ABSTRACT

Title of Thesis:	EVALUATING THE EFFECT OF POTATO LEAFHOPPER *(EMPOASCA FABAE)* FEEDING ON BIOLOGICAL NITROGEN FIXATION IN ALFALFA *(MEDICAGO SATIVA)*
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Aboveground feeding by potato leafhopper (PLH), *Empoasca fabae*, (Hemiptera: Cicadellidae) causes significant injury to alfalfa (*Medicago sativa)*, including disrupting translocation of fixed carbon from leaves to roots. Basal transport of fixed carbon in alfalfa fuels a critical mutualism between roots and nitrogen-fixing bacteria (*Sinorhizobium meliloti*). Above- and belowground nutrient allocation in alfalfa determines perennial persistence across growing seasons, as well as forage quality. Whether leafhopper feeding alters nutrient allocation and subsequently affects nitrogen fixation, however, is not clear. To test this, my objectives were 1) to examine the effect of different management strategies on PLH injury and nitrogen fixation, and 2) to quantify the amount and location of fixed nitrogen in whole alfalfa plants when fed on by leafhoppers. Overall, my work contributes to an understanding of how aboveground pest pressure can disrupt belowground processes in plants and ultimately affect the economic viability of crops for growers.

EVALUATING THE EFFECT OF POTATO LEAFHOPPER *(EMPOASCA FABAE)* FEEDING ON BIOLOGICAL NITROGEN FIXATION IN ALFALFA *(MEDICAGO SATIVA)*

by

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Dedication

To Larry

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Abstract

Nitrogen acquisition and allocation limits the success of perennial crops over multiple growing seasons. Severe pest pressure can reduce the nutritional content of crops, resulting in losses for growers. Potato leafhopper (PLH; *Empoasca fabae*, Hemiptera: Cicadellidae) remains one of the most significant pests of *Medicago sativa*, reducing growth and forage quality through feeding damage. Management strategies, such as planting resistant cultivars and intercropping with grasses, offer ways to control PLH pressure. Whether PLH feeding alters nitrogen acquisition, allocation, and fixation, however, remains unclear. To test this, our objectives were to 1) quantify the effect of PLH injury on nitrogen biomass and allocation across resistant and susceptible cultivars, 2) understand the effect of intercropping on PLH injury across cultivars, and 3) describe how nitrogen fixation is altered across cultivars by PLH injury. Under PLH pressure, resistant cultivars accumulated higher aboveground nitrogen biomass but intercropping with fescue did not affect accumulation. Cultivars varied in levels of nitrogen fixation following PLH injury. Our results advance sustainable management strategies for forage growers by

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comparing the effectiveness of two PLH management strategies in the field and greenhouse.

Introduction

Nitrogen acquisition and availability determines the nutritional value of harvested crops. To acquire nitrogen, crops form specialized interactions with nitrogen-fixing microbes, assimilate inorganic and organic nitrogen directly from the soil (Jones et al. 2005), or rely on a combination of such processes (Thornton and Robinson 2005). Nitrogen assimilation from the soil requires sufficient levels of available nitrogen in the soil, which often results in additional fertilizer inputs of inorganic nitrogen (Miller and Cramer 2005). Although enhanced soil nitrogen levels can dramatically increase crop growth and yield (Spiertz 2009), increased nitrogen content can also increase losses to insect pests (Scriber 1984) and ultimately reduce the nutritional value of crops (Aqueel and Leather 2011). In an effort to limit nitrogen inputs to agroecosystems, nitrogen-fixing crops potentially offer sustainable alternatives (Peoples et al. 1995; Vance 1997) but little is known about how insect pests affect nitrogen fixation and how these effects interact with other pest management strategies. Here, our objective was to understand how pest injury alters nitrogen acquisition and allocation in a nitrogen-fixing forage crop, exploring the use of intercropping and resistant cultivars as pest management strategies.

Medicago sativa, also known as alfalfa or lucerne, is a nitrogen-fixing legume grown primarily as a forage crop across 80 million acres worldwide (Russelle 2001). Referred to as "Queen of Forages," *M. sativa* boasts an agricultural history dating back thousands of years (Russelle 2001). Since its domestication, *M. sativa* was

grown for livestock and is now the prevailing choice of feed for dairy cows (Barnes 1988) because *M. sativa* contains high levels of crude protein and exhibits high digestibility (Balde et al. 1993). As a perennial crop, *M. sativa* stands can persist for 3 to 5 years on average and allow for multiple harvests throughout the growing season, depending on the local climate (Veronesi et al. 2010). Belowground nitrogen allocation significantly influences the success of *M. sativa* across multiple harvests and as a perennial crop (Volenec et al. 1996). Crop health and nitrogen allocation can also be impacted by pest pressure.

A well-studied pest of *M. sativa*, potato leafhopper (PLH; *Empoasca fabae* Harris), is a highly polyphagous (Lamp et al. 1994), migratory North American pest (Chasen et al. 2014). PLH disperses from the southern United States and Mexico northward into Canada during the growing season (Carlson et al. 1992; Taylor and Shields 1995). PLH feeding damage is primarily identified in agricultural fields by the distinctive v-shaped yellowing of *M. sativa* leaves, referred to as 'hopperburn' (Backus et al. 2005). PLH feeding induces a saliva-enhanced wound response in *M. sativa* (Ecale and Backus 1995), resulting in decreased rates of photosynthesis and transpiration (Womack 1984; Flinn et al. 1990) and disrupted basal translocation of photoassimilates (Nielsen et al. 1990; Lamp et al. 2001). Such physiological damage to *M. sativa* ultimately reduces stem elongation (Hutchins and Pedigo 1989) and reduces crude protein content (Hower and Flinn 1986), resulting in yield losses for growers (Cuperus et al. 1983; Lamp et al. 1991).

To combat pest losses, growers often select resistant cultivars, which possess traits that disrupt or halt pest damage. PLH-resistant *M. sativa* cultivars produce

glandular trichomes, which impede movement and feeding of nymphs and decrease adult localization and feeding (Ranger and Hower 2001, 2002). Ranger et al. (2005) used headspace volatile collection to determine how resistant cultivars are less attractive to PLH, and showed different ratios of chemical compounds produced by susceptible and resistant cultivars (Ranger et al. 2005). In a field setting, resistant cultivars show increased forage quality relative to susceptible cultivars (Sulc et al. 2004) and decreased PLH damage under high PLH pressure (Sulc et al. 2001). PLH resistant cultivars allow growers to avoid the use of insecticides when controlling for PLH, which provides an economical and environmentally beneficial pest management strategy.

Intercropping offers another pest management strategy for PLH in *M. sativa*. When intercropping, *M. sativa* and at least one other plant species are heterogeneously seeded and grown together to reduce PLH damage to *M. sativa*. Intercropped fields can reduce the density of *M. sativa* and thus deter PLH feeding. For instance, *M. sativa* fields intercropped with grass decrease PLH feeding (Oloumi-Sadeghi et al. 1987; Lamp 1991) by increasing PLH adult emigration from intercropped fields (Smith et al. 1994; Roda et al. 1997). Increased plant diversity through intercropping can also provide natural enemies with suitable habitat conditions for growth and survival, promoting their colonization and activity in agricultural fields (Landis et al. 2000). Intercropping *M. sativa* with orchardgrass (*Dactylis glomerata*) supported greater predator activity of damsel bugs (*Nabis* spp.) through increased PLH movement, reducing hopperburn and improving yield (Straub et al. 2013, 2014). Manipulating the structure of *M. sativa* fields through

intercropping reduces PLH damage by diluting available amounts of *M. sativa* and supporting natural insect predators.

Here we examined the response of *M. sativa* resistant and susceptible cultivars in monoculture or intercropped with tall fescue grass (*Festuca arundinacea*) to varied PLH densities in a field experiment. Combining cultivar selection and intercropping allowed us to determine the singular or additive effectiveness of management strategies. We focused on understanding the effect of PLH feeding on nitrogen biomass, as well as nitrogen allocation above- and belowground. To determine the effect of PLH feeding on nitrogen biomass, allocation, and fixation across *M. sativa* cultivars, we also performed a controlled greenhouse experiment. Overall, our objectives for this study were to 1) quantify *M. sativa* nitrogen biomass and allocation following PLH injury across resistant and susceptible cultivars, 2) examine the potential to mitigate nitrogen losses to PLH injury with intercropping, and 3) understand the effect of PLH feeding on nitrogen fixation across cultivars.

Methods

Field Experiment

We planted our field experiment in Keedysville, MD, USA on September 1, 2017 to allow for dormancy during the fall and winter prior to production. The field (48.8m x 24.4m) was planted in a randomized complete block split-plot design with a buffer strip (6.1m x 12.2m) of bare ground dividing the field in half. Four blocks (12.2m x 24.4m each) and four main plots (6.1m x 12.2m each) per block were established perpendicular to the buffer strip. Main plots included: 1) Susceptible

(Pioneer ‘55V50’) Monoculture (SM), 2) Resistant (Pioneer ‘55H94’) Monoculture (RM), 3) Susceptible-Fescue Intercropped System (SF), and 4) Resistant-Fescue Intercropped System (RF). We divided each plot in half (6.1m x 6.1m) in order to suppress PLH populations, establishing two subplots per main plot: with or without insecticide. We sprayed an insecticide containing the active ingredient lambda-cyhalothrin (Warrior II) at a rate of 116.91 mL per hectare on designated subplots. In this way, our insecticide treatment acted as our control (low PLH pressure) relative to our unsprayed subplots (high PLH pressure). We applied insecticide 12 (June 14, 2018) and 20 (July 11, 2018) days prior to our two harvests.

We harvested the entire field on May 22, 2018 and began taking weekly sweep net samples the following week. The primary author took five sweeps per plot with a sweep net which was 90 cm in length, 40 cm in diameter, and made of canvas cloth. Contents of sweep samples were placed in paper bags. The paper bags were enclosed in a sealed plastic bucket with 5mL of ethyl acetate (killing agent) to collect PLH. We brought samples to the lab and recorded the number of PLH adults and nymphs for each subplot. Weather-permitting, weekly sweep samples were collected until the conclusion of the experiment in early August 2018.

When the crop had grown for 35 days after cutting, we collected foliage samples within four separate 50x50 cm areas for each subplot. Foliage was cut approximately 5 cm above the soil surface using a handheld grass trimmer to mimic normal harvest practices. Areas were randomly selected at four different locations within each subplot and all plant material was placed into a paper bag. Samples were taken to the laboratory, where we separated *M. sativa*, weeds, and fescue (if

applicable). We then dried our samples in drying ovens for a minimum of 24-36 h at 60° C and measured the dry weight (grams) of each sample component.

We also collected whole plant samples from each subplot. Three to four *M. sativa* plants were dug up from 10 cm below the soil at four random locations within each subplot. We rinsed roots with water in the field and then separated whole plant samples into roots, crowns (nutrient storage organ at the interface between above- and belowground portions), and shoots in the laboratory. Whole plant samples were dried in the drying oven for 24-36 h at 60° C.

Both foliar and whole plant samples were ground using an IKA Mills© A10 Basic grinder, sieved through a 1mm sieve, and weighed out for C/N analysis. C/N analysis was performed with a LECO CN628 Carbon/Nitrogen Determinator in the Department of Environmental Science and Technology at the University of Maryland. The analysis combusts samples to determine relative amounts of CO_2 and NO_x as an estimate of the percentage carbon and nitrogen in samples.

Greenhouse Experiment

We planted seeds of susceptible (Pioneer '55V50') and resistant (Pioneer '55H94') *M. sativa* in small trays at the greenhouse on December 20, 2017. After 17 days, we repotted seedlings of susceptible and resistant *M. sativa* into ceramic pots (14 cm x 15 cm) each filled with 2.75 kg of Sakrete Multipurpose sand. Each pot contained three seedlings of a designated cultivar. In total, we had 64 experimental units (pots). Seedlings were inoculated in a dilution of rhizobia (*Sinorhizobium meliloti*) and water planting. Pots were arranged in a randomized complete block design across two greenhouse benches. In total, we established eight blocks each

containing eight treatments, which included three factors with two levels each, fully crossed. Our three factors included: 1) *M. sativa* cultivar (Susceptible or Resistant), 2) Nitrogen amendment (16mg ^{15}N-labelled potassium nitrate/50mL of water or 16mg of potassium chloride/50mL of water), and 3) PLH (10 Adult PLH or None). We fertilized with potassium nitrate to determine if *M. sativa* could compensate for nitrogen losses from PLH feeding with supplemental soil nitrogen. To account for any effect of additional potassium from our potassium nitrate treatment, we supplied all other pots with potassium chloride. *M. sativa* roots do not readily take up chloride, leaving potassium available in these pots. Plants surrounded by empty cages served as uninjured controls.

Prior to the addition of nitrogen and PLH treatments, we fertilized the pots once a week with nitrogen-free Hoagland's solution. Pots were continuously watered at the greenhouse via hydroponics set up. On March 23, 2018, M-Pede® (Gowan Co., Yuma, AZ, insecticidal soap) was applied to all pots to control for thrips and aphid outbreaks. Three days later, we also applied entomopathogenic nematodes to the soil and predatory mites to pots to control for thrips. All biocontrol was completely removed one month later. Due to a relatively low number of thrips, few predatory mites survived. Nevertheless, all plants were visually inspected prior to PLH application to ensure complete removal of both thrips and mites.

Thirteen weeks after repotting, we simulated a harvest on April 10, 2018 by cutting back plants in four blocks. Plants were cut back to about 2.5cm of stem height. We applied PLH and nitrogen treatments three weeks after cutting (21 days after cutting). We selected 21 days after cutting due to the known increase in nitrogen

fixation at this time of the *M. sativa* growth cycle (Vance et al. 1979; Kim et al. 1993). We removed cages one week later (28 days after defoliation) and then we sacrificed plants the following week (35 days after defoliation), which follows standard harvesting practices in the field (Hendershot and Volenec 1993). We completed the same process for the other four blocks, beginning with simulating a harvest on April 17, 2018 (fourteen weeks after repotting) and cutting back all plants. PLH and fertilization treatments were applied at 21 days, cages were removed at 28 days, and plants were sacrificed at 35 days. When sacrificing the plants, we separated roots, crown, and shoots and we measured the fresh weight (grams) of roots, crown, and shoots for each pot. We placed all samples in the drying oven for a minimum of 24-36 h at 60° C and then measured dry weight (grams) of all samples. Dried samples were ground and weighed out 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 are reported as δ^{15}N ‰ (see Appendix B for further discussion of interpretation of δ^{15}N ‰; see also Werner and Brand 2001 & Coplen 2011 for further discussion of instrumentation and interpretation).

Data Analysis

Analyses were conducted within the program R version 3.5.1 (R Core Team 2018). To analyze sweep samples from the field study, we averaged adult, nymph and total PLH densities for each of the untreated subplots to make comparisons between

cultivars and intercropping with fescue. We analyzed adult, nymph, and total PLH densities of untreated subplots as separate response variables across the growing season using repeated measures analysis of variance (ANOVA). The explanatory variables included cultivar, fescue, and the interaction of cultivar and fescue. We separated our repeated measures ANOVA by sampling period and grouped all sweep samples taken before the first sampling period together and all sweep samples taken after the first sampling period together. We also calculated how PLH numbers (adults, nymphs, total) changed over time in response to cultivar, fescue, and insecticide treatment.

For foliar samples from the field study, we calculated average alfalfa, fescue, and weed dry weights, as well as the total biomass dry weight, for each treatment combination across both sampling periods. To analyze response variables, we used three-way ANOVA accounting for the split plot design. Our ANOVA models contained three explanatory variables: two main plot factors (Cultivar, Fescue) and one subplot factor (Insecticide). We tested for all interactions and present interactions for main plots (Cultivar x Fescue) as well as any significant subplot interactions. We also calculated average percentage nitrogen and nitrogen biomass (Percentage Nitrogen x Alfalfa Dry Weight) for all treatment combinations across both sampling periods. We ran ANOVA with the same model structure for each sampling period to separately test for effects on percentage nitrogen and nitrogen biomass.

For whole-plant samples, we separately analyzed shoot, crown, and root samples and present only the results for the first sampling period (June 26, 2018). For each plant component, we calculated average dry weight, percentage nitrogen, and

nitrogen biomass (Dry Weight x Percentage Nitrogen) across all treatment combinations. We constructed three-way ANOVA models to separately analyze shoots, crowns, and roots and each response variable. Explanatory variables included two main plot factors (Cultivar, Fescue) and one subplot factor (Insecticide). We also combined shoot, crown, and root nitrogen biomass values for each subplot to determine above- and belowground allocation patterns across cultivars and fescue. We determined differences between each plant component for healthy and injured plants across each main plot combination using LSD post-hoc comparison tests.

For the greenhouse study, we separated plant components into shoots, crowns, and roots. We determined average dry weight, percentage nitrogen, nitrogen biomass, and $\delta^{15}N$ ‰ values across eight treatment combinations for each plant component. We used three-way ANOVA models (three factors each with two levels, fully crossed) for each response variable and separated our analyses by shoot, crown, and root. We present effects of cultivar, PLH, and nitrogen as well as all the interactions between these factors. Tukey post-hoc comparisons of $\delta^{15}N$ ‰ values for healthy and injured shoots across fertilization treatments and variety determine effects of PLH on translocation of fixed nitrogen aboveground.

Results

Field Study

PLH Densities

Average PLH densities (adult, nymph, total) throughout the growing season for unsprayed subplots indicated an increase in population density around mid-June

followed by a decline after the June sampling period (Table 1.1). Repeated measure two-way ANOVA models for unsprayed subplots indicated a significant effect of cultivar on adult, nymph, and total PLH density across both sampling periods (Table 1.2). Over all dates, adults, nymphs, and total densities were reduced by 58, 73, and 67% on resistant versus susceptible cultivars. For unsprayed subplots, we did not detect a significant effect of fescue or an effect of the interaction between cultivar and fescue on any PLH densities. Adult densities across sprayed and unsprayed subplots of RM and RF fields, as well as sprayed subplots of SM and SF fields, remained low throughout the growing season (Figure 1.1). Nymph densities followed similar trends to adult densities but showed little recovery in numbers at the end of the growing season across all subplots (Figure 1.2). Total densities also followed similar trends, with peaks in unsprayed subplots of SM and SF fields in mid-June (Figure 1.3).

Yield and Nitrogen Biomass

Foliar samples determined the yield of each subplot across all main plots. ANOVA results for the first sampling period show a significant effect of cultivar ($p=0.03$) on both total biomass and alfalfa dry weight (Table 1.4). We also showed, quite obviously, a significant effect of fescue ($p<0.001$) on fescue dry weight. For the second sampling period, we saw a significant effect of insecticide ($p=0.02$) on total biomass dry weight and a significant effect of cultivar ($p=0.03$) on alfalfa dry weight (Table 1.4). We again saw a significant effect of fescue ($p<0.001$) on fescue dry weight and a significant effect of fescue ($p=0.009$) on weed dry weight. Average alfalfa, fescue, weed, and total biomass dry weight for the first sampling period (June 26, 2018) indicated an increase in alfalfa (24%) and total biomass (18%) dry weight

across plots with resistant alfalfa compared to plots with susceptible alfalfa (Table 1.3). We also observed minimal control of weed growth with fescue intercropping in the first sampling period but significant reductions (72%) during the second sampling period (July 31, 2018) in weed dry weight for intercropped plots (Table 1.3). Additionally, we observed a decrease in alfalfa and total biomass dry weight across all subplots between the first and second sampling periods (Table 1.3).

Results from ANOVA models for the first sampling period showed a significant effect of cultivar ($p<0.001$) and insecticide ($p=0.001$) on percentage nitrogen (Table 1.6). Similarly, we saw a significant effect of cultivar ($p=0.01$) and insecticide ($p=0.03$) on nitrogen biomass (Table 1.6). For the second sampling period, we showed a significant effect of insecticide ($p=0.008$) and an effect of the interaction between fescue and insecticide ($p=0.01$) on percentage nitrogen (Table 1.6). ANOVA model results for nitrogen biomass showed a significant effect of cultivar ($p=0.04$). For the first sampling period, pairwise comparisons between healthy and injured for SM, SF, RM, and RF fields revealed decreases across all subplots in percentage nitrogen (16, 13, 7, and 10%) and nitrogen biomass (38, 17, 11, and 22%) (Table 1.5). We observed similar trends for the second sampling period and also noted an increase in percentage nitrogen across all treatment combinations from the first sampling period to the second sampling period (Table 1.5).

