Symbiosis (2021) 84:209–220
https://doi.org/10.1007/s13199-021-00784-2

The direct and interactive effects of elevated CO_2 and additional nitrate on relative costs and benefits of legume-rhizobia symbiosis

Ryoko Oono[1] · Randy Ho[1] · Andres Jimenez Salinas[1,2]

Received: 18 December 2020 / Accepted: 11 June 2021 / Published online: 10 July 2021

Abstract

Rising concentrations of carbon dioxide (CO_2) is likely to have important effects on growth and development of plants and on their relationship with symbiotic microbes. A rise in CO_2 could increase demand by plant hosts for nutrient resources, which may increase host investments in beneficial symbionts. In the legume-rhizobia mutualism, while elevated CO_2 is often associated with increased nodule growth and investment in N_2-fixing rhizobia, it is yet unclear if this response depends on the mutualistic quality of the rhizobia. To test if host carbon allocation towards more-beneficial nodules are similar to less-beneficial (but still effective) nodules when plant N demand changes, we manipulated plant C and N status with elevated CO_2 and additional nitrate. We used two isogenic *Rhizobium etli* strains that differ in their ability to synthesize an energy reserve compound, poly-beta-hydroxybutyrate (PHB), as well as their efficiencies for nitrogen fixation and nodulation rates, resulting in two *Phaseolus vulgaris* host groups with either large number of small nodules or small number of large nodules. The addition of nitrate negatively affected carbon allocation towards nodules, and elevated CO_2 reversed this effect, as expected. However, this alleviation of nodule inhibition was greater on plants that started with greater numbers of smaller nodules. If smaller nodules indicate less-efficient or low-fixing rhizobia, this study suggests that increased demand for nitrogen in the face of elevated CO_2 has the potential to disproportionately favor less-beneficial strains and increase variation of nitrogen fixation quality among rhizobia.

Keywords *Phaseolus vulgaris* · *Rhizobium etli* · Polyhydroxybutyrate · Climate change · Fertilizer addition

1 Introduction

Nitrogen fixation is one of the most important metabolic reactions for life on Earth (Canfield et al. 2010). This chemical reaction is performed solely by prokaryotic microbes and is further selected by macro-hosts, such as plants or termites, that depend on the symbiotic associations with these microbes for nitrogen (Mylona et al. 1995). For example, plant species in the legume family (Fabaceae) form root nodules in which symbiotic rhizobial bacteria fix atmospheric nitrogen in exchange for the host plant carbon. A single legume plant can associate with multiple rhizobial strains simultaneously and allocates more or less carbon (C) to different nodules based on relative qualities of the nitrogen fixation per nodule mass (i.e., giving more C to nodules that provide more N; Kiers et al. 2006). If host resource allocation to each nodule positively correlates with relative fitness of rhizobial genotypes in those nodules, the host can effectively impose selection for the most-beneficial nitrogen-fixing strains (West et al. 2002). A product of this host selection, or "host sanctions" (sensu West et al. 2002), is the positive alignment (i.e., correlation) between legume and rhizobial fitness during single-strain inoculation because plants with highly-beneficial nodules not only grow more (i.e., have higher fitness) themselves but also allocate more carbon to their nodules (i.e., increasing fitness of the rhizobia; Friesen 2012; Kiers et al. 2013). However, when plants are infected by multiple strains (Kiers et al. 2013) or have alternative sources of nitrogen (Streeter and Wong 1988) or when rhizobia can manipulate host carbon allocation (Ratcliff and Denison 2009), the fitnesses of host and symbiont may not align (i.e., a more fit plant does not necessarily have uniformly larger nodules than

Data Access https://figshare.com/s/af496c1cc16401270bde

✉ Ryoko Oono
ryoko.oono@lifesci.ucsb.edu

[1] Department of Ecology, Evolution, and Marine Biology, University of California, Santa Barbara, CA 93106, USA

[2] Department of Chemistry and Biochemistry, San Diego State University, San Diego, CA 92182, USA

a less fit plant; Sachs and Simms 2006) and the mutualistic traits may no longer be under strong selection for either species (Heath and Stinchcombe 2014).

The fitnesses of a legume host and its rhizobial symbionts may not be positively correlated when traded resources are no longer in high demand (i.e., worth the cost or most important) for one or both partners. For example, legume and rhizobial fitnesses are not aligned in environments with high levels of soil nitrogen (e.g., Streeter and Wong 1988), which decreases plant demand for fixed nitrogen (N) from rhizobia. This is likely because even the most highly-beneficial rhizobia requires more energy to fix N_2 than the energy required of a plant to take up nitrate directly through its roots (Ryle et al. 1979) and legumes become 'less willing' to pay the high cost of N_2 fixation if they can find less-expensive alternatives (Oono et al. 2020; Westhoek et al. 2021). Exceptional cases may be in host species whose demand for N have no clear upper bounds (Camargos and Sodek 2010; Regus et al. 2014; Menge et al. 2015). Other environmental factors, such as herbivory, light availability, and atmospheric CO_2 concentrations, also change the plant demand for fixed N by altering the status of plant carbon (i.e., C:N ratios) and, subsequently, affect rhizobial fitness. For example, plants in the shade with decreased photosynthetic rates and C resources have decreased demand for fixed N and make smaller nodules (Houx et al. 2009; Lau et al. 2012; Taylor and Menge 2018; Friel and Friesen 2019; Heath et al. 2020) whereas plants exposed to elevated CO_2 with increased C resources have increased demand for fixed N and make larger nodules (e.g., Schortemeyer et al. 1999; Ainsworth and Long 2005; Rogers et al. 2006; Bertrand et al. 2007, 2011; Prévost et al. 2010; Sanz-Sáez et al. 2012; Sanz-Sáez et al. 2015). Herbivory, which decreases plant C, like shade, can also decrease investments in symbiotic associations, leading to lower nodule biomass (Vance et al. 1979). However, Heath and Lau (2011) found that herbivory did not affect nodule size and even increased nodule number per plant, suggesting there was an increase in demand for fixed N, perhaps to synthesize more secondary defense compounds (Polley et al. 1989). While a significant body of literature focuses on the direct effects of a particular environmental factor (e.g., elevated CO_2, light availability, soil N) on the legume-rhizobia fitness alignment (e.g., Phillips et al. 1976; Streeter and Wong 1988; Cabrerizo et al. 2001; Heath and Tiffin 2007), fewer studies have tested the interactive effects of two or more factors (but see Wong 1980 for nitrate x sugar; Sa and Israel 1998 for phosphorus x CO_2; Sanz-Sáez et al. 2010 for CO_2 x NH_4NO_3; Thomas et al. 2000; Zhang et al. 2011; Butterly et al. 2016 for nitrate x CO_2; Lau et al. 2012; Friel and Friesen 2019 for light x nitrate). Furthermore, no past studies, to our knowledge, has explicitly explored the interactive effects of nitrate and CO_2 on rhizobial fitness because most are focused on understanding plant yield or growth. In this study, we explored the direct and interactive effects of one environmental factor that directly alters plant C status - atmospheric CO_2 concentrations - with another that directly affects plant N status - supplemental nitrate - on the relative benefits and costs of the symbiosis for two rhizobial strains on bean hosts (*Phaseolus vulgaris*).

The effects of resource availability on benefit-cost ratios for legume-rhizobia symbioses can depend on environmental contexts, such as availability of phosphorus or micronutrients (Niklaus and Körner 2004; Hungate et al. 2004; van Groenigen et al. 2006). Generally, however, nitrate has been shown to decrease nodule biomass (Gibson and Pagan 1977; Streeter and Wong 1988; Heath et al. 2010) and elevated CO_2 has been shown to alleviate any inhibitory effects of soil nitrogen on nodule growth, either average mass per nodule or number of nodules per plant (Thomas et al. 2000; Butterly et al. 2016). What is less clear is how this change in plant investment towards nodules across environmental gradients (e.g., a soil N gradient that shifts plant demand for fixed N) may also depend on the symbiotic quality of the nodule or the rhizobial genotype. For example, Oono et al. (2020) showed that plants differentially allocate resources between highly- and moderately-efficient nodules when plants were given no supplemental nitrate, but this differential allocation (based on relative average weight per nodule) was no longer detectable when plants were given supplemental nitrate (echoing similar results from Kiers et al. 2006), suggesting host selection for high-fixing rhizobia was weakened when plant N demand decreased. We hypothesize that carbon allocation towards more-beneficial nodules are disproportionately penalized relative to less-beneficial (but still effective) nodules when plant N demand decreases. In this study, we decreased plant demand for fixed N by providing supplemental nitrate and also increased demand for fixed N by elevating atmospheric CO_2 in gas-tight transparent chambers in the greenhouse. We then evaluated whether any changes in the correlation between rhizobial and legume fitnesses under each of these conditions were due to the relative benefit-cost ratio of the symbiosis (i.e., rhizobial strain quality) to the host.

