

# Herbivory eliminates fitness costs of mutualism exploiters

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## Summary

- A common empirical observation in mutualistic interactions is the persistence of variation in partner quality and, in particular, the persistence of exploitative phenotypes. For mutualisms between hosts and symbionts, most mutualism theory assumes that exploiters always impose fitness costs on their host.
- We exposed legume hosts to mutualistic (nitrogen-fixing) and exploitative (non-nitrogen-fixing) symbiotic rhizobia in field conditions, and manipulated the presence or absence of insect herbivory to determine if the costly fitness effects of exploitative rhizobia are context-dependent.
- Exploitative rhizobia predictably reduced host fitness when herbivores were excluded. However, insects caused greater damage on hosts associating with mutualistic rhizobia, as a consequence of feeding preferences related to leaf nitrogen content, resulting in the elimination of fitness costs imposed on hosts by exploitative rhizobia.
- Our experiment shows that herbivory is potentially an important factor in influencing the evolutionary dynamic between legumes and rhizobia. Partner choice and host sanctioning are theoretically predicted to stabilize mutualisms by reducing the frequency of exploitative symbionts. We argue that herbivore pressure may actually weaken selection on choice and sanction mechanisms, thus providing one explanation of why host-based discrimination mechanisms may not be completely effective in eliminating nonbeneficial partners.

## Introduction

Mutualisms are defined as interactions in which there are reciprocal fitness benefits gained by two or more interacting species (Bronstein, 2001). Mutualisms occur abundantly in nature, but studies of many mutualistic interactions have repeatedly demonstrated the existence of potentially exploitative partners (Pellmyr *et al.*, 1996; Johnson *et al.*, 1997; Maloof & Inouye, 2000; Bshary & Würth, 2001; Sachs & Simms, 2008; Friesen, 2012). Exploitative partners, frequently referred to as ‘cheaters’, are defined as phenotypes or individuals that gain fitness benefits from their interspecific partner but do not reciprocate the fitness reward and, in doing so, do not pay the cost of a mutualism (Bronstein, 2001). As a consequence of influential theoretical models examining the stability of cooperation within species (Trivers, 1971; Axelrod & Hamilton, 1981; Bull & Rice, 1991), a prevalent view of mutualisms is that they are unstable and susceptible to invasion by exploitative partners (Bull & Rice, 1991). Most models to date have attempted to identify mechanisms that reinforce the mutualism and prevent fixation of exploiters (Sachs & Simms, 2006). The majority of theory assumes that exploiters always impose consistent negative fitness consequences on their interspecific partners (Trivers, 1971; Ferrière *et al.*, 2002; Johnstone & Bshary, 2002; West *et al.*, 2002; Foster & Kokko, 2006; Foster & Wenseleers, 2006; Ferrière *et al.*, 2007; Akçay & Simms, 2011). Here, we provide empirical evidence that the

direct negative fitness effects of an exploiter, relative to a mutualist, on its interspecific partner can be eliminated depending on the presence of other ecological interactions. In doing so, we provide evidence for an ecological condition that can weaken selection on mutualism-stabilizing mechanisms that would otherwise result in a reduction in the frequency of exploitative partners.

The partnership between hosts and their microbial symbionts provides an excellent example of an ancient mutualism that has potential for exploitation (Douglas, 2008). Partner choice (Bull & Rice, 1991), host sanctions (Denison, 2000; West *et al.*, 2002), partner fidelity feedback (Bull & Rice, 1991; Weyl *et al.*, 2010) and screening (Archetti *et al.*, 2011) models predict that mutualisms will be stable if fitness benefits are larger for beneficial symbiotic partners, and smaller for non-beneficial partners. There is some empirical evidence which is consistent with partner choice, host sanctions, partner fidelity feedback and screening mechanisms. Specifically, there is evidence that preferential association with more beneficial partners (Simms & Taylor, 2002; Kiers *et al.*, 2003; Heath & Tiffin, 2009; Gubry-Rangin *et al.*, 2010; Oono *et al.*, 2011) leads to higher fitness rewards to those beneficial partners (Heath & Tiffin, 2009). While there is considerable debate over the interpretation of these results (Edwards, 2009; Weyl *et al.*, 2010; Kiers *et al.*, 2011), at their core, all four of these processes and evolutionary models assume that associations with exploitative partners lead to lower host fitness.

In the mutualism between leguminous plants and rhizobia, soil bacteria fix atmospheric nitrogen into a plant-available form (ammonia) in return for plant-derived carbon metabolites; both partners gain substantial fitness benefits from the association, but the production of these resources incurs a cost to both partners (Pate *et al.*, 1979). The recurrent observation of exploitative, non-nitrogen-fixing rhizobia in soil and infected legume roots (Sachs & Simms, 2008) suggests that evolutionary or ecological forces are preventing their elimination by efficient partner choice or host sanction mechanisms. Theoretical models show that variation in mutualist quality can be maintained by frequency-dependent selection as a result of mixed infections (Friesen & Mathias, 2010), and the presence of correlations between the competitive ability of a symbiont and the rewards it provides (Ferriere *et al.*, 2002). Some models suggest that, rather than destabilizing mutualisms, exploitative partners may actually be required for mutualism stability (Foster & Kokko, 2006; Ferrière *et al.*, 2007). An alternative, simple model that could prevent complete extinction of exploitative mutualist partners is ecologically mediated context-dependent effects on host fitness – under some ecological conditions, exploitative partners are potentially beneficial or their costs are reduced (but not eliminated).

Herbivory, in particular, can impact plant–rhizobium interactions by altering the number of rhizobium–root associations (Butler *et al.*, 1959; Chu & Robertson, 1974; Heath & Lau, 2011), whereas changes in belowground rhizobia – including differing rhizobial genotypes – can indirectly change plant resistance and herbivore performance (Dean *et al.*, 2009; Kempel *et al.*, 2009; Katayama *et al.*, 2010; Pineda *et al.*, 2010). These studies suggest that herbivory might play an important role in altering the costs and benefits of the mutualism but have not yet examined exploitative symbionts, which have been a central focus in mutualism theory. Herbivory may lead to ecologically mediated context dependence if the fitness benefits and costs rhizobium strains confer on their hosts are affected by the frequency of herbivory. For example, exploitative nonfixing bacteria may no longer impose fitness costs by altering plant quality cues, including plant size, tissue quality, or resistance, such that herbivores preferentially visit plants associating with nitrogen-fixing mutualists, or actively avoid patches containing high frequencies of nonfixing bacteria. Similarly, nitrogen-fixing bacterial strains might provide fitness advantages in host populations, sites, or years with low herbivore densities. How frequently the conditions for ecologically mediated context dependence are met is fundamentally an empirical question. Importantly, ecologically mediated context dependence of the fitness effects of exploiters can potentially alter the pattern of selection on stabilizing mechanisms, such as partner choice and host sanctions, potentially providing a way for exploiters to persist.

