants surviving (out of 7 plant replicates per treatment)

interactions were significant (inocuation x fertilizer, $F_{3,191} = 0.8083$, $p = 0.4908$; inoculation x plant line, $F_{3,191} = 0.3547$, $p = 0.7858$; fertilizer x plant line, $F_{9,191} = 38.11$, $p = 0.9431$).

In all cases but one (BMR_05, 0.25 g L^{-1} KNO_3), plants formed more nodules with the effective strain #49 than with the ineffective strain #2 (Fig. 3). Mean nodule count per plant increased from zero added N to 0.25 g L^{-1} KNO_3), and did not further increase when N concentration was raised to 0.5 g L^{-1} KNO_3, but began to decrease at 1.00 g L^{-1} KNO_3. Nodulation was nearly or completely eliminated in the highest two N concentrations (3.00, 5.00 g L^{-1} KNO_3; Fig. 3; Supplemental Table 4).

A. strigosus nodule size

A GLM analysis of mean nodule mass was performed that included only the four lowest fertilizer concentrations, as above for nodule number. The GLM uncovered significant effects of inoculation treatment ($F_{1,192} = 71.82$, $p < 0.0001$) and fertilizer concentration ($F_{3,190} = 2.72$, $p < 0.05$), but not of plant line ($F_{1,192} = 1.621$, $p = 0.1863$), and only the interaction of inoculation x plant line was significant (inoculation x fertilizer, $F_{3,191} = 1.712$, $p = 0.1663$; inoculation x plant line, $F_{3,191} = 3.723$, $p = 0.0125$; fertilizer x plant line, $F_{9,191} = 0.5848$, $p = 0.8092$). *A. strigosus* formed significantly larger nodules with the effective strain #49 than with the ineffective strain #2 (Fig. 3; Supplemental Table 4).

A. strigosus growth benefits from *Bradyrhizobium* nodulation

For growth benefits of nodulation, the GLM uncovered significant effects of inoculation treatment ($F_{1,264} = 41.11$, $p < 0.0001$) and fertilizer concentration ($F_{5,264} = 36.49$, $p < 0.0001$), but not plant line ($F_{3,264} = 1.552$, $p = 0.2015$), and only the interaction of inoculation x plant line was not significant (inoculation x fertilizer, $F_{5,264} = 39.19$, $p < 0.0001$; inoculation x plant line, ($F_{3,264} = 2.238$, $p = 0.0843$; fertilizer x plant line, $F_{15,264} = 2.226$, $p = 0.0060$). All plant lines gained significant benefit from nodulation with the effective strain #49 in zero fertilizer (Fig. 3). Growth benefit from nodulation with strain #49 was eliminated by nitrogen fertilization in most cases (except for three treatment combinations; BMR05, BMR07 × 0.25 g L^{-1} and UCR09 × 1.0 g L^{-1}). No plant line gained significant benefit from nodulation with the ineffective strain #2 in any fertilizer concentration (Fig. 3). Negative growth responses to inoculation were observed in 8/24 treatment combinations for UCR lines and never for BMR lines (Fig. 3; Supplemental Table 4).

Mortality analysis for inoculated *A. strigosus*

A multiple logistic regression of mortality uncovered significant effects of fertilizer ($p < 0.0001$) and plant line ($p < 0.0001$) but not inoculation treatment ($p = 0.1522$; Table 2). We also performed multiple logistic regression of mortality within each inoculation treatment, and main effects of fertilizer and plant line were significant for both strain #2 (fertilizer $p < 0.01$, plant line $p < 0.001$) and strain #49 (fertilizer $p < 0.0001$, plant line $p < 0.001$).

Similar to axenic *A. strigosus*, mortality was negligible in the lowest four fertilizer concentrations (0.00–1.00 g L^{-1} KNO_3), but increased in the highest two fertilizer concentrations (3.00–5.00 g L^{-1} KNO_3; Table 2). One plant line had no mortality (UCR09 strain #2). Similar to axenic plants, BMR05 tended to have greater mortality than other lines regardless of inoculation treatment.

Discussion

Over the past century industrialization has more than doubled global N_r output (Galloway et al. 2004), leading to intense deposition in natural ecosystems (Dentener et al. 2006; Holtgrieve et al. 2011). N_r deposition has enriched soils that were historically nitrogen-limited, potentially saturating plants for mineral nitrogen (Vitousek et al. 1997; Dentener et al. 2006). In southern California, deposition has occurred for more than 70 years (Fenn et al. 2010) and some soils have become greatly enriched for N_r over that time span (Egerton-Warburton et al. 2001). Our atmospheric sampling of gaseous and aerosol nitrogen species largely confirmed models predicting significant variation in N_r deposition across California (Fenn et al. 2010) and uncovered >35× variation in dry deposition statewide. Our data strongly supports the key role of N_r deposition in enhancing soil fertility at sampled sites by showing a significant relationship between N_r deposition and extractable N concentrations in the soils. Previous studies have measured the effects of pollution loads on soils (Padgett and

