IMPACT OF NITROGEN AND RHIZOBIAL SEED INOCULANTS ON SOYBEAN APHID (*APHIS GLYCINES* MATSUMURA) DENSITIES

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By
Samantha Marie Brunner

In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Program:
Natural Resources Management
Entomology Option

July 2012

Fargo, North Dakota

North Dakota State University
Graduate School

Title

IMPACT OF NITROGEN AND RHIZOBIAL SEED INOCULANTS ON SOYBEAN APHID (*APHIS GLYCINES* MATSUMURA) DENSITIES

By

Samantha Marie Brunner

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

Deirdre Prischmann-Voldseth
Chair

R. Jay Goos

Janet Knodel

Jason Harmon

Malcolm Butler

Approved by Department Chair:

1 August 2012
Date

Carolyn Grygiel
Signature

ABSTRACT

Soybeans are able to obtain nitrogen from two different sources, nitrogen found in the soil (e.g. from fertilizers) and biologically fixed nitrogen (from symbiotic bacteria called rhizobia). Nitrogen source and degree of reliance on N-fixation can impact plant nitrogen dynamics, which has the potential to impact above-ground herbivore performance. We examined the impact of nitrogen availability and rhizobial association on soybean aphid biology and reproduction in a series of greenhouse and field experiments. Aphid establishment on plants was not significantly affected in any experiment. However, aphid reproduction was significantly affected by rate of nitrogen fertilization, rhizobial inoculation, and type of rhizobial seed inoculant. In general, aphid densities were not correlated with plant parameters associated with plant nitrogen or N-fixation. Producers commonly use fertilizers and rhizobial seed inoculants, thus it is important to continue exploring the mechanisms underlying how plant nitrogen dynamics impact soybean insect pests.

ACKNOWLEDGEMENTS

I would like to offer many thanks to Dr. Deirdre Prischmann-Voldseth, Dr. R. Jay Goos, Gene Schmidt, Brian Johnson, Frances Podrebarac, Don Carey, Stephanie Swenson, all the undergraduates who have worked in our lab and the staff members at the Carrington Research and Extension Center who helped me with my research. I also thank the North Dakota State Board of Agricultural Research and Education, The North Dakota Soybean Council, and the NDSU Agricultural Experiment Station Graduate Student Fellowship for funding.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX TABLES

LIST OF APPENDIX FIGURES

LITERATURE REVIEW

Introduction

Cultivated soybean, *Glycine max* (L.) Merrill, has been grown in China for over 4,000 years (Wu et al. 2004). Soybeans were first grown in the United States in 1765 and various food products (e.g. soy sauce and soybean noodles) were shipped back to England (Hymowitz and Harlan 1983). Soybean production in the United States slowly increased due to their use as cover crops and animal feed (Hymowitz and Harlan 1983), especially in the 1920's when people began growing it for food and oil rather than for forage (Hymowitz 1990). Since their introduction, soybeans have become one of the most important agricultural commodities in the United States. For example, in 2010, 77.4 million acres were planted to soybeans, which produced 3.33 billion bushels of seed worth approximately $3.7 billion (USDA 2012).

Sources of Nitrogen

In agricultural systems plant growth and yield are often impacted by the supply of biologically available nitrogen (Vidal and Gutierrez 2008). Nitrogen is a macronutrient, which means it is needed in large quantities for proper growth and development of all life forms, including plants (Vidal and Gutierrez 2008). Plants primarily obtain nitrogen from compounds such as nitrate (NO_3^-), ammonium (NH_4^+), or ammonia (NH_3) that are found in the soil or originate from fertilizers (Brooker 2008). Legumes such as soybeans are unique because they can also acquire nitrogen from nitrogen gas (N_2) via the process of biological nitrogen fixation.

Nitrogen Fertilizer

Nitrogen fertilizer comes in several forms, including ammonium nitrate, ammonium sulfate, anhydrous ammonia, ammonia, and urea (Johnson 2011). Each form of nitrogen fertilizer possesses certain qualities that may make it more suitable for certain fields depending on the

conditions (Johnson 2011). Nitrogen is easily lost in agricultural systems, making the addition of fertilizer extremely important for proper plant growth (Johnson 2011). In 2009, farmers in the United States applied over 22 million tons of nitrogen products to agricultural crops (USDA 2011). In 2010, 97% and 90% of acres planted to corn and cotton, respectively, received nitrogen fertilizer (USDA 2011). For comparison, in 2006, only 18% of the acres planted to soybean were fertilized (USDA 2011). This reduced fertilization rate is due to the ability of soybean plants to obtain and utilize biologically fixed nitrogen.

Biologically Fixed Nitrogen

Symbiotic relationships between plants and soil organisms can have dramatic impacts on plant physiology and fitness. One important group of plant symbionts are bacteria called rhizobia (Rhizobiaceae), which have been well studied because of their ability to fix atmospheric nitrogen gas (N_2) and the subsequent positive impacts of this process on plant growth and yield. We refer to fixed nitrogen as nitrogen that has been converted from a biologically unavailable form (e.g. atmospheric nitrogen), to a form that is biologically available to plants for growth and development. Nitrogen can be fixed by abiotic occurrences (e.g. when lightning strikes the soil, Falkowski 1997), or more commonly by biological organisms.

Biological nitrogen fixation is carried out by several species and/or strains of both free-living and symbiotic diazotrophs (bacteria and Archaea) (Postgate 1982, Sprent and Sprent 1990, Raymond et al. 2004). Unlike free-living diazotrophs, most symbiotic taxa (i.e. rhizobia) need to form a mutualistic relationship with plant roots in order to fix nitrogen, and are primarily associated with legumes (Fabaceae) such as peas, beans, clover, and some non-leguminous grasses (Poaceae) (Postgate 1982, Sprent and Sprent 1990). Rhizobia-plant mutualisms are

highly specific, with one rhizobia species usually associated with plants in only a few genera (Postgate 1982, van Rhijn and Vanderleyden 1995).

N-Fixation in Soybean

Bradyrhizobium japonicum (Bradyrhizobiaceae) is a Gram-negative soil rhizobium that associates with cultivated (*G. max*) and wild soybean (*G. soja* Sieb. et Zucc.) (van Rhijn and Vanderleyden 1995). Initially, the bacteria migrate through the soil toward the roots in response to root exudates (Loh and Stacey 2003, Gage 2009). The root exudates, typically flavonoids, trigger the activation of *nod* genes within the bacteria, which are responsible for producing lipochitin oligosaccharides called nodulation, or Nod, factors (Loh and Stacey 2003, Skorupska et al. 2010). The Nod factors cause the root hairs to curl around the bacteria, eventually forming a spherical nodule after the bacteria have attached to the root hairs and formed infection threads (Postgate 1982, Loh and Stacey 2003, Gage 2009, Skorupska et al. 2010). When functioning properly (i.e. fixing nitrogen) the root nodules are pink inside (Ohyama et al. 2009).

Leguminous plants are categorized into two groups depending on the major form in which they transport fixed nitrogen. Soybeans belong to the ureide-transporters, which are typically tropical leguminous species; other legumes transport fixed nitrogen in the form of amides and typically originate in temperate regions (Pate 1980, Schubert 1986, Winkler et al. 1988, Sinclair and Serraj 1995). Clover and peanut are amide-transporters while soybeans and cowpeas are ureide transporters (Schubert, 1986).

Soybean nodules are different from peanut nodules (amide transporter) in that their structure allows more water to flow though the nodule (Winkler et al. 1988). The increased water is believed to be important because ureides are less soluble than asparagine, which is a major product of nitrogen fixation in amide transporters (Winkler et al. 1988). Since soybeans and

other ureide transporting legumes originate in tropical areas they evolved with access to more water than other legumes, and are thus more susceptible to drought conditions (Winkler et al. 1988, Sinclair and Serraj 1995).

Impact of Nitrogen Source on Nodulation, N-Fixation, and Nitrogenous Compounds

Soybeans can obtain virtually all of their nitrogen from N-fixation (Keyser and Li 1992, Peoples et al. 2009), however, more commonly they receive between 25 and 75% of their nitrogen as fixed-N (Deibert et al. 1979, Peoples et al. 2009). Several factors can impact the strength of rhizobial-plant associations and subsequent N-fixation rates, most notably soil nitrogen (e.g. nitrate) levels. When the soil contains adequate nitrogen, either from organic matter or synthetic fertilizer, the plant will use those sources before associating with rhizobia (Evans 1982, Ohyama et al. 2009). A meta-analysis determined that in the absence of fertilizer, N-fixation provides a maximum of 337 kg ha^{-1} of nitrogen, which decreases with the addition of nitrogen fertilizer (Salvagiotti et al. 2008). Using this model, if 300 kg ha^{-1} N fertilizer were added, one could expect only 17 kg ha^{-1} of nitrogen to be fixed (Salvagiotti et al. 2008). Plants supplied with adequate soil nitrogen do form nodules and fix a small amount of nitrogen, however, the amount of nitrogen fixed is much lower than when plants are in a low nitrogen environment and must obtain the majority of nitrogen from N-fixation (Patterson and LaRue 1983a, Herridge and Brockwell 1988). In general, plants that associate with rhizobia are taller and have increased biomass and grain yield versus plants primarily getting their nitrogen from fertilizer (McClure and Israel 1979, Johnson et al. 1987, Herridge et al. 2008, Salvagiotti et al. 2008, Katayama et al. 2011).

Even when in a low nitrogen (nitrate) environment, it takes 3-5 weeks from planting before nodules begin functioning and supplying nitrogen to the plant (Thibodeau and Jaworski

1975). During those first few weeks the plant needs an alternative source of nitrogen (Harper and Hageman 1972). As the plant grows, the level of N-fixation changes and peak activity happens during beginning pod (R3) to beginning seed (R5) growth stages (Thibodeau and Jaworski 1975, Zapata et al. 1987).

The source of nitrogen used by soybeans (from fertilization or N-fixation) can impact the identity and concentration of nitrogenous compounds within plant tissues. Nitrogen fertilizers are absorbed by the roots as NO_3^- or NH_4^+ (Ohyama et al. 2009, Witte 2011). Once in the plant root, most of the NO_3^- moves into the xylem while some is converted into the amino acid asparagine prior to being transported into the xylem (McClure and Israel 1979, Reynolds et al. 1982, Schubert 1986, Winkler et al. 1988, Sprent and Sprent 1990, Ohyama et al. 2009). One study indicated that the distribution of nitrogenous compounds in the xylem sap of non-nodulated soybeans (e.g. plants getting their nitrogen from primarily from fertilizer) was 58% nitrate, 36% asparagine, and 6% ureides (Shelp and Da Silva 1990).

In nodulated soybean plants, *Bradyrhizobium japonicum* fixes N_2 into ammonia (NH_3), which is changed to ammonium (NH_4^+), and then allantoin and allantoate (allantoic acid) within root nodules (Reynolds et al. 1982, Schubert 1986, Sprent and Sprent 1990, Todd et al. 2006, Ohyama et al. 2009, Strodtman and Emerich 2009). The latter two compounds are called ureides and are the primary nitrogenous compounds formed during the process of N-fixation in soybean (Reynolds et al. 1982, Schubert 1986, Sprent and Sprent 1990, Ohyama et al. 2009, Strodtman and Emerich 2009). The root nodule utilizes compounds from phloem sap and mitochondria to complete nitrogen fixation (Reynolds et al. 1982, Schubert 1986, Sprent and Sprent 1990, Ohyama et al. 2009, Strodtman and Emerich 2009). After the nitrogen has been fixed it is transported into the xylem mainly as ureides (allantoin and allantoate), along with some

asparagine and small amounts of other amino acids (especially glutamine, aspartic acid, and arginine), ammonium, and nitrate (Fujihara and Yamaguchi 1978, McClure and Israel 1979, Reynolds et al. 1982, van Berkum et al. 1985, Schubert 1986, Shelp and Da Silva 1990, Sprent and Sprent 1990, Peoples et al. 1991, Ohyama et al. 2009). The Shelp and Da Silva study (1990) found the distribution of nitrogenous compounds in the xylem sap of nodulated soybean was 78% ureides, 20% asparagine, and 2% nitrate. In general, the xylem sap of nodulated soybeans is made of 60-95% ureides (McClure and Israel 1979, Schubert 1981).

Transport and Metabolism of Nitrogenous Compounds

The xylem transports nutrients from the roots to the upper parts of the plant. In young soybean tissues, such as newly expanding shoots (actively growing tissue, i.e. nutrient sinks), nitrogenous compounds (especially amino acids) can pass directly from the xylem to the phloem (direct transfer; Da Silva and Shelp 1990, Shelp and Da Silva 1990). However, in most cases nitrogenous products move from xylem into the mesophyll of mature leaves (i.e. nutrient sources) prior to being metabolized and/or translocated into phloem (i.e. indirect transfer; Ohyama and Kawai 1983, Shelp and Da Silva 1990). Within leaves nitrogenous products are also used to synthesize proteins, some of which (e.g. storage proteins) are later degraded into amino acids and amides and exported via the phloem in times of stress or when nitrogen is needed by specific tissues, such as the roots or developing seeds (Ohyama and Kawai 1983, Staswick 1994, Ohyama et al. 2009).

In leaf mesophyll nitrate (NO_3^-) is reduced by nitrate reductase into nitrite and then ammonium, which is then used to make various amino acids and proteins (Miflin and Lea 1977, Schubert 1986, Lea and Ireland 1999). Nitrate reductase activity varies by leaf and plant age (Harper and Hageman 1972, Tingey et al. 1974, Thibodeau and Jaworski 1975, Li and Gresshoff

1990) and environmental factors often associated with photosynthesis such as light (Nicholas et al. 1976) and CO_2 (Kaiser and Förster 1989).

With regard to ureide metabolism, first the enzyme allantoinase breaks down allantoin into allantoate (reviewed in Todd et al. 2006). The next step is the degradation of allantoate to ureidoglycolate which can happen via two different pathways depending on the cultivar, either by allantoate amidohydrolase or allantoate amidinohydrolase (Vadez and Sinclair 2000, Todd et al. 2006). Ureidoglycolate is then broken down into ammonium (via urea) or glyoxylate (Vadez and Sinclair 2000, Todd et al. 2006). The activity of enzymes that degrade ureides vary depending on plant growth stage (Thibodeau and Jaworski 1975, Thomas and Schrader 1981, Winkler et al. 1988), and can be impacted by environmental factors, especially water stress (Vadez and Sinclair 2000, King and Purcell 2005) and manganese (Lukaszewski et al. 1992).

Ultimately, ureide metabolites are used in the formation of amino acids and proteins (Ohyama et al. 2009), although non-metabolized ureides are also exported in the phloem to some degree (Atkins et al. 1982, Shelp and Da Silva 1990). Petiole girdling experiments that blocked phloem transport showed that the amino acid asparagine is one of the primary forms of nitrogen transported in the phloem of soybean (Ohyama and Kawai 1983, Ohyama et al. 2009) and peanut (*Arachis hypogaea* L., Andersen et al. 2002).

Nitrogenous compounds within the phloem are transported to nutrient sinks, but the allocation of nitrogenous compounds to different parts of the plant can vary depending on plant age and the original source of nitrogen (McNeil and LaRue 1984, Ohyama et al. 2009). One study indicated that although nitrogen source (NO_3^- or N_2) affected the relative quantity of nitrogenous compound in the xylem, the distribution of compounds (amino acids and ureides) in

the phloem sap of select leaflets was similar, although they did not assess specific amino acids and only assessed plants at one point in time (McNeil and LaRue 1984).

Quantifying N-Fixation

Several methods have been developed to quantify the N-fixation activity within soybean plants. Nodule mass is correlated with foliar ureides and N-fixation (van Berkum et al. 1985), although location of the nodules within the root system can impact the degree of N-fixation (Hardarson et al. 1989). In addition, the abundance of ureides in above-ground plant tissue has been used as an indicator of N-fixation (Herridge 1982a, Patterson and LaRue 1983b, van Berkum et al. 1985, Herridge et al. 2008). However, the relationship is not always perfect, as senescing tissues (e.g. cotyledons) can release ureides (Herridge 1982b, Reynolds et al. 1982, Todd et al. 2006), nitrogen can be remobilized from older tissues and used in ureide biogenesis (Díaz-Leal et al. 2012), absorbed ammonium can be converted into ureides in non-nodulated plants (Herridge 1982b), and reduced ureide degradation can cause ureides levels to build up in foliar tissues (Vadez and Sinclair 2000, King and Purcell 2005).

Rhizobial Seed Inoculants

The positive impact of N-fixation on agronomically important factors such as yield is one reason why producers commonly use rhizobial seed inoculants. Soybeans require more nitrogen than many agricultural crops (Sinclair and de Wit 1975), and thus it is important for them to associate with efficient rhizobia. Adding rhizobia to the seed at planting can decrease the time it takes to begin N-fixation and also ensures that the desired rhizobial strain is the dominant rhizobia in the root nodules (Graham 2009). Numerous abiotic and land management factors can impact N-fixing bacteria within the soil, including cropping history (Schippers et al. 1987), tillage (Paul and Clark 1996), soil organic matter (De Brito Alvarez et al. 1995), soil pH

(Schubert et al. 1990, Yang et al. 2001), and pesticide applications (Mallik and Tesfai 1985, Campo et al. 2009). Characteristics of the bacteria themselves can also influence plant-rhizobia associations and subsequent plant physiology, including low mobility of the bacteria, low diversity of native soil rhizobia, and the possibility of less effective rhizobia infecting a majority of the nodules (Graham 2009).

Although many commercial rhizobial inoculants only contain *B. japonicum*, the strain used can vary and inoculants often contain other components that are added to increase plant performance. Some products add another bacteria such as *Azospirillum brasilense* or *Delftia acidovorans*. *Azospirillum brasilense* also fixes nitrogen, however, it does not form root nodules (Groppa et al. 1998). This bacteria is not as species specific as rhizobia, but can associate with a broader range of plants and is often studied for its role in fixing nitrogen for grasses and cereal crops (Bashan and Levanony 1990). Soybean plants co-inoculated with *B. japonicum* and *A. brasilense* have an increased number of nodules, higher N-fixation (as assessed using the acetylene reduction assay), and increased nodule leghemoglobin (i.e. the compound responsible for a nodule's pinkish color, Groppa et al. 1998). The role *D. acidovorans* plays in the rhizobium-plant symbiosis or how it promotes plant growth is not as well represented in the literature. *Delftia acidovorans* is able to degrade the herbicide 2,4-D (Hoffmann et al. 1996, Muller et al. 1999), and some strains can infect humans and cause sepsis (Lang et al. 2012).

Some inoculants contain non-living components, such as lipo-chitooligosaccharides, which are involved with the process of nodule formation (Loh and Stacey 2003, Skorupska et al. 2010). There has been some research of lipo-chitooligosaccharides positively impacting the yield of tomatoes by shortening the time from planting to flowering (Chen et al. 2007). In general,

there has been little research on how the use of rhizobial seed inoculants impacts above-ground herbivores.

Soybean Aphids

Historically, there have been few major soybean insect pests in the United States. However, this changed in 2000, when soybean aphids (Hemiptera: Aphididae: *Aphis glycines* Matsumura), an invasive pest from China, were identified from Wisconsin (Venette and Ragsdale 2004). In 2001, soybean aphids were detected in about 60% of the growing region (Venette and Ragsdale 2004), and as of 2009 they are found in 30 states throughout the Midwest and eastern United States (Ragsdale et al. 2011). Computer models estimate the economic benefits of controlling this pest in the United States can reach over one billion dollars, which encompasses increased production costs (e.g. use of insecticides), lost acreage, and reduced production and yield (Kim et al. 2008).

Soybean aphids are phloem feeding insects that are heteroecious holocyclic, meaning that two different host plants are required to complete reproduction and that they reproduce sexually during part of their life cycle (Ragsdale et al. 2004). In the fall, winged males and females migrate to their primary host, buckthorn (Rhamnaceae: *Rhamnus*, Ragsdale et al. 2004). Soybean aphids have been shown to complete their life cycle on three buckthorn species, *R. cathartica* L. (common buckthorn), *R. alnifolia* L'Her (alderleaf buckthorn) and *R. lanceolata* Pursh (lanceleaf buckthorn) (Voegtlin et al. 2005). After the initial migrants feed on buckthorn, females mate with males and lay eggs that remain on buckthorn throughout the winter (Ragsdale et al. 2004). Eggs hatch in the spring and the females reproduce asexually on buckthorn for at least two generations before producing winged females that migrate to soybean, their preferred summer (secondary) host (Ragsdale et al. 2004).

Several factors and behaviors are involved in host plant selection by aphids (reviewed by Powell et al. 2006). Flying aphids use visual and olfactory cues for long-range detection of potential hosts. After landing, aphids will assess cues on the plant's surface, including epicuticular waxes and trichomes. In general, aphids move around on a leaf and make several shallow probes into the leaf epidermis. If the plant is still deemed acceptable they will probe deeper to find a phloem sieve element and on acceptable host plants will feed from one phloem sieve element for several hours.

Once established on a soybean plant, soybean aphids reproduce parthenogenically throughout the summer, which is when females reproduce asexually (Ragsdale et al. 2004). During this time the female aphids give live birth to more female aphids (Ragsdale et al. 2004). Male aphids do not appear until the fall (Ragsdale et al. 2004). When born, a female aphid has several generations of aphid nymphs already developing inside its body (i.e. telescoping generations; Ragsdale et al. 2004). These reproductive strategies facilitate rapid soybean aphid population growth, and field populations can quickly become high enough to surpass economic thresholds (250 aphids per plant on 80% of the crop with increasing populations, Ragsdale et al. 2007) and cause economic yield loss. Several factors can impact soybean aphid behavior, development, mortality, and reproduction, including host plant quality.

Impact of Plant Nitrogen on Foliar Insect Herbivores

Herbivorous insects feed on plant tissue to meet their nutrient needs for growth and reproduction. Common insect herbivores fall into two main categories: 1) chewing insects, which consume plant tissue such as leaves, stems, flowers, and roots, and 2) sucking insects, which use needle-like mouthparts to suck fluid out of vascular tissues (i.e. xylem and phloem). Herbivores are highly dependent on the nutritional quality of their food source, and plant nitrogen is often a

limiting factor in herbivore growth (reviewed by Mattson 1980). Abiotic and biotic factors can cause the nitrogen content of plant tissues to vary substantially, but in general phloem and xylem sap is much lower in nitrogen than solid plant tissue (e.g. leaves, stems; reviewed by Mattson 1980). Therefore, sucking insects such as aphids are usually more nitrogen limited than chewing insects.

Because phloem does not contain all of the amino acids necessary for growth and development, aphids have come to depend upon symbiotic bacteria in the genus *Buchnera* (Enterobacteriales; Douglas 1998, Douglas and van Emden 2007, Gunduz and Douglas 2009). *Buchnera* live within structures called bacteriocytes (or mycetocytes) within the aphid's haemocoel and they are able to synthesize amino acids for the insect (Douglas 1998, Douglas and van Emden 2007). Recent research has shown that the bacteria actually need some genes from the aphid in order to synthesize many essential and non-essential amino acids (Hansen and Moran 2011). Along with *Buchnera*, soybean aphids have a secondary symbiont, *Arsenophonus* sp. (Enterobacteriales) (Wille and Hartman 2009). Although the exact role of *Arsenophonus* in soybean aphids is unknown, in other aphid species secondary symbionts can contribute to aphid defense against parasitism and influence their tolerance to heat stress (Wille and Hartman 2009). However, even with obligate nutritional symbionts, aphids can still be impacted by the nitrogen content of their host plant.

Nitrogen Fertilizer and Herbivorous Insects

Several studies have shown that leaf nitrogen content increases with increasing nitrogen fertilization levels (Nevo and Coll 2001, Jahn et al. 2005, Chen and Ruberson 2008, Zehnder and Hunter 2009, Hosseini et al. 2010, Nowak and Komor 2010, Sauge et al. 2010). Since nitrogen is often a limiting resource for herbivores, it is no surprise that research in multiple cropping

systems has shown that aphids (Hemiptera: Aphididae), which are common sucking insect pests, show a preference for and have higher fitness when feeding on plants with adequate nitrogen compared to those low in nitrogen (Nowak and Komor 2010). Parameters that can be impacted by increased nitrogen fertilizer include higher aphid densities (Nevo and Coll 2001, Jahn et al. 2005), shorter development time, increased longevity, and increased body weight (Nevo and Coll 2001, Hosseini et al. 2010). However, use of nitrogen fertilizer can have neutral or even negative impacts on aphid fitness when rates become excessive, and studies have demonstrated that plants with intermediate nitrogen levels have the highest aphid densities (Zehnder and Hunter 2009, Sauge et al. 2010).

N-Fixation and Herbivorous Insects

The existing literature suggests that impacts of rhizobial presence and N-fixation on herbivore behavior and fitness is less straightforward than that of nitrogen fertilizers. Effects can vary depending on herbivore feeding habits (chewing versus sucking), and positive effects of increased plant nutrition can be offset or nullified by increased production of N-based plant defensive compounds (Kempel et al. 2009, Pineda et al. 2010) or potentially by phloem sap proteins (Kehr 2006). Several researchers have documented negative effects of rhizobia or factors associated with N-fixation on chewing herbivores. Thamer et al. (2011) found that adult Mexican bean beetles (Coccinellidae: *Epilachna varivestis* Mulsant), an oligophagous insect of a variety of legumes, preferred lima beans (Fabaceae: *Phaseolus lunatus* L.) that were grown without rhizobia to plants with rhizobia (collected from lima bean roots, genetically determined to be *Rhizobium*), even in the absence of visual cues. Likewise, in a separate study Wilson and Stinner (1984) found that *E. varivestis* larvae weighed less and had longer developmental times when fed leaves from plants grown without fertilizer and with rhizobia, and this effect was not

linked to foliar nitrogen levels. In addition, larvae weighed less and took longer to develop when fed artificial diets containing allantoin, a ureide formed during N-fixation in soybeans (Wilson and Stinner 1984). In field experiments with *E. varivestis*, soybean plants with the highest levels of defoliation had the lowest densities of nodules (Wilson and Stinner 1984).

Kempel et al. (2009) discovered moth larvae (Lepidoptera: Noctuidae, *Spodoptera littoralis* Boisduval) weighed more when feeding on a white clover line (*Trifolium repens* L. 'Milkanova') with functioning root nodules. A subsequent study using the same larval species and a strain of clover that is able to produce a defensive compound (hydrogen cyanide) showed that rhizobia no longer had a beneficial impact on larval weight, instead the effect of rhizobia was neutral (Kempel et al. 2009). Aphids (Hemiptera: Aphididae, *Myzus persicae* Sulzer) had marginally higher densities when feeding on white clover exposed to rhizobia regardless of the nodule forming capabilities of the clover line (Kempel et al. 2009). Aphid density was not significantly impacted by the hydrogen cyanide producing plants (Kempel et al. 2009). This study suggests that the plant diverts some of the fixed nitrogen into products that aid in the production of defensive compounds (Kempel et al. 2009). The production of some of these defensive compounds are only triggered by chewing insects, which explains why the aphids (*M. persicae*) were not impacted by the cyanogenic potential of the plant (Kempel et al. 2009).

Soybean plants actively fixing nitrogen had increased species richness and higher densities of chewing insects than a genetically similar non-nodulating isoline (Katayama et al. 2011). However, densities of sucking insects were only significantly more abundant on nodulated plants when soybean aphids were excluded from the analysis (Katayama et al. 2011), as densities of the latter were not affected by plant identity (neutral impact). Another field study found that soybean plants associated with rhizobia already present in the soil had significantly

lower aphid densities than plants grown from seed treated with a commercial inoculant (HiStick 2, Becker-Underwood IA), although total leaf nitrogen was similar between these two treatments (Dean et al 2009). In this study, aphid densities on the commercially inoculated plants were comparable to plants supplied with enough nitrogen to suppress nodulation (Dean et al. 2009).

Interactions between herbivores and rhizobia do not flow in one direction, but herbivory can also impact rhizobia. Heath and Lau (2011) showed that the presence of herbivores (Lepidoptera: Noctuidae, *S. exigua* Hubner) feeding on *Medicago truncatula* Gaertn. (Fabales: Fabaceae) increased nodule density. In contrast, leaf defoliation by soybean looper larvae, *Pseudoplusia includens* (Walker), reduced nodule dry weight and N-fixation on soybean (Layton and Boethel 1989, Russin et al. 1990), and artificial defoliation lowered nodule biomass on *M. sativa* (L.) (Quinn and Hall 1996). However, Techau et al. (2004) did not find any impact of simulated herbivory on pea nodules (Fabales: Fabaceae, *Pisum sativum* L.). With regard to sucking insects, Riedell et al. (2009) found that soybean aphid pressure reduced N-fixation, nodule volume, and dry weight of shoots and roots, although nodule density was not affected. In general, aphid feeding can alter the distribution of nutrients and sink-source relationships within plant tissues, with aphid-infested tissues functioning as strong nutrient sinks (Girousse et al. 2005).

Summary

Plant nitrogen dynamics can dramatically impact the behavior, biology, and population growth of herbivorous arthropods. Legumes are unique because they can obtain nitrogen from the soil (from mineral nitrogen or fertilizer) or from biological N-fixation. The process by which soybeans obtain nitrogen affects the amount and identity of nitrogenous compounds in the plant. However, it is unclear how the level of N-fixation and subsequent effects on biosynthesis,

distribution, and metabolism of nitrogenous compounds, including those associated with plant nutrition and resistance, impacts soybean herbivores such as phloem-feeding soybean aphids. Producers commonly use fertilizers and rhizobial seed inoculants, and therefore it is important to understand how management practices that affect plant nitrogen may impact soybean insect pests.

CHAPTER 1. INTERACTIVE EFFECTS OF NITROGEN FERTILIZER AND A RHIZOBIAL SEED INOCULANT ON SOYBEAN APHID DENSITIES UNDER CONTROLLED CONDITIONS

Introduction

Plant nitrogen can have a dramatic impact on herbivorous pests and is a major factor influencing their behavior and reproductive biology. Most plants obtain nitrogen from compounds found in the soil that originate from fertilizers or organic material (Brooker 2008). However, leguminous plants can also acquire nitrogen through the process of nitrogen (N) fixation, where bacteria convert atmospheric nitrogen (N_2) into compounds that can be utilized by the plant. Soybean (*Glycine max* L.) forms a symbiotic relationship with *Bradyrhizobium japonicum* (Bradyrhizobiaceae), a bacterial species in a group of N-fixing taxa associated with legumes collectively referred to as rhizobia (reviewed by van Rhijn and Vanderleyden 1995).

Rhizobia-plant symbioses are often highly species specific, and the bacteria are housed in structures on plant roots called nodules formed by the plant in response to signals sent by the bacteria (reviewed by van Rhijn and Vanderleyden 1995, Dénarié et al. 1996, Gage 2009). Within soybean nodules, atmospheric nitrogen gas is converted into ureides (allantoin and allantoic acid), which move into the xylem and then to foliar tissues where they are broken down and converted into other nitrogenous compounds and/or exported in the phloem (Shelp and Da Silva 1990, Sprent and Sprent 1990, Ohyama et al. 2009).

If the soil is high in nitrogen (from fertilizers or organic material), nodulation and N-fixation is suppressed, and the primary nitrogenous compounds within the xylem are nitrate and the amino acid asparagine (Evans 1982, Shelp and Da Silva 1990, Salvagiotti et al. 2008, Ohyama et al. 2009, Witte 2011). However, one study indicated that although nitrogen source

(NO_3^- or N_2) affected the relative quantity of nitrogenous compounds in the xylem, the distribution of compounds (amino acids and ureides) in the phloem sap of select leaflets was similar, although they did not assess specific amino acids and only assessed plants at one point in time (McNeil and LaRue 1984). The way in which soybeans obtain nitrogen can also affect the distribution of nitrogenous compounds throughout plant tissues (McNeil and LaRue 1984, Ohyama et al. 2009).

There is often a positive relationship between plant nitrogen and insect herbivore performance, including phloem-feeding aphids (Jahn et al. 2005, Chen and Ruberson 2008, Nowak and Komor 2010). Although aphids have obligate intracellular symbionts (i.e. primary symbionts, *Buchnera*) that synthesize amino acids (Douglas and van Emden 2007), they can still be impacted by the nitrogen content of their host plant. Aphids feeding on fertilized plants tend to weigh more, have shorter development times, and an increased life span (Nevo and Coll 2001, Hosseini et al. 2010). Plants that are supplied with an adequate amount of nitrogen are preferred by aphids over low nitrogen plants, and aphids have higher fitness on the former plants (Nowak and Komor 2010). However, excess levels of plant nitrogen can have detrimental effects on aphids (Zehnder and Hunter 2009, Sauge et al. 2010).

Effects of plant nitrogen on herbivores is not as clear when dealing with leguminous plants that can obtain nitrogen both from fertilizer and N-fixation. Plant-associated bacteria can increase the growth and nutrient content of host plants, but likely also contribute to increased production of plant defense compounds (Kempel et al. 2009, Pineda et al. 2010, Thamer et al. 2011). Chewing insects appear to be affected by plant-bacteria associations to a greater degree than sucking herbivores (Wilson and Stinner 1984, Kempel et al. 2009, Pineda et al. 2010, Katayama et al. 2011). However, legume rhizobia have been shown to affect aphid density,

including soybean aphids (Dean et al. 2009), although effects on aphids can be inconsistent (Kempel et al. 2009) or neutral (Katayama et al. 2011).

We conducted a series of greenhouse experiments to explore how altering the nitrogen source available to soybean plants (i.e. varying rates of fertilizer and rhizobial inoculation) impacted soybean aphid establishment on plants, reproduction, development time, and longevity. We also assessed how treatments impacted plant parameters associated with N-fixation (i.e. root nodules and nitrogenous compounds within above-ground plant tissue) and examined if these parameters were correlated with aphid density.

Materials and Methods

Effects of Nitrogen Source on Aphid Densities

We examined effects of nitrogen source on soybean aphids using potted soybean plants grown in a greenhouse (20-23°C, 75-80 %RH, 16:8 L:D). The first experiment focused on how treatments impacted aphid establishment and reproduction in two locations - when aphids were confined to one fully-expanded trifoliate leaf in clip cages and when aphids were allowed to roam freely over the entire plant. These trials were conducted sequentially on the same plant, with the clip cage trial performed first and the whole-plant trial run immediately afterwards.

Experimental Design. The experiment was designed as a 4 × 2 factorial with four levels of nitrogen (0, 25, 50 and 100 mg of N per pot), and two levels of rhizobia: with rhizobia (+Rhiz) and without rhizobia (No-Rhiz). Thus, there were eight treatments, which were replicated ten times.

Planting Procedures. Pots were 7.5 cm (height) × 11.5 cm (diameter), and were lined with clear plastic bags (17.8 cm × 30.5 cm; Sterile Sample Bags, VWR International LLC, Radnor, PA) prior to filling with a soil and sand planting medium. The soil used in experiments

was naturally low in nitrogen and was collected from Streeter, ND in August 2010 by digging up the upper soil layer using shovels (approx. 0.5 m deep). It was stored in large plastic containers (30 gallon) at ~24°C until used in experiments. The soil was mixed in a 1:1 ratio with 2040 medium silica sand (Twin Cities Concrete, West Fargo, ND) immediately prior to planting. Each pot received 300 g of pasteurized sand and soil. The sand and soil were pasteurized in an autoclave in aluminum cans covered in aluminum foil and heated to 121°C for 30 min. The sand and soil remained in those containers until it cooled and was weighed directly into the plastic bags. Samples of pasteurized and non-pasteurized soil (n = 3 for each type; soil only) were taken to the NDSU soil testing lab for analysis to see how pasteurization impacted various chemical and physical properties (Table 1).

After 300g of pasteurized sand was transferred to a plastic bag, it was mixed with nutrients and the rhizobial inoculant prior to adding the pasteurized soil. First, the sand was mixed with a basal nutrient solution (10 mg of P as potassium phosphate and 10 mg of S as potassium sulfate per pot). Then, the appropriate amount of nitrogen and an inoculum premix (see below) was added to the appropriate treatments. Every pot received the basal nutrient solution. The nitrogen was added as calcium nitrate tetrahydrate [$Ca(NO_3)_2 + 4H_2O$], which was put in the dryer at 60°C for 30 min prior to mixing with distilled H_2O (25 mg N/5 mL solution; ex. 126.61 g calcium nitrate tetrahydrate diluted to 3 L). At planting, only 50 mg of nitrogen was added to the 100 mg treatments and the remaining nitrogen was added ten days after planting (DAP).

The rhizobium was added as a peat based mixture containing *B. japonicum* (at least 2×10^8 viable cells per gram; N-Dure, INTX Microbials, LLC, Kentland IN). Each rhizobia treatment received an inoculant premix and inoculated seeds. The inoculum premix consisted of

10% peat inoculant and 90% pasteurized soil; both were sieved (mesh size: 710 mm) before weighing. Two g of the inoculum premix was weighed into small plastic cups (one for each +rhizobia pot) and sealed with a lid (they were prepared and used within 4 h). All +Rhiz seeds were inoculated simultaneously by moistening a small plastic bag with tap water, adding the inoculant, and shaking until all seeds were coated with the mixture. The bag containing the inoculated seeds was cut open so that the seeds were exposed to air and allowed to air dry before planting.