Our objective was to determine whether herbivory in natural field conditions can change the fitness benefits and costs that exploitative and mutualist rhizobia confer on their hosts. By manipulating the presence or absence of exploitative (non-nitrogen-fixing) and mutualist (nitrogen-fixing) rhizobia and insect herbivores in a factorial design, we show that the costs of plants interacting with a rhizobial community that is partially

comprised of nonfixing rhizobia, as opposed to purely mutualistic strains, are dependent on ecological context. For plants protected from herbivory, nonfixing bacteria are costly, while these fitness costs are eliminated in the presence of herbivores, mainly because our data suggest that nonfixing bacteria probably lead to changes in host leaf tissue quality that make them less attractive to insect herbivores. These trends suggest that ecological interactions such as herbivory have the potential to modify the ecological and evolutionary dynamics of both exploitative bacteria and the plant traits that affect mutualism stability.

## Materials and Methods

### Natural history

*Medicago lupulina* L. (Fabaceae) occurs throughout North America, typically in pastures, lawns, roadsides and other disturbed habitats (Turkington & Cavers, 1979). Several invertebrate herbivores feed on *M. lupulina* – several genera of leaf-chewing beetles (*Sitona*, *Hypera* and *Apion*, Curculionidae), dipteran leaf miners (*Agromyza fontella*, Agromyzidae), and phloem feeders (*Acyrtosiphon pisum*, Aphidoidea) (Brown *et al.*, 1988; Gibson *et al.*, 1992). Herbivore exclusion studies have found that herbivory affects *M. lupulina* survivorship and performance (Brown *et al.*, 1988; Reader, 1992). *Medicago lupulina* typically forms beneficial facultative symbiotic associations with rhizobia in the *Ensifer* genus (formerly *Sinorhizobium*; van Rhijn & Vanderleyden, 1995; Willems, 2007). Two taxonomically distinct strains of rhizobia that occur naturally in southern Ontario are *Ensifer meliloti* and *Ensifer medicae* (Prevost & Bromfield, 2003; Bromfield *et al.*, 2010).

### Microbial strains used

We used four rhizobial strains: two we previously isolated from *M. lupulina* in the same locality where the experiment was conducted, RB1 (*E. meliloti*) and RB7 (*E. medicae*), and two strains originally isolated from *Melilotus alba* (both provided by E. Bromfield at AAFC; Bromfield *et al.*, 2010), T2 (*E. medicae*) and T173 (closely related to *Sinorhizobium morelense*). Phylogenetic analysis indicates that T173 nests within the *Ensifer* clade of mutualist rhizobia, including the mutualist rhizobial strains used in this study (Bromfield *et al.*, 2010; Fig. S1).

The *M. lupulina* strain isolates RB1 and RB7 are beneficial strains that produce small and large nitrogen-fixing nodules, respectively, on *M. lupulina* (A. K. Simonsen, unpublished data). Preliminary inoculation tests showed that T173 forms non-nitrogen-fixing nodules on multiple legume hosts in the *Medicago* genus, including its original host, *Melilotus alba* (Bromfield *et al.*, 2010). Consistent with Bromfield *et al.* (2010), our initial inoculation tests on *M. lupulina* confirmed that T2 is a beneficial strain that produces large pink nitrogen-fixing nodules, while T173 is a nodulating, non-nitrogen-fixing, exploitative rhizobium that produces small white nodules. When T173 was inoculated as a single strain on *M. lupulina* in preliminary glasshouse trials using the same soil conditions as used in this study, we observed 100%

mortality before plants produced their fourth true leaf (on average), well before flowering, and faster plant death compared with inoculated controls (Supporting Information Table S1). We also observed reduced biomass and later flowering even when the same soil was supplemented with high-nitrogen fertilizer (Table S1). Although T2 and T173 were isolated from *M. alba*, Bromfield *et al.* (2010) noted that they were found in fields where *M. alba* and *M. lupulina* co-occur, and thus represent naturally co-occurring strains that *M. lupulina* is likely to encounter in the field.

### Experimental design

We manipulated two ecological factors, rhizobial community and herbivory, in a factorial design. Herbivory was manipulated by excluding ambient invertebrate herbivores. Herbivore presence or absence was fully crossed with five rhizobial communities (10 treatment combinations; 15 replicates per treatment combination): (1) RB1, single inoculation; (2) RB7, single inoculation; (3) T2, single inoculation; (4) a mixture of the three beneficial strains (RB1 + RB7 + T2), hereafter referred to as 'Mix', each strain in equal proportion (i.e. 1 : 1 : 1); (5) a mixture of all three beneficial strains, plus the addition of the exploitative strain (RB1 + RB7 + T2 + T173), hereafter referred to as 'Mix+Exploiter', each strain in equal proportion. The first four belowground rhizobial backgrounds all consist of mutualistic nitrogen-fixing strains, while the fifth rhizobial background contains all the mutualist strains plus the exploitative T173 strain. We included multiple mutualist rhizobial species, including a beneficial strain (T2) from the same originating host as T173, to increase the representation of naturally beneficial strains, differing in host origin and taxonomic assignment. Because preliminary single strain inoculation trials showed that T173 always rapidly kills the host, we did not include single strain inoculations of it in the field.