We conducted single-strain inoculation experiments using one of two isogenic strains of *Rhizobium etli*, CE3 and SAM100 (Cevallos et al. 1996). CE3 is derived from the type strain, CFN42 (Quinto et al. 1982), whereas SAM100 is derived from CE3 but with an insertion in the phaC gene, preventing its synthesis of the lipid poly-β-hydroxybutyrate (PHB). This energy-storage lipid compound allows rhizobial cells to reproduce and survive in the soil (Bergersen and Turner 1990; Anderson and Dawes 1990). Cevallos et al. (1996) found that this PHB-negative SAM100 mutant had prolonged N_2-fixation and ability to increase the biomass of its host, relative to wild-type CE3, suggesting that SAM100 is a more efficient N_2-fixer compared to CE3. Recent work measuring H_2 production and CO_2 respiration from intact root systems also corroborate differences in fixation efficiencies

between the two strains (Oono et al. 2020). However, the PHB-negative SAM100 nodulates far slower, typically forming nodules 7–10 days after CE3, and between 33 and 50% fewer nodules per plant than CE3 during single-strain inoculations. We quantified rhizobial fitness under factorial combinations of plants growing under ambient or elevated CO_2 and with or without 5 mM of nitrate. We measured host shoot N as a proxy for host fitness or host benefit and nodule total biomass as a proxy for host cost. Because rhizobia can use plant carbon for either reproduction or cellular energy storage (Denison 2000), we measured rhizobial fitness by average weight per nodule, which is highly correlated with cells per nodule within strains (Ratcliff et al. 2011), and PHB per cell, shown to fuel cell division and to prolong rhizobial survival in nutrient-limited environments (Ratcliff et al. 2008), using flow cytometry.

2 Materials and methods

2.1 Rhizobial strains

Rhizobium etli were grown in 125-ml Erlenmeyer flasks containing 50 ml of tryptone-yeast medium and respective antibiotics (30 μg/ml of Km and 200 μg/ml of Str for PHB-negative, SAM100, and 200 μg/ml of Str for wild-type, CE3) at room temperature with shaking until the flasks were cloudy. Cells were resuspended into starvation buffer (Wei and Bauer 1998) before inoculation.

2.2 Plant growth conditions and treatments

Seeds of dwarf beans (cv. Royal Burgundy) were surface sterilized with 0.09% hypochlorite (3% commercial bleach) for 5 min, rinsed in deionized water, and allowed to germinate in a Petri dish with wet tissues. Germinated seeds were transferred to 8 in.-deep cone-tainers (160 mL) containing vermiculite within one week of seed preparation and watered daily with 10 mL nitrogen-free Fahraeus nutrient media (Fahraeus 1957). At the end of the experiment, there were no signs of 'root-binding' in the cone-tainers, suggesting that the volume of the cone-tainers were sufficient for this dwarf variety. Beans were placed in one of four continuously-stirred tank reactors (CSTR; Heck et al. 1978) located in a greenhouse bay at University of California - Santa Barbara. Briefly, air from each of the CSTR chambers are sequentially sampled every eight minutes for two minutes each by an infrared gas analyzer (EGM-4, PP Systems International, Inc., Amesbury, MA). Rates of CO_2 gas flow from a gas cylinder are modified electronically to specific chambers until CO_2 concentrations are at target levels. The CSTR received either ambient (averaging 463 ppm) or elevated (averaging 820 ppm) atmospheric CO_2. For the elevated CO_2 treatment, we chose a concentration sufficiently higher than what the ambient chambers occasionally experienced during the day (~600 ppm) because the chambers were not equipped with CO_2 scrubbing. Experiments in open-air chambers in the field or in highly-controlled indoor growth chambers would also have been valuable, although field studies rarely have the ability to control the microbial soil community and indoor chambers may not provide adequate solar radiation to meet the demands of plants growing in elevated CO_2. In this study, we weighed the risk of potential contamination in greenhouse settings to the benefits of testing under natural light. We recorded environmental parameters, such as relative humidity, temperature, photosynthetically activated radiation (PAR), and real-time CO_2 levels for each chamber throughout the experiment (see Suppl. Fig. 1–3). To avoid having plants exposed to prolonged microclimatic differences of individual CSTR chambers, CO_2 treatments were randomly assigned to the four chambers every week and every plant sample was randomly moved to the newly assigned chambers of the respective CO_2 treatment every few days. Each chamber contained 61 plants; 19 controls with no rhizobial inoculations, 21 plants with just PHB-negative (SAM100) strain, and 21 plants with just wild-type (CE3). Because SAM100 cannot synthesize PHB and appear to not survive in the rhizosphere for as long as the wild-type after initial inoculation, we inoculated plants assigned to the SAM100 treatment twice with fresh inoculum, three and eight days after planting to saturate potential nodulation for both strain treatments. We had also observed in preliminary studies that inoculating plants only once with SAM100 could lead to poor survival of the plants due to insufficient nodulation. Furthermore, we did not begin applying the supplemental nitrate treatments (5 mM potassium nitrate to the Fahreaus media) until 2 weeks after initial rhizobial inoculation to encourage nodulation since we were most interested in the effect of nitrate on average nodule size, not nodule numbers. After eight weeks (Oct. 6 -Dec. 7, 2016), we harvested the plants, measured the legume dry weights (shoot and root separately), average nodule mass (based on 10 random nodules), number of nodules per plant, shoot $\delta^{15}N$ for measuring relative contribution of fixed N with the natural abundance method (Unkovich et al. 2008), percent N in shoots, and average PHB per cell for wild-type (CE3) nodules.

2.3 Dry mass and stable isotope analyses

Individual plants were divided into roots and shoots, dried in an oven at 65 °C for at least 48 h, and weighed. Total dried root weights were measured with nodules. Dried samples of shoots were homogenized roughly with scissors before being ground by a Wig-L-Bug Grinding Mill using ball bearings. Approximately 1 mg of the ground samples were packed in tin capsules and their weights were recorded. The isotope ratio mass spectrometry was performed on a Costech ECS 4010

CHNSO at the UCSB Marine Science Institute Analytical Lab. Acetanilide and USGS40 standards were used for isotope analysis of the ground samples. The natural abundance of ^{15}N to ^{14}N, an indication of relative contribution from nitrogen fixation and soil, is reported as $\delta^{15}N$ using atmospheric ^{15}N:^{14}N as the reference ratio (Unkovich et al. 2008). Fifteen bean seeds without seed coats were also destructively analyzed for $\delta^{15}N$ and nitrogen content.

2.4 Rhizobial fitness

Approximately ten random nodules per plant were weighed to estimate average rhizobial fitness per nodule per plant. The nodules were chosen from the roots using a random number generator. Nodules were rinsed with sterile deionized water three times before being crushed in bulk (10 nodules per tube) in ascorbic acid buffer (Arrese-Igor et al. 1992). Rhizobial extracts via centrifugation were stained with Nile red and analysed for mean PHB (pg) per rhizobial cell in the flow cytometer following method in Ratcliff et al. (2008) on a Guava ExpressPlus. Samples were run with seven standards whose PHB concentrations had been determined by a gas chromatograph (min: 0.056 pg/ul, max: 0.334 pg/ul) or with a common set of 20 samples to standardize variations among flow cytometry runs (min: 0.0609, max: 0.1653 pg/uL). For each run, a standardization equation was estimated with the standard samples to calculate the PHB concentration (pg/ul) of the samples. Rhizobial cells were gated with the Guava acquisition software by comparing with a negative control (stained blank sample).