Table 1. Soil test results for pasteurized (+Past) and non-pasteurized (No-Past) soil used in all greenhouse experiments.

		+Past†	No-Past†	*P*-value‡
P	ppm	7.0 ± 0.00	5.7 ± 0.33	**0.016**
K	ppm	91.7 ± 1.67	97.0 ± 5.69	0.419
Zn	ppm	0.88 ± 0.02	0.89 ± 0.19	0.987
Fe	ppm	5.00 ± 0.10	8.47 ± 0.27	**0.001**
Mn	ppm	117.87 ± 9.15	20.87 ± 3.07	**0.001**
Cu	ppm	0.58 ± 0.036	0.65 ± 0.027	0.211
Ca	ppm	1793 ± 35	1720 ± 42	0.250
Mg	ppm	240 ± 11.5	227 ± 6.7	0.374
Na	ppm	51.47 ± 4.50	48.93 ± 5.59	0.742
NO_3-N	lb/A	3.7 ± 0.33	3.0 ± 0.00	0.116
S	lb/A	12.3 ± 1.20	8.0 ± 2.08	0.146
Cl	lb/A	13.43 ± 5.62	11.87 ± 3.92	0.830
pH		7.13 ± 0.03	7.10 ± 0.00	0.374
EC*	mmhos/cm	0.18 ± 0.02	0.10 ± 0.00	**0.005**
OM**	%	2.20 ± 0.00	2.10 ± 0.06	0.158
CEC***	Meq/100g	13.13 ± 0.21	13.14 ± 0.22	0.975

†Values represent mean ± SEM
‡$df_{1,4}$ for all analysis
*Electrical conductivity
**Organic Matter
***Cation Exchange Capacity

Pasteurized soil was mixed in after the basal nutrient solution, nitrogen, and inoculum premix were added to the sand. Once the sand and soil were completely mixed, the top 0.5 in was removed and set aside in a sterile container, 40 mL of water was added, and then three seeds (RG607 RR, Agronomy Seed Farm, Casselton, ND) were placed on the soil surface. The soil that had been set aside was then replaced and another 40 mL of water was added. Each pot was watered gravimetrically on a daily basis. Seedlings were thinned to one per pot 11 DAP.

Cages and Aphid Infestation. Prior to aphid infestation, two clip cages were placed on the two outer leaves of the first trifoliate of each experimental plant. Each clip cage was 2.5 cm diameter × 2 cm tall and made out of clear non-flexible plastic tubing. One side of the cylinder was covered with a fine mesh while the other side was open. One prong of a metal hair clip was glued to the cage while the other prong was glued to a piece of foam that secured the cage to the leaf and prevented aphid movement out of the cage (see Figure 1). Clip cages were supported by a thick copper wire that was secured to the pot.

Each clip cage was infested with two adult aphids 25 DAP when plants were at the V1 growth stage. Soybean aphids originated from a colony maintained at North Dakota State University (NDSU) on potted soybean plants (RG607 RR) at 25 ± 2°C, and 16:8 L:D. The aphid colony was established in 2008 and refreshed yearly using soybean aphids field-collected near Prosper and/or Fargo ND. In order to infest experimental plants, approximately 50 adult aphids were transferred from the lab colony into a Petri dish lined with moist filter paper using a small paintbrush. Petri dishes were transported to the greenhouse in coolers. Aphids were gently transferred onto the abaxial leaf surface using a paintbrush and the clip cages secured so that aphids were restricted to the bottom of the leaf. Establishment of adult aphids (i.e. density of adult aphids) was determined 3 d after aphid infestation (28 DAP) and initial reproduction was

simultaneously assessed by counting the number of immatures per cage and calculating immatures per adult. Aphid density (adults + immatures) per cage was reassessed 9 d after infestation (34 DAP).

After 10 days (35 DAP), one clip cage was removed from the plant, the other clip cage remained with only one immature aphid in it(results not discussed). At this time 8 immature aphids of similar age (determined by size) were left to roam freely over the plant. All other aphids in that cage were removed from the plant. After aphids were left to roam over the plant, it was covered by plastic tube cage (30 cm × 79.5 cm) with mesh panels (8 cm × 5.5 cm; see Figure 2). Aphid density per plant (adults + immatures) was assessed 1-2 times per week until the end of the experiment (48 DAP).

Figure 1. Picture of clip cage used in Effects of Nitrogen Source on Aphid Densities Experiment.

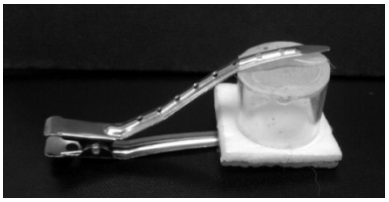
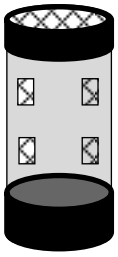


Figure 2. Illustration of tube cage used in Effects of Nitrogen Source on Aphid Densities Experiment.



Plant Parameter Data Collection. Plants were destructively sampled 52 DAP. Stems were cut at the soil surface and roots removed from the pots. Roots were rinsed with tap water, placed in self-sealing plastic bags, and processed within 1 h. Processing involved removing root nodules by hand and counting and weighed while still fresh. Above-ground plant material was placed in paper bags and dried in an oven at 65°C for 2 d. Once dry, samples were weighed and placed in self-sealing plastic bags at 25 ± 2°C out of the sun until ground. Ground material was

placed back into plastic bags and stored as above until the nitrogenous compounds were assessed, about eight months (detailed in a later section).

Effects of Nitrogen Source on Aphid Development and Longevity

This experiment explored how treatments impacted immature aphid development time and longevity for two types of aphids – offspring born to females transferred to experimental plants (cage A) and offspring born on colony plants and transferred to experimental plants (cage B). These trials were conducted simultaneously within two clip cages on the same plant.

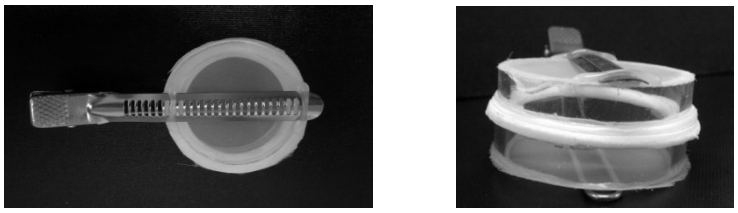
Experimental Design and Planting Procedures. The experimental design was a 4 × 2 factorial with four levels of nitrogen (0, 25, 50 and 100 mg pot^{-1}) and two levels of rhizobia (No-Rhiz, and +Rhiz). The experiment was established using the same methods as detailed previously, with the exception that all seeds were surface sterilized using a 5% Clorox bleach solution for 10 min then rinsed several times with distilled water. Plants were thinned to one plant per pot 11 DAP and 50 mg of nitrogen was added to the 100N treatment 12 DAP.

Cages and Aphid Infestation. There were two clip cages per plant, with each one attached to one leaflet of the first trifoliate. Clip cages were slightly different than those used in the first experiment, and each clip cage consisted of two cylinders 5 cm diameter × 1 cm tall with mesh on one side and a foam ring on the other side. Each cylinder was glued to the prong of a 20.3 cm metal hair clip (Salon Care Professional, Brentwood Beauty Laboratories International, Dallas, TX; see Figure 3) and was supported by a thick copper wire. Clip cages were placed on plants so that aphids could move freely from the top to the bottom of the leaf.

After aphid infestation plants were enclosed within mesh bags (Insect Rearing Bag; 48 cm × 71 cm, Bugdorm; constructed with nylon netting, 94 × 104 mesh/square inch), which were supported by two crisscrossed wires. Pots containing experimental plants rested within a larger

plastic pot (27.3 cm diameter × 14.9 cm tall; HTS1000, ITML Horticultural Products Inc., Middlefield OH), and the mesh bag was cinched underneath the rim of the outer pot.

Figure 3. Pictures of clip cages used in Effects of Nitrogen Source on Aphid Development and Longevity Experiment.



The first clip cage (cage A) was infested with two adult aphids 26-27 DAP when plants were at the V1 growth stage. Adult aphids used to infest cage A were taken from a previously described lab reared colony and were transferred using a small paintbrush. Aphid establishment and aphid density (adults + immatures) was assessed 24 h after the adults were added to the cage, and then all but one immature was removed. If there were no immatures, adults were left in the cage and checked daily until an immature was discovered. After leaving a single immature, cages were monitored daily until that individual began reproducing and subsequently died. Any offspring produced were removed daily.

Aphids transferred to the second clip cage (cage B) were not born on the experimental plants. Initially, adult aphids from the lab colony were placed in clip cages on colony plants (i.e. plants grown in potting soil; LC1, loose fill Sunshine Mix) 22 DAP. Cages were checked 2 d later and all adults removed in order to establish a same-age cohort of immature aphids. Immatures remained on the colony plants for four additional days, and then 2 were transferred to experimental plants (i.e. cage B) 28 DAP when plants were at the V1 growth stage. Aphid establishment was assessed 24 h later and cages checked daily until aphids began reproducing and both aphids died. Any offspring produced were removed daily.

Plant Parameter Data Collection. Relative leaf chlorophyll content was assessed non-destructively using a SPAD meter (SPAD-502 Leaf Chlorophyll Meter, Konica Minolta Optics Inc.; Tokyo Japan), and readings were taken on all leaves of the uppermost fully expanded trifoliate on each plant on 26, 36, 50 and 62 DAP. Because leaf nitrogen is related to leaf chlorophyll content, SPAD meters are useful tools to assess plant nitrogen in many crops (Markwell et al. 1995, Bullock and Anderson 1998).

Density and fresh weight of root nodules and dry weight of above-ground plant biomass were assessed destructively 62 DAP as described in the previous section. After being weighed, the concentration of nitrogenous compounds (total-N, nitrate-N and ureide-N) in above-ground plant material was assessed as described previously and as detailed in the section below.

Analysis of Nitrogenous Compounds within Plant Tissue

Sample Preparation. All analyses were conducted using ground plant material. Total nitrogen, ureide and nitrate content were analyzed for all samples. Ureide-N represents the relative amount of nitrogen fixed in the plant by rhizobia (van Berkum et al. 1985). Nitrates are related to the amount of nitrogen in the plant obtained from nitrogen fertilizer (Ohyama et al. 2009, Witte 2011). Total nitrogen represents all nitrogenous compounds within the plant, these include amino acids, proteins nitrate and ureides. A small sample (0.02 g) of ground plant material was sent to the NDSU Soil Testing lab for analysis of total nitrogen (Kjehldahl digest). For nitrate and ureides analyses, samples from the first experiment were digested by putting 0.2000 ± 0.001 g of ground plant material and 10 mL of distilled water in a 20 x 125 mm screw top vial (Pyrex, VWR International). For the second experiment 0.4000 ± 0.001 g of ground plant material and 20 mL of distilled water was used. The exact weight of each sample was recorded and later used to calculate the ppm of nitrogenous compounds in each sample. Vials

were capped and placed in a water bath at 90° C for 30 min, after which they were allowed to cool at room temperature (~22˚ C) and then filtered (size 2 whatman paper filter, VWR) into a solo cup containing 2 g of H+ resin beads (Dowex® Marathon™ C hydrogen form, Sigma Aldrich).

The resin beads hold a positive charge and will bind with certain nitrogenous compounds (e.g. amino acids) that if not removed will result in erroneously high readings (Patterson et al. 1982). After every use, the resin beads were recharged by adding 100 mL of distilled water and 100 mL of 1M HCl (Sigma Aldrich) to 100 mL resin beads in a small plastic cup and stirred with a metal spatula every 2-3 min for 10 min. About 400 mL of distilled water was then added to the solution and the excess liquid poured off. Another 200 mL of HCl was then added to the beads and was stirred every 2-3 min for 10 min. Distilled water was added and excess liquid poured off to rinse the HCl from the beads. This step was repeated 2-3 more times, then the water and resin beads were poured into a Buchner funnel, rinsed a 2-3 more times with distilled water, then spread out on a clean surface to air dry.

Nitrate Analysis. The nitrate content of the samples was assessed according to the salicylic acid method (Cataldo et al. 1975). Initially, 0.8 mL of a sulfuric/salicylic acid mixture (3 g/60 mL, Sigma Aldrich) was added to a 20 x 125 mm glass screw top vial and 0.2 mL of filtered sample or standard was added to the vial, mixed, and left to react for at least 10 min. Then 19 mL of an NaOH solution (96 g NaOH in 1200 mL distilled water) was added to each tube, which was capped, mixed by hand, and allowed to cool to room temperature(~22˚ C). Once cooled, approximately 2 mL of sample was transferred to a cuvette that had been rinsed with sample immediately prior to filling with sample for the reading. The sample absorbance was read

at 420 nm wavelength in a spectrophotometer (Beckman Coulter DU 530 Life Science UV-Vis Spectrophotometer, Single Cell Module) previously calibrated using a 0 ppm nitrate standard.

The absorbance of five standards (0, 5, 10, 15 and 20 ppm of nitrate) was also determined. To make the standards 0.722 g of nitrate was added to 100 mL water to make a 1000 ppm solution. Then, 50 mL of the 1000 ppm solution was diluted to 500 mL to make a 100 ppm solution. For the 5 ppm standard, 5 mL of the 100 ppm solution was diluted to 100 mL. Likewise, for the remaining standards, 10, 15, or 20 mL of the 100 ppm solution was diluted to 100 mL. The 0 ppm standard was simply distilled water, no nitrate was added. Standards were stored in glass beakers covered (Parafilm M, Bemis Company Inc.; Oshkosh WI) and stored in the refrigerator at 4 ± 2˚C.

Standards were used to develop a linear equation that was used to calculate the ppm nitrate of each sample from the spectrophotometer absorbency readings. Essentially, the absorbency readings of each standard were on plotted against the known ppm nitrate of each standard. For each experimental sample, y (the absorbency reading) was entered and the equation was solved for x (the ppm nitrate in the sample). To determine the ppm of nitrate in the experimental plants, the ppm of each sample was multiplied by the dilution factor, which was 20 mL water × weight of sample used in the digestion (i.e. 20 × 0.400), but note that the exact weight of each sample was used instead of 0.400 g.

Ureide Analysis. We used the Patterson method to determine the ureide content in the soybean tissue (Patterson et al. 1982). Tubes (20 x 125 mm glass screw top vial) received 1.0 mL of sample or standard. Then 1.0 ml of phthalate buffer was added to each tube (40.8456 g potassium hydrogen phthalate per 1000 mL water, Sigma Aldrich), followed by diluted bleach solution (10 mL bleach diluted with 30 mL distilled water) and tubes were mixed by hand. After

5 min 2 mL of color developing solution was added to each tube and left to react for 10 min. The color developing solution contains 15 mL of 20% NaOH and 40 mL of Phenol solution (135 g phenol, 100 mL water, 250 mL methanol). Each sample then received 5.5 mL of distilled water. Approximately 2 mL of sample was transferred into the cuvette, which was read in the spectrophotometer at 625 nm wavelength. The 0 ppm standard was used as a blank.

Six standards were made: 0, 5, 10, 15, 20, and 40 ppm of allantoin (a ureide; Sigma Aldrich). Standards were made by bringing 0.282 g allantoin up to 100 mL with distilled water to make a 1000 ppm solution (the allantoin was dried overnight in a desiccator at 55˚ C before weighing). To make a 100 ppm solution, 50 mL of 1000 ppm solution was diluted to 500 mL. Then, to make the 5 ppm standard, 5 mL of the 100 ppm solution was diluted to 100 mL. Likewise, for the remaining standards, 10, 15, 20, or 40 mL of the 100 ppm solution was diluted to 100 mL. The zero ppm standard contained distilled water with no added allantoin.

The absorbance of the standards was obtained with the spectrophotometer and used to calculate the ppm of ureide in the plant samples the same way as previously described for the nitrate. To calculate the ppm of ureide in the plant, the ppm in the extract was multiplied by the same dilution factor used for nitrates [i.e. (20 × 0.400)]. Again, note that the exact weight of each sample was used instead of the 0.400 g.

Data Analysis. All data were analyzed using SYSTAT® 12 (SYSTAT Software, Inc. 2007). Histograms and Levene's test were to determine if data met the assumptions necessary for parametric statistics. ANOVA was used to compare how pasteurization affected various soil parameters (e.g. pH, % organic matter, nutrients; see Table 1).

Aphid density data for the first experiment was summed across both clip cages and analyzed using a factorial ANOVA with rhizobia inoculation and nitrogen rate as the

independent variables. Tukey's Honest Significant Difference test was used for mean separation if the main effect of nitrogen rate was significant. For aphids that were allowed to roam freely over plants, daily per capita aphid population growth rates were calculated for each sampling period (35 to 37 DAP, 37 to 40 DAP, 40 to 48 DAP) using $[(\lg Nt_2 - \lg Nt_1) / t2 - t1]$, where N = aphid density, t1 = the initial day and t2 = the final day of the sampling period (e.g. Johnson 2008). Population growth rate data from each sampling period were analyzed using a factorial ANOVA with rhizobia inoculation and nitrogen rate as the independent variables.

For the first experiment, strength of linear correlations between log (X+1) transformed free-roaming aphid densities (48 DAP) and plant parameters associated with plant nitrogen (root nodule density and fresh weight, and log (X+1) transformed concentration of nitrogenous compounds within above-ground foliage) were determined using Pearson product-moment correlation coefficients and R^2 (coefficient of determination) values. The significance of the correlations (i.e. the likelihood that the correlation coefficient would occur if there was no relationship between the variables) was determined using Bonferroni probabilities as part of the Pearson correlation analysis (SYSTAT Software, Inc. 2007). Only data from plants receiving rhizobial inoculants were used in correlation analyses involving root nodules.

In the second experiment, aphid establishment in cage A was analyzed separately for adults and immatures prior to the selection of the individual aphid used in development and longevity analyses. Establishment in cage B was analyzed on data collected from the juvenile aphids on experimental plants. In this experiment some aphids never reproduced. Therefore, we explored if the incidence of aphid reproduction within a clip cage (yes, no) was impacted by the experimental treatments using frequency tables and Pearson's chi-square statistic (SYSTAT Software, Inc. 2007). Main effects of each independent variable (rhizobia inoculation and

nitrogen rate) on the occurrence of aphid reproduction was assessed separately using one-way tables, and interactions between variables were assessed using two-way tables.

Data on aphid developmental time, as determined by days until the first offspring was produced within a clip cage, and aphid longevity data (days until death) were analyzed using factorial ANOVA with rhizobia inoculation and nitrogen rate as independent variables followed by Tukey's HSD test for mean separation. For the developmental time data, aphids that never reproduced were omitted from the analysis, and because the data were unbalanced we used Type II sum of squares instead of the default Type III (Langsrud 2003). Because two aphids were added to cage B, aphid longevity data were averaged prior to analysis. Cage A and cage B were analyzed separately.

Plant parameter data (i.e. number of root nodules per plant, fresh weight of all root nodules per plant, dry weight of above ground biomass, total-N, nitrate-N, and ureide-N) were analyzed using a factorial ANOVA with rhizobia inoculation and nitrogen rate as independent variables. Data for the latter three plant parameters in the first experiment only were log (X+1) transformed prior to analysis. SPAD meter reading data were analyzed using a repeated measures factorial ANOVA, followed by a profile analysis (individual ANOVAs on each sampling date) if there were significant time × treatment interactions. Tukey's HSD test was used for mean separation.

Results

Effects of Nitrogen Source on Aphid Densities

Aphids. Aphid establishment was measured 3 d after clip cages were infested with adult aphids (28 DAP), and treatments did not have a significant impact on the number of adults present (N-Rate × Rhizobia, $df_{3, 72}$, P=0.410; Rhizobia, $df_{1, 72}$, P=0.211; N-Rate, $df_{3, 72}$, P=0.446;

Figure 4a). The number of immatures in each clip cage was also assessed 28 DAP and no differences were found between treatments (N-Rate × Rhizobia, $df_{3, 72}$, P=0.867; Rhizobia, $df_{1, 72}$, P=0.132; N-Rate, $df_{3, 72}$, P=0.737; Figure 4b). N-rate and rhizobial inoculation had a moderately significant interactive effect on aphid densities 34 DAP, 9 d after infestation, which was likely driven by similar aphid densities in the 100N treatment in both inoculation regimes but disparate effects at lower N-rates (N-Rate × Rhizobia, $df_{3, 72}$, P=0.067; Figure 5). In general, there were significantly more aphids on inoculated plants (Rhizobia, $df_{1, 72}$, P<0.001), and lower aphid densities on plants in the 100N treatment compared to the 25N (P=0.001) and 0N (P=0.053) treatments (N-Rate, $df_{3, 72}$, P=0.002; Figure 5).

With regard to aphids that were allowed to roam freely over the plant, treatment effects on daily per capita aphid population growth changed as the experiment progressed (Figure 6). Treatment effects on population growth in the initial time period (35-37 DAP) were similar to total aphid densities after 9 d in clip cages, with a marginal interaction between the independent variables (N-Rate × Rhizobia, $df_{3, 72}$, P=0.082). This was likely caused by similar population growth rates at higher N-rates and dissimilar growth rates at lower N-rates, with the greatest population growth on inoculated plants receiving 0 or 25N. There was a marginally significant positive main effect of rhizobial inoculation (Rhizobia, $df_{1, 72}$, P=0.066). However, the main effect of N-rate was not significant (N-Rate, $df_{3, 72}$, P=0.733), and aphid population growth was negative in the 25N no rhizobia treatment. During the second time period (37-40 DAP), none of the treatment effects were significant (N-Rate × Rhizobia, $df_{3, 72}$, P=0.205; Rhizobia, $df_{1, 72}$, P=0.311; N-Rate, $df_{3, 72}$, P=0.277). High aphid population growth rates in the non-inoculated 0N and 25N treatments were likely due to the presence of mutant nodules (see Nodule results section). During the last time period (40-48 DAP), rhizobial inoculation had a significant

negative effect on aphid population growth rates (Rhizobia, $df_{1, 72}$, P=0.005; Figure 6c). In fact, aphid populations on plants in the +Rhizobia 0N and 25N treatments were actually declining.

Figure 4. Mean number of adult and immature aphids per plant. a) adults, 3 days after aphid infestation (28 DAP) according to rhizobial inoculation and nitrogen treatment. b) immatures, 3 days after aphid infestation (28 DAP) according to rhizobial inoculation and nitrogen treatment.

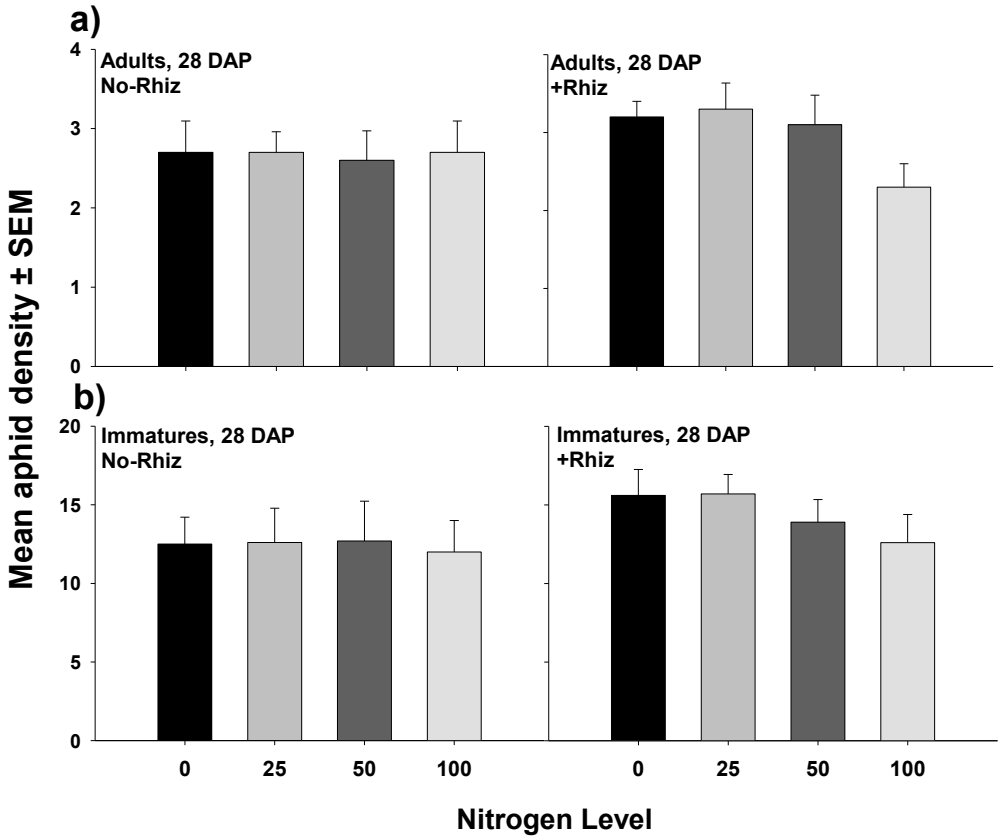


Figure 5. Mean total aphids 9 days after infestation (34 DAP) according to rhizobial inoculation and nitrogen treatment.

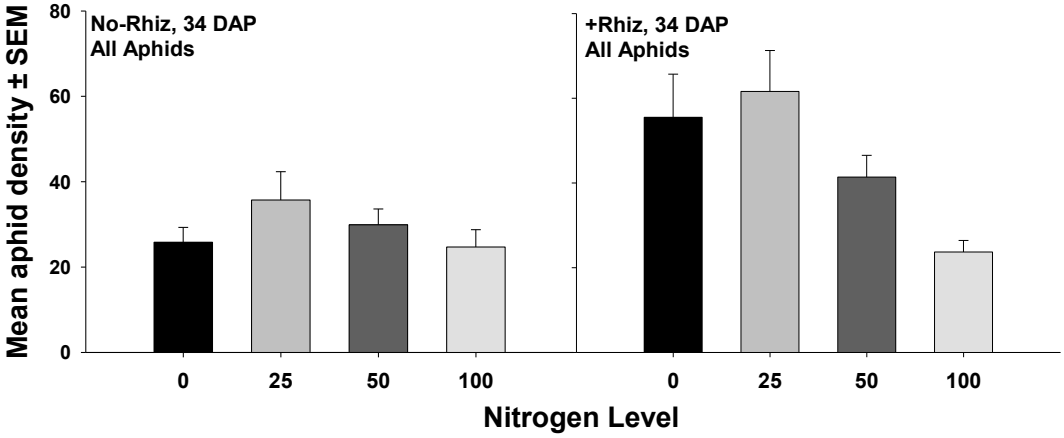
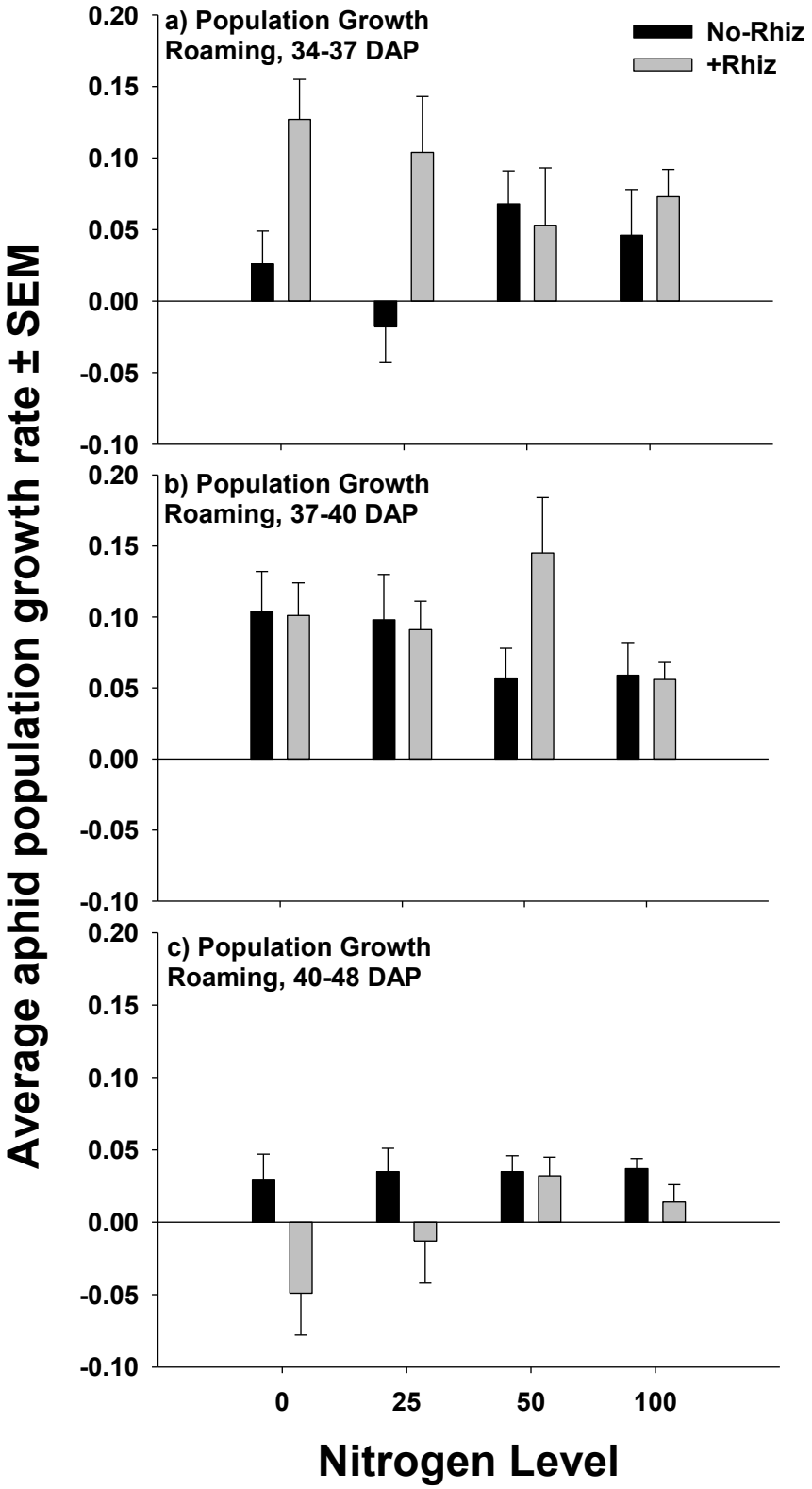


Figure 6. Population growth rates for roaming aphids. a) Growth rate 34-37 DAP (d1-d3 after infestation). b) Growth rate 37-40 DAP (d3-d6). c) Growth rate 40-48 DAP (d6-d14).



Densities of roaming aphids on the last sampling date (48 DAP) were not correlated with nodule parameters (nodule count: R^2=0.031, Chi-Square=1.089, *P*=0.297; nodule weight: R^2=0.009, Chi-Square=0.314, *P*=0.575; Figure 7) or the ureide-N, nitrate-N and total-N content of above-ground plant material (ureide: R^2<0.001, Chi-Square<0.001, *P*=0.991; nitrate: R^2<0.001, Chi-Square=0.070, *P*=0.792; total-N: R^2<0.001, Chi-Square=0.005, *P*=0.944; Figures 8 and 9).

Figure 7. Correlations with roaming aphids from 48 DAP and root nodule parameters (+Rhiz plants only).

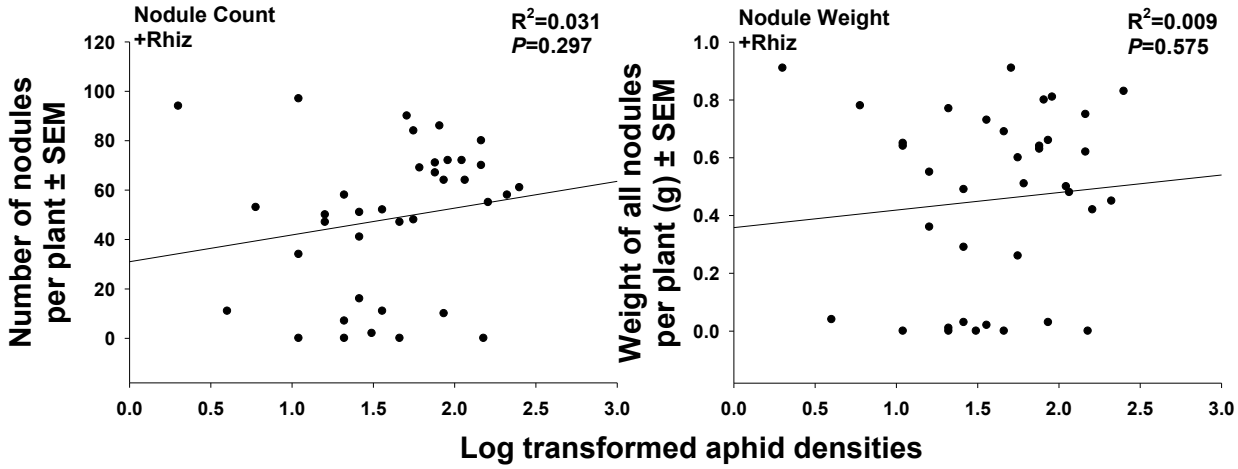
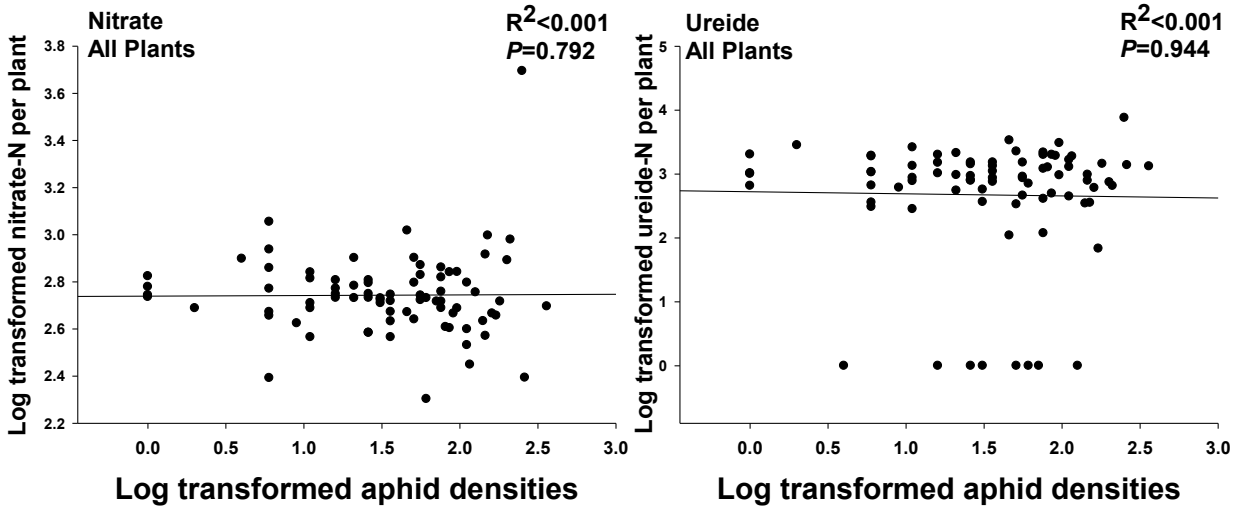
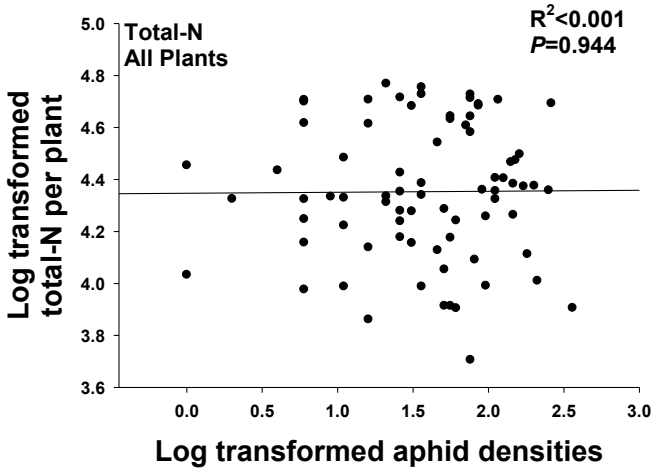


Figure 8. Correlations with roaming aphids from 48 DAP and nitrate and ureide-N compounds (all plants).



Nodules. During the experiment, we noticed that plants under the most severe nitrogen stress (i.e. No-Rhiz plants, 0N and 25N) that had yellowed suddenly began turning green. Plants in the 0N treatment began greening around 34 DAP. At the end of the experiment we discovered that these plants had become contaminated with rhizobia part-way through the experiment and formed 'mutant' root nodules. These nodules were much larger than nodules found in the +Rhiz treatments and were not located near the stem of the plant where most nodules of the +Rhiz treatments were found. These nodules when broken open were still pink on the inside indicating they were likely fixing nitrogen, although the bacterial species associated with the plant may not have been *B. japonicum*.