We used 15 *M. lupulina* maternal plant families, randomly sampled from a natural population from the same locality where the field experiment was performed, that we previously determined to show significantly different nodulation phenotypes from beneficial rhizobial strains (A. K. Simonsen, unpublished data). Before the experiment, each maternal line was selfed for one generation in the glasshouse to equalize maternal effects and assigned to each treatment combination so that all families were distributed evenly across each treatment combination ( $n = 1$  family/treatment combination). We surface-sterilized seeds using bleach (6% sodium hypochlorite; 4-min immersion) and seeds were stratified for 7 d on 1% agar plates at 4°C in darkness. Seeds were placed at 21°C in darkness for 12 h to encourage radicle growth and transplanted into the field in pots containing a 1 : 3 autoclaved mixture of turf (Profile Products LLC, Buffalo Grove, IL, USA) and Sunshine mix#2 (Sun Gro Horticulture, Quincy, MI, USA). Preliminary glasshouse trials with the same soil mix determined that uninoculated plants did not grow beyond their fourth true leaf, and died well before flowering (Table S1). We planted two seedlings in each pot and thinned germinants to one plant per pot after 2 wk of seedling

establishment. We inoculated plants 1 wk following planting with 5 ml of the assigned rhizobial treatment. We prepared liquid cultures by growing each strain in TY medium (Somasegaran & Hoben, 1994) for 36 h. We equalized cell densities across all single strain inoculations by diluting with double-distilled H<sub>2</sub>O to  $c. 10^6$  cells ml<sup>-1</sup> (optical density (OD<sub>600</sub>) = 0.1). To prepare inoculum containing a mixture of rhizobial strains, we mixed each single strain inoculum in equal proportions.

The field experiment was conducted at the Koffler Scientific Reserve (latitude 44°1'57.5328"N; longitude 79°32'5.6832"W; <http://ksr.utoronto.ca/>). To expose plants to experimental rhizobial strains and minimize contact with resident microbes in the field soil, we transplanted retrofitted experimental pots into the soil. We placed each pot in a self-contained wicking water reservoir system that separated the pot from the field soil (Fig. S2). We buried each reservoir so that the top of the pot was 2.54 cm above the ground and spaced 0.5 m from other reservoirs. An early-July survey showed that 2 out of 10 evenly distributed control (uninoculated) pots had nodulated, each with one to two nodules (treatment plants typically had in excess of  $\approx 458$  nodules per plant). The remaining control plants died from a lack of nodulation. Collectively, these data indicate that our reservoir systems reduced contamination from *in situ* soil microbes, and that our inoculations of a large number of rhizobial cells ( $10^6$ ) should predominate.

To manipulate herbivory, we sealed each pot at the rim with a tube-shaped bridal mesh bag 30 cm tall, closed at the top with a rubber band (Fig. S2). To allow herbivores to feed on the plants, we cut six holes 18 mm in diameter into bags, three vertically arranged holes on opposite sides of the mesh facing north and south to minimize biased sun exposure of mesh treatments with holes. Photosynthetically active radiation (PAR) measurements on a subsample of experimental pots showed that light quantity did not significantly differ between pots with and without holes (holes:  $481.6 \pm 48.6495 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; no holes:  $458.1 \pm 44.6599 \mu\text{mol m}^{-2} \text{s}^{-1}$ ;  $t_{14} = -0.36$ ;  $P = 0.7267$ ). Treatment combinations were planted in a randomized block design (five blocks).

### Quantifying plant–insect and plant–rhizobium interactions

In mid-July (52 d after planting), we estimated insect herbivore leaf damage by counting the proportion of leaflets with the presence of leaf-chewing, sap-sucking or leaf-mining damage. In the third week of September, at the sign of first frost, we harvested all plants and measured nodule density (nodule number mg<sup>-1</sup> root) and mean individual nodule mass on the root samples. To do this, we separated each root sample into three components: the main root stem, fine roots, and roots that grew past the pot and into the water reservoir. Each component was dried and weighed separately. To obtain nodule density, we cut and mixed fine roots and counted nodules on a subsampled portion ( $c. 66\%$ ) of the fine hair mass (nodule number mg<sup>-1</sup> subsampled roots). We then estimated mean nodule mass by sampling 16 nodules from the subsampled fine roots. Nonbiased nodule selection was accomplished by cutting the subsampled fine roots into smaller root

segments (*c.* 0.25–1.0-cm pieces), mixing and spreading all the root segments on a 0.25-cm<sup>2</sup> grid, selecting 16 points haphazardly distributed over the grid, and sampling the nodule nearest to or overlapping each point. We dried all 16 nodules for several days in a drying oven at 60°C and weighed all nodules to obtain an average mass of a nodule for each plant.

### Measuring plant performance

When plants were harvested (134 d after planting), we estimated plant fitness and plant size by counting fruit production and weighing dried shoots and roots. To evaluate whether rhizobial communities altered plant traits that affect feeding behaviour in insects, we measured three leaf traits: trichome density, nitrogen per unit leaf area and carbon per unit leaf area in leaves. We subsampled 10 randomly chosen mature leaves from each plant. Trichome density was estimated by counting trichome number using high-magnification photographs (Leica M124; Leica Microsystems, Concord, Ontario, Canada) from the lamina surface of three of these randomly sampled leaves, and dividing by the leaf area captured in the image. We measured the total leaf area of the sampled leaves using scanned images, and the image analysis software IMAGEJ v1.6.0 (<http://rsbweb.nih.gov/ij/>). Scanned leaves were later dried and weighed, before being analysed for nitrogen and carbon content. We measured per cent carbon and nitrogen using elemental combustion analysis (ECS 4010; Costech Analytical Technologies, Valencia, CA, USA) from ground leaf tissue of the original subsampled leaves. As herbivorous insects are nitrogen-limited (Mattson, 1980), we used our estimates of leaf area and dry weight to convert %N into a more ecologically relevant variable – N mg cm<sup>-2</sup> leaf area – that measured the amount of nitrogen a herbivore could obtain per unit of leaf area consumed. The same conversion was performed for %C content in leaf tissue.

### Statistical analysis

To measure the effects of herbivore and rhizobial community treatments on plant performance and other plant traits, we used parametric models in SAS (v9.2; SAS Institute Inc., Cary, NC, USA) on phenotypic data. We analysed numerical measurements (i.e. biomass and carbon per unit leaf area, trichome density, and proportional damage) using standard general linear models (proc glm). We log-transformed any numerical data that produced nonnormal residuals. We analysed count data (i.e. fruit number) using over-dispersed Poisson regression models. We included block, strain and herbivore treatment as fixed effects in all models. Because of a number of zeroes for fruit count, we fitted a model that adjusted the dispersion parameters so that the deviance ratio (a criterion used to assess goodness of fit) approximated 1.

