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CHEMICAL ECOLOGY OF PLANT-MICROBE INTERACTIONS AND EFFECTS ON INSECT HERBIVORES

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Abstract

Plants are central to most community interaction webs and thus the ability to coordinate responses to many simultaneous interactions is an essential adaptation. Interaction with one organism may make the plant more vulnerable or more resistant to attacks by very different organisms. Microorganisms can form intimate associations with plants with direct effects ranging from beneficial to antagonistic, but indirect effects of plant-microbe relationships on plant interactions with other organisms are not well understood. Here, we explore the influences of microorganisms and microbial products on herbivory and resulting plant defenses. We use the legume-rhizobia mutualism as a model-system to explore herbivore-plant interactions by first characterizing the effects of rhizobial inoculation on herbivore feeding and subsequent accumulation of plant defense signaling hormones. We found interactive effects of the legume-rhizobia mutualism on plant-herbivore interactions which were dependent on both the stage of the mutualism and on the feeding style of the herbivore. Next, we explored the effects of association with different sources of rhizobia of soybean on specialist aphid populations in an agricultural setting. We found that particular rhizobia strains can confer greater resistance to their mutualist partners than others. In order to explore the effects of a rhizobia strain-specific trait on herbivory, we focused on the rhizobial product, rhizobitoxine, which can be found in host plant tissues. We found that the presumed presence of this compound decreased herbivore feeding and damage, which could be useful in pest management of legumes. Finally, we utilize genetically modified maize to further explore the application of a microbial product used for insect resistance and its effects on herbivore feeding and induction of plant defenses. Our results contribute to understanding of the evolution of host plant defenses and could facilitate the development of more sustainable management techniques for agriculture that are informed by an understanding of the chemical ecology of plants, microbes, and insects.

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Chapter 1

Introduction

Being primary producers, plants play a central role in most interaction webs, and have thus evolved a wide array of mechanisms to respond to encounters with other organisms. These interactions, which often occur simultaneously and range from beneficial to antagonistic, require the plant to coordinate physiological responses in a manner which minimizes fitness costs. Many recent studies have highlighted the importance of examining plant interactions with multiple organisms simultaneously in order to gain a clearer understanding of the evolution and ecology of plant defenses. Distinct attackers such as herbivores and pathogens elicit unique responses from the host plant, and combinations of attackers often produce surprising results (Cardoza et al., 2003; Cui et al., 2005; Delphia et al., 2007; Rodriguez-Saona et al., 2005; Thaler et al., 2002b; Wurst and van der Putten, 2007). In general, attack by one organism may make the plant more vulnerable or more resistant to future attacks by very different organisms (Morris et al., 2007).

Beneficial organisms can also affect plant resistance to attackers. Several classes of microorganisms engage in mutualisms with plants, in which both partners benefit from the interaction. These complex, coevolved associations between plant and microbe can alter many plant traits, and thus influence other interactions, especially those between plants and herbivores. Studies exploring the effects of microbial mutualists, particularly mycorrhizal fungi and fungal endophytes, on plant-herbivore interactions have found both positive and negative consequences for the host plant and enemy (Bennett and Bever, 2007; Borowicz, 1997; Gange and West, 1994; Goverde et al., 2000; Tintjer and Rudgers, 2006). The mechanisms by which mutualistic microbes can influence herbivory are even less clear, but could include interference of plant signaling networks, changes in plant quality for the consumer, or direct interaction between microbial products and herbivores.

The following collection of studies explores how plant defenses against herbivores are modulated by concurrent interactions with plant-associated microbes. The primary component focuses on the well known mutualism between nitrogen-fixing bacteria (rhizobia) and legumes, and the consequential effects of this relationship on herbivore feeding and plant responses to incurred damage. We examine host plants with different stages and intensities of rhizobial inoculation exposed to feeding by two distinct herbivores in order to gain a broad understanding of these complex, three-way relationships. We also explore the influence of genetic diversity of rhizobia partners on the variation found in effects on plant-herbivore interactions. By using genetically modified maize, we conclude by exploring the effects of a microbial toxin on herbivore-induced volatile production – a component of indirect plant defenses – without the confounding effects of infection itself. This body of work aims to increase our understanding of the chemically mediated interactions between plants, microbes, and herbivores in order to contribute to the goal of sustainably managing agricultural systems through strategies informed by a sophisticated understanding of natural ecological systems.

Plant-Herbivore Interactions

Due to their relatively large size and immobility, plants may appear, at first glance, to be vulnerable targets for the immense diversity of herbivorous insects. Yet plants dominate terrestrial ecosystems in terms of biomass, emphasizing the longstanding question: Why is the world green? A simplistic approach suggests that herbivory is limited by resource limitations (bottom-up effects), control by natural enemies (top-down effects), or more likely, a combination of both (Gruner, 2004; Ode, 2006; Polis, 1999; Terborgh et al., 2001).

Host plant quality from the perspective of an herbivore largely depends on the nutrient composition and defensive chemistry of the plant. These traits can be altered by concurrent interactions between plants and other organisms, which, in turn, drive changes in the behavior and fitness of herbivores (Borowicz, 1997; Cui et al., 2005; Rodriguez-Saona et al., 2005; Selosse et al., 2004; Walling, 2000; Wilson and Stinner, 1984). Nitrogen (N), which is essential for protein building and nucleic acids,

appears to be the most limiting nutritional factor for herbivores. The concentration of total N in plants (typically 2-4%) is much lower than that of primary consumers (about 8-14% N), underlying limitations to herbivore growth (Schoonhoven et al., 2005). The form of N is also of critical importance, as many plant compounds rich in N are unusable by, or even detrimental to, herbivores (Felton, 1996; Mattson, 1980).

Plant defensive chemistry is also a key regulator of herbivore behavior and fitness. Some chemical defenses, such as secondary metabolites and anti-nutritive proteins, have direct negative impacts on herbivores, while others, such as volatile organic compounds, work indirectly by recruiting natural enemies to the host (reviewed in Karban and Baldwin, 1997; Schoonhoven et al., 2005). Plant defenses can either be constitutively present in tissues, or induced upon plant recognition of attack. The induction of defenses requires coordination of signaling pathways within the plant which ultimately lead to a cascade of defense-related events. The phytohormones jasmonic acid (JA) and salicylic acid (SA) are generally associated with plant defense pathways in response to herbivores and pathogens, respectively, and inhibiting the function of either increases plant susceptibility (Walling, 2000). In addition to involvement in many key plant functions, JA plays a pivotal role in many herbivorous attacks and leads to the production of both direct and indirect defensive compounds that can help prevent further damage (Chen et al., 2005; Farmer et al., 1992; Schmelz et al., 2003; Thaler et al., 2002a). However, many factors can modulate herbivore-induced accumulation and action of JA, such as simultaneous induction of SA pathways commonly associated with pathogens (Felton et al., 1999; Thaler et al., 2002b). Moreover, piercing-sucking insects often trigger SA-dependent pathways, while some non-pathogenic, root-colonizing microbes confer a general resistance against diseases through a JA-dependent pathway (Walling, 2000). As interactions with mutualistic microorganisms such as rhizobia also involve the JA and SA plant pathways, plant-herbivore interactions are likely be influenced by the presence of rhizobia.

Legume-Rhizobia Mutualism

Legumes form symbiotic relationships with rhizobia, bacteria which are capable of fixing atmospheric nitrogen and making it available to the plants in a biochemically usable form. In return, the plants provide carbon and a protective root nodule within which the bacteria live. This specialized association allows legumes to thrive where nitrogen is a limiting factor for other plants, contributing to their ecological importance in both natural and agricultural systems. This pairing of legumes and rhizobia is credited with contributing 70 million metric tons of fixed nitrogen (N) into the earth's soils annually (Brockwell et al., 1995). When integrated into cropping systems with non-legumes, the nitrogen transfer results in reduced need for fertilizers (O'Hara et al., 2002). The ease and low cost of inoculating legume crops with rhizobial preparations, coupled with the yield advantages associated with doing so, has made inoculation a common agricultural practice.

The association of legumes with rhizobia, believed to have originated 65 million years ago, involves numerous physical and chemical changes in the host plant in response to inoculation by a compatible strain of rhizobia (Hirsch and LaRue, 1997) (Figure 1.1). Specific flavonoids exuded by legume roots are perceived by rhizobia, resulting in positive chemotaxis and production of Nod factors, various forms of lipochitooligosaccharides, which in turn initiate nodule development on legume roots (Hirsch et al., 2001). In many legumes, an individual bacterium infects the plant via a plant-produced "infection-thread" through the root hair. Rhizobia reproduce inside the infection thread and are eventually released into the plant cell. Many will differentiate into the N-fixing bacteroids, which will become surrounded by a plant-derived peribactoid membrane within the nodule (Lee and Hirsch, 2006). Once the plant no longer requires the N derived from rhizobia, usually as seeds are forming, the nodules are senesced from the roots and into the soil, at which point viable rhizobia cells are ready to infect another host. The mutualism is facultative, meaning each partner can survive and reproduce without the other. However, each partner can receive a substantial increase in fitness benefits by participating in the mutualism, especially under N-limiting conditions (West et al., 2002).

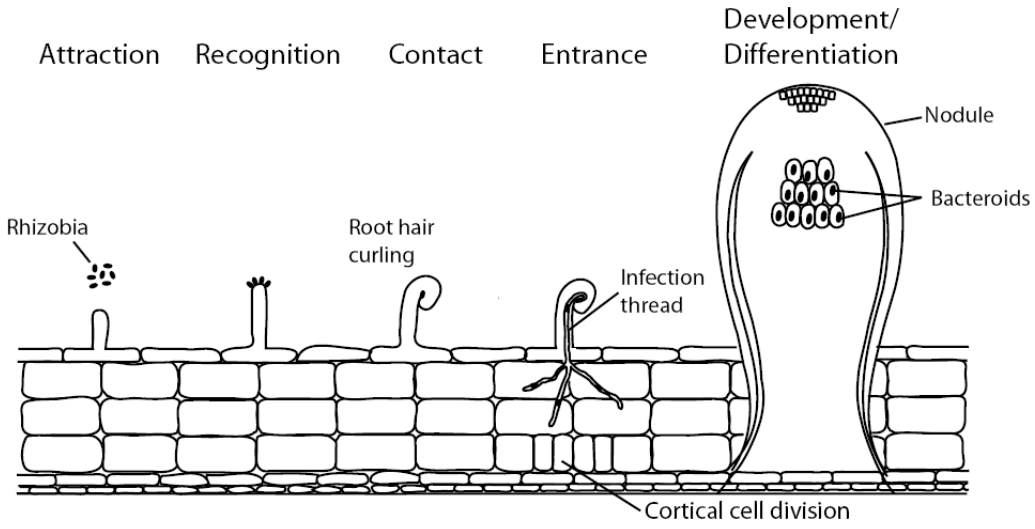

Figure 1.1. Infection and nodulation process of the rhizobia-legume interaction (Lum and Hirsch, 2002).

During nodule development, the host plant may initially respond to rhizobia as a pathogen, as suggested by the induction of anti-pathogen enzyme genes of phenylpropanoid metabolism (Estabrook and Senguptagopalan, 1991), the inhibition of salicylic acid biosynthesis during nodule formation (Martinez-Abarca et al., 1998), and the presence of an oxidative burst in infected roots (Santos et al., 2001). However, further plant defense mechanisms have not been noted, suggesting that the interaction with rhizobia somehow circumvents or suppresses later stages of the response pathway (Baron and Zambryski, 1995; Mithofer, 2002). Additionally, some phytohormones commonly associated with plant defenses against pathogens and herbivores, particularly salicylic acid (SA) and jasmonic acid (JA), appear to be involved in rhizobia-legume interactions, as application of these hormones will inhibit nodulation (Sato et al., 2002; Stacey et al., 2006; Sun et al., 2006).

At later stages of the rhizobia-legume interaction, following nodule establishment, physiological differences have been described between inoculated legumes, and non-inoculated plants receiving supplemental nitrate fertilizers. Inoculated legumes transport nitrogen to the shoots in the form of amides or ureides, as opposed to the inorganic forms that are transported in non-inoculated legumes (Matsumoto et al., 1977b). The addition of nitrate to inoculated soybean plants reduces the amount of

functional nodules and also reduces the proportion of total nitrogen available as ureides while maintaining equivalent total N levels (Matsumoto et al., 1977a; McClure and Israel, 1979).