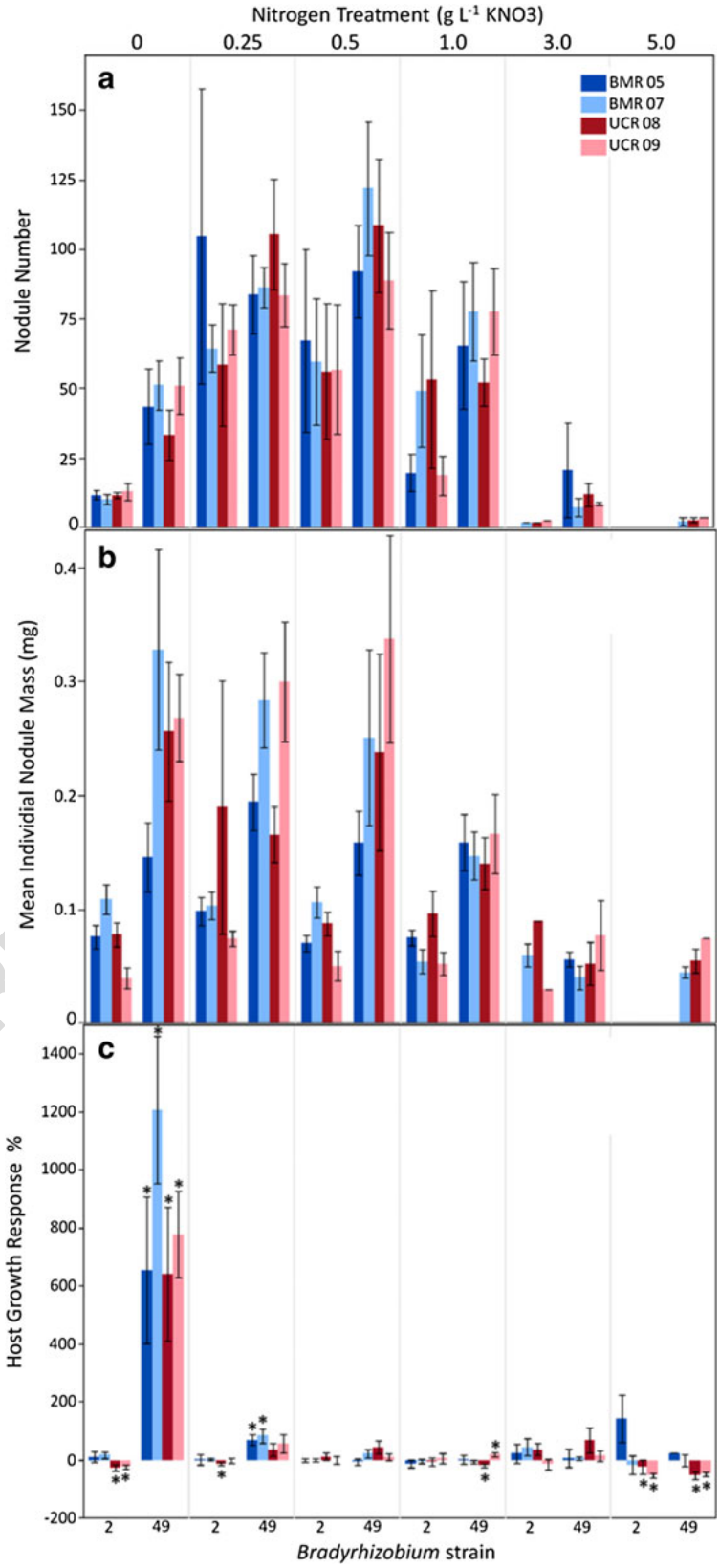

Fig. 3 Nodule status and host percent growth response from symbiosis. Error bars are ± standard error. Asterisks show significant difference from zero in one-sample t-test ($p < 0.05$)

Bytnerowicz 2001; Vourlitis et al. 2007), but with limited field sampling. No previous work that we are aware of has assessed deposition and soil content over such a wide array of field sites and pollution levels.

We analyzed *A. strigosus* seeds, soils, and experimental plant lines from California sites that are minimally (BMR) or highly (UCR) polluted in terms of atmospheric N_r deposition (Fenn et al. 2010) (Fig. 1). Nitrogen isotopic data showed that seeds from UCR are enriched for nitrogen compared to BMR and suggest that the enrichment originates from the N_r polluted soils, given the ~20% reduction in biologically fixed nitrogen incorporated into the UCR seeds (relative to BMR). Analyses of plants inoculated with soil rinsates from each of these sites corroborate the seed data and suggest that soils from BMR are significantly enriched for nitrogen-fixing rhizobia compared to UCR. In total, these data suggest that nitrogen deposition patterns across California can cause legume populations to diverge in nitrogen sources, with some plants largely incorporating biologically fixed N_r and others taking up relatively more N_r from soil that is enriched by anthropogenic deposition.