We tested if the effects of rhizobial inoculation background on plant fitness varied depending on the ecological context of herbivory by conducting planned contrast tests based on *a priori* knowledge of the differing functional effects of rhizobial strains used in our experiment. Within each herbivore treatment, we first compared plant fitness and biomass between mutualist single

rhizobial strain inoculations (i.e. RB1, RB7 and T2) and between single and mixed mutualist (i.e. Mix) strain inoculations. We failed to detect any significant differences among mutualist-only rhizobial backgrounds, in either the presence or absence of herbivory, suggesting that any mutualistic rhizobial backgrounds used in our experiment had very similar functional effects on host fitness. Our primary goal was to test the hypothesis that the presence of exploitative rhizobia (Mix+Exploiter) affects plant fitness and herbivory; we did so with planned contrasts (1) between the Mix and Mix+Exploiter rhizobial treatments, and (2) between the Mix+Exploiter and the mean of the T2, RB1, RB7 and Mix rhizobial treatments. Using these two contrast comparisons, we also investigated whether the change in fitness attributable to the presence of herbivores varied depending on whether the exploiter was present or absent (i.e. the interaction between herbivory and exploiter presence). In total, our analysis included 14 planned contrasts, including contrasts between mutualist-only rhizobial treatments (six tests within each herbivore treatment + two tests for the interaction). To account for multiple contrast tests, we calculated the *q*-values for each hypothesis test, which give the probability that a significant contrast test is a falsely rejected null hypothesis (Storey, 2002).

To investigate plant traits potentially associated with differences in insect damage, we subsequently analysed whether trichome density, nitrogen per unit leaf area, carbon per unit leaf area, nodule size, and nodule density were significantly associated with insect damage. To do this, we regressed damage against all predictors, while controlling for block effects, for the treatment where herbivores were present. We performed a path analysis within herbivore inclusions to disentangle direct effects of insect damage on plant fitness and indirect effects of insect damage through differences in plant traits (see Fig. 3a for the hypothesized model). We also included a path analysis showing direct effects of plant traits on fitness within herbivore exclusions (see Fig. 3b). All path analyses used log-transformed fruit number, log(*Y*+1), to improve model fit as a result of multiple zeroes in fitness data (SAS v9.2, proc calis, ram, method = ml). Both path models contained sufficient degrees of freedom for the specified paths.

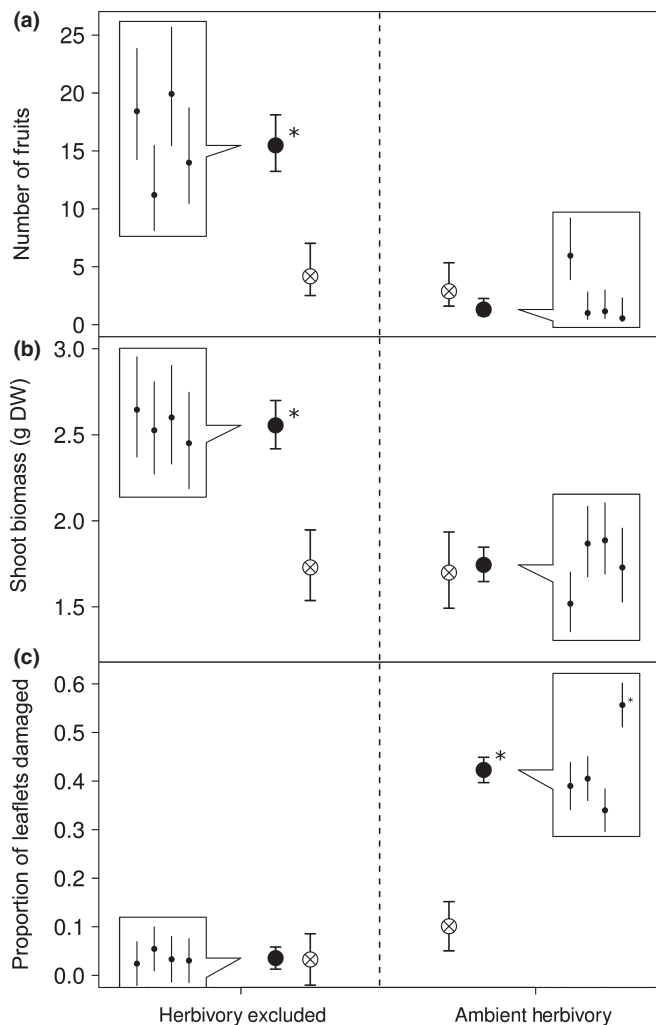
For the last part of our analysis, we investigated whether any of the aboveground plant traits differed between rhizobial strain treatments for any *a priori* contrast comparisons that were significant for damage and fitness, including presence or absence of fruit production as a covariate to account for effects of plant reproductive status on leaf- or root-related traits (Weiner, 2004; Boege & Marquis, 2005). Here, we averaged across both herbivore treatments because we were only interested in the main effects of the rhizobial strain treatments on the traits.

## Results

### Herbivory reduces plant fitness and changes several plant traits

Insect herbivory had a large impact on plant shoot biomass and fitness. Plants exposed to herbivores produced 88.7% less





**Fig. 1** Effects of herbivory and rhizobial strain treatments on (a) fruit production, (b) plant shoot biomass and (c) proportional damage of *Medicago lupulina*. Herbivore treatments consist of plants either excluded from herbivory or exposed to ambient herbivory in field conditions. Large dots compare the responses of plants grown in rhizobial communities containing just mutualists (closed dots) versus those of communities containing i.e. 'mutualists and exploiters' (open dots). The zoom-out panel shows individual mutualist strain treatments from left to right: RB1, RB7, T2 and Mix. Asterisks (\*) beside small closed dots indicate significant contrast tests between mutualist treatments only (mean of RB1, RB7 and T2 vs Mix). Asterisks (\*) beside large dots show significant contrasts between mutualist and mutualist + exploiter treatments. Error bars represent  $\pm 1$  SE.

fruit ( $F_{1,136} = 32.79$ ;  $P < 0.0001$ ; Fig. 1a), were 26.6% smaller in shoot biomass ( $F_{1,118} = 18.51$ ;  $P < 0.0001$ ; Fig. 1b), and were 57.6% smaller in root biomass ( $F_{1,118} = 4.62$ ;  $P = 0.0336$ ). The mesh cloth was very effective at excluding herbivores, as plants in herbivore exclusions sustained significantly less damage ( $F_{1,127} = 118.92$ ;  $P < 0.0001$ ; Fig. 1c). A generalized linear model of fruit production regressed against herbivore damage within the herbivore inclusion treatment indicated that plants with more damage had significantly fewer fruits ( $F_{1,69} = 10.46$ ;  $P = 0.0019$ ). While there was some nominal damage in the herbivore exclusion treatment, damage did

not predict fruit set ( $F_{1,68} = 0.62$ ;  $P = 0.4351$ ). The herbivore treatments had no significant overall impact on leaf carbon content ( $F_{1,103} = 0.07$ ;  $P = 0.7854$ ; Table S2) or leaf nitrogen content ( $F_{1,103} = 0.99$ ;  $P = 0.3216$ ; Table S2). In the herbivore inclusions, there was a trend towards higher trichome density and nodule density (21.9% higher,  $F_{1,108} = 2.95$ ;  $P = 0.09$ , and 39.5% higher,  $F_{1,116} = 2.67$ ;  $P = 0.1049$ , respectively; Table S2). Mean nodule mass was marginally higher on plant roots when herbivores were excluded (23.9% higher;  $F_{1,116} = 3.63$ ;  $P = 0.0592$ ; Table S2).