Many of the physical and chemical changes induced in the host plant by compatible rhizobia are strongly influenced by the source and genotype of the bacterial partner. Different strains of rhizobia can have different effects on features of the mutualism including nitrogen-fixation efficiency, leaf chemistry, and plant fitness (Burdon et al., 1999; Fuhrmann, 1990; Lafavre and Eaglesham, 1986; Parker, 1995; van Berkum et al., 1985). For example, many of the soybean-compatible rhizobia strains commonly encountered in US soils fix less N for, yet are preferentially nodulated by, the host plant as compared to strains introduced from intentional inoculation at the time of planting (Amarger, 2001). Also, some rhizobia strains produce compounds, in addition to fixed-N products, which are translocated to the shoots, affecting leaf chemistry. The production of rhizobitoxine, an enol-ether amino acid, by particular rhizobia strains provides one example of a bacterial product found in host plant tissues that could potentially affect herbivore feeding. To our knowledge, no studies have explored how these variations in host plant effects due to different rhizobial genotypes could influence herbivory.

Model system for studying rhizobia-legume-herbivore interactions

Soybean-herbivore interactions

Legumes are attacked by a wide array of herbivores, and accordingly have evolved a diverse suite of both constitutive and inducible defenses. Soybeans defenses in particular have received a good deal of attention due to their worldwide economic status and breeding programs aimed at producing herbivore resistant cultivars. However, breeding for insect resistance in soybeans in the US has met limited success, and in actuality, many key defensive mechanisms are missing from

cultivated legumes due to selective breeding for other agronomic traits (Edwards and Singh, 2006).

Constitutive defenses of soybean leaves and seeds have been well studied, and include structural protection (trichomes) and chemical defenses (e.g., lectins and flavonoids) (Edwards and Singh, 2006). Soybeans are rich in flavonoids in all plant parts tested (Romani et al., 2003). These compounds are considered to be an important component of defense against many invaders, in addition to serving as critical belowground signals to initiate the nodulation process with compatible rhizobia strains (Kosslak et al., 1987). Isoflavonoids from soybeans have been shown to have negative effects on herbivore behavior, consumption, and growth, and are inducible via herbivore feeding (Hoffmann-Campo et al., 2001; Piubelli et al., 2003; Sharma and Norris, 1991).

Many other components of soybean defenses are also inducible; resulting in reductions in growth of herbivores fed previously damaged leaves. Proteinase inhibitors, which hinder the ability of herbivores to utilize ingested leaf proteins, accumulate in leaves after damage, and are implicated in growth reductions of beetles and lepidopteran larvae (Bi et al., 1994; Kraemer et al., 1987). Components of oxidative stress, such as oxidative enzymes and lipid peroxidases are also increased in soybeans following herbivory, with specific components dependent on the type of herbivore (Bi and Felton, 1995; Felton et al., 1994). Soybean aphids induced volatiles, such as methyl salicylate, which were attractive to predaceous beetles and parasitoids (Wyckhuys and Heimpel, 2007; Zhu, 2005). There is much variation among soybean cultivars in the degree of inducible and constitutive defenses (Underwood et al., 2000).

The strong inducibility of soybeans defenses are hypothesized to have prevented local legume specialists from colonizing this imported crop in the US (Kogan, 1991). Most New World pests of soybeans, such as *Helicoverpa zea*, are very mobile and often highly polyphagous. Until just recently, there was a striking absence of soybean-colonizing aphids, which are one of the most damaging of pests to this crop at its center of origin (China) (Wu et al., 2004). As inducible defenses against herbivores must be very dependent on signaling networks, such as the

octadecanoid pathway, within the host plant, other organisms interacting with the plant such as rhizobia may influence the degree or timing of induction.

Rhizobia

Rhizobia refers to a general class of gram-negative bacteria which have the ability to infect the roots or stems of legumes and provide fixed nitrogen to the host. Nitrogen fixation is accomplished through the action of the enzyme nitrogenase, which breaks the tight triple bond of atmospheric N gas, producing ammonium through a highly energetic process:

$N_2 + 8e^- + 8H^+ + 16ATP \longrightarrow 2NH_3 + 16ADP + 16Pi + H_2$

Oxygen flow in the root nodule is tightly controlled by a plant produced O_2-binding protein (leghemoglobin) since the nitrogenase enzyme is denatured by oxygen, but other essential cellular functions of rhizobia are aerobic (Fisher and Newton, 2002).

The mutualism between rhizobia and legumes is facultative and initiated anew with each generation of host plants; therefore rhizobia exist and reproduce in a free-living state in the soil at some point, or if a suitable host cannot be found, through their entire life. Non-symbiotic individuals do not fix N, and are at a reproductive disadvantage as compared to their legume-associated kin (West et al., 2002).

There is a high degree of specificity in the relationships between legume hosts and their compatible rhizobia, mediated in part by the particular chemical interactions between plant roots and rhizobia in the soil for proper nodulation (Hirsch et al., 2001). However, it is common for a single plant species to form effective associations with numerous strains of a rhizobia species, or even with strains from different genera. For example, soybeans can successfully interact with strains from *Bradyrhizobium japonicum*, *B. elkanii*, *B. lianonigense*, and *Sinorhizobium fredii* (Amarger, 2001).

Many biotic and environmental factors influence the genetic composition of rhizobia populations in the soil over time (Denton et al., 2002; Streeter, 1994; Taylor et al., 1991). Rhizobia populations also become adapted to local environmental conditions and plant genotypes, with resulting effects on mutualisms (Parker, 1995; Thrall et al., 2007). The diversity of rhizobia may also be increased through the

horizontal exchange of genetic material, such as the transfer of symbiotic genes from introduced rhizobia to native, non-symbiotic bacteria in the field (Barcellos et al., 2007; Spratt and Maiden, 1999; Sullivan et al., 1995). One or all of these factors can contribute to high levels of phenotypic and genotypic variation found in rhizobial isolates as compared to parental inoculant strains after many years in the soil without a host plant (Batista et al., 2007).

Human intervention is another factor influencing rhizobial diversity. During the long history of legume cultivation, humans have shuffled rhizobia strains throughout many regions of the world. As a result, diverse populations of soybean-compatible rhizobia are naturally present in many agricultural soils (Amarger, 2001; Ferreira and Hungria, 2002). These "indigenous" strains are now subject to the same influences on genetic composition that occur in undisturbed settings, such as adaptation to local conditions and horizontal gene transfer with native microbes and contribute to the diverse rhizobia populations which legumes may encounter in the soil.

Herbivores

The soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), is a phloem-feeding specialist of soybeans native to areas of Asia from which soybeans origniated. As with other members of this family, soybean aphids use the stylet of their piercing-sucking mouthparts to maneuver through intercellular spaces until reaching the phloem, piercing only the sieve element cells (Powell et al., 2006). Once a suitable food source has been located, the aphid remains sessile and continues to draw out phloem over a long period of time. Since its discovery in the US in 2000, the soybean aphid has since spread throughout much of the soybean growing regions of the US and Canada and is now considered "the most significant insect threat to soybean production in North America" (Ragsdale et al., 2007).

Annual outbreaks of the soybean aphid have occurred since its introduction, causing yield losses between 20 and 25% and increasing pesticide usage (Ragsdale et al., 2007; Rutledge and O'Neil, 2006; Rutledge et al., 2004). While economic thresholds suggest insecticide use after 250 aphids per plant, it is common to find

aphid numbers per plant in the thousands (Landis et al., 2004; Ragsdale et al., 2007)(personal observations). In addition to direct damage inflicted by phloem-feeding, the soybean aphids can also transmit plant diseases such as bean yellow mosaic virus between soybean plants and other economically important viruses between non-host crops (Wang et al., 2006). Although efforts have been made to identify cultivars of soybeans with enhanced resistance against the soybean aphids (Li et al., 2006), very little is known about the mechanisms behind host plant protection. And to date, no studies have explored the possibility of controlling aphids, or other herbivores, through selective inoculation with rhizobia.

Another common pest of soybeans is *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae), a moth species native to North America. The larvae of this species use powerful mandibles to chew through foliage and are highly polyphagous – incorporating many economically important crops into its host range – and have earned an assortment of common names, such as corn earworm, cotton bollworm, and soybean podworm. Most of the agronomic problems associated with *H. zea* are concentrated in the southern and mid-Atlantic regions of the US where successful overwintering occurs, but it is highly dispersive and serves as a secondary crop pest for all but the most northern regions of North America (Capinera, 2000). Although a substantial portion of its pest status stems from larval damage to cotton and corn, *H. zea* can also cause substantial economic losses in soybeans (Herbert et al., 1991; Yu et al., 1993).

The severity of yield loss in soybeans is dependent on the timing and intensity of *H. zea* infestations. Extensive defoliation during the reproductive stages of growth, such as during full flower bloom, can cause up to 50% yield reductions (reviewed in (Timsina et al., 2007). Young larvae can be found in high densities in the developing leaves, while older larvae tend to feed on the forming seed pods (Kraemer et al., 1997). Additionally, soybeans can serve as reservoirs for *H. zea* populations which will infest other crops (Jackson et al., 2008). Development of herbivore resistant soybean cultivars have met limited success, with products not meeting other agronomic standards (Rector et al., 2000). Also, concerns over the development of resistance to insecticides, especially Bt and pyrethroids, warrant the need for more

sustainable means of controlling *H. zea* populations (Jackson et al., 2004; Pietrantonio et al., 2007).

Use of microbial products in agriculture for pest control through genetic modification

Basic research into the evolution and ecology of plant defenses can eventually lead to applied outcomes, particularly towards the ongoing task of developing sustainable strategies for herbivore pest management in agricultural systems. An intimate understanding of multi-level biotic interactions such as those between plants, microbes, and herbivores are essential if control methods are derived from manipulation of these interactions. One such technology which has reached widespread commercial success in some systems is the genetic modification of crop plants to incorporate insect-resistance conferred by foreign genes.

Bacillus thuringiensis (Bt), a common soil bacterium, has been utilized in sustainable farming for many years to combat agricultural pests, namely Lepidopteran larvae. During sporulation, certain Bt strains produce various crystalline inclusions containing endotoxins which are lethal to specific groups of insects (Broderick et al., 2006). Through genetic modification, bacterial genes for the production of endotoxins have been successfully inserted into the genome of some key crops, particularly cotton and maize. The constitutive production of insecticidal endotoxins in plant tissues has the potential to control target pests and consequently reduce insecticide usage.

Bt-maize became commercially available in 1996, and as of 2007, 49% of all maize planted in the US incorporates Bt genes (National Agricultural Statistics Service, 2007). Large-scale implementation of transgenic crop technology in a short time span has sparked substantial debate over the social, economic, and ecological implications of GM agriculture. Ecological concerns of insect-resistant transgenic crops have often focused on resistance development by pests and potential negative effects on natural enemies (Gould, 1998; Shelton et al., 2002). These issues are intricately linked to one another, because natural enemies can influence the rate that pest populations adapt to resistant plants (Gould et al., 1991). The effects of genetic

modification on plant defenses have received little attention, despite the influence of defenses on herbivore and natural enemy behavior (reviewed in Schoonhoven et al., 2005). The insertion of a foreign gene has the potential to cause unexpected phenotypic changes in the complex biochemical pathways leading to plant defenses. Defenses can also be affected through changes in the interaction between herbivore and plant, such as changes in feeding behavior, reduced amounts of herbivory, or alterations to oral secretions.

Overview of chapters

My dissertation research broadly examines the effects of microbe-plant interactions on herbivory and plant defenses against herbivores. The coevolved legume-rhizobia mutualism serves as a model system with which to study the ecology and mechanisms of these tripartite interactions. Also, the use of a genetically modified insect-resistant plant further allowed examination of the roles of microbial products on herbivore behavior and plant defenses.

Chapter 2. Influences of the legume-rhizobia mutualism on herbivore feeding and plant hormone responses

The interactions between plants and a diversity of other organisms has been an important component of research aimed to better understand the evolution of plant defenses. Simultaneous encounters with both beneficial and antagonistic organisms can modulate host plant responses, and thus the plant's ability to defend itself. This study attempts to characterize the effects of the legume-rhizobia mutualism on plant-herbivore interactions by looking at plant responsiveness to feeding via phytohormone accumulation and fitness parameters of two herbivores with distinct modes of feeding. We found interactive effects of the legume-rhizobia mutualism on plant-herbivore interactions and that these effects were dependent on both the stage of the mutualism and on the feeding style of the herbivore.

