Plant Soil
https://doi.org/10.1007/s11104-021-04999-6

REGULAR ARTICLE

Herbivory enhances legume-rhizobia symbioses function, increasing aboveground allocation of biologically fixed nitrogen, but only in soils without additional nitrate

Morgan N. Thompson · William O. Lamp

Received: 28 June 2020 / Accepted: 6 May 2021

Abstract

Purpose Beneficial soil microbes, such as rhizobia, engage in facultative symbioses in the roots of leguminous host plants to exchange nitrogen for products of photosynthesis, and these symbioses can be altered by biotic and abiotic factors. Here, we investigated how soil nitrate supply and aboveground insect herbivory interact to influence biological nitrogen fixation in *Medicago sativa* (alfalfa or lucerne).

Methods Using field and greenhouse experiments, we quantified above- and belowground allocation of rhizobially fixed nitrogen using isotopic nitrogen ratios in plants with different combinations of herbivory and nitrate supplementation. We caged *Empoasca fabae* (potato leafhopper) on fixing and non-fixing cultivars of *M. sativa* and supplemented soils with varied nitrate concentrations.

Results We detected strong changes in legume above- and belowground allocation of fixed nitrogen in response to both herbivory and nitrate supply. Moderate nitrate soils, irrespective of herbivory, induced little to no fixed nitrogen allocation across both field and greenhouse experiments. In the field only, non-supplemented soil increased aboveground allocation of fixed nitrogen following herbivore damage but resulted in no changes belowground. In contrast, non-supplemented and high nitrate soils in the greenhouse increased above- and belowground fixed nitrogen allocation relative to moderate nitrate soils.

Conclusion Our results demonstrate herbivory drives distinct plant allocation strategies across soil nitrate levels, advancing our understanding of how rhizobia influence legumes both above- and belowground. Herbivory-induced changes in rhizobia-legume symbioses are likely widespread across both agricultural and natural ecosystems.

Keywords Leafhopper · *Medicago* · Rhizobia · Aboveground/belowground interactions · Mutualisms · Nitrogen fertilization

Responsible Editor: Katharina Pawlowski.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11104-021-04999-6.

M. N. Thompson (✉) · W. O. Lamp
Department of Entomology, University of Maryland, College Park, 4291 Fieldhouse Drive, College Park, MD 20742, USA
e-mail: mthompson@tamu.edu

M. N. Thompson
Department of Entomology, Texas A&M University, TAMU 2475, College Station, TX 77843-2475, USA

Introduction

Plant roots shape belowground ecosystems. Roots assimilate nutrients and water and deposit exudates during growth, which can alter soil microbiomes (Haichar et al. 2014), nutrient cycling (Tateno et al.

Published online: 31 May 2021

2017), and long-term soil fertility in agricultural systems (Congreves et al. 2015). Abiotic stressors, such as drought or nutrient limitation, and biotic stressors, such as pathogens or root herbivores, can affect root function (Fallath et al. 2017; Kramer-Walter and Laughlin 2017; Naylor and Coleman-Derr 2018; Grunseich et al. 2020). To combat stressors, roots can recruit beneficial soil microbes to increase nutrient uptake (Lum and Hirsch 2002), stimulate growth (Pineda et al. 2010), and decrease drought stress (Vurukonda et al. 2016), and these processes also systemically affect aboveground plant tissues (Grunseich et al. 2019). Systemic changes in aboveground plant tissues following interactions with beneficial microbes often enhance plant defenses against pathogen or herbivore attack, referred to as induced systemic resistance (Pieterse et al. 2014). Induced systemic resistance has been shown to occur following plant root associations with arbuscular mycorrhizal fungi (Mitra et al. 2021), as well as rhizobacteria (Friman et al. 2021) and rhizobia (Tonelli et al. 2020).

Nitrogen-fixing rhizobia form highly specialized interactions with roots in the family Leguminosae (Fabaceae) by inducing root nodulation. Rhizobia fix atmospheric dinitrogen, which is metabolically costly for rhizobia and requires legumes to provide substantial amounts of photosynthetically fixed carbon (Kramer et al. 2012; Ladygina and Hedlund 2010). Despite carbon losses to rhizobia, legumes gain bioavailable nitrogen (Burity et al. 1989), a critical and often growth-limiting plant macronutrient. Legume-rhizobia symbioses do not always result in mutualism, however, and numerous factors are theorized to induce rhizobia parasitism (Denison and Kiers 2004; Porter and Simms 2014; Sachs and Simms 2006) or legume abortion of nodules (Ferguson et al. 2019). For instance, high levels of bioavailable soil nitrogen, such as nitrate, often cause legumes to abort the rhizobia mutualism in favor of less costly sources of nitrogen (Camargos and Sodek 2010; Carroll and Gresshoff 1983; Imsande 1986; Streeter and Wong 1988). In addition, non-fixing parasitic rhizobia have been shown to alter insect herbivory on legumes (Simonsen and Stinchcombe 2014), further implicating the important role of fixed nitrogen in plant–herbivore interactions.

Aboveground nitrogen allocation in plants contributes to the synthesis of important components of primary and secondary metabolism, such amino acids, enzymes, and proteins. Fixed nitrogen allocation aboveground in legumes uniquely shapes their metabolism (Barsch et al. 2006), resulting in nitrogen-rich plants relative to non-fixing plants (Adams et al. 2016; McKey 1994; Wolf et al. 2017). Legumes translocate fixed nitrogen aboveground in the form of ureides (Ladrera et al. 2007) or amino acids, such as asparagine and glutamate (Lodwig et al. 2003), and aboveground fixed nitrogen allocation can both attract nitrogen-limited herbivores (Ballhorn et al. 2017) but also heighten legume anti-herbivore defenses through induced systemic resistance. Nitrogen allocation processes can be strongly affected by herbivory, as shown when rhizobia-associated lima beans increased production of toxic nitrogen-containing compounds after herbivore attack, enhancing cyanogenesis and emission of volatile indole (Ballhorn et al. 2013; Thamer et al. 2011). Similarly, when comparing cyanogenic and non-cyanogenic varieties of *Trifolium repens*, the cyanogenic variety reduced aboveground chewing herbivore growth but did not decrease aphid performance (Kempel et al. 2009), suggesting piercing-sucking herbivores bypass rhizobia-based legume defenses. Piercing-sucking herbivores, such as aphids, can also manipulate and enhance aboveground nitrogen allocation in host plants for their own benefit (Wilson et al. 2011). Indeed, aphid densities correlated with increased ureide concentrations in soybeans (Riedell et al. 2013), suggesting aphids may induce greater fixed nitrogen allocation for their own nutrient supply. Depending on herbivore feeding guild and legume defense strategy, aboveground insect herbivores can differently drive fixed nitrogen allocation in legumes. Here we explore how soil nitrate and aboveground insect herbivory alter legume-rhizobia symbioses, ultimately shaping above- and belowground plant nutrient allocation patterns.

In conjunction with aboveground fixed nitrogen allocation, legumes allocate photosynthetic carbon belowground for both long-term storage and symbioses with rhizobia. Carbon allocation is also affected by aboveground insect herbivory, but herbivory can drive either increases or decreases in belowground carbon. On one hand, to reduce aboveground herbivore access to carbon, carbon allocation can shift belowground following herbivore attack (Kaplan et al. 2008; Schwachtje et al. 2006), which can cascade to enhance root associations with rhizobia (Heath and Lau 2011). Increased legume-rhizobia symbioses

following herbivory can feedback to affect nitrogen fixation and subsequent fixed nitrogen allocation. On the other hand, aboveground insect herbivory can reduce carbon through decreased rates of photosynthesis (Lamp et al. 2004; Velikova et al. 2010) and legume growth (Brunner et al. 2015), diminishing belowground carbon allocation and inhibiting rhizobia nitrogen fixation (Layton and Boethel 1987). How legumes allocate carbon following herbivory, as well as whether or not herbivory suppresses aboveground carbon fixation, determines the outcomes of legume-rhizobia symbioses (Heath and McGhee 2012) but how herbivory influences legume-rhizobia interactions is not fully understood.

In addition to aboveground insect herbivory, soil nitrate levels shape legume-rhizobia symbioses. Essentially, plants take up 'cheaper' sources of nitrogen in the form of soil nitrate rather than allocating carbon to rhizobia, although nitrate effects vary depending on the genotype of both plants and rhizobia (Heath et al. 2010). Legumes balance soil nitrate and rhizobia ammonium uptake depending on the amount of each nitrogen resource available and the needs of the plant at a given time (Regus et al. 2017). How insect herbivory alters the effect of nitrate, however, remains less clear. Extrapolating from the predictions of Vannette and Hunter (2011), legume defense expression after herbivore attack is constrained by rhizobia carbon costs and these costs vary across nitrate levels. Examining the effect of herbivory across different nitrate levels provides novel understanding on how legumes alter interactions with rhizobia and fixed nitrogen allocation.

Further complexity arises when considering legume roots do not interact with rhizobia alone under field conditions. Although rhizobia are typically highly abundant in soil microbial communities surrounding legumes, legumes also interact with many other microbes in the rhizosphere (Tsiknia et al. 2021), as well as endophytically in legume roots or nodules (Xiao et al. 2017; Brown et al. 2020). Soil microbial communities are often shaped by species richness, abundance, and functional diversity (Fierer 2017), all of which can influence legume growth and function. Microbial communities are also not static and legumes can modulate their surrounding microbial communities, or microbiomes, through root exudates (Sasse et al. 2018). Legumes release flavonoids to attract nitrogen-fixing rhizobia, increasing the abundance and activity of rhizobia in microbiomes, but recent evidence suggests legumes promote the growth of other types of microbes as well for their own benefit (Hartman et al. 2017). Intriguingly, microbiomes exert significantly different effects in greenhouse and field settings (Schittko et al. 2016; Heinze and Joshi 2018; Forero et al. 2019), meriting comparisons between the two experimental approaches. For legumes, greenhouse settings can isolate effects of rhizobia and contrast these effects with complex microbiomes present in field settings.