### Herbivory alters the effects of exploitative rhizobia on plant fitness

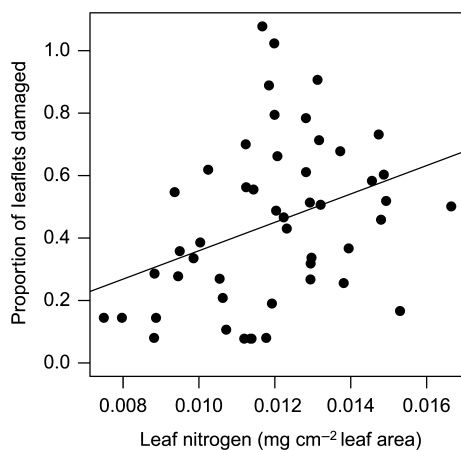
Generally, the rhizobial inoculation treatment had very few main effects on plant traits. The overwhelming trend in our data is that rhizobial treatment effects on plant traits differed depending on herbivore levels, and that the strongest effects were found for comparisons between mutualistic rhizobial communities that either contained or did not contain exploitative rhizobia. In the absence of herbivory, exploitative rhizobia are costly for plant fitness: fruit production was lower for plants with an exploitative rhizobium present in the community, and this comparison held between the mean of RB1, RB7, T2 and Mix vs Mix+Exploiter ( $F_{1,136} = 8.85$ ;  $P = 0.0035$ ;  $q = 0.0162$ ; Fig. 1a) and between Mix vs Mix+Exploiter ( $F_{1,136} = 5.06$ ;  $P = 0.0261$ ;  $q = 0.0468$ ; Fig. 1a). However, when plants were exposed to herbivores, contrast tests showed that there was no significant fitness difference in the presence or absence of exploitative rhizobia (average of RB1, RB7, T2 and Mix vs Mix+Exploiter:  $F_{1,136} = 0.96$ ;  $P = 0.3298$ ;  $q = 0.1384$ ; Mix vs Mix+Exploiter:  $F_{1,136} = 0.96$ ;  $P = 0.1787$ ;  $q = 0.1031$ ; Fig. 1a). Contrast tests of the interaction showed that changes in fitness attributable to herbivory were much larger when exploiters were absent compared with when exploiters were present (average of RB1, RB7, T2 and Mix vs Mix+Exploiter:  $F_{1,136} = 4.28$ ;  $P = 0.0406$ ;  $q = 0.0468$ ; Mix vs Mix+Exploiter:  $F_{1,136} = 4.48$ ;  $P = 0.0361$ ;  $q = 0.0469$ ; Fig. 1a), demonstrating that herbivores eliminated the fitness costs of associating with exploitative rhizobia relative to mutualistic rhizobia. Contrasts of shoot biomass showed similar patterns to fruit counts. When herbivores were excluded, there was higher biomass when exploitative rhizobia were absent (mean of RB1, RB7, T2 and Mix vs Mix+Exploiter:  $F_{1,118} = 8.92$ ;  $P = 0.0034$ ;  $q = 0.0340$ ; Mix vs Mix+Exploiter:  $F_{1,118} = 4.31$ ;  $P = 0.0402$ ;  $q = 0.1960$ ; Fig. 1b). In herbivore inclusions, we found no differences in shoot size in the presence or absence of the exploitative rhizobia (mean of RB1, RB7, T2 and Mix vs Mix+Exploiter:  $F_{1,118} = 0.02$ ;  $P = 0.8781$ ;  $q = 0.7031$ ; Mix vs Mix+Exploiter:  $F_{1,118} < 0.01$ ;  $P = 0.9843$ ;  $q = 0.7031$ ; Fig. 1b). These analyses indicated that, while plant fitness showed the predicted response to exploitative rhizobia in the absence of herbivory, those fitness differences between purely mutualistic and exploitative rhizobial backgrounds were eliminated in the presence of herbivory. In total, our analysis showed that a clearer pattern emerged from fitness differences explicitly due to the presence or absence of exploitative rhizobia (Fig. 1a,b).