Chapter 3. Effects of genetic diversity of rhizobia on soybean aphid populations

Many of the physical and chemical changes induced in the host plant by compatible rhizobia are strongly influenced by the source and genotype of the bacterial partner. We explored how association with different sources of rhizobia of soybean influences the population dynamics of the soybean aphid in the field. We found differences in aphid abundance between plants hosting either a commercial rhizobia mixture or indigenous rhizobia from our field site. Several plant traits influencing fitness and vulnerability to herbivore feeding were assessed and rhizobia strains isolated from our field plants were genotyped.

Chapter 4. Preliminary assessment of the effects of rhizobitoxine production on feeding by *Helicoverpa zea*

One example of a rhizobia strain-specific trait that could potentially affect insect herbivores is the production of rhizobitoxine (Rtx) which is translocated to the leaves of the host plant. We explored possible resistance conferred to the host plant by an Rtx-producing rhizobia strain on herbivory by the soybean podworm. We found reduced growth of larvae and damage incurred by plants, presumably mediated by the presence of Rtx. Further studies are needed to better understand these findings.

Chapter 5. Effects of genetic modification on herbivore-induced volatiles from maize

Insect-resistant transgenic crops have become widespread in agriculture as a result of many years of basic and applied research into the interactions among plants, microorganisms, and insect herbivores. Herbivore-induced volatile emissions from plants are known to attract natural enemies of insect pests, thus changes to this defense system through genetic modification could have implications for insect resistance management and effects on non-target organisms. We examined the influence of the Bt transgene in maize on herbivore feeding behavior and subsequent induction of indirect plant defenses.

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Chapter 2

The legume-rhizobia mutualism influences herbivore feeding and plant hormone responses

Abstract

Diverse interactions between plants and other organisms have shaped the evolution of plant defenses. Simultaneous encounters with beneficial and antagonistic organisms can modulate host plant responses, and thus the plant's ability to defend itself. We explored the effects of mutualistic rhizobia bacteria on plant's susceptibility to feeding by insect herbivores and on plant defense responses. We documented effects that were dependent on both the stage of the mutualism and on the feeding style of the herbivore. During the early stages of mutualism, before the plants began to receive fixed nitrogen (N) from the root nodules, inoculated plants were better protected against the phloem-feeding soybean aphid than non-inoculated plants. This protection appears to be regulated by differences in the levels of phytohormones that lead to activation of plant defenses. However, the presence of rhizobia on these young plants did not significantly affect the fitness of the foliar-feeding soybean podworm or the induction of a JA burst characteristically observed following feeding by lepidopteran larvae. To examine nutritive effects of rhizobia inoculation, we grew older plants with a range of dependency on rhizobia versus N fertilizer for their nitrogen needs. This difference in nitrogen sources results in different ratios of organic ureide N to inorganic nitrate N. Aphid fitness was not apparently affected by these differences, but podworm larvae grew better on plants completely dependent on rhizobia. In response to feeding by aphids and podworms, accumulations of SA and JA, respectively, were more pronounced in plants completely dependent on rhizobia.

Introduction

Plants constantly interact with, and respond to diverse organisms whose effects on plant fitness range from beneficial to antagonistic. Many recent studies have highlighted the importance of examining plant interactions with multiple organisms simultaneously in order to gain a clearer understanding of the evolution and ecology of plant defenses. Different antagonists of plants, such as herbivores and pathogens elicit different responses from the host plant, and combinations of attackers often produce unexpected results (Cardoza et al., 2003; Cui et al., 2005; Delphia et al., 2007; Rodriguez-Saona et al., 2005; Thaler et al., 2002b; Wurst and van der Putten, 2007). Less attention has been given to the effects of plant responses to simultaneous interactions antagonistic and beneficial organisms, though an interesting body of work explores the effects of mycorrhizal fungi on plant herbivores and pathogens (Bennett and Bever, 2007; Borowicz, 1997; Gange and West, 1994; Goverde et al., 2000; Pozo and Azcón-Aguilar, 2007). Few studies have addressed the effects of rhizobia – another important class of plant mutualists – on interactions between plants and other organisms, such as insect herbivores.

Legume plants form symbiotic relationships with rhizobia, bacteria capable of fixing atmospheric nitrogen (N) and making it available to the plants in a biochemically usable form. In return, the plants provide photosynthates and a protective root nodule within which the bacteria live. This specialized association allows legumes to thrive where nitrogen is a limiting factor for other plants, contributing to their ecological importance in both natural and agricultural systems. The association of legumes with rhizobia, believed to have originated around 65 million years ago, involves numerous physical and chemical changes in the host plant in response to inoculation by a compatible strain of rhizobia (Hirsch and LaRue, 1997).

The initial response of host plants to infection by rhizobia resembles the defensive response mounted against pathogens (Estabrook and Senguptagopalan, 1991; Santos et al., 2001; Sato et al., 2002). However, subsequent pathogen defense mechanisms are not induced, suggesting that the interaction with rhizobia somehow circumvents

or suppresses later stages of the response pathways (Baron and Zambryski, 1995; Mithofer, 2002). Additionally, some phytohormones commonly associated with plant defenses against pathogens and herbivores—particularly salicylic acid (SA) and jasmonic acid (JA)—appear to be involved in rhizobia-legume interactions, as application of these hormones inhibits nodulation (Sato et al., 2002; Stacey et al., 2006; Sun et al., 2006).

During later stages of the rhizobia-legume interaction, following nodule establishment, physiological differences have been described between plants reliant on rhizobia to meet N needs and those acquiring inorganic N directly from the soil. Inoculated legumes transport nitrogen to the shoots in the form of amides or ureides rather than the inorganic forms transported in non-inoculated legumes (Matsumoto et al., 1977b). The addition of nitrate to inoculated plants reduces the number of functional nodules and also reduces the proportion of total nitrogen available as ureides in soybean (Matsumoto et al., 1977a; McClure and Israel, 1979). Nitrogen is a key nutrient for herbivores, and the form of nitrogen available can affect their fitness and feeding behavior (Cockfield, 1988; Felton, 1996; Mattson, 1980).

As host plant quality, from the perspective of an insect herbivore, encompasses plant features such as defensive compounds and nutrient composition, the legume-rhizobia mutualism may strongly influence plant-insect interactions. In response to herbivore attack, signaling molecules accumulate within the plant and mediate a cascade of defense-related events. Jasmonic acid is known to play a pivotal role in mediating defenses against many herbivores and elicits the production of defensive compounds that can deter further damage (Chen et al., 2005; Farmer et al., 1992; Schmelz et al., 2003; Thaler et al., 2002a). However, many factors can modulate herbivore-induced accumulation and action of JA, including simultaneous induction of SA pathways commonly associated with pathogens (Felton et al., 1999; Thaler et al., 2002b). Moreover, piercing-sucking insects often trigger SA-dependent pathways, although plant resistance to these herbivores appears to be regulated by the JA pathway (Smith and Boyko, 2007). Since pathogen- and herbivore-response pathways have been shown to exhibit “cross-talk” in some systems, plant-herbivore interactions

are likely be influenced by biochemical changes caused by the presence of rhizobia (Felton et al., 1999).

Differences in the quality of foliar nitrogen between inoculated and non-inoculated plants could also lead to different outcomes for phytophagous insects. The ratio of organic to inorganic nitrogen is likely to be an important aspect of feeding efficiency, as the latter is generally assumed to be unusable by insects (Mattson, 1980; Schoonhoven et al., 2005). However, ureides, the organic forms of nitrogen transported by some inoculated legumes, may not be easily metabolized. For instance, allantoin, a type of ureide found in soybean, has been shown to reduce the performance of lepidopteran larvae when added to artificial diet (Wilson and Stinner, 1984).

This study attempts to characterize the effects of the legume-rhizobia mutualism on plant-herbivore interactions by measuring phytohormone accumulation in plants and the performance of two herbivores with distinct modes of feeding. The soybean podworm (*Helicoverpa zea* Boddie; Lepidoptera) is a leaf-feeding generalist, while the soybean aphid (*Aphis glycines* Matsumura; Hemiptera) is a phloem-feeding specialist. As the effects on these herbivores may be related to differences in both plant defense responses and nutritional quality between inoculated and non-inoculated plants, we have separated these variables as much as possible by employing two distinct growing regimes: Comparing young plants that have been inoculated, but not yet received nitrogen from the rhizobia, with non-inoculated plants allows us to focus on plant defense responses caused by the interaction with the bacteria. To examine nutritive differences we compare inoculated plants that have been fertilized with nutrient solutions containing either 0 mM, 10 mM, or 20 mM of nitrate. Nitrate is an inhibitor of rhizobial nitrogen-fixation, as plants decrease the number of nodules as nitrate concentrations are increased, while maintaining equivalent levels of total leaf nitrogen (Matsumoto et al., 1977a; McClure and Israel, 1979). This regime produced plants varying in the relative amounts of organic nitrogen, derived from rhizobia, and inorganic nitrogen in leaf tissues.

Methods

Plant growth

Soybean seeds (Williams 82) were rinsed with a 10% sodium hypochlorite solution, then with copious amounts of water, and afterwards placed on wet perlite to germinate for 2 to 3 days. Sprouted seeds were planted in an autoclaved sand/ perlite mixture in (small) pots lined with coffee filters. Inoculated plants were given 1 mL of a *Bradyrhizobium* mixture (HiStick Liquid, Becker Underwood), added to the same hole as the seed. For experiments using "young" plants (15 days after planting (DAP) and younger), all plants (inoculated and non-inoculated) were given a diluted, modified Hoagland's nutrient solution lacking nitrogen (McClure and Israel, 1979). "Older" plants (4-5 weeks after planting) used for experiments were watered with similar nutrient solutions supplemented with varying amounts (0 mM, 10 mM, and 20 mM) of nitrogen as calcium nitrate and ammonium nitrate. All plants were maintained in growth chambers on a 16:8 light:dark cycle.

Insects

Soybean aphids were obtained from natural populations at the Russell E. Larson Agricultural Research Center, Rock Springs, PA and maintained in a growth chamber on soybean plants, which were replaced weekly.

Soybean podworm eggs were obtained from the USDA/ARS facility in Tifton, GA, and larvae were reared on an artificial casein-based diet. For most experiments, second-instar larvae which were showing signs of molting were removed from diet the day before the experiment and placed in empty diet cups with sufficient moisture. By the day of the experiment, all larvae had freshly molted into the third-instar with empty guts.

N-fixation determination in young plants

All plants were grown in autoclaved, N-free sand/perlite medium and watered with a N-free nutrient solution, so that any nitrogen present in the plants originated from either the seed endosperm or rhizobia. Nitrogen levels were measured from

inoculated and non-inoculated plants and nodulation assessed in order to establish a timeframe for when nitrogen-fixation began under our growing conditions. Plants for each time point (n = 4) were dried at 50°C and ground for N analysis by the combustion method (PSU Agricultural Analytical Services Laboratory, University Park, PA, USA). Nitrogen levels for inoculated and non-inoculated plants were compared at each time point by a two-sample t-test with Statistix 8.0 (Analytical Software 2003; used for all analyses).

Helicoverpa zea larvae on young plants

The growth rates of larvae were assessed on plants that had either been damaged previously by *H. zea* larvae (induced), or not damaged (uninduced), for both inoculation treatments. Relative growth rates (RGR) represented the difference in initial and final weights of each individual divided by the number of days of feeding weighted by the initial weight (Farrar et al., 1989). Induced plants were fed on by 4th instar larvae 9 DAP for 24 hours, and plants receiving more than 20% damage to the leaves were used for the growth rate trials. At 12 DAP, a weighed, newly molted 3rd instar larva which had been starved for 24 hours was placed on each plant and the plants enclosed in mesh bags. Larvae fed for 48 hours, then were taken off the plants and reweighed after resting for 2 hours to clear the gut. Samples were discarded if larvae died during the experiment, or if insufficient damage had been inflicted on induced plants, resulting in sample sizes between 9 and 17 larvae per treatment. Growth rates for this experiment required natural log transformation to satisfy the assumptions of normality, and treatment means were compared by one-way ANOVA.