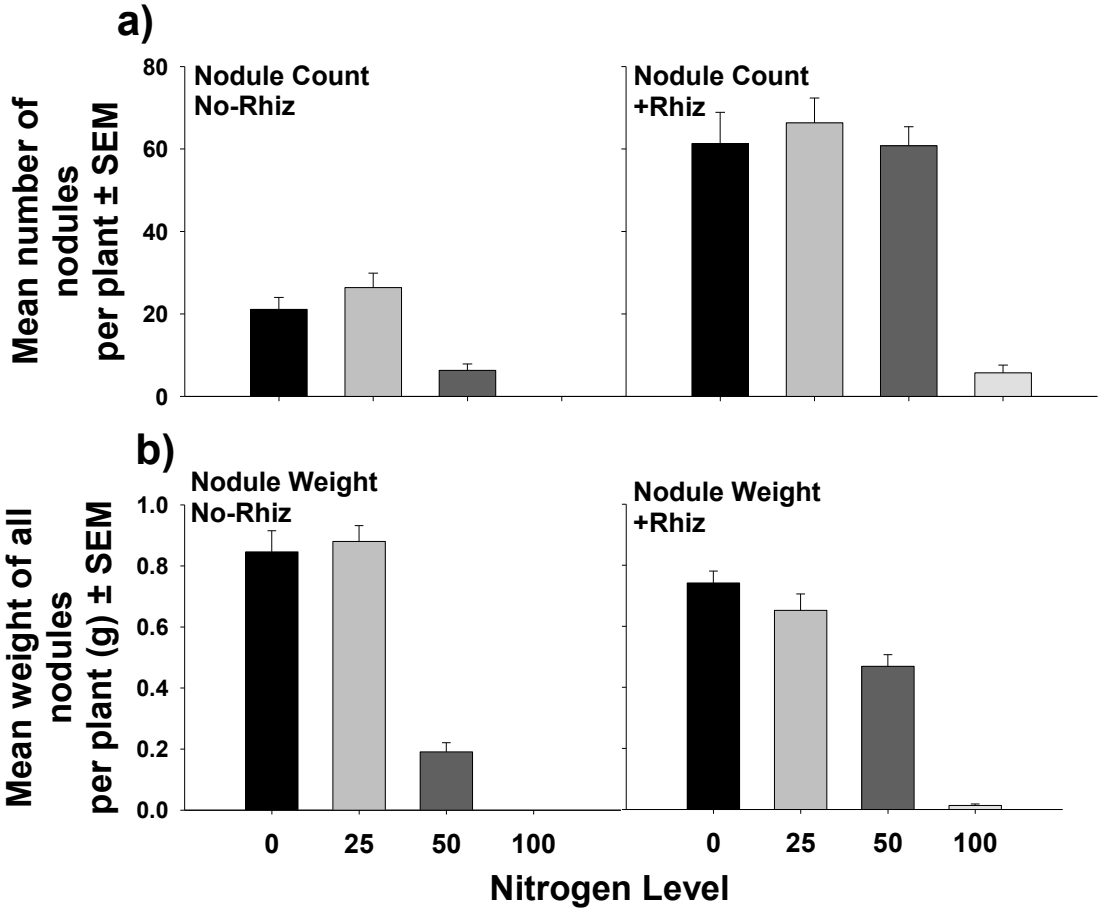
Figure 9. Correlations with roaming aphids from 48 DAP and total-N (all plants).



As expected, +Rhiz plants had more root nodules than No-Rhiz plants, and nodulation appeared to decrease as N-rate increased (Rhizobia, $df_{1, 70}$, P<0.001; N-Rate, $df_{3, 70}$, P<0.001; Figure 10a). There was a significant interaction between N-rate and rhizobial inoculation on nodule density (N-Rate × Rhizobia, $df_{3, 70}$, P<0.001), which was anticipated, because +Rhiz plants had a much higher degree of nodulation in low N-rate treatments, but similar nodulation at the highest N-rate. Treatment effects on total fresh weight of root nodules per plant were similar

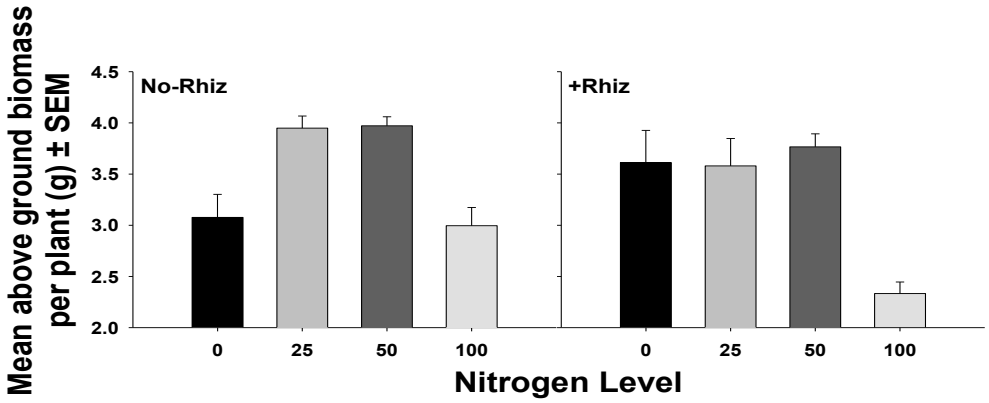
to nodule density (N-Rate × Rhizobia, $df_{3, 70}$, P<0.001; Rhizobia, $df_{1, 70}$, P=0.735; N-Rate, $df_{3, 70}$, P<0.001; Figure 10b). Because of the mutant nodules, the weight of all nodules on the No-Rhiz and +Rhiz plants were similar for the 0N and 25N pots. Although there were fewer mutant nodules per plant, individual mutant nodules were much more massive than normal nodules.

Figure 10. Nodules per plant according to nitrogen level and rhizobia treatment at 52 DAP. a) mean number per plant. b) mean weight of all nodules per plant.



Above Ground Biomass. Plants in the 0N treatment were lighter than the other plants, but only when plants were not inoculated with rhizobia. This led to a significant interaction between N-rate and rhizobia inoculation on dry weight of above ground biomass per plant (N-Rate × Rhizobia, $df_{3, 72}$, P=0.021; Rhizobia, $df_{1, 72}$, P=0.204; Figure 11). Plants receiving excessive nitrogen (100N) tended to be smaller than the other plants (N-Rate, $df_{3, 72}$, P<0.001; 100N vs 0N, 25N, and 50N, P≤0.004; Figure 11).

Figure 11. Mean above ground biomass per plant at 52 DAP according to nitrogen level and rhizobial inoculation.



Plant N Compounds. At the end of the experiment, the parts per million (ppm) of nitrate-N in the above ground plant tissue did not vary by N-Rate (N-Rate, $df_{3, 72}$, P=0.370), however, rhizobial inoculation had a moderately positive impact (Rhizobia, $df_{1, 72}$, P=0.071; N-Rate × Rhizobia, $df_{3, 72}$, P=0.432; Figure 12). There was a significant interactive effect of rhizobia and inoculation on nitrogen rate and ureide N per plant (N-Rate × Rhizobia, $df_{3, 72}$, P=0.053; Figure 13), this was likely due to the presence of nodules on No-Rhiz plants at low N rates. In general, ureide levels were higher when plants were inoculated with N-fixing bacteria and decreased as N-rate increased (Rhizobia, $df_{1, 72}$, P=0.003; N-Rate, $df_{3, 72}$, P<0.001). Ureides are primarily the products of N-fixation (Reynolds et al. 1982, Schubert 1986, Sprent and Sprent 1990, Ohyama et al. 2009, Strodtman and Emerich 2009); however, some ureides were present in the leaf tissue of plants lacking nodules. This may be due to the uptake of ammonium from the soil and subsequent conversion to ureides (Herridge 1982b). The total nitrogen of each plant was not impacted by the addition of rhizobia (N-Rate × Rhizobia, $df_{3, 72}$, P=0.535; Rhizobia, $df_{1, 72}$, P=0.721). However, N-rate did impact the total-N (N-Rate, $df_{3, 72}$, P<0.001; Figure 14), with lower total-N in the 50 N treatment compared to both the 100N and 25N treatments (50N versus:

25N P=0.008; 100N P<0.001; P≥0.05 for all other comparisons). Higher levels of total N in lower N-rate treatments may be partially due to mutant nodules in No-Rhiz plants.

Figure 12. Mean ppm of nitrate-N per plant at 52 DAP. a) according to rhizobia treatment, data combined across all N-rates. b) According to N-rate, data combined across all rhizobia treatments. Different letters indicate significant differences at P=0.071.

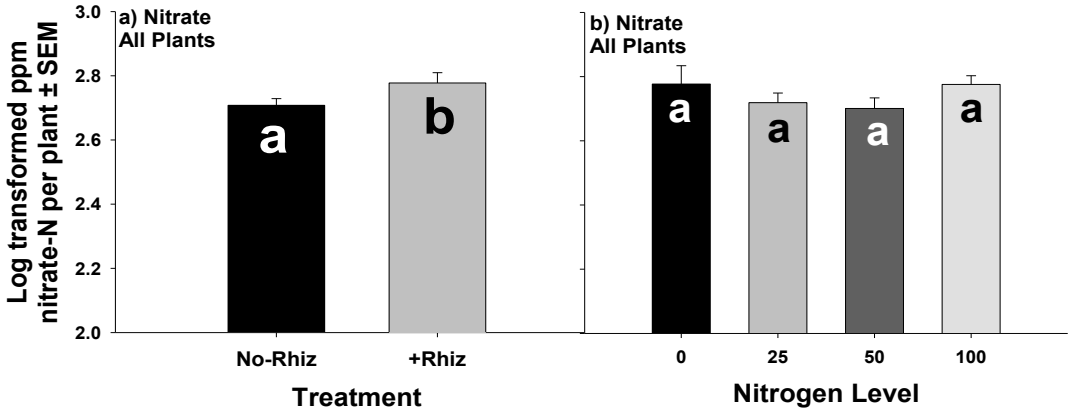


Figure 13. Mean ppm of ureide-N per plant at 52 DAP according to rhizobia inoculation and nitrogen treatment.

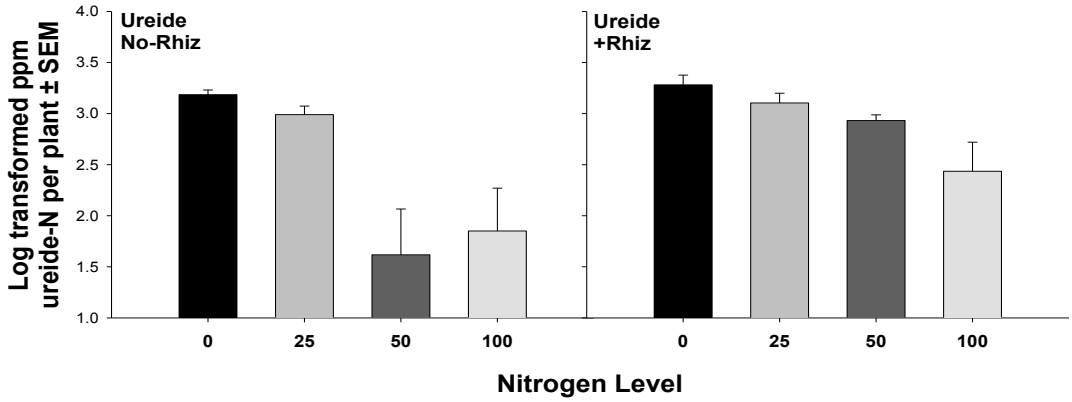
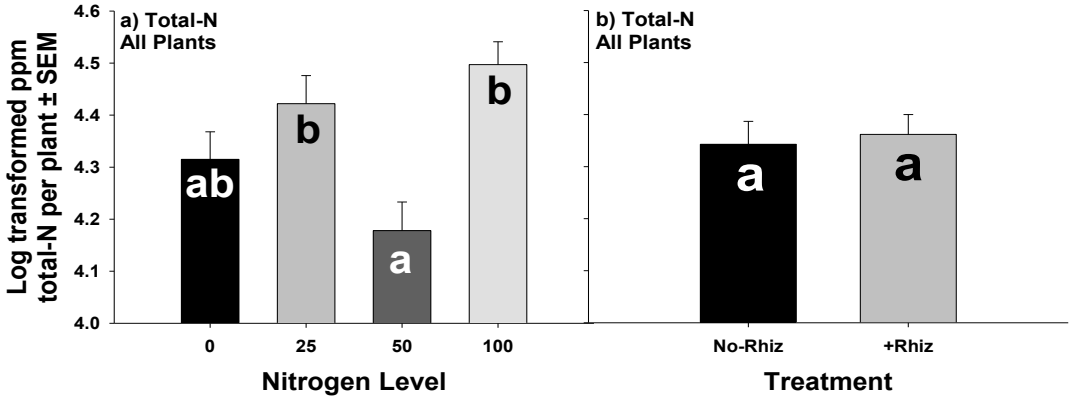


Figure 14. Mean ppm of total-N per plant according to N-rate at 52 DAP. Data combined across all rhizobia treatments. Different letters indicate significant differences at P<0.05.



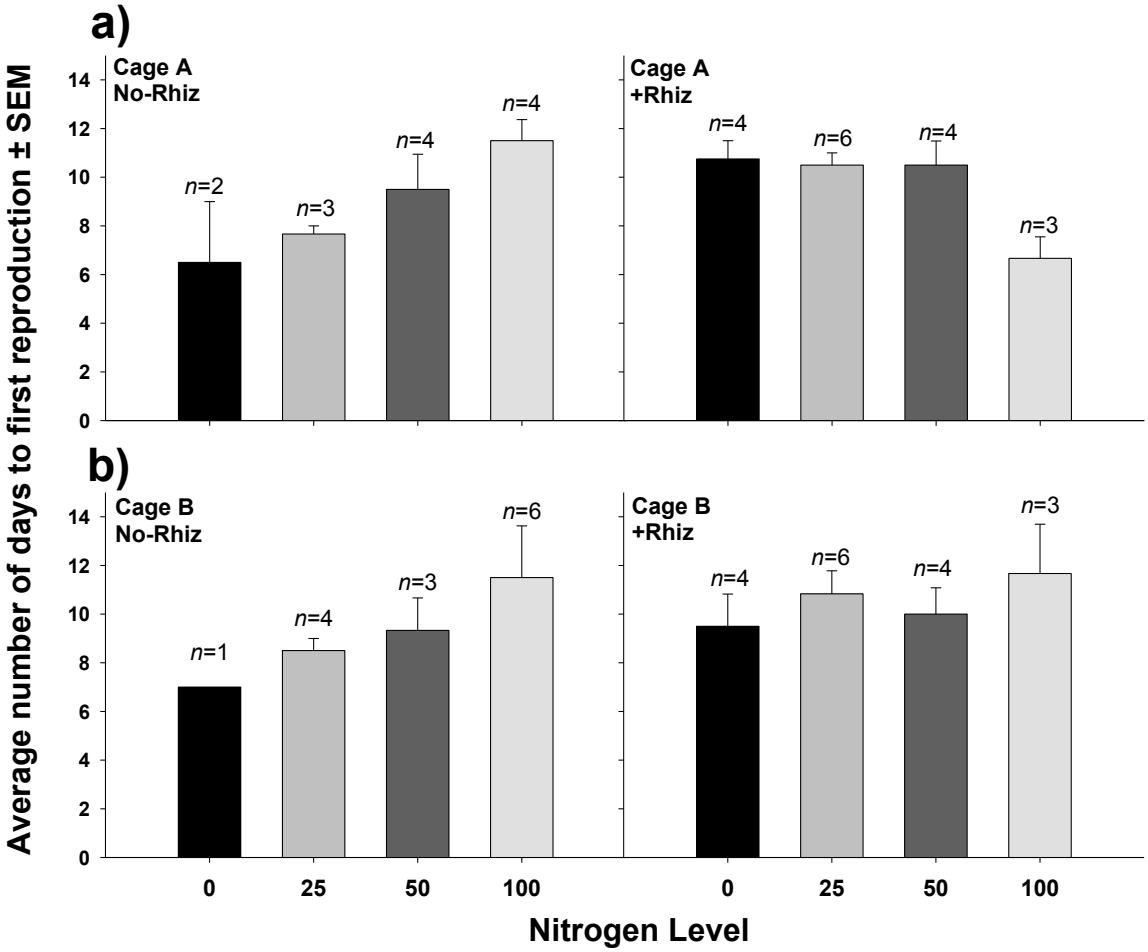
Effects of Nitrogen Source on Aphid Development and Longevity

Aphids. Aphid establishment in Cage A (adults transferred to experimental plants and allowed to reproduce) was assessed 24 h after adults were added to clip cages, and there were no significant treatment effects on adult establishment (N-Rate × Rhizobia, $df_{3, 72}$, P=0.418; Rhizobia, $df_{1, 72}$, P=1.000; N-Rate, $df_{3, 72}$, P=0.699; data not shown). Likewise, there was also no impact on the number of immature aphids present after 24 h (N-Rate × Rhizobia, $df_{3, 72}$, P=0.837; Rhizobia, $df_{1, 72}$, P=0.795; N-Rate, $df_{3, 72}$, P=0.920; data not shown). The treatments did not significantly impact the occurrence of aphid reproduction (N-Rate × Rhiz: Chi-Square=1.299, P=0.729, N-Rate: Chi-Square=0.667, P=0.881, Rhiz: Chi-Square=0.533, P=0.465). There was an interactive effect of rhizobia inoculation and N-Rate on development time of immature aphids born on experimental plants (N-Rate × Rhizobia, $df_{3, 22}$, P=0.001; Rhizobia, $df_{1, 22}$, P=0.321; N-Rate, $df_{3, 22}$, P=0.874; Figure 15a). This was due to longer development time on No-Rhiz plants as nitrogen increased and a decrease in development time on +Rhiz plants at the 100 mg N-rate. Increasing nitrogen rate had a moderately significant impact on aphid life span, with aphids tending not to live as long on low (0)N plants (N-Rate, $df_{3, 71}$, P=0.072; Rhizobia, $df_{1, 71}$, P=0.100; N-Rate × Rhizobia, $df_{3, 71}$, P=0.152; Figure 16a).

Aphid establishment within Cage B (immatures reared on colony plants for 6 d then transferred to experimental plants) was also assessed 24 h after aphid infestation and was not impacted by treatment (N-Rate × Rhizobia, $df_{3, 72}$, P=0.398; Rhizobia, $df_{1, 72}$, P=0.321; N-Rate, $df_{3, 72}$, P=0.398). Similar to Cage A, the occurrence of aphid reproduction in Cage B was not significantly impacted by the treatments (N-Rate × Rhiz: Chi-Square=2.351, P=0.503, N-Rate: Chi-Square=1.733, P=0.630, Rhiz: Chi-Square=0.533, P=0.465). In contrast to Cage A, immature aphid development time in Cage B was not impacted by rhizobia inoculation or N-Rate

(N-Rate × Rhizobia, $df_{3, 23}$, P=0.877; Rhizobia, $df_{1, 23}$, P=0.292; N-Rate, $df_{3, 23}$, P=0.351; Figure 15b). However, there was a significant interactive effect of nitrogen and rhizobia on mean aphid longevity (N-Rate × Rhizobia, $df_{3, 72}$, P=0.001; N-Rate, $df_{3, 72}$, P=0.033; Figure 16b), which in Cage B was due to shorter life spans among aphids on plants receiving 0 or 25 mg N per pot, but only in the absence of rhizobia. In general, aphids lived longer on +Rhiz plants (Rhizobia, $df_{1, 72}$, P=0.001).

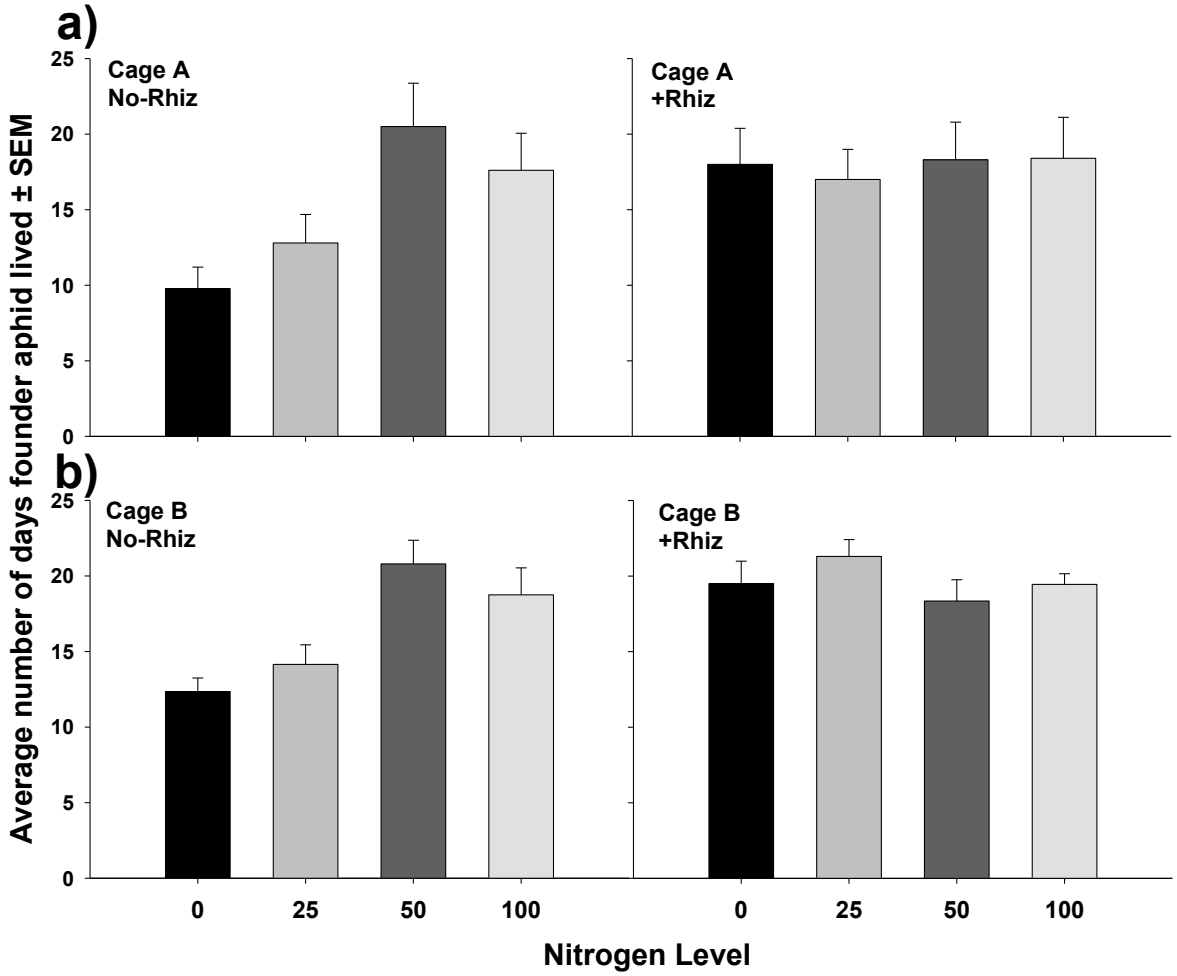
Figure 15. Mean aphid development time (days). a) Cage A according to rhizobial inoculation and nitrogen treatment; 0 days = 27 DAP, 14 days = 41 DAP. b) Cage B according to rhizobial inoculation and nitrogen treatment; 0 days = 24 DAP, 14 days = 38 DAP. The number of cases in each treatment is represented by *n*=X over the corresponding bar.



Nodules. Because we surface sterilized seeds in the second experiment, there was minimal contamination of the No-Rhiz plants by mutant root nodules, and therefore root nodule

data were only analyzed for in the +Rhiz treatments. As expected, as nitrogen rate increased the number of root nodules per plant decreased ($df_{3, 35}$, P<0.001; (0N versus: 25N P=0.414; 50N P<0.001; 100N P<0.001; 25N versus: 50N P=0.004; 100N P<0.001; 50N versus: 100N P<0.001; Figure 17a). There was a similar trend for the total weight of all root nodules per plant, with the exception that nodules weighed significantly more in the 0N treatment than the 25N treatment ($df_{3, 35}$, P<0.001; 0N versus: 25N P=0.003; 50N P<0.001; 100N P<0.001; 25N versus: 50N P<0.001; 100N P<0.001; 50N versus: 100N P<0.001; Figure 17b).

Figure 16. Average number of days founder aphid lived. a) Cage A according to nitrogen treatment and rhizobia inoculation. b) Cage B according to nitrogen treatment, and rhizobia inoculation. Cage A: 0 days = 27 DAP, 25 days = 52 DAP; Cage B: 0 days = 24 DAP, 25 days = 49 DAP.



Above Ground Biomass. There was an interactive effect of N-rate and rhizobia on the dry weight of above ground plant tissue (N-Rate × Rhizobia, $df_{3, 71}$, P<0.001; Rhizobia, $df_{1, 71}$, P<0.001; N-Rate, $df_{3, 71}$, P<0.001; Figure 18), which was driven by smaller plant in the 0N treatment, but only when plants were not inoculated with rhizobia. Among +Rhiz plants above ground biomass decreased with increasing nitrogen, and with the exception of the 0N treatment, the biomass of the No-Rhiz plants followed a similar pattern. Generally, plants inoculated with rhizobia had greater above ground biomass.

Figure 17. Root nodule parameters assessed at 62 DAP. a) mean number of nodules per plant according to nitrogen treatment, +Rhiz treatments only. b) mean weight of all nodules per plant according to nitrogen treatment, +Rhiz treatments only. Different letters indicate significant differences at P<0.05.

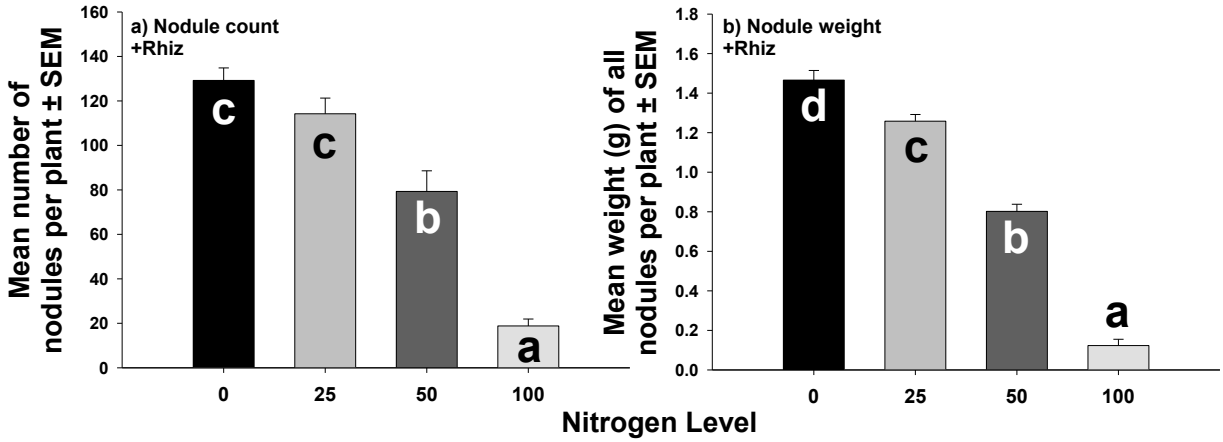
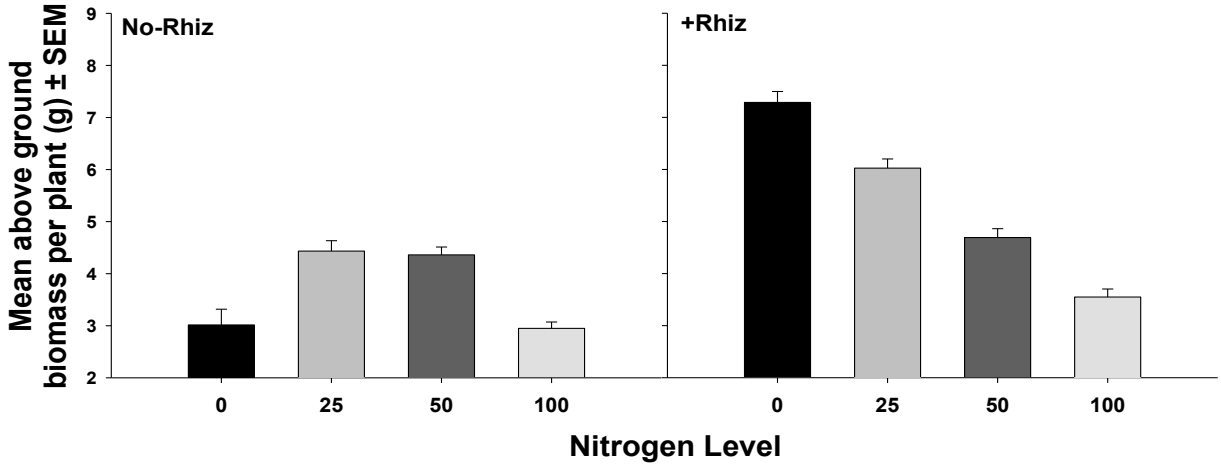


Figure 18. Mean above ground biomass per plant at 62 DAP according to rhizobial inoculation and nitrogen treatment.



Leaf Chlorophyll. Leaf chlorophyll measurements (as assessed indirectly using a SPAD meter) were taken four times throughout the experiment, and can be used as an indicator of nitrogen stress. Treatment effects on SPAD meter readings changed throughout the course of the experiment, as evidenced by significant time by treatment interactions (Time × N-Rate × Rhizobia, $df_{9, 207}$, P=0.013; Time × Rhizobia, $df_{3, 207}$, P<0.001; Time × N-Rate, $df_{9, 207}$, P=0.002; Time, $df_{3, 207}$, P<0.001; N-Rate × Rhizobia, $df_{3, 69}$, P<0.001; Rhizobia, $df_{1, 69}$, P<0.001; N-Rate, $df_{3, 69}$, P<0.001; Figure 19). In general, SPAD meter readings on non-inoculated plants were highest at the highest N-rate (100N), followed by the 50N, 25N, and 0N. With the exception of the 100N treatment, readings for No-Rhiz plants decreased over time, and this likely reflects the fact that plants were steadily using up the available nitrogen in the soil. In contrast, SPAD meter readings on plants receiving inoculants steadily increased over time, indicating an increasing reliance on N-fixation. Significant interactions between treatments were likely caused by similar SPAD readings for 100N plants despite rhizobial inoculation and dissimilarities in the response of 0N plants. The former was expected since high N-rates suppress nodulation and 100N +Rhiz plants were functionally similar to 100N No-Rhiz plants. The latter was also expected, and although 0N +Rhiz plants initially had low readings, by the end of the experiment readings were similar among 0N, 25N, and 50N +Rhiz plants.

Plant N Compounds. There was a marginal interactive effect of rhizobial inoculation and N-rate on the nitrate content (ppm) of above-ground plant tissue (N-Rate × Rhizobia, $df_{3, 71}$, P=0.067; Rhizobia, $df_{1, 71}$, P=0.035; N-Rate, $df_{3, 71}$, P=0.183; Figure 20) due to the extremely low concentration found in No-Rhiz, 0N plants. Ureide content of above-ground plant tissue was impacted by an interaction between rhizobial inoculation and nitrogen treatment (N-Rate × Rhizobia, $df_{3, 71}$, P=0.040; Rhizobia, $df_{1, 71}$, P<0.001; N-Rate, $df_{3, 71}$, P=0.499; Figure 21).

Generally, +Rhiz plants contained more ureide-N except when N-rates were high, when ureide content was similar to the concentration found in No-Rhiz plants. Total-N content of above-ground plant tissue was significantly higher when plants were inoculated with rhizobia, although N-rate did not have a significant impact (Rhizobia, $df_{1, 71}$, P=0.025; N-Rate, $df_{3, 71}$, P=0.340; N-Rate × Rhizobia, $df_{3, 71}$, P=0.299; Figures 22 and 23).

Figure 19. Mean leaf chlorophyll content per plant over time according to treatment as measured using a SPAD meter; unit less measure. Higher values indicate more leaf chlorophyll.

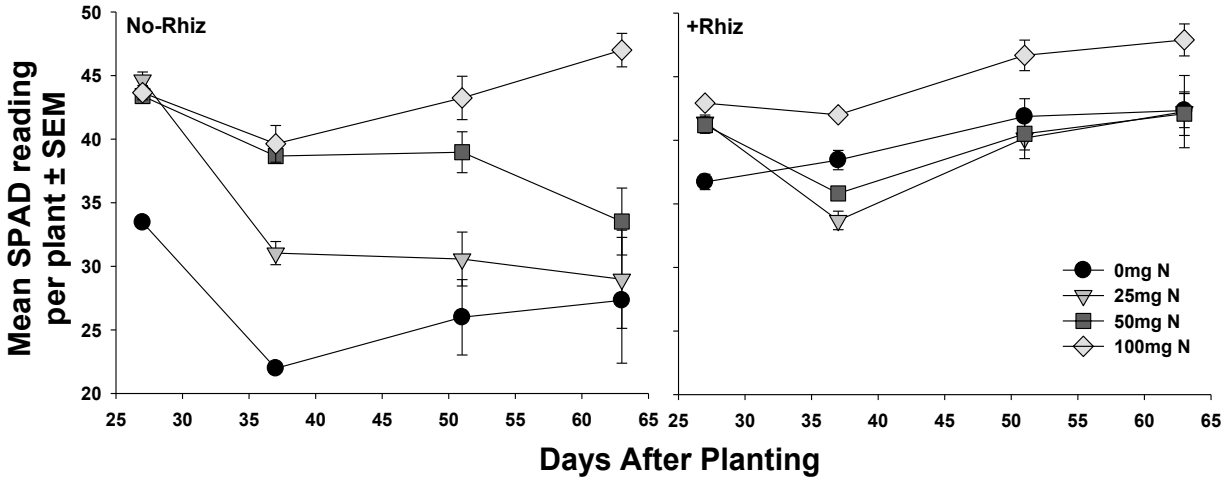


Figure 20. Mean nitrate-N per plant at 62 DAP according to rhizobia inoculation and nitrogen treatment.

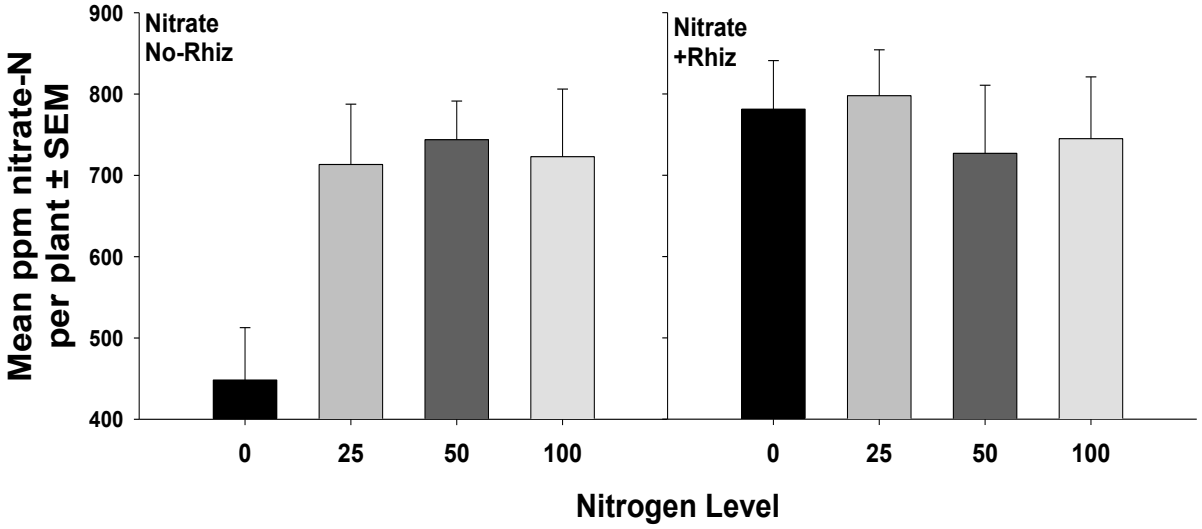


Figure 21. Mean ureide-N ppm per plant at 62 DAP according to rhizobia inoculation and nitrogen treatment.