2.5 Statistics

We used linear models with interaction terms (*lm*; stats package, R 3.5.2) to test for the effects of CO_2 concentration, supplemental nitrate, rhizobial inoculation, and their interactions on plant benefit (root and shoot biomass, shoot nitrogen, shoot $\delta^{15}N$), plant cost (number of nodules, total nodule biomass per plant), and rhizobial benefit (average biomass per nodule and PHB per cell) of the symbiosis. We considered shoot nitrogen, a proxy for plant benefit, to also be a proxy for symbiotic cost for the rhizobia if no supplemental nitrate was provided. We considered the benefit-cost ratio between shoot N and total nodule biomass as the symbiotic efficiency or the symbiotic quality of the strain (Oono and Denison 2010). Because the majority of uninoculated control treatments still produced some nodules, we remain open to the potential that all plants could be infected with both strains. However, we found expected differences in nodule numbers and nodule sizes between the two inoculum treatments under ambient conditions with no nitrate, suggesting that plants were dominated by the strain of the inoculum and had minimal cross-contamination. When group means were significantly different, we conducted *post-hoc* testing with Tukey's HSD at a significance level of 0.05.

3 Results

3.1 Effects of CO_2 and nitrate on plant biomass and nitrogen

Although elevated CO_2 (0.38 ± 0.07 s.d. for aCO_2 vs. 0.47 ± 0.12 for eCO_2; $p = 0.132$) and nitrate (0.38 ± 0.09 no N vs. 0.48 ± 0.11 with N, $p = 0.014$) increased the average shoot biomasses with similar effect sizes, only nitrate had statistically significant effects when accounting for all factors (Table 1). And although additional elevated CO_2 and nitrate both increased the average root biomass (0.21 ± 0.04 for aCO_2 vs. 0.25 ± 0.07 for eCO_2, $p = 0.876$; 0.22 ± 0.06 no N vs. 0.25 ± 0.06 with N, $p = 0.901$), neither effects were statistically significant (Suppl. Table 1). Elevated CO_2 and nitrate had greater

Table 1 Linear model summary testing effect of nitrate (0 vs. 5 mM potassium nitrate), CO_2 (elevated vs. ambient), inoculum (wild-type vs. PHB-negative), and their interactions on shoot dry biomass (residual $df = 127$). Estimates are based on plants growing in no nitrate under ambient CO_2 conditions with the wild-type (CE3) as the reference group. Estimates and standard errors are analyzed from non-normalized values. Shoot nitrogen concentrations were square-root transformed for normal distribution. The *t*-values and *p*-values are based on normalized values

	Shoot biomass				Shoot N%				Shoot N			
	Estimate	Std. Error	*t*-value	*p*	Estimate	Std. Error	*t*-value	*p*	Estimate	Std. Error	*t*-value	*p*
Nitrate	**0.071**	**0.028**	**2.495**	**0.014**	0.042	0.206	0.207	0.837	1.608	0.996	1.615	0.111
CO_2	0.042	0.028	1.516	0.132	−0.331	0.200	−1.540	0.128	−0.266	0.970	−0.274	0.785
Strain	0.045	0.029	1.564	0.120	**−0.544**	**0.196**	**−2.712**	**0.008**	−0.839	0.949	−0.883	0.380
Nitrate * CO_2	**0.084**	**0.040**	**2.104**	**0.037**	−0.137	0.278	−0.536	0.594	0.616	1.345	0.458	0.648
N * Strain	**−0.087**	**0.043**	**−2.027**	**0.045**	**0.859**	**0.295**	**2.609**	**0.011**	1.004	1.426	0.704	0.484
CO_2 * Strain	−0.017	0.039	−0.421	0.675	0.074	0.277	0.062	0.951	0.001	1.339	0.000	1.000
CO_2 * Strain * N	0.098	0.058	1.703	0.091	−0.480	0.400	−0.973	0.334	−0.767	1.935	−0.396	0.693

than additive effects on above- (significant CO_2 x nitrate interaction, $p = 0.037$, Table 1) and below-ground plant biomass (significant CO_2 x nitrate interaction, $p = 0.009$, Suppl Table 1). The positive effect of additional nitrate on shoot biomass also depended on the strain (significant nitrate x strain interaction, $p = 0.045$, Fig. 1a). Beans are reported to have one of the poorest capacities for nitrogen fixation among legumes (Isoi and Yoshida 1991), possibly explaining why we saw an increase in host biomass by nitrate but no increase by elevated CO_2 alone.

We found no significant dilution effect by elevated CO_2 to decrease nitrogen concentrations (N%) in shoot tissues overall (1.86 ± 0.58 s.d. for aCO_2 vs. 1.42 ± 0.40 for eCO_2; $p = 0.128$, Table 1), likely due to large variations within each treatment (Fig. 1b). Plants inoculated with the PHB-negative had lower N% than plants inoculated with the wild-type (1.50 ± 0.61 vs. 1.74 ± 0.42, $p < 0.008$), but this was highly dependent on the nitrate treatment (significant nitrate x strain interaction, $p = 0.011$). Additional nitrate increased shoot N% for plants inoculated with the PHB-negative strain (from 1.27 ± 0.28 to 1.79 ± 0.79 s.d.) but not for plants inoculated with the wild-type strain (from 1.77 ± 0.40 to 1.72 ± 0.45 s.d.). Ultimately, this led to no detectable effects for any of the treatments or their interactions on the total nitrogen in shoots, as calculated by multiplying N% with respective dry shoot biomass (Table 1, Fig. 1c). Plants without supplemental nitrate had lower total shoot nitrogen on average, but the effects were not statistically significant (5.67 ± 1.76 s.d. without N vs. 7.92 ± 2.33 with N; $p = 0.111$, Fig. 1c).

3.2 Effects of CO_2 and nitrate on nodulation and rhizobial fitness

The number of nodules was smallest on plants growing with 5 mM of nitrate under ambient CO_2 conditions and greatest on plants growing without nitrate under ambient CO_2 conditions, with nitrate having a significant effect on its own ($p < 0.001$, Fig. 2a, Table 2). However, a consistent effect on nodule number by elevated CO_2 was not found across all treatments ($p = 0.244$). Elevated CO_2 only had a positive effect on nodule numbers when nitrate was supplied (significant nitrate x CO_2 interaction, $p = 0.002$, Fig. 2a, Table 2). Finally, although we found nodules on uninoculated controls and all plants could potentially be co-infected, we found a strong effect by the inoculum treatment on nodule number ($p < 0.001$). As expected from a prior study (Oono et al. 2020), plants inoculated with PHB-negative strain had fewer nodules, averaging 32 nodules (s.d. ±19), while plants inoculated with the wild-type strain averaged 94 nodules per plant (s.d. ±38). If plants inoculated with the PHB-negative strain were contaminated with the wild-type strain, we would expect much greater numbers of nodules per plant. Since the PHB-negative strain is a poor nodulator, we would not expect plants inoculated with the wild-type strain to be significantly contaminated with the

