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Effective rhizobia enhance legume growth during subsequent drought despite water costs associated with nitrogen fixation

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Abstract

Purpose Drought induces mortality in plants; however, how symbioses mediate the plant drought response remains understudied and may be crucial to understanding how shifts in symbioses under climate change scenarios may impact plant drought induced mortality.

Methods Drought was experimentally imposed in parallel on two legume hosts, *Acmispon strigosus* and *Lotus japonicus*, whose roots are nodulated by nitrogen fixing rhizobia in different genera. Within each host taxon, greenhouse experiments factorially combined legume genotypes varying in root nodule investment with rhizobia varying in nitrogen fixation capacity, to investigate how costs and benefits of symbiosis modulate plant performance under drought.

Results Net benefits of symbiosis with nitrogen fixing symbionts, namely increased number of leaves and shoot mass, were maintained under drought irrespective of nodulation level or host species, mitigating the carbon strain during drought. Net benefits of symbiosis occurred despite increased water cost associated with the maintenance of nitrogen fixation, costs that were evident even in pre drought baseline conditions.

Conclusion Our results demonstrate that benefits of root nodule symbiosis can be robust to environmental perturbations, and potentially mediate carbon starvation during drought induced mortality events. The investment in symbiosis and its impacts on biomass suggest that in long term droughts legumes may be more limited by carbon than water. Ongoing stability of these associations is predicted, even under extended drought conditions.

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Introduction

Climate change is rapidly transforming the environmental pressures that shape plant communities, but our understanding has focused on individual species

responses, with little account for their symbiotic associations (Six 2009). Under modified environmental conditions, symbiotic partnerships might shift to become harmful for host species, require novel symbiotic partners, or be completely abandoned by hosts depending on the net benefits and costs of the association (Sachs & Simms 2006; Kiers et al. 2010). Anthropogenic warming and drought are among the most destructive components of climate change for plant communities (Williams et al. 2020). Defined as extreme climate events over land with below normal precipitation, droughts are predicted to increase in frequency and intensity in the upcoming decades (Dai 2011). Drought impacts natural and managed plant populations, increasing stress and mortality, negatively impacting yields, and increasing susceptibility to disease (Dai 2011; McDowell et al. 2008). Interactions between plants and soil microbiota can ameliorate some negative effects of drought (Grover et al. 2014; Thilakarathna & Raizada 2017). Root associations with fungi can increase the effective surface area of roots, thus enhancing the transport of water and nutrients to the plant (Allen 2007), improving survivorship (Bast et al. 2016), growth (Li et al. 2015), and reproduction during drought (Koide and Dickie 2002). Associations with bacteria have received less attention as they do not typically form a hyphal network and are not known to directly assist with water acquisition. The number of nodules and the nitrogen fixation of rhizobia may decrease during drought conditions (King and Purcell 2001; Marquez-Garcia et al. 2015), although the negative effects of drought can be ameliorated in the presence of nitrogen fixing rhizobia (Kiriziy et al. 2022). Despite the importance of beneficial soil bacterial symbionts for natural and agricultural plant communities (Mueller and Sachs 2015), their effects on plant drought response remain poorly understood.

The physiological mechanisms of drought-induced stress are attributed to a combination of hydraulic failure and carbon starvation (McDowell et al. 2008; McDowell 2011). When in sufficient supply, water moves through xylem by the cohesion of water molecules and the negative water potential, or tension, from evaporation through open stomata (Dixon and Joly 1894; Lambers et al. 1998). Hydraulic failure occurs when xylem vessels reach a critically low water potential that allows air entry, forming an embolism and blocking subsequent transport of water to leaves (Sevanto et al. 2014). Carbon starvation occurs when plants lack or are unable to transport non-structural carbohydrates through phloem to support growth and metabolism throughout the plant (McDowell et al. 2008). Hydraulic failure and carbon starvation are coupled (McDowell 2011). Insufficient water can cause the photosynthetic output to slow or stop (as can insufficient sunlight, or other nutrients), resulting in a photosynthate deficit. The photosystem functions decline over the course of drought, and the damage to the xylem cannot be repaired without photosynthate (Tomasella et al. 2017). Interdependence of hydraulic failure and carbon starvation is recognized as a catastrophic drop in plant water status that limits photosynthetic carbon uptake, transport, and utilization, and a reduction in non-structural carbohydrates that limits the plant's ability to repair xylem damage further reinforcing water stress (McDowell et al. 2013).

Plant associations with symbiotic bacteria involve marked benefits that could alter plant drought responses. Rhizobia are root nodulating bacteria that fix nitrogen, interact primarily with legumes, and represent one of the most extensively studied systems of beneficial plant-microbial interactions (Masson-Boivin and Sachs 2018). This symbiosis is initiated when legume roots and compatible rhizobia exchange signals in the soil, leading to rhizobia becoming encased by plant cells to form a root nodule (Sachs et al. 2018). Within the nodule, the bacteria fix atmospheric nitrogen for the plant in exchange for photosynthate (White et al. 2007). Increased access to nitrogen could impact several aspects of leaf gas exchange, including greater photosynthetic carbon gain for a given rate of water loss in plants. The effect of rhizobia on a plant's drought response is poorly understood, but could involve more than just delivery of fixed nitrogen. The sourcing of nitrogen from symbionts or directly from soil incur differing costs or benefits that might alter physiological responses under other costly scenarios (Pfau et al. 2018). Symbiont derived nitrogen can increase stomatal conductance, net photosynthesis, and internal CO_2 concentrations compared to soil derived nitrogen, which might benefit plant drought response by ameliorating carbon starvation (Frechilla et al. 2000). Rhizobia also directly impact host root system architecture (Concha and Doerner 2020) increasing the amount of growth directed towards roots (Li et al. 2015), potentially

enhancing nutrient and water access. Moreover, rhizobia can delay leaf senescence (Staudinger et al. 2016), thus ameliorating carbon starvation by increasing the length of time plants can fix carbon.

Bacterial symbioses might also impose substantial costs to host plants under drought. To gain a net benefit from symbiosis, legumes must invest substantial metabolic resources into nodule formation and maintenance (Krussell et al. 2002; Quides et al. 2021) and the supplementation of photosynthates to support nitrogen fixation within nodules (White et al. 2007). Although increased nitrogen access is generally beneficial to host plants, it might exacerbate water stress during drought. This could occur directly through water costs associated with uptake and movement of nitrogen (Cramer et al. 2009), as well as indirectly through decreased plant investment in roots as nutrient needs are met (Concha and Doerner 2020) thereby reducing the surface area of roots for water during drought. In addition to water stress, the maintenance of the symbiosis directly incurs a carbon cost. Because legumes can vary genotypically in the magnitude of host resources invested into nodules (Wendlandt et al. 2019), this could lead to varied costs of associating with rhizobia (Quides et al. 2021). There is also substantial variation in how much nitrogen is fixed and shared by root nodulating rhizobia in natural populations (Gano-Cohen et al. 2020), a symbiotic service that the plant host must support with photosynthates.

Here, we experimentally manipulated the benefits and costs of root nodulating symbiosis for legumes, to investigate how rhizobial associations affect legume drought response. To test the effects of nitrogen fixation (i.e., benefits), we employed *Bradyrhizobium* genotypes that naturally vary in their capacity to fix atmospheric nitrogen with *Acmispon strigosus*, as well as wildtype and mutant (non-nitrogen fixing) *Mesorhizobium loti* that associate with *Lotus japonicus*. To test the effects of host-investment into symbiotic root tissues (i.e., costs), we employed host genotypes that varied in the number of nodules produced, including natural variants of *A. strigosus* and lab generated mutants of *L. japonicus*. By including distinct levels of host investment into root nodulation, contrasting nitrogen fixing capacities by associated rhizobia, and two diverged host-plant species, our experimental framework can distinguish between symbiotic nutrient fluxes that might mitigate or intensify the effects of drought. We test how rhizobial symbiosis interacts with plant drought response based on the physiological mechanisms of drought induced mortality, using shoot water potentials to infer water limitation, and chlorophyll fluorescence, leaf retention, and shoot mass to examine carbon status. Our goals are to investigate i) the net effects of rhizobial symbioses on plants during drought events and ii) quantify how the net costs and benefits of symbioses can mediate the host plants drought response.