Plant Components

We separated whole plant samples into components of shoots, crowns, and roots. For shoot samples, ANOVA results showed a significant effect of cultivar ($p=0.006$) on dry weight (Table 1.8). We also determined a significant effect of

cultivar ($p=0.04$), insecticide ($p<0.001$), and an interaction between cultivar and insecticide ($p=0.003$) on percentage nitrogen. Similarly, nitrogen biomass results indicated a significant effect of cultivar ($p=0.006$) and insecticide ($p=0.04$). We observed a decrease in dry weight, percentage nitrogen, and nitrogen biomass for SM, SF, and RM unsprayed subplots compared to sprayed subplots (Table 1.7). In contrast, RF fields showed small increases in dry weight, percentage nitrogen, and nitrogen biomass in unsprayed versus sprayed subplots.

For crowns, ANOVA results for dry weight showed a significant effect of cultivar ($p=0.002$) and insecticide ($p=0.04$), and percentage nitrogen showed the same response (Table 1.8). Cultivar had a significant effect ($p=0.01$) on nitrogen biomass. Averages for crowns showed reductions in dry weight and nitrogen biomass in unsprayed subplots compared to sprayed subplots for SM, SF, RM, and RF fields (Table 1.7). However, across SM, SF, RM, and RF fields, percentage nitrogen increased in unsprayed versus sprayed subplots (Table 1.7).

ANOVA results for root dry weight showed a significant effect of cultivar ($p=0.001$), insecticide ($p=0.01$), and an interaction between cultivar, fescue, and insecticide ($p=0.04$) (Table 1.8). ANOVA model for percentage nitrogen revealed no significant effects. Cultivar had a significant effect ($p=0.002$) on nitrogen biomass. Root sample averages showed a reduction in dry weight (43%) between susceptible and resistant cultivars (Table 1.7). For SM, SF, RM, and RF fields, percentage nitrogen increased (14, 10, 5, and 9%) in injured plants compared to healthy plants and nitrogen biomass showed minimal differences across field comparisons of healthy and injured plants.

To examine nitrogen allocation, we combined nitrogen biomass (grams of nitrogen) averages for shoots, crowns, and roots from each of the eight treatment combinations (Figure 1.4). Nitrogen biomass incorporates the size of plants into how plants distribute nitrogen above- and belowground. Results from LSD post-hoc comparison tests for each plant component showed no significant differences between healthy and injured nitrogen biomass across SM, SF, RM, and RF fields. Overall, susceptible plants produced less nitrogen (65%) than resistant plants. Injured plants in SM, SF, and RM fields showed decreases (46, 46, and 26%) in aboveground nitrogen biomass and minimal decreases (0, 20, and 20%) in belowground nitrogen biomass compared to healthy plants. In contrast, RF injured plants showed an increase (26%) in aboveground nitrogen biomass and almost no change in belowground nitrogen biomass compared to healthy plants.

Greenhouse Experiment

Nitrogen Biomass

Three-way ANOVA results for shoot dry weight indicated significant effects of cultivar ($p=0.04$), PLH ($p=0.02$), and a significant interaction effect of cultivar and PLH ($p=0.03$) (Table 1.10). Tukey post-hoc comparisons, however, revealed no significant differences between comparisons of interest: (1)S, -N, -PLH vs. S, -N, +PLH (2) R, -N, -PLH vs. R, -N, +PLH (3) S, +N, -PLH vs. S, +N, +PLH and (4) R, +N, -PLH vs. R, +N, +PLH. For percentage nitrogen content, we detected a significant effect of PLH ($p=0.0002$) and a significant three-way interaction effect between cultivar, nitrogen, and PLH ($p=0.04$). Tukey post-hoc comparisons revealed a significant decrease in percentage nitrogen content for when PLH were added to

susceptible plants fertilized with nitrogen (p=0.0044). For nitrogen biomass, we showed a significant effect of cultivar (p=0.03) and a significant interaction between cultivar and PLH (p=0.009). Tukey post-hoc comparisons revealed no differences between comparisons of interest to the study. Aboveground shoots showed inconsistent trends across treatment combinations (Tables 1.9). PLH injury decreased (8%) percentage nitrogen across all cultivar and nitrogen fertilizer combinations. PLH injury decreased dry weight (10%) in unfertilized susceptible plants and increased dry weight in unfertilized resistant plants (28%), fertilized susceptible plants (12%), and fertilized resistant plants (16%). Nitrogen biomass values followed similar trends from non-uniform percentage nitrogen and dry weight values. δ^{15}N ‰ values increased (99%) in pots with nitrogen fertilization across both cultivars.

Plant Components

ANOVA model results for crown samples indicated a significant effect of cultivar (p=0.02) and nitrogen fertilizer (p=0.02) on dry weight (Table 1.10). Percentage nitrogen responded to an interaction between cultivar and PLH (p=0.03). Results for the nitrogen biomass model showed a significant effect of cultivar (p=0.02) and nitrogen fertilizer (p=0.04). Nitrogen fertilizer had a significant effect (p<0.001) on δ^{15}N ‰ values. Averages for crown samples revealed increased dry weight in fertilized injured plants across both cultivars (Table 1.9). Percentage nitrogen decreased in fertilized (16%) and unfertilized (1%) injured susceptible plants and increased across fertilized (6%) and unfertilized (13%) injured resistant plants. We saw increased nitrogen biomass in injured resistant plants, both fertilized (14%) and unfertilized (31%). Nitrogen biomass did not change across healthy and injured

unfertilized susceptible plants and decreased (30%) across fertilized susceptible plants. $\delta^{15}N$ ‰ values increased in pots with nitrogen fertilization across both cultivars.

Across all ANOVA models for roots, we detected a significant effect of cultivar ($p=0.03$) on nitrogen biomass and a significant effect of nitrogen fertilizer ($p<0.001$) on $\delta^{15}N$ ‰ values (Table 1.10). Dry weight increased across root samples from injured plants for all treatment combinations except for fertilized resistant plants (Table 1.9). Percentage nitrogen decreased (2%) for unfertilized injured susceptible plants and remained unchanged for fertilized susceptible plants. Percentage nitrogen increased in fertilized (7%) and unfertilized (12%) injured resistant plants. Nitrogen biomass increased for injured unfertilized resistant plants (86%) and fertilized susceptible plants (15%), decreased for fertilized resistant plants (16%), and remained unchanged for unfertilized susceptible plants. $\delta^{15}N$ ‰ values increased in pots with nitrogen fertilization across both cultivars.

Source of Nitrogen

$\delta^{15}N$ ‰ values across susceptible and resistant cultivars with and without added nitrogen revealed drastic increases (99%) in $\delta^{15}N$ ‰ values for fertilized experimental units, regardless of cultivar and PLH treatment (Figures 1.5 and 1.6). Such high $\delta^{15}N$ ‰ values indicate little nitrogen fixation and Tukey post-hoc comparison tests revealed no differences between each cultivar with and without PLH. We noted contrasting trends across cultivars: susceptible shoots showed an increase (57%) in $\delta^{15}N$ ‰ with the addition of PLH and resistant shoots showed a

decrease (31%) in $\delta^{15}N$ ‰ with the addition of PLH. Further, despite the orders of magnitude difference between our fertilized and unfertilized experimental units, we observed the same trend in our results, although again a non-significant trend. These results suggest a decrease in nitrogen fixation for susceptible plants when PLH are present and the exact opposite trend in resistant plants.

Discussion

We aimed to understand how PLH pressure affects nitrogen acquisition and accumulation of *M. sativa* resistant and susceptible cultivars in monoculture and intercropped with fescue. Specifically, we executed field and greenhouse experiments to 1) compare resistant and susceptible cultivars in terms of nitrogen biomass accumulation and allocation following PLH injury, 2) determine if intercropping with fescue can reduce nitrogen losses, and 3) understand alterations across cultivars to nitrogen fixation in response to PLH injury. These experiments demonstrate differences in nitrogen biomass allocation across cropping systems, as well as contrasting responses of nitrogen fixation to PLH injury. Ultimately, perturbations to nitrogen acquisition and allocation affect long-term perennial persistence and economic viability of *M. sativa*.

Our resistant cultivar showed increased benefits in biomass accumulation in the field but not the greenhouse. Regardless of insecticide or fescue treatments, resistant foliar samples showed greater total biomass, as well as *M. sativa* biomass, than susceptible foliar samples, and whole plant samples followed similar trends. Additionally, resistant-containing fields sustained lower PLH populations. Previous studies also indicated increased benefits from the use of resistant cultivars, such as

reduced yield loss and increased forage quality (Sulc et al. 2001, 2004). In contrast, we did not observe biomass differences between cultivars in the greenhouse experiment. Rather, we saw a significant effect of cultivar, PLH, and an interaction between cultivar and PLH on shoot dry weight, indicating cultivars are responding in contrasting ways to PLH damage. When examining the response of resistant and susceptible cultivars to PLH injury, Lamp et al. (2014) demonstrated decreased rates of photosynthesis and transpiration but a greater decrease in susceptible compared to resistant cultivars. Our results support proposed differences in physiological and molecular responses of resistant and susceptible cultivars to PLH injury.

Further, we found significant effects on nitrogen biomass accumulation and allocation across cultivars in response to PLH injury. In our field study, cultivar and insecticide both had significant effects on nitrogen biomass of foliar and whole plant samples. Shoots from whole plant samples collected in sprayed RM fields accumulated the most aboveground nitrogen biomass (Fig 1.4). However, when comparing shoots from whole plants collected in sprayed SM fields to shoots from unsprayed RM fields, we saw comparable levels of aboveground nitrogen biomass. Our results align closely with the findings of Hansen et. al (2002), which showed decreased hopperburn and PLH activity in unsprayed resistant fields compared to sprayed susceptible fields but variable responses in yield and nitrogen content. Interestingly, in this study, unsprayed resistant fields initially showed greater nitrogen content when compared to sprayed susceptible fields but this trend reversed over time and unsprayed resistant fields showed significantly less nitrogen content. Hansen et al. concluded resistant cultivars may reduce visually observable effects of PLH, while

simultaneously exhibiting reduced forage quality relative to sprayed susceptible cultivars. Examining multiple metrics of forage production determined unanticipated differences in the response of cultivars to PLH injury.

We also sought to quantify the contributions of intercropping with fescue to PLH injury across *M. sativa* cultivars. Fescue treatments showed no significant effect on any response variables measured except weed dry weight during the second sampling period. SF and RF fields both benefited from intercropping with fescue late in the growing season in terms of reduced weed pressure. The benefits of intercropping for weed suppression are well-established in the literature (Liebman and Dyck 1993; Hauggaard-Nielsen et al. 2001; Bilalis et al. 2010). Although we were not specifically testing weed suppression in this study, we contend intercropping may offer a useful management tool for *M. sativa* growers struggling with late-season weed growth. Broad-leaf weeds, for instance, can elevate PLH densities in fields and increase damage on *M. sativa* (Oloumi-Sadeghi et al. 1987). Therefore, intercropping with fescue can reciprocally benefit weed and PLH management.

Moreover, we predicted intercropping with fescue would reduce nitrogen losses to PLH injury across cultivars, as grasses repel PLH (Roda et al. 1997) and promote natural enemies (Straub et al. 2013, 2014). Instead we observed decreases in aboveground nitrogen biomass when intercropping with fescue across both cultivars when compared to monoculture fields of the same cultivar. It is interesting to note injured shoots from whole plant samples collected in RF fields showed slightly greater amounts of aboveground nitrogen biomass compared to healthy shoots, as well as comparable amounts to injured RM shoots (Fig 1.4). Reductions in nitrogen

biomass accumulation when intercropping may relate to nitrogen fixation of *M. sativa*.

Intercropping with a nitrogen-fixing crop often results in increased nitrogen transfer to the non-fixing crops (Ledgard et al. 1985; Hauggaard-Nielsen et al. 2009). The goal is often to increase nitrogen content of the non-fixing crop. However, we were uninterested in nitrogen transfer from *M. sativa* to fescue, as fescue was intended only to repel PLH activity. Therefore, competition between *M. sativa* and fescue for nitrogen (or other macro- and micronutrients in the soil) may have resulted in decreased *M. sativa* nitrogen biomass accumulation when grown with fescue (Xie et al. 2015). For instance, sufficient amounts of bioavailable phosphorus are required for nitrogen fixation, as phosphorus fuels the production of ATP, an energy source for nitrogen-fixing microbes (Liu et al. 2018). If fescue roots outcompeted *M. sativa* roots for phosphorus, nitrogen fixation may have been inhibited, diminishing aboveground nitrogen biomass accumulation.

Concurrently, physiological differences between cultivars in responding to PLH injury may have influenced nitrogen transfer between *M. sativa* and fescue. Results from our greenhouse experiment detailed contrasting responses of cultivars in nitrogen fixation across whole plant samples. Although we did not detect any significant differences, we observed decreases in nitrogen fixation of injured susceptible plants compared to healthy susceptible plants, regardless of the nitrogen fertilizer treatment. Contradictorily, injured resistant plants showed increases in nitrogen fixation compared to healthy resistant plants, also irrespective of fertilizer. Increases in nitrogen fixation of resistant plants under PLH pressure may explain our

field results, as injured RF fields maintained comparable levels of nitrogen biomass to healthy RF fields. Additionally, our greenhouse results could also account for field results of decreases in nitrogen biomass accumulation in injured susceptible plants. However, increased nitrogen fixation of resistant plants fails to explain differences between healthy and injured plants in RM fields, as we saw drastic decreases in nitrogen biomass in injured plants.

One possible explanation for the discrepancy between RM and RF fields in nitrogen biomass accumulation may be different amounts of realized PLH feeding damage. We detected similar PLH densities across RM and RF fields, however, densities may not translate into the actual amount of PLH damage occurring on resistant plants. Perhaps plants from RM fields sustained greater amounts of PLH feeding damage, surpassing the amount of PLH damage experienced by RF and greenhouse plants, and altering the plant response in nitrogen fixation. Increased plant diversity increases PLH host-searching behavior, enhancing vulnerability to predators (Straub et al. 2013, 2014) and reducing time spent feeding by PLH (Roda et al. 1997). Thus, similar PLH densities across RM and RF fields may result in varying amounts of PLH injury and cascading effects on nitrogen fixation.

Overall, our study demonstrates benefits of resistant cultivars and varying effects of intercropping with fescue on PLH injury to *M. sativa*. We found increased nitrogen biomass accumulation in resistant cultivars compared to susceptible cultivars, regardless of PLH pressure or fescue addition. Nitrogen biomass relates directly to crude protein content and forage quality of *M. sativa*, and we recommend planting resistant cultivars to growers, regardless of other management practices.

Further research is needed to determine how nitrogen fixation varies in a field setting, particularly across cultivars and intercropping. Our study demonstrates nitrogen fixation varies across *M. sativa* cultivars, which suggests nitrogen transfer to non-fixing crops may also vary depending on the companion plant species. Determining differences across intercropping plant species and *M. sativa* cultivars enhances management strategies to control PLH populations and maintain high forage quality.

Table 1.1 Sweep samples throughout growing season for field study. Numbers represent means +/- standard deviation; SM = Susceptible Monoculture, SF = Susceptible-Fescue, RM = Resistant Monoculture, RF = Resistant-Fescue; DAS = Days After Sampling; June and July sampling periods coincided with sweep samples 35 DAS; Adult Density = Adults Per Sweep, Nymph Density = Nymphs Per Sweep, Total Density = Adults and Nymphs Per Sweep

Growth Period	Densities	SM	SF	RM	RF
11 DAS	Adult	0.30 ± 0.12	0.40 ± 0.14	0.13 ± 0.13	0.33 ± 0.15
1-Jun-18	Nymph	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Total	0.30 ± 0.12	0.40 ± 0.14	0.13 ± 0.13	0.33 ± 0.15
15 DAS	Adult	0.68 ± 0.25	0.48 ± 0.33	0.55 ± 0.35	0.63 ± 0.22
5-Jun-18	Nymph	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Total	0.68 ± 0.25	0.48 ± 0.33	0.55 ± 0.35	0.63 ± 0.22
22 DAS	Adult	3.40 ± 0.50	2.78 ± 1.14	2.25 ± 1.12	2.05 ± 1.15
12-Jun-18	Nymph	0.10 ± 0.16	0.43 ± 0.43	0.18 ± 0.17	0.10 ± 0.2
	Total	3.50 ± 0.55	3.20 ± 1.17	2.43 ± 1.12	2.15 ± 1.15
30 DAS	Adult	8.40 ± 1.48	6.75 ± 3.16	1.20 ± 0.91	1.80 ± 0.59
20-Jun-18	Nymph	8.80 ± 3.63	7.55 ± 3.68	1.85 ± 1.25	1.30 ± 0.48
	Total	17.20 ± 4.20	14.30 ± 5.86	3.05 ± 1.86	3.10 ± 0.35
35 DAS	Adult	6.10 ± 0.74	5.10 ± 2.16	2.95 ± 1.80	1.85 ± 0.57
25-Jun-18	Nymph	6.65 ± 0.44	4.35 ± 3.56	2.15 ± 0.81	1.85 ± 0.81
(June Sampling)	Total	12.75 ± 0.93	9.45 ± 5.66	5.10 ± 1.05	3.70 ± 1.16
15 DAS	Adult	1.00 ± 0.28	0.55 ± 0.53	0.75 ± 0.47	0.25 ± 0.25
10-Jul-18	Nymph	0.15 ± 0.19	0.10 ± 0.12	0.10 ± 0.20	0.00 ± 0.00
	Total	1.15 ± 0.10	0.65 ± 0.53	0.85 ± 0.53	0.25 ± 0.25
22 DAS	Adult	0.75 ± 0.50	0.80 ± 0.37	0.15 ± 0.19	0.20 ± 0.28
17-Jul-18	Nymph	0.30 ± 0.26	0.40 ± 0.28	0.25 ± 0.30	0.40 ± 0.23
	Total	1.05 ± 0.74	1.20 ± 0.33	0.40 ± 0.37	0.60 ± 0.43
35 DAS	Adult	1.85 ± 1.40	1.85 ± 2.29	0.50 ± 0.38	0.40 ± 0.43
30-Jul-18	Nymph	1.80 ± 1.70	1.85 ± 1.34	0.15 ± 0.19	0.35 ± 0.57
(July Sampling)	Total	3.65 ± 3.00	3.70 ± 3.54	0.65 ± 0.44	0.75 ± 0.97

Table 1.2 Repeated measures two-way ANOVA results for sweep samples of unsprayed subplots from the first sampling period (1-Jun-18 through 25-Jun-18) and second sampling period (10-Jul-18 through 30-Jul-18). Adult Density = Adults Per Sweep, Nymph Density = Nymphs Per Sweep, Total Density = Adults and Nymphs Per Sweep

		June – First Sampling Period					*July – Second Sampling Period*				
Parameter	**Source**	**df**	**SS**	**MS**	**F value**	**p-value**	**df**	**SS**	**MS**	**F value**	**p-value**
Adult Density	Residuals (Date)	1	217.50	217.50			1	2.76	2.76		
	Cultivar	1	85.28	85.28	29.70	<0.001	1	6.90	6.90	9.72	0.003
	Fescue	1	2.89	2.89	1.00	0.32	1	0.30	0.30	0.42	0.52
	Cultivar x Fescue	1	1.74	1.74	0.61	0.44	1	0.008	0.008	0.011	0.92
	Residuals (Within)	75	215.38	2.87			43	30.53	0.71		
Nymph Density	Residuals (Date)	1	271.10	271.10			1	7.69	7.69		
	Cultivar	1	83.60	83.64	17.23	<0.001	1	3.74	3.74	7.07	0.01
	Fescue	1	3.40	3.44	0.71	0.40	1	0.041	0.041	0.08	0.78
	Cultivar x Fescue	1	1.10	1.06	0.22	0.64	1	0.007	0.007	0.01	0.91
	Residuals (Within)	75	364.1	4.85			43	22.75	0.53		
Total Density	Residuals (Date)	1	974.40	974.40			1	19.67	19.67		
	Cultivar	1	337.80	337.80	26.74	<0.001	1	20.8	20.80	9.66	0.003
	Fescue	1	12.60	12.60	1.00	0.32	1	0.12	0.12	0.06	0.81
	Cultivar x Fescue	1	5.50	5.50	0.44	0.51	1	0.00	0.00	0.00	1.00
	Residuals (Within)	75	947.70	12.60			43	92.61	2.154		