To assess the accumulation of phytohormones during initial feeding, we allowed larvae to feed on young plants. One of the two primary leaves fully expanded on plants 10 DAP was enclosed in a plastic diet cup with a cardboard lid with a slit through which the petiole fit. One larva was added to each cup and allowed to feed for up to 4 hours; on control plants a cup enclosed the test leaf, but no larvae were present. At each of 6 time points (0, 1, 2, 4, 9, and 24 hours), the test leaf was removed from selected plants if the it had received between 25 and 40% damage and was immediately frozen in liquid N. Plants reserved for time points for the 9 and 24

hour timepoints had the plastic diet cups and larvae removed at 4 hours. Also, due to the high degree of variation in hormone levels caused by herbivory, twice as many samples were collected for the podworm-damaged plants (about 6 replicates) compared to the control plants (3-4 replicates). Samples were stored at -80°C until phytohormone analysis. Hormone levels for all experiments were analyzed by one-way analysis of variance at each time point.

Aphids on young plants

The reproductive potential of the soybean aphid was compared on uninfested inoculated and non-inoculated soybean plants. An individual 6-day old aphid was placed on the upper surface of a primary leaf of a young plant (7 DAP). The aphid was allowed to roam freely over the plant and reproduce for 8 days, at which point the plant was examined and all aphids counted. Plants with no aphids were discarded (n = 29). Differences between the number of offspring produced on inoculated and non-inoculated plants were analyzed with a two-sample t-test.

To evaluate aphid fitness and phytohormone induction on heavily infested plants, young plants (7 days after planting) were infested with 50 apterous adult aphids on the upper surface of one of the two primary leaves. Aphids were transferred from colony plants to experimental plants with a small paintbrush. Control plants, which were used for the phytohormone analyses below, were touched with the paintbrush to mimic the disturbance of transferring aphids. Differences between the final aphid populations were analyzed with a two-sample t-test (n = 4).

Individual plants were harvested every other day over 7 days for aphid counts and phytohormone analyses. The timeframe for this experiment was limited to the time between when primary leaves were large enough to support the initial aphid infestation and the point at which the inoculated plants began to receive nitrogen from rhizobia. Each day, infested and non-infested plants of both inoculated and non-inoculated treatments were randomly selected for leaf collection. After counting aphids on infested plants, the majority of the insects were removed by gently rolling the sticky side of a piece of tape over the surface of the initially infested leaf. The leaf was then detached from the plant at the petiole, rolled into a cylinder, and placed in a

labeled, 2.0 mL Eppendorf tube in liquid nitrogen for flash freezing. Control plants were collected in a similar manner, with tape rolled over the leaves to mimic aphid removal. Samples were stored at -80°C until phytohormone processing (n=4).

Older plants

To look at the effects of rhizobia which are actively fixing nitrogen on herbivore feeding and subsequent phytohormone induction, we used 5-week old plants that ranged in their dependence on rhizobia for nitrogen needs. All plants were inoculated as above, and received one of three nutrient solutions containing either high or low amounts of inorganic nitrogen (in the forms of nitrate and ammonium) or no nitrogen. Dried plants were evaluated for percent total nitrogen and the dry weights of nodules per plant were assessed.

Helicoverpa zea larvae on older plants

The ability of *H. zea* larvae to distinguish between plants receiving different ratios of nitrogen from rhizobia was examined. Preference tests were conducted in 6-well plates, in which leaf disks (#8 cork borer ~ 100 mm^2) were pinned to foam covered with moist filter paper. Each arena contained three disks, one from a similar-aged leaf of plants of each nutrient treatment. One larva was allowed to feed in each arena for 5.5 hours. During this time, larvae typically tasted each leaf disk in the arena, and by the end of the time had consumed about half of one of the disks. Leaf disks were either taken from the youngest fully expanded leaf or the next youngest leaf, which was still expanding. At the end of the test, the larvae were removed and the leaf disks scanned digitally for area analysis (SigmaScan Pro 5, SPSS Inc.). Data was analyzed by comparing the amount consumed from each disk as a proportion of total consumption in each trial, with individual larvae used as blocks for analysis of variance (n = 30).

The growth rates of third instar larvae on soybean leaves from plants of the different nutrient treatments were determined in no-choice assays. Leaves from two age classes (first fully expanded leaf and the next youngest leaf) were detached and placed in diet cups containing 1.5% agar to retain moisture. Freshly molted larvae were weighed before being placed in cups, allowed to feed for 48 hours, and then

weighed again. Dead larvae were excluded from the final analyses, resulting in approximately 20 replicates per treatment. RGR was compared among the treatments by one-way ANOVA. The amount of leaf area consumed was determined by digitally scanning the remaining material.

Phytohormone levels in older plants were evaluated over 8 days of *H. zea* feeding (n = 5). Five freshly hatched neonates were enclosed on the first fully expanded leaf by a plastic clip cage with mesh ends which permitted air flow through the leaf. The initial weights of neonates were estimated to be zero, as individuals weighed less than could be measured accurately, given the precision of the balance. Control plants had clip cages placed on the equivalent leaf. After the first day, the clip cage and enclosed larvae were moved to the next younger leaf, and the damaged leaf was frozen for hormone analyses. Cages and larvae were moved again on the third day. On the fourth day, the clip cages were removed and the clipped leaf collected. The plants were then covered with mesh bags, and the final leaf from each plant was harvested on day 8 of the experiment. At this time, remaining larvae from each plant were collected and weighed, and the remaining leaves scanned for leaf area determination. Some control plants were discarded after being fed upon by escaped aphids or *H. zea* larvae. Since variation in hormone levels in control plants was extremely low, controls from each nutrient treatment were combined to make one overall control group for data analyses (n = 5).

Aphids on older plants

The reproductive potential of aphids on older plants was evaluated by placing 15 adult females of similar size on the upper side of the youngest, fully expanded leaf of plants from each nutrient treatment (n = 4). After four days, each plant, including uninfested controls, was covered in a mesh bag to limit wandering of the rapidly growing populations. Individuals on each plant were counted after 8 days and differences among the population means compared with one-way ANOVA.

Phytohormone levels were evaluated in aphid-infested and control plants 8 days after aphids began feeding. Leaves were collected as above for freezing and JA/SA analysis. A high degree of variation in the JA levels of these plants

necessitated a natural log transformation to satisfy the assumptions of normality. As with the aphid experiment on older plants, the control plants in this experiment were combined from each nutrient treatment to make one overall control group for data analyses (n = 5).

Phytohormone analyses

Phytohormones were analyzed from leaf tissue using a method developed by Schmelz et al. (2004) and modified by Tooker and De Moraes (2007). Between 100 and 200 mg of leaf tissue was added to FastPrep tubes containing Zirmil ceramic beads. An acidified 1-propanol extraction solution and phytohormone internal standards (100 ng each of isotope-labeled 2H6-SA and dihydrojasmonic acid) were added to the samples. The samples were homogenized by agitation on a FastPrep for 40 seconds. Methylene chloride was added and each tube shaken a second time, after which they were centrifuged. The organic phase containing phytohormones was transferred to a 4 mL glass tube, from which the methylene chloride evaporated under a stream of N_2. The carboxylic acids were derivatized to methyl esters with 1:9 MeOH:diethyl ether and trimethylsilyldiazomethane (2 M in hexane) for 25 minutes, at which point the derivatization reaction was stopped by addition of 88:12 hexane:acetic acid. The methyl esters were collected by vapor phase extraction by heating the products to 200°C and pulling air (1 LPM) through filters containing an adsorbent polymer (SuperQ) for 2 minutes. The filters were eluted with dichloromethane and the resulting methyl esters were analyzed by GC-MS with isobutene chemical ionization in the selected-ion mode. Hormones were quantified relative to the internal standards and retention times confirmed by standard curves of pure compounds.

Results

N-fixation determination in young plants

Since nitrogen was not supplied to the plants through watering or in the growth media, the point at which plants began to receive nitrogen from rhizobia was measured as the point when the total nitrogen concentration of inoculated plants surpassed that of non-inoculated plants. At 15 DAP and before, the nitrogen levels

were similar between inoculated and non-inoculated plants (Figure 2.1). Also, at these time points, the few small nodules that were present on the roots were white inside, instead of the red-pink color indicative of active nitrogen fixation. By 18 DAP, the inoculated plants had a higher nitrogen concentration than non-inoculated plants, indicating that rhizobia had begun to supply nitrogen to their host plants.

Helicoverpa zea larvae on young plants

Weight gain by podworm larvae was significantly affected by prior damage, with induced plants supporting reduced weight gain in caterpillars compared to undamaged plants (Figure 2.2a; $F_{3, 47} = 30.9$; $p < 0.001$). While there was a general trend within each damage treatment of reduced growth on non-inoculated plants, no significant inoculation effect was observed (Figure 2.2a).

Larval feeding caused JA levels to increase compared to undamaged control plants at 4 hours, which was the peak of JA production (Figure 2.2b; $F_{3, 25} = 14.3$, $p < 0.001$). There was no significant difference in the intensity of the JA burst between damaged inoculated and non-inoculated plants. SA levels were not affected by *H. zea* feeding damage in either inoculated or non-inoculated plants at any of the time points (Figure 2.2c).

Aphids on young plants

Aphids placed singly on non-inoculated or inoculated young plants exhibited no difference in the amount of offspring produced after 8 days (two sample t-test; $t_{56} = 0.12$; $p = 0.904$). When plants received a heavy infestation, the aphid populations grew consistently on both treatments until 7 days after the initial aphids were added, at which point there were significantly more aphids on the non-inoculated than inoculated plants (Figure 2.3a; $t_6 = -.382$; $p = 0.009$).

Aphid infestation caused the salicylic acid levels to increase over the aphid-free controls in both treatments by Day 7 after infestation (Figure 2.3b; $F_{3, 12} = 20.4$; $p < 0.001$). Furthermore, the overall increase in SA levels from aphid feeding was higher in non-inoculated than inoculated plants at this time. Levels of JA were extremely variable, showing no treatment effects until the last time point. On Day 7,

inoculated, aphid-infested plants had higher JA levels than infested non-inoculated plants or control inoculated plants (Figure 2.3c; $F_{3, 12} = 12.71$; $p = 0.004$).

Older plant growth

By varying concentrations of nitrate supplied in fertilizer, we produced plants differing in their dependence on rhizobia to maintain total plant nitrogen levels. Plants receiving no fertilizer nitrogen (NoN) were completely dependent on rhizobia, as reflected by their intensely nodulated roots (Figure 2.4b; $F_{2,15} = 225.3$; $p < 0.001$), while plants receiving the highest nitrogen concentrations (HighN) had very few nodules, yet exhibited similar percent total nitrogen concentration (Figure 2.4a; $F_{2,27} = 1.52$; $p = 0.236$). Plants fertilized with low amounts of nitrogen (LowN) exhibited an intermediate level of rhizobial dependence and nodulation.

Helicoverpa zea on older plants

The ability of *H. zea* to discriminate between leaves of soybean receiving different nitrogen treatments was assessed through three-way choice assays. While the larvae took bites from each type of leaf, there was a distinct preference for HighN over NoN leaves in both leaf ages (Figure 2.5a; $F_{2, 179} = 7.95$; $p = 0.001$). Expanding LowN leaves appeared to be an intermediate choice for the larvae, but older LowN leaves elicited a similar response from larvae as the older NoN leaves (Figure 2.5a).

No-choice tests were used to assess the growth rates of larvae on leaves from plants with different nitrogen treatments. Larvae grew more when fed young leaves from NoN plants than leaves from HighN plants, while larvae fed LowN leaves grew an intermediate amount (Figure 2.5b; $F_{2, 119} = 5.12$; $p = 0.008$). When fed older leaves, these differences diminish and overall growth rate decreases (Figure 2.5b).