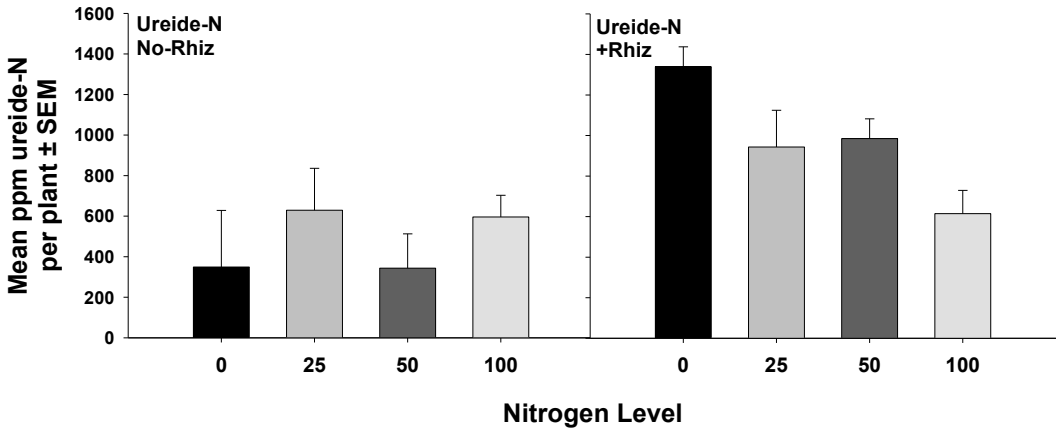


Figure 22. Mean total-N per plant at 62 DAP according to rhizobial inoculation and nitrogen treatments.

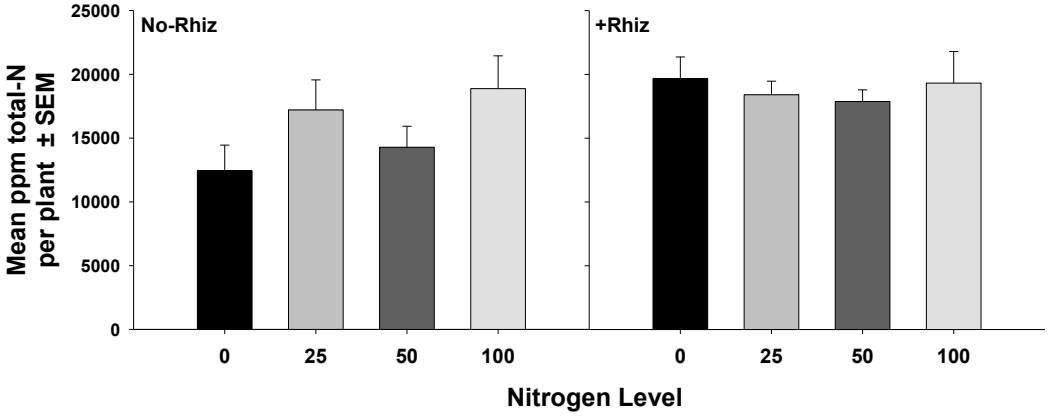
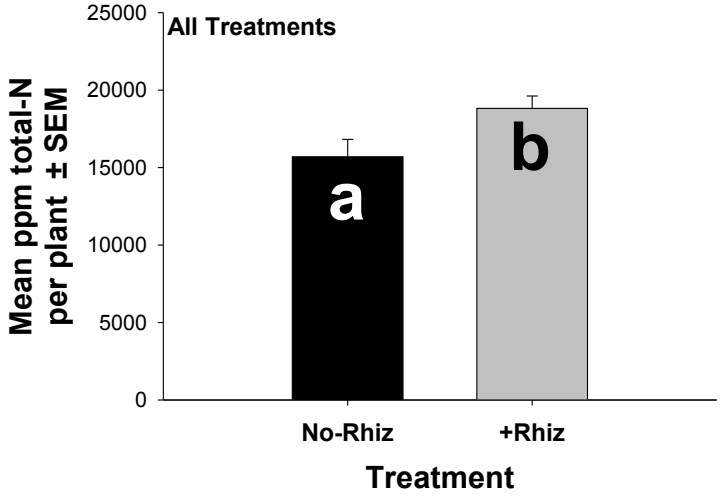


Figure 23. Mean total-N per plant according to rhizobial inoculation at 62 DAP, data combined across all nitrogen treatments. Different letters indicate significant differences at $P<0.05$.



Conclusion

Nitrogen is often a limiting factor for the growth and development of many living organisms, including many herbivorous insects (reviewed by Mattson 1980). Because of this the nitrogen content of their host plant is extremely important to their biology and population growth. Many plants rely on the nitrogen content of the soil, from fertilizers or the decomposition of organic matter, for growth and reproduction. Legumes, however, have developed a specialized relationship with rhizobia, bacteria that can fix atmospheric nitrogen, to meet their nitrogen needs. This relationship is dependent on exogenous nitrogen present in the soil (e.g. fertilizer), and high soil nitrogen results in low N-fixation (Evans 1982, Ohyama et al. 2009). Rhizobia can positively impact above ground plant parameters, such as nitrogen content of foliage and plant defensive compounds (McClure and Israel 1979, Johnson et al. 1987, Herridge et al. 2008, Salvagiotti et al. 2008, Katayama et al. 2011), which can subsequently impact insect herbivores. We used greenhouse studies to explore how differential nitrogen sources (i.e. rhizobia and nitrogen fertilizer) impacted phloem-feeding soybean aphid establishment on plants, reproduction, development time, and lifespan.

In the first experiment, when aphids were confined to one trifoliate, we found that the amount of nitrogen fertilizer (N-rate) and rhizobia inoculation did not impact aphid establishment on plants, nor did it impact the number of offspring produced in the first few days (24 to 72 h). However, after 9 d aphid densities were higher on inoculated plants with the lowest levels of nitrogen fertilizer. To aid in understanding how our treatments affected plant growth and parameters associated with N-fixation through time, we conducted a similar experiment with no aphids when plants were destructively sampled at various time points (see Appendix 1). Based on those results, the nodulation pattern of inoculated soybeans 9 d after aphid infestation

(34 DAP) decreased as N-rate increased (Tables A4-A5, Figures A4-A5). Therefore, in our aphid experiment it appeared that plants with the most nodules, and presumably the highest N-fixation rates, had the highest aphid densities. Kempel et al. (2009) found that aphid densities on clover were marginally higher on plants with rhizobia, although this was likely due to factors other than N-fixation, as they were working with a plant line that could not form functioning nodules. Our results are different from Katayama et al. (2011), where soybean aphid densities were similar between nodulating and genetically-similar non-nodulating soybean lines in a common garden experiment with natural arthropod infestation.

With regard to N-rate, aphids on plants receiving intermediate levels of fertilizers (25mg N per pot) had the highest densities. It is not surprising that aphids on non-inoculated plants receiving 0N did poorly, as over time these plants showed signs of nitrogen deficiency (i.e. were yellowing). We also saw that effects of N-rate on aphid densities were non-linear; aphid performance was poor on plants receiving the highest amount of fertilizer. Previous experiments have demonstrated that as applied nitrogen increases so does the nitrogen content of the foliar tissue, however, excess nitrogen can harm herbivorous insects as indicated by reduced aphid densities (Zehnder and Hunter 2009, Sauge et al. 2010).

After removing clip cages we looked at how treatments affected aphid population growth when aphids were allowed to roam freely over the plant. Initially, aphid performance was similar to what was observed at the end of our clip cage experiment, with highest population growth on plants that presumably had the highest N-fixation rates. However, it is important to note that by the end of the experiment aphid population growth rates on those plants actually became negative. It is unlikely that this is related to density-dependent limitations on population growth, as mean aphid densities at the end of the experiment were not excessive (~100 aphids per plant).

Declines in aphid population growth in specific treatments may reflect an initial benefit of increased plant nutrients, followed by subsequent negative effects due to the production of defensive compounds or some other factor. Population growth of aphids on non-inoculated low N plants was not consistent through time, because these plants, which were initially yellowed and nitrogen deficient, developed mutant nodules mid-way through the experiment that began fixing-N. We do not believe this contamination impacted the aphids in clip cages, since plants only began to turn green after that portion of the experiment was completed. However, this contamination certainly affected plant parameter results.

Aphid densities at the end of the experiment were not correlated with plant parameters relating to plant nitrogen and N-fixation (i.e. nodules, ureide-N and nitrate-N). The lack of correlation we found between nitrogen compounds and aphid densities parallels the results from Dean et al. (2009), although they only looked at total-N content of the foliage. Our results contrast those of Noma et al. (2010), who showed that as soybean leaf nitrogen content increased, so did aphid densities. Lack of significant correlations between final aphid densities and nitrate-N are not necessarily surprising because nitrogen fertilizer was only at the beginning of the experiment and therefore the amount available in the soil decreased over time as the plants grew. Looking at data from 48 DAP and the no aphid experiment (see Appendix), initial differences in plant nitrate declined until the effect of N-rate was no longer significant and nitrate levels in plant tissue converged among treatments. In addition, due to the mutant nodules, towards the end of the experiment nodulation and ureide-N were similar among treatments. It may be more informative to look at how changes in plant nodules and nitrogenous compounds are related to changes in aphid densities, rather than examine data from a single time point. Furthermore, plant parameters assessed were used as indices of N-fixation; we did not actual

measure N-fixation activity or nitrogenous compounds within xylem or phloem sap. Therefore, it is possible that this level of resolution was too coarse and/or these compounds are not relevant, instead we may need to relate aphid densities to changes in nitrogenous compounds (i.e. amino acids) found within the phloem. Another possibility is that effects of rhizobia on aphid densities are unrelated to nodulation or N-fixation.

For the second experiment we took more precautions to prevent contamination of the No-Rhiz treatments, including sterilizing the seeds, and had minimal contamination of non-inoculated plants. Treatment effects on aphid developmental time and longevity depended to some degree on what host plant environment juvenile aphids (and potentially their mothers) had experienced, although overall differences were minor. In general, it appeared that there was a positive relationship between nitrogen fertilizer rate and aphid development time. However, one notable exception was that aphids born on experimental plants (i.e. Cage A) matured faster on high nitrogen plants (100N), but only when plants were inoculated with rhizobia. Our results do not correlate with those of Wilson and Stinner (1984) who found that Mexican bean beetle larvae developed faster on nitrogen fertilized plants than on rhizobia inoculated plants. Aphid longevity was lower on nitrogen deficient plants (i.e. receiving the lowest amount of nitrogen), but this effect was ameliorated when plants were inoculated with rhizobia, as presumable these plants were obtaining adequate nitrogen via the process of N-fixation. Effects of the latter on aphid longevity were more pronounced for aphids that had been on experimental plants for the shortest period of time (i.e. Cage B). It is unclear if differences between Cage A and B are due to the previous experience of the aphid itself or of its mother.

Overall, our treatments did not impact aphid establishment on plants. In contrast, nitrogen source did affect aphid biology and reproduction, and there was evidence that early juvenile

and/or maternal environment influenced the response. In general, aphids did best (i.e. higher reproduction) on plants inoculated with rhizobia at intermediate levels of fertilization. It appeared that effects were most pronounced on plants that presumably had the highest degree of N-fixation, although the type of effect (positive versus negative) on population growth rates appeared to change over time. At the end of the experiment there were no correlations between aphid densities and plant parameters associated with nitrogen dynamics. It is unclear if differential effects of nitrogen source on aphids are due to alterations in plant nutrition or some other factor(s). Effects of changing host plant nutrition on aphids may be minimal due to their internal nutritional symbionts (i.e. *Buchnera*). In contrast, there is a growing body of literature that suggests rhizobia-mediated effects on above ground herbivores are related to defensive compounds.

There are several factors that can influence the density of a population including establishment, reproduction, development and longevity. To reach maximum densities individuals within a population need to have high establishment, high reproduction, short development time and have longer life spans. In this series of experiments establishment was high for all treatments. Reproduction, measured 9 d after infestation, was highest on plants presumably fixing the most nitrogen. Development time was the shortest on plants that were presumably deficient in nitrogen. Longevity was longest on all treatments assumed to have adequate to excessive nitrogen (i.e. all but the plants appearing to be deficient in N, the No-Rhiz 0N and 25N). Population growth, before nodule contamination, was similar to reproduction where it was the highest in the plants presumably fixing the most nitrogen. Development and longevity do not conflict with this; all the factors interact and impact each other to result in final population densities. Certain factors can have a stronger impact on final densities than other

factors. For example in this series of experiments it appears that reproduction had a stronger impact on aphid densities than development time.

CHAPTER 2. IMPACT OF COMMERCIAL SOYBEAN SEED INOCULANTS ON SOYBEAN APHIDS IN THE FIELD

Introduction

Plants routinely form close mutualistic associations with soil microorganisms such as bacteria and fungi. Although both partners directly benefit from the relationship, the implications for interactions with organisms in higher trophic levels are only beginning to be explored in depth. Many root-associated microorganisms aid plants by increasing their resistance to aboveground herbivores via defensive compounds (Kempel et al. 2009; reviewed in Pineda et al. 2010, Partida-Martinez and Heil 2011, Eisenhauer 2012). In contrast, the increase in plant growth and nutrition due to the mutualistic association can make plants more susceptible to herbivores (reviewed in Pineda et al. 2010, Partida-Martinez and Heil 2011, Eisenhauer 2012). The factors impacting plant nutrition and defensive compounds can interact which makes this a difficult topic of study.

One of the best known plant-soil bacteria mutualisms is that between legumes and nitrogen (N) fixing bacteria. Commercial soybean (*Glycine max* L.) production requires less nitrogen input than non-leguminous crops due to this plant's ability to obtain biologically fixed nitrogen via a symbiotic relationship with *Bradyrhizobium japonicum* (Bradyrhizobiaceae), a bacterial species in a group of N-fixing taxa associated with legumes collectively referred to as rhizobia (reviewed by van Rhijn and Vanderleyden 1995). The bacteria are located within root nodules and convert atmospheric nitrogen gas (N_2) into nitrogenous compounds called ureides (allantoin and allantoic acid; reviewed by van Rhijn and Vanderleyden 1995).

Rhizobia are an integral part of the soil microbial community, and can persist in this environment for several years (Bottomley 1992). However, because the most suitable rhizobia

species may not occur in high densities, many soybean growers use commercially-available rhizobial seed inoculants to increase N-fixation and boost yield (Sprent and Sprent 1990, Bottomley 1992, Keyser and Li 1992). These commercial inoculants are typically applied to seeds before planting and contain *B. japonicum*, but they often have additional components intended to further increase yield (e.g. other biological organisms or growth promoting factors; see Table 2). The other chemical and biotic components in the inoculants may affect plant physiology in various ways, including enhancing seedling emergence, nodulation, N-fixation, and/or plant growth (Dénarié et al. 1996, Yesmin et al. 2004, Cassán et al. 2009, Rodriguez-Navarro et al. 2010, Sindhu et al. 2010).

Rhizobial species identity, plant nodulation, and N-fixation have been shown to impact the behavior and biology of herbivorous arthropods, including those that feed on legumes (Wilson and Stinner 1984, Dean et al. 2009, Kempel et al. 2009, Thamer et al. 2011, Katayama et al. 2011), although there are surprisingly few studies in the literature. Effects of rhizobia on herbivores may depend on the latter's feeding habit, as chewing herbivores seem to be affected to a greater degree than sucking herbivores (Kempel et al. 2009, Katayama et al. 2011). Although there is mixed evidence for effects of rhizobia on aphids (Dean et al. 2009, Kempel et al. 2009, Katayama et al. 2011), one study indicated that effects may depend on the species identity of rhizobia associating with the plant (Dean et al. 2009). Thus, it is possible that specific inoculants may differentially affect host plant quality for herbivorous insects, including invasive soybean aphids (Hemiptera: Aphididae: *Aphis glycines* Matsumura).

To investigate the impact of commercially-available rhizobial seed inoculants on soybean aphid establishment and reproduction on soybean plants, we conducted a two year field study using four inoculants, a non-inoculated control (i.e. only native rhizobia), and a high soil

nitrogen control intended to suppress nodulation and N-fixation while still providing adequate nitrogen (as nitrate) for plant growth. Two cages were erected in each field plot (one with aphids and one without aphids) to assess how treatments impacted parameters associated with plant quality and yield potential independent of aphid presence (e.g. height, number of pods, and above-ground biomass). We also assessed nodule weight and the nitrogen content of above-ground plant tissue (total-N, nitrate-N, ureide-N) in order to determine how the treatments affected foliar nitrogen levels and reliance on N-fixation, and also to explore if these parameters were correlated with aphid density.

Materials and Methods

Experimental Design

Experiments were conducted in 2010 and 2011 in Carrington ND at the North Dakota State University Research and Extension Center (CREC) adjacent to a long-term study evaluating impacts of seed inoculants on soybean agronomic properties. Each experimental plot was 1.5 x 7.6 m and was separated by buffer plots planted with soybeans (1.5 x 7.6 m). Therefore, each replicate consisted of a series of experimental and buffer plots, with each replicate separated by a 1.5 m fallow strip that ran the length the entire replicate (Figure 24). Treatments were randomly assigned to plots within each replicate. In late June of both years ammonium sulfate (2.3 L/ha) was mixed with glyphosate (3.5 L/ha) and applied to plots for weed control. Plots were not sprayed with any other chemicals, including insecticides, for the duration of the experiment.

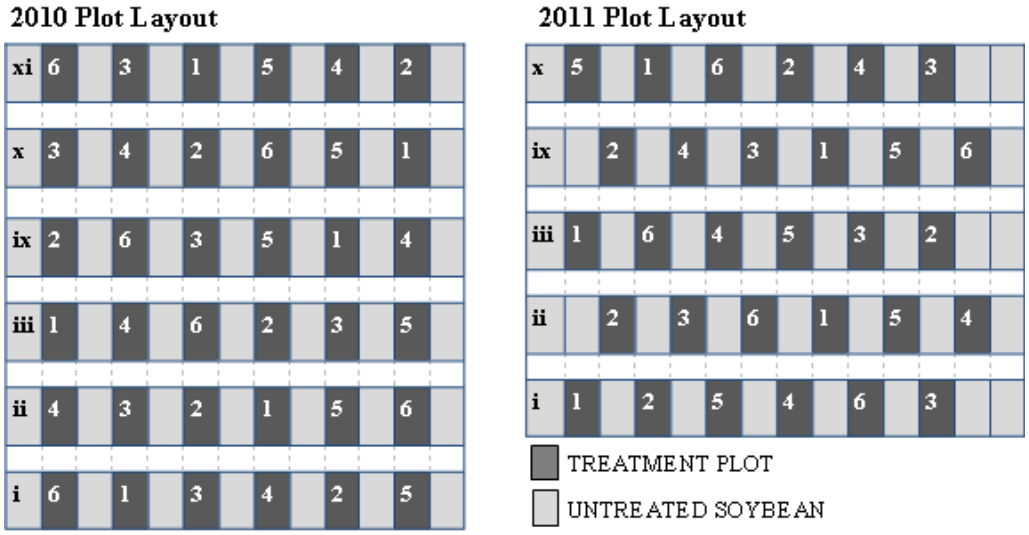
In 2010, there were six experimental treatments replicated six times: a non-inoculated control (Check), a non-inoculated high soil nitrogen control (N), and four commercial inoculants: N-Dure (INTX Microbials, LLC, Kentland IN), Optimize 400 (Opt, EMD Crop Bioscience, now

Novozymes, Brookfield WI), Primo (INTX Microbials) and BioBoost Plus (BB, BrettYoung, Winnipeg, Manitoba, Canada). In 2011, there were only five replicates of each treatment.

Table 2. List of select commercial inoculants and active ingredients or growth promoting factors contained within the product.

Product	Company	Form	Components
N-Dure	INTX Microbials	Peat	*B. japonicum*
BioBoost Plus	Brett Young	Peat	*B. japonicum* & *Delftia acidovorans*
Optimize 400	Novozymes	Peat	*B. japonicum* & lipo-chitooligosaccharide
Primo	INTX Microbials	Liquid	*B. japonicum* & *Azospirillum brasilense*

Figure 24. Plot layout maps for 2010 and 2011. Roman numerals indicate replicates, treatments are indicated by numbers: Check-1, N-2, N-Dure-3, BB-4, Opt-5 and Primo-6.



Dairyland 401 Roundup Ready soybeans (Dow AgroSciences, Indianapolis IN) were used in both 2010 and 2011. Inoculants were applied to seeds 1 d prior to planting in 2010 and within 4 h of planting in 2011. Seeds were inoculated by shaking them with the inoculant in a plastic bag according to package instructions. In 2010, seeds were stored out of the sun at room temperature until planting and in 2011 they were stored in a cooler. Soybean seeds were planted on 20-May 2010 and 2-June 2011 using a Hege 1000 research plot planter at a rate of 220,000

seeds per acre with 17.8 cm between rows and 10.4 cm within-row seed spacing. For high nitrogen control plots, 67.26 kg N fertilizer hectare^{-1} was applied by measuring out the appropriate amount of urea for each plot and hand broadcasting across the whole plot plus 0.76 m on all sides of each plot. Urea was added to plots after planting on 21-May 2010 and 7-June 2011, and therefore it was not manually incorporated into the soil. Instead, rainfall events were relied upon to mix the urea into the soil.

On 23-June 2010 and 18-July 2011, two green mesh field cages (0.6 w × 0.6 d × 1.4 m h; collapsible field cages; Bioquip, Rancho Dominguez CA) were erected over metal frames in each experimental plot. Cages were centered in plots so that approximately 0.3 m separated cages from each other and from plot edges. To access plants, each cage had a 1 m slit in the center of one side that was secured with a 2.5 cm wide Velcro® strip (Velcro USA Inc., Manchester NH) in 2010 and a zipper in 2011. Bottom edges of each cage were buried approximately 8 cm in the soil. When cages were erected, plants were thinned to two plants per cage in both years. All cages were sprayed with PyGanic® (active ingredients: pyrethrins; 1 oz. /1.25 gal; MGK, Minneapolis MN) on 23-June 2010 and 18-July 2011 in order to eliminate any arthropod predators or pests in cages prior to aphid infestation.

Aphid Infestation

One cage in each plot was infested with soybean aphids (designated as +Aphid cages) on 28-June 2010 and 21-July 2011. Five adult soybean aphids were added to both plants within a cage. Aphids originated from a colony maintained at NDSU on potted soybean plants (RG607 RR, Agronomy Seed Farm, Casselton, ND) at 25 ± 2°C, and 16:8 L:D. The aphid colony was established in 2008 and refreshed yearly using soybean aphids field-collected near Prosper and/or Fargo ND. On 28-June 2010 and 21-July 2011, aphids were removed from infested leaves

using a small paintbrush and then transferred to small plastic cups containing moist cotton and a partial soybean leaf. Cups were maintained in a refrigerator at 4 ± 2°C and transported to the field in a cooler. Plants in +Aphid cages were checked for arthropods, and then each plant was infested with five aphids by draping the leaf piece over the second trifoliate. Any aphids remaining in the cup were gently transferred to the second trifoliate using a small paintbrush.

Data Collection

Soybean aphid densities were recorded 24 h after infestation to determine the rate of insect establishment on host plants. In 2010, aphid density and plant height were non-destructively assessed for both plants within +Aphid and No-Aphid cages on 29-June, 6-July, 20-July, 4-August, and 19-August. In 2011, aphid density was non-destructively assessed for both plants within +Aphid and No-Aphid cages on 22-July, 4-August, and 23-August. In both years, final aphid counts were done after plants had been destructively sampled and frozen (i.e. 28-August 2010 and 8-September 2011). After aphids were counted on frozen plants, plants were placed back in the freezer so that the dry weight and nitrogen content of the foliage could be assessed at a later date.

In 2010, except for a few cages on 4-August and 19-August, arthropod contaminants were rarely observed and seemed to be found only in cages with very high aphid densities. When encountered they were removed by hand. In 2011, there was a natural infestation of soybean aphids in the general region, and a few aphids remained in field cages after spraying with PyGanic®, which we attempted to remove by hand on 21-July. On 27-July, No-Aphid cages were sprayed again with PyGanic® (1 oz. /1.25 gal) in an attempt to eliminate the soybean aphid contaminants. In addition, the presence of an aphid pathogen was noted in all experimental cages late in the season (23-August 2011).

On 28-August 2010 and 8-September 2011, all plants in +Aphid and No-Aphid cages were destructively sampled, which involved counting the number of pods, cutting stems at the soil surface, and placing each plant in a separate plastic bag (49.2 L in 2010 and 7.6 L freezer bag in 2011). Plant foliage samples were then transported to the lab in coolers and stored in the freezer at -20°C.

In 2010, data on root nodules (weight of nodules per plant) was obtained from CREC collaborators sampling non-caged, non-aphid infested plants from the adjacent long-term inoculant study. In 2011, root nodules were collected from the actual +Aphid and No-Aphid experimental plants. After the above-ground foliage was removed, the top 15 cm of the root system was dug out of the soil, and all soil and root material was placed in a self-sealing plastic bag and stored in a refrigerator (4 ± 2˚C) for up to 1 wk. To assess nodules, each sample was placed in a sieve (710 µm opening, U.S.A Standard Testing Sieve, No. 25) to remove soil and find any loose root nodules. All roots and root nodules were then washed to remove any excess soil and nodules still attached to the roots were removed by hand. All nodules from one plant were placed in a small metal dish, counted, dried for 24 h at 60°C, and weighed on a digital scale (Sartorius type 1412; Goettingen Germany).

In order to quantify the dry weight of the above-ground biomass, plants were removed from the freezer, immediately placed in paper bags, and dried for one week at 120°C in 2010 and at 96°C in 2011. In 2011, the lower temperature in the drying chamber resulted in a longer drying time, allowing mold to grow on the plants. Once this was discovered the plants were moved to a different chamber and dried an additional week at 120°C. In 2010, vegetative (i.e. all above-ground plant material except pods) and reproductive biomass (i.e. all pods) were assessed separately, although in 2011 only total above-ground biomass was determined. Dried plants were

then weighed on a digital scale (Sartorius type 1412) and stored in self-sealing plastic bags (3.8 L), contained within black plastic garbage bags at room temperature (~20°C) in the lab until the nitrogenous compounds within the above-ground plant tissue were assessed.

Analysis of Nitrogenous Compounds within Above-Ground Plant Tissue

Sample Preparation. Samples were prepared as described in Chapter 1 with a few modifications. Dried plants (all above-ground plant material) were ground then stored in an airtight container. A small sample (0.2-0.4 g) of ground plant material was sent to the NDSU Soil Testing lab for analysis of total nitrogen (Kjeldahl method). For nitrate and ureides analyses, samples were digested by putting 0.400 ± 0.001 g of ground plant material and 20 ml of distilled water in a 20 x 125 mm screw top vial (Pyrex, VWR International).

Nitrate Analysis. The nitrate (NO_3^-) content of the samples was assessed according to the salicylic acid method (Cataldo et al. 1975). As all experimental samples could not be processed at the same time, they were processed by replicate. Absorbance readings for all standards were taken with each batch of samples, and therefore a unique linear equation was generated for each replicate.

Ureide Analysis. We used the Patterson method to determine the ureide content in the soybean tissue (Patterson et al. 1982). Plant extracts needed to be diluted for the analysis, so each tube (20 x 125 mm glass screw top vial) received 0.5 mL of distilled water and 0.5 mL of filtered sample. The standards were not diluted, so each tube received 1.0 mL of standard. To calculate the ppm of ureide in the plant, the ppm in the extract was multiplied by the same dilution factor used for nitrates (20 mL water × weight of sample), multiplied by two for the diluted samples [i.e. (20 × 0.400 g) ×2]. Again, note that the exact weight of each sample was used instead of 0.400 g.

Data Analysis

Data from the two plants within each cage were averaged for analyses and all data were analyzed using SYSTAT® 12 (SYSTAT Software, Inc. 2007). Histograms and Levene's test were to determine if data met the assumptions necessary for parametric statistics. We used repeated measures ANOVA to assess the impacts of treatments on explanatory variables that were non-destructively assessed throughout the season. Due to differences in timing of sampling between years, data from parameters assessed non-destructively were analyzed separately for each year. For 2010 plant height analyses, a factorial repeated measures ANOVA was used, with aphid infestation and inoculant treatment as the independent variables. The word inoculant in analyses encompasses all soil treatments (i.e. non-inoculated control plants associated with native soil rhizobia, non-inoculated plants in plots with excess nitrogen to suppress nodulation, and plants that receiving each of the four types of commercially-available rhizobial inoculants at planting). In 2011, plant height was only assessed twice, and therefore data from each date were analyses using factorial ANOVA with aphid infestation and inoculant treatment as the independent variables.

Data on mean aphid density per plant and cumulative aphid days (CAD) from both years were log (X+1) transformed to meet the assumptions of parametric statistics prior to analysis. CAD were calculated using the equation formulated by Hanafi et al. (1989). For 2010 aphid analyses, aphid density or CAD were the explanatory variables and inoculant treatment was the independent variable. However, in 2011, No-Aphid cages became contaminated with resident aphids, and therefore aphid infestation was included as an independent variable in the model to assess differences between treatments and possible implications for plant parameters. If Time × Treatment interactions were significant, a profile analysis was done, which consisted of

conducting separate ANOVAs on data from each sampling date. Fisher's LSD test was used for mean separation if the main *P*-value was significant.

Data from explanatory variables that were assessed once at the end of both seasons (i.e. pod density, dry weight of total above-ground biomass) were analyzed using factorial ANOVA with year, aphid infestation, and inoculant treatment as the independent variables. Data on parameters only assessed for one year [i.e. vegetative and reproductive biomass (2010 only), nitrogen content of above-ground biomass (i.e. total-N, nitrate-N, and ureide-N; 2010 only) and nodule weight (2011 only)] were assessed using factorial ANOVA with aphid infestation and inoculant treatment as the independent variables. Because data on 2010 nodule weight was collected from adjacent CREC plots lacking the aphid infestation treatment, these data were analyzed using ANOVA with inoculant treatment as the sole independent variable. If the overall *P*-value was significant, Fisher's LSD test was used for mean separation among treatments.

Strength of linear correlations between variables were determined using Pearson product-moment correlation coefficients and R^2 (coefficient of determination) values. The significance of the correlations (i.e. the likelihood that the correlation coefficient would occur if there was no relationship between the variables) was determined using Bonferroni probabilities as part of the Pearson correlation analysis (SYSTAT Software, Inc. 2007). Log (X+1) transformed aphid densities from the last sampling date were used in these analyses.

Results

Plant Parameters

Root Nodules. In 2010, root nodule data was collected by CREC personnel, and at the end of the season the total weight of nodules per plant in the N treatment was significantly lower than all other treatments ($df_{5, 30}$, $P<0.001$; N versus: Check $P<0.001$, N-Dure $P<0.001$, BB

$P<0.001$, Opt $P<0.001$, Primo $P<0.001$; $P\geq0.05$ for all other comparisons; Figure 25). This demonstrates that the excess nitrogen applied to these plots successfully suppressed nodulation. However, in 2011, there was no significant treatment effect on total nodule weight per plant (Inoculant × Aphid, $df_{5, 36}$, $P=0.649$; Inoculant, $df_{5, 36}$, $P=0.652$; Figure 26), indicating that the nitrogen treatment did not effectively suppress nodulation. In 2011, effects of aphid infestation on nodule weight was not significant (Aphid, $df_{1, 36}$, $P=0.352$), which was likely due to contamination of No-Aphid cages by naturally occurring aphid populations (Figure 26).

Figure 25. Mean weight (grams) of all nodules per plant in 2010 collected from open plots by the CREC staff according to treatment. Different letters indicate significant differences at $P<0.05$.

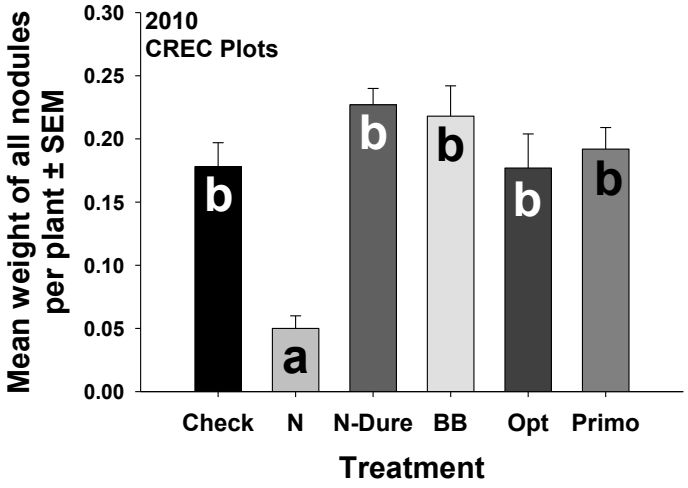
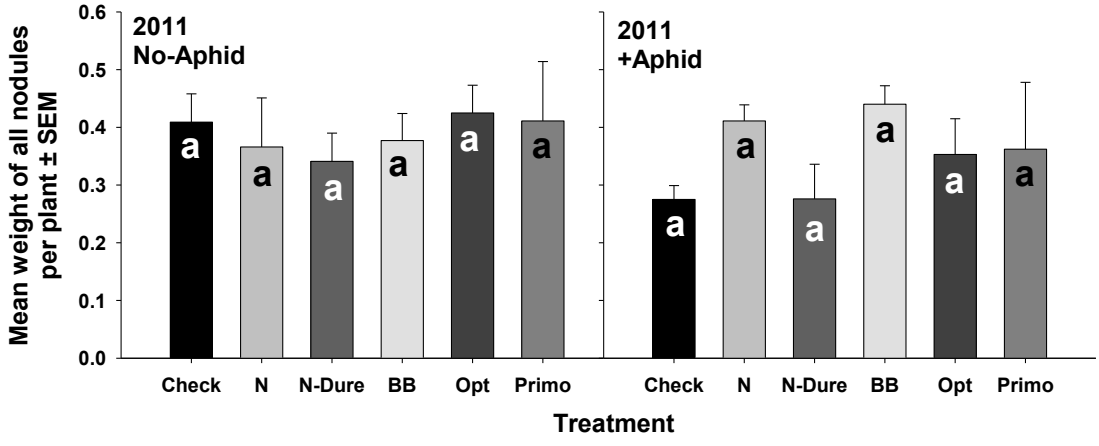


Figure 26. Mean weight (grams) of all nodules per plant in 2011 according to treatment and aphid infestation. Different letters indicate significant differences at $P<0.05$.



Plant Height. In 2010, plant height increased steadily in the beginning of the season before leveling off 36 d after the start of the experiment (Time, $df_{5, 295}$, $P<0.001$). Impacts of treatments on plant height were consistent throughout the season (Time × Aphid × Inoculant, $df_{25, 295}$, $P=0.811$; Time × Aphid, $df_{5, 295}$, $P=0.056$; Time × Inoculant, $df_{25, 295}$, $P=0.523$). Aphid infestation persistently had a negative impact on plant height (24 h: Aphid × Inoculant, $df_{5, 59}$, $P=0.929$; Aphid, $df_{1, 59}$, $P=0.029$; Figure 28). The significant impact of inoculant on plant height (24 h: Inoculant, $df_{5, 59}$, $P=0.049$) was driven by plants in the N treatment being shorter than all other treatments except BB (24 h after aphid infestation, N versus: check $P=0.035$; N-Dure $P=0.006$; Opt $P=0.035$; Primo $P=0.001$; $P\geq0.05$ for all other comparisons; Figure 27).

In 2011, because the experiment was started later in the season, plants were generally taller at the start of the experiment (22-July, 13.05 ± 0.20 inches) than in 2010 (29-June, 6.02 ± 0.06 inches). In 2011, none of the treatments had a significant impact on plant height, either at the start (24 h) or end (49 d) of the experiment (24 h: Inoculant × Aphid, $df_{5, 48}$, $P=0.835$; Aphid, $df_{1, 48}$, $P=0.747$; Inoculant, $df_{5, 48}$, $P=0.702$; 49 d: Inoculant × Aphid, $df_{5, 48}$, $P=0.598$; Aphid, $df_{1, 48}$, $P=0.822$; Inoculant, $df_{5, 48}$, $P=0.713$; Figures 28 and 29).

Figure 27. Mean height per plant 24 h and 61 d after aphid infestation in 2010 according to inoculation treatment. Data combined across all aphid infestation treatments. Different letters indicate significant differences at $P<0.05$.

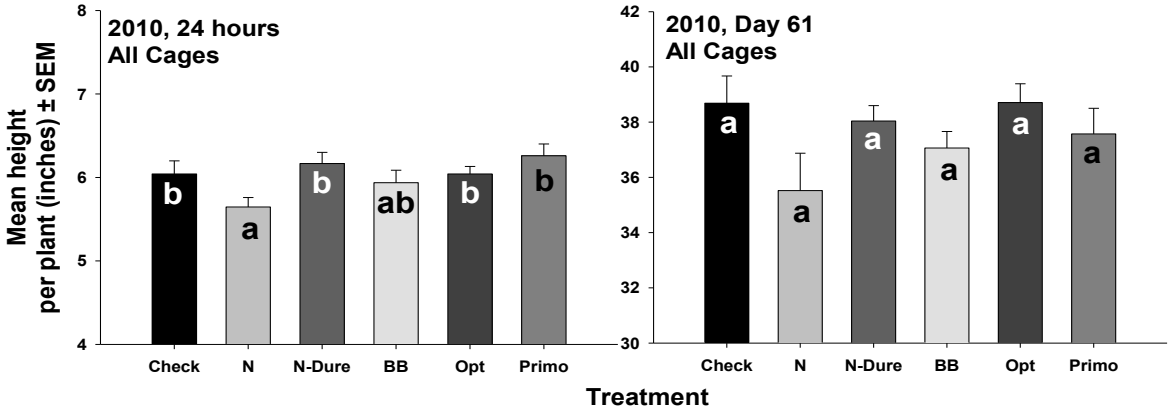


Figure 28. Mean height per plant 24 h and 49 d after aphid infestation in 2011 according aphid infestation treatment. Data combined across all inoculant treatments. Different letters indicate significant differences at $P<0.05$.