Materials and methods

Biological materials

We selected two host genera, *Acmispon* and *Lotus* as they have distinct evolutionary histories, having diversified in North America and Eurasia, respectively (Allan and Porter 2000). Two *A. strigosus* inbred lines, hereafter BMR-highnod and Yucca-lownod, were used that vary in nodulation response when clonally inoculated with a panel of rhizobia strains and harvested eight weeks after inoculation. The higher nodulation response host (BMR04, collected from Bodega Marine Reserve, Sonoma County, CA) produces an average of 24.24 ± 2.46 nodules when inoculated with nitrogen fixing rhizobia, 28.55 ± 3.16 with non-fixing strains or ~150% more nodules than the lower nodulation response host (Yuc03, collected from Burns Pinyon Ridge Nature Reserve at Yucca Valley, San Bernardino County, CA) which produces an average of 20.20 ± 2.39 nodules with nitrogen fixing strains, or 14.86 ± 2.62 nodules with non-fixing strains (supplemental data). The BMR site is coastal and mesic, with average rainfall of 86 cm per year, and temperature ranges from 6 °C to 18 °C, while the Yucca site is inland and arid, with average rainfall of 25 cm per year and temperature ranges from -1.1 °C to 35 °C. *A. strigosus* hosts were inoculated with one of two *Bradyrhizobium* strains, that differ in their capacity to fix nitrogen. *Bradyrhizobium* strains were collected from *A. strigosus* at BMR in 2005 (Sachs et al. 2009). While both strains have the capacity to form nodules, strain #56 fixes nitrogen, and significantly enhances growth of *A. strigosus* hosts in the absence of extrinsic sources of nitrogen, whereas

strain #2 does not fix nitrogen and does not enhance host growth (Sachs et al. 2010; Regus et al. 2017).

Two *L. japonicus* genotypes were used, including the wildtype MG-20 and its near-isogenic hypernodulating mutant *plenty,* which forms ~ 250% the number of nodules relative to MG-20, when inoculated with the nitrogen fixing *M. loti* strain MAFF303099 (hereafter MAFF; Quides et al. 2017, 2021). *L. japonicus* hosts were inoculated with one of two *M. loti* strains including MAFF, that fixes substantial nitrogen for *L. japonicus*, and its near-isogenic mutant STM6 that has a transposon inserted in the nitrogenase gene *nifD* (strain ID 17T02d02) and is incapable of fixing nitrogen (Regus et al. 2017; Shimoda et al. 2008). In previous experiments, wherein plants were inoculated and harvested five weeks later, MG-20 formed 11.42 ± 1.32 nodules with MAFF, and 8.05 ± 0.81 nodules with STM6 compared to *plenty*, which formed 23.75 ± 2.39 nodules with MAFF and 19.84 ± 1.67 nodules when inoculated with STM6 (Quides et al. 2021).

Inoculation experiment

A. strigosus and *L. japonicus* seeds were germinated in sterile conditions following published protocols from 22–29 January 2017 (Sachs et al. 2009). Seedlings were planted in sterilized conetainers™ two weeks later (SC10; Steuwe & Sons, Tangent, OR, USA) filled with sterilized calcined clay (Turface® Pro League®, Turface Athletics, Buffalo Grove, Illinois, USA), and maintained in a controlled growth room with daily misting for two weeks. After true leaves developed, seedlings were fertilized with 5 mL of Jensen solution supplemented with 0.5 g/L potassium nitrate weekly (Somasegaran and Hoben 1994). Two weeks after true leaves developed, seedlings were moved to the greenhouse to harden behind shade cloth designed to block 50% of light. Plants in the greenhouse also received daily misting. Within each block, twelve seedlings of *A. strigosus* and twelve *L. japonicus* seedlings were randomly assigned to one of three treatments, including inoculation with the compatible nitrogen fixing strain for each host species, the non-fixing strain for each host, or sterilized water as a control, on March 5th, 2017. Rhizobia were plated from pure stocks, incubated at 29 °C until lawns formed, scraped from plates, and

resuspended in liquid MAG to estimate concentration via optical density. Cells were gently centrifuged to remove media (4000 g, 20 min.) and resuspended in sterile water at 10^8 cells mL-1.
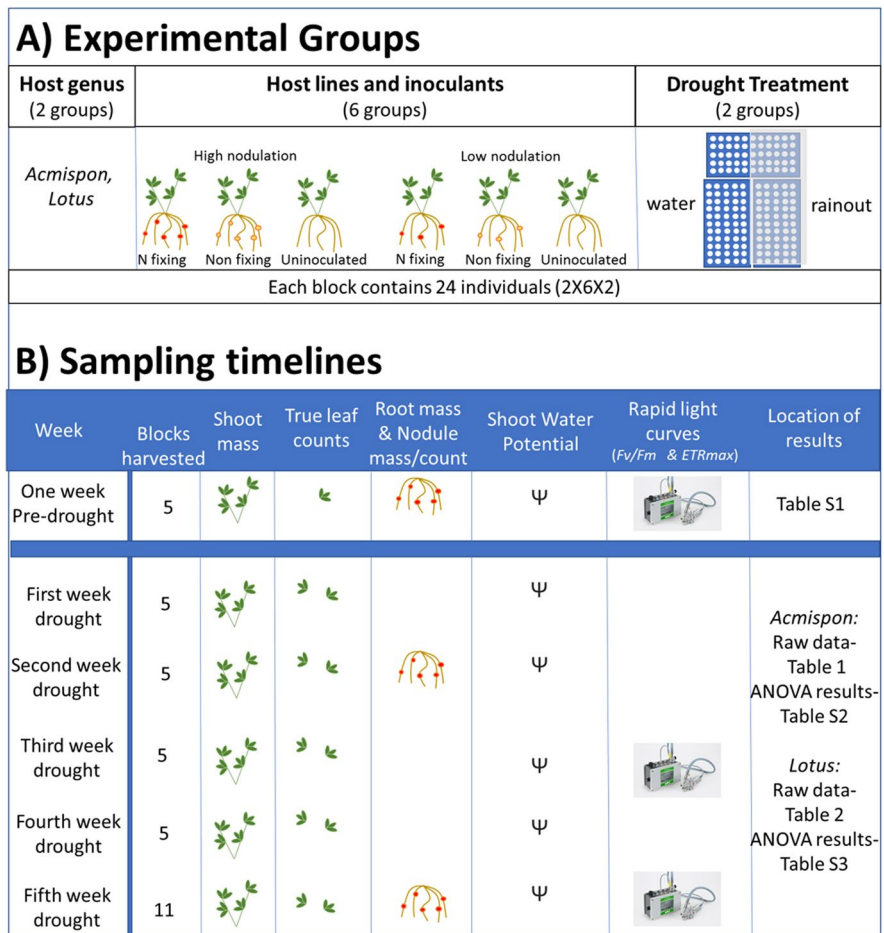
(Gano-Cohen et al. 2020). Seedlings were drip inoculated directly into the soil with 5 mL of inoculum, with a concentration of 10^8 cells per mL for a total of 5×10^8 cells for inoculation treatments.

After inoculation, plants were arranged in the greenhouse using a randomized split plot and factorial design with a main effect of watering treatments forming our split plot (watered versus drought), with three inoculations (N fixing, nonfixing, uninoculated), and two levels of host nodulation (high nodulation and low nodulation) for a total of twelve experimental groups (Fig. 1). One replicate of each treatment group was included within each block, totaling 24 plants per block with twelve of each species. Thirty-six blocks in total were established to allow for sampling responses over time, five blocks every week for six weeks with an additional six blocks to conduct final biomass measures on shoots that did not undergo water potential measures for a total of 864 plants. The drought treatment began on April 30, 2017 (8 weeks after inoculation) and consisted of placing rainout shelters during morning mist watering (6:30AM for six minutes every day) which were immediately removed post watering. Placement of the rainout shelters ensured the drought treatment did not receive water for the duration of the experiment, thus simulating a truncated rainy season as all plants were germinated and initially grown under watered conditions. We applied the drought treatment for a total of five weeks, hereafter referred to as weeks post drought.

Plant growth and physiology

Five randomly selected blocks were harvested every week for the five weeks of drought treatment and once prior to treatment to establish a baseline. Due to time constraints, we did not collect all types of data on each harvest week; however, we collected all data types in the pre-drought baseline harvest sampling event (Fig. 1). We measured shoot mass at every week post drought and whole plant biomass (roots + shoots + nodules) at two and five weeks post drought (Fig. 1). For whole plant biomass dissections, plants were removed from pots, washed free of soil

Fig. 1 Experimental design. Host genera were analyzed separately (i.e., *Acmispon*, *Lotus*). Within each genus, experimental groups included host line, inoculation treatment, and drought treatment, totaling 24 individuals per block, with twelve experimental groups per genus (**A**). Sampling timelines are depicted for response variables (**B**) where each week had a sample size of five. Seedlings were 5 weeks old when moved to the greenhouse, were inoculated at 6 weeks, and exposed to drought at 14 weeks. The drought experiment (week 0) thus was initiated when the plants were 14 weeks old. Data collection did not include all metrics in every week, due to time constraints. Pre-drought baseline measures were analyzed separately from the remaining data

A) Experimental Groups

Host genus (2 groups)	Host lines and inoculants (6 groups)	Drought Treatment (2 groups)
Acmispon, Lotus	High nodulation — N fixing, Non fixing, Uninoculated; Low nodulation — N fixing, Non fixing, Uninoculated	water / rainout

Each block contains 24 individuals (2X6X2)

B) Sampling timelines

Week	Blocks harvested	Shoot mass	True leaf counts	Root mass & Nodule mass/count	Shoot Water Potential	Rapid light curves (*Fv/Fm* & *ETRmax*)	Location of results
One week Pre-drought	5	✓	✓	✓	Ψ	✓	Table S1
First week drought	5	✓	✓		Ψ		*Acmispon:* Raw data- Table 1 ANOVA results- Table S2 *Lotus:* Raw data- Table 2 ANOVA results- Table S3
Second week drought	5	✓	✓	✓	Ψ		
Third week drought	5	✓	✓		Ψ	✓	
Fourth week drought	5	✓	✓		Ψ		
Fifth week drought	11	✓	✓	✓	Ψ	✓	

and dissected into shoot, root, and nodule portions. Nodules were counted and photographed. Biomass was measured by drying tissues in an oven at 60 °C for at least 48 h. In the fifth week post drought, we used a separate set of six blocks independent of the blocks used for physiology measures to perform final complete biomass measures on plants that were still intact (Fig. 1).