The goal of this study was to examine how herbivory alters above- and belowground nutrient allocation in legumes across varied soil conditions in both field and greenhouse settings. Here, we explored how an aboveground piercing-sucking herbivore (*Empoasca fabae*) and nitrate supplementation interact to alter fixed nitrogen and carbon allocation in a legume (*Medicago sativa*). Building on previous work that showed *E. fabae* herbivory decreases photosynthesis rates (Flinn et al. 1990; Womack 1984) and belowground carbon allocation (Lamp et al. 2001; Nielsen et al. 1990), we measured fixed nitrogen allocation in *M. sativa* and predicted that herbivory would limit nitrogen fixation through reduced carbon supply to rhizobia. We also tested how varying soil nitrate levels change systemic fixed nitrogen allocation in *M. sativa* in the field and greenhouse, and we predicted legume-rhizobia interactions would follow a quadratic relationship. Non-supplemented soils were predicted to constrain legume primary metabolism, as photosynthesis is limited by nitrogen inputs. Moderate nitrate supplementation was predicted to alleviate constraints on primary metabolism, allowing for increased photosynthesis despite herbivore feeding. Increased photosynthesis was theorized to feed back to legume-rhizobia symbioses and increase systemic fixed nitrogen allocation at moderate nitrate levels (Friesen and Friel 2019). We tested high nitrate soils in the greenhouse only and predicted *M. sativa* would assimilate soil nitrogen alone, reducing *M. sativa* reliance on rhizobia. Finally, as a strategy to tolerate herbivory, we predicted allocation of fixed nitrogen away from herbivore attack to belowground tissues, regardless of nitrogen supplementation. Our study changes current thinking on plant allocation as a tolerance strategy to insect herbivory by including

the influence of plant-associated microbes, further elucidating the role of microbes in plant tolerance/resistance strategies to insect herbivores.

Methods and materials

Study system

We used the legume *Medicago sativa* L. (Family Fabaceae, alfalfa or lucerne), which forms root nodules when interacting with nitrogen-fixing bacteria (*Ensifer meliloti*), to explore the effect of herbivory and soil nitrate on fixed nitrogen allocation. Based on preliminary experiments, we selected two near isogenic *M. sativa* cultivars (Barnes et al. 1990): one capable of nitrogen fixation (Saranac '2425') and one that is not (Saranac '2393'). We henceforth refer to these as 'fixing' and 'non-fixing,' respectively. The non-fixing cultivar is homozygous recessive for the '*in*' gene, which controls nitrogen fixation in *M. sativa* (Peterson and Barnes 1981). For both field and greenhouse experiments, the non-fixing cultivar was subjected to the same treatment combinations as the fixing cultivar but the non-fixing cultivar was used only to determine nitrogen fixation levels in the fixing cultivar, as described below.

Empoasca fabae (Family Cicadellidae), which are well-studied insect herbivores of *M. sativa*, were used for aboveground herbivory treatments. *E. fabae* induces significant damage to *M. sativa* including reduced rates of photosynthesis (Lamp et al. 2004), decreased stem elongation (Hutchins and Pedigo 1989), and reduced basal translocation of photoassimilates (Nielsen et al. 1990). For the greenhouse experiment only, *E. fabae* were collected from *M. sativa* fields in Keedysville, MD, USA and reared on fava beans (*Vicia faba*) in BugDorm mesh cages (BugDorm-44545F Insect Rearing Cage, Megaview Science, Taiwan) in a growth chamber (60% relative humidity, 16:8 L:D cycle). The colony was maintained for six months prior to experimentation, which corresponds to 6–7 *E. fabae* generations.

As a perennial forage crop, *M. sativa* stands last for multiple growing seasons, and successful stands rely on both root and crown (transitional structure between roots and shoots) storage of nutrients (Márquez-Ortiz et al. 1999). *M. sativa* is also continuously harvested every 35 d during the growing season. Between harvests of *M. sativa*, levels of nitrogen fixation in *M. sativa* vary, peaking after 21 d of regrowth (Vance et al. 1979). After 21 d of regrowth, for both field and greenhouse experiments, herbivory treatments were applied. In the field, herbivores were caged on plants until the conclusion of the experiment at 35 d and we repeated the experiment across two harvests, or sampling periods. In the greenhouse, herbivores were caged on plants for one week and removed for the final week of growth before we sacrificed plants at 35 d.

Field cage experiment

To determine how varied nitrate supplementation treatments and herbivory affect *M. sativa* fixed nitrogen allocation in a field setting, plots were seeded on 5-Sept-2017 in Hagerstown Silt Loam with 3 to 8% slope at the Western Maryland Research and Education Center (WMREC) in Keedysville, Maryland, USA (39.4862° N, 77.6997° W) following wheat (*Triticum aestivum*). Wheat was grown without spring applications of fertilizer to promote uptake of ambient soil nitrogen prior to planting *M. sativa*, which allowed us to manipulate soil nitrogen levels relative to non-supplemented control plots. The design was a randomized complete block split-plot with four blocks and four main plots per block. Main plots (3 m × 6 m) were inoculated with rhizobia from Welter Seed and Honey Company (Onslow, IA, USA) and seeded at a rate of 20 kg/hectare. Main plots included the following combinations of *M. sativa* cultivar and nitrate supplementation: 1) the fixing cultivar with no nitrate, 2) the fixing cultivar with moderate nitrate supply, 3) the non-fixing cultivar with no nitrate, and 4) the non-fixing cultivar with moderate nitrate supply. Main plots were divided in half (3 m × 3 m) to establish two subplots per main plot: cages either with or without herbivores. Across all plots, emergent spring growth was mowed to a height of 4 cm on 22-May-2018 and subsequent analyses were conducted on regrowth of *M. sativa*. Three days later, we collected soil samples from each block and applied nitrate supplementation treatments to each designated subplot at a rate of 0.20 g of ^{15} N-labelled potassium nitrate diluted in 120 mL of deionized water (Schmitt et al. 2013). Nitrate supplementation treatments were sprayed directly on soil surface with a plastic spray bottle. Heavy nitrogen (^{15} N) was utilized to distinguish

the contribution of soil nitrogen to plants relative to atmospheric, or fixed, nitrogen (^{14} N). We applied nitrate supplementation treatments once throughout the entire experiment as heavy nitrogen is retained by soils for long periods of time (Epstein et al. 2001). Field cages ($n=32$) were erected fifteen days later to provide 1×1 m of plant material to herbivores and neem oil organic insecticide (Eight Insect Control; Bonide Products, Inc., Oriskany, NY) was sprayed inside cages to reduce any outbreak of unwanted pests. Five days later (20 d after spring cutback), we added 100 adult herbivores to designated cages, which is comparable to economically damaging field densities in 1×1 m area. Herbivores were collected by D-Vac from adjacent *M. sativa* fields at WMREC, aspirated from mesh fabric cages ($60\times60\times92$ cm), and released into designated field cages. Two weeks after herbivores were caged on plants, we removed cages and clipped plant foliage from the entire plot to 4 cm above soil surface with a handheld grass trimmer. The timing of clipping followed a typical harvest cycle of *M. sativa* (Tracy et al. 2016), as described above. Plant samples were taken to the lab where we separated weeds from *M. sativa* and placed all material in a forced air oven at 68 degrees Celsius until dry. Samples of *M. sativa* were ground and weighed (4 mg) for nitrogen isotope analysis. Sample processing was conducted by the Colorado Plateau Stable Isotope Laboratory (Flagstaff, Arizona, USA). Samples were processed using a DELTA V Advantage Isotope Ratio Mass Spectrometer (Thermo Fisher™ Instruments, USA) coupled with an Elemental Analyzer (Carlo Erba Instruments, Milan, Italy) through a Finnigan™ ConFlo III. Nitrogen isotope values were reported as δ^{15}N ‰ and we used δ^{15}N ‰ values to calculate the nitrogen percentage derived from the atmosphere (%Ndfa).

Plots for the second sampling period were prepared following the same methodology as the first sampling period. Subplots receiving herbivore treatments were rerandomized to avoid any additive effects of herbivory. Following methodology from the first sampling period, shoots were clipped after 35 d. We also collected belowground tissue by digging up *M. sativa* crowns and roots (including nodules) at 10 cm below the soil surface, which encompassed the primary biomass of the roots. We brought all samples to the lab, placed samples in the forced air oven until dry, and followed the same procedure to grind and prepare samples for nitrogen isotope analysis. Shoots from the second sampling period degraded in the lab prior to nitrogen isotope analysis and were removed from statistical analysis.