As indicated by the no-choice tests, larvae grew better on NoN plants than HighN plants (Figure 2.6a; $F_{2,50} = 3.95$; $p = 0.026$). After four days of larval feeding, JA levels from HighN and LowN plants are significantly higher than levels from NoN and control plants (Figure 2.6b; $F_{3, 16} = 14.9$; $p < 0.001$). While there are no significant differences in JA levels on Day 8 among the damaged treatments, there is a general trend of increased JA as the plants' dependency on rhizobia for N increases, with NoN plants having significantly higher levels of JA than the controls (Figure

2.6b). SA levels also increased in damaged plants as compared to the control, but only on Day 8 (Figure 2.6c; $F_{3, 16} = 8.72$; $p = 0.001$). There is a general trend towards decreased SA as the plants dependency on rhizobia increases, which is supported by the reduced difference between NoN plants and the control as compared to the significant differences between HighN and LowN plants and the control. The amount of larval damage incurred by the plants was similar among the different nutrient treatments, both over the entire plant, which lost between 3 and 4% of total leaf area ($F_{2, 12} = 2.50$; $p = 0.124$) and for the leaf sampled on Day 8 ($F_{2, 12} = 0.89$; $p = 0.436$).

Aphids on older plants

The initial populations of 15 aphids per plant increased approximately 40 fold, with no differences in final aphid numbers between the treatments (Figure 2.7a; $F_{2, 9} = 0.24$; $p = 0.792$). However, nutrient treatment did have a significant effect on hormone levels. There was a general trend of increased SA as the plant's dependency on rhizobia for N increased, with SA levels of NoN plants significantly higher than aphid-free plants (Figure 2.7b; $F_{3, 13} = 5.18$; $p = 0.014$). The reverse trend is shown in JA levels, with decreased levels as the plant's dependency on rhizobia increased (Figure 2.7c; $F_{3, 13} = 4.76$; $p = 0.019$), consistent with the significantly higher JA levels observed in HighN plants over the controls.

Discussion

Plants are central to most community interaction webs, and thus their ability to coordinate responses to many simultaneous interactions is an essential adaptation. Interaction with beneficial organisms, such as mycorrhizal fungi, can have a wide range of effects on plant-herbivore interactions (Bennett and Bever, 2007; Borowicz, 1997; Gange and West, 1994; Goverde et al., 2000; Morris et al., 2007). Nitrogen-fixing bacteria, another class of plant mutualists, have received little attention concerning their effects on plant-herbivore interactions. This study explores the effects of rhizobia on herbivory and plant responses to herbivores, while controlling for differences in plant nitrogen concentration. We found effects of the legume-

rhizobia mutualism on plant-herbivore interactions and that were dependent on both the stage of the mutualism and on the feeding style of the herbivore.

Many aspects of interactions between legume plants and rhizobia are well-characterized due to the ecological and economic importance of this mutualism (Lee and Hirsch, 2006). However, most previous studies have focused on either early or late stages of the interaction. We examined herbivore performance and hormone accumulation during two stages of the mutualism to gain a better understanding of the overall effects of rhizobia on herbivores. Prior to 15 days after planting and inoculation, unfertilized plants had similar total nitrogen concentrations, regardless whether they were inoculated with rhizobia or not. Nitrogen present in the plants at this stage derived from the initial nutrients supplied by the seed endosperm. On inoculated plants, small nodules were visible by 15 DAP, but were white inside, instead of the reddish color associated with the oxygen-binding protein leghemoglobin, which is required for N-fixation to occur (Jones et al., 2007). By 18 DAP, inoculated plants had significantly higher levels of N than non-inoculated plants, confirming active N fixation. During this initial stage—after bacteria-root contact but before active nitrogen-fixation begins and consequently benefits the host—the plant must recognize the rhizobia as beneficial and permit nodule formation. Many studies have reported defense-like responses in host plants at this stage (Estabrook and Senguptagopalan, 1991; Martinez-Abarca et al., 1998; Santos et al., 2001). However, the absence of ensuing pathogen defense mechanisms suggests that the interaction with rhizobia somehow circumvents or suppresses later stages of the response pathways (Baron and Zambryski, 1995; Mithofer, 2002), leaving open the possibility of defense pathway-mediated effects on herbivores.

We used the soybean podworm and soybean aphid as model herbivores due to their distinct feeding mechanisms, in addition to their economic impacts on soybean cultivation. Plants differentially regulate responses to various attackers, with the timing and quantity of hormone accumulations creating a “signal signature” specific to plant-antagonist combinations (De Vos et al., 2005). In resistant plants, these phytohormones then activate a cascade of defense-related genes, resulting in the production of plant compounds that deter feeding, thus reducing further damage. We

did not find a significant difference in *H. zea* growth rates when fed inoculated or non-inoculated young plants, although there was a trend towards reduced growth on non-inoculated plants. There was a significant reduction in overall growth rate in larvae fed plants that had been previously damaged 3 days prior as compared to those fed undamaged plants, confirming previous studies demonstrating inducibility of defenses in soybean, mainly in the form of proteinase inhibitors (Bi et al., 1994; Srinivas et al., 2001). Chewing herbivores such as Lepidopteran larvae typically initiate plant defense responses linked to the lipoxygenase pathway (Walling, 2000). Immediately following initial damage, plants typically accumulate a brief wound-induced "burst" of JA, which is amplified when wounded tissue comes in contact with herbivore-derived elicitors (Baldwin et al., 1997; Schmelz et al., 2003). We found no differences in the accumulated JA burst during initial herbivore feeding. This may be due to leaves from each treatment receiving similar damage amounts as leaves collected for hormone analysis. We have yet to examine the induced accumulation of JA on subsequent days, which would offer more information on the induction of plant defenses during prolonged feeding.

Responses to aphid feeding are less defined and more subtle, presumably due to their stealthy mode of feeding. Aphids use their stylets to maneuver through intercellular spaces until reaching the phloem, piercing only the sieve element cells (Powell et al., 2006). Once a suitable food source has been located, the aphid remains sessile while consuming phloem. In susceptible plants, hormonal responses to aphids are often similar to those initiated in response to pathogen attack, with involvement of the SA pathways and suppression of JA (Smith and Boyko, 2007). However, in resistant plant-aphid combinations, it is the JA pathways that are induced, products of which appear to contribute to decline in aphid fitness. In general, our young soybean plants were very susceptible to soybean aphid attack, with the initial populations increasing between 10- and 17-fold within 7 days. Yet inoculated plants appear to be somewhat more resistant than non-inoculated plants against aphid infestations, as the final populations were higher on the latter, although no differences in reproduction were noticed when individual aphids were placed on healthy plants. SA was induced by aphid feeding in both treatments, but to a higher degree in non-inoculated plants.

On day 7, JA appeared to be somewhat suppressed in the non-inoculated, aphid-infested plants, in contrast to the high level of JA induced in inoculated plants. Studies suggest that aphids and other phloem-feeding insects that activate SA pathways may benefit from the inhibitory crosstalk of SA on JA-mediated defenses (Zarate et al., 2007). Here, the presence of rhizobia may be preventing the SA levels from increasing too much, possibly due to the negative effect of elevated SA on nodulation during the early stages of the mutualism, thus lessening crosstalk inhibition of the JA pathway.

We examined the influence of foliar nutritional differences on plant-herbivore interactions by using older plants with well-established rhizobial relationships. Addition of inorganic nitrogen to the growing media has been shown to reduce the plant's dependency on rhizobia, and thus proportion of ureide N in the leaves, in a dose-dependent manner (Matsumoto et al., 1977a; McClure and Israel, 1979). As we increased fertilizer N, our plants reduced the number of nodules, but maintained a consistent level of total nitrogen. This allowed us to investigate insect feeding and hormone induction of plants over a continuum of rhizobial dependency.

As both N quality and quantity is critical to herbivore development, the changes in N forms in our older plants are likely to influence feeding. The ability of insects to efficiently metabolize nitrogenous compounds such as ureides remains unclear. Wilson and Stinner (1984) concluded *H. zea* larvae can not efficiently utilize ureides as a N source due to decreased pupal weights and longer development times when allantoin was added to artificial diet. However, in our experiments—both the no-choice tests with detached developing leaves, and tests on whole plants—*H. zea* larvae grew best on plants completely dependent on rhizobia for N. The discrepancy between these results may stem from differences in feeding studies conducted with artificial diet versus actual leaves, as the artificial diet may be missing particular phytocompounds necessary for proper utilization of ureides. Three-way choice tests revealed that *H. zea* prefer to feed on plants receiving mostly fertilizer N, a surprising result considering the larvae grew best on the least preferred choice.

In the whole plant experiment, both JA and SA were induced over 8 days of *H. zea* feeding; however, there were differences in the amount of hormones

accumulated among the different nutrient treatments. There was a general trend toward increased JA, and decreased SA, as the plant's dependency on rhizobia increased, although the amount of damage was similar across the treatments. This is notable because we would expect an increase in defense induction and thus decrease in larval growth with higher levels of JA. However, the JA levels in NoN plants were lower than the other two nutrient treatments on day 4, so perhaps this slower induction of JA related pathways was critical to the increased larval growth. It may be necessary to allow *H. zea* to feed on the same plant until adulthood to get a more complete understanding of rhizobial effects on herbivore fitness.

The ureides which are transported in the xylem and accumulate in the shoots are eventually degraded in the leaves and the by-products assimilated. By the time rhizobial N reaches the phloem, no differences in ureide concentration have been noted between nitrate fed or inoculated soybean (Pate et al., 1984). Similar quality of phloem may explain why we saw no differences in aphid populations among plants with different nutrient treatments. As with the larval feeding on older plants, there was a general trend of plants completely dependent on rhizobia to accumulate more phytohormones commonly associated with the attacking herbivore, in this case an increase in SA and decrease in JA with aphid feeding. It is unclear why herbivore feeding elicits a more pronounced response in plants receiving N only from rhizobia, perhaps the greater intensity of nodulation, and thus rhizobia contact, keeps these plants in a slightly more primed state.

Our results showing that the rhizobia-legume mutualism can mediate some plant-herbivore interactions contribute to a growing area of research exploring plant responses to multiple organisms. The shifts we found in effects on herbivores and plant responses to feeding as the relationship between host and rhizobia developed highlight some of the complexity of these interactions. This study, which is the first to compare responses to distinct herbivores during different stages of a plant-microbe mutualism, increases our understanding of the processes between host-mutualist and host-antagonist interactions.

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Figure 2.1.

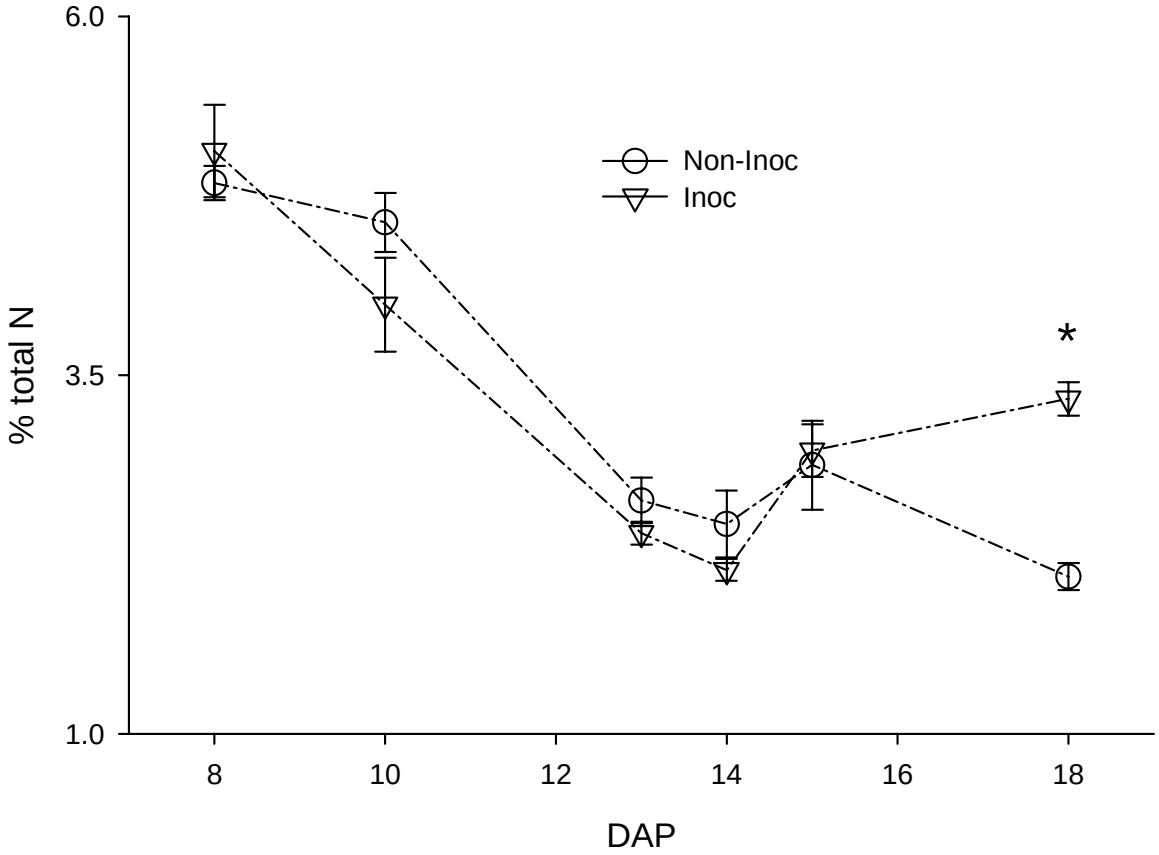

Figure 2.1. Percent total nitrogen (determined by combustion) of young soybean plants grown without nitrogen fertilizer. Inoculated plants were given a rhizobial preparation at the time of planting. Nitrogen levels were similar between inoculated and non-inoculated plants until after 15 days after planting, at which point inoculated plants began to receive N from their associated rhizobia. An asterisk indicates significant differences between the percent N of inoculated and non-inoculated plants on that day ($p < 0.05$).