Pre-dawn shoot water potentials were measured using a Scholander pressure chamber (Model 600D; Plant Moisture Stress Instruments, Albany, OR, USA) over two consecutive mornings each week of the experiment. We measured shoot water potential on the five blocks designated for harvest each week. Sampling events began at midnight to allow plant water status to equilibrate with soil moisture, and blocks were placed in opaque polyethylene bags before being taken to the lab to minimize transpiration. Water potential measures were destructive to the samples, as these plants are too small to utilize a single leaf. In preliminary trials we found the stems of our samples snapped in the gasket closure. We wrapped strips of parafilm across the base of the stem to protect the stem from breaking under pressure and made a new longer gasket to spread the pressure of the gasket across a greater area thereby preventing stem breakage within the gasket. Gas was metered carefully and slowly into the chamber due to the delicate nature of the shoots and to increase the accuracy of our measures.

Chlorophyll fluorescence was quantified using a portable chlorophyll fluorometer (mini-PAM; Waltz, Effeltrich, Germany) to measure photosystem response. Measures were taken at 3 weeks and 5 weeks post drought beginning at least one hour after sunset to ensure leaves were dark adapted. Variable fluorescence/maximal fluorescence (*Fv/Fm*) was calculated as a metric of photosystem II stress

where greater values equate to less photosystem stress (Maxwell and Johnson 2000). Maximum electron transport rate (ETRmax) was calculated as an index of photosynthetic capacity, where higher values indicate greater capacity for photosynthesis, with rapid light curves across nine values of photon flux density ranging from 0–2500 μmol m^{-2} s^{-1} (Maxwell and Johnson 2000) and derived using Photosynthesis Assistant (ver. 1.1; Dundee Scientific, Dundee, UK).

Data analysis

Data were transformed when necessary to achieve normal distributions of model residuals to satisfy the assumptions of ANOVA. We used a square root transformation for *Acmsipon* nodule counts, and *Acmispon* and *Lotus* nodule weights, and water potentials. We used a log10 transformation for *Acmispon* and *Lotus* shoot mass, true leaf counts, and ETRmax values. In *Lotus*, the pre-drought measures of true leaf counts were not transformed, and the *Fv/Fm* values were log10 transformed. The percent leaf nitrogen and carbon in both *Acmispon* and *Lotus* were log10 transformed. Pre-drought plant measures (i.e., baseline) were analyzed separately from the post-drought measures. This allowed us to determine that the effects of drought were due to the implementation of the drought treatment and to set a baseline for the physiological metrics. Pre-drought data were analyzed using a three-way ANOVA to test the fixed and interactive effects of host line, inoculant, and drought. The denominator degrees of freedom were adjusted to account for the split-plot design by including an error term with drought treatment nested within block. Post drought measures were analyzed in the same manner, but with the additional fixed effect of week. All analyses were performed in SAS (version 9.4). Correlations between nodulation and water stress were executed using the base R package *corr()* function.

Results

Acmispon hosts

Host nodulation and growth response: BMR-highnod and Yucca-lownod hosts had growth and nodulation responses that were consistent with previous experiments, both in pre-drought measures (Supplemental data, Table S1; Yoshida et al. 2010; Sachs et al. 2010) and during the drought treatment, with BMR-highnod forming more nodules with greater mass than Yucca-lownod hosts, independent of inocula type (Table 1, Table S2). Three out of 45 plants formed nodules in the uninoculated control group. All were BMR-highnod hosts, with two in the watered treatment forming one or two nodules, and one in the drought group forming five nodules.

During the drought exposure, BMR-highnod hosts had significantly greater shoot and root biomass compared to Yucca-lownod (shoot host $p < 0.0001$ $n = 369$, root host $p < 0.0001$ $n = 132$, Fig. S1) and formed significantly more true leaves (host $p < 0.0001$ $n = 359$), consistent with positive fitness feedbacks from greater investment into symbiosis. Hosts inoculated with the nitrogen fixing strain had the greatest average shoot biomass and most true leaves compared to uninoculated hosts and hosts inoculated with non-fixing strains, indicating a significant benefit received from nitrogen fixing symbionts (shoot inoculant $p < 0.0001$ $n = 369$, true leaf inoculant $p < 0.0001$ $n = 359$, Fig. 2).

Hosts subjected to the drought treatment had significantly lower shoot biomass (drought $p = 0.0015$ $n = 369$, Fig. 2) and fewer leaves (drought $p = 0.0048$ $n = 359$, Fig. 2) compared to the watered hosts, indicating that the drought treatment induced biologically significant water stress. Hosts inoculated with the nitrogen fixing strain had greater shoot mass in the watered treatment, and even in drought, hosts inoculated with the fixing strain had greater shoot mass than the hosts inoculated with nonfixing strains and uninoculated hosts (interaction drought X inoculum $p = 0.0005$ $n = 369$, Fig. 2). These results indicate that the presence of nitrogen fixing rhizobia enhanced host growth during the stressful drought period. The interaction between host and inoculation treatments was significant for shoot dry mass and true leaf counts (interaction host X inoculant shoot $p < 0.0001$ $n = 369$, true leaf count $p < 0.0001$ $n = 359$), also consistent with fitness feedbacks from investing in symbiosis.

Physiological response to drought: Drought-treated plants had significantly reduced water potentials compared to the watered plants (drought $p < 0.0001$ $n = 272$, Fig. 3), an effect that intensified over the course of the drought treatment reaching an average of -4.4 MPa for all host lines and inocula in

Table 1 Averages and standard error of untransformed data for response metrics of experimental groups over the course of drought for *Acmispon*. Not all measures were taken in every week, and were left blank in this table, please refer to Fig. 1 for more information on experimental design and when measures were performed

Time	Line[1]	Inoc.[2]	Drgt[3]	Shoot mass (g)	Root mass (mg)	True leaf count	Water Ptl.[4] (Mpa)	Nodule count	Nodule Mass (mg)	ETRmax	Fv/Fm
1	B	C	D	0.024±0.004		18.6±1.94	-1.70±0.25				
1	B	C	W	0.027±0.008		23.2±5.10	-0.95±0.42				
1	B	F	D	0.052±0.013		40.8±9.57	-2.38±0.43				
1	B	F	W	0.069±0.022		40±6.75	-1.13±0.18				
1	B	NF	D	0.024±0.005		23.6±2.44	-1.64±0.26				
1	B	NF	W	0.038±0.004		28.4±2.25	-0.59±0.08				
1	Y	C	D	0.015±0.005		21±4.56	-1.62±0.33				
1	Y	C	W	0.019±0.005		21.4±3.6	-0.88±0.32				
1	Y	F	D	0.023±0.008		22.6±4.02	-1.67±0.58				
1	Y	F	W	0.021±0.004		21.8±2.42	-0.79±0.25				
1	Y	NF	D	0.019±0.005		20.8±2.48	-1.86±0.35				
1	Y	NF	W	0.024±0.008		23.2±4.14	-0.80±0.29				
2	B	C	D	0.032±0.007	15.9±3.26	19.0±7.78	-2.48±0.65	0±0			
2	B	C	W	0.037±0.005	18.2±2.43	30.0±6.68	-1.02±0.42	0.2±0.2			
2	B	F	D	0.067±0.013	17.9±3.81	45.8±12.28	-3.47±0.36	13.2±3.44	5.58±1.74		
2	B	F	W	0.068±0.016	17.5±4.37	47.5±10.5	-2.13±0.50	13.2±3.56	6.65±1.99		
2	B	NF	D	0.025±0.004	15.9±3.87	19.2±4.25	-3.06±0.45	33±9.32	1.62±0.56		
2	B	NF	W	0.027±0.005	12.6±2.40	25.3±3.99	-1.50±0.46	36.8±12.3	2.36±0.92		
2	Y	C	D	0.017±0.006	8.42±2.49	13.5±4.41	-2.99±0.63	0±0			
2	Y	C	W	0.014±0.002	6.03±0.74	15.5±3.30	-1.71±0.72	0±0			
2	Y	F	D	0.030±0.011	7.06±3.10	16.8±2.50	-3.16±0.42	13.8±4.53	1.22±0.70		
2	Y	F	W	0.054±0.014	10.8±3.21	27.0±3.65	-1.40±0.73	22.8±2.52	2.03±0.57		
2	Y	NF	D	0.024±0.005	9.64±2.46	20.3±3.12	-2.04±0.23	0.8±0.37	0.27±0.11		
2	Y	NF	W	0.024±0.003	10.9±1.70	19.5±2.72	-0.74±0.12	0.6±0.40	1.39±0.77		
3	B	C	D	0.027±0.005		28.6±4.27	-2.5±0.13			11.6±5.32	0.65±0.13
3	B	C	W	0.048±0.006		30.02±5.43	-0.70±0.08			43.4±17.6	0.70±0.06
3	B	F	D	0.048±0.006		33.6±4.37	-3.70±0.63			57.6±19.7	0.81±0.03
3	B	F	W	0.135±0.020		66.2±5.21	-1.90±0.42			61.4±21.9	0.82±0.02
3	B	NF	D	0.025±0.004		27.8±2.08	-3.60±1.04			22.7±5.76	0.71±0.04
3	B	NF	W	0.032±0.005		32.4±4.17	-0.70±0.34			23.2±11.9	0.73±0.03
3	Y	C	D	0.018±0.003		21.0±1.92	-2.32±0.70			35.3±11.7	0.73±0.06
3	Y	C	W	0.021±0.009		22.6±6.01	-1.41±0.82			65.2±51.3	0.84±0.03
3	Y	F	D	0.029±0.005		24.2±2.67	-2.25±0.59			44.7±12.0	0.83±0.04
3	Y	F	W	0.050±0.009		29.4±3.44	-0.46±0.09			47.4±15.4	0.86±0.01
3	Y	NF	D	0.023±0.004		22.8±4.31	-2.01±0.51			66.5±34.4	0.76±0.02