Statistical analyses were conducted within the program R version 3.5.1 (R Core Team 2018). Since we did not collect whole plant samples on the same date (shoots collected separately from crowns and roots), we analyzed each plant component independently. Additionally, we only analyzed fixing plants, as non-fixing plants were only included in the experiment to calculate nitrogen fixation. Response variables for fixing plants were tested using linear mixed effects models with the "lmer" function from package lme4 (Bates et al. 2015). When necessary, response variables were transformed to meet assumptions of normality and equal variance. Data presented in figures, however, is not transformed. Our models included nitrate, herbivory, and the interaction of nitrate and herbivory as fixed effects. A random term (block by nitrate) accounted for our block and split-plot in our randomized complete block split-plot design. Significances of the model terms were tested using analysis of deviance in the car package (Fox et al. 2012). For multiple means comparisons, least-square means were compared using the "ls_means" function from package lmerTest (Kuznetsova et al. 2015) to determine any significant differences within nitrate treatment levels for plants with and without herbivores.

Greenhouse experiment

To further investigate the effect of nitrate supplementation and herbivory on fixed nitrogen allocation in *M. sativa*, we conducted a greenhouse experiment comparing non-supplemented plants to two rates of nitrate supplementation (moderate and high). We included an additional rate of nitrate supplementation to determine the quantity of soil nitrogen needed to induce different nutrient acquisition strategies in *M. sativa.* To test our predictions, seeds of fixing and non-fixing cultivars of *M. sativa* were planted in standard potting mixture and placed in a growth chamber (60% relative humidity, 16:8 L:D cycle). We kept plants in the growth chamber for the duration of the experiment. We repotted seedlings ($n=96$), both fixing ($n=48$) and non-fixing ($n=48$), after three weeks. All seedling roots were dipped in a solution of 4.00 g rhizobia/500 mL water using

the same rhizobia inoculant as the field experiment. Seedlings were placed in 'cone-tainers' (3.8 cm in diameter × 21 cm deep) containing 50/50 mixture of sterilized sphagnum peat moss and sand (Sakrete Multi-Purpose Sand; Sakrete, Charlotte, NC), totaling 130 g of sand-peat mixture per cone-tainer. We fertilized plants once per week with 10 mL of full-strength nitrogen-free Hoagland's solution (Hoagland and Arnon 1950). Cone-tainers were arranged in a randomized complete block design containing eight blocks and twelve treatment combinations. Our treatment combinations contained two factors with two levels (cultivar, herbivory) and one factor with three levels (nitrate supplementation), fully crossed. Nitrate supplementation treatments were applied once a week following repotting until the conclusion of the experiment and consisted of three different levels: full rate (High Nitrate), 25% rate (Moderate Nitrate), or none (No Nitrate). Using an estimate of 67 kg of nitrogen fertilizer per hectare for small grain production, we calculated the full rate of nitrogen supplementation to be 4.3 mg per pot. For the full rate (High Nitrate), we added 3.03×10^{-3} mmol/L (5 mL) of ^{15} N-labelled potassium nitrate diluted in deionized water each week. To account for any effect of potassium, we equilibrated the amount of potassium added across all other supplementation treatments with potassium chloride amendments. Hence, the 25% full rate (Moderate Nitrate) supplementation consisted of 7.60×10^{-4} mmol/L (1.5 mL) of ^{15} N-labelled potassium nitrate diluted in deionized water and 3.09×10^{-3} mmol/L (3.5 mL) of potassium chloride diluted in deionized water per week. For non-supplementation (No Nitrate), we added 3.00×10^{-3} mmol/L (5 mL) potassium chloride diluted in deionized water per week. Plants were watered daily with 10–20 mL of deionized water as needed.

After twelve weeks of growth, plants were clipped to simulate a harvest and herbivores were caged on regrown *M. sativa* 21 days later. Following the same procedure as the field experiment, herbivores were applied 21 days after clipping due to known increases in nitrogen fixation in *M. sativa* at this time (Vance et al. 1979). We placed 2 nymphs (fourth instar) in designated plastic cages (4 cm in diameter × 30–60 cm in height; mesh fabric glued to plastic connector and placed on top to allow for gas exchange). After 7 d of feeding, nymphs were removed from plants and all cages were removed. Plants grew for seven more days to reach 35 d of regrowth after our simulated harvest. We sacrificed plants and separated roots (including nodules), crowns, and shoots, and placed samples in the forced air oven at 68 degrees Celsius until dry and measured dry weight of all samples. Dried samples were ground and weighed (4 mg) for nitrogen isotope analysis following the same procedure described for the field experiment.

For statistical analyses, we tested each plant component independently and combined shoots, crowns, and roots for each plant sample to test whole plant responses. Again, only fixing plants were analyzed. Response variables were tested using linear mixed effects models with the "lmer" function from package lme4 (Bates et al. 2015). When necessary, response variables were transformed to meet assumptions of normality and equal variance. Data presented in figures, however, is not transformed. Our models included nitrate, herbivory, and the interaction of nitrate and herbivory as fixed effects. A random term (block) accounted for block effect in our randomized complete block design. Significances of the model terms were tested using analysis of deviance in the car package (Fox et al. 2012). For multiple means comparisons, least-square means were compared using the "ls_means" function from package lmerTest (Kuznetsova et al. 2015) to determine significant differences between nitrate treatment levels, as well as within nitrate treatment levels on plants with and without herbivores.

Calculating biological nitrogen fixation and fixed nitrogen biomass

To determine the amount of biological nitrogen fixation in *M. sativa*, we used natural nitrogen isotope ratios (expressed as δ^{15}N values) of fixing and non-fixing plants. Nitrogen fixation is expressed as the nitrogen percentage derived from the atmosphere (%Ndfa). Non-fixing reference plants account for the contribution of soil nitrogen to the isotopic signature of fixing plants. In other words, the δ^{15}N value of the fixing plant should fall somewhere between the δ^{15}N value of the non-fixing plant, which relies entirely on soil nitrogen, and the δ^{15}N value of the atmosphere, as 99.636% of N in the atmosphere is ^{14} N. Two different equations were used to calculate %Ndfa, depending on nitrate supplementation treatment. For experimental units that did not receive supplemental

nitrate, we used the ^{15} N Natural Abundance Equation (Shearer and Kohl 1986):

$$\%\text{Ndfa} = \frac{\delta^{15}\text{N of reference plant} - \delta^{15}\text{N of } N_2 - \text{fixing legume}}{\delta^{15}\text{N of reference plant} - \text{B}} \times \frac{100}{1}$$

In this equation, 'B' represents the δ^{15}N of nitrogen derived from fixation for a given plant species. Following similar methodology as West et al. (2005), we set B equal to 0. Additionally, for each treatment combination, we averaged δ^{15}N values of non-fixing reference plants (Tables S3 and S6) and used the average per treatment combination value for calculations of %Ndfa in each fixing replicate.

For experimental units that received supplemental nitrate, we calculated %Ndfa using the ^{15} N Isotope Dilution Equation (McAuliffe et al. 1958):

$$\%\text{Ndfa} = \left(1 - \frac{\text{atom}\%^{15}\text{N excess } N_2\text{\_fixing legume}}{\text{atom}\%^{15}\text{N excess reference plant}}\right) \times 100$$

'Atom%^{15} N excess' is similar to δ^{15}N values but reflects the ^{15} N enrichment above background. We used the ^{15} N Natural Abundance Equation because soil nitrogen content was artificially enriched relative to the atmosphere, allowing us to disregard the B value because enrichment exceeds natural variation of ^{15} N (Unkovich et al. 2008). We averaged atom%^{15} N excess values for all reference plants under specific nitrate supplementation and herbivory treatment combinations and used those average per treatment values to calculate %Ndfa of each fixing replicate.

To calculate fixed nitrogen biomass, we determined nitrogen biomass (Dry Biomass x N Percentage) and used the following formula:

$$\text{Fixed Nitrogen Biomass} = \frac{(\text{Nitrogen Biomass} \times \%\text{Ndfa})}{100}$$

Results

Field experiment: above- and belowground responses to herbivory and nitrate supplementation

Soil nutrient analyses revealed low levels of soil nitrate prior to nitrate supplementation treatments (Table S1). Nitrate supplementation, herbivory, and their interaction had no effect on shoot total biomass, carbon percentage, or nitrogen percentage (Table 1; Table S2). However, herbivory had a significant effect on shoot C:N ratios ($F_{1,6}=10.07$, $P=0.02$; Table 1) and, under moderate nitrate supplementation only, induced an 11% increase ($P=0.02$; Fig. 1a) in the C:N ratio of shoots. Shoot nitrogen biomass was not affected by either herbivory or nitrate (Table 1; Table S2). Shoot %Ndfa was affected by nitrate supplementation ($F_{1,6}=15.21$, $P=0.008$; Table 1) and herbivory ($F_{1,6}=21.53$, $P=0.004$; Table 1) treatments, as well as the interaction of nitrate supplementation and herbivory ($F_{1,6}=37.33$, $P<0.001$; Table 1). Specifically, under no nitrate conditions, we detected a 150% increase ($P<0.001$; Fig. 1b) in shoot %Ndfa following herbivory when compared to undamaged controls. Moderate nitrate conditions induced low %Ndfa regardless of herbivory (Fig. 1b). When we tested shoot fixed nitrogen biomass, our model detected an effect of nitrate ($F_{1,6}=13.90$, $P=0.01$; Table 1). Fixed nitrogen biomass increased 130% ($P=0.04$; Fig. 1c) in shoots with herbivory under no nitrate supplementation compared to shoots without herbivory, indicating biomass differences alone did not drive the pattern seen in %Ndfa. Shoot fixed nitrogen biomass in moderate nitrate soils remained low with and without herbivory (Fig. 1c).