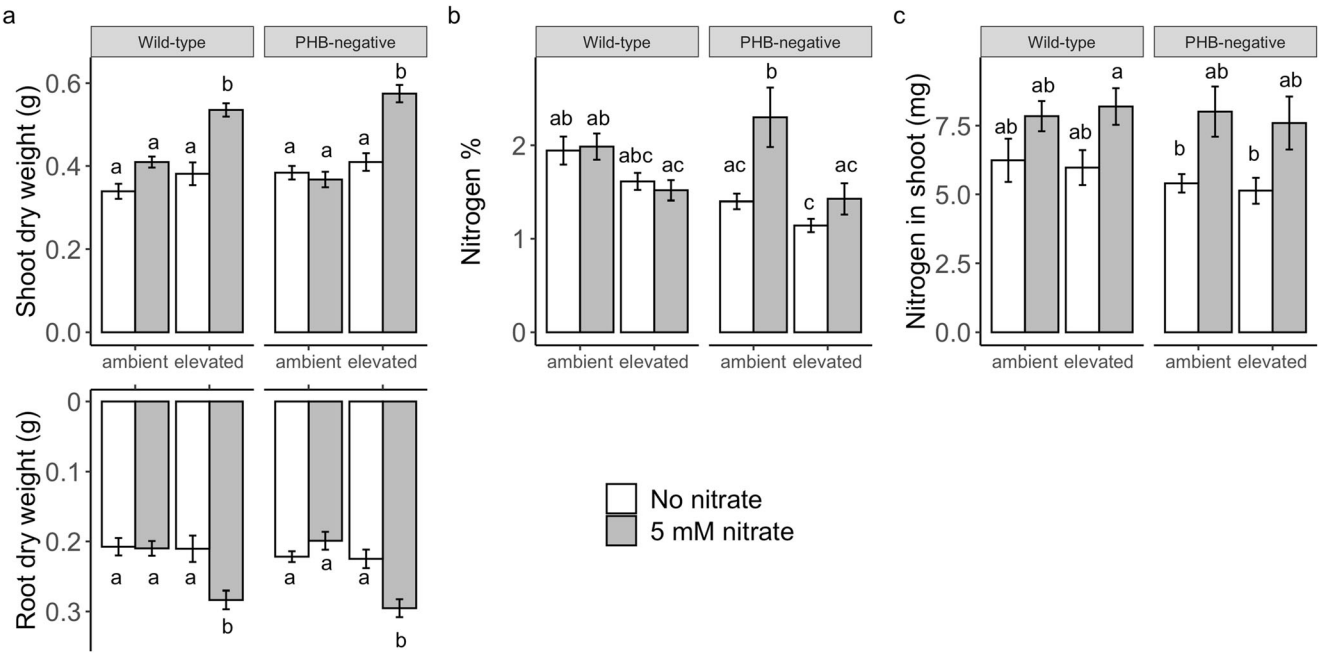

Fig. 1 Effects of CO_2, nitrate and inoculum treatments on shoot and root dry weights, shoot nitrogen percent and total nitrogen. a) Dry weights of above- (shoot) and below-ground (root) host tissues were dried and weighed. Bars are standard errors (15–20 plants per group). b) Shoot tissues were analyzed for nitrogen with isotope mass spectrometer. Bars are standard errors (7–12 plants per group, sample numbers are lower than dry mass because not all samples were analyzed by the mass spectrometer) c) Total shoot N was calculated by multiplying percent nitrogen with total dried shoot biomass. Letters in all panels indicate results from post-hoc Tukey's HSD tests after overall significances were confirmed with linear models with interaction terms (Table 1, Suppl. Table 1)

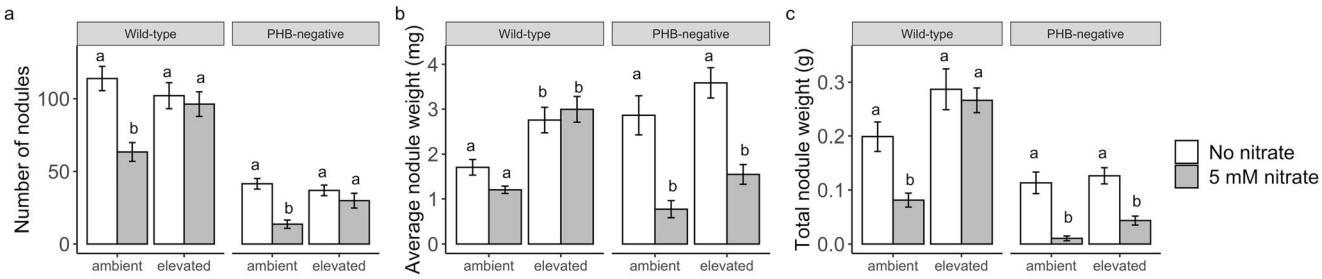

Fig. 2 Effects of CO_2 and nitrate treatments on nodule number and nodule wet weights per plant. Bars are standard errors (15–20 plants per group). Letters indicate results from post-hoc Tukey's HSD tests after linear models tested for significant differences between treatments. All tests were performed separately for each strain treatment

PHB-negative strain. Furthermore, the alleviating effect of CO_2 on the inhibition of nodule number by additional nitrate was consistent between the rhizobial strains (no significant strain x CO_2 x nitrate interaction, $p = 0.643$, Table 2). For the wild-type strain, nitrate decreased nodule numbers by 44% on average under ambient conditions and only by 6% under elevated CO_2 conditions. For PHB-negative strain, nitrate decreased nodule numbers by 68% on average under ambient conditions and only by 19% under elevated CO_2 conditions.

Nitrate did not have a consistent effect on average nodule weights ($p = 0.144$) whereas CO_2 levels increased average nodule weights for both inoculum (on average ± s.d., from 1.5 ± .6 to 2.8 ± 1.1 mg for wildtype or from 2.1 ± 1.8 to 2.6 ± 1.6 mg for PHB-negative) and nitrate (from 2.4 ± 1.5 to 3.2 ± 1.4 mg for no N or from 1.0 ± 0.6 to 2.3 ± 1.3 mg with N) treatments ($p = 0.009$; Fig. 2b, Table 2). Nitrate only decreased average nodule weights for plants inoculated by the PHB-negative strain (from 3.3 ± 1.7 to 1.3 ± 1.0 mg), and had little effect for plants inoculated by the wild-type strain (from 2.2 ± 1.1 to 2.1 ± 1.2 mg; nitrate x strain, $p = 0.001$). Similar to nodule numbers, we found expected differences in average nodule weights depending on inoculum treatment ($p = 0.004$). Plants inoculated with the PHB-negative strain had significantly larger nodules (3.0 mg ±1.8) than those inoculated with the wild-type strain (1.7 mg ± 0.7) under conditions of no nitrate and ambient CO_2 levels, which agrees with all preliminary studies when comparing these strains. The difference in nodule size is likely due to differences in nitrogen fixation efficiency between the strains (Oono et al. 2020) and because nodules with greater fixation efficiencies receive more plant carbon (Cevallos et al. 1996). Consistent and large differences in nodule weight between the two strains further suggest that our plants inoculated with the PHB-negative strain were unlikely to be significantly contaminated with the wild-type strain.

The total nodule weight per plant, as calculated by multiplying nodule number by the average weight per nodule, decreased with additional nitrate ($p < 0.001$), increased under elevated CO_2 ($p = 0.035$) and was generally less on plants inoculated with the PHB-negative strain ($p = 0.009$; Table 2, Fig. 2c). The inhibitory effect of nitrate on total nodule weight depended on the CO_2 condition, where total nodule weights

Table 2 Linear model summary testing effect of nitrate (0 vs. 5 mM potassium nitrate), CO_2 (elevated vs. ambient), inoculum (wild-type vs. PHB-negative), and their interactions on nodule number, average weights per nodule and total nodule biomass per plant for each strain treatment separately (residual $df = 128$). Estimates are based on plants growing in no nitrate under ambient CO_2 conditions with the wild-type (CE3) as the reference group. Estimates and errors are based on non-transformed analyses. Nodule number of PHB-negative strain was square-root transformed for normal distribution. The t-values and p-values are based on normalized values

	Nodule number				Average nodule weight				Total nodule weight			
	Estimate	Std. Error	t-value	p	Estimate	Std. Error	t-value	p	Estimate	Std. Error	t-value	p
Nitrate	**−50.59**	**9.28**	**−4.821**	**<0.001**	−0.503	0.402	−1.469	0.144	**−0.118**	**0.031**	**−4.168**	**<0.001**
CO_2	−11.86	9.15	−1.171	0.244	**1.049**	**0.396**	**2.647**	**0.009**	**0.088**	**0.031**	**2.133**	**0.035**
Strain	**−72.53**	**9.28**	**−7.297**	**<0.001**	**1.291**	**0.402**	**2.949**	**0.004**	**−0.079**	**0.031**	**−2.641**	**0.009**
Nitrate * CO_2	**44.77**	**13.04**	**3.165**	**0.002**	0.742	0.564	1.506	0.134	**0.097**	**0.044**	**2.964**	**0.004**
N * Strain	22.65	14.00	−0.056	0.955	**−1.706**	**0.606**	**−3.455**	**0.001**	0.011	0.047	−1.330	0.186
CO_2 * Strain	7.27	12.79	0.327	0.744	−0.461	0.554	−0.866	0.388	−0.081	0.043	−1.278	0.203
CO_2 * Strain * N	−23.92	18.84	−0.464	0.643	−0.491	0.815	−0.129	0.898	−0.072	0.063	−0.919	0.360

decreased more under ambient conditions (from 0.159 ± 0.105 to 0.055 ± 0.055 g, on average) than under elevated conditions (from 0.202 ± 0.144 to 0.150 ± 0.131 g, on average; significant nitrate x CO_2 interactions, $p = 0.004$).