Table 1 (continued)

Time	Line[1]	Inoc.[2]	Drgt[3]	Shoot mass (g)	Root mass (mg)	True leaf count	Water Ptl.[4] (Mpa)	Nodule count	Nodule Mass (mg)	ETRmax	Fv/Fm
3	Y	NF	W	0.047±0.021		25.8±2.48	-0.51±0.05			35.7±16.0	0.81±0.05
4	B	C	D	0.022±0.003		18.8±2.58	-4.58±0.88				
4	B	C	W	0.032±0.007		27.8±4.44	-0.79±0.31				
4	B	F	D	0.087±0.013		50.8±7.33	-4.08±0.66				
4	B	F	W	0.137±0.031		64.2±11.4	-1.42±0.22				
4	B	NF	D	0.020±0.002		16.0±2.26	-3.09±0.32				
4	B	NF	W	0.025±0.002		22.0±2.17	-0.96±0.38				
4	Y	C	D	0.020±0.005		21.4±3.72	-2.11±0.02				
4	Y	C	W	0.020±0.002		26.2±0.97	-0.54±0.12				
4	Y	F	D	0.031±0.004		25.2±2.15	-3.44±0.51				
4	Y	F	W	0.067±0.024		31.0±6.45	0.50±0.10				
4	Y	NF	D	0.024±0.005		22.6±2.62	-2.57±0.88				
4	Y	NF	W	0.015±0.006		21.4±4.50	-0.87±0.12				
5	B	C	D	0.031±0.008	13.9±3.45	16.1±2.62	-4.33±0.42	0.83±0.83	0.15±0.10	33.6±10.5	0.55±0.11
5	B	C	W	0.041±0.006	15.6±3.60	29.5±3.62	-0.41±0.06	0.33±0.33		73.2±27.2	0.73±0.08
5	B	F	D	0.059±0.010	13.8±2.67	37.9±5.76	-5.03±0.60	31.7±13.1	5.61±1.71	36.7±27.0	0.48±0.17
5	B	F	W	0.172±0.054	24.0±9.22	70.6±14.6	-1.6±0.38	16.2±3.16	7.69±3.26	44.5±22.9	0.81±0.02
5	B	NF	D	0.024±0.004	14.6±1.99	16.6±2.09	-3.41±0.17	43.3±15.7	2.85±1.27	13.2±3.98	0.52±0.13
5	B	NF	W	0.036±0.004	16.1±2.41	25.7±3.09	-1.12±0.46	61.7±22.3	4.62±1.86	29.1±10.4	0.72±0.08
5	Y	C	D	0.019±0.003	10.4±1.82	16.5±1.92	-1.27±0.0				
5	Y	C	W	0.025±0.004	11.2±3.83	22.0±3.24	-0.56±0.20	9.67±3.55	1.66±0.69	62.3±21.7	0.65±0.10
5	Y	F	D	0.035±0.009	6.10±0.93	20.5±3.36	-5.10±1.31	15.5±6.85	1.69±0.67	18.4±7.66	0.50±0.25
5	Y	F	W	0.051±0.013	7.00±1.46	25.1±4.90	-0.77±0.18			50.6±13.9	0.68±0.06
5	Y	NF	D	0.023±0.004	12.9±2.56	17.5±1.93	-5.38±0.82	0.33±0.21	0.12±0.07	87.7±81.2	0.33±0.13
5	Y	NF	W	0.030±0.005	15.0±4.01	22.0±3.17	-0.69±0.28	0.33±0.33		66.8±11.1	0.75±0.02

1. Line = Hosts Lines: BMR = B, Yucca = Y
2. Inoc. = Inoculation Treatments (Inoc.): Control = C, Fixing = F, Nonfixing = NF
3. Drgt. = Drought Treatment: Water = W, Drought = D
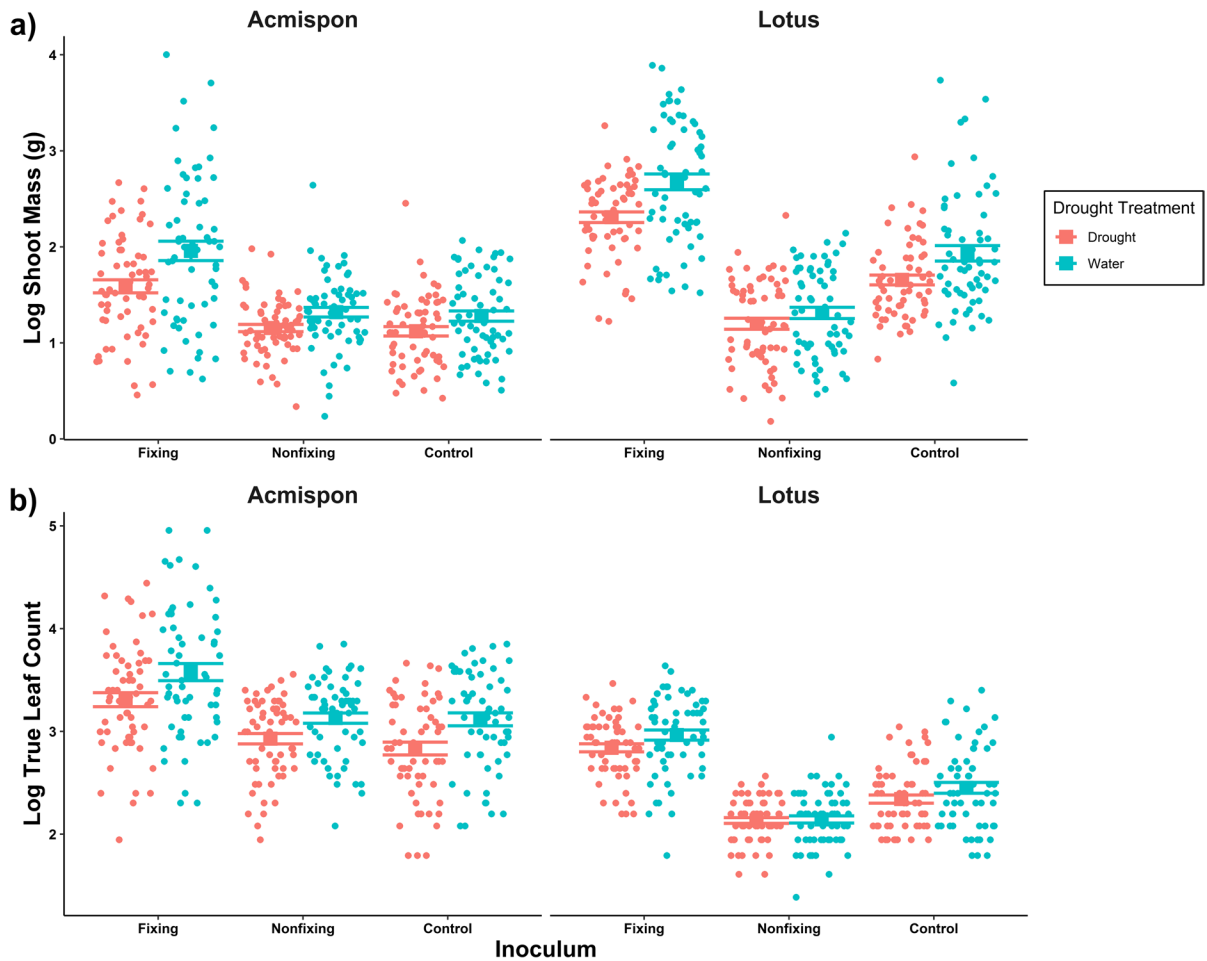4. Water Ptl. = Water potential