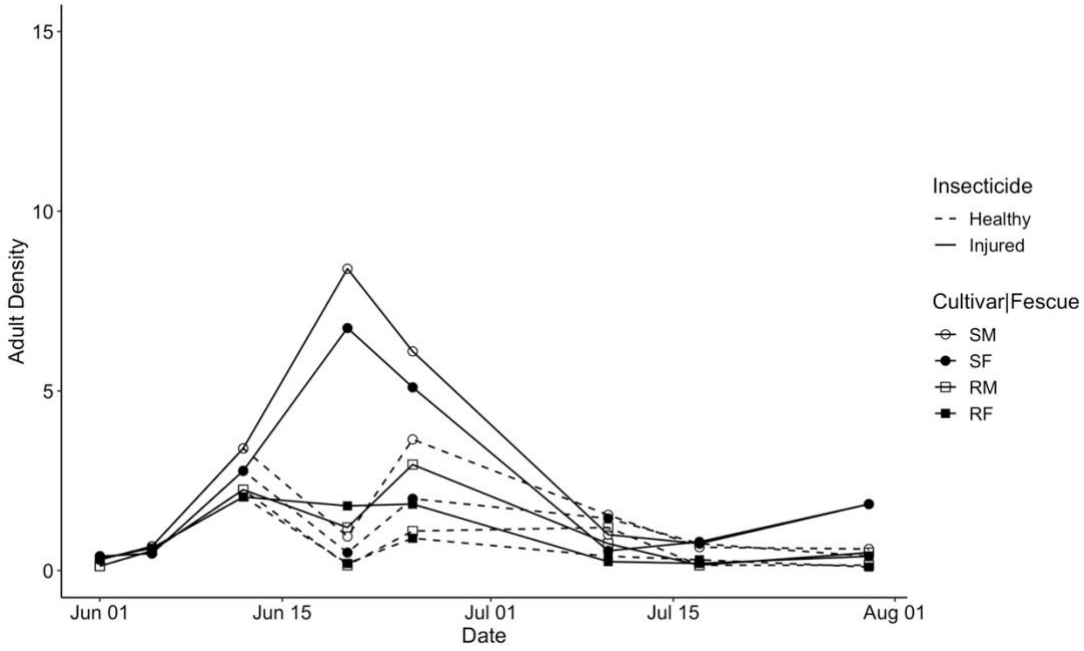

Figure 1.1 Adult densities (measured as adults per sweep) across the entire growing season of 2019 for the field study. SM = Susceptible Monoculture, SF = Susceptible-Fescue, RM = Resistant Monoculture, RF = Resistant-Fescue; Healthy = Insecticide Sprayed, Injured = No Insecticide Sprayed; Sampling Periods = June 26, July 31

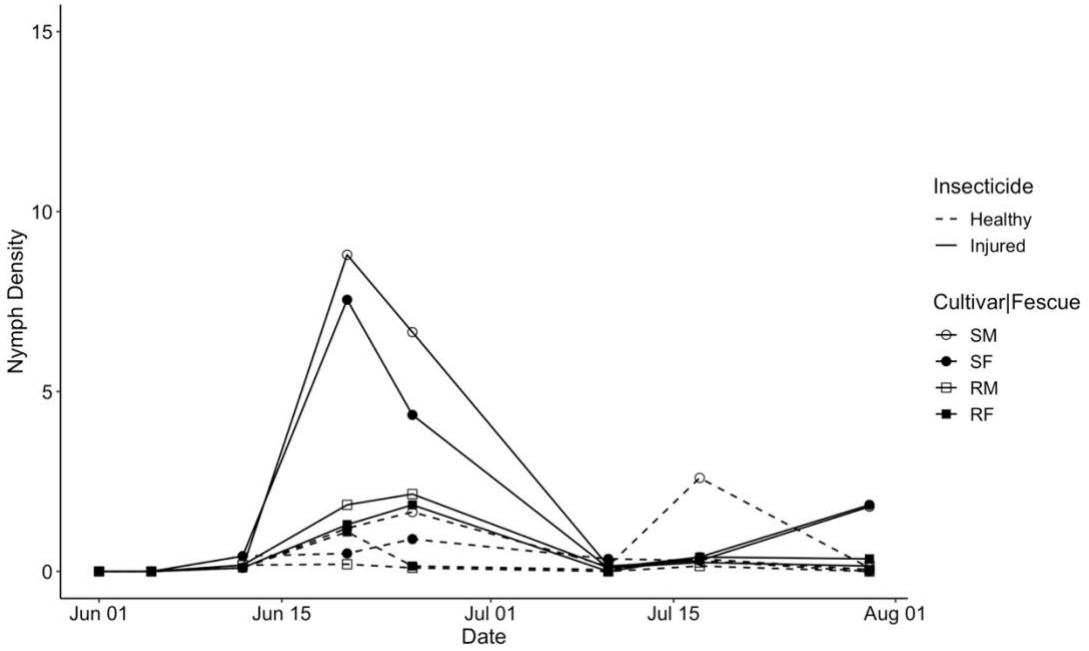

Figure 1.2 Nymph densities (measured as nymphs per sweep) across the entire growing season of 2019 for the field study. SM = Susceptible Monoculture, SF = Susceptible-Fescue, RM = Resistant Monoculture, RF = Resistant-Fescue; Healthy = Insecticide Sprayed, Injured = No Insecticide Sprayed; Sampling Periods = May 22, June 26, July 31

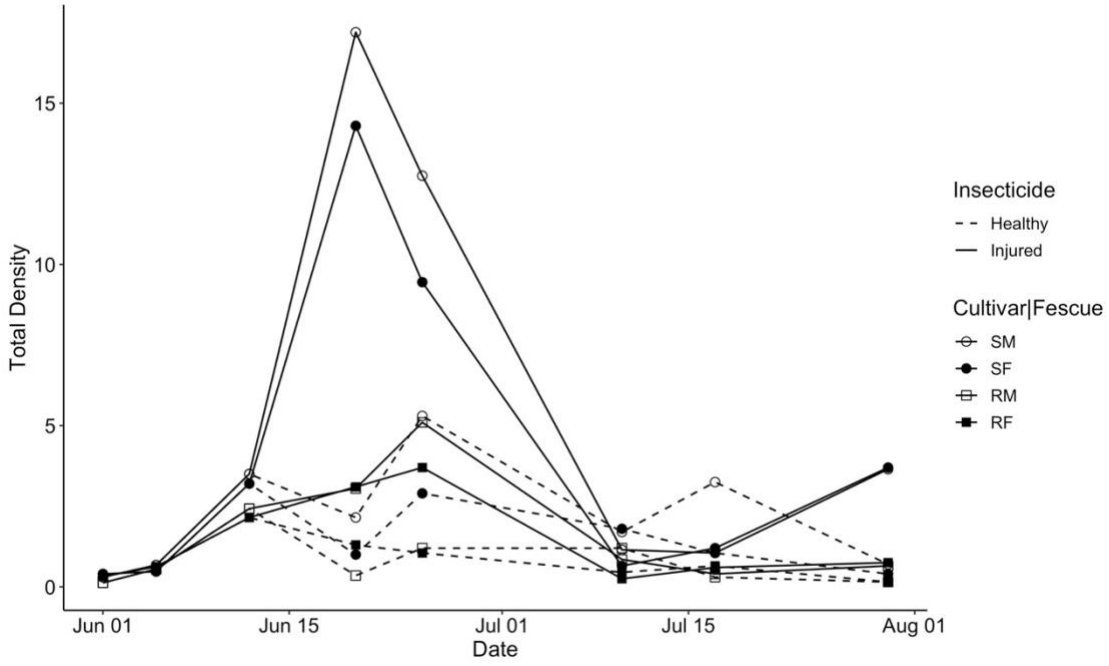

Figure 1.3 Total densities (measured as adults and nymphs per sweep) across the entire growing season of 2019 for the field study. SM = Susceptible Monoculture, SF = Susceptible-Fescue, RM = Resistant Monoculture, RF = Resistant-Fescue; Healthy = Insecticide Sprayed, Injured = No Insecticide Sprayed; Sampling Periods = June 26, July 31

Table 1.3 Foliar samples from first and second sampling periods for the field study collected on June 26, 2018 and July 31, 2018, respectively. Numbers represent means +/- standard deviation; Healthy = Insecticide Sprayed, Injured = No Insecticide Sprayed

	Susceptible				**Resistant**			
	Monoculture		*Fescue*		*Monoculture*		*Fescue*	
	Healthy	Injured	Healthy	Injured	Healthy	Injured	Healthy	Injured
June – First Sampling Period								
Total Biomass Dry Weight (g)	21.7 ± 3.7	18.2 ± 2.5	21.5 ± 4.6	21.3 ± 3.4	23.7 ± 4.2	24.1 ± 6.8	28.6 ± 8.7	24.4 ± 3.7
Alfalfa Dry Weight (g)	20.4 ± 4.8	16.2 ± 3.3	16.3 ± 3.4	15.0 ± 4.4	22.4 ± 5.4	23.4 ± 7.2	23.7 ± 9.0	19.6 ± 3.2
Fescue Dry Weight (g)	N/A	N/A	4.7 ± 2.1	6.0 ± 1.3	N/A	N/A	4.7 ± 0.9	4.5 ± 1.2
Weed Dry Weight (g)	1.2 ± 1.3	2.0 ± 3.1	0.4 ± 0.2	0.3 ± 0.2	1.3 ± 1.5	0.9 ± 1.3	0.3 ± 0.2	0.4 ± 0.2
July – Second Sampling Period								
Total Biomass Dry Weight (g)	15.9 ± 2.2	16.0 ± 2.5	18.9 ± 2.0	16.7 ± 1.7	22.1 ± 3.2	20.5 ± 3.5	20.2 ± 6.1	17.6 ± 3.3
Alfalfa Dry Weight (g)	11.1 ± 4.8	11.1 ± 5.1	13.0 ± 3.7	9.4 ± 3.3	18.5 ± 6.1	18.1 ± 5.4	16.8 ± 6.0	12.5 ± 4.6
Fescue Dry Weight (g)	N/A	N/A	5.3 ± 2.7	5.3 ± 1.9	N/A	N/A	2.9 ± 0.5	4.1 ± 2.0
Weed Dry Weight (g)	4.8 ± 2.7	4.9 ± 3.5	0.9 ± 0.3	2.0 ± 1.5	4.8 ± 3.1	2.4 ± 2.1	0.6 ± 0.3	1.3 ± 1.3

Table 1.4 Split plot ANOVA (2 main plot factors, 1 subplot factor) results for foliar samples from first and second sampling periods for the field study collected on June 26, 2018 and July 31, 2018, respectively.

			June – First Sampling Period				*July – Second Sampling Period*			
Parameter	**Source**	**df**	**SS**	**MS**	**F value**	**p-value**	**SS**	**MS**	**F value**	**p-value**
Total Biomass Dry Weight (grams)	*Main Effects*									
	Cultivar	1	165.15	165.15	6.39	0.03	83.75	83.75	4.28	0.06
	Fescue	1	32.68	32.68	1.27	0.28	0.63	0.63	0.03	0.86
	Cultivar x Fescue	1	2.80	2.80	0.11	0.75	36.73	36.73	1.88	0.20
	Residuals	12	310.00	25.83			234.92	19.58		
	Subplot Effects									
	Insecticide	1	27.43	27.43	1.09	0.32	20.26	20.26	8.00	0.02
	Cultivar x Insecticide	1	0.01	0.01	0.00	0.99	2.30	2.30	0.91	0.36
	Fescue x Insecticide	1	0.74	0.74	0.03	0.87	5.79	5.79	2.29	0.16
	Cultivar x Fescue x Insecticide	1	29.92	29.93	1.19	0.30	0.80	0.80	0.31	0.59
	Residuals	12	301.61	25.13			30.39	2.53		
Alfalfa Dry Weight (grams)	*Main Effects*									
	Cultivar	1	222.30	222.33	6.19	0.03	228.10	228.12	5.67	0.03
	Fescue	1	30.60	30.64	0.85	0.37	25.40	25.42	0.63	0.44
	Cultivar x Fescue	1	3.80	3.83	0.11	0.75	28.70	28.65	0.71	0.42
	Residuals	12	431.40	35.95			482.80	40.23		
	Subplot Effects									
	Insecticide	1	37.65	37.65	1.61	0.23	35.47	35.47	3.93	0.07
	Cultivar x Insecticide	1	2.76	2.76	0.12	0.74	0.80	0.80	0.09	0.77
	Fescue x Insecticide	1	2.40	2.40	0.10	0.75	28.13	28.13	3.12	0.10
	Cultivar x Fescue x Insecticide	1	32.31	32.31	1.38	0.26	0.06	0.06	0.01	0.94
	Residuals	12	280.51	23.38			108.36	9.03		
Fescue Dry Weight (grams)	*Main Effects*									
	Cultivar	1	1.23	1.23	1.18	0.32	6.29	6.29	2.00	0.18
	Fescue	1	197.34	197.34	188.70	<0.001	155.29	155.29	49.44	<0.001
	Cultivar x Fescue	1	1.23	1.23	1.18	0.30	6.29	6.29	2.00	0.18
	Residuals	12	12.55	1.05			37.69	3.14		
	Subplot Effects									
	Insecticide	1	0.60	0.60	0.56	0.49	0.80	0.80	1.40	0.26
	Cultivar x Insecticide	1	0.95	0.95	0.88	0.37	0.69	0.69	1.21	0.29
	Fescue x Insecticide	1	0.60	0.60	0.56	0.47	0.80	0.80	1.40	0.26
	Cultivar x Fescue x Insecticide	1	0.95	0.95	0.88	0.37	0.69	0.69	1.21	0.29
	Residuals	12	12.95	1.08			6.83	0.57		
Weed Dry Weight (grams)	*Main Effects*									
	Cultivar	1	0.90	0.90	0.34	0.57	11.86	11.86	1.67	0.22
	Fescue	1	7.82	7.82	2.94	0.11	67.44	67.44	9.50	0.009
	Cultivar x Fescue	1	0.68	0.68	0.26	0.62	3.24	3.24	0.46	0.51
	Residuals	12	31.86	2.66			85.17	7.10		
	Subplot Effects									
	Insecticide	1	0.02	0.02	0.02	0.91	0.32	0.32	0.10	0.75
	Cultivar x Insecticide	1	0.58	0.58	0.56	0.47	2.10	2.10	0.70	0.42
	Fescue x Insecticide	1	0.01	0.01	0.01	0.94	4.01	4.01	1.33	0.27
	Cultivar x Fescue x Insecticide	1	1.41	1.41	1.34	0.27	0.10	0.10	0.03	0.86
	Residuals	12	12.62	1.05			36.33	3.03		

Table 1.5 Foliar samples from first and second sampling periods for the field study collected on June 26, 2018 and July 31, 2018, respectively. Numbers represent means +/- standard deviation; Healthy = Insecticide Sprayed, Injured = No Insecticide Sprayed

	Susceptible				**Resistant**			
	Monoculture		*Fescue*		*Monoculture*		*Fescue*	
	Healthy	Injured	Healthy	Injured	Healthy	Injured	Healthy	Injured
June – First Sampling Period								
Nitrogen (%)	3.7 ± 0.1	3.1 ± 0.3	3.7 ± 0.1	3.2 ± 0.1	3.9 ± 0.2	3.6 ± 0.1	3.9 ± 0.2	3.5 ± 0.3
Nitrogen Biomass (grams of N)	0.8 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.9 ± 0.3	0.8 ± 0.2	0.9 ± 0.4	0.7 ± 0.1
July – Second Sampling Period								
Nitrogen (%)	4.3 ± 0.4	4.0 ± 0.3	4.1 ± 0.3	4.1 ± 0.3	4.4 ± 0.2	4.2 ± 0.3	4.2 ± 0.3	4.2 ± 0.4
Nitrogen Biomass (grams of N)	0.5 ± 0.3	0.4 ± 0.2	0.5 ± 0.2	0.4 ± 0.2	0.8 ± 0.3	0.8 ± 0.3	0.7 ± 0.3	0.5 ± 0.2

Table 1.6 Split plot ANOVA (2 main plot factors, 1 subplot factor) results for foliar samples from first and second sampling periods for the field study collected on June 26, 2018 and July 31, 2018, respectively.

			June – First Sampling Period				*July – Second Sampling Period*			
Parameter	**Source**	**df**	**SS**	**MS**	**F value**	**p-value**	**SS**	**MS**	**F value**	**p-value**
Nitrogen (%)	*Main Effects*									
	Cultivar	1	0.62	0.62	20.85	<0.001	0.15	0.15	0.85	0.37
	Fescue	1	0.01	0.01	0.19	0.67	0.04	0.04	0.24	0.63
	Cultivar x Fescue	1	0.03	0.03	0.98	0.34	0.0005	0.0005	0.003	0.96
	Residuals	12	0.36	0.03			2.11	0.18		
	Subplot Effects									
	Insecticide	1	1.45	1.45	34.86	<0.001	0.14	0.14	10.12	0.008
	Cultivar x Insecticide	1	0.08	0.08	1.93	0.19	0.01	0.01	0.45	0.52
	Fescue x Insecticide	1	0.0001	0.0001	0.003	0.95	0.12	0.12	8.82	0.01
	Cultivar x Fescue x Insecticide	1	0.01	0.01	0.22	0.65	0.03	0.03	2.20	0.16
	Residuals	12	0.50	0.04	1.93	0.19	0.17	0.01		
Nitrogen Biomass (g of N)	*Main Effects*									
	Cultivar	1	0.47	0.47	8.67	0.01	0.48	0.48	5.24	0.04
	Fescue	1	0.05	0.05	0.87	0.37	0.07	0.07	0.73	0.41
	Cultivar x Fescue	1	0.002	0.002	0.03	0.86	0.05	0.05	0.56	0.47
	Residuals	12	0.65	0.05			1.09	0.09		
	Subplot Effects									
	Insecticide	1	0.21	0.21	6.54	0.03	0.09	0.09	0.11	0.75
	Cultivar x Insecticide	1	0.004	0.004	0.12	0.74	0.001	0.001	0.07	0.80
	Fescue x Insecticide	1	0.004	0.004	0.11	0.74	0.03	0.03	1.64	0.23
	Cultivar x Fescue x Insecticide	1	0.05	0.05	1.55	0.24	0.002	0.002	0.11	0.75
	Residuals	12	0.39	0.03	0.12	0.74	0.23	0.02		

Table 1.7 Whole plant samples collected on June 26, 2018 for the field study. Numbers represent means +/- standard deviation; Healthy = Insecticide Sprayed, Injured = No Insecticide Sprayed

	Susceptible				**Resistant**			
	Monoculture		*Fescue*		*Monoculture*		*Fescue*	
	Healthy	Injured	Healthy	Injured	Healthy	Injured	Healthy	Injured
Shoots								
Dry Weight (grams)	6.7 ± 4.5	3.6 ± 0.7	4.7 ± 2.0	3.1 ± 1.0	10.2 ± 3.5	7.9 ± 2.1	6.0 ± 1.4	7.2 ± 3.5
Nitrogen (%)	3.6 ± 0.3	3.1 ± 0.1	3.8 ± 0.1	3.1 ± 0.1	3.7 ± 0.1	3.4 ± 0.3	3.6 ± 0.2	3.6 ± 0.2
Nitrogen Biomass (g of N)	0.2 ± 0.2	0.1 ± 0.02	0.2 ± 0.1	0.1 ± 0.03	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
Crowns								
Dry Weight (grams)	2.1 ± 0.6	1.7 ± 0.6	2.0 ± 0.6	1.2 ± 0.5	4.1 ± 1.4	3.1 ± 0.7	3.5 ± 1.7	2.8 ± 1.3
Nitrogen (%)	1.9 ± 0.2	2.0 ± 0.3	1.9 ± 0.3	2.2 ± 0.1	1.7 ± 0.1	1.7 ± 0.2	1.8 ± 0.1	1.9 ± 0.1
Nitrogen Biomass (g of N)	0.04 ± 0.01	0.03 ± 0.02	0.04 ± 0.02	0.03 ± 0.01	0.07 ± 0.02	0.05 ± 0.01	0.06 ± 0.03	0.05 ± 0.03
Roots								
Dry Weight (grams)	2.4 ± 0.3	2.1 ± 0.6	2.8 ± 0.9	1.9 ± 0.5	4.7 ± 0.7	3.6 ± 0.7	3.9 ± 1.2	3.9 ± 1.6
Nitrogen (%)	1.9 ± 0.4	2.2 ± 0.3	1.9 ± 0.2	2.1 ± 0.5	2.1 ± 0.2	2.3 ± 0.1	2.0 ± 0.4	2.1 ± 0.2
Nitrogen Biomass (g of N)	0.05 ± 0.02	0.05 ± 0.01	0.05 ± 0.02	0.04 ± 0.02	0.10 ± 0.02	0.08 ± 0.01	0.08 ± 0.02	0.08 ± 0.03

Table 1.8 Split plot ANOVA (2 main plot factors, 1 subplot factor) results for whole plant samples collected on June 26, 2018 for the field study.