## Exploitative rhizobia reduce herbivory by altering plant nitrogen

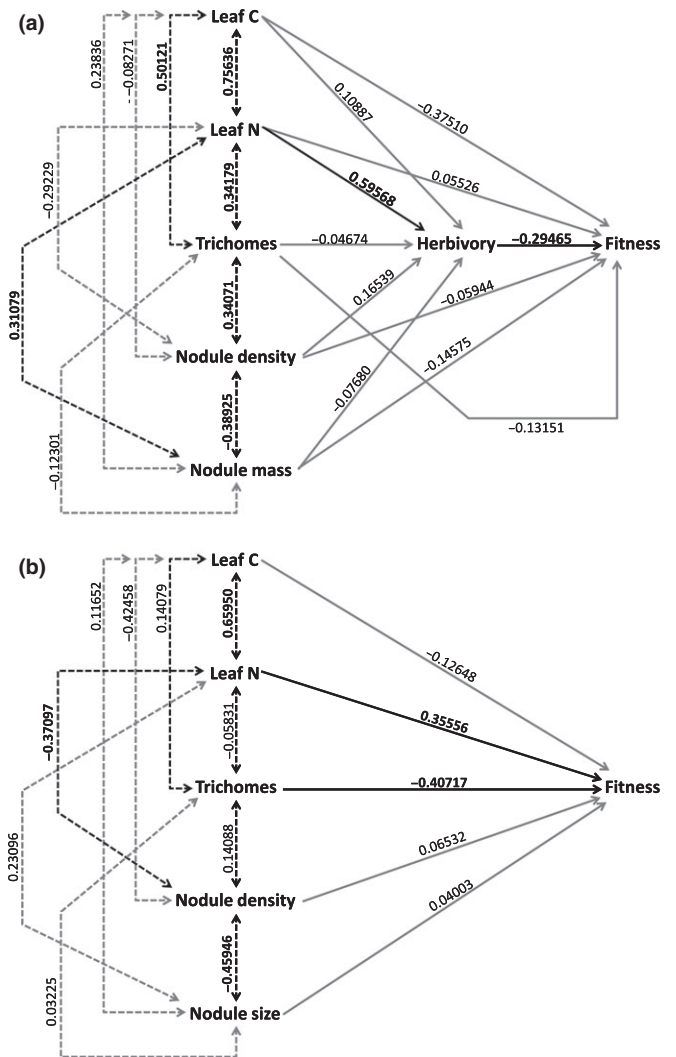
Analysis of mid-season insect damage data revealed several mechanistic hypotheses for the observed fitness effects of the exploitative rhizobia. Plants in the herbivore inclusion treatment, when interacting with purely mutualist rhizobia, sustained significantly greater damage compared with plants associating with exploitative rhizobia (mean of RB1, RB7, T2 and Mix vs Mix+Exploiter:  $F_{1,127} = 33.57$ ;  $P < 0.0001$ ;  $q < 0.00047$ ; Mix vs Mix+Exploiter:  $F_{1,127} = 45.11$ ;  $P < 0.0001$ ;  $q < 0.00047$ ; Fig. 1c). We found no difference in damage between any single strain treatments. We detected significantly greater insect damage in the mixed mutualist treatment compared with the mean damage across single strain mutualist treatments (mean of RB1, RB7 and T2 vs Mix;  $F_{1,127} = 11.6$ ;  $P = 0.0009$ ;  $q = 0.00284$ ; Fig. 1c).

Multiple regression analysis showed that nitrogen content per unit leaf area was the only significant predictor of insect damage in the presence of herbivores, while all other traits (nodule density, mean nodule mass, trichome density and carbon content per unit leaf area) were not significant. Nor did the effects of nitrogen content per unit leaf area on herbivore damage depend on any other traits (i.e. no significant nitrogen content per unit leaf area  $\times$  trait terms, such as nitrogen content per unit leaf area  $\times$  trichome density). We found that insect damage tended to increase as nitrogen content per leaf area increased ( $F_{1,38} = 6.76$ ;  $P = 0.0130$ ; Fig. 2). Path analysis within herbivore inclusions confirmed that leaf nitrogen content only affected host fitness indirectly through insect damage (Fig. 3a). Within herbivore exclusions, additional path analysis showed that leaf nitrogen had a significant positive association with host fitness, suggesting that higher nitrogen was beneficial to plant fitness but only when herbivores were absent (Fig. 3b).

Nitrogen content per unit leaf area was 12.2% higher in leaves from plants grown in mutualist treatments (mean of RB1, RB7, T2 and Mix vs Mix+Exploiter:  $F_{1,102} = 5.70$ ;  $P = 0.0189$ ; Mix vs

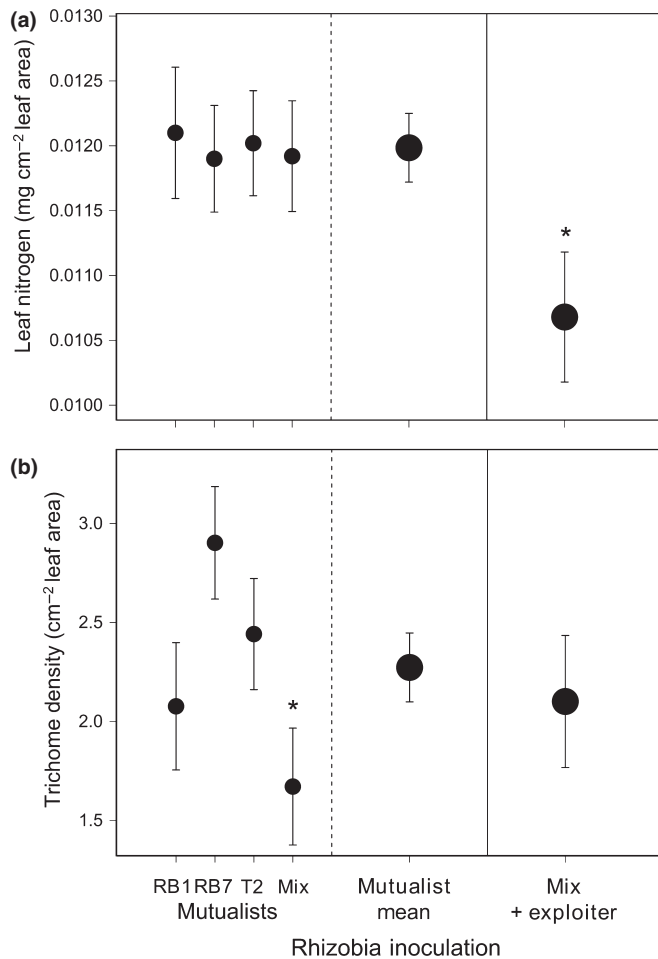


**Fig. 2** Partial leverage plot between proportional leaf damage from insects and leaf nitrogen of *Medicago lupulina* obtained from general linear model residuals that account for block effects. Mean trait values of leaf damage and leaf nitrogen were added to each residual value for axis interpretability. Leaf nitrogen significantly predicts insect damage ( $F_{1,44} = 4.79$ ;  $P = 0.034$ ; block included in model).



**Fig. 3** Hypothesized causal relationships between plant traits and fitness. (a) Path diagram within herbivore inclusions showing hypothesized (1) direct relationship paths between fitness ( $\log(\text{fruit number} + 1)$ ) and five plant traits in *Medicago lupulina*: leaf nitrogen and carbon content, trichome density, nodule density and mean individual nodule mass, and (2) indirect relationships between plant traits and fitness, through insect herbivory (proportional leaflet damage). Leaf nitrogen content significantly affects host fitness indirectly through herbivore damage (total indirect effect:  $-0.17552$ ). (b) Path diagram within herbivore exclusions showing direct effects of plant traits on fitness. Significant path coefficients are denoted by bold solid arrows. Solid single-headed arrows denote hypothesized causal relationships between variables, and correlations between variables are denoted by dashed double-headed arrows. Error variances of each individual variable were included in the model, but are not shown.