PHB concentrations in the wild-type CE3 cells significantly decreased in the presence of additional nitrate ($p < 0.001$), but was not affected by elevated CO_2 alone ($p = 0.71$; Fig. 3, Table 3). The decreasing effect of nitrate on PHB concentrations per cell diminished marginally under elevated CO_2 conditions from 37% to 21% on average (nitrate x CO_2, $p = 0.118$). A small subsample of PHB-negative nodules were analyzed but no significant fluorescence signals could be obtained that could be distinguished from negative controls (unstained cells), as expected. This, again, substantiates a lack of significant cross-contamination between the inoculation treatments.

3.3 Effects of CO_2 and nitrate on symbiotic benefit-cost ratio

Total shoot nitrogen was positively correlated with total nodule weight in all conditions (Suppl. Fig. 5a–c) except when plants were inoculated with the PHB-negative mutant and treated with additional nitrate (Suppl. Fig. 5d). Many plants inoculated with PHB-negative mutant and treated with additional nitrate failed to nodulate sufficiently (often less than 10 nodules per plant) and lacked enough variability in total nodule mass to compare with total shoot nitrogen. Since nodulation was not always successful by the PHB-negative mutant when plants were given additional nitrate, we analyzed the benefit-cost ratio of the symbiosis to the host (shoot N: total nodule biomass) just among the plants without any additional nitrate. We confirmed that while the PHB-negative mutant continued to give similar benefit per cost ratios across CO_2 treatments, the wild-type gave less benefit per cost under elevated CO_2 (significant interaction term CO_2 x strain, $p = 0.029$, Table 4, Fig. 4), suggesting that inoculation with the less-efficient wild-type strain did not meet the greater N demand by the host under elevated CO_2 conditions despite increased nodule investment.

The stable isotope signatures of nitrogen were significantly different between the nitrate treatments ($p < 0.001$), but was also different between inoculum treatments ($p = 0.009$; Suppl. Table 2, Suppl. Fig. 4). Hence, we were unable to compare the changes in relative contribution between biological nitrogen fixation and nitrate from ambient to elevated CO_2 conditions between the two inoculums. Shoot $\delta^{15}N$ signatures were maintained between inoculum treatments ($p = 0.009$), likely due to different nodulation rates, further suggesting any cross-contamination was minimal. Additional sources of variation in $\delta^{15}N$ among plants with no nitrate are likely due to contributions from seed N since we found significant variation among bean seeds ($\delta^{15}N$ averaged 0.24 ‰ with standard deviation of 1.14 ‰, $n = 15$).

Table 3 Linear model summary testing effect of nitrate, CO_2, and their interaction on PHB per cell of wild-type CE3 rhizobia (residual $df = 65$). Estimates are based on plants growing in no nitrate under ambient CO_2 conditions as the reference group. PHB per cell were log-transformed for normality

		Wild-type only			
		Estimate	Std. Error	*t*-value	*p*
PHB per cell	Nitrate	**−0.427**	**0.102**	**−4.21**	**<0.001**
	CO_2	0.037	0.1000	0.37	0.712
	Nitrate * CO_2	0.226	0.1425	1.58	0.118

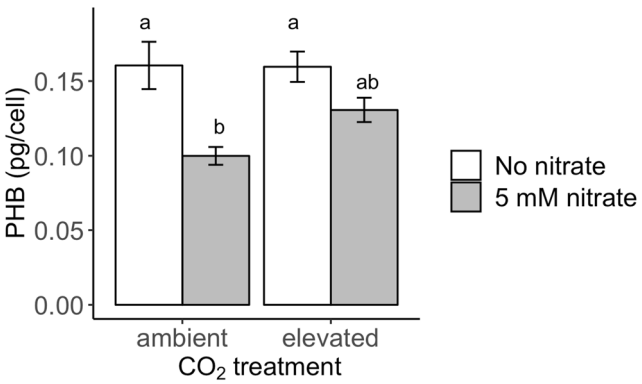

Fig. 3 Effects of CO_2 and nitrate treatments on PHB concentrations in wild-type (CE3) cells. Bars represent standard errors (9–12 PHB measures per group, sample numbers are lower because not all samples were analyzed by the flow cytometer). Letters indicate results from post-hoc Tukey's HSD tests after linear models tested for significant differences between treatments

4 Discussion

To understand how the value of traded resources affect resource mutualisms, we manipulated atmospheric CO_2 and supplemental nitrate to directly affect the carbon and nitrogen status, respectively, of plant hosts associated with N_2-fixing rhizobia. The addition of nitrate negatively affects nodule size for the highly-efficient strain, but had little to no effect on the wild type, leading to a misalignment between legume and rhizobial fitness. Elevated CO_2 alleviated this misalignment to some degree, by increasing plant N demand and reversing the effect of nitrate inhibition on total nodule weight, mainly by increasing nodule numbers. However, this alleviation was greater on plants that started with greater numbers of smaller nodules, indicative of the less-efficient or less-beneficial strain, than for plants with smaller numbers of larger nodules

Table 4 Linear model summary testing effect of nitrate, CO_2, and their interaction on benefit:cost ratio of the symbiosis, based on shoot N per total nodule mass (residual $df = 70$). Estimates are based on plants inoculated with the wild-type strain growing under ambient CO_2 conditions as the reference group. Shoot N per nodule mass was log-transformed to achieve normal distribution

		No nitrate only			
		Estimate	Std. Error	*t*-value	*p*
Shoot N per nodule mass	Strain	0.169	0.2772	0.61	0.546
	CO_2	**−0.726**	**0.2833**	**−2.56**	**0.015**
	Strain * CO_2	**0.892**	**0.3910**	**2.28**	**0.029**

(indicative of the high-efficient or highly-beneficial strain, summarized in Fig. 5).

4.1 Nitrate and elevated CO_2 on legume benefit

Even though plants inoculated with the wild-type strain formed 2.7–4.8 times more nodules than the PHB-negative strain within each treatment category, we did not see significant differences in host growth between strains, even under elevated CO_2. While this may be due to potential cross-contamination, it is more likely because the more-beneficial PHB-negative strain is a poor nodulator and the less-beneficial wild-type strain is a fast nodulator (Oono et al. 2020). The lack of difference in host growth between strains with substantially different nodulation and fixation traits echoes findings from Sanz-Sáez et al. (2015), where inoculation by an efficient rhizobia (fixing more N per C) did not improve soybean yield under elevated CO_2 conditions any more than when plants were inoculated by a less-efficient rhizobia. Sanz-Sáez et al. (2015) suggested this was due to the presence of less-efficient native rhizobia that co-infected plants with the more-efficient rhizobial strain in the field. However, they still found no differences in the magnitude of the positive effect of elevated CO_2 on host growth between more-efficient inoculum treatment and field soil treatment, assumed to consist of many less-efficient strains, when plants were grown in sterile growth chambers under elevated CO_2.