For the above- and belowground interface (crowns) and belowground (roots) tissue, we detected few effects of herbivory, nitrate supplementation, or the interaction of nitrate and herbivory (Table 1; Table S2). To calculate %Ndfa for crowns and roots, we used non-fixing δ^{15}N and Atom%^{15} N excess values (Table S3). For crowns, we detected no significant effects across all response variables (Table 1). The interaction of nitrate supplementation and herbivory affected carbon percentage of roots ($F_{1,6}=8.71$, $P=0.03$; Table 1). For all other response variables, we detected no significant effects for roots (Table 1).

Greenhouse experiment: above- and belowground responses to herbivory and nitrate supplementation

We found that nitrate supplementation and herbivory treatments differentially altered the response

Table 1 Linear mixed effects model analysis of variance results of *M. sativa* samples taken from field experiment. Shoots were collected on 25-Jun-2018, and crowns and roots were collected on 30-Jul-2018. Bolded values indicate significant effects

			Shoots		Crown		Roots	
Parameter	Source	df	*F* value	*p*-value	*F* value	*p*-value	*F* value	*p*-value
Total Biomass	Nitrate	1	0.11	0.75	4.02	0.07	3.22	0.12
	Herbivory	1	3.34	0.12	1.55	0.24	0.54	0.49
	Herbivory *Nitrate	1	2.14	0.19	1.49	0.24	1.90	0.22
Percent Carbon (%)	Nitrate	1	3.13	0.13	1.26	0.31	1.14	0.33
	Herbivory	1	0.80	0.41	2.98	0.08	2.87	0.14
	Herbivory *Nitrate	1	0.12	0.74	0.84	0.36	**8.71**	**0.03**
Percent Nitrogen (%)	Nitrate	1	0.03	0.88	1.83	0.22	0.08	0.79
	Herbivory	1	2.40	0.17	0.03	0.86	3.29	0.12
	Herbivory *Nitrate	1	0.03	0.86	1.78	0.23	0.08	0.78
C:N Ratio	Nitrate	1	0.91	0.38	0.16	0.70	1.19	0.32
	Herbivory	1	**10.07**	**0.02**	1.15	0.30	0.02	0.89
	Herbivory *Nitrate	1	0.63	0.46	0.52	0.48	1.61	0.25
Nitrogen Biomass	Nitrate	1	0.11	0.76	2.50	0.14	3.62	0.11
	Herbivory	1	3.15	0.13	1.55	0.24	1.24	0.31
	Herbivory *Nitrate	1	1.28	0.30	0.74	0.41	2.27	0.18
Fixed Nitrogen (%)	Nitrate	1	**15.21**	**0.008**	0.01	0.94	0.54	0.48
	Herbivory	1	**21.53**	**0.004**	0.01	0.92	0.38	0.55
	Herbivory *Nitrate	1	**37.33**	**< 0.001**	0.22	0.65	0.38	0.55
Fixed Nitrogen Biomass	Nitrate	1	**13.90**	**0.01**	0.11	0.75	0.49	0.50
	Herbivory	1	2.49	0.17	0.08	0.79	0.33	0.58
	Herbivory *Nitrate	1	4.10	0.09	0.32	0.58	0.31	0.59

of shoots, crowns, and roots in the greenhouse (Table 2; Table S4). Nitrate and herbivory treatments did not change percent carbon in shoots, crowns, or roots (Table 2; Table S5). We detected an effect of nitrate ($F_{1,35} = 3.76$, $P = 0.03$; Table 2) and herbivory ($F_{1,35} = 8.07$, $P = 0.007$; Table 2) on percent

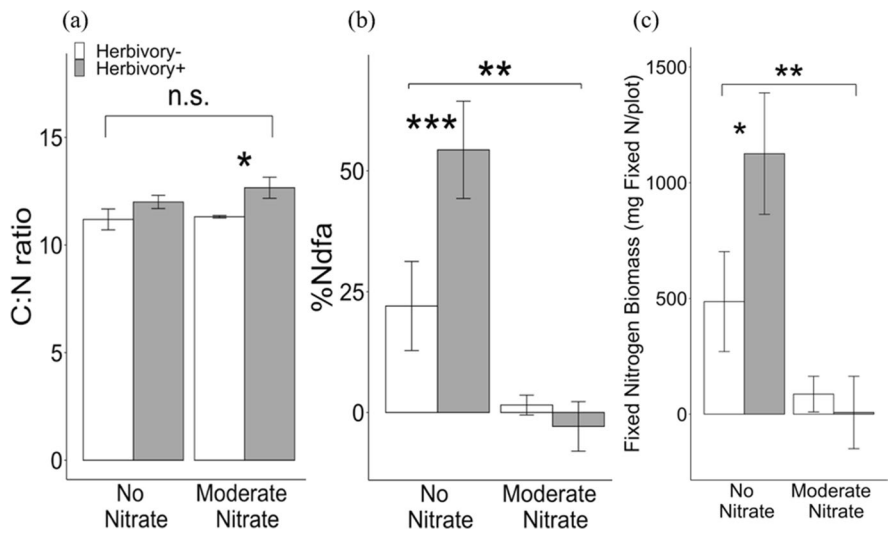

Fig. 1 Herbivory in the field increased aboveground nitrogen fixation and fixed nitrogen allocation in non-supplemented soils. Shoot (stems and leaves) means (± SE). Brackets indicate differences between nitrate levels. '*' $P < 0.05$ '**' $P < 0.01$ '***' $P < 0.001$; **a** %Ndfa = Nitrogen percentage derived from the atmosphere **b** Fixed nitrogen biomass

Table 2 Linear mixed effects model analysis of variance results of whole plants of *M. sativa* from greenhouse experiment. Shoots, crowns, and roots were collected simultaneously and combined for whole plant analyses. Bolded values indicate significant effects

			Shoots		Crown		Roots		Whole Plant	
Parameter	Source	df	*F* value	*p*-value	*F* value	*p*-value	*F* value	*p*-value	*F* value	*p*-value
Total Biomass	Nitrate	2	0.69	0.51	0.06	0.95	0.04	0.96	0.21	0.81
	Herbivory	1	1.45	0.24	0.02	0.90	0.31	0.58	0.05	0.82
	Herbivory *Nitrate	2	2.87	0.07	1.46	0.24	1.86	0.17	1.83	0.17
Percent Carbon (%)	Nitrate	2	1.45	0.98	3.14	0.06	0.59	0.56	0.23	0.80
	Herbivory	1	5.78	0.97	3.50	0.07	0.21	0.65	1.07	0.31
	Herbivory *Nitrate	2	1.20	0.98	0.24	0.78	0.76	0.47	0.94	0.40
Percent Nitrogen (%)	Nitrate	2	**3.76**	**0.03**	0.40	0.68	0.81	0.46	1.22	0.31
	Herbivory	1	**8.07**	**0.007**	1.34	0.26	0.01	0.92	2.88	0.10
	Herbivory *Nitrate	2	1.89	0.17	2.47	0.10	0.34	0.71	1.65	0.21
C:N Ratio	Nitrate	2	2.67	0.08	0.89	0.42	1.18	0.32	0.25	0.78
	Herbivory	1	**14.11**	**< 0.001**	0.66	0.42	0.01	0.92	0.34	0.56
	Herbivory *Nitrate	2	1.36	0.27	2.83	0.07	0.34	0.71	1.10	0.34
Nitrogen Biomass	Nitrate	2	0.34	0.71	0.19	0.83	0.14	0.87	0.26	0.78
	Herbivory	1	0.30	0.59	0.16	0.69	0.23	0.64	0.29	0.60
	Herbivory *Nitrate	2	0.97	0.39	0.50	0.61	1.15	0.33	1.00	0.38
Fixed Nitrogen (%)	Nitrate	2	**559.24**	**< 0.001**	**607.27**	**< 0.001**	**52.07**	**< 0.001**	**76.71**	**< 0.001**
	Herbivory	1	**5.92**	**0.02**	0.53	0.47	0.87	0.36	0.29	0.59
	Herbivory *Nitrate	2	0.43	0.66	0.71	0.50	0.47	0.63	1.43	0.25
Fixed Nitrogen Biomass	Nitrate	2	**16.99**	**< 0.001**	**23.50**	**< 0.001**	**21.83**	**< 0.001**	**54.52**	**< 0.001**
	Herbivory	1	0.08	0.78	0.01	0.99	0.18	0.67	3.17	0.08
	Herbivory *Nitrate	2	0.81	0.45	0.86	0.43	0.33	0.72	0.03	0.97

nitrogen of shoots but not percent nitrogen of crowns or roots. C:N ratio of shoots ($P < 0.05$; Fig. 2a) and crowns ($P < 0.05$; Fig. 2a) under no nitrate conditions increased by 18% and 32%, respectively, in response to herbivory. Herbivory also increased C:N ratio of high nitrate shoots by 12% ($P < 0.05$; Fig. 2a). C:N ratio of shoots, crowns, and roots under moderate nitrate conditions showed no significant response to herbivory (Fig. 2a). Nitrate supplementation affected %Ndfa ($P < 0.001$; Table 2) and fixed nitrogen biomass ($P < 0.001$; Table 2) of shoots, crowns, and roots. Additionally, an effect of herbivory on %Ndfa of shoots was detected as well ($F_{1,35} = 5.92$, $P = 0.02$; Table 2). Fixed nitrogen biomass increased across shoots, crowns, and roots under herbivory without nitrate supplementation (Fig. 2b). In contrast, fixed nitrogen biomass decreased in high nitrate shoots, crowns, and roots under herbivory (Fig. 2b). Shoots, crowns, and roots in moderate nitrate conditions showed no changes in fixed nitrogen biomass (Fig. 2b).