Fig. 2 Effects of inoculum and drought on shoot growth and leaf retention. Log transformed values of shoot biomass and leaf counts are shown for *Acmispon* (left) and *Lotus* (right) during the drought treatment. The shoot growth graph displays the interaction between inocula and drought (*Acmispon* $p = 0.0005$, $n = 369$, *Lotus* $p = 0.0002$ $n = 367$). The leaf count figure displays the interaction between inocula and drought (*Acmispon* $p = 0.5953$, $n = 359$, *Lotus* $p = 0.4813$, $n = 359$). Circles are data points within groups, and squares are averages for the group with standard error bars. Scales are different in each plot. Datapoints are jittered to minimize overlap

week 5 of the drought treatment (interaction week X drought, $p < 0.0001$ $n = 272$, Fig. 3). This indicates physiologically significant water stress that increased with length of drought treatment. BMR-highnod hosts had significantly lower shoot water potentials than Yucca-lownod hosts (host $p < 0.0001$ $n = 272$, Fig. 4) regardless of drought treatment, suggesting that the formation of additional nodules by BMR-highnod hosts was associated with water stress. Similarly, hosts inoculated with the fixing strain had lower shoot water potentials than those inoculated with the non-fixing strain and uninoculated hosts (inoculant $p < 0.0001$ $n = 272$

Fig. S2), indicating that nitrogen fixation enhances water stress under these conditions. BMR-highnod hosts had lower water potentials than Yucca-lownod hosts when inoculated with the fixing strain of *Bradyrhizobium*, (interaction host X inoculant $p = 0.0261$ $n = 275$). Neither nodule counts nor nodule mass were correlated with water potential during the drought period, indicating no clear link between increased water stress and nodulation (Pearson's product-moment correlation, $p = 0.852$ $n = 130$, $p = 0.647$ $n = 79$, respectively). We interpret these data to suggest that nitrogen fixation itself is the main driver of water stress to the host, rather than
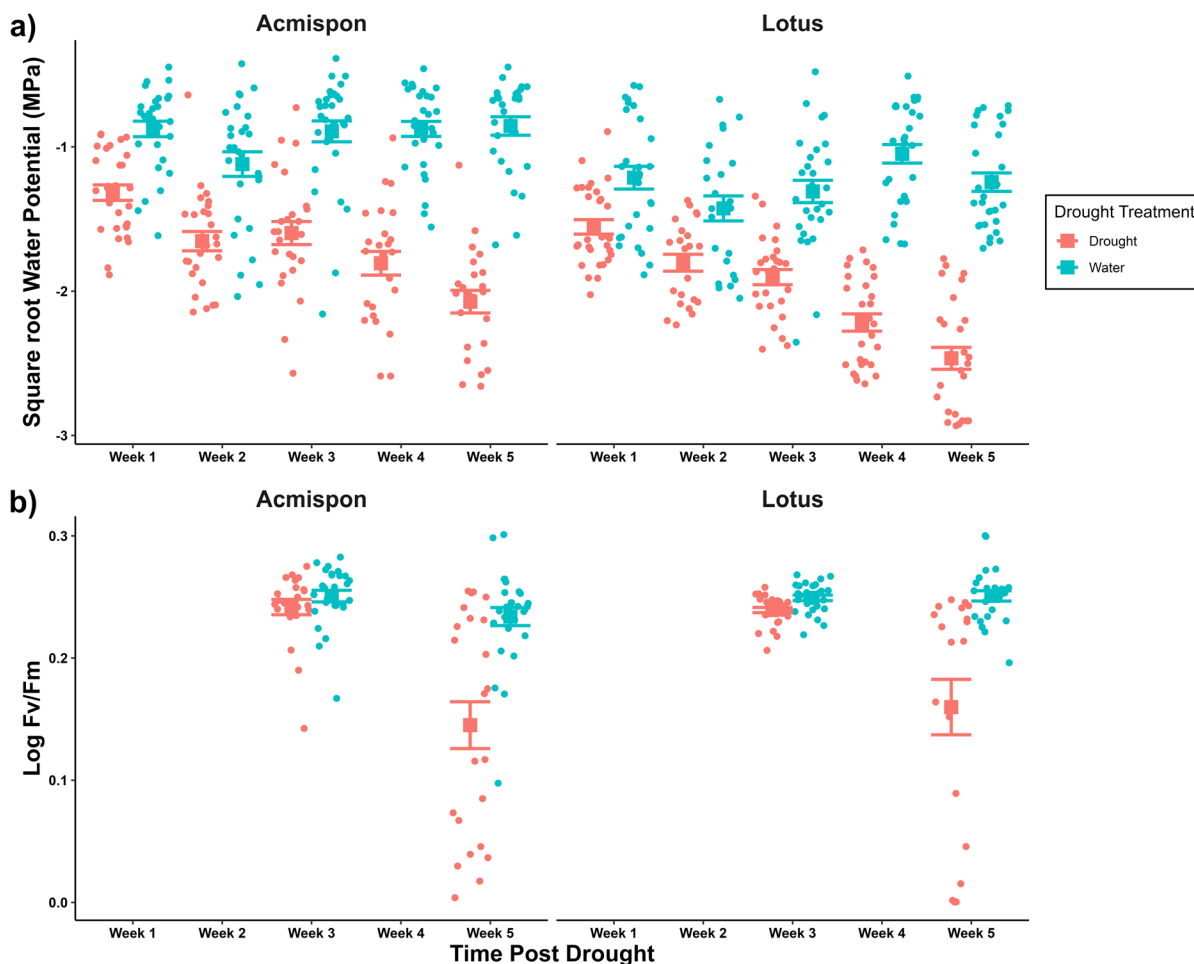
Fig. 3 Effects of time and drought on shoot water potentials and *Fv/Fm*. Values of water potential (MPa, Square root transformed) and chlorophyll fluorescence (*Fv/Fm*, log transformed) are shown for *Acmispon* (left) and *Lotus* (right) plants during the drought treatment. The shoot water potential graph displays the interaction between time and drought treatment (*Acmispon* $p < 0.0001$, $n = 272$, *Lotus* $p < 0.0001$, $n = 273$). The *Fv/Fm* graph displays the interaction between time and drought treatment (*Acmispon* $p = 0.0024$, $n = 100$, *Lotus* $p < 0.0001$, $n = 109$). Circles are data points within groups, and squares are averages for the group with standard error bars. Scales are different in each plot. Datapoints are jittered to minimize overlap

the formation of additional nodules or nodule tissue, at least in the hosts and inoculant strains used. Importantly, the increased water stress due to nitrogen fixation is weak relative to the water stress associated with drought, and nitrogen fixation remains a net positive effect on hosts. Rhizobia strains in nature generate a continuum of benefits to hosts as well as resources extracted from hosts (Gano-Cohen et al. 2020) and could also vary in promoting or protecting hosts from drought stress.

Host maximum electron transport rate (ETRmax) did not differ between host lines (host