			Shoots				*Crown*				*Roots*			
Parameter	**Source**	**df**	**SS**	**MS**	**F value**	**p-value**	**SS**	**MS**	**F value**	**p-value**	**SS**	**MS**	**F value**	**p-value**
Dry Weight (grams)	*Main Effects*													
	Cultivar	1	87.91	87.91	11.11	0.006	20.26	20.26	15.67	0.002	23.32	23.32	17.97	0.001
	Fescue	1	26.54	26.54	3.35	0.09	1.13	1.13	0.88	0.37	0.05	0.05	0.04	0.85
	Cultivar x Fescue	1	2.59	2.59	0.33	0.58	0.01	0.01	0.01	0.92	0.19	0.19	0.15	0.71
	Residuals	12	94.97	7.91			15.51	1.29			15.58	1.30		
	Subplot Effects													
	Insecticide	1	16.30	16.30	2.69	0.13	4.15	4.15	5.16	0.04	2.92	2.92	9.12	0.01
	Cultivar x Insecticide	1	6.12	6.13	1.01	0.33	0.09	0.09	0.11	0.75	0.02	0.02	0.07	0.79
	Fescue x Insecticide	1	12.48	12.48	2.06	0.18	0.00	0.00	0.00	0.98	0.14	0.14	0.44	0.52
	Cultivar x Fescue x Insecticide	1	1.81	1.82	0.30	0.59	0.34	0.34	0.42	0.53	1.71	1.71	5.35	0.04
	Residuals	12	72.67	6.06			9.64	0.80			3.84	0.32		
Nitrogen (%)	*Main Effects*													
	Cultivar	1	0.19	0.19	4.77	0.04	0.36	0.36	8.35	0.01	0.05	0.05	0.39	0.54
	Fescue	1	0.02	0.02	0.51	0.49	0.12	0.12	2.79	0.12	0.10	0.10	0.75	0.40
	Cultivar x Fescue	1	0.001	0.001	0.02	0.89	0.002	0.002	0.04	0.85	0.002	0.002	0.01	0.91
	Residuals	12	0.47	0.04			0.52	0.04			1.55	0.13		
	Subplot Effects													
	Insecticide	1	1.04	1.04	33.67	<0.001	0.12	0.12	8.02	0.02	0.27	0.27	3.68	0.08
	Cultivar x Insecticide	1	0.43	0.43	14.00	0.003	0.02	0.02	1.08	0.32	0.04	0.04	0.52	0.48
	Fescue x Insecticide	1	0.02	0.02	0.51	0.49	0.02	0.02	1.22	0.29	0.02	0.02	0.29	0.60
	Cultivar x Fescue x Insecticide	1	0.10	0.10	3.14	0.10	0.02	0.02	1.20	0.29	0.001	0.001	0.01	0.93
	Residuals	12	0.37	0.03			0.18	0.02			0.89	0.07		
Nitrogen Biomass (g of N)	*Main Effects*													
	Cultivar	1	0.12	0.12	10.82	0.006	0.005	0.005	8.95	0.01	0.01	0.01	16.72	0.002
	Fescue	1	0.03	0.03	2.92	0.11	0.00008	0.00008	0.16	0.69	0.0002	0.0002	0.35	0.56
	Cultivar x Fescue	1	0.004	0.004	0.33	0.58	0.00002	0.00002	0.04	0.86	0.0002	0.0002	0.37	0.56
	Residuals	12	0.13	0.01			0.006	0.001			0.008	0.001		
	Subplot Effects													
	Insecticide	1	0.04	0.04	4.81	0.04	0.0009	0.0009	3.69	0.08	0.0005	0.0005	2.34	0.15
	Cultivar x Insecticide	1	0.01	0.01	1.21	0.29	0.00001	0.00001	0.04	0.85	0.00001	0.00001	0.01	0.94
	Fescue x Insecticide	1	0.02	0.02	2.57	0.14	0.00001	0.00001	0.01	0.93	0.00005	0.00005	0.24	0.64
	Cultivar x Fescue x Insecticide	1	0.005	0.005	0.62	0.45	0.0001	0.0001	0.28	0.61	0.0006	0.0006	3.19	0.10
	Residuals	12	0.09	0.01			0.003	0.0003			0.002	0.0002		

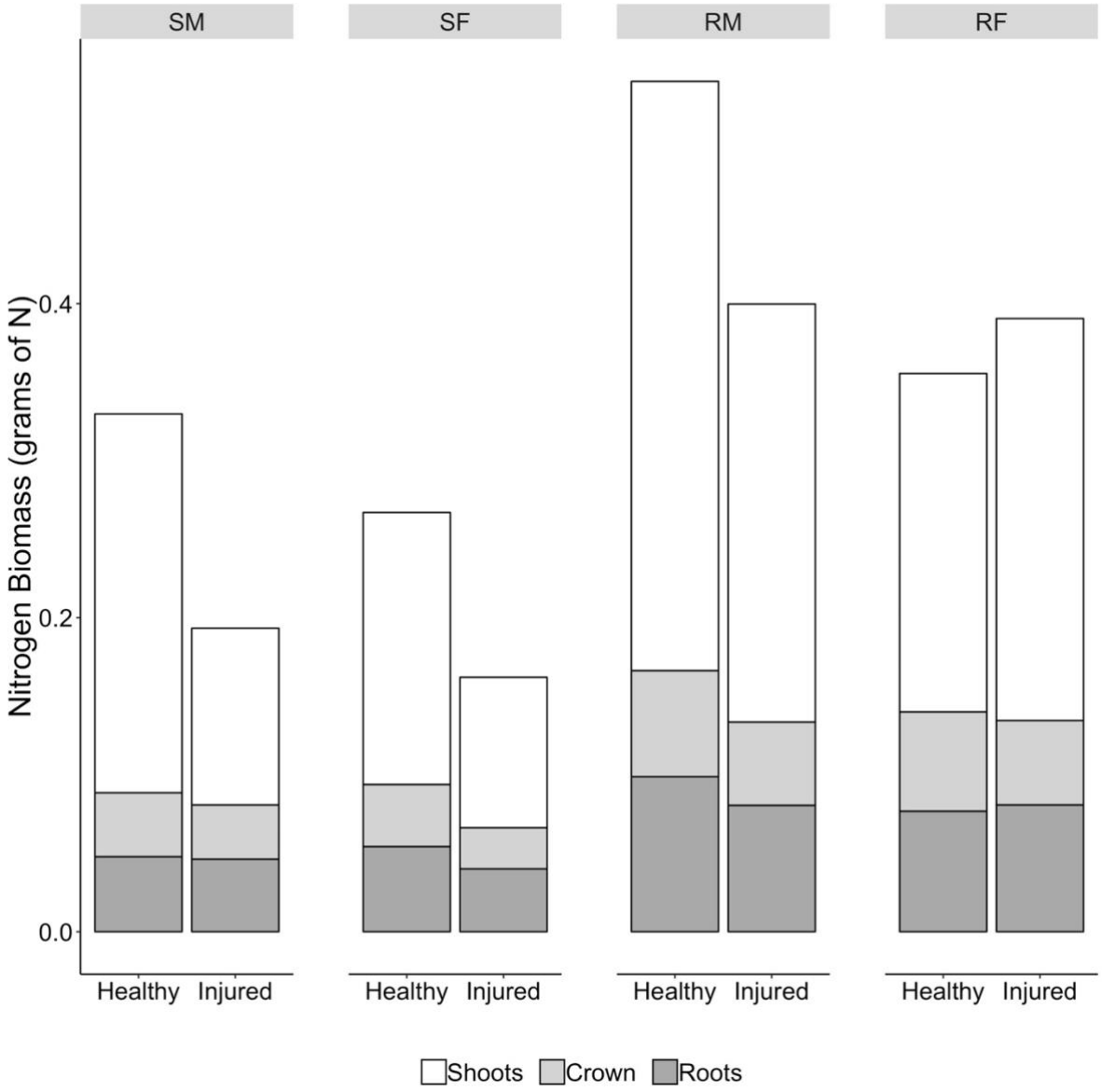

Figure 1.4 Nitrogen biomass (grams of nitrogen) allocation across whole plant samples. SM = Susceptible Monoculture, SF = Susceptible-Fescue, RM = Resistant Monoculture, RF = Resistant-Fescue; Healthy = Insecticide Sprayed, Injured = No Insecticide Sprayed; SM Healthy – Injured Shoots p-value = 0.0736, Crown p-value = 0.658, Roots p-value = 0.919; SF Healthy – Injured Shoots p-value = 0.271, Crown p-value = 0.351, Roots p-value = 0.339; RM Healthy – Injured Shoots p-value = 0.126, Crown p-value = 0.308, Roots p-value = 0.223; RF Healthy – Injured Shoots p-value = 0.562, Crown p-value = 0.502, Roots p-value = 0.786

Table 1.9 Whole plant samples for greenhouse study. Numbers represent means +/- standard deviation; Healthy = No PLH Added, Injured = PLH Added

	Susceptible				**Resistant**			
	No Nitrogen Added		*Nitrogen Added*		*No Nitrogen Added*		*Nitrogen Added*	
	Healthy	Injured	Healthy	Injured	Healthy	Injured	Healthy	Injured
Shoots								
Dry Weight (g)	4.1 ± 1.1	3.7 ± 1.5	3.7 ± 1.1	4.2 ± 1.7	3.4 ± 1.4	4.7 ± 1.4	4.3 ± 0.9	5.1 ± 1.7
Nitrogen (%)	3.7 ± 0.3	3.5 ± 0.4	4.0 ± 0.2	3.4 ± 0.2	3.9 ± 0.3	3.6 ± 0.3	3.7 ± 0.4	3.6 ± 0.4
Nitrogen Biomass (g of N)	0.15 ± 0.03	0.13 ± 0.05	0.15 ± 0.04	0.14 ± 0.06	0.13 ± 0.05	0.17 ± 0.05	0.16 ± 0.03	0.18 ± 0.05
$\delta^{15}N$ (‰)	1.3 ± 1.32	3.7 ± 6.12	847.2 ± 304.9	987.9 ± 255.9	1.2 ± 0.9	0.7 ± 0.8	1110.8 ± 320.1	891.7 ± 294.0
Crowns								
Dry Weight (g)	1.26 ± 0.91	1.25 ± 0.69	1.60 ± 0.59	1.39 ± 0.62	1.26 ± 0.76	1.72 ± 1.06	2.06 ± 0.71	2.30 ± 1.06
Nitrogen (%)	2.34 ± 0.31	2.09 ± 0.67	2.31 ± 0.15	1.95 ± 0.84	2.01 ± 0.83	2.30 ± 0.31	2.13 ± 0.39	2.26 ± 0.20
Nitrogen Biomass (g of N)	0.028 ± 0.020	0.028 ± 0.018	0.037 ± 0.013	0.026 ± 0.017	0.027 ± 0.021	0.039 ± 0.022	0.044 ± 0.018	0.051 ± 0.020
$\delta^{15}N$ (‰)	16.76 ± 15.11	5.92 ± 3.11	461.77 ± 186.79	448.92 ± 212.41	4.76 ± 5.59	3.29 ± 1.90	568.70 ± 173.18	519.65 ± 222.06
Roots								
Dry Weight (g)	2.39 ± 1.26	2.40 ± 1.64	2.44 ± 1.17	2.95 ± 1.60	2.76 ± 1.23	2.71 ± 1.31	3.53 ± 1.48	2.64 ± 1.24
Nitrogen (%)	2.39 ± 0.24	2.35 ± 0.43	2.19 ± 0.32	2.19 ± 0.40	2.30 ± 0.58	2.60 ± 0.26	2.26 ± 0.49	2.43 ± 0.36
Nitrogen Biomass (g of N)	0.055 ± 0.026	0.054 ± 0.031	0.052 ± 0.025	0.061 ± 0.033	0.059 ± 0.021	0.069 ± 0.029	0.074 ± 0.017	0.062 ± 0.024
$\delta^{15}N$ (‰)	28.91 ± 28.69	20.21 ± 14.68	1338.01 ± 429.96	1475.98 ± 623.50	10.79 ± 4.01	13.58 ± 7.13	1436.18 ± 312.44	1821.61 ± 488.49

Table 1.10 Three-way ANOVA results for whole plant samples from the greenhouse study.

		Shoots					*Crowns*					*Roots*			
Parameter	**Source**	**df**	**SS**	**MS**	**F value**	**p-value**	**df**	**SS**	**MS**	**F value**	**p-value**	**df**	**SS**	**MS**	**F value**	**p-value**
Dry Weight (grams)	Block	7	60.55	8.65	9.75	<0.001	7	10.65	1.52	2.75	0.02	7	51.77	7.40	6.67	<0.001
	Cultivar	1	3.80	3.80	4.28	0.04	1	4.22	4.22	7.63	0.01	1	2.15	2.15	1.94	0.17
	PLH	1	4.77	4.77	5.38	0.02	1	0.07	0.07	0.12	0.73	1	0.17	0.17	0.15	0.70
	Nitrogen	1	1.91	1.91	2.15	0.15	1	2.78	2.78	5.04	0.03	1	1.67	1.67	1.51	0.23
	Cultivar*PLH	1	4.51	4.51	5.08	0.03	1	0.95	0.95	1.72	0.20	1	2.15	2.15	1.94	0.17
	Cultivar*Nitrogen	1	1.28	1.28	1.44	0.24	1	0.98	0.98	1.78	0.19	1	0.01	0.01	0.01	0.92
	PLH*Nitrogen	1	0.17	0.17	0.19	0.66	1	0.29	0.29	0.52	0.47	1	0.11	0.11	0.10	0.75
	Cultivar*PLH*Nitrogen	1	1.82	1.82	2.05	0.16	1	0.02	0.02	0.03	0.86	1	1.74	1.74	1.57	0.22
	Residuals	49	43.47	0.89			47	25.99	0.55			49	54.30	1.11		
Nitrogen (%)	Block	7	1.46	0.21	2.48	0.03	7	1.94	0.28	2.69	0.02	7	3.30	0.47	4.11	<0.001
	Cultivar	1	0.02	0.02	0.27	0.60	1	0.01	0.01	0.10	0.76	1	0.22	0.22	1.96	0.17
	PLH	1	1.35	1.35	16.06	<0.001	1	0.03	0.03	0.31	0.58	1	0.18	0.18	1.53	0.22
	Nitrogen	1	0.00	0.00	0.01	0.94	1	0.04	0.04	0.40	0.53	1	0.32	0.32	2.82	0.10
	Cultivar*PLH	1	0.13	0.13	1.49	0.23	1	0.34	0.34	3.29	0.08	1	0.27	0.27	2.33	0.13
	Cultivar*Nitrogen	1	0.09	0.09	1.12	0.30	1	0.08	0.08	0.81	0.37	1	0.02	0.02	0.21	0.65
	PLH*Nitrogen	1	0.05	0.05	0.54	0.46	1	0.04	0.04	0.34	0.56	1	0.01	0.01	0.07	0.79
	Cultivar*PLH*Nitrogen	1	0.37	0.37	4.45	0.04	1	0.001	0.001	0.01	0.91	1	0.03	0.03	0.22	0.64
	Residuals	49	4.11	0.08			47	4.83	0.10			49	5.62	0.11		
Nitrogen Biomass (g of N)	Block	7	0.07	0.010	10.10	<0.001	7	0.005	0.001	2.46	0.03	7	0.02	0.00	8.48	<0.001
	Cultivar	1	0.005	0.005	4.94	0.03	1	0.002	0.002	6.64	0.01	1	0.002	0.002	4.97	0.03
	PLH	1	0.001	0.001	1.10	0.30	1	0.00003	0.00003	0.12	0.73	1	0.0001	0.0001	0.06	0.81
	Nitrogen	1	0.002	0.002	1.89	0.17	1	0.001	0.001	3.99	0.05	1	0.0002	0.0002	0.44	0.51
	Cultivar*PLH	1	0.007	0.007	7.43	0.009	1	0.001	0.001	2.10	0.15	1	0.0001	0.0001	0.32	0.57
	Cultivar*Nitrogen	1	0.001	0.001	0.89	0.35	1	0.0003	0.0003	1.07	0.31	1	0.0001	0.0001	0.06	0.81
	PLH*Nitrogen	1	0.00001	0.00001	0.01	0.93	1	0.0001	0.0001	0.48	0.49	1	0.0001	0.0001	0.35	0.56
	Cultivar*PLH*Nitrogen	1	0.001	0.001	0.71	0.40	1	0.00003	0.00003	0.12	0.73	1	0.001	0.001	2.80	0.10
	Residuals	49	0.05	0.001			47	0.013	0.0003			49	0.02	0.0004		
$\delta^{15}N$ (‰)	Block	7	510409	72916	1.86	0.10	7	117327	16761	1.03	0.42	7	957605	136801	1.24	0.30
	Cultivar	1	27035	27035	0.69	0.41	1	28601	28601	1.76	0.19	1	175612	175612	1.59	0.21
	PLH	1	5837	5837	0.15	0.70	1	7226	7226	0.45	0.51	1	267813	267813	2.42	0.13
	Nitrogen	1	14674033	14674033	374.29	<0.001	1	3978590	3978590	244.97	<0.001	1	35979408	35979408	325.32	<0.001
	Cultivar*PLH	1	131515	131515	3.35	0.07	1	9657	9657	0.60	0.45	1	67056	67056	0.61	0.44
	Cultivar*Nitrogen	1	29046	29046	0.74	0.39	1	14722	14722	0.91	0.35	1	219538	219538	1.99	0.17
	PLH*Nitrogen	1	6440	6440	0.16	0.69	1	378	378	0.02	0.88	1	280168	280168	2.53	0.12
	Cultivar*PLH*Nitrogen	1	127306	127306	3.25	0.08	1	12927	12927	0.80	0.38	1	55681	55681	0.50	0.48
	Residuals	49	1921029	39205			47	763329	16241			49	5419200	110596		