Greenhouse experiment: combined whole plant responses

Since all tissue types responded similarly to treatment combinations in the greenhouse, shoots, crowns, and roots were combined to analyze changes in whole plant responses to nitrate supplementation, herbivory, and the interaction of herbivory and nitrate (Table S7). We found a 17% reduction ($P = 0.02$; Fig. 3a) in the nitrogen percentage of whole plants under herbivory in conditions without nitrate supplementation. Interestingly, plants without nitrate supplementation or herbivory accumulated the greatest nitrogen percentage relative to all other treatment combinations (Fig. 3a). An effect of nitrate ($F_{2,35} = 76.71$, $P < 0.001$; Table 2) on %Ndfa of whole plants was documented, although no significant pairwise comparisons between plants with and without herbivores were detected (Fig. 3b). Nitrate supplementation had an effect on fixed nitrogen biomass of whole

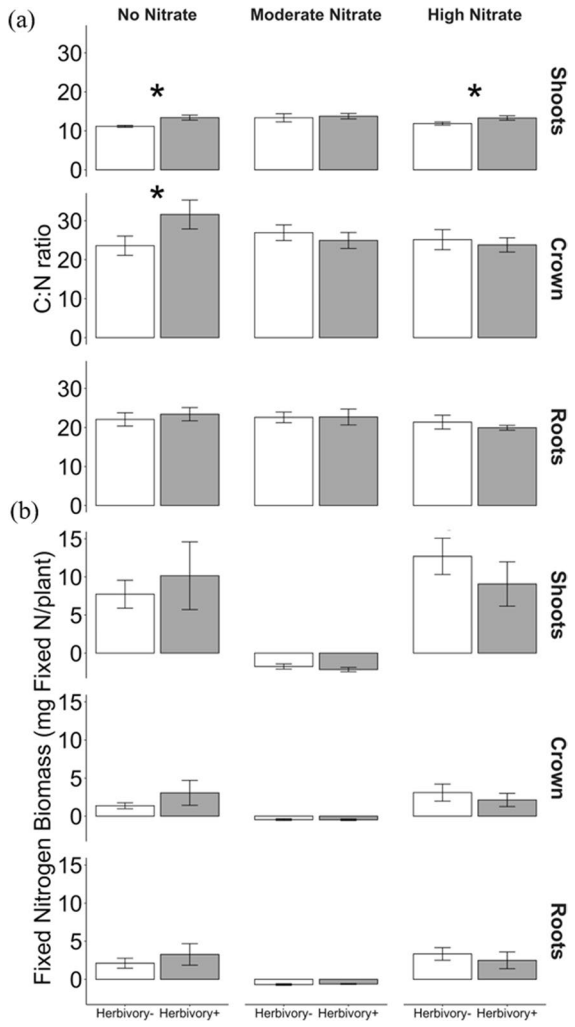

Fig. 2 Nitrate supplementation in the greenhouse did not alter nitrogen percentage but changed fixed nitrogen allocation above- and belowground. Shoot (stems and leaves), crown, and root means (±SE). Brackets indicating differences between nitrate levels removed for clarity. '*' $P<0.05$ '**' $P<0.01$ '***' $P<0.001$; **a** Nitrogen (%), significant nitrate effect for shoots: None-Moderate ($P=0.01$) **b** Fixed nitrogen biomass of shoots (leaves and stems), crowns, and roots, significant nitrate effect for shoots, crowns, and roots: None-Moderate ($P<0.001$) and Moderate-High ($P<0.001$)

plants ($F_{2,35}=54.52$, $P<0.001$; Table 2). Whole-plant fixed nitrogen allocation was marginally significant ($F_{2,35}$ 3.17, $p=0.08$; Table 2) and showed differential responses to herbivory across nitrate levels: increases with no nitrate supplementation, none at moderate nitrate, and decreases with high nitrate supplementation (Fig. 3c).

Discussion

Determining outcomes of plant interactions with beneficial soil microbes remains challenging but critical as these symbioses often enhance plant growth and defense (Pineda et al. 2010; Heinen et al. 2018; Friman et al. 2021), and harnessing the full potential of beneficial microbes is predicted to boost agroecosystem productivity (Pineda et al. 2017; Yadav et al. 2020; French et al. 2021). Plant interactions in both above- and belowground environments can influence soil microbe symbioses, requiring examination of systemic changes in plants. Here, we tested how *M. sativa* plants respond to nitrate supplementation and aboveground herbivory, examining both above- and belowground plant resource allocation. We predicted herbivory would decrease legume carbon provided to rhizobia, as previous research demonstrated herbivory reduced photosynthesis and belowground carbon allocation (Nielsen et al. 1990). Reduced belowground carbon was predicted to limit nitrogen fixation, and we predicted these effects would be evident in non-supplemented soils. In contrast, moderate nitrate soils were predicted to allow legumes to overcome carbon losses to herbivores, as nitrate would boost photosynthesis. High nitrate supply was predicted to reduce legume reliance on rhizobia, allowing legumes to access nitrogen necessary for growth and defense through the soil. Across nitrate levels, we predicted allocation of fixed nitrogen belowground, away from the aboveground herbivore feeding.

Overall, we detected the opposite response in *M. sativa*: non-supplemented and high nitrate soils stimulated fixed nitrogen allocation, although patterns of above- and belowground allocation differed between the field and greenhouse, and moderate nitrate soils allocated comparatively low levels of fixed nitrogen. Across soil nitrate levels in both the field and greenhouse, we detected very minimal effects of herbivory on belowground tissues. In non-supplemented field soils only, herbivory increased aboveground fixed nitrogen allocation (Fig. 1b-c).

Above- and belowground resource allocation

To tolerate herbivory, plants can allocate resources above- or belowground away from the attacking herbivore (Kaplan et al. 2008). Tolerance allows plants to withstand herbivory but not necessarily directly deter

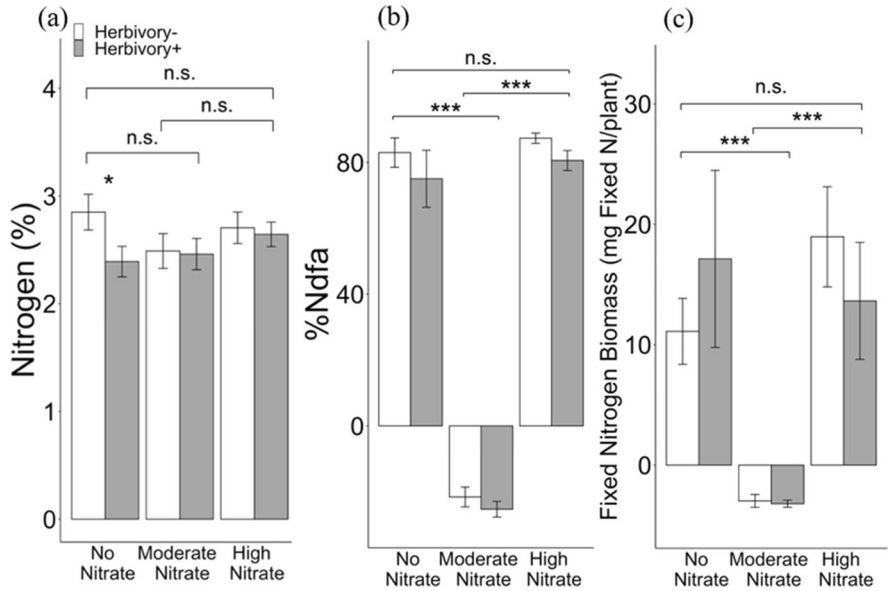

Fig. 3 Nitrogen fixation and fixed nitrogen allocation varied in the greenhouse across nitrate supplementation, but not nitrogen percentage. Whole plant means (±SE). Brackets indicate differences between nitrate levels. '*' $P<0.05$ '**' $P<0.01$ '***' $P<0.001$; **a** Nitrogen (%) **b** %Ndfa = Nitrogen percentage derived from the atmosphere **c** Fixed nitrogen biomass

the herbivore. In contrast to tolerance, plants can also allocate resources to defense against herbivores, and rhizobia are thought to play important roles in supplying nitrogen for plant production of aboveground defense compounds (Thamer et al. 2011). We found *M. sativa* allocated greater amounts of fixed nitrogen aboveground following herbivory in non-supplemented field soils (Fig. 1b-c). To determine fixed nitrogen allocation, we used nitrogen isotope methods, which have some limitations including underestimating fixed nitrogen allocation, as we could not account for nitrogen lost to plant volatiles or root exudates, and subjecting fixing and non-fixing cultivars to herbivory. The cultivars may respond to herbivory in contrasting ways but we used the non-fixing cultivar, in essence, as a tool to estimate nitrate uptake in fixing plants with nitrate supplementation treatments. Increased aboveground fixed nitrogen allocation could indicate *M. sativa* utilizes fixed nitrogen aboveground for defense or a compensatory growth response that is independent of herbivore response pathways due to aboveground tissue loss. Dean et al. (2014) found no changes in defensive phytohormones of soybeans following aphid feeding but recent findings indicate this effect is dependent on the identity of aphid bacterial endosymbionts (Pandharikar et al. 2020). To subvert plant defenses, insect herbivores can employ different counter-defense strategies to access host-plant nutrients (Karban and Agrawal 2002). Aphids, as well as other piercing-sucking herbivores, rely on endosymbionts in their salivary glands to manipulate host-plant defense (Wang et al. 2020) and access nutrients (Goggin 2007). Indeed, pea aphid feeding redirects nitrogen movement in *M. sativa* (Girousse et al. 2005), which suggests piercing-sucking herbivores could enhance fixed nitrogen allocation aboveground for their own benefit. Pea aphids are co-evolved with *M. sativa* whereas *E. fabae* is native to North America and *M. sativa* is not. Although *E. fabae* and *M. sativa* are not co-evolved, *E. fabae* feeds on a wide range of host plants and shows a clear preference for Fabaceae (Lamp et al. 1994). It is possible that *E. fabae* can manipulate fixed nitrogen movement in co-evolved species within Fabaceae and the same mechanisms are at play with *M. sativa*. Recent work on the transcriptomics of *E. fabae* salivary glands identified numerous candidate genes that may shed light on the mechanism underlying *E. fabae* host-plant manipulation (DeLay et al. 2012). Further research is needed to determine if the increase in aboveground fixed nitrogen allocation is driven by plant or herbivore.