Figure 2.2

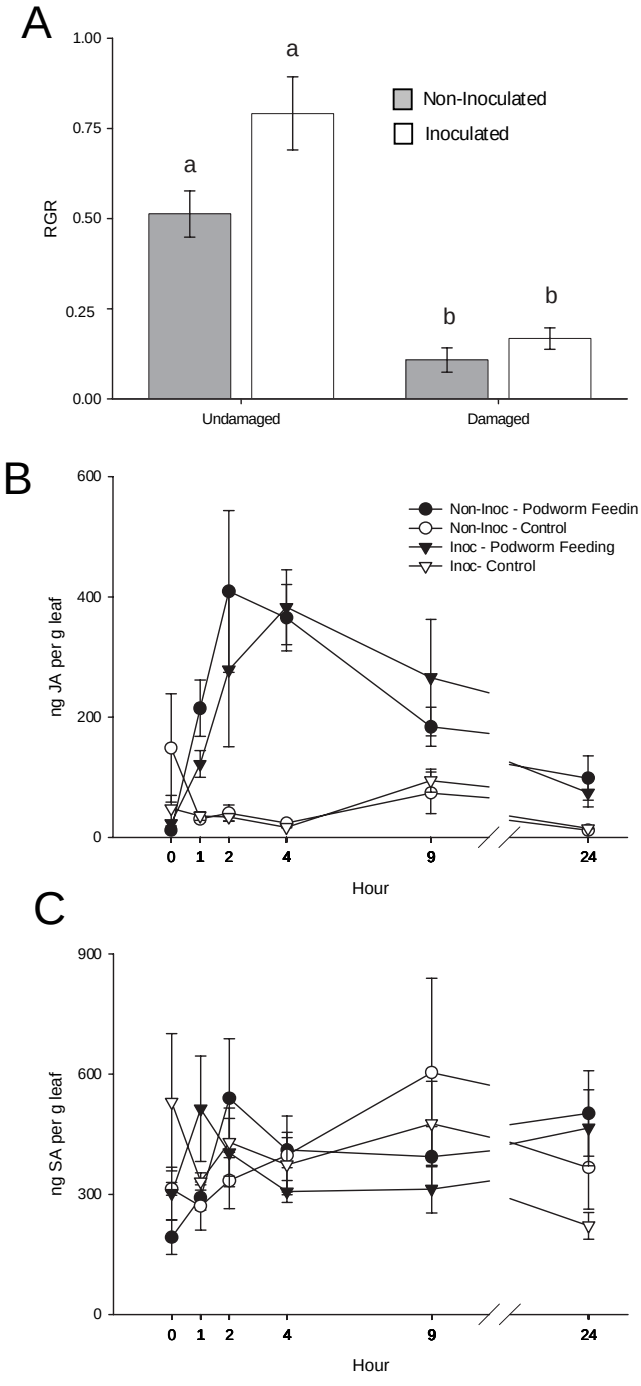

Figure 2.2. Effects of rhizobial inoculation on soybean podworm feeding on young plants (before active nitrogen fixation) and plant responses. a) Relative growth rate of larvae feeding over 48 hours on inoculated and non-inoculated plants that had either been undamaged or previously damaged by other larvae 72 hours prior. Different letters above the bars indicate significant differences among the means ($p < 0.05$). b) Accumulation of JA in primary leaves of plants inoculated (triangles) or non-inoculated (circles), with H. zea feeding (dark shapes) or undamaged control (open shapes). c) Accumulation of SA. Results shown as means ± S.E.

Figure 2.3

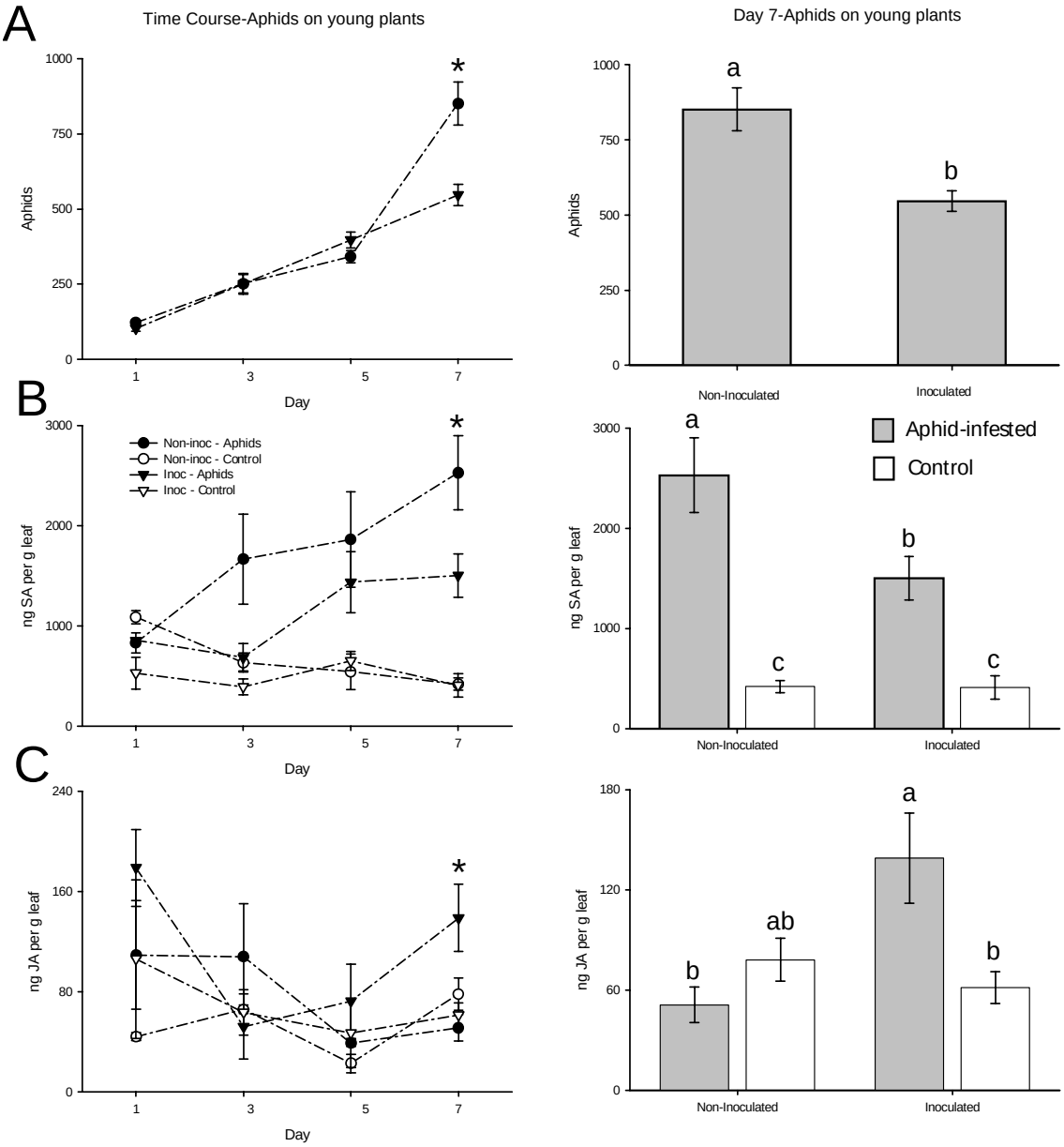

Figure 2.3. Effects of rhizobia inoculation on soybean aphid feeding on young plants (before active nitrogen fixation) and plant responses during the course of experiment and on Day 7. a) Increase of aphids from an initial population of 50 adult females in inoculated (circles) and non-inoculated (triangles) plants. b) Accumulation of SA in primary leaf of plants infested with aphids (closed shapes) and aphid-free controls (open shapes) through the course of the week and on Day 7. c) Accumulation of JA in primary leaf of plants infested with aphids and aphid-free controls. For both time courses of SA and JA, an asterisk indicates significant differences between the inoculated and non-inoculated aphid infested plants on that day ($p < 0.05$). For all bar graphs of Day 7, different letters above the bars indicate significant differences among the means for that comparison ($p < 0.05$). Results shown as means ± S.E.

Figure 2.4

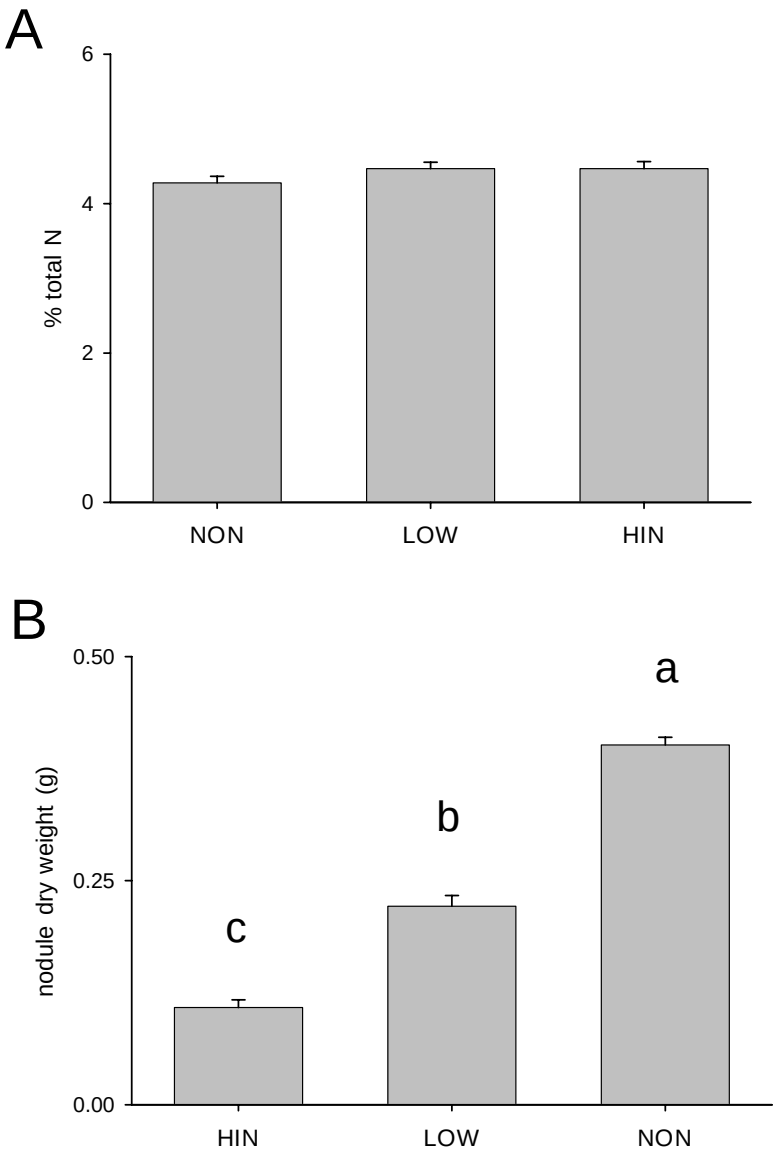

Figure 2.4. Characteristics of 5-week old plants inoculated with rhizobia, but given different levels of nitrogen fertilizer. a) Percent total nitrogen; b) Nodule dry weights. HIN = High N fertilizer, LOW = Low N fertilizer, and NON = fertilizer with no N. Different letters above the bars indicate significant differences among the means ($p <$ 0.05). Results shown as means ± S.E.