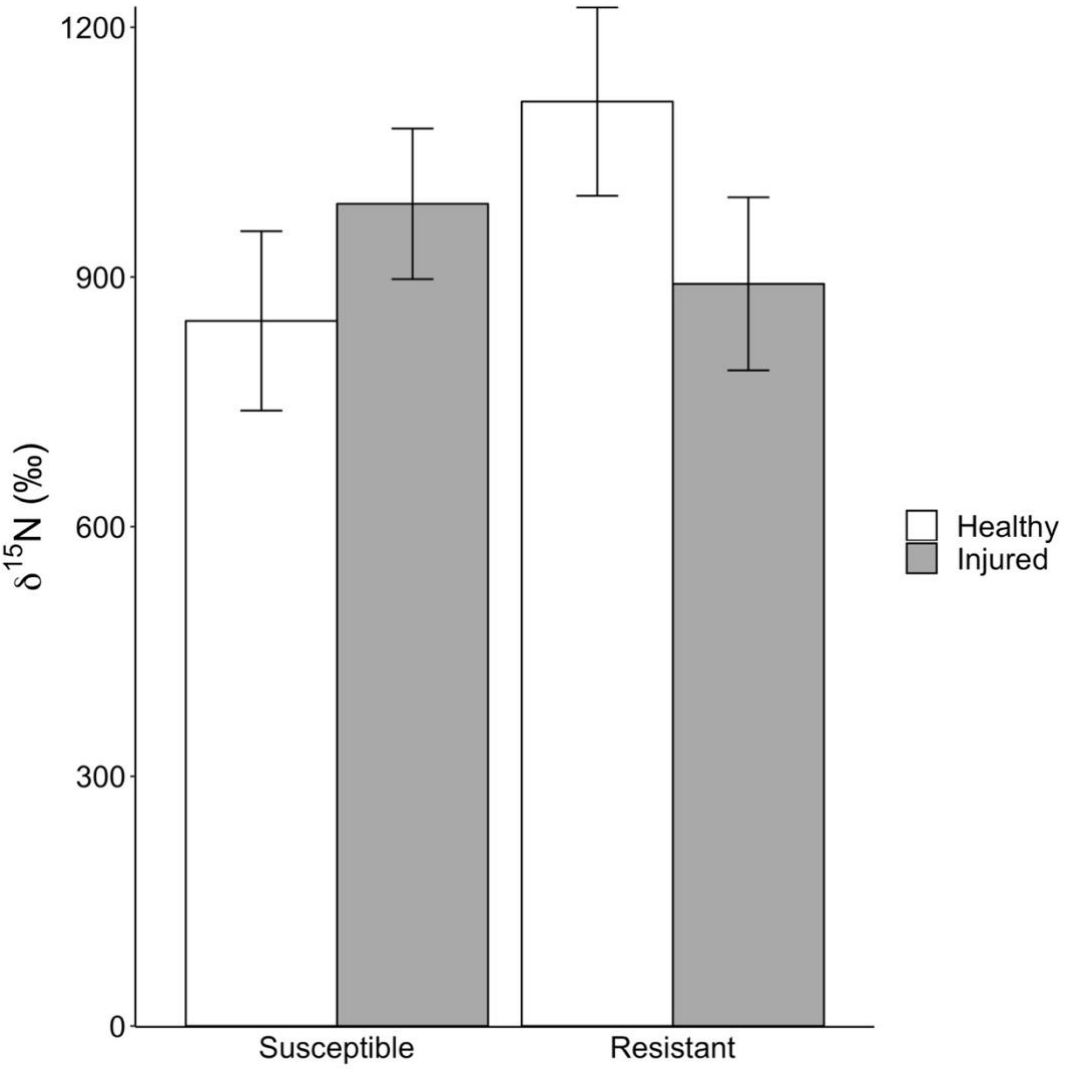

Figure 1.5 Aboveground amount of fixed nitrogen for pots with added ^{15}N; Healthy = No PLH Added, Injured = PLH Added

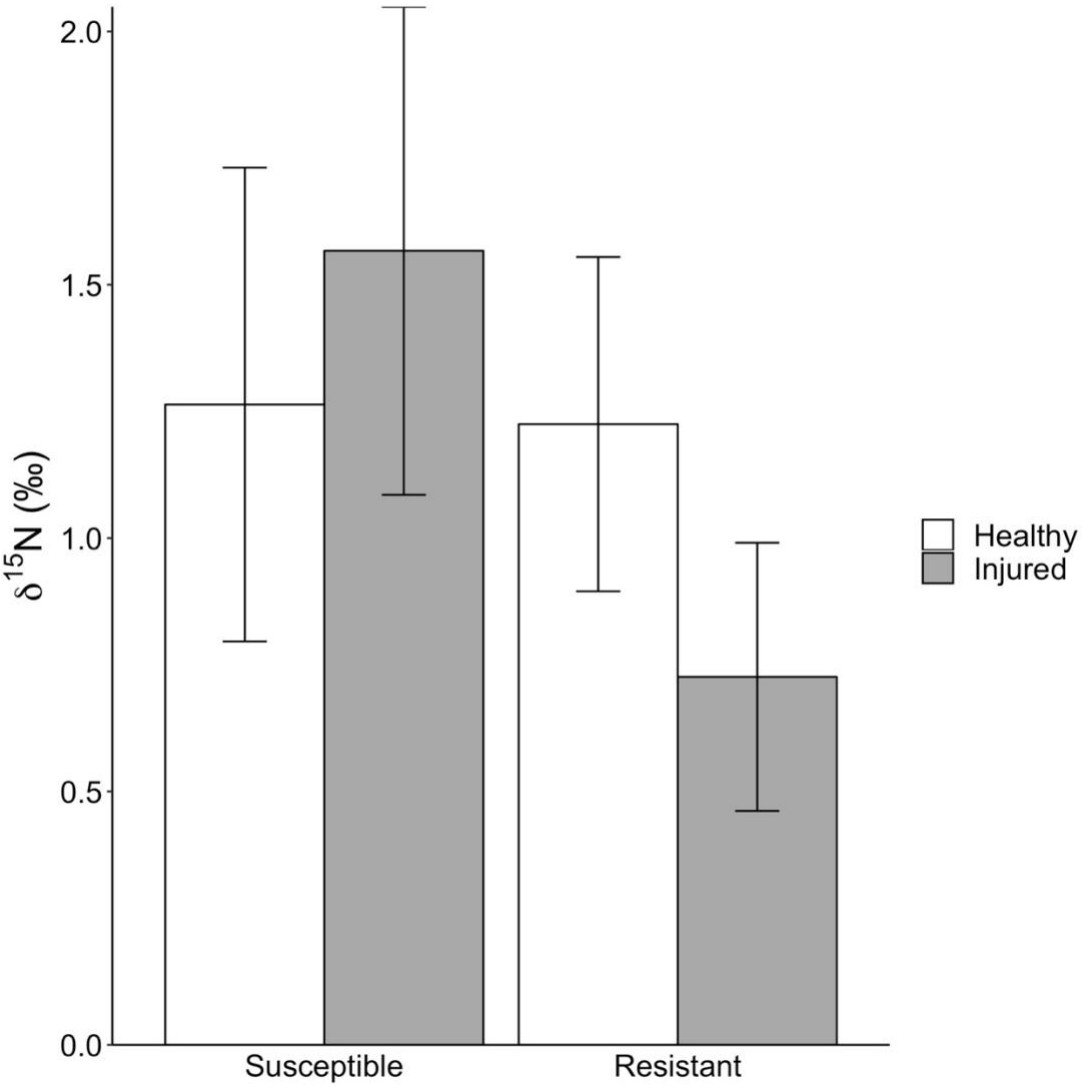

Figure 1.6 Aboveground amount of fixed nitrogen for pots without added ^{15}N; Healthy = No PLH Added, Injured = PLH Added

Chapter 2 Aboveground herbivory induces increased nutrient acquisition in a nitrogen fixing plant[2]

Abstract

Beneficial soil microbes engage in mutualisms with plant roots, aiding plants in nutrient acquisition. In return, plants donate photosynthate as an energy source for microbes. Nitrogen-fixing plants, for instance, live symbiotically with mutualistic microbes, such as *Rhizobium* and *Frankia*, which extract inert nitrogen gas from the atmosphere in exchange for carbon. Disrupted basal translocation of fixed carbon from leaves to roots, however, could negatively impact plant-rhizobia interactions. Aboveground insect herbivory can reduce photosynthate production, which may cascade to alter belowground interactions. Whether aboveground herbivory indirectly alters belowground nitrogen fixation, however, remains unclear. To test this, my objectives were to 1) determine differences in fixed nitrogen allocation across whole plants in response to herbivory, and 2) identify if plants can recover from herbivore-induced losses to nitrogen fixation with additional soil nitrogen. Overall, our work advances our understanding of how herbivory can indirectly influence interactions of plants with beneficial organisms.

Introduction

As sessile organisms, plants rely on bioavailable nutrient pools in surrounding soil environments. A plant acquires nutrients belowground (Chapman et al. 2012) and

[2] Prepared for submission to Oecologia

allocates these nutrients primarily aboveground, depending on biological needs across the whole plant (Reynolds and Chen 1996; Linker and Johnson-Rutzke 2005). Vegetative growth, for instance, requires different amounts of energy and nutrient inputs than reproductive growth (Bloom et al. 1985), and both of these processes trade-off with defense allocation (Züst and Agrawal 2017) throughout plant ontogeny (Boege and Marquis 2005; Barton and Koricheva 2010). Acquiring and allocating nutrients determines the growth and survival of plants, ultimately affecting plant persistence across ecological and evolutionary time (Farnsworth 2004; Weiner 2004).

To enhance nutrient acquisition, plants often depend on symbiotic mutualisms with beneficial soil microbes (Shtark et al. 2010). Beneficial microbes donate bioavailable macronutrients to plant roots (Lum and Hirsch 2002). In return, plant roots offer organic matter derived from photosynthates, which fuels costly nutrient acquisition processes for microbes (Ladygina and Hedlund 2010; Kramer et al. 2012). The specificity of such plant-microbe interactions varies across plant species. Mycorrhizal fungi and plant growth-promoting rhizobacteria associate with numerous plant families (van der Heijden et al. 2008; Berg 2009) whereas nitrogen-fixing microbes (rhizobia) form highly specialized interactions with plants in the family Leguminosae (Fabaceae) (Andrews and Andrews 2017). Rhizobia transform atmospheric nitrogen gas (N_2) into ammonium (NH_4^+), which plants incorporate into amino acids for transport throughout their vascular systems (Liu et al. 2018). Plants generally transport fixed nitrogen aboveground (Collier and Tegeder 2012), resulting in nitrogen-rich plants relative to non-fixing plants (McKey 1994; Adams et al. 2016; Wolf et al. 2017).

Nitrogen-rich plants attract insect herbivores, as insects are nitrogen-limited organisms (Mattson 1980; Strong, Lawton, and Southwood 1984; Slansky and Scriber 1985; Fagan et al. 2002). Host plant location and exploitation varies across herbivorous insect feeding guilds (Peeters et al. 2007), as well as the degree to which an herbivore is specialized on a particular host (Ali and Agrawal 2012). Sap-feeding insects, such as aphids, leafhoppers, froghoppers, and scale insects, demonstrate increased growth and reproduction on nitrogen-rich host plants (Awmack and Leather 2002). Aphids, for instance, show increased localization to and success on meristematic and young plant tissues high in nitrogen content (Giordanengo et al. 2010) and can, in some cases, manipulate nutrient flow in plants (Way and Cammell 1970; Inbar et al. 2004). Sap-feeding insects access nutritional resources (soluble nitrogen) through direct feeding on vascular plant tissues, imbibing nutrients in transit and avoiding defensive compounds produced in other plant tissues (Huberty and Denno 2004). Hence, nitrogen-fixing legumes offer exploitable high-quality resources for sap-feeding insects.

Feeding damage by insect herbivores across feeding guilds, however, alters aboveground plant physiology (Schwachtje and Baldwin 2008), reducing rates of photosynthesis (Lamp et al. 2004; Velikova et al. 2010) and plant growth (Huang et al. 2014). Additionally, plant nutrient allocation patterns can change in response to insect herbivory (Orians et al. 2011). Plants often allocate resources belowground in response to aboveground herbivory, physically limiting insect herbivores from accessing such resources (Schwachtje et al. 2006; Kaplan et al. 2008). Nutrient allocation and reallocation in plants occurs in the vascular system, from which sap-

feeding insects directly imbibe. Hence, disrupted aboveground nutrient allocation in legumes may alter belowground interactions with rhizobia and reduce the ability of roots to acquire nitrogen.

In this experiment, we evaluated how an aboveground sap-feeding herbivore (*Empoasca fabae*) alters nitrogen fixation in a legume (*Medicago sativa*). We predicted herbivory would decrease nitrogen fixation due to well-documented perturbations to *M. sativa* physiology in response to herbivory, such as reductions in photosynthesis (Womack 1984; Flinn et al. 1990) and photosynthate translocation (Nielsen et al. 1990; Lamp et al. 2001). To quantify fixed nitrogen, we utilized naturally occurring nitrogen isotope ratios ($^{15}N/^{14}N$) and a non-fixing reference plant. Our reference plant determined soil nitrogen fractionation within our fixing plant and allowed us to assess alterations in the percentage of nitrogen derived from the atmosphere (%Ndfa, i.e. fixed nitrogen) in response to herbivory. We also measured changes in fixed nitrogen allocation across above- and belowground plant components following herbivory. This work expands our understanding of ecological connections by linking aboveground processes to belowground inter-species interactions, contributing to our knowledge of plant-soil feedbacks and herbivory.

Methods

Study System

We selected *Medicago sativa* L. (Family Fabaceae, alfalfa or lucerne) as our nitrogen-fixing plant. *M. sativa* relies on mutualistic interactions with nitrogen-fixing bacteria (*Sinorhizobium meliloti*) to meet nitrogenous demands (Vance et al. 1979). In a preliminary greenhouse experiment, we evaluated six Saranac cultivars of alfalfa:

two cultivars capable of nitrogen fixation and four cultivars that were not capable of nitrogen fixation. For both field and greenhouse experiments, we selected two cultivars of *M. sativa*: Saranac '2425' and Saranac '2393,' henceforth referred to as 'fixing' and 'non-fixing,' respectively. Both cultivars exhibited high levels of germination (77.6 ± 9.6% and 73.6 ± 8.3%) and large numbers of nodules (8.2 ± 5.4 and 11.4 ± 3.8), although the nodules were non-functional in the non-fixing plants. The non-fixing cultivar allowed us to understand changes to nitrogen fixation in our fixing cultivar (Appendix C).

Potato leafhoppers (PLH; Family Cicadellidae, *Empoasca fabae* Harris) were collected from alfalfa fields in Keedysville, MD, USA and reared on fava beans (*Vicia faba*) for our greenhouse study in the lab. PLH were kept in BugDorm mesh cages in a growth chamber at the University of Maryland in the Entomology Department. PLH is a well-studied phloem-feeding insect herbivore of *M. sativa*. PLH induces significant damage to plants 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).

Field Cage Experiment

We seeded our field study on September 5, 2017 at the Western Maryland Research and Education Center (WMREC) in Keedysville, Maryland, USA. We set up a randomized complete block split-plot design with four blocks and four main plots per block. Main plots (3m x 6m) were seeded at a rate of 8 kg/acre. Main plots included: 1) Fixing x Non-Fertilized, 2) Fixing x Fertilized, 3) Non-Fixing x Non-Fertilized, and 4) Non-Fixing x Fertilized. We divided main plots in half (3m x 3m)

to establish two subplots per main plot: with PLH or without PLH. Across all plots, we cut back emergent spring growth on May 22, 2018 and applied our nitrogenous fertilizer treatment three days later. Each designated subplot received 0.20067g of ^{15}N-labelled potassium nitrate diluted in 120.16mL of RO water. Nitrogen fertilizer was sprayed directly on the soil surface with a plastic spray bottle. We fertilized only once throughout the entire experiment as heavy nitrogen (^{15}N) persists for long periods of time in the environment (Epstein et al. 2001). Due to a limited number of available field cages, two blocks received 1m x 1m x 1m (small) cages and the other two blocks received 2m x 2m x 2m (large) cages. To standardize the amount of plant material available to PLH across small and large cages, we nailed down a border of weed cloth in the large cages to allow for the same amount of *M. sativa* growth as the small cages. We erected field cages (sixteen small cages and sixteen large cages with weed cloth) on June 6, 2018 and applied Neem Oil organic insecticide inside cages to reduce any outbreak of unwanted pests. Five days later (20 days after spring cutback), we added 100 PLH adults to designated cages. PLH adults were collected by D-Vac from adjacent *M. sativa* fields at the Keedysville farm, placed in mesh cages, and aspirated from mesh cages into designated field cages. Thirty-four days after the initial spring cutback, we removed cages and cutback plots to 4 cm with a handheld grass trimmer, which followed a typical harvest cycle of *M. sativa*. Plant samples were taken to the lab where we separated weeds from *M. sativa* and placed all material in a drying oven for 24 to 48 h. We weighed and ground samples 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 are reported as δ^{15}N ‰ (see Appendix B for further discussion of interpretation of δ^{15}N ‰; see also Werner and Brand 2001 & Coplen 2011 for further discussion of instrumentation and interpretation). We used δ^{15}N ‰ values to calculate the percentage nitrogen derived from the atmosphere (%Ndfa; see Appendix C).

Nine days after our first sampling period, we prepared plots for our second sampling period. We applied an organic insecticide with a low residual time to all plots to reduce pest outbreak. Seven days later we erected field cages and applied Neem Oil. Seven days after this, we added PLH to field cages using the same methodology as our previous sampling period. To account for any additive effects from PLH feeding on nitrogen fixation across sampling periods, we varied PLH treatments across cages (half of the cages received the same treatment across both sampling periods, half received two different treatments). We removed field cages and cutback the plots 35 days after our first sampling period, following the same procedure. After cutting back the plots, we also collected belowground plant samples by digging up alfalfa crowns and roots at 2.5 cm below the soil surface. Due to an unusually high presence of weeds, we were unable to collect whole plant samples for this study. Instead, we collected foliar samples of the entire plot and dug up crowns and roots from three random locations in the plots. We brought all samples to the lab, dried samples for 24-48 h in the drying oven, and followed the same procedure to grind and prepare samples for nitrogen isotope analysis.

Greenhouse Experiment

We planted seeds of fixing and non-fixing *M. sativa* in standard potting mixture on October 4, 2018 and placed the seeds in a growth chamber at the University of Maryland in the Department of Entomology. We repotted 48 seedlings of fixing *M. sativa* and 48 seedlings of non-fixing *M. sativa* on October 25, 2018. Seedling roots were dipped in rhizobia-water dilution (4.00g rhizobia/500 mL of water) and placed in cone pots containing 50/50 mixture of Sphagnum peat moss and Sakete Multipurpose sand, totaling 130g of soil-peat mixture per cone pot. We used a mixture in an effort to reduce root exposure to ambient nutrients but also provide non-fixing, unfertilized plants with an environment suitable for growth. We fertilized plants once per week with 10 mL of nitrogen-free Hoagland's solution (Appendix A). Cone pots were arranged in a randomized complete block design containing eight blocks and twelve treatment levels. Our treatment combinations contained two factors with two levels (Cultivar, PLH) and one factor with three levels (Nitrogen Fertilizer), fully crossed. Our twelve treatment combinations included: 1) Fixing x With PLH x High Nitrogen, 2) Fixing x With PLH x Low Nitrogen, 3) Fixing x With PLH x No Nitrogen, 4) Fixing x No PLH x High Nitrogen, 5) Fixing x No PLH x Low Nitrogen, 6) Fixing x No PLH x Low Nitrogen, 7) Non-Fixing x With PLH x High Nitrogen, 8) Non-Fixing x With PLH x Low Nitrogen, 9) Non-Fixing x With PLH x No Nitrogen, 10) Non-Fixing x No PLH x High Nitrogen, 11) Non-Fixing x No PLH x Low Nitrogen, and 12) Fixing x No PLH x Low Nitrogen. Nitrogen fertilizer treatments were applied once a week following repotting and consisted of three different levels: full rate, 0.25x full rate, or none. Using an estimate of 67kg of nitrogenous fertilizer

per hectare for small grain production, we measured the surface area of a cone pot (6.5 cm^2) and calculated the full rate of nitrogen fertilization to be 4.3mg per pot. Rather than applying our nitrogenous fertilizer treatment once, we applied fertilizer treatments once a week from repotting to the conclusion of the experiment. For the full rate, we applied 0.306 mg of ^{15}N-labelled potassium nitrate diluted in 5 mL of water per week. The 0.25x full rate application consisted of 0.0768 mg of ^{15}N-labelled potassium nitrate diluted in 1.5 mL of water and 0.168mg of potassium chloride diluted in 3.5mL of water per week. To account for any effect of potassium, we equilibrated the amount of potassium added across fertilization treatments with potassium chloride amendments. Hence, for the no-nitrogen fertilization treatment, we added 0.224 mg potassium chloride diluted in 5 mL of water per week. The final fertilization treatment was applied one week before the experiment ended. In conjunction with nitrogen fertilizer applications, we applied nitrogen-free Hoagland's solution once a week. Plants were watered daily with 10-20 mL of water as needed. We cut plants back on December 27, 2018 to simulate a harvest and caged PLH on January 17, 2019 to manipulate PLH presence or absence. We placed 2 fourth-instar PLH nymphs from our lab colony in to designated plastic cages. After seven days of feeding, PLH nymphs were removed from plants and all cages were removed. Plants grew for seven more days to reach 35 days of regrowth after our simulated harvest. We sacrificed plants and separated roots, crowns, and shoots, and placed all samples in a drying oven for 24 h and measured dry weight (grams) of all samples. Dried samples were ground and weighed for nitrogen isotope analysis following the sample procedure described for the field study.