Soil nitrate and legume-rhizobia symbioses

Legume-rhizobia symbioses typically result in mutualism, as legumes provide carbon in exchange for fixed nitrogen from rhizobia. Mutualisms can

breakdown, however, when rhizobia 'cheat' by inducing nodulation but not fixing nitrogen, which is often detected and sanctioned against by legumes (Kiers et al. 2003). Enhanced soil nitrate levels can also drive legumes to opt-out of rhizobia mutualisms (Kiers et al. 2006), as legumes assimilate nitrogen directly from the soil (Streeter and Wong 1988). Numerous lines of recent evidence call into question the generality of nitrate effects on legume-rhizobia symbioses, such as maintaining nitrogen fixation with increased nitrate fertilization (Regus et al. 2014; Wolf et al. 2017; Forrester and Ashman 2018) and varying responses of different genotypes of both rhizobia and *M. sativa* to nitrate supply (Heath et al. 2010). Further, previous studies reported *M. sativa* maintained nitrogen fixation despite nitrogen fertilization at rates comparable to our high nitrate supply (Kelner et al. 1997; Lamb et al. 1995), suggesting a capacity for legumes to simultaneously participate in rhizobia mutualisms and assimilate soil nitrate. Since legumes assimilate and transport fixed nitrogen and soil nitrate in contrasting ways (Ciesiołka et al. 2005; Katayama et al. 2010), different nitrogen sources may play distinct roles in legume growth and defense. Our field and greenhouse experiments showed moderate nitrate supply decreased both nitrogen fixation (Figs. 1b and 3b) and fixed nitrogen biomass (Figs. 1c and 3c) relative to legumes in non-supplemented soils. In the field, we also observed moderate nitrate supply increased shoot C:N ratio (Fig. 1a) following herbivory. Changes in C:N ratio may reflect increased carbon-based defenses (Burghardt 2016), which, coupled with low fixed nitrogen allocation, support differential contributions of nitrogen sources to legume defense (Pandharikar et al. 2020). In the greenhouse, high nitrate soils increased fixed nitrogen biomass relative to moderate nitrate soils (Figs. 2b and 3c), restoring fixed nitrogen allocation to levels indistinguishable from non-supplemented soil (Fig. 3c). Following herbivory in high nitrate soils, we also documented non-significant reductions in fixed nitrogen biomass (Figs. 2b and 3c), providing additional evidence nitrate plays a distinct role in legume defense that differs from fixed nitrogen. One possible explanation for restored nitrogen fixation in high nitrate soils could be nutrient co-limitation. For nitrogen fixation, legume-rhizobia mutualisms require specific amounts of macro- and micronutrients in soils (Bonilla and Bolanos 2009), and legume roots may also require certain nutrients to maintain nitrate assimilation as well. Alternatively, rhizobia can also utilize soil nitrate (Breitenbeck and Bremner 1989), which may indicate free-living rhizobia outcompeted *M. sativa* for nitrate in high-nitrate soils (Kaye and Hart 1997), forcing legumes to rely on nitrogen fixation.

Differential legume responses across field and greenhouse soils

An intriguing finding from our study was the difference between field and greenhouse experiments in fixed nitrogen allocation in non-supplemented soils. In our field experiment, herbivory stimulated increased fixed nitrogen allocation to shoots (Fig. 1c) but did not impact allocation to crowns or roots (Table S2). In greenhouse non-supplemented soils, herbivory did not change fixed nitrogen allocation across shoots, crowns, and roots (Fig. 2b). Rhizobia diversity could drive different patterns between the field and greenhouse, as Dean et al. (2009) detected differential responses of aphids feeding on soybeans inoculated with commercial rhizobia compared to naturally occurring rhizobia. Although we inoculated *M. sativa* with commercial rhizobia for our field experiment, it is possible naturally occurring rhizobia nodulated *M. sativa* as well or even outcompeted the commercial strain. Variation between rhizobia strains can differentially affect legume growth and nutrient allocation (Heath et al. 2020), and even cascade to influence ecosystem function (Taylor et al. 2020), indicating legume soil microbiome diversity could be an underappreciated tool in agroecosystems. Other beneficial microbes, such as arbuscular mycorrhizal fungi, can also influence aboveground legume defense against pathogens (Ballhorn et al. 2014) and herbivores (Selvaraj et al. 2020), and these additional microbes may account for contrasting patterns across the field and greenhouse. Root herbivores can also play hidden roles in belowground interactions, such as the clover root weevil enhancing its performance by feeding on legume nodules (Wolfson 1987) and disrupting aboveground resource allocation in legumes (Johnson and McNicol 2010). Additional research is needed to identify the specific cause of the difference between field and greenhouse results.

Conclusions

Our study demonstrates herbivory increases aboveground fixed nitrogen allocation in legumes but only in soils not supplemented with additional nitrate. Overall, our results align with proposed non-linear responses of plants to nutrient availability and belowground mutualists (Treseder and Allen 2002; Vannette and Hunter 2011), as theory suggests a quadratic relationship between nutrient gradients and root colonization by beneficial microbes. Nutrient-free and high nutrient levels are predicted to decrease plant associations with microbes whereas moderate nutrient levels increase microbial colonization. Our results with legume-rhizobia symbioses show the opposite pattern, as non-supplemented and high nitrate soils increased fixed nitrogen allocation and moderate nitrate soils showed little fixed nitrogen allocation. Different types of plant–microbe symbioses alter plant traits in contrasting ways (Gibert et al. 2019), indicating more research is needed to determine how herbivores alter each symbiosis across soil nutrient conditions.

We conclude the response of *M. sativa* to herbivory is altered by nitrate supplementation, changing above- and belowground fixed nitrogen allocation. Considering nitrogen fixation drives increased production of specific amino acids (Liu et al. 2018), future research should focus on discerning the identity of the proteins or compounds *M. sativa* incorporates fixed nitrogen into in response to herbivory and their role in plant defense which may help to determine the mechanism behind the observed responses. Our work advances current knowledge on how herbivory affects plant–microbe symbioses across varying abiotic conditions, which has important implications for our understanding of these tripartite interactions across natural and agricultural ecosystems.

Acknowledgements We are grateful to the Western Maryland Research and Education Center staff and greenhouse staff at the University of Maryland for their support and help in executing these experiments, as well as members of the Lamp Lab (Alina Avanesyan, Dylan Kutz, Kevin Clements, Kimmy Okada, Emily Mast, Nina McGranahan, Jessica Ho, Sami Louguit). We would like to thank Drs. Kelly Hamby, Daniel Gruner, and Karin Burghardt for feedback on an earlier version of this manuscript, as well as two anonymous reviewers. We also extend gratitude and appreciation to Dr. Alan Leslie for substantial guidance on statistical analyses and writing. This project was supported by a graduate research award provided to M. Thompson from Northeastern Sustainable Agriculture Research and Education (Award Number GNE18-187-32231), as well as by the Hatch Project MD-ENTM-180.

References

Adams MA, Turnbull TL, Sprent JI, Buchmann N (2016) Legumes are different: Leaf nitrogen, photosynthesis, and water use efficiency. Proc Natl Acad Sci 113:4098–4103

Ballhorn DJ, Elias JD, Balkan MA, Fordyce RF, Kennedy PG (2017) Colonization by nitrogen-fixing Frankia bacteria causes short-term increases in herbivore susceptibility in red alder (*Alnus rubra*) seedlings. Oecologia 184:497–506

Ballhorn DJ, Kautz S, Schädler M (2013) Induced plant defense via volatile production is dependent on rhizobial symbiosis. Oecologia 172:833–846

Ballhorn DJ, Younginger BS, Kautz S (2014) An aboveground pathogen inhibits belowground rhizobia and arbuscular mycorrhizal fungi in *Phaseolus vulgaris*. BMC Plant Biol 14

Barnes D, Heichel G, Vance C, Peaden R (1990) Registration of 'ineffective agate' and 'ineffective saranac' non-N_2-fixing alfalfa germplasms. Crop Sci 30:752–753

Barsch A, Tellström V, Patschkowski T, Küster H, Niehaus K (2006) Metabolite profiles of nodulated alfalfa plants indicate that distinct stages of nodule organogenesis are accompanied by global physiological adaptations. Mol Plant Microbe Interact 19:998–1013

Bates D, Maechler M, Bolker B, Walker S, Christensen RHB, Singmann H, Dai B, Grothendieck G, Eigen C, Scheipl F (2015) Package 'lme4.' Convergence 12