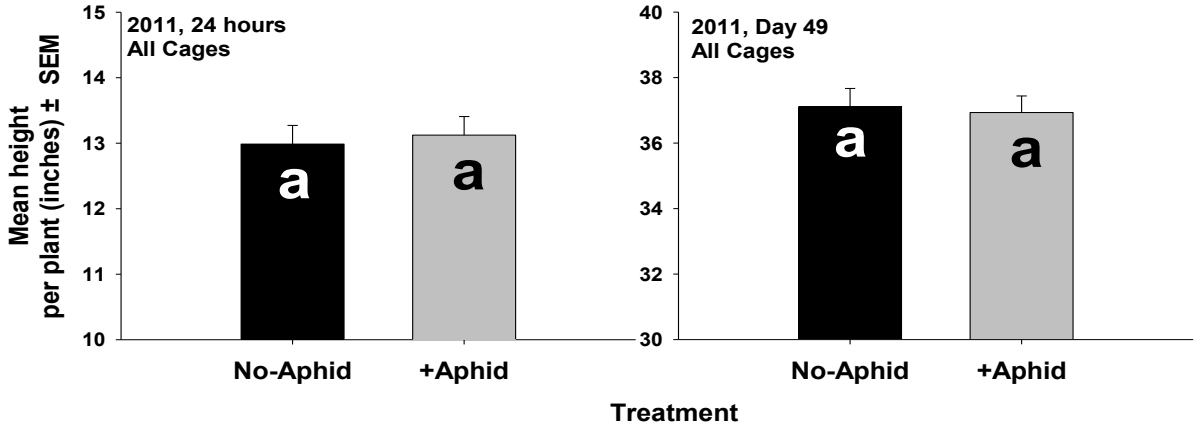
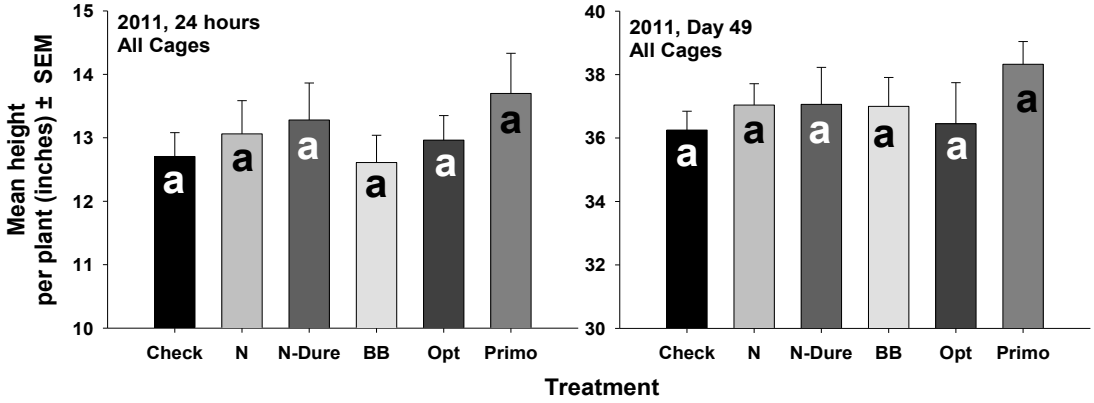


Figure 29. Mean height per plant 24 h and 49 d after aphid infestation in 2011 according to inoculation treatment. Data combined across all aphid infestation treatments. Different letters indicate significant differences at $P<0.05$.



Above-Ground Biomass. In 2010, above-ground biomass was separated into vegetative and reproductive biomass. Vegetative biomass was negatively affected by aphid infestation (Aphid × Inoculant, $df_{5, 60}$, $P=0.789$; Aphid, $df_{1, 60}$, $P=0.001$; Figure 30). Inoculant treatment also impacted vegetative biomass, with plants in the excess nitrogen treatment having significantly less vegetative biomass than all other treatments except BB inoculated plants (Inoculant, $df_{5, 60}$, $P=0.038$; N versus: Check $P=0.012$, N-Dure $P=0.048$, Opt $P=0.005$, Primo $P=0.015$, $P\geq0.05$ for all other comparisons; Figure 31).

Figure 30. Mean vegetative biomass (grams) per plant in 2010 according to aphid infestation treatment. Data combined across all inoculation treatments. Different letters indicate significant differences at $P<0.05$.

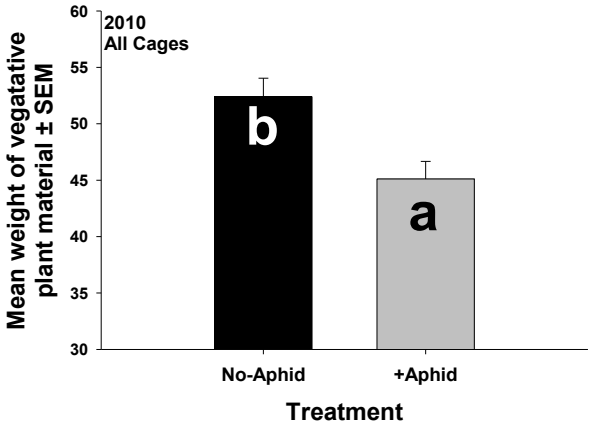
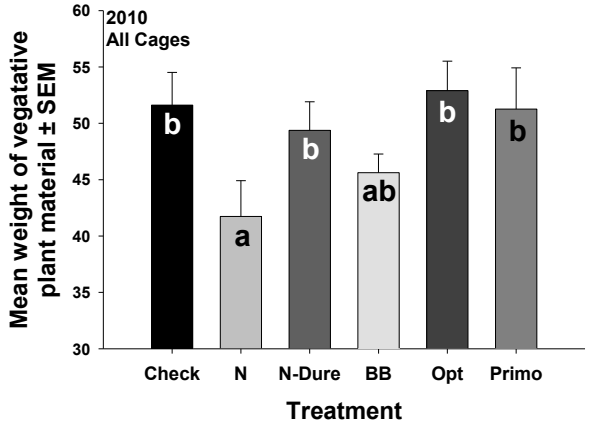


Figure 31. Mean vegetative biomass (grams) per plant in 2010 according to inoculation treatment. Data combined across all aphid infestation treatments. Different letters indicate significant differences at $P<0.05$.



Reproductive biomass was also significantly impacted by aphid infestation resulting in lower pod biomass in +Aphid cages than in No-Aphid cages (Aphid × Inoculant, $df_{5, 60}$, $P=0.519$; Aphid, $df_{1, 60}$, $P<0.001$; Figure 32). Similar to vegetative biomass, reproductive biomass was also impacted by the inoculation treatment in with plants in plots receiving excess nitrogen having significantly lower biomass than plants in all other treatments, except BB inoculated plants (Inoculant, $df_{5, 60}$, $P=0.029$; N versus: Check $P=0.018$, N-Dure $P=0.001$, Opt $P=0.018$, Primo $P=0.021$, $P\geq0.05$ for all other comparisons; Figure 33). In 2011, above-ground biomass was not separated into vegetative and reproductive biomass.

Figure 32. Mean reproductive biomass (grams) per plant in 2010 according to aphid infestation treatment. Data combined across all inoculation treatments. Different letters indicate significant differences at $P<0.05$.

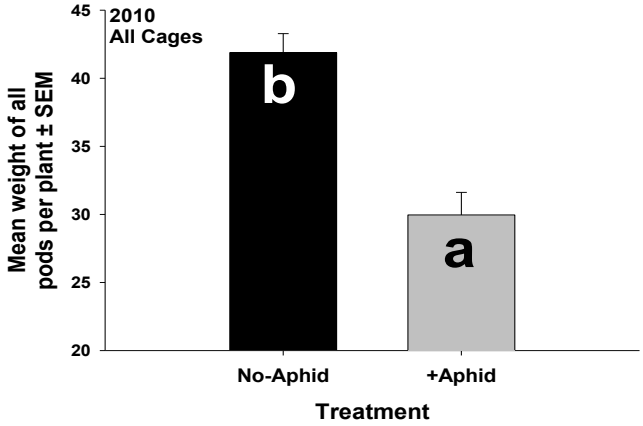
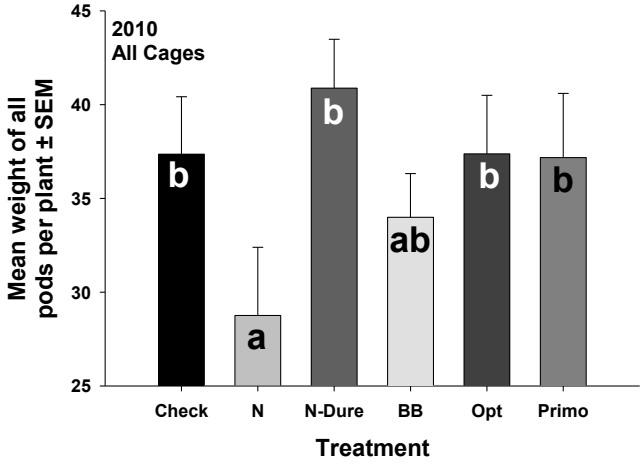


Figure 33. Mean reproductive biomass (grams) per plant in 2010 according to inoculation treatment. Data combined across all aphid infestation treatments. Different letters indicate significant differences at $P<0.05$.



The impact of aphid infestation on total above-ground biomass (vegetative + reproductive) varied from year to year, and so data from 2010 and 2011 were analyzed separately (Year × Aphid × Inoculant, $df_{5, 105}$, $P=0.744$; Year × Aphid, $df_{1, 105}$, $P=0.004$; Year × Inoculant, $df_{5, 105}$, $P=0.233$; Year, $df_{1, 105}$, $P<0.001$; Figures 34 and 35). In 2010, aphid infestation negatively impacted total above-ground biomass regardless of inoculation treatment (Aphid × Inoculant, $df_{5, 57}$, $P=0.510$; Aphid, $df_{1, 57}$, $P<0.001$; Figure 34). Additionally, inoculation treatment had a significant impact on above-ground biomass (Inoculant, $df_{5, 57}$, $P=0.018$; Figure

35), which was driven by significantly lower biomass in the N treatment compared to the check, N-Dure, Opt, and Primo treatments (N versus: Check P=0.018, N-Dure P=0.004, Opt P=0.004, Primo P= 0.003, $P \geq 0.05$ for all other comparisons). In 2011, there was no impact of either inoculant or aphid infestation on the total above-ground plant biomass (Aphid × Inoculant, $df_{5, 48}$, P=0.994; Aphid, $df_{1, 48}$, P=0.458; Inoculant, $df_{5, 48}$, P=0.935; Figures 34 and 35).

Figure 34. Mean weight of above-ground biomass (grams) per plant according to aphid infestation treatment and year. Data combined across all inoculant treatments. Different letters indicate significant differences at P<0.05.

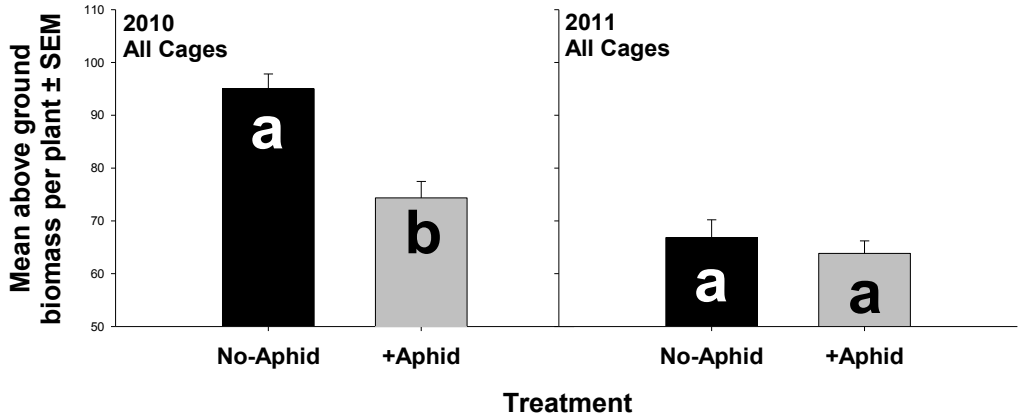
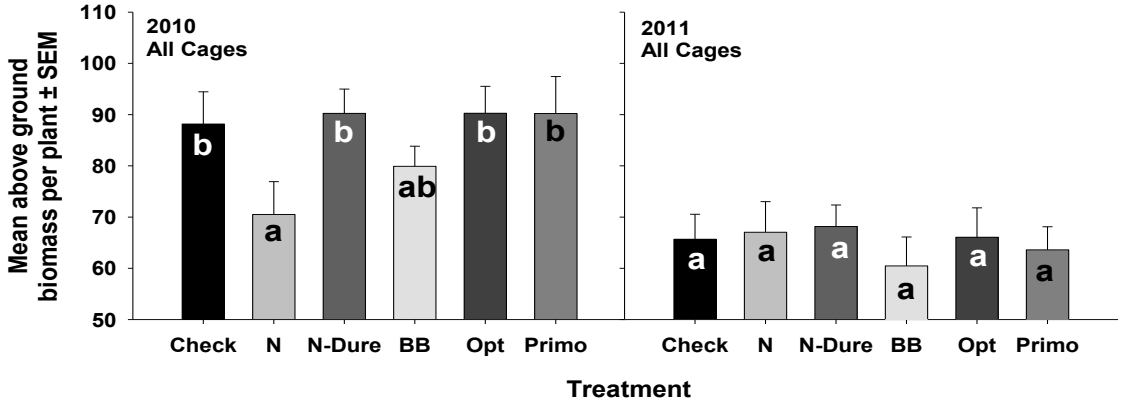


Figure 35. Mean weight of above-ground biomass (grams) per plant according to treatment and year. Data combined across all aphid infestation treatments. Different letters indicate significant differences at P<0.05.



Number of Pods. Effects of aphid infestation on the number of pods per plant varied from year to year, and so data from 2010 and 2011 were analyzed separately (Year × Aphid × Inoculant, $df_{5, 108}$, P=0.610; Year × Aphid, $df_{1, 108}$, P=0.001; Year × Inoculant, $df_{5, 108}$, P=0.298;

Aphid × Inoculant, $df_{5, 108}$, P=0.841; Year, $df_{1, 108}$, P=0.204; Aphid, $df_{1, 108}$, P<0.001; Inoculant, $df_{5, 108}$, P=0.098). In 2010, aphid infestation had a strong negative effect on the number of pods per plant, regardless of inoculant treatment (Aphid × Inoculant, $df_{5, 60}$, P=0.540; Aphid, $df_{1, 60}$, P<0.001; Figure 36). In addition, there was a significant effect of inoculant on pod density (Inoculant, $df_{5, 60}$, P=0.038) that was driven by lower numbers of pods in the N treatment compared to the N-Dure treatment (P=0.035, P≥0.05 for all other comparisons; Figure 37). In 2011, there were no significant effects of aphid or inoculant treatments on pod densities (Aphid × Inoculant, $df_{5, 48}$, P=0.902; Aphid, $df_{1, 48}$, P=0.674; Inoculant, $df_{5, 48}$, P=0.708; Figures 36 and 37).

Figure 36. Mean number of pods per plant in 2010 and 2011 according to aphid infestation treatment. Data combined across all inoculant treatments. Different letters indicate significant differences at P<0.05.

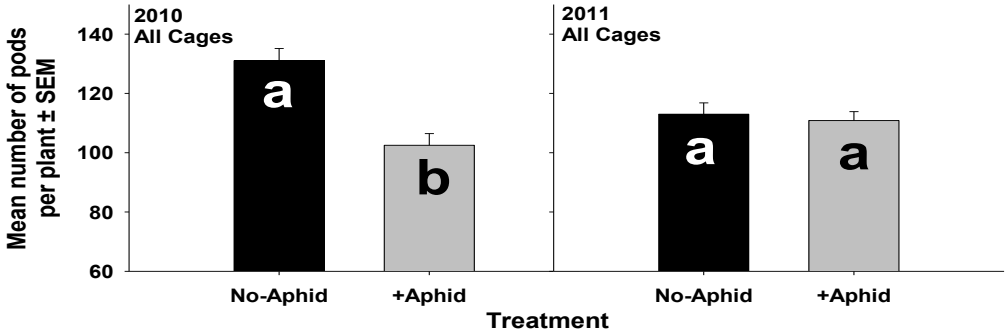
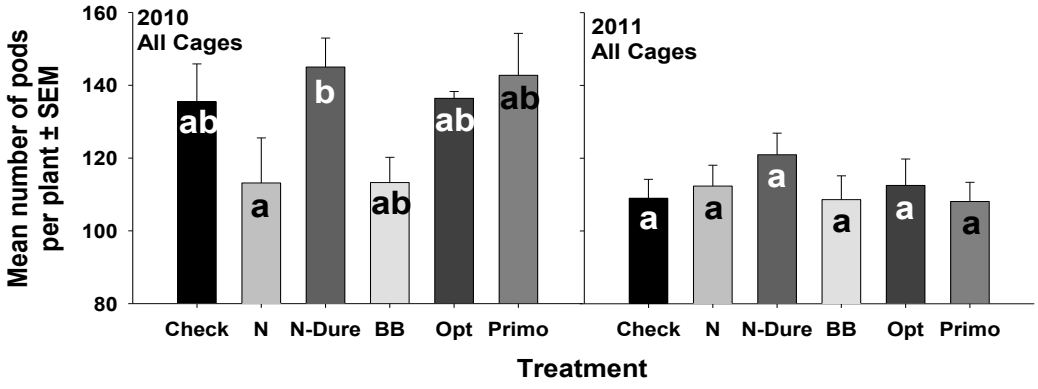


Figure 37. Mean number of pods per plant in 2010 and 2011 according to inoculation treatment. Data combined across all aphid infestation treatments. Different letters indicate significant differences at P<0.05 (the N-Dure vs. BB P=0.071).



Nitrogenous Plant Compounds. Nitrogenous compounds within above-ground plant tissue were only assessed in 2010 due to problems with mold on 2011 plant samples. Inoculation treatment did not have a significant impact on total-N in 2010 (Inoculant × Aphid, $df_{5, 60}$, P=0.863; Inoculant, $df_{5, 60}$, P=0.784; Figure 38). However, the No-Aphid plants contained significantly more total-N than the +Aphid plants (Aphid, $df_{1, 60}$, P=0.047). The ureide and nitrate content of the plants did not differ significantly among inoculation or aphid infestation treatments (ureide-N: Inoculant × Aphid, $df_{5, 60}$, P=0.664; Inoculant, $df_{5, 60}$, P=0.462; Aphid, $df_{1, 60}$, P=0.091; nitrate-N: Inoculant × Aphid, $df_{5, 60}$, P=0.151; Inoculant, $df_{5, 60}$, P=0.134; Aphid, $df_{1, 60}$, P=0.097; Figures 39 and 40).

Figure 38. Mean ppm of total-N per plant in 2010 according to treatment and aphid infestation.

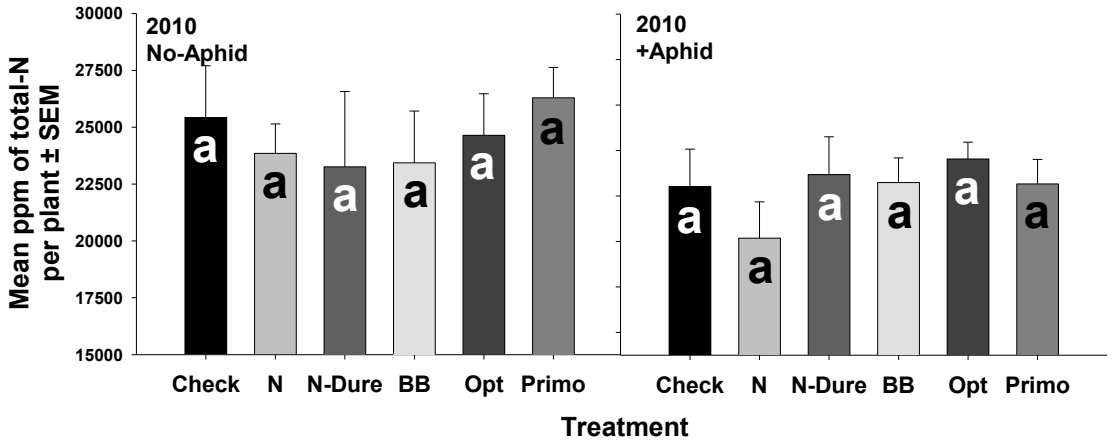


Figure 39. Mean ppm of ureide-N per plant in 2010 according to treatment and aphid infestation.

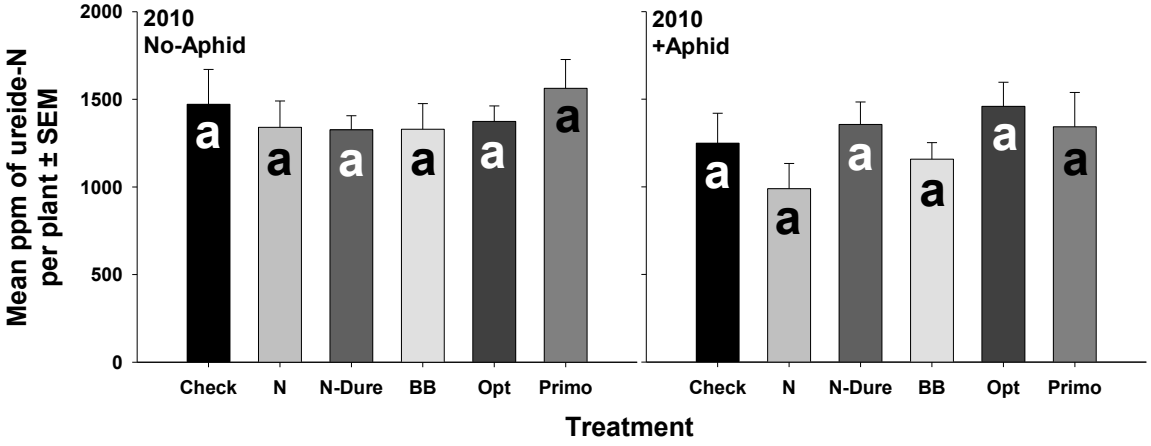
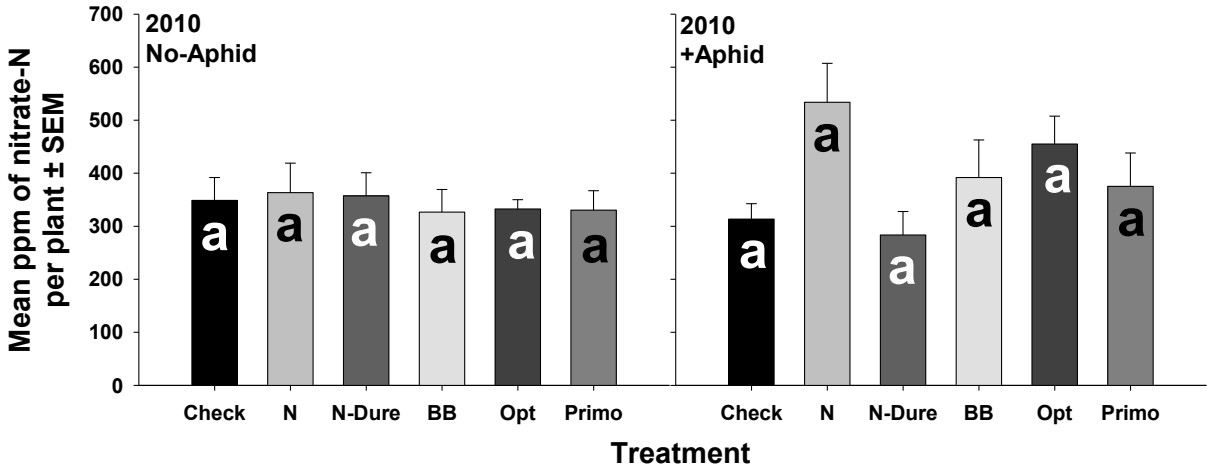


Figure 40. Mean ppm of nitrate-N per plant in 2010 according to treatment and aphid infestation.



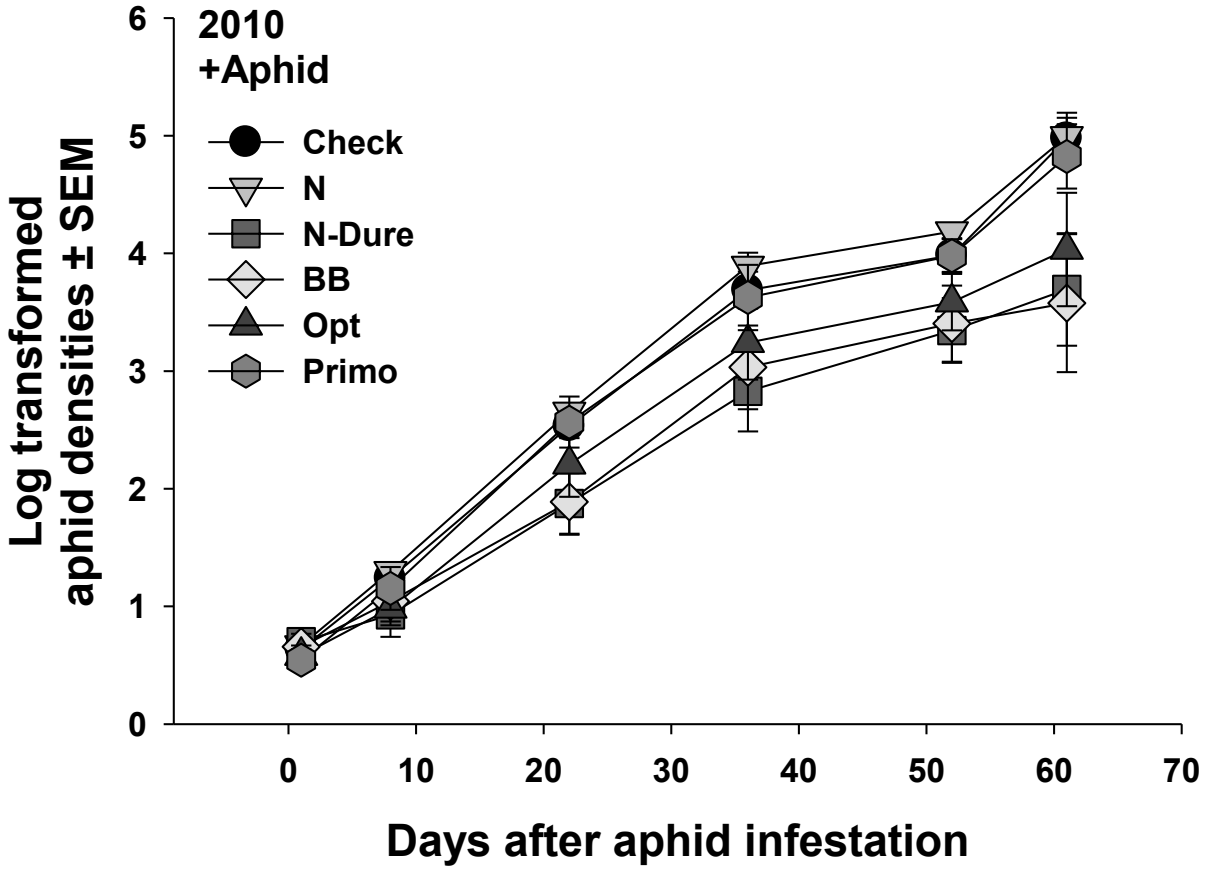
Soybean Aphid Density

In 2010, the impact of inoculant treatment on aphid densities in +Aphid cages was not consistent over time, leading to a significant interaction (Time × Inoculant, $df_{25, 145}$, P=0.050; Time, $df_{5, 145}$, P<0.001; Figure 41), and therefore data from each date were explored separately. Inoculant treatment did not significantly impact aphid establishment on experimental plants, as there were no significant differences in aphid densities among treatments one d (24 h) later (Figure 42). However by day 22 inoculant treatment significantly impacted aphid densities ($df_{5, 30}$, P=0.046; Figure 43), with lower densities on plants in the N-Dure and BB treatments compared to the check, N, and Primo treatments (see Table 3 for mean separation). This pattern continued throughout the season, although the strength of the main effect of inoculant varied somewhat among sampling dates (day 36: $df_{5, 30}$, P=0.072; day 56: $df_{5, 30}$, P=0.045; day 61: $df_{5, 30}$, P=0.032; Figure 43. Mean separation can be found in Table 3).

In 2011, No-Aphid cages were contaminated with naturally-occurring aphids that were not eliminated by the Pyganic insecticide, and so aphid infestation was included as an

independent variable in analyses. The impact of treatments on aphid densities did not remain consistent over the summer (Time × Aphid × Inoculant, $df_{15, 144}$, P=0.963, Time × Aphid, $df_{3, 144}$,

Figure 41. Mean aphid density per plant (log transformed) in 2010, +Aphid cages.



P<0.001; Time × Inoculant, $df_{15, 144}$, P=0.071; Time, $df_{3, 144}$, P<0.001; Figure 44). One d (24 hrs) after aphid infestation there was no impact of either aphid infestation or inoculant treatment on aphid densities (Inoculant × Aphid, $df_{5, 48}$, P=0.627; Aphid, $df_{1, 48}$, P=0.211; Inoculant, $df_{5, 48}$, P=0.830; Figure 45). The No-Aphid cages were sprayed with Pyganic insecticide after this sampling date. This spraying resulted in significantly more aphids in the +Aphid cages 14 d after aphid infestation, however, inoculation treatment had no impact on aphid densities on this date (Aphid × Inoculant, $df_{5, 48}$, P=0.909; Aphid, $df_{1, 48}$, P<0.001; Inoculant, $df_{5, 48}$, P=0.223; Figure 46). This trend continued on 33 d after aphid infestation (Aphid, $df_{1, 48}$, P<0.001; Inoculant, $df_{5,}$

$_{48}$, P=0.511; Aphid × Inoculant, $df_{5, 48}$, P=0.954; Figure 46). On the last sampling date aphid densities declined due to the pathogen discovered 33 d after aphid infestation. Both aphid infestation and inoculant treatment significantly impacted aphid densities on this date, although this could be due to the treatments, the pathogen, or a combination of the two (Aphid × Inoculant, $df_{5, 48}$, P=0.751; Aphid, $df_{1, 48}$, P=0.022; Inoculant, $df_{5, 48}$, P=0.021). On this date, aphid densities were significantly lower in the No-Aphid cages than in the +Aphid cages (Figure 46). The significant impact of inoculant treatment was due to aphid densities on plants in the N treatment being significantly lower than the Check, Opt and Primo treatments, and aphid densities on plants in the BB treatment being significantly lower than the Check (N versus: Check P=0.001; Opt P=0.030; Primo P=0.007; BB versus: Check P=0.037; $P\geq$0.05 for all other comparisons; Figure 47).

Figure 42. Mean aphid densities per plant (log transformed) in 2010 taken 24 h after infestation according to treatment, +Aphid cages. Different letters indicate significant differences at P<0.05.

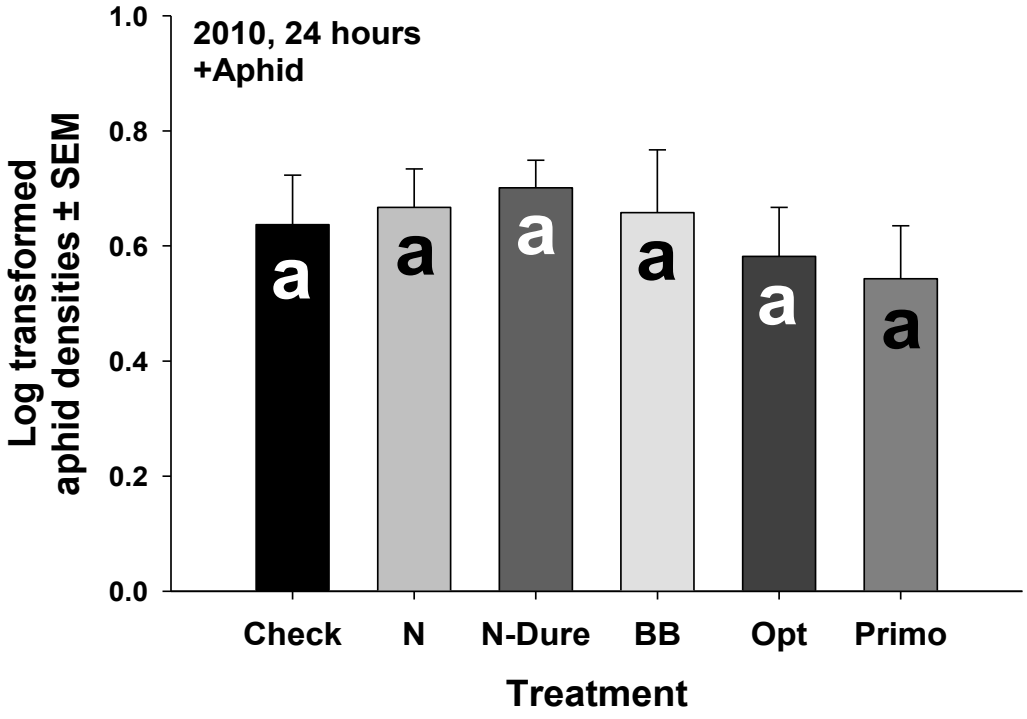


Figure 43. Mean aphid density per plant (log transformed) in 2010 according to date and inoculation treatment, +Aphid cages. Different letters indicate significant differences at $P<0.05$.

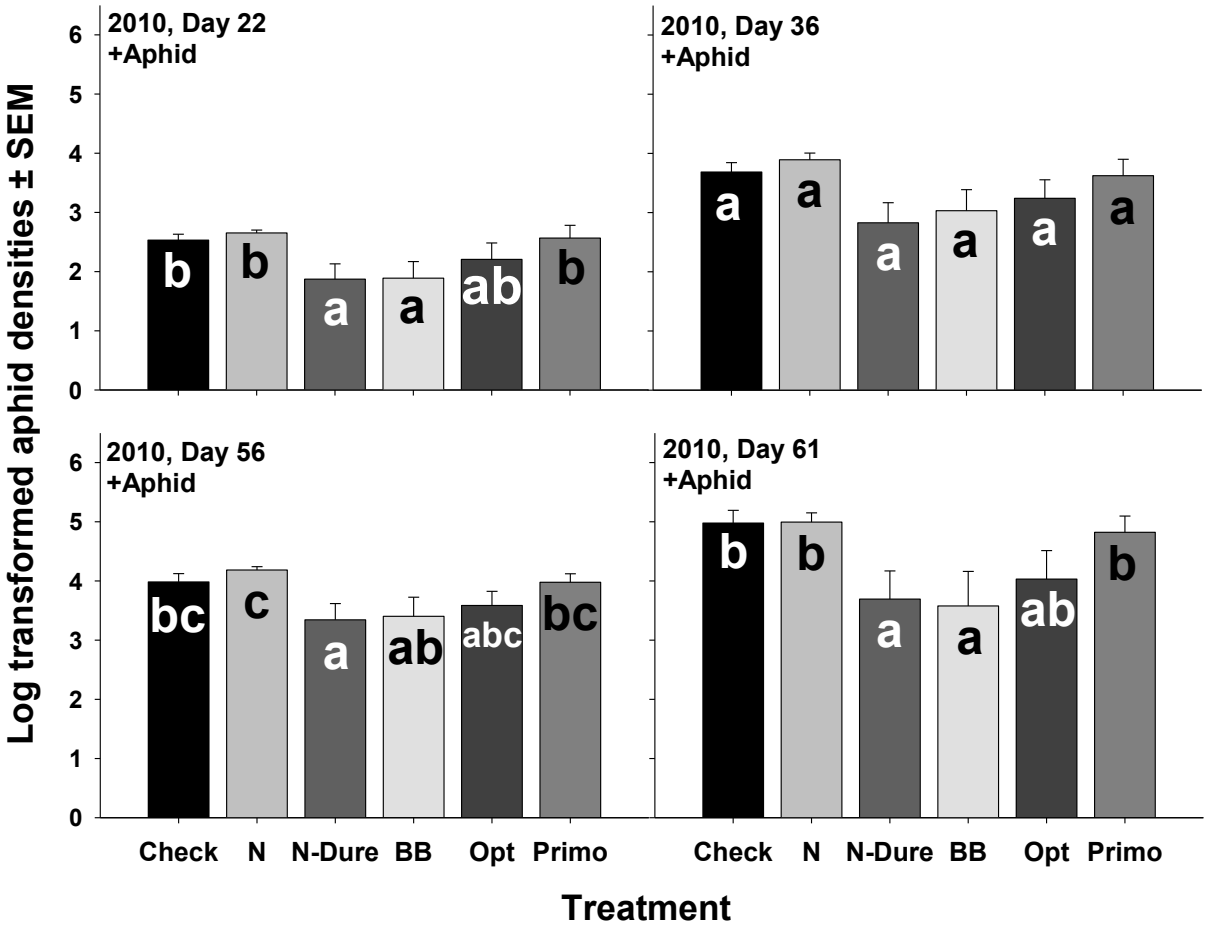


Table 3. Significant LSD values for mean aphid density per plant according to date. All other LSD values are $P>0.05$.