ETRMax $p = 0.0591$ $n = 100$), inoculum (inoculant ETRmax $p = 0.2846$ $n = 100$), or drought (drought $p = 0.2494$ $n = 100$), indicating that the maximum rate of photosynthesis remained consistent across experimental groups. Host photosystem stress (*Fv/Fm*) was not affected by host line (host $p = 0.0709$ $n = 100$, Fig. 4), indicating that host investment did not influence the degree of photosystem stress. Hosts inoculated with the fixing strain had the highest *Fv/Fm* values, which were greater than or equal to hosts inoculated with the nonfixing strain and the uninoculated hosts had the lowest *Fv/Fm* values,
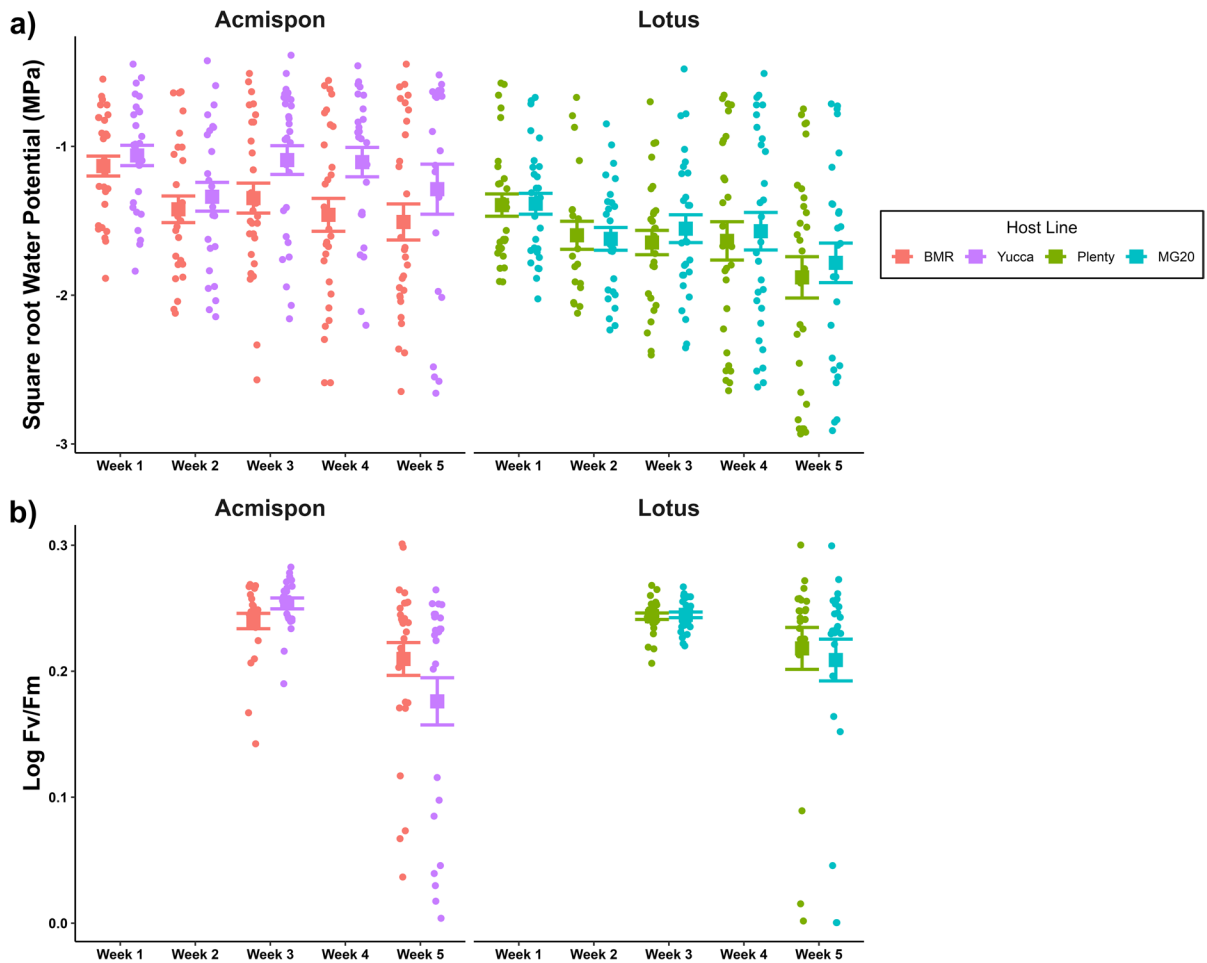
Fig. 4 Effects of plant host line and time on shoot water potentials and *Fv/Fm*. Values of water potential (Mpa, Square root transformed) and photosystem stress (*Fv/Fm*, log transformed) are shown for *Acmispon* (left) and *Lotus* (right) plants during the drought treatment. The shoot water potential graph displays the interaction between host line and time post drought (*Acmispon p=0.2265, n=272, Lotus p=0.7291,* *n=273*). The *Fv/Fm* graph displays the interaction between time and host line (*Acmispon p=0.0642, n=100, Lotus p=0.1147 n=109*). Circles are data points within groups, and squares are averages for the group with standard error bars. Scales are different in each plot. Datapoints are jittered to minimize overlap

indicating the greatest degree of photosystem stress (inoculum *p=0.0094 n=100*). Hosts subjected to the drought treatment had lower *Fv/Fm* values compared to watered hosts (drought *p=0.0003 n=100*, Fig. 3). Hosts had lower *Fv/Fm* values regardless of drought treatment as the experiment progressed (week *p<0.0001 n=100*, Fig. 3); however, this decrease is due to the severe decrease in *Fv/Fm* values of the drought treated hosts in week five with a drought treatment average of 0.43 compared to the watered average of 0.72 (interaction week X drought *p=0.0024 n=100*, Fig. 3, Table S2).

Lotus hosts

Host nodulation and growth response: There was low-level contamination in many of the uninoculated control treatments, with ~75% of harvested plants forming at least one nodule. However, the contamination levels per plant were minimal (in terms of the number of nodules formed on control plants) and expected treatment effects were not obscured. Baseline measures of plant responses (taken before drought was imposed) were consistent with previous experiments, based on expected differences in host

line nodulation response (Krusell et al. 2002; Supplemental data), and on expected differences in growth effects of the different rhizobia strains (Krusell et al. 2002). Hosts inoculated with the fixing strain experienced lower water potentials prior to drought application (inocula $p < 0.0001$ $n = 56$, Table S1), indicating that nitrogen fixing symbionts increase water stress even in watered conditions. During the drought exposure period, the *plenty* hosts had greater nodule counts and nodule mass than wildtype hosts (Table 2, Table S2), consistent with previous work (Quides et al. 2021). Hosts inoculated with the fixing strain (MAFF) had greater nodule dry mass than hosts inoculated with the non-fixing strain (STM6) and uninoculated hosts, indicating increased host investment in the nitrogen fixing strain. Shoot mass was positively correlated with nodule mass (Pearson's product-moment correlation $p < 0.0001$ $n = 55$) but not nodule counts ($p = 0.148$ $n = 60$).

Despite the widespread nature of the contamination, nodulation of the uninoculated controls generally occurred at low levels, and we detected the expected differences among the host lines and rhizobia treatment groups. The MG-20 hosts had greater shoot and root biomass than *plenty* hosts (shoot host $p < 0.0001$ $n = 367$, root host $p < 0.0001$ $n = 131$, Fig. S1), consistent with previous work showing costs of additional nodule production in the mutant host (Quides et al. 2021). MG-20 hosts also had significantly more true leaves than *plenty* (host $p = 0.0002$ $n = 359$, Fig. S1). Notably, the relationship between nodulation and shoot biomass are opposite in *L. japonicus* compared to *A. strigosus*. Hosts inoculated with the fixing strain had significantly more shoot and root biomass and more true leaves compared to hosts inoculated with the non-fixing strain (shoot inoculant $p < 0.0001$ $n = 367$, root inoculant $p < 0.0001$ $n = 131$, leaf count inoculant $p < 0.0001$ $n = 359$; Fig. 2). Growth of hosts inoculated with the fixing strain increased with time for shoot and root biomass as well as leaf counts (interaction week X inoculum shoot $p = 0.0002$ $n = 367$, root $p = 0.0385$ $n = 131$, true leaf count $p = 0.0011$ $n = 359$). Hosts subjected to the drought treatment had significantly lower shoot biomass compared to watered hosts (shoot drought $p = 0.0003$ $n = 367$, Fig. 2) indicating that, similar to the *Acmispon* hosts, drought treatment induced biologically significant water stress. Hosts inoculated with the fixing strain had the greatest biomass in the

watered treatment, and greater biomass in the drought treatment than hosts inoculated with the nonfixing strain and control in the watered treatment (interaction inocula X drought $p < 0.0001$ $n = 367$, Fig. 2). Thus, just as in the *Acmispon* hosts, the presence of nitrogen fixing rhizobia enhanced host growth during drought.

Physiological response to drought: Lotus host line did not influence the shoot water potential independent of drought treatment (host $p = 0.1537$ $n = 273$, Fig. 4). Hosts inoculated with the fixing strain had the lowest shoot water potentials regardless of drought treatment (host $p < 0.0001$ $n = 273$, Fig. S2), again supporting the hypothesis that nitrogen fixation is a key source of water stress (even if that stress is small in magnitude compared to the drought treatment). Drought treatment significantly reduced shoot water potentials in all hosts compared to the watered treatment (drought $p < 0.0001$ $n = 273$, Fig. 3), an effect which increased over the length of the drought where week five had an average shoot water potential of -6.23 MPa independent of inocula type or host line (interaction week X drought $p < 0.0001$, Fig. 3). These data are also parallel with the results from *Acmispon* hosts and indicate that the drought induced significant water stress.