Bonilla I, Bolanos L (2009) Mineral nutrition for legume-rhizobia symbiosis: B, Ca, N, P, S, K, Fe, Mo, Co, and Ni: A review, in: Organic Farming, Pest Control and Remediation of Soil Pollutants. Springer 253–274

Breitenbeck GA, Bremner JM (1989) Ability of free-living cells of *Bradyrhizobium japonicum* to denitrify in soils. Biol Fertil Soils 7:219–224

Brown SP, Grillo MA, Podowski JC, Heath KD (2020) Soil origin and plant genotype structure distinct microbiome compartments in the model legume *Medicago truncatula*. Microbiome 8:139

Brunner SM, Goos RJ, Swenson SJ, Foster SP, Schatz BG, Lawley YE, Prischmann-Voldseth DA (2015) Impact of nitrogen fixing and plant growth-promoting bacteria on a phloem-feeding soybean herbivore. Appl Soil Ecol 86:71–81

Burghardt KT (2016) Nutrient supply alters goldenrod's induced response to herbivory. Funct Ecol 30:1769–1778

Burity H, Ta T, Faris M, Coulman B (1989) Estimation of nitrogen fixation and transfer from alfalfa to associated grasses in mixed swards under field conditions. Plant Soil 114:249–255

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

Carroll BJ, Gresshoff PM (1983) Nitrate Inhibition of Nodulation and Nitrogen Fixation in White Clover. Z Für Pflanzenphysiol 110:77–88

Ciesiołka D, Muzquiz M, Burbano C, Altares P, Pedrosa M, Wysocki W, Folkman W, Popenda M, Gulewicz K (2005) An effect of various nitrogen forms used as fertilizer on *Lupinus albus* L. yield and protein, alkaloid and α-galactosides content. J Agron Crop Sci 191:458–463

Congreves KA, Hayes A, Verhallen EA, Van Eerd LL (2015) Long-term impact of tillage and crop rotation on soil health at four temperate agroecosystems. Soil Tillage Res 152:17–28

Dean J, Mescher M, De Moraes C (2014) Plant dependence on rhizobia for nitrogen influences induced plant defenses and herbivore performance. Int J Mol Sci 15:1466–1480

Dean JM, Mescher MC, De Moraes CM (2009) Plant–rhizobia mutualism influences aphid abundance on soybean. Plant Soil 323:187–196

DeLay B, Mamidala P, Wijeratne A, Wijeratne S, Mittapalli O, Wang J, Lamp W (2012) Transcriptome analysis of the salivary glands of potato leafhopper, *Empoasca fabae*. J Insect Physiol 58:1626–1634

Denison RF, Kiers ET (2004) Lifestyle alternatives for rhizobia: mutualism, parasitism, and forgoing symbiosis. FEMS Microbiol Lett 237:187–193

Epstein HE, Burke IC, Mosier AR (2001) Plant effects on nitrogen retention in shortgrass steppe 2 years after ^{15}N addition. Oecologia 128:422–430

Fallath T, Rosli AB, Kidd B, Carvalhais LC, Schenk PM (2017) Toward plant defense mechanisms against root pathogens, in: Agriculturally Important Microbes for Sustainable Agriculture. Springer 293–313

Ferguson BJ, Mens C, Hastwell AH, Zhang M, Su H, Jones CH, Chu X, Gresshoff PM (2019) Legume nodulation: The host controls the party. Plant Cell Environ 42:41–51

Fierer N (2017) Embracing the unknown: disentangling the complexities of the soil microbiome. Nat Rev Microbiol 15:579–590

Flinn P, Hower A, Knievel D (1990) Physiological response of alfalfa to injury by *Empoasca fabae* (Homoptera: Cicadellidae). Environ Entomol 19:176–181

Forero LE, Grenzer J, Heinze J, Schittko C, Kulmatiski A (2019) Greenhouse- and field-measured plant-soil feedbacks are not correlated. Front Environ Sci 7:184

Forrester NJ, Ashman T-L (2018) Nitrogen fertilization differentially enhances nodulation and host growth of two invasive legume species in an urban environment. J Urban Ecol 4

Fox J, Weisberg S, Adler D, Bates D, Baud-Bovy G, Ellison S, Firth D, Friendly M, Gorjanc G, Graves S (2012) Package 'car.' Vienna R Found Stat Comput

French E, Kaplan I, Iyer-Pascuzzi A, Nakatsu CH, Enders L (2021) Emerging strategies for precision microbiome management in diverse agroecosystems. Nat Plants 1–12

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

Friman J, Pineda A, Loon JJA, Dicke M (2021) Bidirectional plant-mediated interactions between rhizobacteria and shoot-feeding herbivorous insects: a community ecology perspective. Ecol Entomol 46:1–10

Gibert A, Tozer W, Westoby M (2019) Plant performance response to eight different types of symbiosis. New Phytol 222:526–542

Girousse C, Moulia B, Silk W, Bonnemain J-L (2005) Aphid infestation causes different changes in carbon and nitrogen allocation in alfalfa stems as well as different inhibitions of longitudinal and radial expansion. Plant Physiol 137:1474–1484

Goggin FL (2007) Plant–aphid interactions: molecular and ecological perspectives. Curr Opin Plant Biol 10:399–408

Grunseich JM, Thompson MN, Aguirre NM, Helms AM (2019) The role of plant-associated microbes in mediating host-plant selection by insect herbivores. Plants 9:6

Grunseich JM, Thompson MN, Hay AA, Gorman Z, Kolomiets MV, Eubanks MD, Helms AM (2020) Risky roots and careful herbivores: Sustained herbivory by a root-feeding herbivore attenuates indirect plant defences. Funct Ecol 34:1779–1789

Haichar FZ, Santaella C, Heulin T, Achouak W (2014) Root exudates mediated interactions belowground. Soil Biol Biochem 77:69–80

Hartman K, van der Heijden MG, Roussely-Provent V, Walser J-C, Schlaeppi K (2017) Deciphering composition and function of the root microbiome of a legume plant. Microbiome 5

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

Heath KD, McGhee KE (2012) Coevolutionary constraints? the environment alters tripartite interaction traits in a legume. PLoS ONE 7:e41567

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

Heinen R, Biere A, Harvey JA, Bezemer TM (2018) Effects of soil organisms on aboveground plant-insect interactions in the field: patterns, mechanisms and the role of methodology. Front Ecol Evol 6:106

Heinze J, Joshi J (2018) Plant–soil feedback effects can be masked by aboveground herbivory under natural field conditions. Oecologia 186:235–246

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Circ Calif Agric Exp Stn 347

Hutchins S, Pedigo L (1989) Potato leafhopper-induced injury on growth and development of alfalfa. Crop Sci USA

Imsande J (1986) Inhibition of nodule development in soybean by nitrate or reduced nitrogen. J Exp Bot 37:348–355

Johnson SN, McNicol JW (2010) Elevated CO_2 and aboveground–belowground herbivory by the clover root weevil. Oecologia 162:209–216

Kaplan I, Halitschke R, Kessler A, Rehill BJ, Sardanelli S, Denno RF (2008) Physiological integration of roots and