Our experimental N_r deposition gradient tested concentrations that span and exceed current levels of mineral nitrogen in the sampled soils. Our greenhouse experiments revealed that even modest concentrations of N_r can eliminate the growth benefit of rhizobial nodulation. Nodulation with the effective strain #49 actually caused significant growth decreases in three instances for UCR08 in 1.0 g L^{-1} and for both UCR lines at 5.0 g L^{-1}, suggesting the possibility of costs associated with hosting rhizobia in high N_r contexts. Nodulation with the ineffective strain #2 caused a growth decrease only for UCR at the two ends of the simulated deposition gradient (Fig. 3) suggesting that mineral nitrogen availability can reduce the impacts of exploitative rhizobia in low N_r contexts. This pattern could be the manifestation of the significant fertilizer x plant line interaction effect that we uncovered. All negative growth responses were observed in UCR plant lines and both *Bradyrhizobium* used in this study were isolated from *A. strigosus* at BMR (Sachs et al. 2009), so it is possible that negative growth responses were influenced by host-symbiont specificity interactions between plant host and allopatric rhizobia (i.e., G x G interactions). It is worth noting that in the highest fertilizer concentration, both UCR lines experienced negative growth effects from inoculation with strain #2 but did not form any nodules, suggesting that halting nodulation is not without systemic costs for legume hosts. Previous work has found that induced systemic resistance to pathogens was costly in terms of growth and seed production (Heil et al. 2000).

Several *A. strigosus* sites that we studied exhibit mineral N_r soil concentrations comparable to the middle treatments used in this study (0.5 and 1.0 g L^{-1}; ~10–30 ppm), at which plants gained little or no benefit from nodulation by *Bradyrhizobium* strain #49. Among several *Bradyrhizobium* strains that have been tested on *A. strigosus*, strain #49 provides among the highest levels of growth benefit and nitrogen fixation (Sachs et al. 2010a, b; Regus et al. 2014, 2015). We hypothesize from these data and from the isotopic analyses that *A. strigosus* populations at the Bernard Field Station, the Motte-Rimrock Reserve, and UC Riverside often gain a greatly reduced benefit from *Bradyrhizobium* symbiosis compared to the unpolluted sites. We find it fascinating that both in our experiment and in the field sites we nonetheless observe that *A. strigosus* plants are always highly nodulated. If hosts are gaining little or no benefit from rhizobia but continue to allow nodulation, this could lead to the evolutionary degradation of host traits that differentiate beneficial from ineffective rhizobia (Sachs and Simms 2006; Kiers et al. 2010), as has been suggested by research on soybean (Kiers et al. 2007) and experimental populations of clover (Weese et al. 2015). An important caveat for our work is that we did not assess benefits that UCR lines gain from sympatric *Bradyrhizobium* strains.

Based on the limited number of plant lines analyzed, we did not uncover any evidence that *A. strigosus* plants from southern California (i.e., two UCR lines) have adapted evolutionarily in terms of increased growth rate across the spectrum of N_r concentrations tested (relative to Northern California lines). Shoot growth universally increased for all plant lines up through N_r concentrations currently experienced by UCR plant populations (i.e. 1.0 g L^{-1}; all plant lines, axenic and inoculated) and then decreased in N_r concentrations greater than observed at UCR (3.0 g L^{-1}, 5.0 g L^{-1}), consistent with toxicity. The BMR05 line had significantly greater mortality than other lines, and also had more dead plants in the highest two fertilizer concentrations for both axenic plants and inoculated plants (Table 2). Since plant lines were generated in greenhouse conditions, it is unlikely that seed quality or other maternal effects explain the mortality response in BMR05.

We experimentally assessed mineral N_r concentrations beyond those observed for *A. strigosus* to model predicted increases in the intensity of N_r deposition (Galloway et al. 2008). Some regions, particularly in China, can experience nitrogen deposition rates more than 5× that of California (Fenn et al. 2010; Ti et al. 2012; Tu et al. 2014). For comparison, the middle two of our six N_r treatments (0.5 and 1.0 g L^{-1}) bracketed concentrations observed at the high deposition *A. strigosus* site in this study, and the highest fertilizer treatment represented approximately 6× the observed concentrations. While the UCR site has experienced significant N_r deposition for more than 70 years (Fenn et al. 2010), we found little or no evidence of differential adaptation to high soil N_r by *A. strigosus* from UCR. Because global N_r deposition is predicted to continue increasing (Galloway et al. 2008) we must understand the effects of extreme nitrogen enrichment on biological nitrogen fixation. Reduction or elimination of symbiosis by legumes would remove a major global contributor to N_r cycling (Galloway et al. 2008) and a replacement of natural cycles with anthropogenic ones.

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