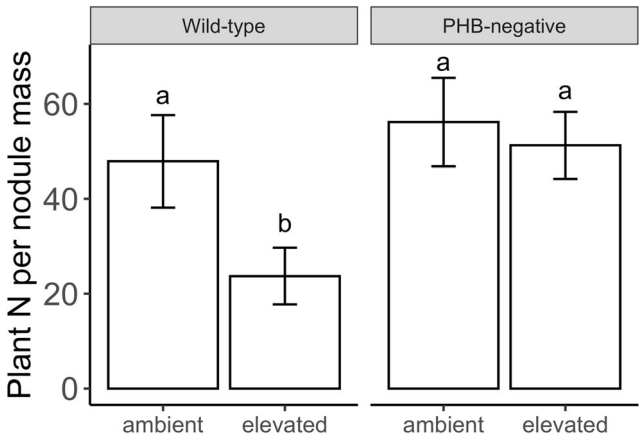

Fig. 4 Effects of elevated CO_2 treatments on the benefit:cost ratio of the symbiosis for plant hosts. Bars represent standard errors (9–11 plants per group). Letters indicate results from post-hoc Tukey's HSD tests after linear models tested for significant differences between treatments. Strain identity was a factor in this linear model. Only plant samples whose nitrogen contents were analyzed are included in analysis

We suggest two possible reasons why plant growth does not necessarily increase with more-beneficial strains under elevated CO_2. One reason is that nodules occupied by more-beneficial strains may be limited in their growth potential. For example, the average nodule size of the wild-type strain increased by 58% from 1.7 to 2.7 mg while the more-efficient PHB-negative strain only increased by 29% from 2.8 to 3.6 mg from ambient to elevated CO_2 conditions without nitrate. The smaller proportional increase may be due to constraints of nodule physiology (e.g., increasing N_2 or O_2 gas barrier with decreasing nodule surface area to volume ratios) rather than a lack of host resource allocation. Secondly, more-beneficial strains, like the PHB-negative strain in this study or USDA110 used in Sanz-Sáez et al. (2015)'s study, may have greater N_2-fixation but poorer ability to nodulate (i.e., under-nodulate). A trade-off between PHB-synthesis and N_2-fixation (shown in Hahn and Studer 1986; Willis and Walker 1998; Aneja et al. 2005) can further explain the trade-off between N_2-fixation and nodulation because PHB reserves are positively correlated with fitness in free-living environments (Ratcliff et al. 2008). Furthermore, rhizobial traits considered beneficial for symbiosis are not selected under free-living conditions (Burghardt et al. 2018). Hence, although plants under elevated CO_2 have increased demand for fixed N, increasing fixed N from only highly-beneficial strains may have limitations if the plant cannot find them thriving in the soil to form new nodules. A similar explanation could be that, instead of the PHB-negative strain under-nodulating, the wild-type strain is hyper-nodulating. Using strains that would not realistically survive long in the soil due to a mutation in a key metabolic pathway (e.g., PHB-negative) may be a limitation of this study. We also note that variations in host growth responses to nitrate and elevated CO_2 are plant species-dependent (West et al. 2005) and may also be genotype-dependent.

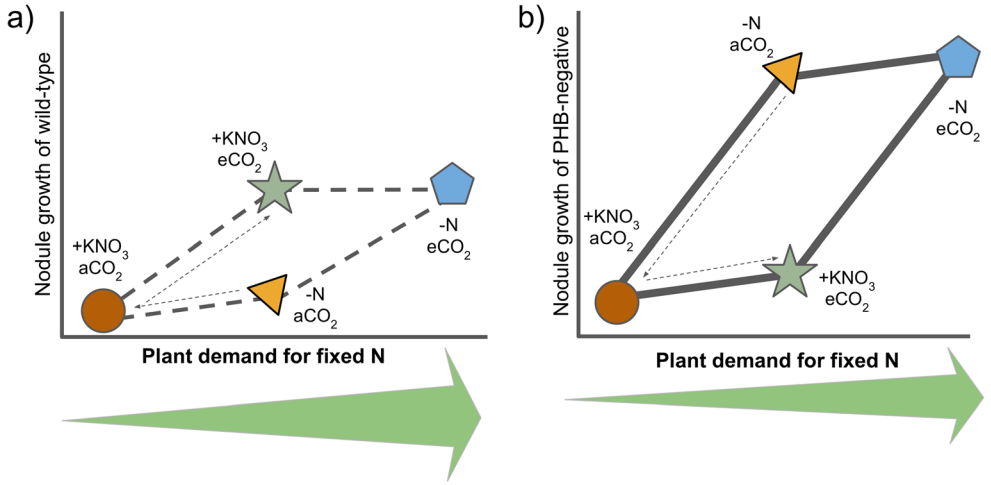

Fig. 5 Summary graph of changes in relative fitness as plant demand for fixed N changes under new environmental conditions. Treatments for supplemental nitrate (+KNO_3), no nitrate (-N), elevated CO_2 (eCO_2) and ambient CO_2 (aCO_2) are positioned along the x-axis denoting plant demand for fixed N. We assume that plants with nitrate under ambient CO_2 have the lowest demand for fixed N and plants without supplemental nitrate under elevated CO_2 have the greatest demand for fixed N. We did not distinguish whether plants under elevated CO_2 with nitrate had greater or lesser demand for fixed N than plants under ambient CO_2 without nitrate. Hence, these two treatments occupy an intermediate space on the x-axis. Since our study only included single-strain inoculations, we only plot the nodule growth among treatments for each strain separately (**a**) for mediocre wild-type and (**b**) for more-efficient PHB-negative strain. Dotted arrow indicates path by which host legumes inhibit nodule growth under nitrate supplementation but then reverses this effect under elevated CO_2. Nodule size of PHB-negative strain increases under elevated CO_2 but this effect is not a complete rescue as is for the wild-type strain. While the effect of each treatment has similar trends (e.g., increasing or decreasing) on nodule growth regardless of strain identity, the relative changes could depend on strain or benefit-cost ratio of the nodules

4.2 Nitrate and elevated CO_2 on rhizobial benefit

An increase in total nodule weight under elevated CO_2 conditions echos previous results by Thomas et al. (2000) and Butterly et al. (2016). This common pattern may indicate that the only mechanism to increase nitrogen supply from rhizobia is to increase nodule biomass (and number of rhizobial cells therein) perhaps because the maximum rate or efficiency of nitrogenase activity per cell is more or less fixed for each rhizobial genotype (as suggested in Cen and Layzell 2004; Sugawara and Sadowsky 2013). Interestingly, while both CO_2 and nitrate treatments had significant effects on the total nodule weights, CO_2 levels affected total nodule weights by influencing the average weights per nodule per plant whereas nitrate affected total nodule weights by influencing the number of nodules per plant. That said, total nodule weight or total nodule number in single-strain inoculation studies are not helpful proxies for rhizobial fitness because plants would be nodulated by a diverse community of rhizobial genotypes in the field. While a strain may form numerous nodules on its host under single-inoculation conditions, this strain would likely form significantly fewer in competition with others, deeming absolute number of nodules formed or total nodule weights during single-strain inoculation irrelevant as a rhizobial fitness proxy for real-world scenarios. Hence, we prefer to compare average mass per nodule, which is typically correlated with cells per nodule for the same strain (Ratcliff et al. 2011), or PHB accumulation per cell per nodule.

The negative effect of additional nitrate on the average mass of each nodule depended on the strain. The lower demand for fixed N with the supplemental nitrate prompted hosts to severely cut carbon resources towards more-efficient nodules but not necessarily towards less-efficient nodules. When plant N demand returned under elevated CO_2, highly-efficient strains regained some host resources, while less-efficient strains gained even more resources than under ambient CO_2 conditions (arrows in Fig. 5). This supports our hypothesis that carbon allocation towards more-beneficial nodules are disproportionately penalized relative to less-beneficial (but still effective) nodules when plant N demand decreases (summarized in Fig. 5).

While nitrate did not significantly suppress average nodule mass of wild-type nodules, nitrate significantly suppressed PHB accumulation, similar to results in Oono et al. (2020). Hence, we demonstrate that rhizobia cannot necessarily hoard more PHB just because their hosts have greater biomass with additional nitrate. If rhizobial PHB can be controlled by plant hosts, and rhizobia from larger plants are not any more likely to gain more PHB and survive longer in the soil than rhizobia from smaller plants, this weakens a key mechanism of cheating by rhizobia.

4.3 Nitrate and elevated CO_2 on legume-rhizobia fitness alignment

Host and symbiont fitnesses generally remained positively correlated in our single-strain conditions, except when plants

were inoculated with the PHB-negative strain and given 5 mM of nitrate (Suppl. Fig. 5d). The nodule size for the PHB-negative strain remained considerably inhibited even under elevated CO_2 (Fig. 2b, Fig. 5). This cannot be explained by a compensation with nodule numbers or PHB accumulation because the PHB-negative strain forms fewer nodules than the wild-type and cannot synthesize or accumulate PHB. This further suggests that the strength of selection for mutualistic quality depends on the quality (i.e., benefit-cost ratio) of the rhizobial strain and the value of traded resources.