Figure 2.5

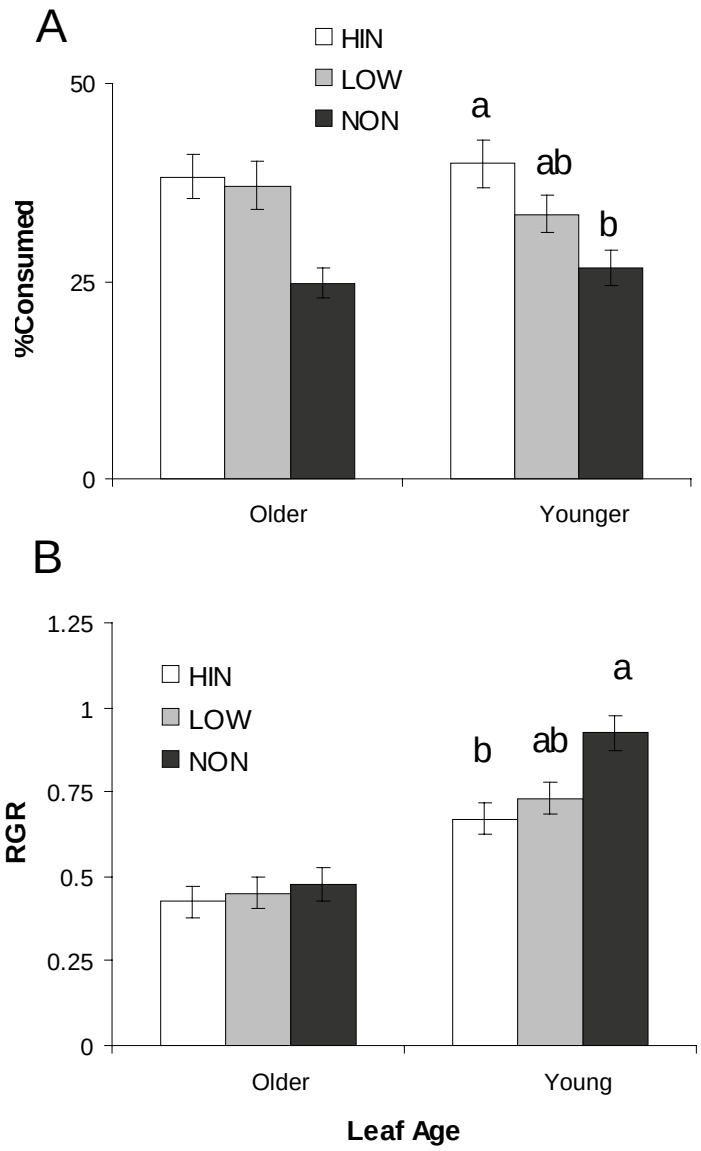

Figure 2.5. Effects of dependence on rhizobia N in 5-week old soybean on the behavior and growth of H. zea larvae. A) In a 3-way choice test, larvae prefer to feed on leaves from plants receiving high amounts of nitrogen fertilizer (HIN - white bars) over plants relying solely on rhizobia (NON - black bars), while plants receiving low-nitrogen fertilizer (LOW - grey bars) represented an intermediate choice. B) In no-choice tests with expanding leaves (B), larvae grew better on NON plants than HIN plants, with LOW plants supporting an intermediate amount of growth. These differences disappear with fully expanded leaves (A). Different letters above the bars indicate significant differences among the means ($p < 0.05$). All results are shown as means ± SE.

Figure 2-6

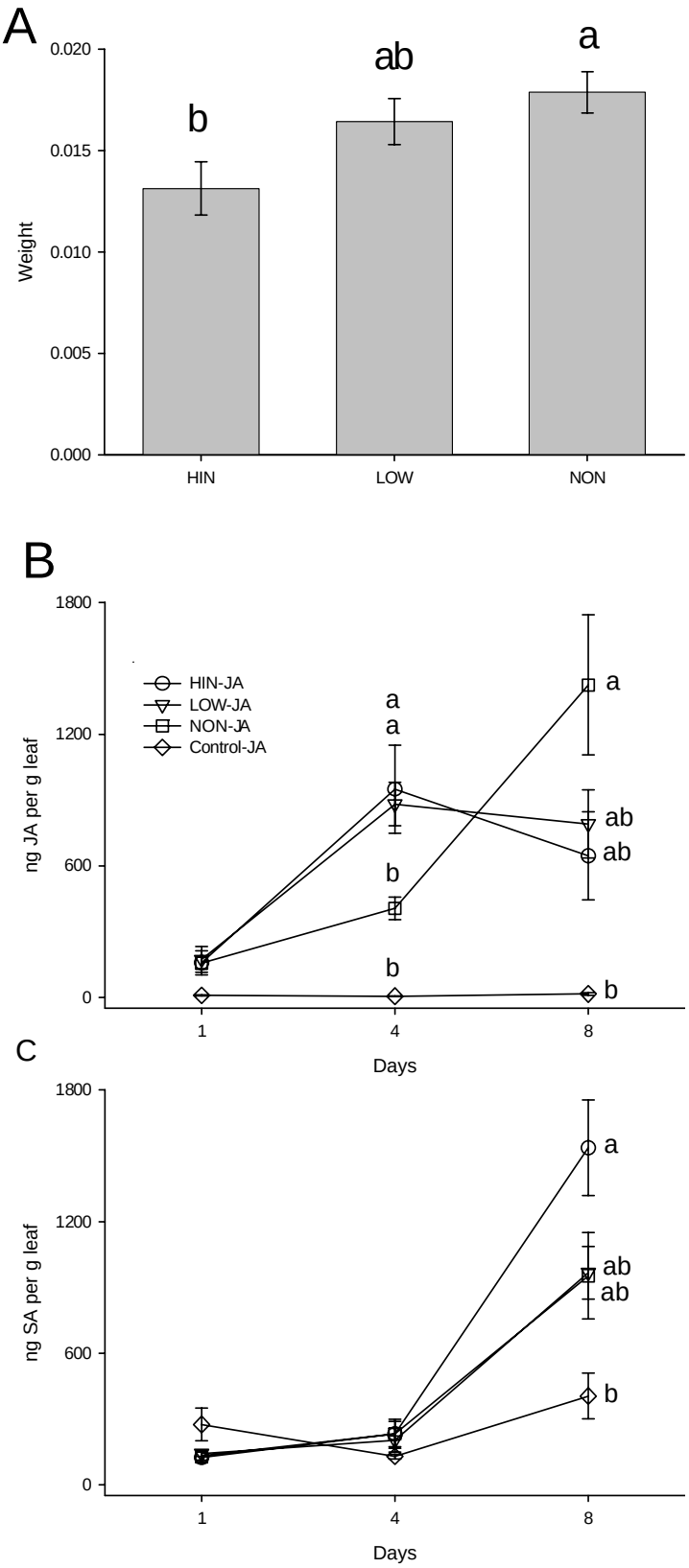

Figure 2.6. Effects of dependence on rhizobia N in 5-week old soybean on growth rate of *H. zea* and plant responses. A) Final weight of *H. zea* larvae (which began as neonates) after feeding on soybean plants for 8 days. Different letters above the bars indicate significant differences among the means ($p < 0.05$). B) Accumulation of JA during the course of *H. zea* feeding. C) Accumulation of JA during the course of *H. zea* feeding. HIN = High N fertilizer received, LOW = Low N fertilizer received, and NON = fertilizer with no N received. Results shown as means ± S.E.

Figure 2-7

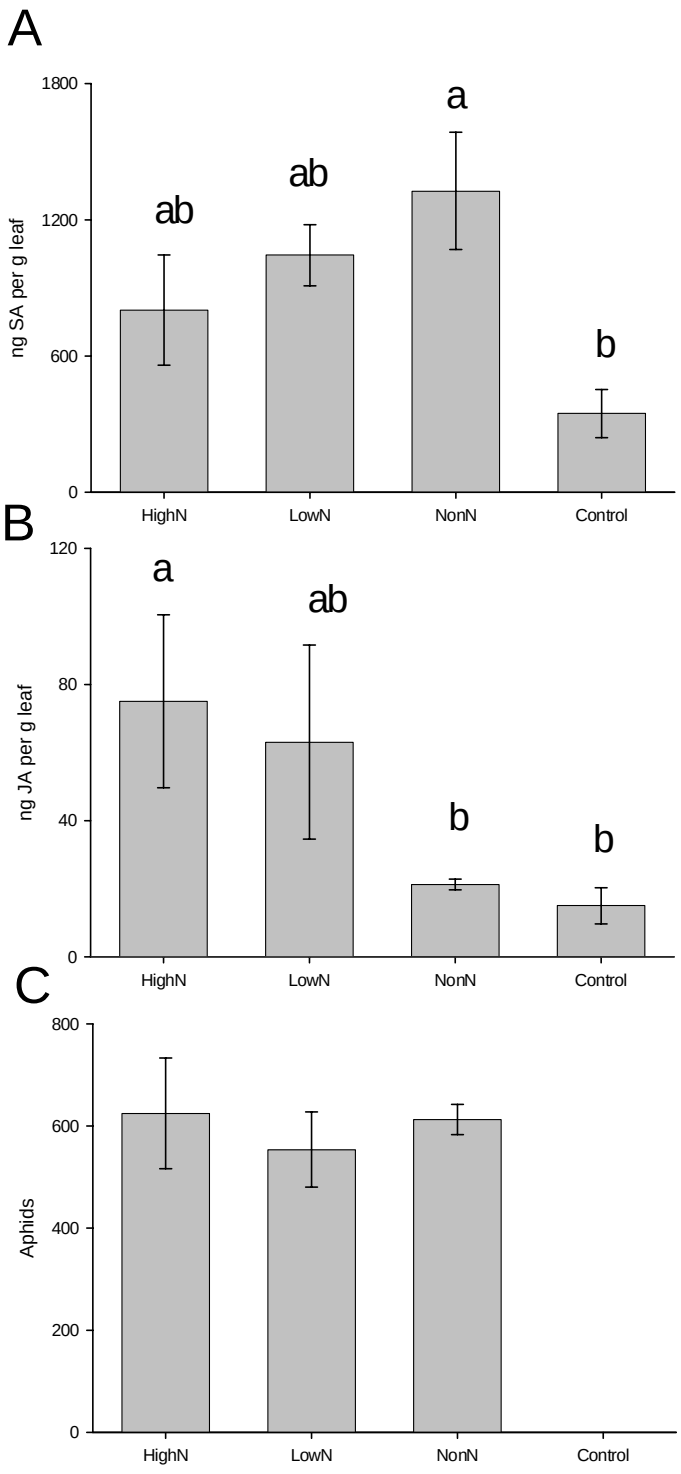

Figure 2.7. Effects of dependence on rhizobia N in 5-week old soybean on reproductive rate of soybean aphids and plant responses. A) Final populations of soybean aphids after 8 days from transferring initial population of 50 aphids per plant. B) Accumulation of SA after 8 days of aphid feeding. C) Accumulation of JA after 8 days of aphid feeding. HIN = High N fertilizer received, LOW = Low N fertilizer received, and NON = fertilizer with no N received. Different letters above the bars indicate significant differences among the means ($p < 0.05$). Results shown as means ± S.E.