ETRmax was not affected by host line, inoculum, or drought treatment (host $p = 0.1362$ $n = 103$, inoculant $p = 0.4441$ $n = 103$, drought $p = 0.7363$ $n = 103$), indicating the maximum rate of photosynthesis remained consistent between experimental groups. *Fv/Fm* was not affected by host line (host $= 0.1537$ $n = 109$; Fig. 4), indicating that the host lines did not experience different levels of photosystem stress. Hosts inoculated with the fixing strain had the lowest *Fv/Fm* values independent of drought treatment (inoculant $p < 0.0001$, $n = 109$, Fig. S3). Within the drought treatment, hosts inoculated with fixing strains experienced the lowest *Fv/Fm* values (interaction inoculant X drought $p < 0.0001$ $n = 109$, Fig. S3). The drought treatment significantly reduced *Fv/Fm* values (drought $p < 0.0001$ $n = 109$, Fig. 3). All drought treated hosts had higher *Fv/Fm* values in the third week of drought compared to the fifth, and in week five the hosts inoculated with fixing strains had the lowest *Fv/Fm* values with an average of 0.29 (interaction week X inoculant X drought $p < 0.0001$ $n = 109$), implying that hosts inoculated with the nitrogen

Table 2 Averages and standard error of untransformed data for response metrics of experimental groups over the course of drought for *Lotus*. Not all measures were taken in every week, and were left blank in this table, please refer to Fig. 1 for more information on experimental design and when measures were performed

Time	Line[1]	Inoc.[2]	Drgt[3]	Shoot mass (g)	Root mass (mg)	True leaf count	Water Ptl.[4] (Mpa)	Nodule count	Nodule Mass (mg)	ETRmax	Fv/Fm
1	M	C	D	0.054±0.012		11.2±1.86	-2.45±0.30				
1	M	C	W	0.058±0.009		10.8±1.07	-1.13±0.29				
1	M	F	D	0.125±0.011		19.6±0.75	-2.81±0.55				
1	M	F	W	0.129±0.018		18.8±1.16	-2.44±0.37				
1	M	NF	D	0.035±0.004		9.6±0.92	-2.15±0.40				
1	M	NF	W	0.044±0.006		9.4±0.40	-1.21±0.36				
1	P	C	D	0.032±0.006		9.6±1.40	-2.00±0.17				
1	P	C	W	0.035±0.004		8.4±0.68	-1.32±0.57				
1	P	F	D	0.100±0.008		19.0±0.78	-3.07±0.18				
1	P	F	W	0.088±0.008		18±1.14	-2.82±0.15				
1	P	NF	D	0.014±0.004		8.4±0.68	-2.46±0.42				
1	P	NF	W	0.013±0.002		9.6±2.36	-0.78±0.20				
2	M	C	D	0.051±0.010	17.7±3.18	11.8±2.78	-3.05±0.43	8.8±3.26	3.29±1.26		
2	M	C	W	0.062±0.022	16.5±4.08	15.0±3.58	-1.90±0.52	9.6±4.03	5.47±1.28		
2	M	F	D	0.098±0.015	20.0±4.08	17.8±2.56	-4.58±0.21	34.8±6.98	8.32±2.16		
2	M	F	W	0.136±0.035	32.3±6.37	22.0±3.03	-2.84±0.61	44.2±9.56	7.88±2.02		
2	M	NF	D	0.039±0.005	13.9±2.37	9.25±1.11	-2.57±0.39	56.4±9.78	3.12±0.52		
2	M	NF	W	0.039±0.006	16.2±2.93	9.75±0.75	-1.93±0.61	58.8±11.08	2.62±0.42		
2	P	C	D	0.030±0.004	11.4±2.76	9.75±1.11	-2.88±0.39	24.8±10.50	6.75±1.61		
2	P	C	W	0.042±0.01	13.4±1.90	9.50±1.85	-1.73±0.37	66.6±24.80	11.1±1.94		
2	P	F	D	0.097±0.029	20.4±4.69	19.0±2.48	-4.18±0.18	91.6±15.20	11.9±2.76		
2	P	F	W	0.064±0.024	14.6±6.12	16.0±2.35	-3.11±0.79	61.2±9.71	8.03±1.50		
2	P	NF	D	0.012±0.003	3.40±1.04	8.00±0.71	-2.54±0.21	81.4±17.12	6.64±1.92		
2	P	NF	W	0.015±0.003	3.20±0.47	7.50±0.50	-1.97±0.68	92.6±9.63	8.18±1.17		
3	M	C	D	0.044±0.004		11.0±1.14	-3.16±0.41			8.84±2.71	0.73±0.02
3	M	C	W	0.063±0.013		12.2±2.20	-1.66±0.47			14.9±4.31	0.79±0.03
3	M	F	D	0.082±0.022		18.8±1.43	-3.76±1.06			30.7±19.4	0.73±0.02
3	M	F	W	0.209±0.057		20.0±3.54	-3.01±0.88			35.1±12.3	0.79±0.02
3	M	NF	D	0.038±0.002		9.20±0.58	-3.39±0.11			14.0±3.63	0.78±0.00
3	M	NF	W	0.045±0.004		10.6±0.75	-0.97±0.20			12.0±1.08	0.74±0.02
3	P	C	D	0.055±0.017		12.8±2.11	-3.85±0.56			26.3±7.39	0.73±0.01
3	P	C	W	0.051±0.013		10.0±0.95	-1.88±0.27			16.0±4.25	0.76±0.03
3	P	F	D	0.102±0.01		20.2±1.36	-4.92±0.35			13.0±4.10	0.71±0.03
3	P	F	W	0.160±0.024		21.4±2.42	-2.24±0.13			17.6±7.02	0.80±0.02
3	P	NF	D	0.014±0.003		8.00±0.45	-3.35±0.29			14.2±1.92	0.74±0.02

Table 2 (continued)

Time	Treatments			Shoot mass	Root mass	True leaf count	Water Ptl.[4]	Nodule count	Nodule Mass	ETRmax	Fv/Fm
	Line[1]	Inoc.[2]	Drgt[3]	(g)	(mg)		(Mpa)		(mg)		
3	P	NF	W	0.019±0.001		8.00±0.45	-1.17±0.38			31.0±7.17	0.78±0.01
4	M	C	D	0.043±0.007		10.4±1.25	-4.47±0.47				
4	M	C	W	0.053±0.007		13.6±2.54	-0.92±0.44				
4	M	F	D	0.122±0.021		18.0±2.12	-6.51±0.16				
4	M	F	W	0.224±0.014		23.8±1.50	-1.71±0.19				
4	M	NF	D	0.036±0.004		9.60±0.68	-3.86±0.27				
4	M	NF	W	0.056±0.004		11.6±0.51	-0.70±0.13				
4	P	C	D	0.046±0.01		11.0±1.52	-4.39±0.67				
4	P	C	W	0.050±0.011		10.4±0.93	-1.04±0.45				
4	P	F	D	0.096±0.016		21.6±3.14	-6.42±0.23				
4	P	F	W	0.264±0.039		28.0±3.21	-1.63±0.24				
4	P	NF	D	0.013±0.002		8.00±0.32	-4.47±0.73				
4	P	NF	W	0.013±0.001		6.60±0.40	-1.30±0.44				
5	M	C	D	0.05±0.01	19.7±3.31	11.2±1.20	-6.52±0.75	15.8±5.17	3.07±1.04	13.9±9.40	0.58±0.12
5	M	C	W	0.13±0.04	34.8±10.1	16.3±1.89	-1.81±0.40	20.5±9.43	9.62±2.62	19.0±5.24	0.80±0.03
5	M	F	D	0.10±0.02	25.7±5.84	16.2±2.23	-5.15±1.14	35.0±3.81	6.61±1.30	106±105	0.04±0.04
5	M	F	W	0.20±0.05	20.9±8.09	18.0±2.73	-2.29±0.19	32.4±6.62	6.27±2.06	27.0±10.6	0.79±0.09
5	M	NF	D	0.04±0.01	19.9±2.62	9.91±0.58	-5.73±0.84	77.0±11.40	3.54±0.30	11.8±4.46	0.70±0.06
5	M	NF	W	0.05±0.00	17.0±2.23	9.09±0.61	-0.94±0.27	58.0±9.37	2.86±0.44	24.4±3.28	0.73±0.02
5	P	C	D	0.05±0.01	14.7±2.67	10.6±1.37	-6.72±1.01	35.7±14.3	6.30±2.48	42.0±34.7	0.51±0.24
5	P	C	W	0.11±0.032	19.9±3.34	14.0±2.25	-1.68±0.31	71.8±19.55	9.04±3.64	38.3±19.4	0.78±0.02
5	P	F	D	0.08±0.013	14.5±1.25	14.3±1.20	-8.10±0.28	65.6±9.81	9.68±1.50	214±213	
5	P	F	W	0.17±0.032	24.5±9.75	22.1±2.66	-2.13±0.38	80.8±14.9	13.4±4.21	65.8±24.8	0.82±0.07
5	P	NF	D	0.02±0.002	3.43±0.59	6.91±0.39	-5.09±0.77	83.2±10.5	6.61±0.90	12.5±0.94	0.59±0.09
5	P	NF	W	0.012±0.00	3.23±0.62	7.55±0.76	-1.26±0.37	105±22.10	7.15±1.62	27.6±9.74	0.80±0.03

1. Line = Hosts Lines: BMR = B, Yucca = Y

2. Inoc. = Inoculation Treatments (Inoc.): Control = C, Fixing = F, Nonfixing = NF

3. Drgt. = Drought Treatment: Water = W, Drought = D

4. Water Ptl. = Water potential

fixing strain experienced greater photosystem stress late in drought.