Mix+Exploiter:  $F_{1,102} = 3.59$ ;  $P = 0.0611$ ; Fig. 4a). Mix vs Mix+exploiter yielded marginal  $P$ -values, probably because of increased standard errors from lower statistical power, but still showed a consistent directional response in leaf nitrogen compared with contrasts between the mean of RB1, RB7, T2 and Mix and Mix+Exploiter. The presence or absence of exploitative rhizobia had no effect on mean nodule mass, trichome density, carbon content per unit leaf area or nodule density. Although herbivore damage was significantly higher in the mixed mutualist



**Fig. 4** Effects of rhizobial strain treatment on herbivore defence-related plant traits of *Medicago lupulina* in both herbivore treatments: (a) leaf nitrogen content and (b) trichome density. An asterisk (\*) beside a small dot indicates significant contrast tests between mutualist treatments only (mean of RB1, RB7 and T2 vs Mix). An asterisk (\*) beside a large dot shows significant contrasts between mutualist and mutualist + exploiter treatments. Error bars represent  $\pm 1$  SE.

treatment compared with the single mutualist inoculations (Fig. 1c), we failed to detect differences in any plant traits between Mix and the mean of RB1, RB7 and T2, with the exception of trichome density, which was 44% lower in mixed inoculations (mean of RB1, RB7 and T2 vs Mix;  $F_{1,108} = 4.88$ ;  $P = 0.0293$ ; Fig. 4b).

In total, these analyses indicate that trichome density, nodule mass and nodule density cannot explain differences in herbivore damage observed between the presence and absence of exploitative rhizobia. Our data more robustly show that lower damage on plants exposed to exploitative rhizobia was related to leaf nitrogen, suggesting that exploitative rhizobia reduced leaf tissue quality.

## Discussion

How mutualisms remain stable in the face of exploitative individuals or phenotypes has been a major debate in ecology and evolutionary biology. A persistent challenge has been to explain why

exploitative partners have neither swept to fixation nor been eliminated by mechanisms that are theoretically expected to stabilize the mutualism between hosts and symbionts of variable partner quality. Fundamental to mutualism theory has been the largely untested assumption that exploitative individuals or phenotypes always cause fitness reductions in their mutualistic partners (Trivers, 1971; Axelrod & Hamilton, 1981; Bull & Rice, 1991; Weyl *et al.*, 2010). One major result emerges from our field experiment: associating with exploitative bacteria entails relative fitness costs, but not under all conditions. Ecological context – namely the presence or absence of insect herbivores – changes whether associating with exploitative rhizobia is detrimental for plant hosts. We discuss this result as it relates to the relative impacts of herbivory versus exploitative rhizobia on legume hosts, and the consequences of conditional exploitation for evolutionary dynamics of host traits predicted to stabilize the mutualism (i.e. partner choice, host sanctions and partner fidelity feedback).

## Herbivory and the costs and benefits of rhizobial interactions

Decades of research have shown that herbivory has large top-down impacts on plant fitness, plant community structure and composition, range limits, and ecosystem function (e.g. productivity or nutrient cycling; Schmitz, 2008). Long-term studies have shown that herbivory is an important driver of legume fecundity and abundance compared with other plant taxa (Ritchie & Tilman, 1995). In our experiment, herbivory resulted in a nearly 27% decrease in biomass, and a 89% decrease in fruit production. Our results are consistent with those of previous studies that found *M. lupulina* to be intolerant of herbivore damage (Goertzen & Small, 1993; Reader & Bonser, 1998). Given that legumes also interact directly with rhizobia – also commonly known to affect plant condition and fecundity – it is perhaps unsurprising that previous studies have observed indirect interactions between insect herbivores and rhizobia, mediated by changes in plant traits (Dean *et al.*, 2009; Kempel *et al.*, 2009; Katayama *et al.*, 2010; Heath & Lau, 2011). Quantitative genetic data suggest that responses to herbivory and rhizobia share, at least in part, a common genetic basis and signalling response pathway (Heath & McGhee, 2012). Furthermore, jasmonic acid, a well-known signalling molecule associated with induced herbivore resistance (reviewed in Kessler & Baldwin, 2002), also plays a role in nodulation with rhizobia (reviewed in Hause & Schaarschmidt, 2009), suggesting that rhizobia could affect defences against herbivores.

Herbivory may influence legume–rhizobium interactions more generally because of the importance of nitrogen in both insect–plant and rhizobium–legume interactions. We have shown that exploitative rhizobial bacteria and herbivory both affect plant fitness for *M. lupulina* in the field. There is substantial evidence that insect herbivores are nitrogen-limited and prefer to feed on nitrogen-enriched plants (Price *et al.*, 2011) – especially those enriched with nitrogen derived from nitrogen-fixing rhizobia (Katayama *et al.*, 2010) – and that legumes have higher leaf nitrogen content compared with other plant taxa (McKey, 1994) as a

consequence of their association with beneficial nitrogen-fixing rhizobia (Went, 1973). Our study shows that insect herbivores cause more damage to individual plants that associate with beneficial rhizobia compared with plants that associate with a mixture of mutualist and exploitative symbionts. An explanation that is consistent with the findings of previous studies is that beneficial rhizobia fix more nitrogen and thus their hosts are more palatable. Alternatively, increased damage could have caused leaf nitrogen content to increase when beneficial rhizobia were present. Regardless, our study provides the first evidence, to our knowledge, that the negative fitness effects of associating with exploitative rhizobia relative to mutualistic rhizobia can be counteracted by indirect gains in fitness (or, alternatively, the elimination of direct fitness costs) as a result of reductions in herbivory.

Interestingly, we found that plants associated with a mixture of beneficial strains received greater damage and had lower trichome density compared with plants associating with a single inoculation of the same beneficial strains. Dean *et al.* (2009) also found that differences in the community of beneficial rhizobia alter plant resistance to herbivores. While we cannot robustly infer what traits may be causing greater damage, as trichomes were not correlated with herbivore damage, future studies can address the differential effects of beneficial strains, either singly or in mixtures, which may have more subtle and complex effects on ecologically important traits and fitness.

Ultimately, our experiment demonstrates how influential herbivory can be in modifying the costs and benefits legume hosts acquire from their rhizobial partners. Because spatial and temporal heterogeneity in herbivory is ubiquitous (Wiens, 1976; Rand, 2002), these results suggest that herbivory may play an even larger role in the evolutionary dynamics between legumes and rhizobia than previously thought, and has important consequences for the evolution of legume–rhizobium interactions (see next section).