shoots in plant defense strategies links above- and belowground herbivory. Ecol Lett 11:841–851
Karban R, Agrawal AA (2002) Herbivore offense. Annu Rev Ecol Syst 33:641–664
Katayama N, Nishida T, Zhang ZQ, Ohgushi T (2010) Belowground microbial symbiont enhances plant susceptibility to a spider mite through change in soybean leaf quality. Popul Ecol 52:499–506
Kaye JP, Hart SC (1997) Competition for nitrogen between plants and soil microorganisms. Trends Ecol Evol 12:139–143
Kelner DJ, Vessey JK, Entz MH (1997) The nitrogen dynamics of 1-, 2- and 3-year stands of alfalfa in a cropping system. Agric Ecosyst Environ 64:1–10
Kempel A, Brandl R, Schädler M (2009) Symbiotic soil microorganisms as players in aboveground plant-herbivore interactions - the role of rhizobia. Oikos 118:634–640
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, Rousseau RA, West SA, Denison RF (2003) Host sanctions and the legume–*Rhizobium* mutualism. Nature 425:78–81
Kramer S, Marhan S, Ruess L, Armbruster W, Butenschoen O, Haslwimmer H, Kuzyakov Y, Pausch J, Scheunemann N, Schoene J, Schmalwasser A, Totsche KU, Walker F, Scheu S, Kandeler E (2012) Carbon flow into microbial and fungal biomass as a basis for the belowground food web of agroecosystems. Pedobiologia 55:111–119
Kramer-Walter KR, Laughlin DC (2017) Root nutrient concentration and biomass allocation are more plastic than morphological traits in response to nutrient limitation. Plant Soil 416:539–550
Kuznetsova A, Brockhoff PB, Christensen RHB (2015) Package 'lmertest.' R Package Version 2
Ladrera R, Marino D, Larrainzar E, González EM, Arrese-Igor C (2007) Reduced carbon availability to bacteroids and elevated ureides in nodules, but not in shoots, are involved in the nitrogen fixation response to early drought in soybean. Plant Physiol 145:539–546
Ladygina N, Hedlund K (2010) Plant species influence microbial diversity and carbon allocation in the rhizosphere. Soil Biol Biochem 42:162–168
Lamb JFS, Barnes DK, Russelle MP, Vance CP, Heichel GH, Henjum KI (1995) Ineffectively and effectively nodulated alfalfas demonstrate biology nitrogen fixation continues with high nitrogen fertilization. Crop Sci 35:153
Lamp W, Nielsen G, Quebedeaux B, Wang Z (2001) Potato leafhopper (Homoptera: Cicadellidae) injury disrupts basal transport of ^{14}C-labelled photoassimilates in alfalfa. J Econ Entomol 94:93–97
Lamp WO, Nielsen GR, Danielson SD (1994) Patterns among host plants of potato leafhopper, *Empoasca fabae* (Homoptera: Cicadellidae). J Kans Entomol Soc 354–368
Lamp WO, Nielsen GR, Fuentes CB, Quebedeaux B (2004) Feeding site preference of potato leafhopper (Homoptera: Cicadellidae) on alfalfa and its effect on photosynthesis. J Agric Urban Entomol 21:25–38
Layton MB, Boethel DJ (1987) Reduction in N_2 fixation by soybean in response to insect-induced defoliation. J Econ Entomol 80:1319–1324
Liu A, Contador CA, Fan K, Lam H-M (2018) Interaction and regulation of carbon, nitrogen, and phosphorus metabolisms in root nodules of legumes. Front in Plant Sci 9
Lodwig EM, Hosie AHF, Bourdès A, Findlay K, Allaway D, Karunakaran R, Downie JA, Poole PS (2003) Amino-acid cycling drives nitrogen fixation in the legume–rhizobium symbiosis. Nature 422:722–726
Lum MR, Hirsch AM (2002) Roots and their symbiotic microbes: strategies to obtain nitrogen and phosphorus in a nutrient-limiting environment. J Plant Growth Regul 21:368–382
Márquez-Ortiz JJ, Lamb JFS, Johnson LD, Barnes DK, Stucker RE (1999) Heritability of crown traits in alfalfa. Crop Sci 39:38–43
McAuliffe C, Chamblee D, Uribe-Arango H, Woodhouse W (1958) Influence of inorganic nitrogen on nitrogen fixation by legumes as revealed by ^{15}N dilution methods. Plant Soil 102:149–160
McKey D (1994) Legumes and nitrogen: the evolutionary ecology of a nitrogen-demanding lifestyle. Adv Legume Syst 5:211–228
Mitra D, Djebaili R, Pellegrini M, Mahakur B, Sarker A, Chaudhary P, Khoshru B, Gallo MD, Kitouni M, Barik DP, Panneerselvam P, Mohapatra PKD (2021) Arbuscular mycorrhizal symbiosis: plant growth improvement and induction of resistance under stressful conditions. J Plant Nutr 1–37
Naylor D, Coleman-Derr D (2018) Drought stress and root-associated bacterial communities. Front Plant Sci 8
Nielsen GR, Lamp WO, Stutte GW (1990) Potato leafhopper (Homoptera: Cicadellidae) feeding disruption of phloem translocation in alfalfa. J Econ Entomol 83:807–813
Pandharikar G, Gatti J-L, Simon J-C, Frendo P, Poirié M (2020) Aphid infestation differently affects the defences of nitrate-fed and nitrogen-fixing *Medicago truncatula* and alters symbiotic nitrogen fixation. Proc R Soc B Biol Sci 287:20201493
Peterson M, Barnes D (1981) Inheritance of ineffective nodulation and non-nodulation traits in alfalfa. Crop Sci 21:611–616
Pieterse CM, Zamioudis C, Berendsen RL, Weller DM, Van Wees SC, Bakker PA (2014) Induced systemic resistance by beneficial microbes. Annu Rev Phytopathol 52:347–375
Pineda A, Kaplan I, Bezemer TM (2017) Steering soil microbiomes to suppress aboveground insect pests. Trends Plant Sci 22:770–778
Pineda A, Zheng S-J, van Loon JJA, Pieterse CMJ, Dicke M (2010) Helping plants to deal with insects: the role of beneficial soil-borne microbes. Trends Plant Sci 15:507–514
Porter SS, Simms EL (2014) Selection for cheating across disparate environments in the legume-rhizobium mutualism. Ecol Lett 17:1121–1129
R Core Team (2018) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria

Regus J, Wendlandt C, Bantay R, Gano-Cohen K, Gleason N, Hollowell A, O'Neill M, Shahin K, Sachs J (2017) Nitrogen deposition decreases the benefits of symbiosis in a native legume. Plant Soil 414:159–170

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

Riedell WE, Beckendorf EA, Catangui MA (2013) Relationships between soybean shoot nitrogen components and soybean aphid populations. Arthropod-Plant Interact 7:667–676

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

Sasse J, Martinoia E, Northen T (2018) Feed your friends: do plant exudates shape the root microbiome? Trends Plant Sci 23:25–41

Schittko C, Runge C, Strupp M, Wolff S, Wurst S (2016) No evidence that plant-soil feedback effects of native and invasive plant species under glasshouse conditions are reflected in the field. J Ecol 104:1243–1249

Schmitt A, Pausch J, Kuzyakov Y (2013) C and N allocation in soil under ryegrass and alfalfa estimated by ^{13}C and ^{15}N labelling. Plant Soil 368:581–590

Schwachtje J, Minchin PE, Jahnke S, van Dongen JT, Schittko U, Baldwin IT (2006) SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. Proc Natl Acad Sci 103:12935–12940

Selvaraj A, Thangavel K, Uthandi S (2020) Arbuscular mycorrhizal fungi (*Glomus intraradices*) and diazotrophic bacterium (*Rhizobium* BMBS) primed defense in blackgram against herbivorous insect (*Spodoptera litura*) infestation. Microbiol Res 231:126355

Shearer G, Kohl DH (1986) N_2-fixation in field settings: estimations based on natural ^{15}N abundance. Funct Plant Biol 13:699–756

Simonsen AK, Stinchcombe JR (2014) Herbivory eliminates fitness costs of mutualism exploiters. New Phytol 202:651–661

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

Tateno R, Taniguchi T, Zhang J, Shi W-Y, Zhang J-G, Du S, Yamanaka N (2017) Net primary production, nitrogen cycling, biomass allocation, and resource use efficiency along a topographical soil water and nitrogen gradient in a semi-arid forest near an arid boundary. Plant Soil 420:209–222

Taylor BN, Simms EL, Komatsu KJ (2020) More than a functional group: diversity within the legume–rhizobia mutualism and its relationship with ecosystem function. Diversity 12:50

Thamer S, Schädler M, Bonte D, Ballhorn DJ (2011) Dual benefit from a belowground symbiosis: nitrogen fixing rhizobia promote growth and defense against a specialist herbivore in a cyanogenic plant. Plant Soil 341:209–219

Tonelli ML, Figueredo MS, Rodríguez J, Fabra A, Ibañez F (2020) Induced systemic resistance -like responses elicited by rhizobia. Plant Soil 448:1–14

Tracy BF, Albrecht K, Flores J, Hall M, Islam A, Jones G, Lamp W, MacAdam JW, Skinner H, Teutsch C (2016) Evaluation of alfalfa–tall fescue mixtures across multiple environments. Crop Sci 56:2026

Treseder KK, Allen MF (2002) Direct nitrogen and phosphorus limitation of arbuscular mycorrhizal fungi: a model and field test. New Phytol 155:507–515

Tsiknia M, Tsikou D, Papadopoulou KK, Ehaliotis C (2021) Multi-species relationships in legume roots: From pairwise legume-symbiont interactions to the plant – microbiome – soil continuum. FEMS Microbiol Ecol 97

Unkovich M, Herridge D, Peoples M, Cadisch G, Boddey B, Giller K, Alves B, Chalk P (2008) Measuring plant-associated nitrogen fixation in agricultural systems. Australian Centre for International Agricultural Research (ACIAR)

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

Vannette RL, Hunter MD (2011) Plant defence theory re-examined: nonlinear expectations based on the costs and benefits of resource mutualisms: Nonlinear effects of mutualism on defence. J Ecol 99:66–76

Velikova V, Salerno G, Frati F, Peri E, Conti E, Colazza S, Loreto F (2010) Influence of feeding and oviposition by phytophagous pentatomids on photosynthesis of herbaceous plants. J Chem Ecol 36:629–641

Vurukonda SSKP, Vardharajula S, Shrivastava M, SkZ A (2016) Enhancement of drought stress tolerance in crops by plant growth promoting rhizobacteria. Microbiol Res 184:13–24

Wang Q, Yuan E, Ling X, Zhu-Salzman K, Guo H, Ge F, Sun Y (2020) An aphid facultative symbiont suppresses plant defence by manipulating aphid gene expression in salivary glands. Plant Cell Environ 43:2311–2322

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

Wilson ACC, Sternberg LdSL, Hurley KB (2011) Aphids alter host-plant nitrogen isotope fractionation. Proc Natl Acad Sci 108:10220–10224

Wolf AA, Funk JL, Menge DNL (2017) The symbionts made me do it: legumes are not hardwired for high nitrogen concentrations but incorporate more nitrogen when inoculated. New Phytol 213:690–699

Wolfson JL (1987) Impact of *Rhizobium* nodules on *Sitona hispidulus*, the clover root curculio. Entomol Exp Appl 43:237–243

Womack CL (1984) Reduction in photosynthetic and transpiration rates of alfalfa caused by potato leafhopper (Homoptera: Cicadellidae) infestations. J Econ Entomol 77:508–513

Xiao X, Chen W, Zong L, Yang J, Jiao S, Lin Y, Wang E, Wei G (2017) Two cultivated legume plants reveal the enrichment process of the microbiome in the rhizocompartments. Mol Ecol 26:1641–1651

Yadav AN, Singh J, Rastegari AA, Yadav N (2020) Plant microbiomes for sustainable agriculture. Springer