Treatments	Day 22	Day 56	Day 61
Nitrogen: BB	0.018	0.016	0.018
N: N-Dure	0.016	0.010	0.022
BB: Check	0.044	NS	0.019
BB: Primo	0.035	NS	0.035
Check: N-Dure	0.040	0.045	0.023
N-Dure: Primo	0.031	0.046	0.044

Figure 44. Mean aphid densities per plant (log transformed) in 2011 according to inoculation treatment and aphid infestation. The placement of "Pathogen" in the graph indicates when the aphid pathogen was initially discovered.

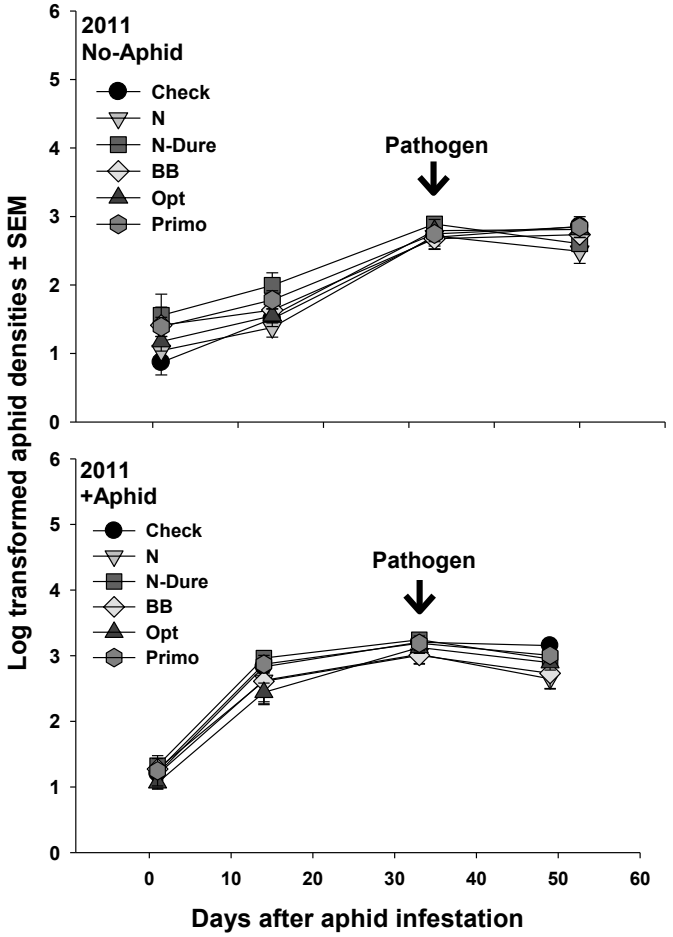


Figure 45. Mean aphid density per plant 24 h after aphid infestation in 2011 according to inoculation treatment. Different letters indicate significant differences at $P<0.05$.

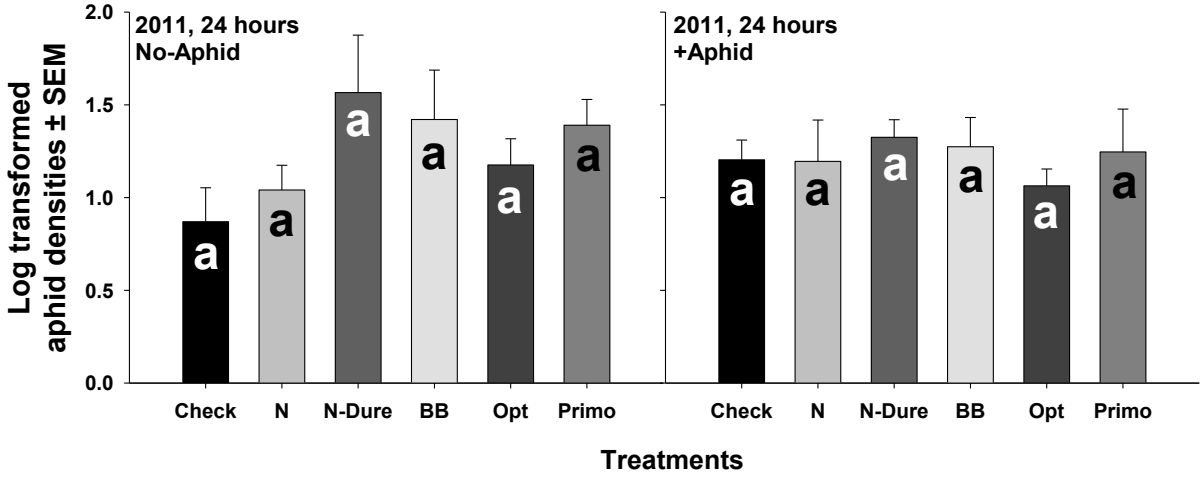


Figure 46. Mean log transformed aphid densities per plant in 2011 according to aphid infestation. The placement of "Pyganic" in the graph indicates when Pyganic insecticide was sprayed in the No-Aphid cages. Asterisks indicate significant differences at $P<0.05$.

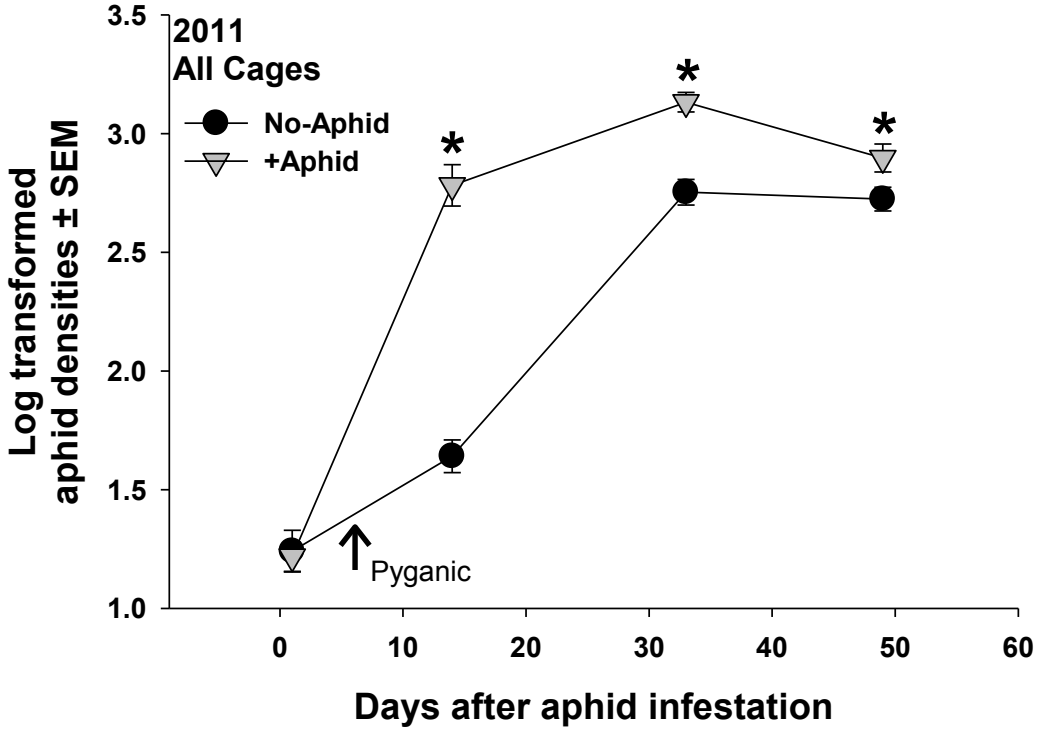
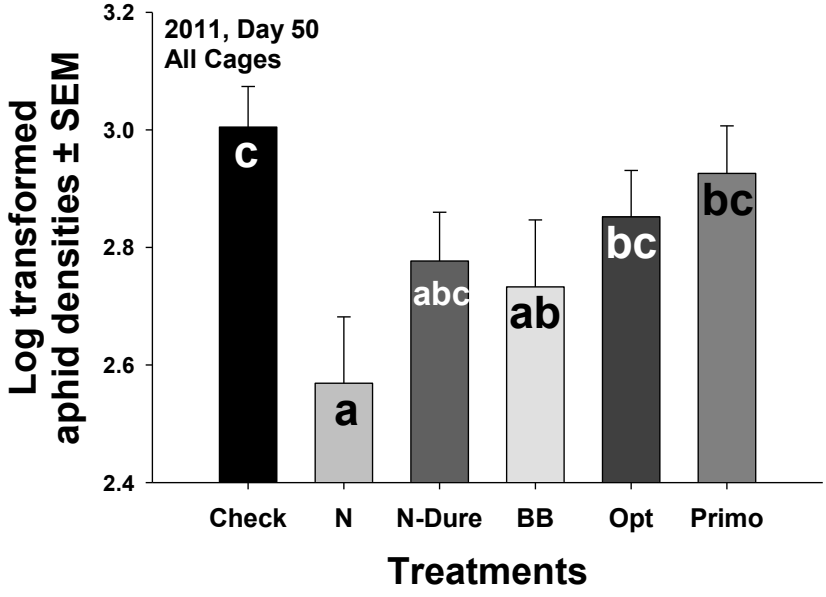


Figure 47. Mean aphid density per plant 50 d after aphid infestation in 2011 according to inoculation treatment. Different letters indicate significant differences at $P<0.05$.



Cumulative aphid days (CAD) were calculated for both 2010 and 2011. In 2010, CAD reached an average of 803,854 ± 159,814 CAD across all +Aphid cages, in 2011 18,936 ± 1,784 CAD across all No-Aphid cages and 48,508 ± 3,099 CAD across all +Aphid cages in 2011. Inoculant treatment had a significant impact on CAD in 2010 (Inoculant, $df_{5, 30}$, P=0.035), with significantly fewer CAD in the N-Dure treatment compared to the Check, N and Primo treatment, and CAD in the BB treatment were also significantly lower than the Check and N treatments (N-Dure versus: N P=0.009, Check P=0.019, Primo P=0.030; BB versus: N P=0.023, Check P=0.046; $P \geq 0.05$ for all other comparisons; Figure 48). In 2011, there was no impact of inoculation treatment on CAD (Inoculant, $df_{5, 48}$, P=0.360; Aphid × Inoculant, $df_{5, 48}$, P=0.979; Figure 49). As expected, CAD were significantly higher in cages intentionally infested with aphids (Aphid, $df_{1, 48}$, P<0.001; Figure 49).

Figure 48. Mean log transformed cumulative aphid days in 2010 according to inoculation treatment, +Aphid cages. Different letters indicate significant differences at P<0.05.

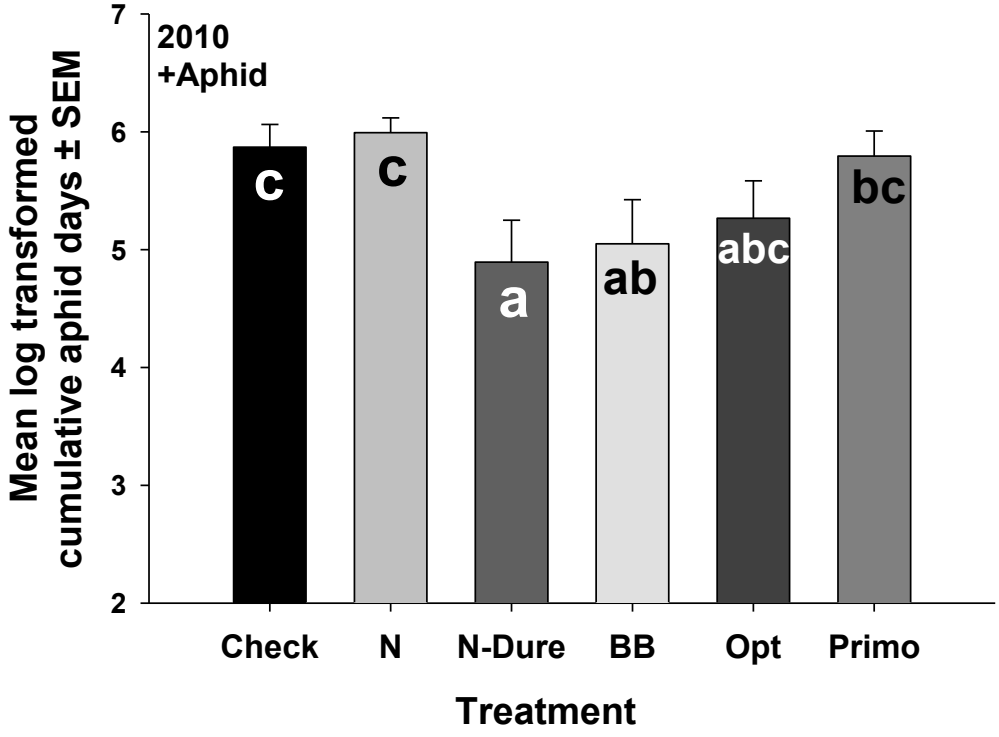
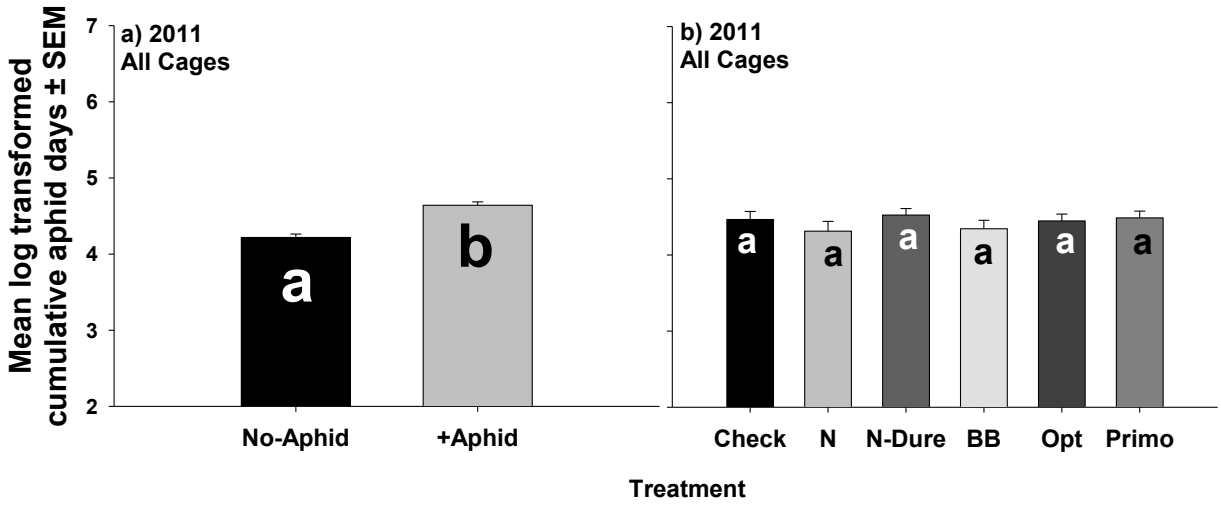


Figure 49. Mean log transformed cumulative aphid days in 2011 according to a) aphid infestation and b) inoculation treatment. Different letters indicate significant differences at P<0.05.

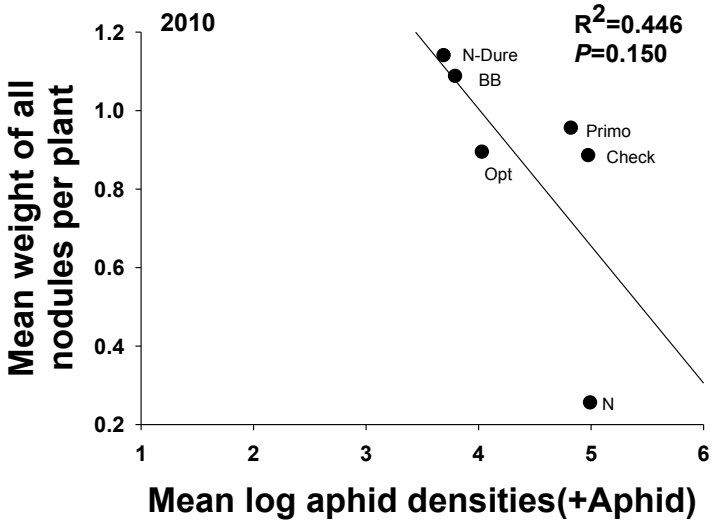


Correlations. In both 2010 and 2011, we examined correlations between the total weight of root nodules and mean aphid density at the end of the season. In 2010, because nodule data was collected from adjacent CREC plots lacking aphids, we plotted means for each treatment rather than means from each cage. Thus, the correlation examined how nodule weight per plant in the absence of aphids related to final aphid density on +Aphid plants. There was a slight negative correlation between aphid densities and total nodule weight, although it was non-significant (R^2=0.446, Bartlett Chi-Square=2.072, *P*=0.150; Figure 50). All commercial inoculants and the check treatment were close to the predicted line, however, the N-treatment did not follow the same pattern due to the very low nodule weight and high aphid densities. Although the N-treatment did not follow the same pattern, removing the N-treatment from the analysis did not greatly alter the results (R^2=0.565, Bartlett Chi-Square=2.085, *P*=0.149). In 2011, root nodules were collected from experimental plants, and so the average weight of root nodules per plant and the log transformed aphid densities, from 23-Aug, were plotted instead of treatment means. There was not a strong correlation between the No-Aphid root nodule weight

and the No-Aphid aphid densities (R^2=0.021, Bartlett Chi-Square=0.462, *P*=0.497; Figure 51). There was a negative correlation among the +Aphid root nodule weight and aphid densities, +Aphid (R^2=0.384, Bartlett Chi-Square=10.444, *P*=0.001; Figure 51).

In 2010, we examined correlations between the concentrations of above-ground nitrogenous compounds from +Aphid plants and log transformed aphid densities (last sampling date). There was no real relationship between total-N and aphid densities (R^2=0.058, Bartlett Chi-Square=1.997, *P*=0.158; Figure 52) or ureide-N and aphid densities (R^2=0.020, Bartlett Chi-Square=0.690, *P*=0.406; Figure 53b). There was a positive relationship between nitrate-N and aphid densities, although the correlation was fairly weak (R^2=0.119, Bartlett Chi-Square=4.254, *P*=0.039; Figure 53a).

Figure 50. Scatterplot of mean weight of all nodules per plant (collected from CREC plots) and log transformed aphid densities from +Aphid cages, in 2010, according to inoculation treatment. Aphid densities are from the last sampling date. The line and R^2 value represents the correlation between the two variables.



In 2010 correlations between the nitrate-N content and ureide-n content of the plant were examined. Among No-Aphid plants there was a slight negative correlation among nitrate-N and ureide-N (R^2=0.082, Bartlett Chi-Square=2.879, *P*=0.090; Figure 54). There was no correlation

between the two forms of nitrogen among the +Aphid plants (R^2<0.001, Bartlett Chi-Square=0.002, *P*=0.962; Figure 54)

Figure 51. Scatterplot of mean weight (grams) of all nodules per plant and log transformed aphid densities per plant from 23-Aug 2011. The line and R^2 value represents the correlation between the two variables.

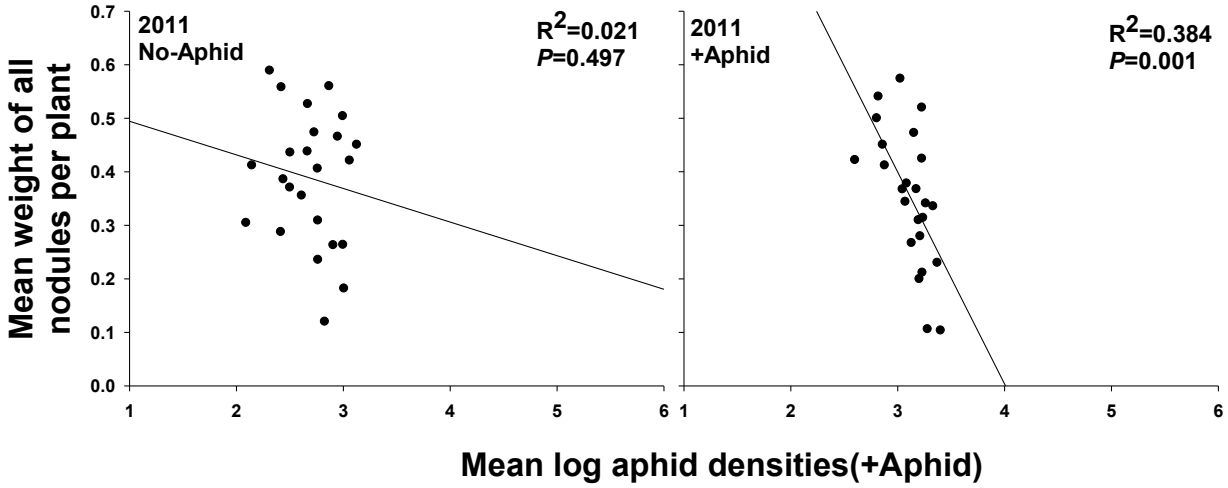


Figure 52. 2010 Scatterplot of total-N (+Aphid) and log aphid densities (+Aphid). All aphid density is from the last sampling date. The line and R^2 values on each graph represent the correlation between the two variables.

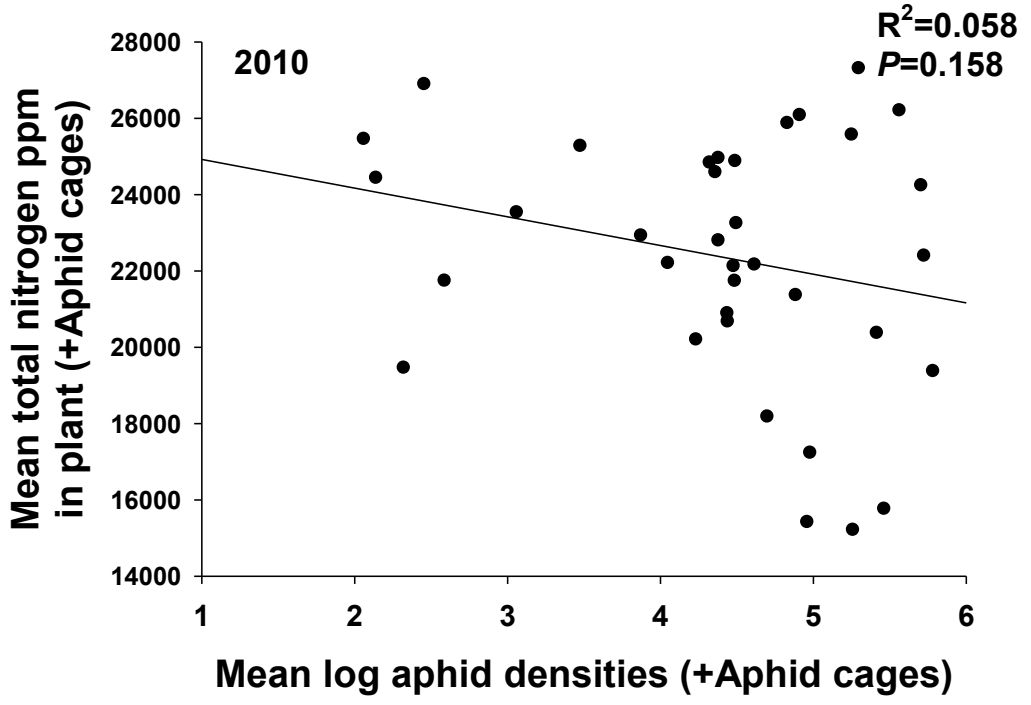


Figure 53. Scatterplot of 2010 log aphid densities and select nitrogenous compounds, nitrate and ureide. a) nitrate-N (+Aphid) and log aphid densities (+Aphid). b) ureide-N (+Aphid) and log aphid densities (+Aphid). All aphid density is from the last sampling date. The line and R^2 values on each graph represent the correlation between the two variables.

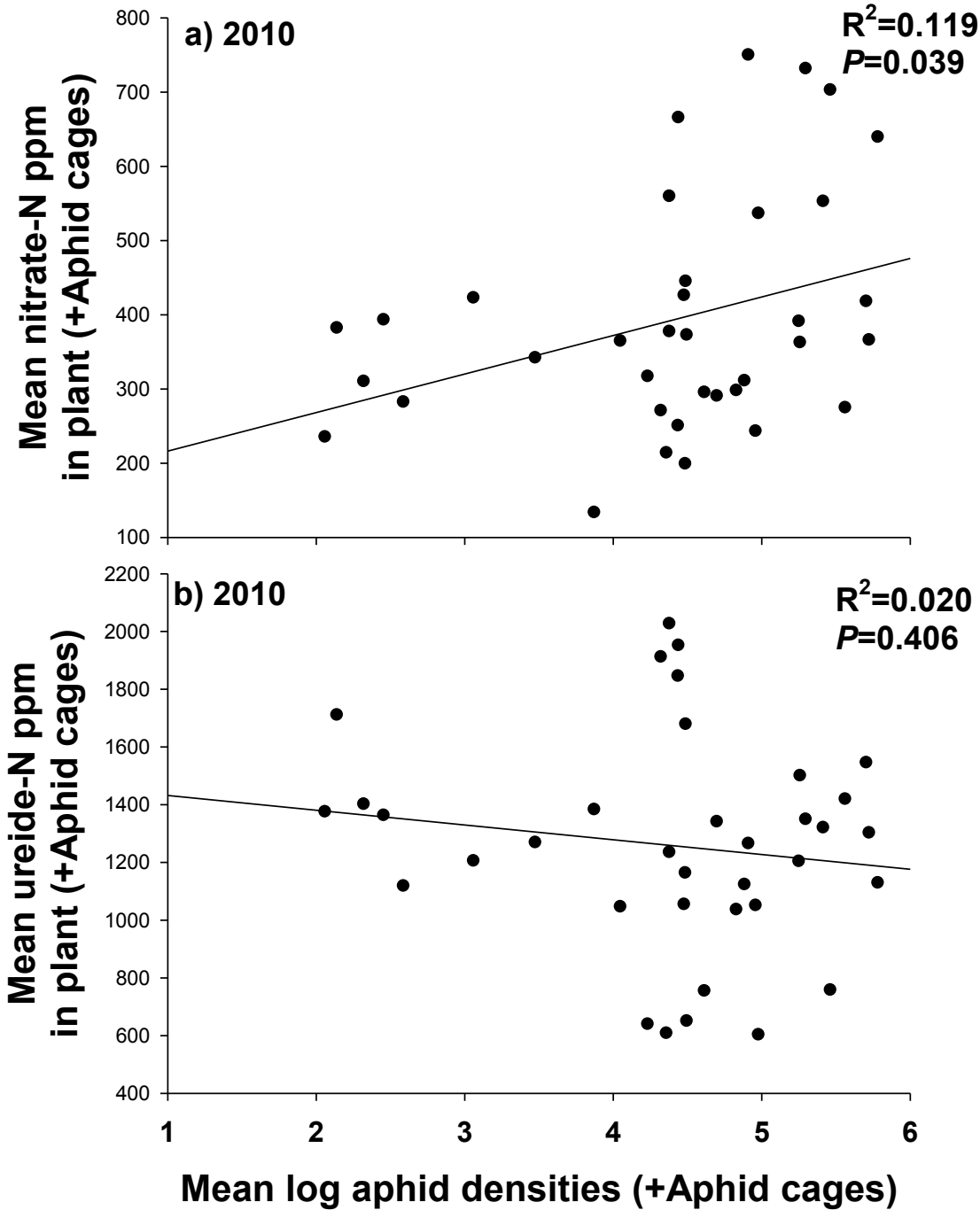
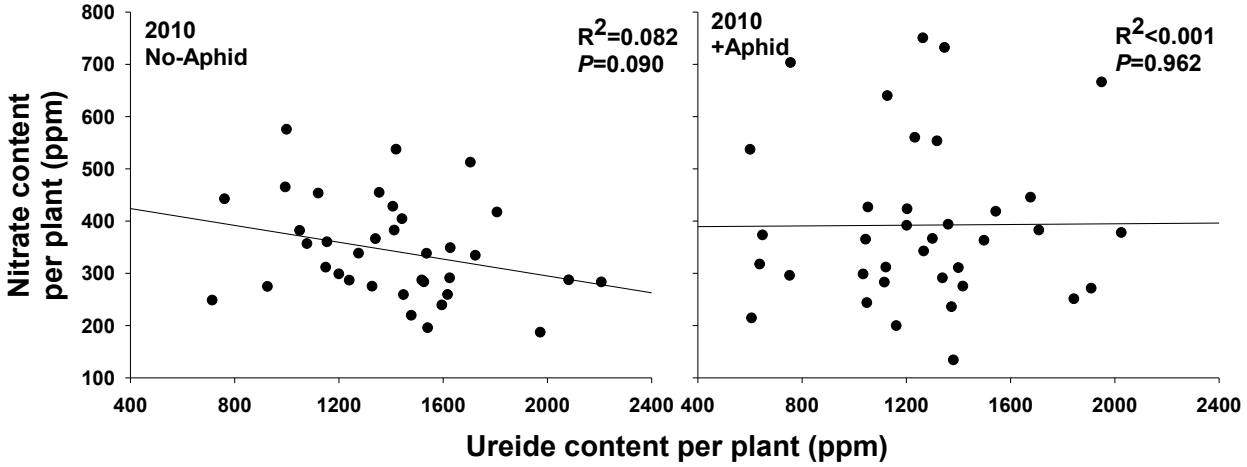


Figure 54. Scatterplot of nitrate and ureide content per plant in 2010 according to aphid infestation treatment. The line and R^2 values on each graph represent the correlation between the two variables.



Conclusion

Microorganisms associated with plant roots have important impacts on plant growth and physiology, including enhancing nutrient absorption and availability or increasing tolerance to abiotic stress (Pineda et al. 2010, Eisenhauer 2012). However, positive effects on plants can also benefit insect herbivores, often by increasing the nutritional quality of the host plant (Pineda et al. 2010, Partida-Martinez and Heil 2011, Eisenhauer 2012). In contrast, plant-associated root microorganisms can also protect plants against herbivory by inducing plant resistance genes and/or contributing to the production of toxic compounds (Pineda et al. 2010, Partida-Martinez and Heil 2011, Eisenhauer 2012). For leguminous plants, the presence of N-fixing rhizobia can impact above-ground phytophagous insects, although the effects vary depending on feeding habits and rhizobial species (Dean et al. 2009). Soybean producers often use rhizobial seed inoculants that contain *B. japonicum* along with other soil microbes or compounds, which are used in order to promote plant growth, enhance N-fixation, and increase yield. We compared the effects of four rhizobial seed inoculants, native soil rhizobia, and a high nitrogen control with

suppressed N-fixation on soybean plants and the population growth of phloem-feeding soybean aphids in a replicated field experiment.

In our study, adding fertilizer to suppress root nodulation had detrimental effects on several plant parameters in comparison to control plants associated with native rhizobia and inoculated plants, although there was no impact on concentrations of nitrogenous compounds within foliar tissues. In 2011, comparisons with the high N treatment were invalid because nodule weight data from plants in fertilized plots indicated that the treatment was not successfully established. It is unclear why nodulation was not suppressed, but may have to do the urea being leached out of the soil, as there were several major rain events in 2011 where plots received 14.17 cm of rainfall within a 2 wk period (14-27 June, NDAWN – North Dakota Agricultural Weather Network, http://ndawn.ndsu.nodak.edu/) that resulted in standing water within the plots. For the duration of the experiment in 2010 (20-May to 28-Aug) a weather station set up near the plots recorded an estimated 19.79 cm of rainfall, in contrast, in 2011 (2-June to 8-Sept) the same station recorded an estimated 39.27 cm of rainfall (NDAWN; Carrington Station).

Outside of plants receiving excess nitrogen, plants inoculated with BioBoost were shorter, lighter, and less reproductive than plants receiving the other commercial inoculants, although there were few significant differences in plant parameters among native (control) and commercial rhizobia (i.e. N-DURE, BioBoost, Optimize, and Primo). Several factors can affect the strength and effectiveness of rhizobia-plant associations, including cropping history, soil physical and chemical attributes, temperature, water, and the native microbial community (Lie et al. 1976, Sprent and Sprent 1990, Graham 2009). Since this experiment was conducted on land

previously planted to soybean and used in rhizobial inoculation testing, differential effects of inoculants applied in 2010 and 2011 on plants and aphids may be subtle.

In 2010, aphid densities in +Aphid cages greatly surpassed the economic injury level (674 ± 94 aphids per plant, Ragsdale et al. 2007) by 36 d after infestation (6,284 ± 1,052 across all treatments). The Primo treatment surpassed this level by day 22 with 762 ± 366 aphids and the nitrogen treatment had 602 ± 84 aphids. Under normal conditions (not caged and exposed to predators) aphid populations would probably never reach such high densities. Aphid presence had a strong negative impact on all plant parameters, including total nitrogen in above-ground plant tissue, although concentrations of specific nitrogenous compounds within the foliage (i.e. nitrate-N and ureide-N) were not significantly affected. In 2011, aphid densities in the +Aphid cages exceeded the economic injury level after 14 d (826 ± 85 across all treatments). In addition, a natural infestation that we were unable to successfully control affected No-Aphid cages, and although significantly lower than +Aphid cages, aphid densities in these cages still surpassed economic injury levels after 33 d (687 ± 76 across all treatments). Therefore, it was not surprising that in 2011 aphid infestation did not significantly affect any plant parameters.

The presence of a pathogen had detrimental impacts on aphid populations in the second year of the study, and may have been due to increased rainfall. The excess rain resulted in prolonged moisture within field cages, creating what appeared to be a more humid environment than seen in the previous year or in open field plots. The pathogen was discovered in virtually all cages across all inoculant and aphid infestation treatments and was observed to a lesser degree in open field plots. It is unknown if inoculation treatments interacted with the pathogen in any way, but soybean nodulation status can affect the presence of predaceous arthropods (Katayama et al. 2011).

In both years, inoculant treatment identity did not affect aphid establishment on plants, although since this was essentially a no-choice study we did not assess aphid attraction to or preference for plants grown with specific inoculants. However, in 2010, inoculant treatment had a significant impact on aphid population growth, with effects emerging approximately 2 wks after aphids were added to plants and remaining fairly consistent throughout the season. Aphid densities on plants with suppressed nodulation (excess nitrogen treatment) were similar to those on plants associated with native rhizobia (check) and plants inoculated with *B. japonicum* + *A. brasilense* (Primo). Plants with *B. japonicum* + lipo-chitooligosaccharides (Optimize) had intermediate aphid densities, while the lowest aphid densities were on plants inoculated with *B. japonicum* (N-DURE) and *B. japonicum* + *D. acidovorans* (BioBoost). *Azospirillum brasilense* is a known nitrogen fixer and some research has indicated that it also enhances the effectiveness of B. *japonicum* (Groppa et al. 1998). Lipo-chitooligosaccharides are Nod factors that are normally produced by the bacterium and aid in nodule formation (Loh and Stacey 2003, Skorupska et al. 2010). These molecules have been shown to positively impact non-legume plants (Chen et al. 2007). *Delftia acidovorans* is able to break down an herbicide (2,4-D; Hoffmann et al. 1996, Muller et al. 1999), however, there is little research demonstrating positive impacts on plant growth. Dean et al. (2009) also found that rhizobial inoculants can impact soybean aphid populations, although in contrast to our study they found the lowest aphid densities on plants associated with naturally occurring rhizobia compared to a nitrogen fertilizer treatment and a commercial inoculant (*B. japonicum*; HiStick 2, Becker-Underwood IA).

Unfortunately, in 2011, effects of the inoculant treatment on aphid densities were unclear, primarily due to the apparent failure of the excess nitrogen to suppress nodulation and the presence of an aphid pathogen, which was widespread 33 d after aphid infestation. Although the

relative impact of the control, BioBoost, Optimize, and Primo treatments on aphid densities appeared to be similar between years, the performance of N-DURE was not consistent. Variations in effects of native rhizobia and commercial inoculants that only contain *B. japonicum* could be due to multiple factors, including differences in bacterial strain, bacterial densities, presence of other biotic organisms and abiotic soil properties (Lie et al. 1976, Sprent and Sprent 1990, Groppa et al. 1998, Dean et al. 2009, Graham 2009). Dean et al. (2009) suggested that the identity of the strain of rhizobia present could influence the amount of protection against herbivores that the rhizobia convey to the plant.