Data Analysis

Analyses were conducted within the program R version 3.5.1 (R Core Team 2018). For the field study, to analyze our foliar data from both sampling periods, we first calculated averages for measured response variables. For our first sampling period (June 26, 2018), we used a three-way analysis of variance (ANOVA) accounting for the split-plot design in the model. We used two main plot factors (Cultivar, Nitrogen Fertilizer) and one subplot factor (PLH), which served as our explanatory variables. We ran three separate ANOVAs with the same explanatory variables and three different response variables: dry weight, percentage nitrogen, and nitrogen biomass. We performed similar analyses for our second sampling period (July 31, 2018) with a modified ANOVA model. Due to missing data points, samples from plots fertilized by nitrogen were removed from analysis. To analyze percentage nitrogen derived from the atmosphere (%Ndfa) and fixed nitrogen biomass (%Ndfa x Nitrogen Biomass), we paired non-fixing plants that received the same nitrogen fertilizer and PLH treatment as fixing plants within the same block. Hence, we dropped 'Cultivar' as an explanatory variable for ANOVAs examining dependent variables: %Ndfa and Fixed Nitrogen Biomass. Here we used the split plot ('sp.plot') function in the agricolae package in R 4/23/2019 4:50:00 PM. We ran LSD post-hoc comparisons for total percentage nitrogen and %Ndfa in plots that did not receive PLH (Healthy) and those that did receive PLH (Injured) across nitrogen fertilizer treatments. We repeated the same analyses for belowground plant samples collected on July 31, 2018.

We followed similar analyses for the greenhouse study. We report here only on the plants that received no nitrogen treatments (Non-Fixing x Without PLH, Non-Fixing x With PLH, Fixing x Without PLH, Fixing With PLH). First, we determined average values across shoots, crowns, and roots for our response variables: dry weight, percentage nitrogen, and nitrogen biomass. We then ran two-way ANOVAs with cultivar (fixing or non-fixing) and PLH (with or without) and the interaction between the two as our explanatory variables. To analyze percentage nitrogen derived from the atmosphere (%Ndfa) and fixed nitrogen biomass (%Ndfa x Nitrogen Biomass), we again paired non-fixing plants that received the same PLH treatment as fixing plants within the same block and dropped 'Cultivar' as an explanatory variable for ANOVAs. We used these ANOVAs to understand dependent variables: %Ndfa and Fixed Nitrogen Biomass. We ran t-tests to compare total percentage nitrogen and %Ndfa in fixing plants that did not receive PLH (Healthy) and those that did receive PLH (Injured).

Results

Field Cage Experiment

Foliar samples from our first and second sampling periods differed across all measured variables (Tables 2.1). Due to heavy rainfall during June 2018, our field plots experienced extensive invasion from weeds after the first sampling period, reflected in the increase in weed dry weight between the two sampling periods. Additionally, percentage nitrogen and nitrogen biomass decreased between the two sampling periods. ANOVA models for the first sampling period (Table 2.2) indicated

a significant effect of cultivar (p=0.02) and PLH (p=0.03) on dry weight. We detected on significant effects on percentage nitrogen but we determined a significant effect of cultivar (p=0.03) and PLH (p=0.03) on nitrogen biomass. In comparison, we found no significant effects of any explanatory variables across all models for the second sampling period (Table 2.2). When we examined response variables (%Ndfa and Fixed Nitrogen Biomass), we found a significant effect of nitrogen fertilizer (p=0.02) and a significant interaction between nitrogen fertilizer and PLH (p=0.05) on %Ndfa for the first sampling period (Table 2.3). We found no significant effect of any explanatory variables on fixed nitrogen biomass. For the second sampling period, we found no significant effect of any explanatory variables on either response variable (Table 2.3). Through LSD post-hoc comparisons of foliar samples from the first sampling period, we observed no significant differences in percentage nitrogen between healthy and injured fixing plants across both nitrogen fertilizer treatments (Figure 2.1) but we found a significant difference in %Ndfa between healthy and injured unfertilized fixing plants (p=0.0121) and no difference in fertilized fixing plants (Figure 2.2). Foliar samples from the second sampling period showed contrasting, non-significant trends in percentage nitrogen when compared to the first sampling period (Figure 2.3) and we observed no significant differences in LSD post-hoc comparisons for %Ndfa (Figure 2.4).

Belowground samples showed similar averages of response variables across crown and roots (Table 2.4). Crown and root samples exhibited lower dry weights than foliar samples, as well as lower percentages of nitrogen and less nitrogen biomass. Our results indicate the plants translocated most nitrogen aboveground.

ANOVA models for crowns from the second sampling period showed no significant effects of any explanatory variables on dry weight or nitrogen biomass but did show a significant effect ($p=0.008$) of cultivar on percentage nitrogen (Table 2.5). Results for roots mirrored crown results, showing no significant effect of any explanatory variables on dry weight or nitrogen biomass but a significant effect ($p<0.001$) of cultivar on percentage nitrogen (Table 2.5). Both %Ndfa and fixed nitrogen biomass of crown samples from fixing plants revealed a significant effect ($p=0.02$) of the interaction between nitrogen fertilizer and PLH (Table 2.6). In contrast, there were no significant effects of any explanatory variables on %Ndfa and fixed nitrogen biomass of root samples (Table 2.6). LSD post-hoc comparisons showed no significant differences between percentage nitrogen of healthy and injured crown samples across nitrogen fertilizer treatments (Figure 2.5). We detected a significant difference ($p=0.0272$) in %Ndfa between healthy and injured crown samples from unfertilized plots but no significant difference in fertilized plots (Figure 2.6). LSD post-hoc comparisons of healthy and injured root samples revealed no significant differences in percentage nitrogen nor %Ndfa across fertilizer treatments (Figures 2.7 and 2.8).

Greenhouse Experiment

Shoot samples revealed differences across cultivars in terms of dry weight, percentage nitrogen, and nitrogen biomass (Table 2.7). Two-way ANOVA results for shoot samples also revealed a significant effect of cultivar ($p<0.001$) on all three response variables (Table 2.8). We also detected an effect of PLH ($p=0.003$) and an interaction effect of cultivar and PLH ($p=0.04$) on percentage nitrogen (Table 2.8). T-tests on the percentage nitrogen of fixing shoots revealed a significant difference

($p=0.0151$) between healthy and injury samples (Figure 2.9) but no significant differences in terms of %Ndfa (Figure 2.10).

Crown samples showed differences in response variable averages across cultivars (Table 2.7). All ANOVA models showed a significant effect of cultivar on dry weight ($p=0.004$), percentage nitrogen ($p<0.001$), and nitrogen biomass ($p=0.01$) (Table 2.8). T-tests revealed no significant differences between crowns from healthy and injured fixing plants in terms of percentage nitrogen (Figure 2.11) and %Ndfa (Figure 2.12). There is, however, a non-significant trend towards a decrease in percentage nitrogen and an increase in %Ndfa when PLH are introduced.

Root samples followed shoot and crown samples as there were differences between cultivars in terms of dry weight and nitrogen biomass but less of a drastic difference for percentage nitrogen (Table 2.7). Our ANOVA models for root samples revealed a significant effect of cultivar ($p<0.001$) across all three response variables (Table 2.8). T-tests revealed no significant differences between healthy and injured fixing plants in terms of percentage nitrogen (Figure 2.13) and %Ndfa (Figure 2.14).

We compiled results for fixed nitrogen biomass (%Ndfa x Nitrogen Biomass) for shoot, crown, and root samples across healthy and injured fixing plants (Figure 2.15). We ran t-tests to compare each plant component separately and found no significant differences between healthy and injured fixing plants. Despite no significant differences in fixed nitrogen biomass, there is a clear trend for more fixed nitrogen biomass in injured fixing plants.

Discussion

Our results, across both field and greenhouse studies, completely contradicted our predictions. We anticipated PLH feeding would disrupt interactions between *M. sativa* and rhizobia, leading to decreased nitrogen fixation. Instead, plants fed on by PLH increased accumulation of fixed nitrogen aboveground and maintained belowground amounts of fixed nitrogen. Our results demonstrate an increased allocation of fixed nitrogen aboveground in response to insect herbivory. This work contributes to rapidly expanding knowledge on interactions between herbivores, plants, and soil microbes, and highlights the underexamined effect of herbivory on plant-microbe mutualisms (as noted in Pineda et al. 2010).

Due to known effects of PLH feeding on *M. sativa* physiology, we predicted reductions in photosynthesis (Womack 1984; Flinn et al. 1990) and basal translocation of photosynthates (Nielsen et al. 1990; Lamp et al. 2001) caused by PLH injury would ultimately disrupt belowground nitrogen fixation. Although we observed significant reductions in the overall percentage nitrogen in *M. sativa* shoots, we simultaneously observed significant increases in %Ndfa of shoots and crowns across both field and greenhouse experiments. Hence, aboveground plant components contained less nitrogen but more of that nitrogen was derived from nitrogen fixation.

Further, we used a nitrogen fertilization treatment in the field experiment to determine if *M. sativa* could recover from losses in nitrogen fixation due to PLH injury. We predicted *M. sativa* would assimilate available soil nitrogen, increasing the nitrogen biomass of fertilized plants despite PLH injury. However, we observed no increase in nitrogen biomass of fertilized plants compared to unfertilized plants across

shoots, crowns, and roots, with or without PLH. We also found nitrogen fertilizer reduced %Ndfa to almost zero, regardless of PLH treatment and across all plant components. A complete lack of nitrogen fixation in fertilizer *M. sativa* was a surprising result, as previous studies reported *M. sativa* maintained moderate levels of nitrogen fixation despite high levels of available soil nitrogen (Lamb et al. 1995; Kelner et al. 1997). However, other studies saw decreases in nitrogen fixation of *M. sativa* with increased soil nitrogen (Streeter and Wong 1988) and posit *M. sativa* may preferentially assimilate soil nitrogen as it is less costly for plants than donating photosynates to rhizobia. Further, plants assimilate and transport fixed and soil nitrogen in contrasting ways (Ciesiołka et al. 2005; Katayama et al. 2010). Halting nitrogen fixation in *M. sativa* could result in altered biochemical pathways, which may cascade to affect longer term plant growth and survival.

We did not observe any reallocation of fixed nitrogen belowground, suggesting *M. sativa* preferentially allocated fixed nitrogen aboveground in response to nitrogen losses. Allocation above- or belowground may depend on other abiotic or biotic stressors present in the environment of a given plant (Kaplan et al. 2008) and could explain a lack of reallocation in the field experiment. Essentially, other stressors could influence the movement of fixed nitrogen across the whole plant, confounding any effect of PLH injury aboveground.

One potential abiotic stressor was extensive periods of rainfall prior to the second sampling period, which resulted in increased weed growth across all field plots. Weed pressure may have increased belowground competition for nutrients, as other key macro- and micronutrients, such as phosphorous, influence not only *M.*

sativa growth and survival but also nitrogen fixation (Liu et al. 2018). However, we did not observe any fixed nitrogen reallocation in the greenhouse, where *M. sativa* plants were grown individually and did not compete with other species or conspecifics for nutrients. Therefore, we conclude *M. sativa* does not retain or reallocate more fixed nitrogen belowground in response to aboveground nitrogen losses.

Increased %Ndfa aboveground may derive from a generalized wound response in *M. sativa*. When detecting nitrogen losses—from insect herbivores or otherwise—*M. sativa* could translocate greater amounts of fixed nitrogen aboveground. During plant senescence, source-sink dynamics regulate nutrient flow between young and old leaves. Aging leaves accumulate greater amounts of nitrate and ammonium while losing amino acids and carbohydrates to younger leaves during senescence (Masclaux et al. 2000). Hence, senescence alters the movement of various forms of nitrogen throughout plants and can, in some cases, increase nitrogen fixation (Fischinger et al. 2006). If *M. sativa* responds to PLH injury in affected tissues as generalized senescence, *M. sativa* may alter the movement of nitrogen throughout the plant, resulting in greater amounts of fixed nitrogen aboveground. Since we did not test the effect of feeding from other insect herbivores nor physical damage (i.e. cutting) on *M. sativa*, we cannot conclude if the response is specific to PLH or a ubiquitous senescence response.

Moreover, plant defense offers another possible explanation for our results. Following colonization of plant roots by microbes, beneficial soil microbes stimulate induced systemic resistance (ISR) in host plants (Kloepper et al. 2004). In other

words, microbes interact with plant roots to systemically upregulate phytohormones involved in plant defense or otherwise prime plants for defense against antagonists (Pineda et al. 2010). Therefore, induction of plant defense occurs prior to herbivory.

In contrast, other legumes benefit from direct increases in the amount of available nitrogen via fixation, which legumes can incorporate into nitrogen-based defense compounds. Thamer et al. (2011) demonstrated an increase in cyanogenesis of lima beans associating with rhizobia and resulting decreases in herbivore performance (Thamer et al. 2011). Analysis of volatile organic compounds released subsequent to aboveground herbivory also revealed an increase in the production of indole, a nitrogen-based defense compound, in lima beans associating with rhizobia (Ballhorn et al. 2013). Additionally, the first study to document an effect of rhizobia on aboveground plant-insect interactions showed reduced larval growth of a chewing herbivore (*Spodoptera littoralis*) on a cyanogenic strain of *Trifolium repens* associating with rhizobia (Kempel, Brandl, and Schädler 2009). Rhizobia increased nitrogen available for cyanogenesis of nitrogen-based defense compounds. However, in the same study, the authors detected no difference in the performance of aphids on cyanogenic plants associating with rhizobia and suggest aphids are able to bypass plant defenses with piercing-sucking mouthparts.

Although piercing-sucking insect herbivores are generally thought to bypass plant defenses produced in leaf tissues, such herbivores can actually modulate the immune response of plants through effectors, or small molecules released from saliva into plant cells (Hogenhout and Bos 2011). Effectors can trigger an immune response, such as the upregulation of defense or release of volatile organic compounds to

recruit natural enemies. Therefore, although the effect on performance or survival of piercing-sucking insects is often variable, defensive responses by plants may still occur. Previous research shows reduced growth and disrupted physiology of *M. sativa* in response to PLH feeding but has not yet examined the role of plant defense.

However, one experiment on *M. sativa* demonstrated soil applications of synthetic methyl jasmonate (MeJA) increased nitrogen accumulation in roots (Meuriot et al. 2004). Although this study did not analyze changes in nitrogen fixation of *M. sativa* in response to MeJA, the results could indicate increased amounts of fixed nitrogen localized to an area where *M. sativa* detected injury. Hence, nitrogen contributions from rhizobia could contribute to formulating molecules involved in plant defense of *M. sativa,* which are transported by plants to affected areas. Our results of increased fixed nitrogen accumulation aboveground in response to PLH injury align with such a proposed mechanism of plant defense compound production in *M. sativa*.

Although we cannot definitively conclude whether the response of *M. sativa* to PLH injury is related to senescence or defense, this experiment demonstrates *M. sativa* transports fixed nitrogen aboveground following PLH herbivory. Future research should focus on discerning the identity of the proteins or compounds *M. sativa* incorporates fixed nitrogen into in response to herbivory, which may help to determine the mechanism driving the response. Our work advances current knowledge on how aboveground herbivory affects the contribution of beneficial soil microbes to plant physiology, which has important implications across ecological and agricultural systems.

Table 2.1 Foliar samples from first and second sampling periods for the field study collected on June 26, 2018 and July 31, 2018, respectively. Numbers represent means +/- standard deviation; Healthy = No PLH Added, Injured = PLH Added

	Non-Fixing				**Fixing**			
	No Added Nitrogen		*Nitrogen Added*		*No Added Nitrogen*		*Nitrogen Added*	
	Healthy	Injured	Healthy	Injured	Healthy	Injured	Healthy	Injured
June – First Sampling Period								
Alfalfa Dry Weight (g)	36.2 ± 28.9	28.5 ± 27.1	40.6 ± 36.7	22.3 ± 17.0	93.7 ± 58.7	67.9 ± 41.4	95.0 ± 54.5	92.2 ± 64.6
Weed Dry Weight (g)	12.4 ± 1.5	19.8 ± 21.1	21.5 ± 22.4	11.5 ± 7.5	3.0 ± 2.8	4.7 ± 7.3	1.1 ± 0.8	1.7 ± 0.9
Nitrogen (%)	3.7 ± 0.3	3.6 ± 0.4	3.9 ± 0.2	3.6 ± 0.1	3.8 ± 0.6	3.5 ± 0.3	3.8 ± 0.04	3.5 ± 0.4
Nitrogen Biomass (g of N)	1.4 ± 1.2	1.1 ± 1.1	1.6 ± 1.6	0.8 ± 0.6	3.55 ± 2.2	2.5 ± 1.6	3.6 ± 2.1	3.4 ± 2.7
July – First Sampling Period								
Alfalfa Dry Weight (g)	8.6 ± 10.3	8.9 ± 8.8	14.1 ± 8.0	6.0 ± 5.9	29.4 ± 32.2	27.2 ± 20.1	41.3 ± 25.9	29.6 ± 27.5
Weed Dry Weight (g)	66.2 ± 56.9	89.7 ± 80.0	48.6 ± 14.1	184.8 ± 66.4	33.4 ± 26.9	81.5 ± 76.5	34.0 ± 23.7	25.6 ± 21.2
Nitrogen (%)	3.4 ± 0.1	3.6 ± 0.3	3.7 ± 0.5	3.6 ± 0.1	3.4 ± 0.2	3.5 ± 0.5	3.4 ± 0.2	3.1 ± 0.4
Nitrogen Biomass (g of N)	0.3 ± 0.4	0.3 ± 0.3	0.5 ± 0.3	0.2 ± 0.2	1.0 ± 1.1	0.9 ± 0.6	1.4 ± 0.9	0.9 ± 0.9

Table 2.2 Split plot ANOVA (2 main plot factors, 1 subplot factor) results for foliar samples from first and second sampling periods for the field study collected on June 26, 2018 and July 31, 2018, respectively. For the first sampling period, residuals and interaction terms (Cultivar x PLH, Nitrogen Fertilizer x PLH, Cultivar x Nitrogen Fertilizer x PLH) were non-significant and removed for clarity. For the second sampling period, due to missing data points, samples from plots fertilized by nitrogen were removed from analysis. ANOVA results are from unfertilized plots only.

		June – First Sampling Period					*July – Second Sampling Period*				
Parameter	**Source**	**df**	**SS**	**MS**	**F value**	**p-value**	**df**	**SS**	**MS**	**F value**	**p-value**
Dry Weight (grams)	*Main Effects*										
	Cultivar	1	24489	24489	6.75	0.02	1	1530	1530.2	3.35	0.12
	Nitrogen	1	284	284	0.08	0.78				-	-
	Cultivar x Nitrogen	1	373	373	0.10	0.75				-	-
	Residuals	12	43556	3630			6	2740	456.7		
	Subplot Effects										
	PLH	1	1502.3	1502.3	6.10	0.03	1	3.40	3.40	0.009	0.93
	PLH x Cultivar	1	3.30	3.30	0.01	0.91	1	6.30	6.30	0.018	0.90
	PLH x Nitrogen	1	76.20	76.20	0.31	0.59					
	Cultivar x Nitrogen x PLH	1	564.30	564.30	2.29	0.16					
	Residuals	12	2953.4	246.10			6	2136.1	356		
Nitrogen (%)	*Main Effects*										
	Cultivar	1	0.007	0.007	0.05	0.83	1	0.03	0.03	0.35	0.58
	Nitrogen	1	0.02	0.02	0.14	0.72				-	-
	Cultivar x Nitrogen	1	0.002	0.002	0.01	0.91				-	-
	Residuals	12	1.62	0.14			6	0.46	0.08		
	Subplot Effects										
	PLH	1	0.35	0.35	4.28	0.06	1	0.07	0.07	0.56	0.48
	PLH x Cultivar	1	0.02	0.02	0.28	0.61	1	0.0003	0.0003	0.003	0.96
	PLH x Nitrogen	1	0.04	0.04	0.53	0.48					
	Cultivar x Nitrogen x PLH	1	0.01	0.01	0.17	0.68					
	Residuals	12	0.97	0.08			6	0.71	0.12		
Nitrogen Biomass (g of N)	*Main Effects*										
	Cultivar	1	33.13	33.13	5.95	0.03	1	1.68	1.68	3.11	0.13
	Nitrogen	1	0.46	0.46	0.08	0.78				-	-
	Cultivar x Nitrogen	1	0.49	0.49	0.09	0.77				-	-
	Residuals	12	66.87	5.57			6	3.24	0.54		
	Subplot Effects										
	PLH	1	2.93	2.93	5.91	0.03	1	0.01	0.01	0.03	0.86
	PLH x Cultivar	1	0.02	0.02	0.04	0.84	1	0.02	0.02	0.05	0.83
	PLH x Nitrogen	1	0.05	0.05	0.10	0.75					
	Cultivar x Nitrogen x PLH	1	0.92	0.92	1.85	0.20					
	Residuals	12	5.94	0.50			6	2.35	0.39		

Table 2.3 Split plot ANOVA (1 main plot factor, 1 subplot factor) results for for foliar samples from first and second sampling periods for the field study collected on June 26, 2018 and July 31, 2018, respectively.