4.4 Plant benefit-cost ratio influences rhizobial fitness

By examining the effects of elevated CO_2 and soil N on the relationship between plant benefit-cost (plant N: nodule biomass) ratio and rhizobial fitness, we assessed how legume hosts may differentially reward rhizobial strains of varying symbiotic quality (Fig. 4). Under elevated CO_2, the average weights per nodule are greater or the same for the wild-type nodules from ambient to elevated CO_2 conditions (Fig. 2b), even though the nodules collectively returned the same amount of fixed N (Fig. 1c). This supports the hypothesis that strains that provide lower benefit-cost ratios to their hosts will be at a greater advantage than those that provide higher benefit-cost ratios when their host has increased carbon supply and increased demand for fixed N (Fig. 4). These results are also similar to those of Sanz-Sáez et al. (2015), who found nodule dry weight increased in both effective and less-effective strains under elevated CO_2, but the increase in nodule dry weight for the more-effective strain was less, suggesting that elevated CO_2 will not give highly-beneficial strains an advantage over mediocre ones.

5 Conclusion

Plants appear to "loosen their hold" of their carbon in the face of elevated CO_2, as exemplified by the increase in nodule weights without detectable increases in fixed nitrogen in plants that were inoculated with the less-efficient strain (Fig. 5). Interpretations of single-strain experiments to understand the evolution of a rhizobial trait, however, have limitations, especially when strains differ in nodulation rates (Kiers et al. 2013). We do not anticipate that the relative fitness of a less-beneficial strain would ever be greater than that of a more-beneficial strain on the same plant, but conclude that the gap in their fitnesses will shrink, rather than increase or stay the same. Future studies will benefit from dual-inoculations or controlled inoculations that vary the ratio of nodule number by different strains to determine the effect of increased plant carbon and nitrogen on rhizobial competition.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13199-021-00784-2.

Acknowledgements We thank Miguel Cevallos for sharing the strains developed in his study. We profusely thank Sam Ray for his generous contributions in constructing the CSTRs for the UCSB Greenhouse and developing the code for running the software to manipulate and monitor CO_2 levels in the chambers. We also thank Miranda Ayad, Connor Leggett, Timothy Ho for early contributions in constructing and testing the CSTRs. We also thank Roy Cho for assistance during the experiment. We thank Angelina Huynh, Derek Tang, Simer Chima and Carina Salaiz for help preparing samples for stable isotope analyses, which were run at the Analytical Lab in the UCSB Marine Science Institute and we thank Georges Paradis for his guidance throughout the experiment. A. J. S. was supported by the Edison McNair Summer Fellowship, UCSB Undergraduate Research Creative Activities grant, and numerous funds from the UCSB Faculty Research Assistance Program. The authors have no conflicts of interest to disclose.

Author's contributions RO conceived and designed the experiments. AJS and RH performed the experiments. AJS and RO analyzed the data. AJS and RO wrote the manuscript.

References

Ainsworth EA, Long SP (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. The New Phytologist 165:351–371

Anderson AJ, Dawes EA (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev 54:450–472

Aneja P, Zachertowska A, Charles TC (2005) Comparison of the symbiotic and competition phenotypes of *Sinorhizobium meliloti* PHB synthesis and degradation pathway mutants. Can J Microbiol 51: 599–604

Arrese-Igor C, Royuela M, Aparicio-Tejo PM (1992) Denitrification in lucerne nodules and bacteroids supplied with nitrate. Physiol Plant 84:531–536

Bergersen FJ, Turner GL (1990) Bacteroids from soybean root nodules: accumulation of poly-β-hydroxybutyrate during supply of malate and succinate in relation to N_2 fixation in flow-chamber reactions. *Proceedings of the Royal Society of London. B. Biological Sciences* 240:39–59

Bertrand A, Prévost D, Bigras FJ, Lalande R, Tremblay GF, Castonguay Y, Bélanger G (2007) Alfalfa response to elevated atmospheric CO_2 varies with the symbiotic rhizobial strain. Plant Soil 301:173–187

Bertrand A, Prévost D, Juge C, Chalifour F-P (2011) Impact of elevated CO_2 on carbohydrate and ureide concentrations in soybean

inoculated with different strains of *Bradyrhizobium japonicum*. Botany 89:481–490

Burghardt LT, Epstein B, Guhlin J, Nelson MS, Taylor MR, Young ND, Sadowsky MJ, Tiffin P (2018) Select and resequence reveals relative fitness of bacteria in symbiotic and free-living environments. Proc Natl Acad Sci U S A 115:2425–2430

Butterly CR, Armstrong R, Chen D, Tang C. 2016. Free-air CO_2 enrichment (FACE) reduces the inhibitory effect of soil nitrate on N_2 fixation of *Pisum sativum*Ann Bot 117: 177–185

Cabrerizo PM, Gonzalez EM, Aparicio-Tejo PM, Arrese-Igor C (2001) Continuous CO_2 enrichment leads to increased nodule biomass, carbon availability to nodules and activity of carbon-metabolising enzymes but does not enhance specific nitrogen fixation in pea. Physiol Plant 113:33–40

Camargos LS, Sodek L (2010) Nodule growth and nitrogen fixation of *Calopogonium mucunoides* L. show low sensitivity to nitrate. Symbiosis 51:167–174

Canfield DE, Glazer AN, Falkowski PG (2010) The evolution and future of Earth's nitrogen cycle. Science 330:192–196

Cen Y-P, Layzell DB (2004) Does oxygen limit nitrogenase activity in soybean exposed to elevated CO_2? Plant Cell Environ 27:1229–1238

Cevallos MA, Encarnación S, Leija A, Mora Y, Mora J (1996) Genetic and physiological characterization of a *Rhizobium etli* mutant strain unable to synthesize poly-beta-hydroxybutyrate. J Bacteriol 178: 1646–1654

Denison RF (2000) Legume sanctions and the evolution of symbiotic cooperation by rhizobia. Am Nat 156(6):567–576

Fahraeus G (1957) The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J Gen Microbiol 16:374–381

Friel CA, Friesen ML (2019) Legumes modulate allocation to rhizobial nitrogen fixation in response to factorial light and nitrogen manipulation. Front Plant Sci 10:1316

Friesen ML (2012) Widespread fitness alignment in the legume-rhizobium symbiosis. The New Phytologist 194:1096–1111

Gibson AH, Pagan JD (1977) Nitrate effects on the nodulation of legumes inoculated with nitrate-reductase-deficient mutants of *Rhizobium*. Planta 134:17–22

van Groenigen K-J, Six J, Hungate BA, de Graaff M-A, van Breemen N, van Kessel C (2006) Element interactions limit soil carbon storage. *Proc Natl Acad Sci USA* 103:6571–6574

Hahn M, Studer D (1986) Competitiveness of nif- *Bradyrhizobium japonicum* mutant against the wild-type strain. FEMS Microbiol Lett 33:143–148

Heath KD, Lau JA (2011) Herbivores alter the fitness benefits of a plant–rhizobium mutualism. Acta Oecol 37:87–92

Heath KD, Podowski JC, Heniff S, Klinger CR, Burke PV, Weese DJ, Yang WH, Lau JA (2020) Light availability and rhizobium variation interactively mediate the outcomes of legume-rhizobium symbiosis. Am J Bot 107:229–238

Heath KD, Stinchcombe JR (2014) Explaining mutualism variation: a new evolutionary paradox? Evolution 68:309–317

Heath KD, Stock AJ, Stinchcombe JR (2010) Mutualism variation in the nodulation response to nitrate. J Evol Biol 23:2494–2500

Heath KD, Tiffin P (2007) Context dependence in the coevolution of plant and rhizobial mutualists. Proc R Soc B Biol Sci 274:1905–1912

Heck WW, Philbeck RB, Dunning JA. 1978. A continuous stirred tank reactor (CSTR) system for exposing plants to gaseous air contaminants. Agricultural Research Service, US Department of Agriculture in cooperation with North Carolina State University Agricultural Experiment Station

Houx JH, McGraw RL, Fritschi FB, Navarrete-Tindall NE (2009) Effects of shade on growth and nodulation on three native legumes with potential for use in agroforestry. Native Plants Journal 10:233–238

Hungate BA, Stiling PD, Dijkstra P, Johnson DW, Ketterer ME, Hymus GJ, Hinkle CR, Drake BG (2004) CO_2 elicits long-term decline in nitrogen fixation. Science 304:1291