Soybean aphid densities varied significantly among plants inoculated with different commercially available rhizobial seed inoculants, however, the mechanism(s) underlying this phenomenon are unclear. Other researchers have found that root nodulation and factors associated with N-fixation can impact the biology and density of soybean herbivores (Wilson and Stinner 1984, Kempel et al. 2009, Katayama et al. 2011), although Katayama et al. (2011) did not find this to be the case for the soybean aphid. Because aphid biology is influenced by nitrogen supply (Mattson 1980, Nowak and Komor 2010), one might think that differences in N-fixation rates, and subsequently the amount and identity of nitrogenous compounds (e.g. amino acids, defensive proteins) among plants receiving different inoculants, could be responsible for differential aphid reproduction. In one study (Dean et al. 2009), several factors relating to N-fixation and plant quality (i.e. shoot dry weight, seed number and dry weight, percent total N within the foliage, nodule dry weight) were similar among treatments with significantly different soybean aphid densities, and they suggested the form of nitrogen present (i.e. amino acid composition of the phloem) and/or defensive compounds may play an important role.

In our study we did not directly quantify N-fixation, but used total nodule mass per plant and ureide-N content of the foliage as indicators of N-fixation rates (Herridge 1982a, Patterson and LaRue 1983b, van Berkum et al. 1985, Herridge et al. 2008). Plot-wide correlations (2010) and plant-level correlations (2011, +Aphid plants) supported the idea that plants with greater nodulation had lower aphid densities, although there was no correlation between nodule mass and aphid densities on plants where aphids were unintentional contaminants (i.e. 2011, No-Aphid plants).

There was no relationship between the concentration of ureides or total nitrogen in above-ground plant tissue and aphid densities. This is in contrast to the results of Noma et al. (2010), who found a positive relationship between soybean aphid densities and total leaf nitrogen, although they sampled when plants started to flower. However, there are several important caveats to consider, including that we assessed nitrogenous compounds in above-ground plant tissue, not xylem or phloem sap, and we only assessed compounds at one point in time. In addition, although nodule biomass is related to N-fixation rates, nodule location on the root system can also impact this parameter (Hardarson et al. 1989). Using the ureide content of above-ground tissue as an indicator of N-fixation can be unreliable due to certain factors that can cause fluctuations in the concentration of ureides present in the plant. Some of these factors include the accumulation of ureides due to reduced ureide degradation (Vadez and Sinclair 2000, King and Purcell 2005), remobilization of ureides from senescing tissue (Herridge 1982b, Reynolds et al. 1982, Todd et al. 2006), ureide biogenesis from reallocation of nitrogen from older tissues (Diaz-Leal et al. 2012), and potential conversion of ammonium into ureides (Herridge 1982b). In our study it was surprising that the correlation between ureide and nitrate content was so weak, especially in non-aphid infested plants. Finally, because soybean nodule

volume and ureide-N content (in addition to other plant parameters) are also affected by aphid density (Riedell et al. 2009), it is difficult to determine what is driving the relationship.

Although the relationship was relatively weak, aphid densities were positively related to nitrate content of above-ground foliage, which provides some indication that plants obtaining their nitrogen from fertilizer may be slightly better hosts than plants obtaining nitrogen from N-fixation. Another point to consider is that we did not assess how treatments impacted the concentration of amide-N in the plant, and aphid densities are likely influenced by the identity and amount of amino acids in the phloem. Asparagine may be of particular interest, because it is an amino acid exported from the xylem regardless of whether the plants obtains nitrogen from N-fixation or fertilizer, although more is present in the xylem when the plant obtains its nitrogen primarily from fertilization (Shelp and Da Silva 1990). Alterations in phloem asparagine content have been suggested as a mechanism underlying the positive effects of soil potassium deficiency on soybean aphid reproduction (Myers et al. 2005, Myers and Gratton 2006, Walter and DiFonzo 2007).

In summary, this research shows that the identity of rhizobial seed inoculants can affect soybean aphid population growth, although effects were generally small and aphids reached high densities in all treatments. It appears that some factors associated with nitrogen acquisition (i.e. nodulation, nitrate content of above-ground tissue) are influencing aphid reproduction, although the impact was minimal and it is likely that alterations in other factors not assessed in this study (i.e. amino acids within phloem, phloem sap proteins, or defensive compounds) could also be impacting these herbivores. Additionally, intracellular nutritional symbionts (*Buchnera*) could buffer effects of variable nitrogen availability on aphid reproduction, or aphids could redistribute themselves within plants to optimize nitrogen acquisition. Since the mechanisms underlying our

results are unclear, currently we cannot make strong recommendations about the brand and composition of inoculants best suited for use in soybean aphid IPM programs. Additionally, it is important to explore multi-season and multi-location variability and the impacts of different commercial inoculants on aphids in open field plots where they are exposed to natural enemies.

GENERAL CONCLUSIONS

Soybean aphids have been plaguing soybean crops in the United States for over 12 years (Venette and Ragsdale 2004). They are phloem feeding insects whose growth and development are often limited by the nitrogen content of the plants they are feeding on (reviewed by Mattson 1980). Their secondary host, soybean, can obtain nitrogen from two different sources, nitrogen present in the soil (from decomposition or fertilizers) and from biologically fixed nitrogen (by symbiotically associating with bacteria that fix atmospheric nitrogen into plant available forms). The source of nitrogen supplied to the plant impacts the forms of nitrogen transported through the plant and thus potentially influences soybean aphids.

In a series of experiments, we explored how nitrogen fertilizer and N-fixing rhizobia impacted various facets of aphid biology contributing to aphid population growth under specific environmental conditions. In both greenhouse and field experiments host plant acceptance (i.e. aphid establishment) was not influenced by treatments. However, nitrogen source did have a significant impact on aphid biology and population growth. In greenhouse experiments aphid densities were initially highest on plants with potentially the highest amount of fixed nitrogen. However, peculiarities in the experiments (i.e. rhizobial contamination of non-inoculated treatments in the first experiment and the declining concentration of available nitrogen through time) complicated our ability to relate aphid performance to plant parameters associated with N-fixation (foliar nitrate, ureide, total-N and root nodules).

In the field, rhizobial seed inoculant identity impacted soybean aphid population growth, although overall effects on aphid densities were generally minor and variable between years. Aphid densities on plants treated with specific inoculants (i.e. Optimize 400 and Primo), were similar to aphid densities on plants associated with naturally occurring rhizobia species and on

plants obtaining their nitrogen primarily from fertilizer. We did not find significant correlations between aphid densities and levels of total foliar nitrogen or ureides (products of N-fixation). However, there was a weak positive relationship between foliar nitrate and aphid densities.

Soybean aphid biology and population growth is clearly influenced by available nitrogen and N-fixing rhizobia. However, the exact mechanisms by which aphid population growth is affected is still unclear. In addition, strength of the effects may be highly dependent on plant age at time of infestation and environmental conditions. Any type of management recommendations would need to be developed after more extensive research.

REFERENCES CITED

Andersen PC, Brodbeck BV, Herzog DC. 2002. Girdling-induced nutrient accumulation in above ground tissue of peanuts and subsequent feeding by *Spissistilus festinus*, the three-cornered alfalfa hopper. Entomol. Exp. Appl. 103: 139-149.

Atkins CA, Pate JS, Ritchie A, Peoples MB. 1982. Metabolism and translocation of allantoin in ureide-producing grain legumes. Plant Physiol. 70:476-482.

Bashan Y, Levanony H. 1990. Current status of *Azospirillum* inoculation technology: *Azospirillum* as a challenge for agriculture. Can. J. Microbiol. 37:591-608.

Bottomley PJ. 1992. Ecology of *Bradyrhizobium* and *Rhizobium*, pp. 293-348. In: Stacey G, Burris RH, Evans HJ (eds.), Biological Nitrogen Fixation. Chapman & Hall, New York, NY. 943 pp.

Brooker RJ, Widmaier EP, Grahm LE, Stiling PD. 2008. Biology. McGraw-Hill Higher Education, New York pp. 788-797.

Bullock DG, Anderson DS. 1998. Evaluation of the Minolta SPAD-502 chlorophyll meter for nitrogen management in corn. J. Plant Nutrition. 21(4): 741-755.

Campo RJ, Araujo RS, Hungria M. 2009. Nitrogen fixation with the soybean crop in Brazil: compatibility between seed treatment with fungicides and bradyrhizobial inoculants. Symbiosis. 48: 154-163.

Cassán F, Perrig D, Sgroy V, Masciarelli O, Penna C, Luna V. 2009. *Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109, inoculated singly or in combination, promote seed germination and early seedling growth in corn (*Zea mays* L.) and soybean (*Glycine max* L.). European J of Soil Biol. 45: 28-35.

Cataldo DA, Haroon M, Schrader LE, Youngs VL. 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun. Soil Sci. Plant Anal. 6:71-80.

Chen C, McIver J, Yang Y, Bai Y, Schultz B, McIver A. 2007. Foliar application of lipo-chitooligosaccharides (Nod factors) to tomato (Lycopersicon esculentum) enhances flowering and fruit production. Can. J. Plant Sci. 87:365-372.

Chen Y, Ruberson JR. 2008. Impact of variable nitrogen fertilization on arthropods in cotton in Georgia, USA. Agriculture, Ecosystems and Environment 126:281-288

Da Silva MC, Shelp BJ. 1990. Xylem-to-phloem transfer of organic nitrogen in young soybean plants. Plant Physiol. 92: 797-801.

De Brito Alvarez MA, Gagne S, Antoun H. 1995. Effect of compost on rhizosphere microflora of the tomato and on the incidence of plant growth-promoting rhizobacteria. Appl. Environ. Microbiol. 61: 194-199.

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

Deibert EJ, Bijeriego M, Olson RA. 1979. Utilization of ^{15}N fertilizer by nodulating and non-nodulating soybean isolines. Agronomy Journal 71:717-723.

Dénarié J, Debellé F, Promé J-C. 1996. Rhizobium lipo-chitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis. Annu. Rev. Biochem. 65: 503-535.

Díaz-Leal JL, Gálvez-Valdivieso G, Fernández J, Pineda M, Alamillo JM. 2012. Developmental effects on ureide levels are mediated by tissue-specific regulation of allantoinase in *Phaseolus vulgaris* L. J. Exp. Bot. 1-12. doi: 10.1093/jxb/ers090

Douglas AE, van Emden HF. 2007. Nutrition and symbiosis, pp. 115-134. In: van Emden HF, Harrington R (eds.), Aphids as Crop Pests. CABI International, Wallingford, United Kingdom.

Douglas AE. 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic *Buchnera*. Annu. Rev. Entomol. 43:17-37.

Eisenhauer N. 2012. Aboveground-belowground interactions as a source of complementarity effects in biodiversity experiments. Plant Soil 35:1-22.

Evans J. 1982. Response of soybean-rhizobium symbioses to mineral nitrogen. Plant and Soil 66:439-442.

Falkowski PG. 1997. Evolution of the nitrogen cycle and its influence on the biological sequestration of CO2 in the ocean. Nature 387:272-275.

Fujihara S, Yamaguchi M. 1978. Probable site of allantoin formation in nodulating soybean plants. Phytochemistry 17:1239-1243.

Gage D. 2009. Nodule development in Legumes, pp. 1-24. In: Emerich DW, Krishnan HB (eds.), Nitrogen fixation in crop production Agronomy. Monograph no. 52, American Society of Agronomy, Madison, WI.

Girousse C, Moulia B, Silk W, Bonnemain J.-L. 2005. Aphid infestation causes different changes in carbon and nitrogen allocation in alfalfa stems as well as different inhibitions of longitudinal and radial expansion. Plant Physiol. 137: 1474-84.

Graham PH. 2009. Soil biology with an emphasis on symbiotic nitrogen fixation, pp. 171-209. In: Emerich DW, Krishnan HB (eds.), Nitrogen fixation in crop production. Agronomy. Monograph no. 52, American Society of Agronomy, Madison, WI.

Groppa MD, Zawonzink MS, Tomaro ML. 1998. Effect of co-inoculation with *Bradyrhizobium japonicum* and *Azospirillum brasilense* on soybean plants. Eur. J. Soil Biol. 34:75-80.

Gunduz EA, Douglas AE. 2009. Symbiotic bacteria enable insect to use a nutritionally inadequate diet. Proc. R. Soc. B. 276:987-991.

Hanafi A, Radcliffe EB, Ragsdale DW. 1989. Spread and control of potato leafroll virus in Minnesota. J. Econ. Entomol 82:1201-1206.

Hansen AK, Moran NA. 2011. Aphid genome expression reveals host-symbiont cooperation in the production of amino acids. PNAS 108:2849-2854.

Hardarson G, Golbs M, Danso SKA. 1989. Nitrogen fixation in soybean (*Glycine max* L. Merrill) as affected by nodulation patterns. Soil Biol. Biochem. 21: 783-7.

Harper JE, Hageman RH. 1972. Canopy and seasonal profiles of nitrate reductase in soybeans (*Glycine max* L. Merr.). Plant Physiol. 49: 146-154.

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

Herridge DF, Brockwell J. 1988. Contributions of fixed nitrogen and soil nitrate to the nitrogen economy of irrigated soybean. Soil Biol. Biochem. 20:711-717.

Herridge DF, Peoples MB, Boddey RM. 2008. Global inputs of biological nitrogen fixation in agricultural systems. Plant Soil 311:1-18.

Herridge DF. 1982a. Relative abundance of ureides and nitrate in plant tissues of soybean as a quantitative assay of nitrogen fixation. Plant Physiol. 70: 1-6.

Herridge DF. 1982b. Use of the ureide technique to describe the nitrogen economy of field-grown soybeans. Plant Physiol. 70: 7-11.

Hoffmann D, Muller RH, Kiesel B, Babel W. 1996. Isolation and characterization of an alkaliphilic bacterium capable of growing on 2,4-dichlorophenoxyacetic acid and 4-chloro-2-methylphenoxyacetic acid. Acta Biotechnol. 16:121-131.

Hosseini M, Ashouri A, Enkegaard A, Goldansaz SH, Mahalati MN, Hosseininaveh V. 2010. Performance and population growth rate of the cotton aphid, and associated yield losses in cucumber, under different nitrogen fertilization regimes. International Journal of Pest Management 56:127-135.

Hymowitz T, Harlan JR. 1983. Introduction of soybean to North America by Samuel Bowen in 1765. Economic Botany. 37:371-379.

Hymowitz T. 1990. Soybeans: the success story, pp. 195-163. In: Janick J, Simon JE (eds.), Advances in new crops. Timber Press Portland, OR.

Jahn GC, Almazan LP, Pacia JB. 2005. Effect of nitrogen fertilizer on the intrinsic rate of increase of *Hysteroneum setariae* (Thomas) (Homoptera: Aphididae) on rice (*Oryza sativa* L.). Environmental Entomology 34:938-943.

Johnson JW. 2011. Selecting forms of nitrogen fertilizer. Ohio State University Extension Service, Columbus, Ohio. Available at: http://ohioline.osu.edu/agf-fact/0205.html. Accessed on December 16, 2011.

Johnson MTJ. 2008. Bottom-up effects of plant genotype on aphids, ants, and predators. Ecology. 89: 145-154.

Johnson ND, Liu B, Bentley BL. 1987. The effects of nitrogen fixation, soil nitrate and defoliation on the growth, alkaloids and nitrogen levels of *Lupinus succluntus* (Fabaceae). Oecologia 74:425-431.

Kaiser WM, Förster J. 1989. Low CO_2 prevents nitrate reduction in leaves. Plant Physiol. 91: 970-974.

Katayama N, Zhang ZQ, Ohgushi T. 2011. Community-wide effects of below-ground rhizobia on above-ground arthropods. Ecological Entomology 36:43-51.

Kehr J. 2006. Phloem sap proteins: their identities and potential roles in the interaction between plants and phloem-feeding insects. J. Exp. Bot. 57: 767-774.

Kempel A, Brandl R, Schadler M. 2009. Symbiotic soil microorganisms as players in aboveground plant-herbivore interactions- the role of rhizobia. Oikos 118:634-640.

Keyser HH, Li F. 1992. Potential for increasing biological nitrogen fixation in soybean. Plant and Soil 141: 119-135.

Kim CS, Schaible G, Garrett L, Lubowski R, Lee D. 2008. Economic impacts of the U.S. soybean aphid infestation: a multi-regional competitive dynamic analysis. Agricultural and Resource Economics Review 37:227-242.

King CA, Purcell LC. 2005. Inhibition of N_2 fixation in soybean is associated with elevated ureides and amino acids. Plant Physiol. 137: 1389-1396.

Lang KJ, Chinzowu T, Cann KJ. 2012. *Delftia acidovorans* as an unusual causative organism in line-related sepsis. Indian J. Mirobiol. 52:102-103.

Langsrud O. 2003. ANOVA for unbalanced data: use Type II instead of Type III sums of squares. Statistics and Computing 13: 163-167.

Layton MB, Boethel DJ. 1989. Response of soybean growth and N_2-fixing ability to progressive insect-inducted defoliation. J. Econ. Ent. 82: 275-80.

Lea PJ, Ireland RJ. 1999. Nitrogen metabolism in higher plants, pp. 1-47. In: Singh BK (eds.) Plant amino acids, biochemistry and biotechnology. Marcel Dekker, New York.

Li ZZ and Gresshoff PM. 1990. Developmental and biochemical regulation of 'constitutive' nitrate reductase activity in leaves of nodulating soybean. J. Exp. Bot. 41:1231-1238.

Lie TA, Hille D, Lambers R, Houwers A. 1976. Symbiotic specialization in pea plants: some environmental effects on nodulation and nitrogen fixation, pp. 319-333. In: Nutman PS (ed.), Symbiotic nitrogen fixation in plants. Cambridge University Press, London,

Loh J, Stacey G. 2003. Nodule gene regulation in Bradyrhizobium japonicum: a unique integration of global regulatory circuits. Applied and Environmental Microbiology 69:10-17.

Lukaszewski KM, Blevins DG, Randall DD. 1992. Asparagine and boric acid cause allantoate accumulation in soybean leaves by inhibiting manganese-dependent allantoate amidohydrolase. Plant Physiol. 99: 1670-6.

Mallik MAB, Tesfai K. 1985. Pesticidal effect on soybean-rhizobia symbiosis. Plant Soil 85: 33-41.

Markwell J, Osterman JC, Mitchell JL. 1995. Calibration of the Minolta SPAD-502 leaf chlorophyll meter. Photosynthesis Research 46: 467-472.

Mattson WJ. 1980. Herbivory in relation to plant nitrogen content. Annual Review of Ecology and Systematics 11:119-161.

McClure PR, Israel DW. 1979. Transport of nitrogen in xylem of soybean plants. Plant Physiol. 64:411-416.

McNeil DL, LaRue TA. 1984. Effect of nitrogen source on ureides in soybean. Plant Physiol. 74:227-232.

Miflin BJ, Lea PJ. 1977. Amino acid metabolism. Ann. Rev. Plant Physiol. 28:299-329.

Mousseau TA, Dingle H. 1991. Maternal effects in insect life histories. Annu. Rev. Entomol. 36:511-534.

Muller RH, Jorks S, Kleinsteuber S, Babel W. 1999. *Comamonas acidovorans* strain MC1: a new isolate capable of degrading the chiral herbicides dichloroproop and mecoprop and the herbicides 2,4-D and MCPA. Microbiol. Res. 154:241-246.

Myers SW, Gratton C, Wolkowski RP, Hogg DB, Wedberg JL. 2005. Effect of soil potassium availability on soybean aphid (Hemiptera: Aphididae) population dynamics and soybean yield. J Econ Entomol 98: 113-120.

Myers SW, Gratton C. 2006. Influence of potassium fertility on soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), population dynamics at a field and regional scale. Environ Entomol 35: 219-227.

Nevo E, Coll M. 2001. Effect of nitrogen fertilization on *Aphis gossypii* (Homoptera: Aphididae): variation in size, color and reproduction. J. Econ. Entomol. 64:27-32.

Nicholas JC, Harper JE, Hageman RH. 1976. Nitrate reductase activity in soybeans (*Glycine max* [L.] Merr.). I. Effects of light and temperature. Plant Physiol. 58: 731-735.

Noma T, Gratton C, Colunga-Garcia M, Brewer MJ, Mueller E, Wyckhuys KAG, Heimpel GE, O'Neal ME. 2010. Relationship of soybean aphid (Hemiptera: Aphididae) to soybean plant nutrients, landscape structure, and natural enemies. 39: 31-41.

Nowak H, Komar E. 2010. How aphids decide what is good for them: experiments to test aphid feeding behavior on *Tanacetum vulgare* (L.) using different nitrogen regimes. Oecologia 163:973-984.

Ohyama T, Kawai S. 1983. Nitrogen assimilation and transport in soybean leaves: investigation by petiole girdling treatment. Soil Sci. Plant Nutr. 29: 227-132.

Ohyama T, Ohtake N, Sueyoshi K, Tewari K, Takahashi Y, Ito S, Nishiwaki T, Nagumo Y, Ishii S, Sato T. 2009. Nitrogen fixation and metabolism in soybean plants. Nova Science Publishers, New York.

Partida-Martinez LP, Heil M. 2011. The microbe-free plant: fact or artifact? Frontiers in Plant Science 2:1-16.

Pate JS. 1980. Transport and partitioning of nitrogenous solutes. Ann. Rev. Plant Physiol. 31:313-340.

Patterson T, Glenister R, LaRue T. 1982. Simple estimation of ureides in soybean tissue. Anal. Biochem. 119:90-95.

Patterson TG, LaRue TA. 1983a. N_2 fixation (C_2H_2) and ureide content of soybeans: environmental effects an source-sink manipulations. Crop Science 23:819-824.

Patterson TG, LaRue TA. 1983b. N_2 fixation (C_2H_2) and ureide content of soybeans: ureides as an index of fixation. Crop Science 23:825-831

Paul EA, Clark FE. 1996. Soil microbiology and biochemistry, 2nd ed. Academic Press Inc., San Diego, CA.

Peoples MB, Atkins CA, Pate JS, Chong K, Faizah AW, Suratmini P, Nurhayati DP, Bagnall DJ, Bergersen FJ. 1991. Reevaluation of the role of ureides in the xylem transport of nitrogen in the arachis species. Physiologia Plantarum 83:560-567.

Peoples MB, Brockwell J, Herridge DF, Rochester IJ, Alves, BJR, Urquiaga S, Boddey RM, Dakora FD, Bhattarai S, Maskey SL, Sampet C, Rerkasem B, Khan DF, Hauggaard-Nielsen H, Jensen ES. 2009. The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. Symbiosis 48:1-17.

Pineda A, Zheng S-J, van Loon JJA, Pieterse CMJ, Dicke M. 2010. Helping plants to deal with insects: the role of beneficial soil-borne microbes. Trends in Plant Science 15:507-514.

Postgate JR. 1982. The fundamentals of nitrogen fixation. Cambridge University Press, New York, NY.

Powell G, Tosh CR, Hardie J. 2006. Host plant selection by aphids: behavioral, evolutionary, and applied perspectives. Annu. Rev. Entomol. 51:309-330.

Quinn MA, Hall MH. 1996. Compensatory growth response of the legume, *Medicago sativa*, to defoliation and demodulation. Ent. Exp. Appl. 78: 243-252.

Ragsdale DW, Landis DA, Brodeur J, Heimpel GE, Desneux N. 2011. Ecology and management of the soybean aphid in North America. Annu. Rev. Entomol. 56:375-399.

Ragsdale DW, McCornack BP, Venette RC, Potter BD, Macrae IV, Hodgson EW, O'Neal ME, Johnson KD, O'Neal RJ, Difonzo CD, Hunt TE, Glogoza PA, Cullen EM. 2007. Economic threshold for soybean aphid (Hemiptera: Aphididae). J. Econ. Entomol. 100:1258-1267.

Ragsdale DW, Voegtlin DJ, O'Neil RJ. 2004. Soybean aphid biology in North America. Ann Entomol. Soc. Am. 97:204-208.

Ramirez CC, Niemeyer HM. 2000. The influence of previous experience and starvation on aphid feeding behavior. Journal of Insect Behavior 13:699-709.

Raymond J, Siefert JL, Staples CR, Blankenship RE. 2004. The natural history of nitrogen fixation. Mol. Biol. Evol. 21:541-554.

Reynolds PHS, Boland MJ, Blevins DG, Randall DD, Schubert KR. 1982. Ureide biogenesis in leguminous plants. Trends Biochem. Sci. 10: 366-368.

Riedell WE, Catangui MA, Beckendorf EA. 2009. Nitrogen fixation, ureide, and nitrate accumulation responses to soybean aphid injury in *Glycine max*. Journal of Plant Nutrition 32:1674-1686.

Rodriguez-Navarro DN, Oliver IM, Contreras MA, Ruiz-Sainz JE. 2011. Soybean interactions with soil microbes, agronomical and molecular aspects. Agronomy for Sustainable Development. 31: 173-190.

Ruiz-Montoya L, Nunez-Farfan J. 2009. Natural selection and maternal effects in life history traits of *Brevicoryne brassicae* (Homoptera: Aphididae) on tow sympatric closely related hosts. Florida Entomological Society 92:635-644.

Russin JS, Layton MB, Boethel DJ, McGawley EC, Snow JP, Berggren GT. 1990. Growth, nodule development, and N_2-fixing ability in soybean damaged by an insect-fungus-nematode pest complex. J. Econ. Ent. 83: 247-254.

Salvagiotti F, Cassman KG, Specht JE, Walters DT, Weiss A, Dobermann A. 2008. Nitrogen uptake, fixation and response to fertilizer N in soybeans: a review. Field Crops Research 108:1-13.

Sauge M-H, Grechi I, Poessel JL. 2010. Nitrogen fertilization effects on *Myzus persicae* aphid dynamics on peach: vegetative growth allocation or chemical defense?. Entomologia Experimentalis et Applicata 136:123-133.

Schippers B, Bakker AW, Bakker PAHM. 1987. Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. Ann. Rev. Phytopathol. 25: 339-358.

Schubert KR. 1981. Enzyme of purine biosynthesis and catabolism in Glycine max I. comparison of activities with N2 fixation and composition of xylem exudate during nodule development. Plant Physiol. 68:1115-1122.

Schubert KR. 1986. Products of biological nitrogen fixation in higher plants: synthesis, transport and metabolism. Ann. Rev. Plant Physiol. 37:539-574.

Schubert E, Mengel K, Schubert S. 1990. Soil pH and calcium effect on nitrogen fixation and growth of broad bean. Agron. J. 82: 969-972.

Shelp BJ, Da Silva MC. 1990. Distribution and metabolism of xylem-borne ureido and amino compounds in developing soybean shoots. Plant Physiol. 94: 1505-1511.

Sinclair TR, Seraj R. 1995. Legume nitrogen fixation and drought. Nature 378:344.

Sinclair TR, de Wit CT. 1975. Photosynthate and nitrogen requirements for seed production by various crops. Science 189:565-567.

Sindhu SS, Dua S, Verma MK, Khandelwal A. 2010. Growth promotion of legumes by inoculation of rhizosphere bacteria, pp. 195-235. In: Khan MS, Zaidi A, Musarrat J (eds.), Microbes for Legume Improvement, Springer, Vienna, Austria.

Skorupska A, Wielbo J, Kidaj D, Marek-Kozaczuk M. 2010. Enhancing Rhizobium-legume symbiosis using signaling factors, pp. 27-54. In: Khan MS, Zaidi A, Musarrat J (eds.), Microbes for Legume Improvement, Springer-Verlag/Wien, Germany.535 pp.

Sprent and Sprent 1990 Sprent JI and Sprent P. 1990. Nitrogen fixing organisms: pure and applied aspects. Chapman and Hall, New York, NY.

Staswick PE. 1994. Storage proteins of vegetative plant tissues. Annu. Rev. Plant Physiol. Plant Mol. Biol. 45: 303-22.

Strodtman, DN, Emerich DW. 2009. Nodule metabolism, pp. 95-124. In: Emerich DW, Krishnan HB (eds.), Nitrogen fixation in crop production Agronomy. Monograph no. 52, American Society of Agronomy, Madison, WI.

SYSTAT Software, Inc. 2007. SYSTAT®12 Statistics – I, SYSTAT Software, Inc., San Jose CA.

Techau MEC, Bjornlund L, Christensen S. 2004. Simulated herbivory effects on rhizosphere organisms in pea (*Pisum sativum*) depend on phosphate. Plant and Soil 264:185-194.

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

Thibodeau PS, Jaworski EG. 1975. Patterns of nitrogen utilization in the soybean. Planta (Berl.). 127: 133-147.

Thomas RJ, Schrader LE. 1981. The assimilation of ureides in shoot tissues of soybeans. Plant Physiol. 67: 973-6.

Tingey DT, Fites RC, Baharsjah J. 1974. Factors influencing nitrate reduction in soybean foliage. New Phytologist 73: 21-29.

Todd CD, Tipton PA, Blevins DG, Piedras P, Pineda M, Polacco JC. 2006. Update on ureide degradation in legumes. J. Exp. Bot. 57: 5-12.

UDSA. 2011. Fertilizer use and price. Available at: http://www.ers.usda.gov/Data/FertilizerUse/. Accessed on December 16, 2011.

USDA. 2012. Table 2: Soybeans: Acreage planted, harvested, yield, production, value, and loan rate, U.S., 1960-2011. Found at: http://www.ers.usda.gov/data-products/oil-crops-yearbook.aspx. Accessed on July 13, 2012.

Vadez V, Sinclair TR. 2000. Ureide degradation pathways in intact soybean leaves. J. Exp. Bot. 51: 1459-65.

van Berkum P, Sloger C, Weber DF, Cregan PB, Keyser HH. 1985. Relationship between ureide N and N2 fixation, aboveground N accumulation, acetylene reduction, and nodule mass in greenhouse and field studies with *Glycine max* L. (Merr). Plant Physiology 77:53-58.

van Rhijn P, Vanderleyden J. 1995. The rhizobium-plant symbiosis. Microbiological Reviews 59:124-142.

Venette RC, Ragsdale DW. 2004. Assessing the invasion by soybean aphid (Homoptera: Aphididae): where will it end? Ann. Entomol. Soc. Am. 97:219-226.

Vidal EA, Gutierrez RA. 2008. A systems view of nitrogen nutrient and metabolite responses in Arabidopsis. Plant Biology 11:521-529.

Voegtlin DJ, O'Neil RJ, Graves WR, Lagos D, YOO HJS. 2005. Potential winter hosts of soybean aphid. Ann. Entomol. Soc. Am. 98:690-693.

Walter AJ, DiFonzo CD. 2007. Soil potassium deficiency affects soybean phloem nitrogen and soybean aphid populations. Environ Entomol 36: 26-33.

Wille BD, Hartman GL. 2009. Two species of symbiotic bacteria present in the soybean aphid (Hemiptera: Aphididae). Environ. Entomol. 38:110-115.

Wilson KG, Stinner RE. 1984. A potential influence of rhizobium activity on the availability of nitrogen to legume herbivores. Oecologia (Berlin) 61:337-341.

Winkler RG, Blevins DG, Polacco JC, Randall DD. 1988. Ureide catabolism in nitrogen-fixing legumes. Trends Biochem. Sci. 13: 97-100.

Witte C-P. 2011. Urea metabolism in plants. Plant Science 180:431-438.

Wu Z, Schenk-Hamlin D, Zhan W, Ragsdale DW, Heimpel GE. 2004. The soybean aphid in China: a historical review. Ann. Entomol. Soc. Am. 97:209-218.

Yang SS, Bellogin RA, Buendia A, et al. 2001. Effect of pH and soybean cultivars on the quantitative analyses of soybean rhizobia populations. J. Biotechnol. 91: 243-255.

Yesmin L, Oresnik I, Banerjee MR. 2004. Optimizing legume production using sulfur oxidizing bacteria and Rhizobium consortia. Proceedings for the 4th International Crop Science Congress, Brisbane, Australia, 26 September-1 October 2004.

Zapata F, Danso SKA, Hardarson G, Fried M. 1987. Time course of nitrogen fixation in field-grown soybean using nitrogen-15 methodology. Agron J. 79: 172-176.

Zehnder CB, Hunter MD. 2009. More is not necessarily better: the impact of limiting and excessive nutrients on herbivore population growth rates. Ecological Entomology 334:535-543.

Zhang Y, Wu K, Wyckhuys KAG, Heimpel GE. 2009. Trade-offs between flight and fecundity in the soybean aphid (Hemiptera: Aphididae). Journal of Economic Entomology 102:133-138.

APPENDIX. EFFECTS OF NITROGEN SOURCE ON SOYBEANS IN THE ABSENCE OF APHIDS

In order to gain a better understanding of how treatments in our greenhouse experiments that impact soybean plant parameters related to growth and N-fixation changed over time, we conducted an experiment where plants were not infested with aphids and were destructively sampled at multiple time points. The experiment was designed as a 4 × 2 × 2 factorial with four levels of nitrogen (0, 25, 50 and 100 mg of N per pot), two levels of rhizobia (with rhizobia, +Rhiz, N-DURE and without rhizobia, No-Rhiz), and two levels of pasteurization (planting medium pasteurized, +Past and not pasteurized, No-Past). There were four replicates of each treatment that were processed at five separate time points: 19, 21, 28 and 33 days after planting (DAP) to match the time frame of our other experiments. All seeds (RG607 RR, Agronomy Seed Farm, Casselton, ND) were sterilized for 10 min in a 5% bleach (Clorox) solution then rinsed several times with deionized water. All other aspects of experimental establishment and data collection were identical to experiments described in Chapter 1.

All data was analyzed using SYSTAT® 12 (SYSTAT Software, Inc. 2007). Since at each time point plants were destructively sampled, SPAD readings from each date consisted of data from all surviving plants (i.e. Day 14 consisted of plants from all time points, Day 28 consisted of readings on plants in the last two time points). Plant parameters (i.e. number of root nodules, weight of root nodules, SPAD readings, plant height, dry weight of above-ground biomass, total-N, and nitrate-N) were analyzed using factorial ANOVA with nitrogen rate, rhizobia inoculation, and pasteurization as the independent variables. Due to technical difficulties, we are not presenting ureide-N data. Tukey's HSD test was used for mean separation.

Figure A1. Leaf chlorophyll per plant over time as measured using a SPAD meter. Higher SPAD meter values are unit less and indicative of more leaf chlorophyll.

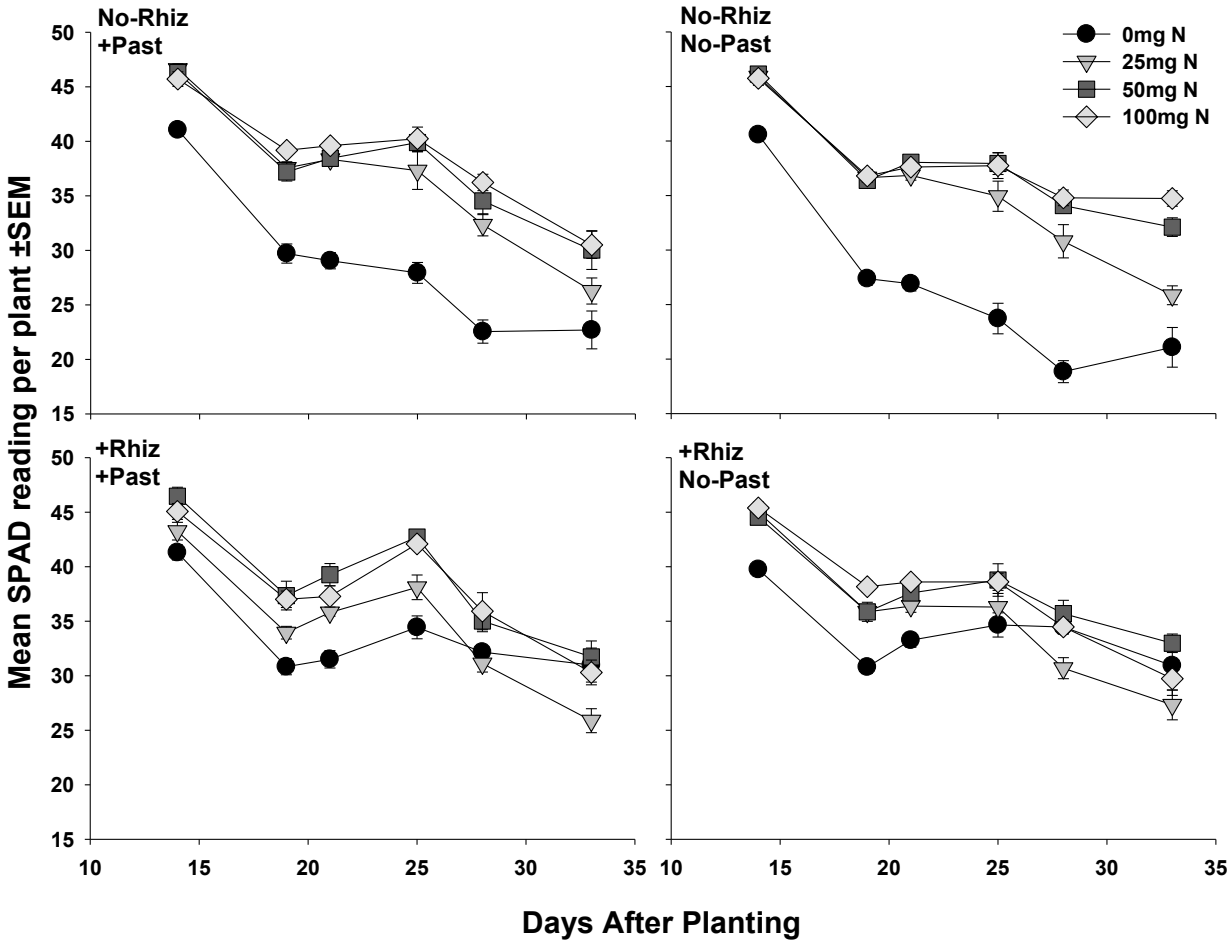


Table A1. Leaf chlorophyll per plant over time as measured using a SPAD meter (mean ± SEM), unit less. Days are DAP.