		June – First Sampling Period					*July – Second Sampling Period*			
Parameter	**Source**	**df**	**SS**	**MS**	**F value**	**p-value**	**SS**	**MS**	**F value**	**p-value**
%Ndfa	*Main Effects*									
	Block	3	699.8	233.3	0.79	0.58	1849	616	2.39	0.31
	Nitrogen	1	5322	5322	17.92	0.02	5.9	5.9	0.02	0.89
	Residuals	3	891	297			515	258		
	Subplot Effects									
	PLH	1	858.3	858.3	3.46	0.11	1579	1579	5.98	0.07
	Nitrogen x PLH	1	1414.8	1414.8	5.70	0.05	1131	1131	4.29	0.11
	Residuals	6	1489.6	248.3			1056	264		
Fixed Nitrogen Biomass (g of N_{fixed})	*Main Effects*									
	Block	3	0.89	0.30	0.81	0.57	0.21	0.07	1.56	0.41
	Nitrogen	1	2.46	2.46	6.75	0.08	0.02	0.02	0.51	0.55
	Residuals	3	1.09	0.36			0.09	0.05		
	Subplot Effects									
	PLH	1	0.30	0.30	2.93	0.14	0.20	0.20	5.98	0.07
	Nitrogen x PLH	1	0.14	0.14	1.35	0.29	0.18	0.18	5.57	0.08
	Residuals	6	0.61	0.10			0.13	0.03		

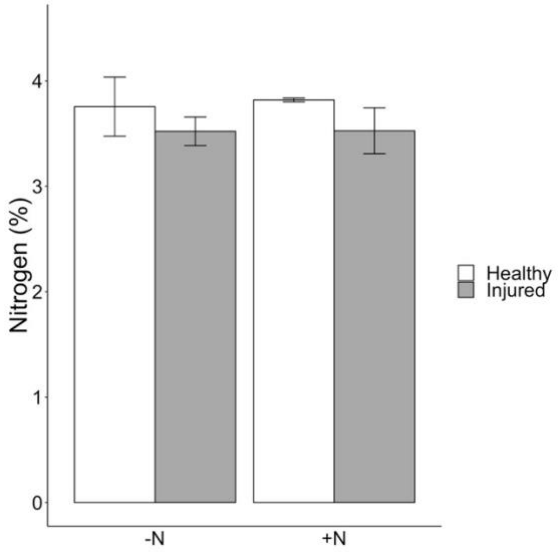

Figure 2.1 Percentage nitrogen for foliar samples of fixing plants without nitrogen fertilizer (-N) and with (+N) collected on June 26, 2018; Healthy = No PLH Added, Injured = PLH Added; -N Healthy – Injured p-value = 0.41, +N Healthy – Injured p-value = 0.299

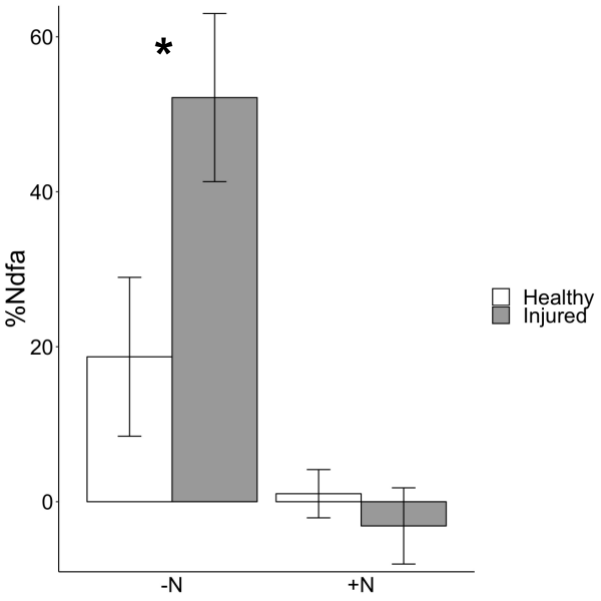

Figure 2.2 Percentage nitrogen derived from the atmosphere (%Ndfa) for foliar samples of fixing plants without nitrogen fertilizer (-N) and with (+N) collected on June 26, 2018; Healthy = No PLH Added, Injured = PLH Added; -N Healthy – Injured p-value = 0.0121, +N Healthy – Injured p-value = 0.72; * denotes significant difference ($p < 0.05$)

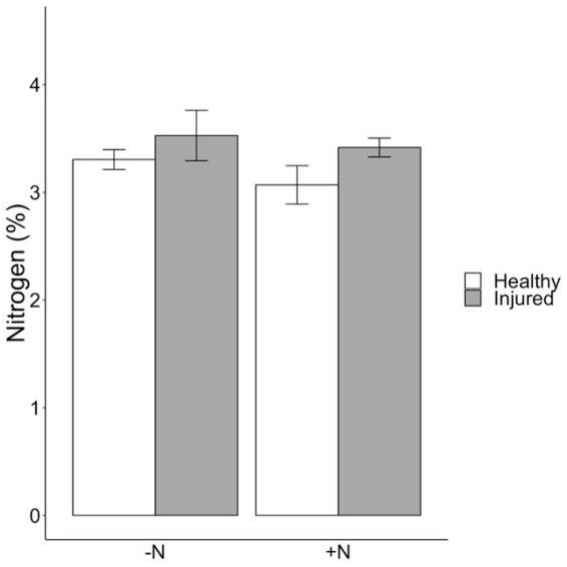

Figure 2.3 Percentage nitrogen for foliar samples of fixing plants without nitrogen fertilizer (-N) and with (+N) collected on July 31, 2018; Healthy = No PLH Added, Injured = PLH Added; -N Healthy – Injured p-value = 0.576, +N Healthy – Injured p-value = 0.205

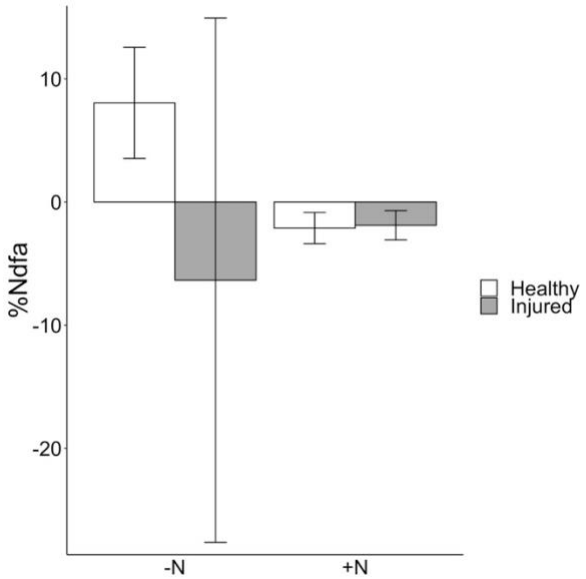

Figure 2.4 Percentage nitrogen derived from the atmosphere (%Ndfa) for foliar samples of fixing plants without nitrogen fertilizer (-N) and with (+N) collected on July 31, 2018; Healthy = No PLH Added, Injured = PLH Added; -N Healthy – Injured p-value = 0.0947, +N Healthy – Injured p-value = 0.991

Table 2.4 Belowground samples for field study collected on July 31, 2018. Numbers represent means +/- standard deviation; -Fix= Non-Fixing Cultivar, +Fix = Fixing Cultivar; -N = No Nitrogen Added, +N = Nitrogen Added; Healthy = No PLH Added, Injured = PLH Added

	Non-Fixing				**Fixing**			
	No Added Nitrogen		*Nitrogen Added*		*No Added Nitrogen*		*Nitrogen Added*	
	Healthy	Injured	Healthy	Injured	Healthy	Injured	Healthy	Injured
Crowns								
Dry Weight (g)	0.7 ± 0.2	0.9 ± 0.2	1.0 ± 0.3	0.7 ± 0.3	1.4 ± 0.4	0.9 ± 0.6	0.7 ± 0.3	0.7 ± 0.5
Nitrogen (%)	1.8 ± 0.5	1.9 ± 0.2	1.9 ± 0.1	2.0 ± 0.3	2.0 ± 0.4	2.2 ± 0.2	2.4 ± 0.1	2.3 ± 0.4
Nitrogen Biomass (g of N)	0.01 ± 0.001	0.02 ± 0.003	0.02 ± 0.01	0.01 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Roots								
Dry Weight (g)	0.7 ± 0.2	0.9 ± 0.2	1.0 ± 0.3	1.0 ± 0.5	1.4 ± 0.4	0.9 ± 0.6	0.6 ± 0.5	0.8 ± 0.5
Nitrogen (%)	1.8 ± 0.3	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.2	2.2 ± 0.4	2.1 ± 0.3	2.1 ± 0.2	2.0 ± 0.3
Nitrogen Biomass (g of N)	0.01 ± 0.002	0.01 ± 0.002	0.02 ± 0.01	0.01 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01

Table 2.5 Split plot ANOVA (2 main plot factors, 1 subplot factor) results for belowground samples from field study collected on July 31, 2018.

			Crowns				*Roots*			
Parameter	**Source**	**df**	**SS**	**MS**	**F value**	**p-value**	**SS**	**MS**	**F value**	**p-value**
Dry Weight (g)	*Main Effects*									
	Cultivar	1	0.11	0.11	0.69	0.42	0.02	0.02	0.09	0.77
	Nitrogen	1	0.30	0.30	1.95	0.19	0.11	0.11	0.49	0.50
	Cultivar x Nitrogen	1	0.57	0.57	3.68	0.08	0.92	0.92	4.13	0.07
	Residuals	12	1.86	0.15			2.68	0.22		
	Subplot Effects									
	PLH	1	0.25	0.25	1.90	0.19	0.03	0.03	0.22	0.65
	PLH x Cultivar	1	0.09	0.09	0.70	0.42	0.10	0.10	0.76	0.40
	PLH x Nitrogen	1	0.004	0.004	0.03	0.87	0.10	0.10	0.75	0.40
	Cultivar x Nitrogen x PLH	1	0.54	0.54	4.02	0.07	0.37	0.37	2.74	0.12
	Residuals	12	1.61	0.13			1.61	0.13		
Nitrogen (%)	*Main Effects*									
	Cultivar	1	0.83	0.83	10.15	0.01	2.82	2.82	35.35	<0.001
	Nitrogen	1	0.17	0.17	2.11	0.17	0.12	0.12	1.51	0.24
	Cultivar x Nitrogen	1	0.04	0.04	0.47	0.51	0.04	0.04	0.51	0.49
	Residuals	12	0.98	0.08			0.96	0.08		
	Subplot Effects									
	PLH	1	0.01	0.01	0.08	0.79	0.22	0.22	7.61	0.02
	PLH x Cultivar	1	0.0003	0.0003	0.003	0.96	0.02	0.02	0.62	0.45
	PLH x Nitrogen	1	0.06	0.06	0.60	0.45	0.02	0.02	0.65	0.43
	Cultivar x Nitrogen x PLH	1	0.07	0.07	0.71	0.42	0.04	0.04	1.25	0.28
	Residuals	12	1.11	0.09			0.35	0.03		
Nitrogen Biomass (g of N)	*Main Effects*									
	Cultivar	1	0.0002	0.0002	2.86	0.12	0.0003	0.0003	4.37	0.06
	Nitrogen	1	0.0001	0.0001	0.98	0.34	0.0001	0.0001	1.61	0.23
	Cultivar x Nitrogen	1	0.0002	0.0002	3.65	0.08	0.0003	0.0003	3.87	0.07
	Residuals	12	0.0008	0.0001			0.0009	0.0001		
	Subplot Effects									
	PLH	1	0.0001	0.0001	1.30	0.28	0.0001	0.0001	1.22	0.29
	PLH x Cultivar	1	0.0001	0.0001	1.03	0.33	0.0001	0.0001	0.93	0.35
	PLH x Nitrogen	1	0.0001	0.0001	0.00	0.98	0.0001	0.0001	1.54	0.24
	Cultivar x Nitrogen x PLH	1	0.0002	0.0002	2.16	0.17	0.0001	0.0001	2.45	0.14
	Residuals	12	0.0009	0.0001			0.0007	0.0001		

Table 2.6 Split plot ANOVA (1 main plot factor, 1 subplot factor) results for belowground samples of fixing plants from field study collected on July 31, 2018.

			Crowns				*Roots*			
Parameter	**Source**	**df**	**SS**	**MS**	**F value**	**p-value**	**SS**	**MS**	**F value**	**p-value**
%Ndfa	*Main Effects*									
	Block	3	1837	612	0.37	0.78	1200	400	0.70	0.61
	Nitrogen	1	526	526	0.32	0.61	2065	2065	3.60	0.15
	Residuals	3	4914	1638			1723	574		
	Subplot Effects									
	PLH	1	3374	3374	5.76	0.06	11.10	11.08	0.01	0.94
	Nitrogen x PLH	1	7696	7696	13.13	0.02	8.50	8.54	0.004	0.95
	Residuals	6	2931	586			10206	2041		
Fixed Nitrogen Biomass (g of N_{fixed})	*Main Effects*									
	Block	3	0.0003	0.0001	0.62	0.65	0.0001	0.0001	1.05	0.48
	Nitrogen	1	0.0001	0.0001	0.06	0.82	0.0001	0.0001	2.05	0.25
	Residuals	3	0.0005	0.0002			0.0001	0.0001		
	Subplot Effects									
	PLH	1	0.0002	0.0002	4.59	0.09	0.0001	0.0001	0.0008	0.98
	Nitrogen x PLH	1	0.0005	0.0005	10.70	0.02	0.0001	0.0001	0.007	0.94
	Residuals	6	0.0002	0.00005			0.0007	0.0001		

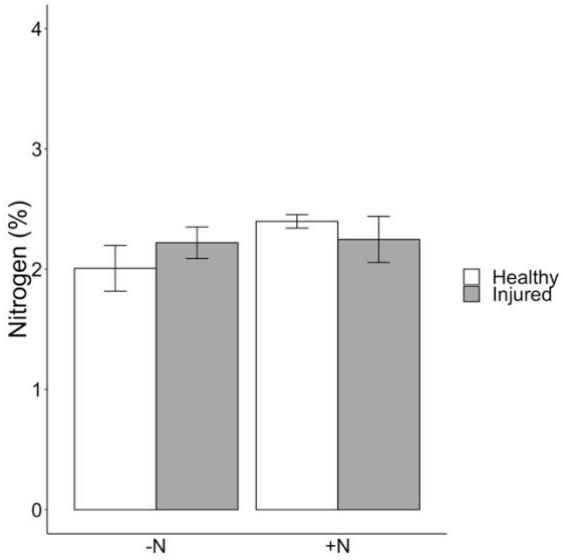

Figure 2.5 Percentage nitrogen for crown samples of fixing plants without nitrogen fertilizer (-N) and with (+N) collected on July 31, 2018; Healthy = No PLH Added, Injured = PLH Added; -N Healthy – Injured p-value = 0.38, +N Healthy – Injured p-value = 0.50

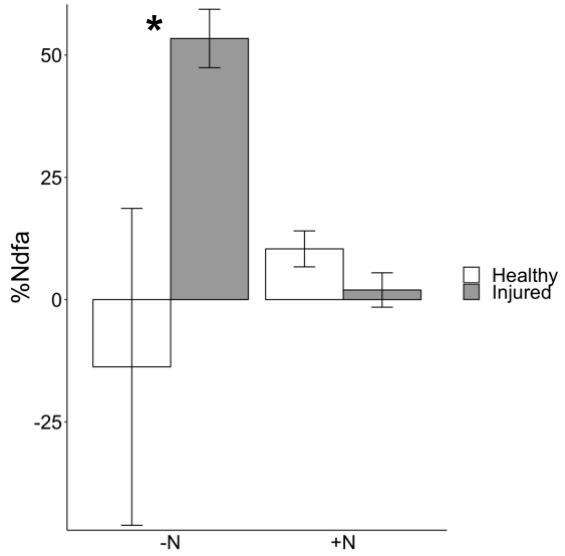

Figure 2.6 Percentage nitrogen derived from the atmosphere (%Ndfa) for crown samples of fixing plants without nitrogen fertilizer (-N) and with (+N) collected on July 31, 2018; Healthy = No PLH Added, Injured = PLH Added; -N Healthy – Injured p-value = 0.0272, +N Healthy – Injured p-value = 0.737; * denotes significant difference ($p < 0.05$)

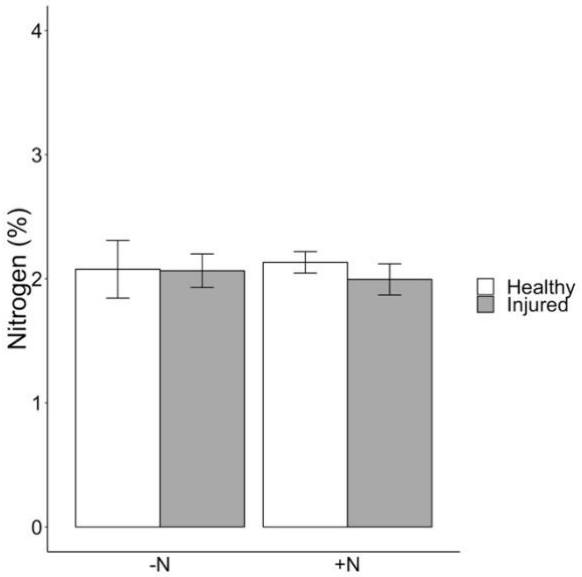

Figure 2.7 Percentage nitrogen for root samples of fixing plants without nitrogen fertilizer (-N) and with (+N) collected on July 31, 2018; Healthy = No PLH Added, Injured = PLH Added; -N Healthy – Injured p-value = 0.956, +N Healthy – Injured p-value = 0.492

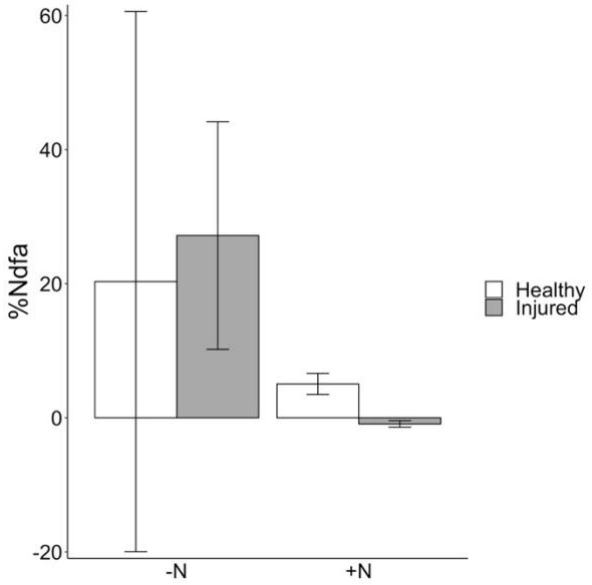

Figure 2.8 Percentage nitrogen derived from the atmosphere (%Ndfa) for root samples of fixing plants without nitrogen fertilizer (-N) and with (+N) collected on July 31, 2018; Healthy = No PLH Added, Injured = PLH Added; -N Healthy – Injured p-value = 0.80, +N Healthy – Injured p-value = 0.812

Table 2.7 Whole plant samples from the greenhouse study. Numbers represent means +/- standard deviation; Healthy = No PLH Added, Injured = PLH Added

	Non-Fixing		**Fixing**	
	Healthy	Injured	Healthy	Injured
Shoots				
Dry Weight (g)	0.03 ± 0.02	0.03 ± 0.01	0.2 ± 0.1	0.4 ± 0.3
Nitrogen (%)	0.8 ± 0.2	0.6 ± 0.1	3.8 ± 0.2	3.2 ± 0.5
Nitrogen Biomass (g of N)	0.0002 ± 0.0002	0.0002 ± 0.00008	0.009 ± 0.005	0.01 ± 0.01
Crowns				
Dry Weight (g)	0.03 ± 0.02	0.02 ± 0.01	0.09 ± 0.05	0.2 ± 0.2
Nitrogen (%)	0.6 ± 0.07	0.7 ± 0.1	1.8 ± 0.6	1.3 ± 0.5
Nitrogen Biomass (g of N)	0.0002 ± 0.0001	0.0001 ± 0.0001	0.002 ± 0.001	0.003 ± 0.005
Roots				
Dry Weight (g)	0.05 ± 0.02	0.03 ± 0.02	0.1 ± 0.08	0.2 ± 0.2
Nitrogen (%)	1.0 ± 0.1	1.1 ± 0.2	1.9 ± 0.5	1.8 ± 0.4
Nitrogen Biomass (g of N)	0.0005 ± 0.0003	0.0003 ± 0.0003	0.003 ± 0.002	0.004 ± 0.004

Table 2.8 Two-way ANOVA results for whole plant samples from the greenhouse study.