Discussion

Bacterial symbionts can enhance fundamental aspects of plant growth, development, and fitness (Friesen et al. 2011). But there has been little work to understand how these partnerships affect plant responses to drought, an increasingly important perturbation under climate change (Dai 2011). Our results suggest three broad conclusions about the effects of rhizobia on plant drought response. First, the net benefits of symbiotic nitrogen fixation appeared early in the growing season, before simulated late season drought was imposed. These benefits were broadly maintained under drought conditions. Symbiotic nitrogen fixation increased host biomass, true leaf counts, and leaf retention under drought, with little variation contributed by host nodulation response or species, and with little change over the course of the drought. Second, despite the net benefits, symbiosis can cause additional water stress. In the *Acmispon* experiments, the host lineage influenced the degree of water stress experienced, whereas in *Lotus* the host line did not affect shoot water potential. Water stress was greatly intensified during drought when hosts were infected with nitrogen fixing symbionts, likely because the uptake and movement of nitrogen into plants requires water (Cramer et al. 2009) and the maintenance of photosynthesis during drought conditions from increased nitrogen access (McDowell 2011). Despite the marginal costs of nitrogen fixation that we detected, the presence of nitrogen fixing rhizobia was always associated with a net growth benefit to plant hosts. Third, the main costs and benefits of rhizobia were associated with nitrogen fixation rather than nodulation, as the water costs of associating with rhizobia largely disappeared in associations with non-fixing rhizobial symbionts, and there is no significant correlation between nodulation count or biomass on water stress (Pearson's product-moment correlation, $p=0.852$, $p=0.647$). Thus, the costs of maintaining symbiosis with rhizobia during drought are likely dependent upon the amount of rhizobial nitrogen fixation as hosts inoculated with ineffective rhizobia in our experiment did not experience greater water stress. The amount of nitrogen fixed by rhizobia can vary with both the strain of rhizobia and host genotype (Heath et al. 2010) and certain strains of rhizobia are ineffective at fixing nitrogen (Sachs et al. 2018). Parallel to our results on rhizobia, fungal symbionts can also enhance plant growth during drought (Li et al. 2014), but notably the mechanism of enhanced growth in fungal symbioses is different than in rhizobial symbioses. However, fungal symbionts do not appear to impose additional water stress during drought as we observed here, likely because the surface area of hyphal networks can substantially increase water access for a plant (Allen 2007) and would also promote the transfer of nutrients to the host.

The benefits of rhizobia under drought

Nitrogen fixation increased retention and production of true leaves in both *Lotus* and *Acmispon* regardless of drought treatment. This greater retention of leaves might in turn favor long term survival under drought by mitigating the degree of carbon starvation experienced by hosts. We interpret these data as suggesting that the increased retention of leaves in herbaceous plants is associated with prioritization of mitigating carbon starvation and would be on the anisohydric end of the drought response spectrum, wherein plants maintain stomatal conductance when soil water diminishes compared to isohydric plants that minimize stomatal conductance at the cost of increased carbon stress (McDowell et al. 2008). Increases in shoot growth also suggest that plant hosts have greater carbon stores prior to drought when associating with nitrogen fixing symbionts. Increased growth can reduce carbon starvation because plant hosts have more photosynthate to partition into storage, which is reflected in molecular signals to reduce respiration, and up-regulation of carbohydrate flux to storage (McDowell 2011). Thus, growth benefits from rhizobia prior to a drought might result in enhanced carbon storage during drought periods.

Nodulation by rhizobia might be favored by natural selection even under drought conditions, since the increased growth can result in greater carbon stores prior to droughts to offset carbon stress. Host line influenced shoot growth for both *Acmispon* and *Lotus*, but in opposing ways. In *Acmispon*, higher nodulation was correlated with increased host shoot growth, and the BMR-highnod ecotype formed more nodules and gained

significantly more growth benefits from rhizobial infection than the Yucca-lownod ecotype. The BMR-highnod host line is from a wetter, cooler place and this ecotype has potentially evolved to invest more into symbiosis and growth than the Yucca-lownod host line which is from a drier and hotter habitat. The rhizobia strains used were sourced from BMR-highnod hosts, and adaptation to the symbionts could also have favored them (Wendlandt et al. 2019). In the *Lotus* hosts, the high nodulation response mutant *plenty* experienced reduced shoot growth. The *plenty* mutant is dysregulated for control over nodulation, and produces significantly more nodules than the wildtype MG-20 host from which it is derived, and experimental work shows that it produces more nodules than is optimal given the fixed costs but diminishing benefits of forming nodules (Quides et al. 2021). The maintenance of symbiosis involves costs that can cause a carbon sink during drought conditions (Quides et al. 2021). Host sanctions against inefficient rhizobia might reduce the effects of carbon loss for hosts (Regus et al. 2017), leading to the mitigation of any fitness costs to hosts. Thus, despite variation in the magnitude of growth benefits from symbiosis, both hosts maintained net growth benefits from symbiosis during drought.

Physiological response under drought

Carbon limitation can contribute to plant drought induced mortality (McDowell et al. 2008), especially if photosystems are damaged. Our rapid light curve data suggests the host plants reached the late-stage pathways of plant drought induced mortality, where *Fv/Fm* values in the last week of drought reached an average of 0.48 in *Lotus* and 0.42 in *Acmispon,* where 0.8 is the unstressed measure of *Fv/Fm* (Maxwell and Johnson 2000). *Lotus* plant photosystems were stressed during the drought, and especially so in the late periods of drought. Hosts inoculated with the nitrogen fixing strains experienced the least amount of photosystem stress in *Acmispon* indicating nitrogen fixing symbionts can decrease the stress during drought and potentially ameliorate carbon starvation in some hosts. Conversely, *Lotus* experienced the greatest amount of photosystem stress with nitrogen fixing symbionts suggesting that the *Lotus* hosts are fixing less carbon when inoculated

with nitrogen fixing symbionts, and the benefits of nitrogen fixing rhizobial symbionts occur prior to drought and through increased retention of leaves possibly due to thresholds with water stress damaging the photosystem in *Lotus*. Changes in photosystem function are critical components of the carbon limitation induced by drought and might mitigate the rate of carbon starvation and resulting plant death during drought. Our findings of photosynthetic stress in both hosts during drought suggests that the pre-drought growth stores, and retention of leaves are the primary factors that can ameliorate carbon limitation experienced by hosts.

The second and coupled component of drought induced mortality is water limitation (McDowell et al. 2008). The type of symbiont influenced the degree of water stress experienced by plant hosts, where hosts inoculated with nitrogen fixing rhizobia having greater water stress in both host species, suggesting a water cost associated with maintaining nitrogen fixing symbiosis. In *A. strigosus*, this response was influenced by host line, where the host line that invested more in symbiosis (i.e., BMR-highnod) displayed greater water stress than hosts that invested less (i.e., Yucca-lownod), and the interaction between host and inocula type suggest the influence of nodulating symbionts on water stress is affected by investment, or host line itself. However, genetic differences among hosts due to adaptation to cooler, wetter habitats (i.e., BMR-highnod) versus the desert, (i.e., Yucca-lownod) might be influencing the host-line mediation of water stress present in *A. strigosus*. Experiments with both *A. strigosus* and *L. japonicus* suggest additional water costs associated with maintaining nitrogen fixation under drought conditions. Our work here is one of the first times that physiological water potentials have been used in a water stress experiment on herbaceous plants, and our findings suggest additional mechanisms beyond plant physiology metrics, namely the presence of nitrogen fixing symbionts, are influencing the degree of water stress experienced by plant hosts. Importantly, the physiology framework of drought induced mortality was developed in woody plants (McDowell et al. 2008; McDowell 2011) and may not directly translate to herbaceous plants due to differences in hydraulic architecture (Skelton et al. 2017).

Conclusions

Our results indicate that legume growth benefits acquired from rhizobia can affect survival in drought through enhancing growth pre-drought increasing carbon stores, with the cost of increased water stress especially in nitrogen fixing rhizobia. Overall, the two genera demonstrated consistent responses to inoculations with nitrogen fixing symbionts, suggesting that the effects of beneficial symbioses and drought are likely general in the *Lotaea* family. Plants inoculated with nitrogen fixing rhizobia had greater biomass, and greater amounts of true leaves retained throughout the drought period in both host genera. Additionally, the photosystem stress was lower with nitrogen fixing symbionts partway through drought conditions. However, in *Lotus* hosts inoculated with effective rhizobia had the greatest amount of photosystem stress in the drought treatment. The second component of drought induced mortality investigated in our experiment is the role of water, where inocula directly impacted water status of host plants. We found the water potentials decreased during the course of the drought period, and the decrease in water potentials was consistently greater in hosts inoculated with nitrogen fixing rhizobia.

Benefits from rhizobia detected pre-drought were maintained under drought conditions, despite additional water stress associated with symbiotic nitrogen fixation. Plants that maintain photosynthesis at low or stressed levels during prolonged droughts have greater survival than hosts that conserve water at the cost of increased carbon stress, implying that carbon starvation may play a greater role than water limitation for plant death in long term drought. Symbionts increased plant growth prior to drought and increase retention of leaves during drought in our experiment. Each of these symbiont impacts on plant hosts might mitigate carbon strain on hosts during drought by increasing carbon stores prior to drought, and by reducing carbon losses during drought through increased photosynthetic potential despite increased photosynthetic stress. The plant growth benefits, combined with the increased importance of carbon starvation during prolonged drought suggests that the rhizobial mutualism will be maintained under drought conditions, despite increased water costs that may be driving the increased photosystem stress.

Data availability The data that supports the findings of this study are available in the supplementary material of this article.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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