Isoi T, Yoshida S (1991) Low nitrogen fixation of common bean (*Phaseolus vulgaris* L.). Soil Science and Plant Nutrition 37:559–563

Kiers ET, Rousseau RA, Denison RF (2006) Measured sanctions: legume hosts detect quantitative variation in rhizobium cooperation and punish accordingly. Evol Ecol Res 8:1077–1086

Kiers ET, Ratcliff WC, Denison RF (2013) Single-strain inoculation may create spurious correlations between legume fitness and rhizobial fitness. The New Phytologist 198:4–6

Lau JA, Bowling EJ, Gentry LE, Glasser PA, Monarch EA, Olesen WM, Waxmonsky J, Young RT (2012) Direct and interactive effects of light and nutrients on the legume-rhizobia mutualism. Acta Oecol 39:80–86

Menge DNL, Wolf AA, Funk JL (2015) Diversity of nitrogen fixation strategies in Mediterranean legumes. Nature Plants 1:15064

Mylona P, Pawlowski K, Bisseling T (1995) Symbiotic nitrogen fixation. Plant Cell 7(869):885

Niklaus PA, Körner C (2004) Synthesis of a six-year study of calcareous grassland responses to *in situ* CO_2 enrichment. Ecol Monogr 74: 491–511

Oono R, Denison RF (2010) Comparing symbiotic efficiency between swollen versus nonswollen rhizobial bacteroids. Plant Physiol 154: 1541–1548

Oono R, Muller KE, Ho R, Jimenez Salinas A, Denison RF. 2020. How do less-expensive nitrogen alternatives affect legume sanctions on rhizobia? Ecology and Evolution 10: 10645–10656

Phillips DA, Newell KD, Hassell SA, Felling CE (1976) The effect of CO_2 enrichment on root nodule development and symbiotic N_2 reduction in *Pisum sativum* L. Am J Bot 63:356–362

Polley HW, Wayne Polley H, Detling JK (1989) Defoliation, nitrogen, and competition: effects on plant growth and nitrogen nutrition. Ecology 70:721–727

Prévost D, Bertrand A, Juge C, Chalifour FP (2010) Elevated CO_2 induces differences in nodulation of soybean depending on *Bradyrhizobial* strain and method of inoculation. Plant Soil 331: 115–127

Quinto C, de la Vega H, Flores M, Fernández L, Ballado T, Soberón G, Palacios R (1982) Reiteration of nitrogen fixation gene sequences in *Rhizobium phaseoli*. Nature 299:724–726

Ratcliff WC, Denison RF (2009) Rhizobitoxine producers gain more poly-3-hydroxybutyrate in symbiosis than do competing rhizobia, but reduce plant growth. The ISME journal 3:870–872

Ratcliff WC, Kadam SV, Denison RF (2008) Poly-3-hydroxybutyrate (PHB) supports survival and reproduction in starving rhizobia. FEMS Microbiol Ecol 65:391–399

Ratcliff WC, Underbakke K, Denison RF (2011) Measuring the fitness of symbiotic rhizobia. Symbiosis 55:85–90

Regus JU, Gano KA, Hollowell AC, Sachs JL (2014) Efficiency of partner choice and sanctions in *Lotus* is not altered by nitrogen fertilization. Proc R Soc B Biol Sci 281:20132587

Rogers A, Gibon Y, Stitt M, Morgan PB, Bernacchi CJ, Ort DR, Long SP (2006) Increased C availability at elevated carbon dioxide concentration improves N assimilation in a legume. Plant Cell Environ 29: 1651–1658

Ryle GJU, Powell CE, Gordon AJ (1979) The respiratory costs of nitrogen fixation in soyabean, cowpea, and white clover: II. Comparisons of the cost of nitrogen fixation and the utilization of combined nitrogen. *J Exp Bot* 30:145–153

Sachs J, Simms E (2006) Pathways to mutualism breakdown. Trends Ecol Evol 21:585–592

Sa T, Israel DW (1998) Phosphorus-deficiency effects on response of symbiotic N_2 fixation and carbohydrate status in soybean to atmospheric CO_2 enrichment. J Plant Nutr 21:2207–2218

Sanz-Sáez Á, Erice G, Aguirreolea J, Irigoyen JJ, Sánchez-Díaz M (2012) Alfalfa yield under elevated CO_2 and temperature depends on the *Sinorhizobium* strain and growth season. Environ Exp Bot 77:267–273

Sanz-Sáez A, Erice G, Aranjuelo I, Nogués S, Irigoyen JJ, Sánchez-Díaz M (2010) Photosynthetic down-regulation under elevated CO_2 exposure can be prevented by nitrogen supply in nodulated alfalfa. J Plant Physiol 167:1558–1565

Sanz-Sáez Á, Heath KD, Burke PV, Ainsworth EA (2015) Inoculation with an enhanced N_2-fixing *Bradyrhizobium japonicum* strain (USDA110) does not alter soybean (*Glycine max* Merr.) response to elevated [CO_2]. Plant Cell Environ 38:2589–2602

Schortemeyer M, Atkin OK, McFarlane N, Evans JR (1999) The impact of elevated atmospheric CO_2 and nitrate supply on growth, biomass allocation, nitrogen partitioning and N_2 fixation of *Acacia melanoxylon*. Funct Plant Biol 26:737

Streeter J, Wong PP (1988) Inhibition of legume nodule formation and N_2 fixation by nitrate. Crit Rev Plant Sci 7(1–23):1

Sugawara M, Sadowsky MJ (2013) Influence of elevated atmospheric carbon dioxide on transcriptional responses of *Bradyrhizobium japonicum* in the soybean rhizoplane. Microbes and environments / JSME 28:217–227

Taylor BN, Menge DNL (2018) Light regulates tropical symbiotic nitrogen fixation more strongly than soil nitrogen. Nature Plants 4:655–661

Thomas RB, Bashkin MA, Richter DD (2000) Nitrogen inhibition of nodulation and N_2 fixation of a tropical N_2-fixing tree (*Gliricidia sepium*) grown in elevated atmospheric CO_2. New Phytol 145:233–243

Unkovich M, Herridge D, Peoples M, Cadisch G, Boddey R, Giller K, Alves B, Chalk P. 2008**.** Measuring plant-associated nitrogen fixation in agricultural systems ACIAR Monograph No 136, 258 pp.

Vance CP, Heichel GH, Barnes DK, Bryan JW, Johnson LE (1979) Nitrogen fixation, nodule development, and vegetative regrowth of alfalfa (*Medicago sativa* L.) following harvest. Plant Physiol 64:1–8

Wei X, Bauer WD (1998) Starvation-induced changes in motility, chemotaxis, and flagellation of *Rhizobium meliloti*. Appl Environ Microbiol 64:1708–1714

West JB, HilleRisLambers J, Lee TD, Hobbie SE, Reich PB (2005) Legume species identity and soil nitrogen supply determine symbiotic nitrogen-fixation responses to elevated atmospheric [CO_2]. New Phytol 167:523–530

West SA, Kiers ET, Simms EL, Denison RF (2002) Sanctions and mutualism stability: why do rhizobia fix nitrogen? *Proceedings*. *Biol Sci/Royal Soc* 269:685–694

Westhoek A, Clark LJ, Culbert M, Dalchau N, Griffiths M, Jorrin B, Karunakaran R, Ledermann R, Tkacz A, Webb I, James EK, Poole PS, Turnbull LA (2021) Conditional sanctioning in a legume-*Rhizobium* mutualism. Proc Natl Acad Sci 118:e2025760118

Willis LB, Walker GC (1998) The phbC (poly-beta-hydroxybutyrate synthase) gene of *Rhizobium*http://paperpile.com/b/jyhR1e/Nu7i(*Sinorhizobium*) *meliloti* and characterization of phbC mutants. Can J Microbiol 44:554–564

Wong PP (1980) Nitrate and carbohydrate effects on nodulation and nitrogen fixation (acetylene reduction) activity of lentil (*Lens esculenta* Moench). Plant Physiol 66:78–81

Zhang L, Wu D, Shi H, Zhang C, Zhan X, Zhou S. 2011. Effects of elevated CO_2 and N addition on growth and N_2 fixation of a legume subshrub (*Caragana microphylla* Lam.) in temperate grassland in China. *PLoS ONE* 6: e26842