### Evolutionary consequences of conditional exploitation for mutualism-stabilizing traits

Partner fidelity feedback, partner choice, host sanctions, and partner screening are mechanisms that are hypothesized to maintain the stability of mutualisms (Bull & Rice, 1991; Denison, 2000; Weyl *et al.*, 2010; Archetti *et al.*, 2011). When completely efficient at partner discrimination, the action of all of these processes predicts that exploitative or nonfixing strains would be eliminated from populations. A common explanation for the persistence of nonbeneficial bacteria is that choice or sanctioning mechanisms are physiologically limited in their ability to completely reduce infection of nonbeneficial strains. For example, host sanctioning mechanisms may not be able to impose fitness costs on nonbeneficial strains if root nodules contain a mixture of both beneficial and nonbeneficial strains (Kiers *et al.*, 2007), and mixed infections can be found in varying degrees of frequency across several legume species (Rolfe & Gresshoff, 1980; Trinick *et al.*, 1983; Demezas & Bottomley, 1986; Sachs *et al.*, 2010). The existence of ‘imperfect’ sanctioning or choice mechanisms, however, does not address the ultimate question of why selection

does not favour more universally effective choice or sanctioning mechanisms, if exploitative strains are always costly for host fitness. Plant–herbivore studies have examined the broad question of what evolutionary forces maintain intermediate levels of plant defences (cf. Simms & Rausher, 1993; Tiffin & Rausher, 1999; Fornoni *et al.*, 2004; Nunez-Farfan *et al.*, 2007) and the same question applies to sanctioning or host choice traits. It is possible that hosts have reached their evolutionary limits to evolve completely efficient partner discrimination despite strong selection, perhaps because of pleiotropic costs of choice or sanctioning mechanisms (Heath, 2010) or rhizobial response feedbacks that modify plant choice or sanctioning (Akçay & Simms, 2011). It is also possible that selection on choice or sanctioning mechanisms becomes weak when infections by nonbeneficial rhizobia no longer harm their hosts, such as if they are rare or mixed with other beneficial rhizobia within a nodule (Kiers *et al.*, 2007; Friesen & Mathias, 2010). Another simple, alternative explanation, supported by our experiment, is that the effects of nonbeneficial partners are ecologically context-dependent and do not always result in lower host fitness.

The opportunity for natural selection, on any trait, is equal to the variance in relative fitness (Crow, 1958; Arnold & Wade, 1984). In the absence of herbivory, there is variation in the number of fruits produced depending on whether the mutualist rhizobial community contains an exploiter or not, suggesting that it is possible for selection to act on mutualism-stabilizing traits (Fig. 1a). By contrast, in the presence of herbivory, the variance in fruit production is substantially lower, indicating less opportunity for selection on mutualism-stabilizing traits. In addition to reducing the strength of selection on mutualism-stabilizing traits, herbivory may particularly reduce the deleterious fitness consequences of exploitative strains when they are rare, because herbivores will have a substantially larger fraction of healthier, more nitrogen-rich tissue nearby in plants that lack exploiters. Context-dependent effects of nonbeneficial symbionts on host fitness can provide a potential evolutionary explanation of why host-based choice mechanisms are not completely effective at eliminating exploiters, in addition to theoretically explored mechanisms such as frequency-dependent selection as a result of mixed infections (Friesen & Mathias, 2010), relationships between competitive ability and mutualistic rewards provided (Ferriere *et al.*, 2002), and negotiation feedbacks from symbionts (Akçay & Simms, 2011).

Context dependence in rhizobial partner quality has been found in numerous studies, which show that fitness rewards of a strain classified as a ‘beneficial nitrogen–fixer’ can vary tremendously depending on species, genetic composition of strain and host, and abiotic conditions (Abel & Erdman, 1964; Carter *et al.*, 1978; Streeter & Wong, 1988; Eaglesham, 1989; Heath & Tiffin, 2007). However, to our knowledge, conditional exploitation through reductions in herbivore damage that neutralize harmful exploiter effects on host fitness has not been described previously. Context-dependent exploitation has been observed in several other interactions, although most examples are of normally beneficial mutualists becoming parasitic in certain circumstances (Bronstein, 1994, 2001). For example, in the mutualism



between yuccas (*Yucca filamentosa*) and yucca moth (*Tegeticula cassandra*) pollinators, Segraves (2003) found that increases in humidity can increase the degree of over-exploitation by yucca moths, by increasing the survivorship of larvae deposited by moths on the flowers. Conditional exploitation has also been proposed in parasitology, where parasites may provide some indirect fitness benefits to their animal hosts, which may compensate for or even reverse their direct fitness costs (Thomas *et al.*, 2000). Thomas *et al.* (2000) suggested that parasites may provide protection from predation if predators avoid infected individuals. Similarly, we find that, in the *M. lupulina*–*Ensifer* system, it appears that exploitative rhizobia reduce the concentration of nitrogen in their host plants, resulting in less palatable plants (Fig. 1c and 4a). Collectively, our work and other previous studies (Bronstein, 1994; Thomas *et al.*, 2000; Segraves, 2003; Stanton, 2003) highlight the importance of ecological context in assessing fitness impacts of mutualistic and exploitative partners on their hosts.

## Conclusions

Understanding the role of exploitative symbionts is a major challenge in evolutionary ecology. Theoretical models that have examined conditions necessary for mutualism stability have made the fundamental assumption that exploiters are always harmful to their host. Yet, empirical observations show that exploitative and ineffective symbionts persist in natural populations. Our empirical results help to reconcile these two contradictory findings by demonstrating ecological conditions where the assumption of harmful exploiters holds – when herbivores are absent – and conditions where it does not hold – when herbivores are present. More generally, our results suggest that reconciling the theoretical literature on mutualism exploitation and observations in nature may be aided by broadening our perspective on mutualistic interactions and integrating them into the wider web of ecological interactions of which they are a part.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Phylogenetic tree (16S) of 26 rhizobia strains, including the four strains used in the current study, RB1, RB7, T173 and T2.

**Fig. S2** Schematic diagram of the self-contained wicking water reservoir system placed in the field.

**Table S1** Preliminary single inoculation trials of the exploiter strain T173

**Table S2** Mean plant trait values within each herbivore treatment

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