			Shoots				*Crowns*				*Roots*			
Parameter	**Source**	**df**	**SS**	**MS**	**F value**	**p-value**	**SS**	**MS**	**F value**	**p-value**	**SS**	**MS**	**F value**	**p-value**
Dry Weight (g)	Cultivar	1	0.65	0.65	20.63	<0.001	0.11	0.11	9.61	0.004	0.14	0.14	18.03	<0.001
	PLH	1	0.05	0.05	1.58	0.22	0.02	0.02	1.49	0.23	0.01	0.01	0.72	0.40
	Cultivar x PLH	1	0.05	0.05	1.58	0.22	0.02	0.02	1.92	0.18	0.02	0.02	2.12	0.16
	Residuals	28	0.88	0.03			0.32	0.01			0.21	0.01		
Nitrogen (%)	Cultivar	1	61.8	61.8	669.86	<0.001	7.22	7.22	43.65	<0.001	4.55	4.55	39.13	<0.001
	PLH	1	0.98	0.98	10.59	0.003	0.40	0.40	2.40	0.13	0.00	0.00	0.00	0.97
	Cultivar x PLH	1	0.41	0.41	4.46	0.04	0.63	0.63	3.83	0.06	0.09	0.09	0.80	0.38
	Residuals	28	2.58	0.09			4.63	0.17			3.26	0.12		
Nitrogen Biomass (g of N)	Cultivar	1	0.001	0.001	17.42	<0.001	0.0001	0.0001	7.44	0.01	0.0001	0.0001	14.49	<0.001
	PLH	1	0.00004	0.00004	0.73	0.40	0.0001	0.0001	0.76	0.39	0.0001	0.0001	0.69	0.41
	Cultivar x PLH	1	0.00004	0.00004	0.76	0.39	0.0001	0.0001	0.79	0.38	0.0001	0.0001	1.08	0.31
	Residuals	28	0.002	0.0001			0.0001	0.0001			0.0001	0.0001		

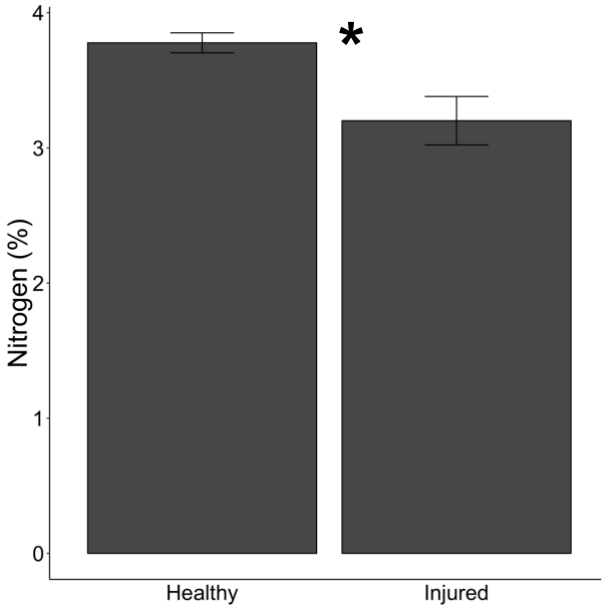

Figure 2.9 Percentage nitrogen for shoot samples of fixing plants from the greenhouse study; Healthy = No PLH Added, Injured = PLH Added; Healthy – Injured p-value = 0.0151; * denotes significant difference ($p < 0.05$)

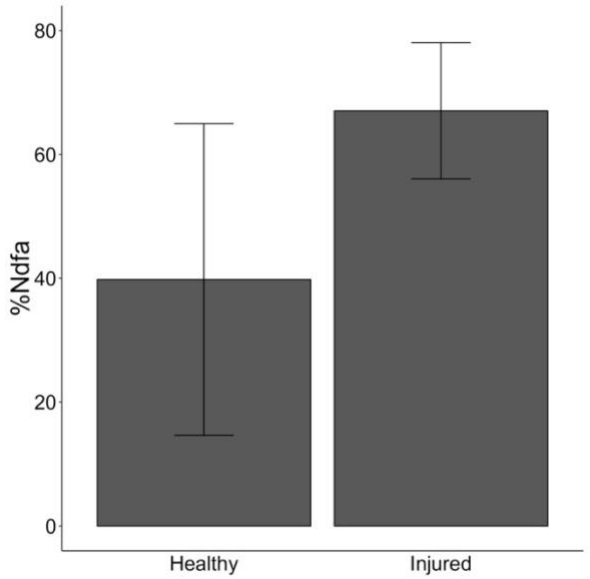

Figure 2.10 Percentage nitrogen derived from the atmosphere (%Ndfa) for shoot samples of fixing plants from greenhouse study; Healthy = No PLH Added, Injured = PLH Added; Healthy – Injured p-value = 0.3451

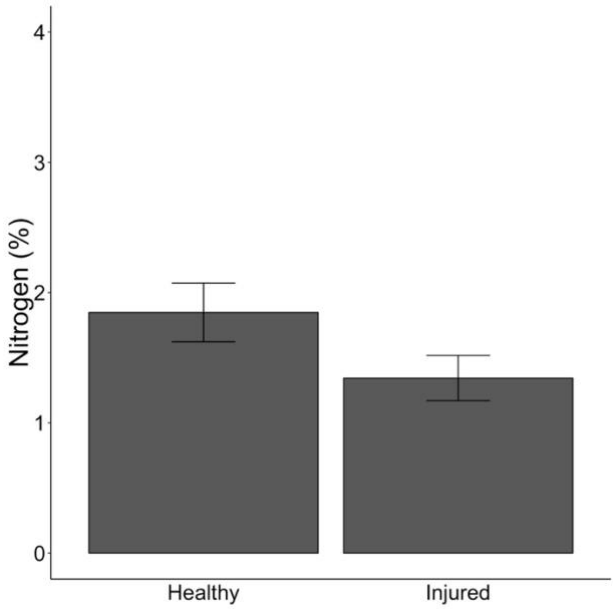

Figure 2.11 Percentage nitrogen for crown samples of fixing plants from the greenhouse study; Healthy = No PLH Added, Injured = PLH Added; Healthy – Injured p-value = 0.09962

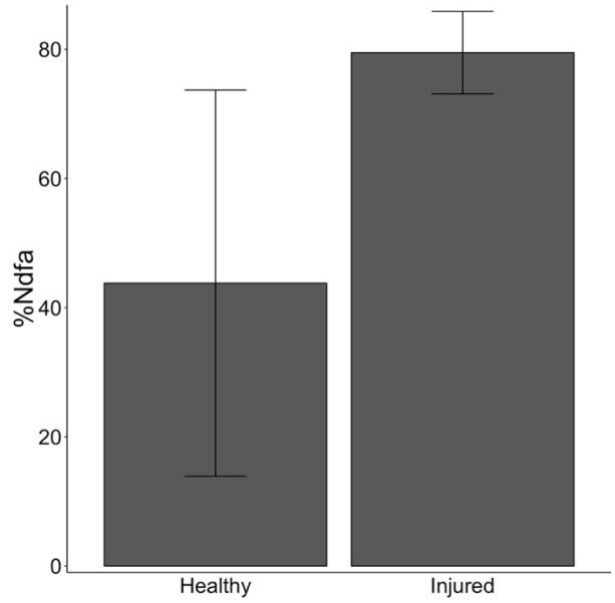

Figure 2.12 Percentage nitrogen derived from the atmosphere (%Ndfa) for crown samples of fixing plants from greenhouse study; Healthy = No PLH Added, Injured = PLH Added; Healthy – Injured p-value = 0.278

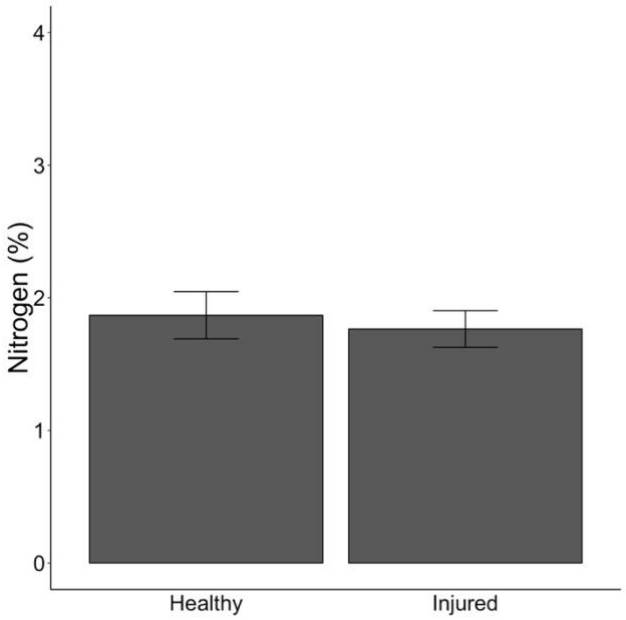

Figure 2.13 Percentage nitrogen for root samples of fixing plants from the greenhouse study; Healthy = No PLH Added, Injured = PLH Added; Healthy – Injured p-value = 0.6524

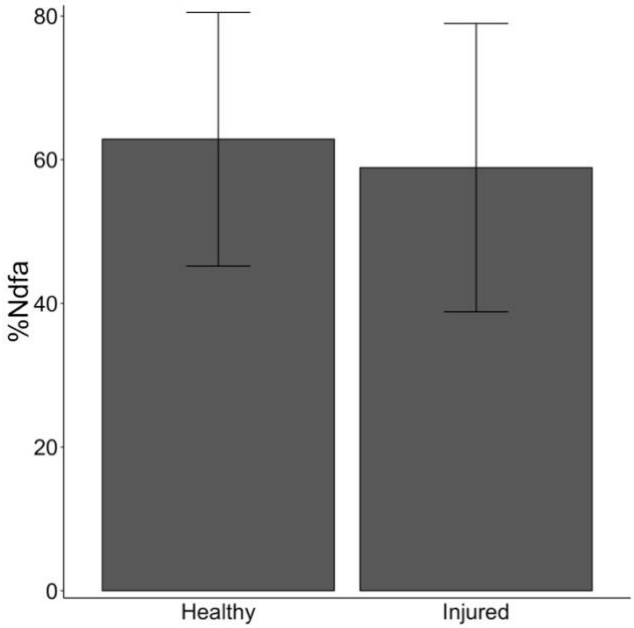

Figure 2.14 Percentage nitrogen derived from the atmosphere (%Ndfa) for root samples of fixing plants from greenhouse study; Healthy = No PLH Added, Injured = PLH Added; Healthy – Injured p-value = 0.8843

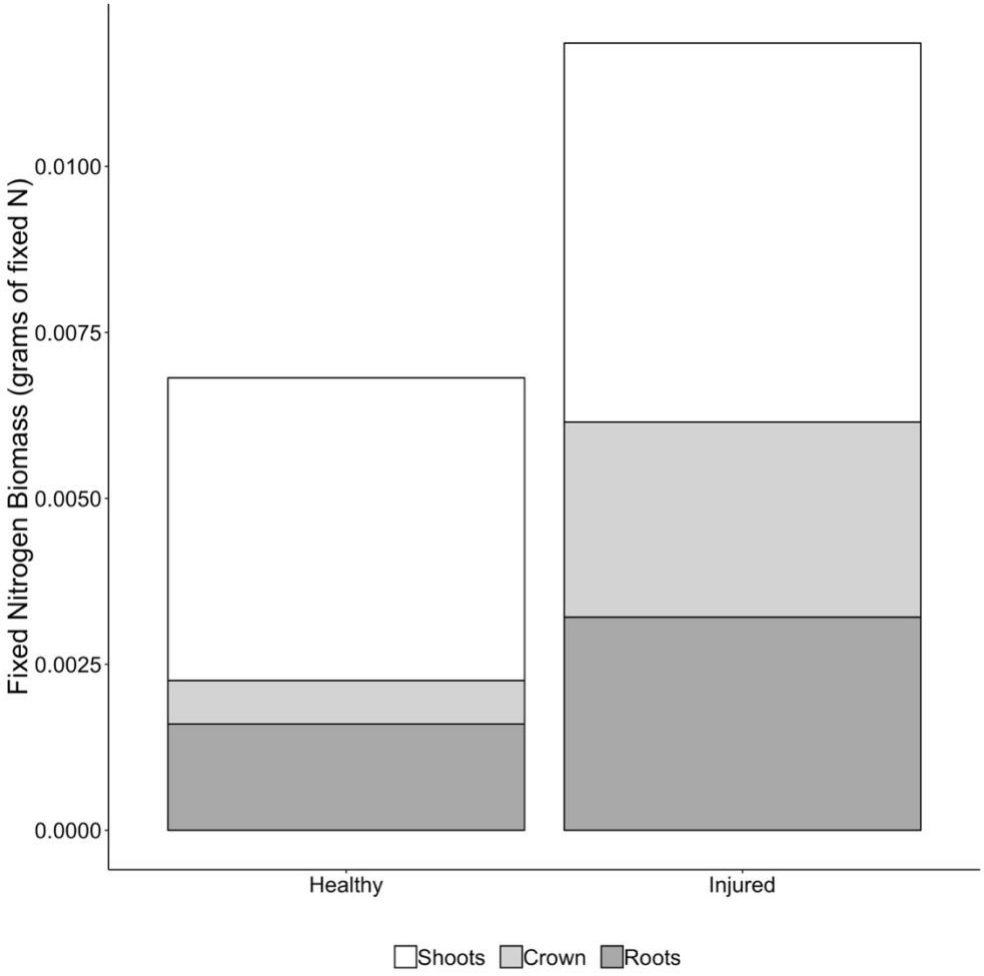

Figure 2.15 Fixed nitrogen biomass (grams of fixed nitrogen) and allocation across whole plant samples; Healthy = No PLH Added, Injured = PLH Added; Healthy Shoots – Injured Shoots p-value = 0.7032; Healthy Crowns – Injured Crowns p-value = 0.2003; Healthy Roots – Injured Roots p-value = 0.3236

Appendices

Appendix A: Nitrogen-free Hoagland's Solution

Stock solutions:

1. KH_2PO_4 – In a one liter Erlenmeyer flask, dissolve 136.1 g. potassium phosphate monobasic (KH_2PO_4) in small aliquots in ca. 800 mL HPLC grade water. Pour into a one liter volumetric and adjust volume with HPLC grade water. Store in refrigerator door.
2. $MgSO_4$ – In a one liter Erlenmeyer flask, dissolve 82.3 g. magnesium sulfate heptahydrate ($MgSO_4\,7H_2O$) in ca. 800 mL HPLC grade water. Pour into a one liter volumetric and adjust volume with HPLC grade water. Store in refrigerator door.
3. $FeSO_4$/EDTA – In a one liter Erlenmeyer flask, dissolve 2.5425 g. ferrous sulfate ($FeSO_4\,7H_2O$) and 1.85750 g. ethylene diamine tetra-acetic acid (EDTA) to ca. 800 mL HPLC grade water. Pour into a one liter volumetric and adjust volume with HPLC grade water. Store in refrigerator door.
4. Micronutrients - In a one liter Erlenmeyer flask, dissolve 3.728 g. potassium chloride (KCl), 1.544 g. boric acid (H_3BO_3), 0.339 g. manganese sulfate monohydrate ($MnSO_4\,H_2O$), 0.576 g. zinc sulfate ($ZnSO_4\,7H_2O$), 0.124 g. cupric sulfate ($CuSO_4\,5H_2O$), 0.08 g. molybdic acid (H_2MoO_4 (85% MoO_8)), 0.088 g. cobalt chloride hexahydrate ($CoCl_2\,6H_2O$) in ca. 800 mL HPLC grade water. Pour into a one liter volumetric and adjust volume with HPLC grade water. Store in refrigerator door.

Final solution in a 20 liter plastic jug:

1. Add RO water to 12-16 L mark.
2. Aerate water with glass tube throughout entire procedure to mix well.
3. Add 120 mL KH_2PO_4 stock solution, aerate for 5 minutes.
4. Add 60 mL $MgSO_4$ stock solution, aerate for 5 minutes.
5. Add 80 mL $FeSO_4$/EDTA stock solution, aerate for 5 minutes.
6. Add 20 mL Micronutrient stock solution, aerate for 5 minutes.
7. Add 10.94 g. calcium sulfate anhydrous ($CaSO_4$) in small quantities to allow for dissolving.
8. Fill with RO water to 20 L mark
9. Aerate for 60 minutes to completely dissolve

Appendix B: Discussion of natural nitrogen isotope ratios

Isotopes are defined as atoms of the same element containing equal numbers of protons but different numbers of neutrons. Hence, isotopes of the same element differ slightly in their atomic masses, resulting in relatively 'heavier' and 'lighter' isotopes. The two naturally occur nitrogen isotopes are ^{14}N and ^{15}N. To determine the isotopic properties of a material, $\delta^{15}N$ values are measured and reported as parts per thousand or per mil (‰), as seen in the equation below:

$$\delta^{15}N\ (‰) = \left(\frac{R_s - R_{ref}}{R_{ref}}\right) = \left(\frac{R_s}{R_{ref}} - 1\right) \times 1000$$

R_s and R_{ref} refer to the sample and reference isotopic ratios ($^{15}N/^{14}N$). The nitrogen isotope ratio of air is the international standard for R_{ref}. In the atmosphere, 99.636% of all nitrogen isotopes are ^{14}N (and 0.364% are ^{15}N). Therefore, $R_{ref} = {}^{15}N/{}^{14}N = 0.364/99.636 = 0.0036533$.

R_s is determined by GC-IRMS (gas chromatography isotope ratio mass spectrometry), compared to R_{ref}, and reported as $\delta^{15}N$ values. Differences between R_{ref} and R_s can be relatively minute which is why the values are reported as parts per thousand (‰). For instance, consider the following example:

$$\delta^{15}N\ (‰) = \left(\frac{R_s}{R_{ref}} - 1\right) = \left(\frac{0.0036520}{0.0036533} - 1\right) = -0.00035584 \times 1000 = -0.3558\ ‰$$

This example highlights how representing values as parts per thousand makes the differences between R_s and R_{ref} easier to discern, particularly when both R values are similar. It also illustrates how one may obtain negative $\delta^{15}N$ values. Additionally, it is important to note that for organisms engaging primarily in biological nitrogen fixation to meet their nitrogen demands, the atmosphere is their dominant source of nitrogen. Therefore, R_s values for nitrogen fixing organisms should closely resemble the atmosphere R, which is also R_{ref}. Nitrogen-fixers, hence, typically possess $\delta^{15}N$ values very close to 0‰.

Appendix C: Calculating %Ndfa (% Nitrogen derived from the atmosphere) in plants

Plants generally obtain nitrogen from the soil but can also obtain nitrogen from specialized interactions with nitrogen-fixing microbes, such as Rhizobia. Rhizobia extract inert nitrogen gas (N_2) from the atmosphere and use enzymes (nitrogenase) to ultimately produce ammonia (NH_3), which the plant takes up and assimilates primarily into amino acids. Plants may transport amino acids aboveground or utilize these molecules belowground, depending on the biological needs of the plant.

A non-fixing reference plant accounts for the contribution of soil nitrogen to the isotopic signature of the fixing plant. In other words, the $\delta^{15}N$ value of the fixing plant should fall somewhere between the $\delta^{15}N$ value of the non-fixing plant (which relies entirely on soil nitrogen) and the $\delta^{15}N$ value of the atmosphere. Essentially, rather than measuring soil nitrogen contributions, we can measure the non-fixing reference plant as a proxy for soil nitrogen.

^{15}N Natural Abundance Equation:

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

^{15}N Isotope Dilution Equation:

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

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