Treatment			Day 14	Day 19	Day 21	Day 25	Day 28	Day 33
No-Rhiz	+Past	0	41.05±0.46	29.71±0.88	29.05±0.75	27.93±0.95	22.55±1.07	22.70±1.73
		25	46.62±0.51	37.56±0.57	38.34±0.50	37.31±1.73	32.34±1.01	26.28±1.20
		50	46.31±0.64	37.19±0.83	38.43±0.58	39.88±0.82	34.54±1.26	30.03±1.78
		100	45.71±0.67	39.18±0.38	39.60±0.40	40.23±1.08	36.23±0.75	30.50±1.24
	No-Past	0	40.58±0.44	27.38±0.61	26.91±0.66	23.27±1.40	18.85±1.01	21.08±1.82
		25	45.90±.051	36.64±0.45	36.84±0.43	34.93±1.39	30.8±1.52	25.85±0.87
		50	46.14±0.43	36.39±0.59	38.05±0.56	37.93±1.01	34.08±0.62	32.10±0.85
		100	45.75±0.64	36.80±0.59	37.60±0.46	37.73±1.18	34.79±0.72	34.73±0.71
+Rhiz	+Past	0	41.30±0.70	30.83±0.75	31.51±0.80	34.43±1.05	32.16±0.38	30.98±1.56
		25	43.25±0.81	33.94±0.58	35.80±0.34	38.11±1.13	31.11±0.77	25.88±1.10
		50	46.46±0.83	37.35±1.32	39.27±1.02	42.72±0.59	35.02±0.99	31.73±1.46
		100	45.07±0.73	37.01±0.75	37.27±0.98	42.08±0.45	35.92±1.70	30.30±1.13
	No-Past	0	39.75±0.45	30.80±0.57	33.26±0.65	34.66±1.11	34.46±0.63	30.93±2.73
		25	45.01±0.45	35.86±0.87	36.38±0.56	36.30±1.24	30.69±0.96	27.33±1.37
		50	44.54±0.55	35.86±0.65	37.59±0.59	38.78±1.49	35.70±1.21	32.98±0.85
		100	45.39±0.55	37.17±0.52	38.60±0.49	38.61±1.79	34.48±0.63	29.73±1.05

	df	*P*	*df*	*P*	*df*	*P*	*df*	*P*	*df*	*P*	*df*	*P*
Rhiz	1, 279	**0.003**	1, 282	0.596	1, 218	0.053	1, 162	**<0.001**	1, 107	**<0.001**	1, 48	**0.006**
N-Rate	3, 279	**<0.001**	3, 282	**<0.001**	3, 218	**<0.001**	3, 162	**<0.001**	3, 107	**<0.001**	3, 48	**<0.001**
Past	1, 279	0.262	1, 282	0.111	1, 218	0.106	1, 162	**<0.001**	1, 107	0.134	1, 48	0.274
Rhiz × N-Rate	3, 279	0.099	3, 282	**<0.001**	3, 218	**<0.001**	3, 162	**<0.001**	3, 107	**<0.001**	3, 48	**<0.001**
Rhiz × Past	1, 279	0.971	1, 282	**0.007**	1, 218	**0.002**	1, 162	0.685	1, 107	**0.041**	1, 48	0.705
N-Rate × Past	3, 279	0.138	3, 282	0.309	3, 218	0.815	3, 162	0.908	3, 107	0.751	3, 48	0.531
Rhiz × N-Rate × Past	3, 279	0.058	3, 282	0.181	3, 218	**0.026**	3, 162	0.269	3, 107	0.139	3, 48	0.333

Figure A2. Mean height (inches) per plant according to treatment over time.

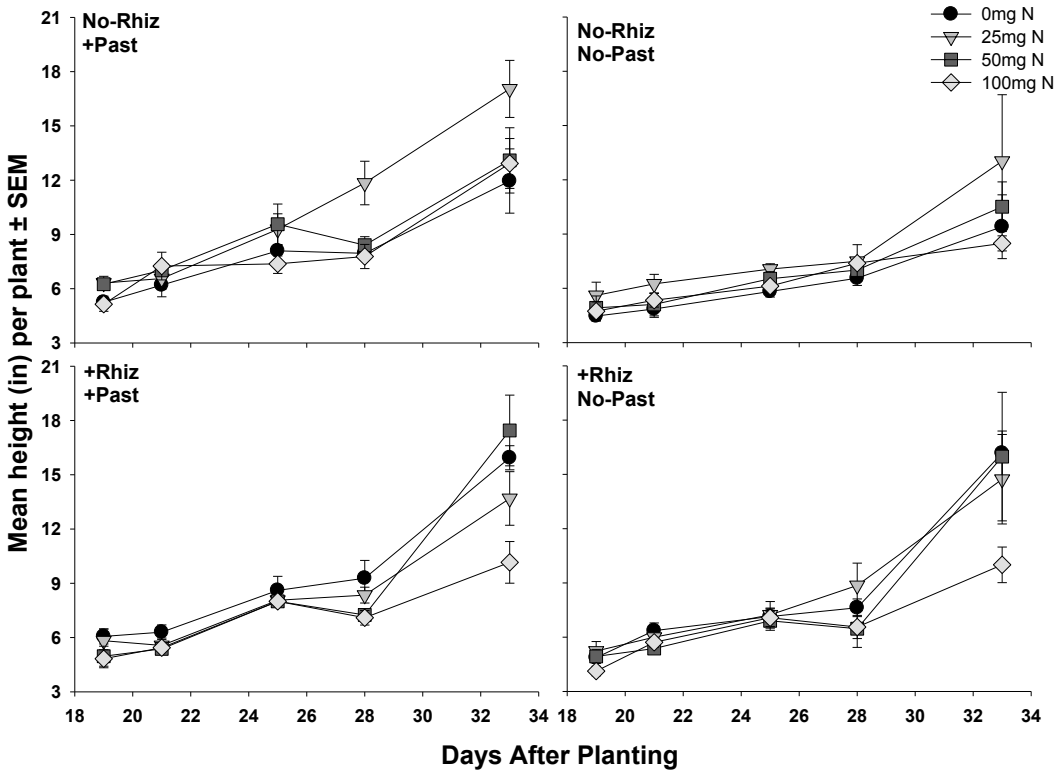


Table A2. Plant height (inches) (mean ± SEM) for each time point. Days are DAP.

Treatment			Day 19	Day 21	Day 28	Day 33
No-Rhiz	+Past	0	5.25±0.34	6.20±0.65	7.95±0.37	11.95±1.78
		25	6.31±0.37	6.56±0.61	11.84±1.21	17.05±1.58
		50	6.25±0.30	7.02±0.23	8.41±0.47	13.09±1.80
		100	5.14±0.39	7.25±0.77	7.78±0.67	12.92±1.38
	No-Past	0	4.47±0.29	4.86±0.46	6.56±0.40	9.41±1.76
		25	5.61±0.73	6.25±0.52	7.50±0.15	13.03±3.67
		50	4.92±0.66	5.11±0.63	7.02±0.58	10.51±1.37
		100	4.73±0.51	5.34±0.38	7.39±1.02	8.48±0.42
+Rhiz	+Past	0	6.06±0.42	6.30±0.40	9.28±0.98	15.94±0.67
		25	5.83±0.64	5.56±0.41	8.34±0.44	13.69±1.48
		50	4.97±0.55	5.38	7.25	17.45±1.96
		100	4.83±0.50	5.44	7.09±0.41	10.16±1.15
	No-Past	0	4.88±0.21	6.36±0.43	7.63±0.49	16.17±1.23
		25	5.23±0.52	6.00±0.26	8.86±1.24	14.73±2.47
		50	4.94±0.39	5.38±0.13	6.47±1.03	15.98±3.56
		100	4.13±0.27	5.72±0.28	6.56±0.64	10.00±0.98

	df	*P*	*df*	*P*	*df*	*P*	*df*	*P*
Rhiz	1, 48	0.332	1, 40	0.254	1, 43	0.386	1, 48	**0.025**
N-Rate	3, 48	**0.027**	3, 40	0.809	3, 43	**0.005**	3, 48	**0.012**
Past	1, 48	**0.003**	1, 40	**0.034**	1, 43	**0.005**	1, 48	0.075
Rhiz × N-Rate	3, 48	0.234	3, 40	0.082	3, 43	0.155	3, 48	**0.033**
Rhiz × Past	1, 48	0.708	1, 40	**0.006**	1, 43	0.143	1, 48	0.090
N-Rate × Past	3, 48	0.923	3, 40	0.503	3, 43	0.325	3, 48	0.974
Rhiz × N-Rate × Past	3, 48	0.558	3, 40	0.779	3, 43	0.750	3, 48	0.891

Figure A3. Mean dry weight (g) of above-ground biomass per plant according to nitrogen level, rhizobia and pasteurization treatment.

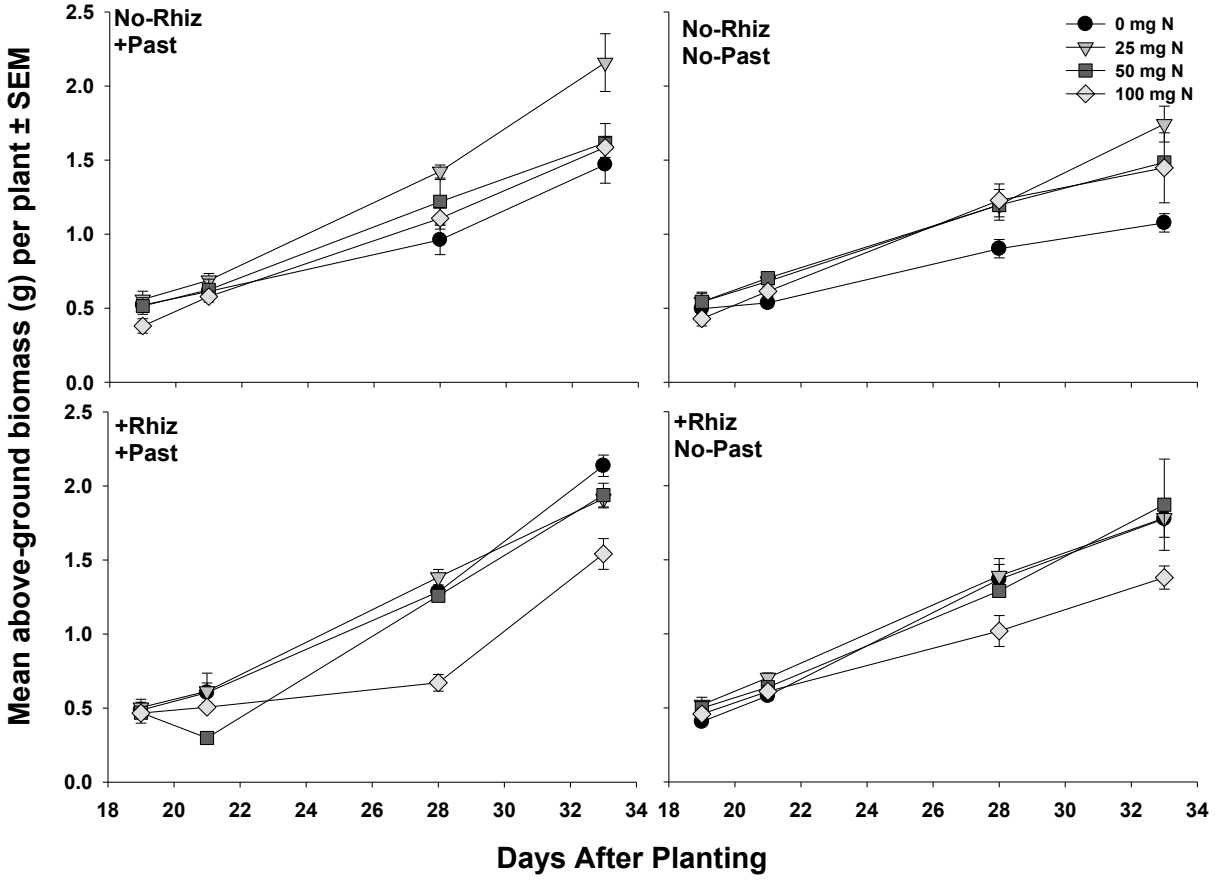


Table A3. Dry weight of above-ground biomass (g) (mean ± SEM) for each time point. Days are DAP.

Treatment			Day 19	Day 21	Day 28	Day 33
No-Rhiz	+Past	0	0.521±0.040	0.613±0.023	0.961±0.099	1.470±0.126
		25	0.559±0.056	0.688±0.046	1.423±0.044	2.158±0.195
		50	0.515±0.056	0.625±0.071	1.219±0.151	1.617±0.130
		100	0.380±0.049	0.578±0.037	1.107±0.073	1.586±0.068
	No-Past	0	0.497±0.034	0.536±0.030	0.902±0.062	1.076±0.062
		25	0.534±0.058	0.681±0.022	1.198±0.104	1.743±0.121
		50	0.543±0.065	0.703±0.039	1.196±0.042	1.484±0.039
		100	0.429±0.049	0.614±0.034	1.228±0.111	1.448±0.236
+Rhiz	+Past	0	0.486±0.034	0.605±0.065	1.285±0.035	2.136±0.072
		25	0.504±0.056	0.614±0.122	1.384±0.052	1.914±0.062
		50	0.469±0.071	0.298	1.258	1.938±0.080
		100	0.466±0.017	0.506	0.671±0.056	1.541±0.104
	No-Past	0	0.407±0.020	0.582±0.031	1.368±0.100	1.777±0.023
		25	0.521±0.051	0.704±0.032	1.392±0.116	1.782±0.130
		50	0.498±0.010	0.640±0.010	1.290±0.040	1.872±0.308
		100	0.458±0.039	0.611±0.040	1.019±0.104	1.380±0.078

	df	*P*	*df*	*P*	*df*	*P*	*df*	*P*
Rhiz	1, 48	0.359	1, 40	**0.036**	1, 43	0.274	1, 48	**0.002**
N-Rate	3, 48	**0.032**	3, 40	**0.021**	3, 43	**<0.001**	3, 48	**0.001**
Past	1, 48	0.983	1, 40	**0.017**	1, 43	0.469	1, 48	**0.002**
Rhiz × N-Rate	3, 48	0.283	3, 40	0.064	3, 43	**<0.001**	3, 48	**<0.001**
Rhiz × Past	1, 48	0.687	1, 40	**0.033**	1, 43	**0.100**	1, 48	0.510
N-Rate × Past	3, 48	0.628	3, 40	**0.017**	3, 43	0.088	3, 48	0.476
Rhiz × N-Rate × Past	3, 48	0.558	3, 48	0.882	3, 40	0.573	3, 43	0.921

Figure A4. Mean number of nodules per plant, +Rhiz plants only, according to nitrogen treatment and sampling date, data combined across all pasteurization levels. Different letters indicate significant differences at $P<0.05$. Days are DAP.

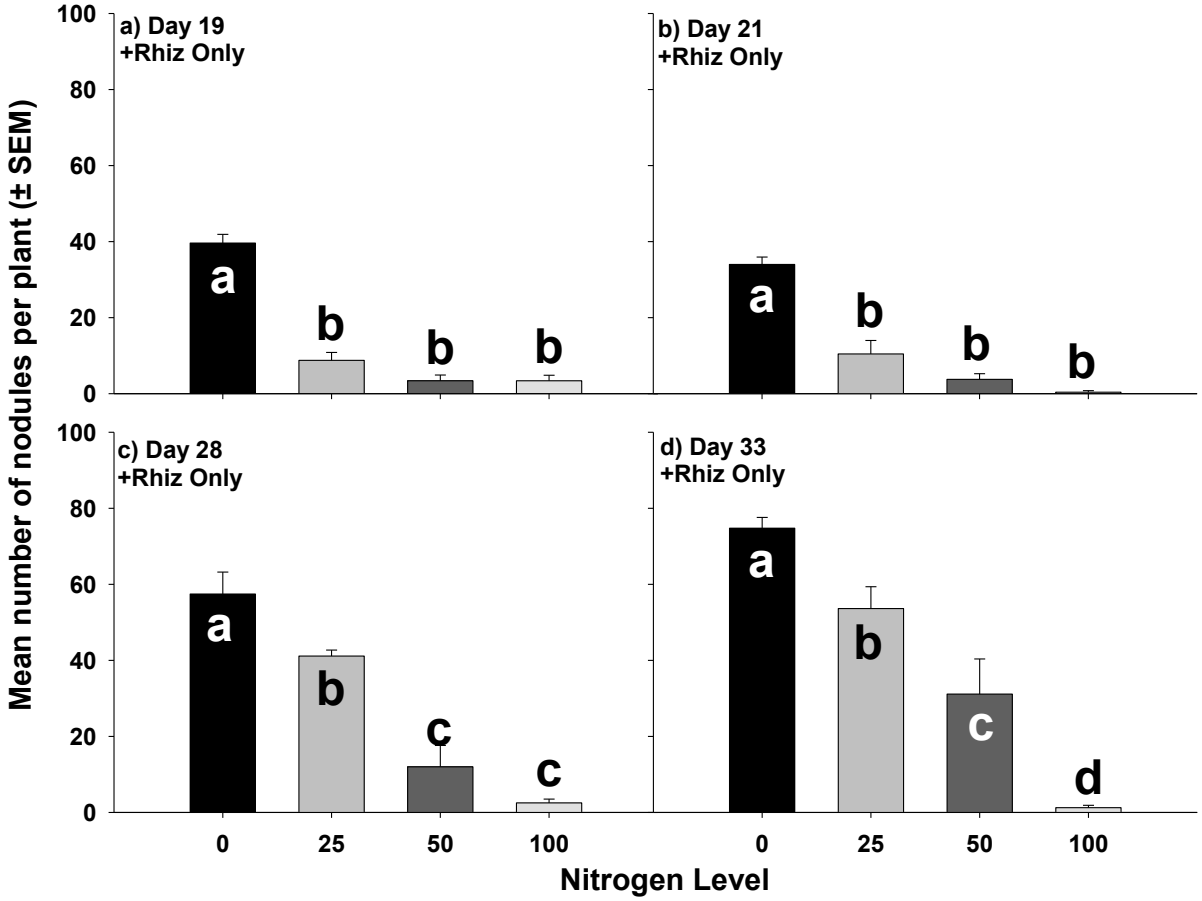


Table A4. Mean number of nodules per plant according to treatment and date. Days are DAP. The analysis on the bottom of the table does not include the No-Rhiz treatments.

Treatment		mg N	Day 19	Day 21	Day 28	Day 33
No-Rhiz	+Past	0	0.00±0.00	0.00±0.00	9.50±6.06	4.25±4.25
		25	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
		50	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
		100	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	No-Past	0	0.75±0.48	0.25±0.25	1.75±0.63	2.75±0.63
		25	0.00±0.00	0.00±0.00	0.00±0.00	0.75±0.75
		50	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
		100	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
+Rhiz	+Past	0	38.00±1.47	34.75±2.87	54.00±8.40	67.5±1.32
		25	5.75±2.23	11.33±8.95	41.00±2.80	42.00±3.72
		50	4.00±1.68	0.00	0.00	27.75±18.32
		100	1.25±0.75	0.00	1.00±1.00	2.00±1.16
	No-Past	0	41.25±4.52	33.25±2.96	62.00±8.72	82.00±1.41
		25	11.75±3.07	9.75±1.89	41.25±1.93	65.25±7.03
		50	2.75±2.75	5.00±1.16	15.00±6.14	34.50±7.31
		100	5.50±2.53	0.50±0.50	3.25±1.32	0.50±0.29
			df	*P*	*df*	*P*	*df*	*P*	*df*	*P*
N-Rate			3, 24	**<0.001**	3, 16	**<0.001**	3, 18	**<0.001**	3, 24	**<0.001**
Past			1, 24	0.110	1, 16	0.855	1, 18	0.176	1, 24	0.056
N-Rate × Past			3, 24	0.561	3, 16	0.897	3, 18	0.706	3, 24	0.419

Figure A5. Mean weight of all nodules per plant (g), +Rhiz plants only, according to nitrogen treatment and sampling date, data combined across all pasteurization levels. Different letters indicate significant differences at $P<0.05$. Days are DAP.

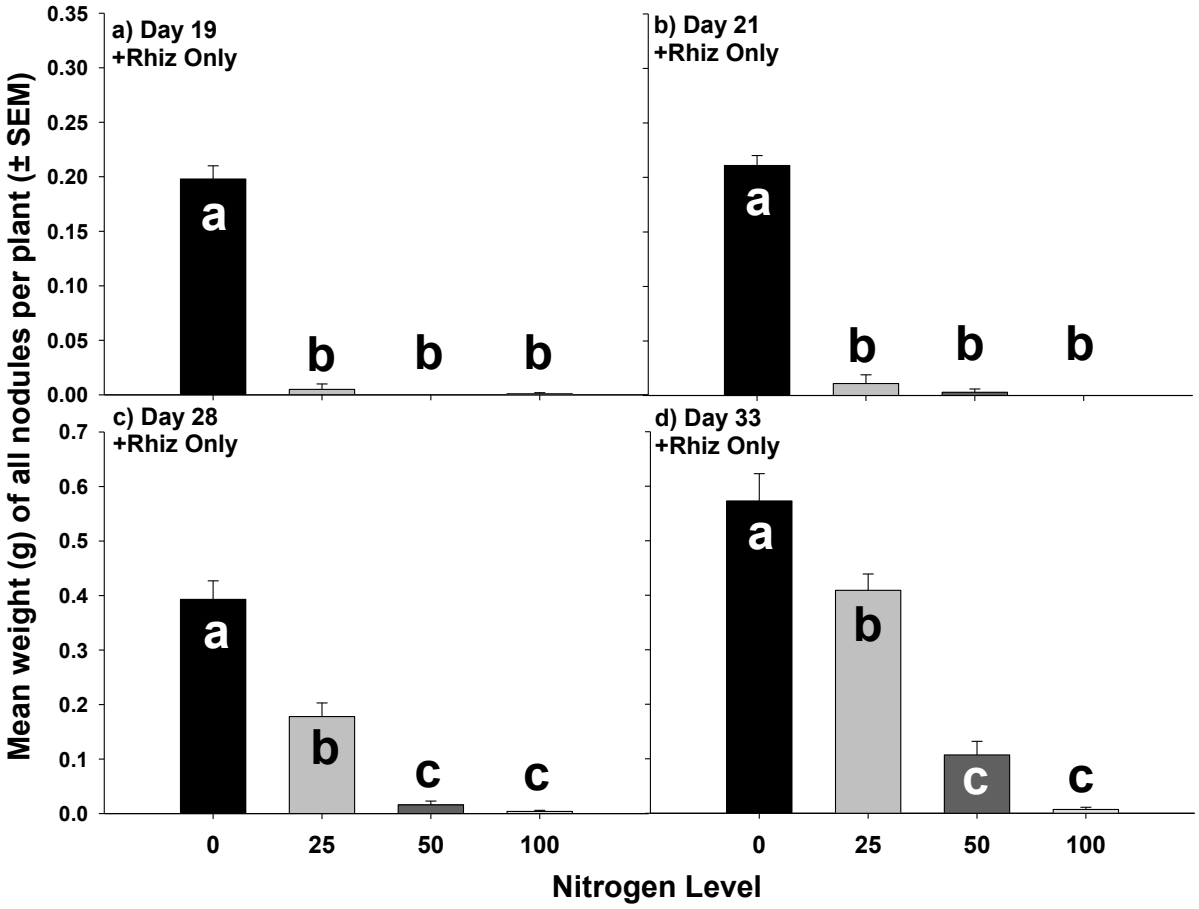


Table A5. Mean weight of all nodules per plant (g) according to date and treatment. Days are DAP. The analysis at the bottom of the table does not include the No-Rhiz treatments.

Treatment			Day 19	Day 21	Day 28	Day 33
No-Rhiz	+Past	0	0.000±0.00	0.000±0.00	0.146±0.06	0.109±0.11
		25	0.000±0.00	0.000±0.00	0.000±0.00	0.000±0.00
		50	0.000±0.00	0.000±0.00	0.000±0.00	0.000±0.00
		100	0.000±0.00	0.000±0.00	0.000±0.00	0.000±0.00
	No-Past	0	0.007±0.01	<0.005±0.00	0.129±0.05	0.132±0.05
		25	0.000±0.00	0.000±0.00	0.000±0.00	0.020±0.02
		50	0.000±0.00	0.000±0.00	0.000±0.00	0.000±0.00
		100	0.000±0.00	0.000±0.00	0.000±0.00	0.000±0.00
+Rhiz	+Past	0	0.188±0.01	0.225±0.01	0.389±0.05	0.542±0.11
		25	<0.005±0.00	0.010±0.01	0.152±0.04	0.368±0.05
		50	<0.005±0.00	0.00	0.00	0.083±0.04
		100	<0.005±0.00	0.00	0.002±0.00	0.012±0.00
	No-Past	0	0.207±0.02	0.198±0.01	0.398±0.06	0.605±0.01
		25	0.010±0.01	0.013±0.01	0.203±0.04	0.451±0.03
		50	<0.005±0.00	0.003±0.00	0.020±0.00	0.131±0.03
		100	<0.005±0.00	0.00	0.002±0.00	0.012±0.00

	df	*P*	*df*	*P*	*df*	*P*	*df*	*P*
N-Rate	3, 24	**<0.001**	3, 16	**<0.001**	3, 18	**<0.001**	3, 24	**<0.001**
Past	1, 24	0.232	1, 16	0.558	1, 18	0.476	1, 24	0.173
N-Rate × Past	3, 24	0.713	3, 16	0.411	3, 18	0.907	3, 24	0.771

Figure A6. Mean nitrate (ppm) per plant according to date and treatment.

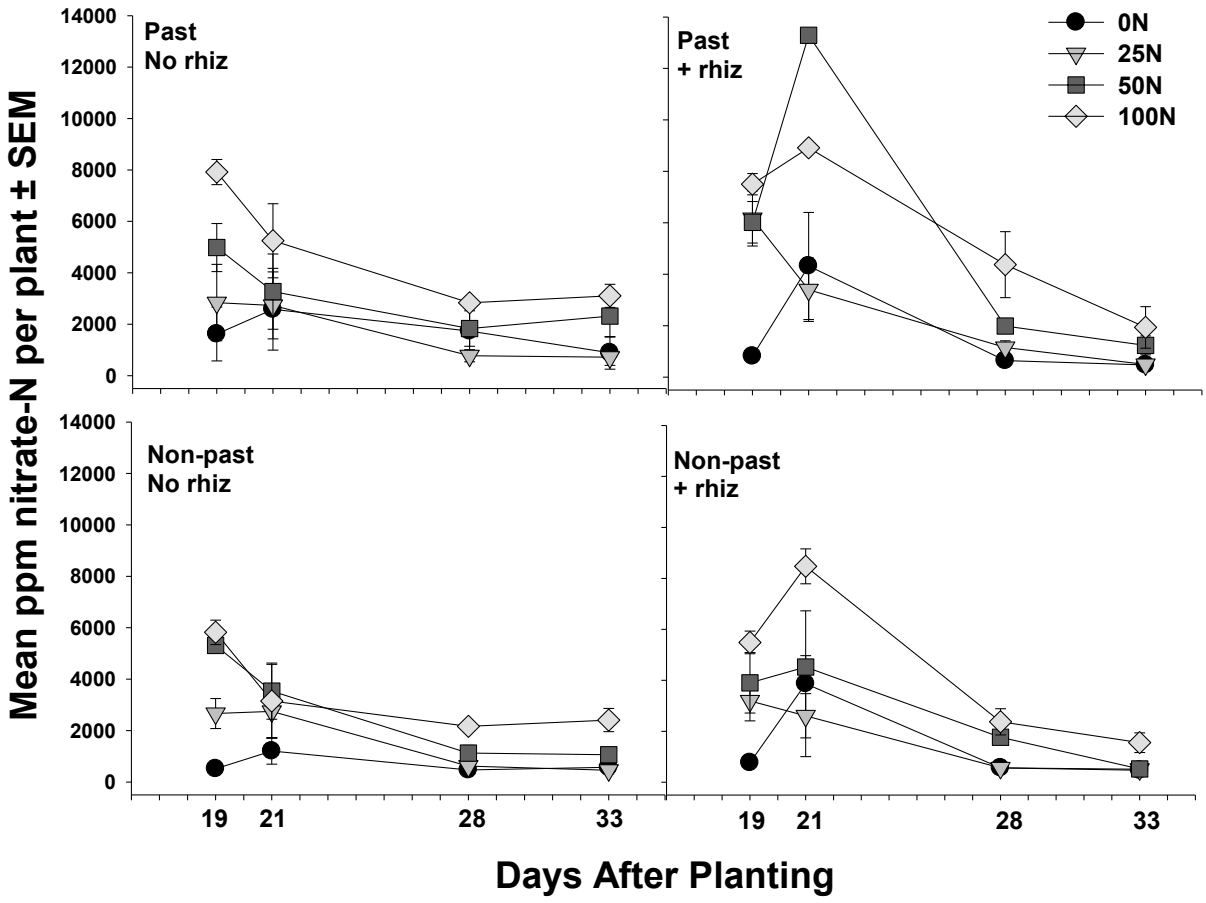


Table A6. Mean nitrate-N per plant (ppm) according to date and treatment. Days are DAP.

Treatment		mg N	Day 19	Day 21	Day 28	Day 33
No-Rhiz	**+Past**	0	1619±1046	2581±1590	1732±944	887±634
		25	2841±1488	2732±1302	773±237	718±317
		50	4981±936	3269±1463	1831±689	2314±814
		100	7919±488	5246±1443	2831±167	3105±449
	No-Past	0	513±153	1206±512	467±55	571±210
		25	2664±585	2747±1048	620±149	456±139
		50	5311±176	3536±1092	1125±301	1059±55
		100	5821±476	3452±1422	2168±152	2409±453
+Rhiz	**+Past**	0	812±131	4321±2083	644±122	473±2
		25	6166±1070	3396±1232	1152±260	490±95
		50	6019±809	13290	1986	1235±367
		100	7500±416	8915	4374±1283	1933±810
	No-Past	0	768±87	3866±2858	566±68	473±92
		25	3194±784	2610±869	565±68	511±94
		50	3902±1187	4523±444	1764±259	524±58
		100	5487±444	8476±684	2372±514	1554±385

	df	*P*	*df*	*P*	*df*	*P*	*df*	*P*
Rhiz	1, 47	0.486	1, 40	**0.001**	1, 39	0.330	1, 47	**0.007**
N-Rate	3, 47	**<0.001**	3, 40	**0.004**	3, 39	**<0.001**	3, 47	**<0.001**
Past	1, 47	**0.002**	1, 40	**0.053**	1, 39	**0.005**	1, 47	**0.024**
Rhiz × N-Rate	3, 47	0.118	3, 40	0.144	3, 39	0.207	3, 47	0.288
Rhiz × Past	1, 47	0.194	1, 40	0.296	1, 39	0.958	1, 47	0.349
N-Rate × Past	3, 47	0.530	3, 40	0.447	3, 39	0.475	3, 47	0.355
Rhiz × N-Rate × Past	3, 47	0.228	3, 40	0.178	3, 39	0.242	3, 47	0.995

Figure A7. Mean total-N (ppm) per plant according to nitrogen level, data combined across all rhizobia and pasteurization levels. a) 21 DAP; b) 28 DAP; c) 33 DAP. Different letters indicate significant differences at $P<0.05$.

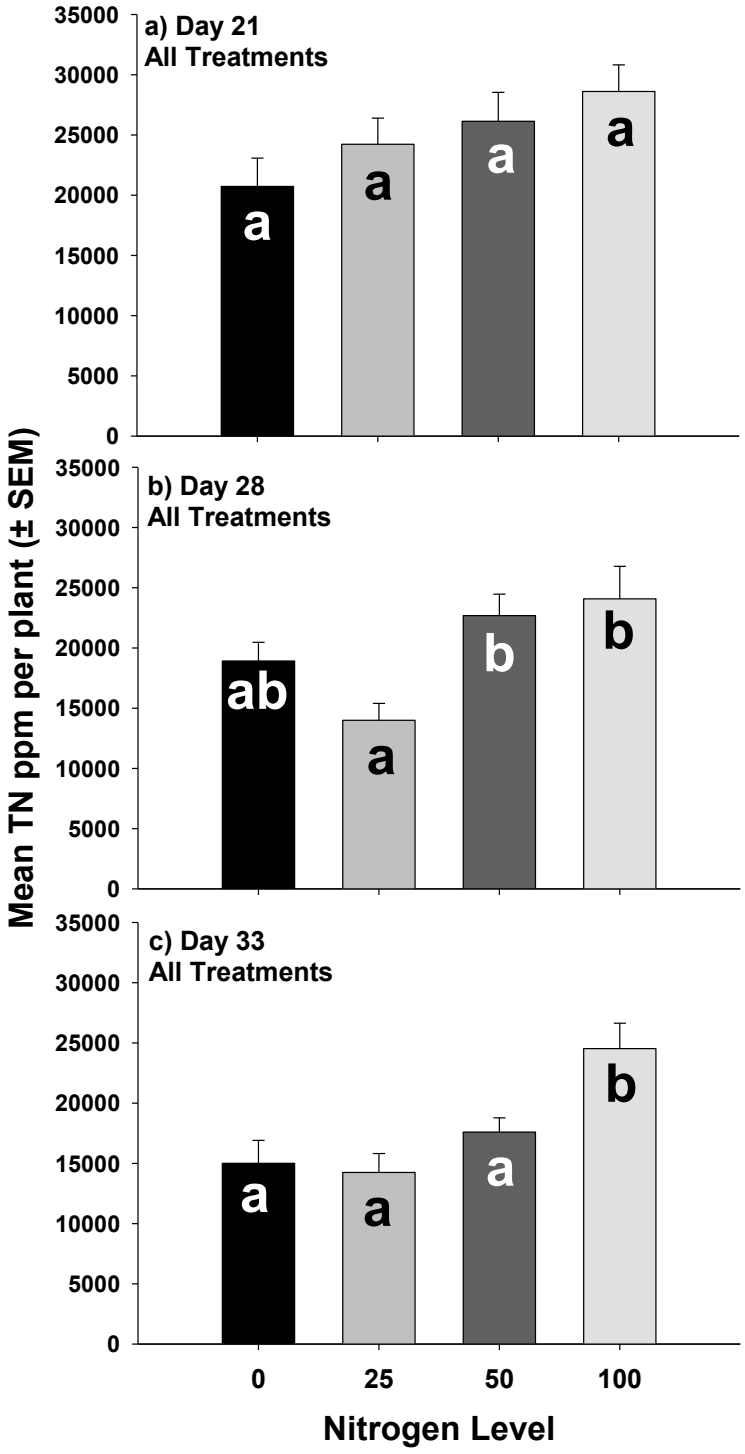


Table A7. Mean total nitrogen per plant (ppm) according to date and treatment. Days are DAP.

Treatment		mg N	Day 21	Day 28		Day 33	
No-Rhiz	**+Past**	0	20825±5644	12178±3069		8806+698	
		25	22998±4728	20180±4066		14366+3942	
		50	21391±3741	11635±1584		12485+3552	
		100	30995±3265	17783±2728		13800+3360	
	No-Past	0	14200±4331	30347+4096		18196+2486	
		25	26572±4913	20396+3352		18049+2391	
		50	26297±4628	16191+6216		25234+1779	
		100	23318±3850	26086+3486		23081+1484	
+Rhiz	**+Past**	0	2533±4995	20494+927		17005+2982	
		25	27414±700	22710+606		19829+5055	
		50	.	13961+2360		13636+3032	
		100	19931	12151+4719		17066+3523	
	No-Past	0	22543±3549	27846+699		14691+2462	
		25	21508±4236	20561		19462+2388	
		50	32560±616	26383+4740		27427+7874	
		100	33666±3481	31200+5603		22368+4081	
				df	*P*	*df*	*P*
Rhiz				1, 42	0.053	1, 48	0.227
N-Rate				3, 42	**0.001**	3, 48	**0.001**
Past				1, 42	0.159	1, 48	0.464
Rhiz × N-Rate				3, 42	0.317	3, 48	0.451
Rhiz × Past				1, 42	0.096	1, 48	0.923
N-Rate × Past				3, 42	0.287	3, 48	0.445
Rhiz × N-Rate × Past				3, 42	0.992	3, 48	0.833