Chapter 3

Plant-rhizobia mutualism influences aphid abundance on soybean

Abstract

The mutualism between legumes and nitrogen-fixing bacteria (rhizobia) is a key feature of many ecological and agricultural systems, yet little is known about how this relationship affects plant-herbivore interactions. We investigated the effects of different rhizobial and nitrogen sources on the abundance of a specialized legume herbivore. Abundance of the soybean aphid (*Aphis glycines*) was measured on soybeans that were either (1) treated with a commercial rhizobial inoculant, (2) associating solely with indigenous rhizobia, or (3) given nitrogen fertilizer. Several plant traits influencing fitness and vulnerability to herbivore feeding were assessed. Rhizobia isolates cultured from field plants were sequenced to determine genetic relatedness between commercial rhizobia and indigenous strains. Plants associating with indigenous rhizobia strains supported reduced aphid populations compared to plants inoculated with a commercial rhizobial preparation or given nitrogen fertilizer. Plant size, total leaf nitrogen content, and the general intensity of nodulation were similar among rhizobia-associated treatments and so cannot explain the observed differences in aphid abundance. Genetic analyses confirmed that the commercial rhizobia strains were distinct from indigenous strains. These results suggest that plant-rhizobia interactions can influence plant resistance to herbivores and that particular rhizobia strains can confer greater resistance to their mutualist partners than others.

Introduction

The relationship between legume plants and nitrogen-fixing rhizobia is one of the best known examples of biological mutualism and plays an important role in many natural and agricultural ecosystems. Inoculation of a legume plant by a compatible rhizobia strain induces physical and chemical changes in the host plant that are strongly influenced by the genotype of the bacterial partner. Different strains of rhizobia can have different effects on features of the mutualism including nitrogen-fixation efficiency, leaf chemistry, and plant fitness (Burdon et al., 1999; Fuhrmann, 1990; Lafavre and Eaglesham, 1986; Parker, 1995; van Berkum et al., 1985). These strain-specific effects may, in turn, be expected to affect the plant's interactions with other organisms, such as herbivores (Schoonhoven et al., 2005). Studies exploring the effects of mycorrhizal fungi—another class of microbial mutualists—on plant-herbivore interactions have found both positive and negative effects dependent on type of herbivore and the species composition of fungi interacting with the host plant (Bennett and Bever, 2007; Borowicz, 1997; Gange and West, 1994; Goverde et al., 2000). However, few studies have explored the effects of rhizobial inoculation on plant-herbivore interactions, and the effects of genetic variation among rhizobia strains on host plant defenses have not previously been documented. Understanding the effects of variation among rhizobia strains on interactions between plants and other organisms is potentially important, as legume plants often encounter a variety of rhizobial strains in the surrounding soil, including both naturally occurring bacteria and strains introduced by agricultural inoculations.

Many factors influence the genetic composition of rhizobia populations in the soil over time. Soil characteristics, such as nutrient levels, soil particle size, pH, and temperature can constrain or enhance particular plant-rhizobia associations (Denton et al., 2002; Streeter, 1994; Taylor et al., 1991). Rhizobia populations also become adapted to local environmental conditions and plant genotypes, with resulting effects on mutualisms. For instance, cross inoculation experiments demonstrated that legumes inoculated with rhizobia from their native site had higher fitness levels than those inoculated with rhizobia from a different population of the same plant (Parker, 1995; Thrall et al., 2007). The diversity of rhizobia may also be increased through

the horizontal exchange of genetic material, such as the transfer of symbiotic genes from introduced rhizobia to native, non-symbiotic bacteria in the field (Barcellos et al., 2007; Spratt and Maiden, 1999; Sullivan et al., 1995).

Human intervention is another factor influencing rhizobial diversity. During the long history of legume cultivation, humans have shuffled rhizobia strains throughout many regions of the world. As a result, diverse populations of soybean-compatible rhizobia are naturally present in many agricultural soils (Amarger, 2001; Ferreira and Hungria, 2002). These "indigenous" strains are now subject to the same influences on genetic composition that occur in undisturbed settings, such as adaptation to local conditions and horizontal gene transfer with native microbes. Host plant interactions with these indigenous rhizobia can be quite different than interactions with strains from current commercial inoculants which have been selected for high yield. For example, indigenous rhizobia are often more competitive for infection sites on host roots, but can be less efficient nitrogen-fixers, than commercial rhizobia strains selected for agronomically-desired traits (Streeter, 1994).

Cultivated legumes, such as soybeans (*Glycine max*) encounter a diverse rhizobia community in the soil, including rhizobia strains from commercial inoculants supplied at the time of planting and those from indigenous populations. Our research investigated the effects of these different rhizobia sources on plant resistance to aphid herbivory. Since its discovery in the US in 2000, the soybean aphid (*Aphis glycines*),, has spread throughout most of the soybean growing regions of the US and Canada and is now considered the most significant insect threat to soybean production in North America (Ragsdale et al., 2007). We monitored the abundance of this phloem-feeding specialist on soybeans that were either inoculated with a commercial rhizobial preparation, given nitrogen fertilizers to suppress nodulation, or allowed to associate with indigenous rhizobia populations. We also scored plant attributes such as total nitrogen level, leaf dry weight, nodulation intensity, and seed production throughout the season. Rhizobia strains associating with soybean in the field were isolated and genotyped to determine their phylogentic relationships to one another and to known strains that have previously been sequenced.

Materials and Methods

Field Design

To determine the effect of inoculation and rhizobial source on aphid abundance, soybean plants (Garst 2918, Garst Seed Company, IA, USA) were grown from seed at the Russell E. Larson Agricultural Research farm (Rock Springs, PA, USA) and subjected to one of three treatments. In the first, seeds were coated at planting with a peat-based commercial rhizobial (CR) inoculant (HiStick 2, Becker-Underwood, IA, USA) according to the recommended labeled amount and received no fertilizer throughout the season. The other two treatments employed seeds from the same batch planted without rhizobial inoculant. One of these received supplemental nitrogen fertilizer (NF) to suppress nodulation, while the other received no fertilizer, facilitating association with indigenous rhizobia (IR).

Soybeans were planted on June 8, 2005 in a randomized complete block design with four replicates in a field that had not been planted with soybeans for at least ten years. Plots were 9 m by 9.8 m with 76 cm row spacing, allowing 12 rows for each plot at a rate of 350,000 seeds per hectare. Fertilizer was applied to the NF treatment by broadcasting 3.6 kg of ammonium nitrate per plot by hand, resulting in 145.6 kg ha^{-1} of nitrogen at 9 and 34 days after planting (DAP), matching agronomic recommendations for corn (Beegle, 2007).

Weekly counts of soybean aphids were conducted from the time reproducing colonies were found on plants (July 22, 2005 – 44 DAP) until the soybean leaves began to senesce (September 1, 2005 – 85 DAP). Sampling occurred in a 3.0 m by 3.7 m subplot in the center of each main plot (in order to minimize interaction with other treatments) within which three new plants were randomly selected each week for aphid counts (12 total plants per treatment per time point). The total number of aphids from each plant was recorded.

Each week, two plants from each plot (4 plots per treatment) were carefully removed from the ground (along with as much of the root structure and surrounding soil as possible) for nodule and plant-nitrogen evaluations. The intensity of

nodulation was visually estimated weekly and quantified (nodule weight and number) as the aphid populations peaked (59 to 62 DAP). Leaf dry weight was also quantified from plants on this date, after drying the aboveground portion at 60°C for 72 hours. Total nitrogen of leaves (one sample per plot from two combined plants) was determined by the PSU Agricultural Analytical Services Laboratory (University Park, PA, USA) using the combustion method. Soybean yield was evaluated, after most leaves had senesced and the seeds had dried on the plant, by determining the number and dry weight of seeds per plant (24 total plants per treatment).

Rhizobial genotyping

In order to confirm that distinct rhizobia strains had inoculated the IR and CR plants and to determine the phylogentic placement of these bacteria, a random sample of root nodules was harvested from field plants to culture individual isolates, from which a region of ribosomal RNA was sequenced. Nodules sampled from the roots of CR and IR plants (8 per treatment) were surfaced sterilized and crushed in sterile water following the methods of Vincent (1970). The crushed material was streaked to isolation in Petri dishes containing yeast-mannitol agar and incubated at 28°C until isolated colonies formed, at which point plates were stored at 4°C until used. Samples successfully cultured without contamination were used for the final sequencing, resulting in a total of 6 and 7 isolates from CR and IR plants, respectively. Reference strains (USDA 110, 123, 94, and 76) were obtained from the USDA-ARS National Rhizobium Germplasm Collection (Beltsville, MD, USA) and grown under the same conditions.

The internal transcribed spacer (ITS) region between 16S and 23S rRNA was sequenced to establish the genotypic relationships between the field isolates, reference strains, and previously sequenced strains of *Bradyrhizobium*. One isolated colony was picked from each plate (representing one nodule) and added to a mixture of Promega GoTaq Green master mix and the forward and reverse primers developed by Kwon et al. (2005). The primers were constructed by the PSU Nucleic Acid Facility (University Park, PA, USA). The ITS region was amplified by polymerase chain reaction using a thermal cycler (2720, Applied BioSystems, CA, USA) with the following settings: 94°C for 12 min (for breakdown of bacterial wall); 30 cycles of

94°C for 1 min, 58°C for 1 min, and 72°C for 2min; 72°C for 10 min (extension step); then lowered to 4°C. The size and quantity of the PCR products were assessed via gel electrophoresis (1.5% agarose in Tris-acetate EDTA buffer with SYBR Safe DNA Gel stain [Invitrogen, Ltd., Paisley, UK]) at a constant 125 volts for 30 minutes. Visualization under ultraviolet light verified that all field isolates and reference strains produced one band approximately 1000 kDa in size when restricted with the primers. The amplification products were then cleaned with ExoSAP-It (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and heated in a thermocycler (37°C for 20 min, then 80°C for 15 min, followed by 4°C).

Purified ITS regions were sequenced by the PSU Nucleic Acid Facility with an ABI Hitachi 3730XL DNA Analyzer using the same forward and reverse primers described above. The resulting ITS sequences of field isolates and sequences of other *Bradyrhizobium* strains from GenBank (accessions from van Berkum and Fuhrmann, 2000) were then aligned using MEGA 3.1 (Kumar et al., 2004). Maximum parsimony and neighbor-joining phylogentic trees with bootstraps were constructed with the same software.

Statistical analyses

Aphid population trends over the sampling period were examined with analysis of variance via Proc Mixed of SAS version 9.1 (SAS Institute, Inc., NC, USA), using block, treatment, and date as variables, with plot as a repeated measure. The last the sampling date (85 DAP) was excluded from analyses because leaves had begun to senesce and total leaf nitrogen from NF plants had dropped to the equivalent of other treatments by this time point. Number of aphids per plant was log-transformed to satisfy the assumptions of analysis of variance. Treatment means were compared using orthogonal contrasts. Total leaf nitrogen was analyzed separately with the same model. Aphid abundance, nitrogen content, nodule dry weight and plant dry weight for the single-day-treatment comparison (prior to aphid peak) and yield at the end of the season were analyzed by analysis of variance with a randomized complete block design using the Tukey's HSD post-hoc test for all pair-wise comparisons (Statistix version 8.0, Analytical Software, FL, USA). Number and

dry weight of seeds per plant were log-transformed to satisfy the assumptions of analysis of variance.

Results

Populations of soybean aphids in all treatments fluctuated in a manner typical of this species in Pennsylvania and similar to the seasonal dynamics of other aphid species (Aponte and Calvin, 2004; Karley et al., 2004). Starting as small colonies distributed throughout the field, aphid populations spread quickly, with numbers per plant growing exponentially until about mid-summer (65 DAP), after which the populations declined rapidly (Figure 3.1). Although these general population trends were similar for each treatment, the levels of infestation were significantly different, with aphid numbers lower on IR plants than on CR and NF plants throughout the season (treatment*time: $F_{2, 206} = 4.07$; $P = 0.019$; Figure 3.1). Shortly before the mid-summer aphid population peak (59 DAP), the average population on IR plants was nearly half that of the other treatments ($F_{2, 30} = 7.02$; $P = 0.003$; Figure 3.2a).

Plants from the two unfertilized treatments (CR and IR) associated with rhizobia throughout the season, as indicated by the presence of nodules on the roots. Nodulation of IR plants occurred more slowly at the beginning of the season, but reached levels similar to CR plants by the second week of aphid sampling (data not shown). There was no significant difference in nodule dry weight by the sampling date prior to the aphid peak for rhizobia-associated treatments; however, the application of a high rate of nitrogen fertilizer to the NF plants effectively suppressed nodulation ($F_{2, 18} = 41.82$; $P < 0.001$; Figure 3.2d). Total leaf nitrogen was similar for nodulated plants (CR and IR) throughout the aphid season (treatment*time: $F_{2, 64} = 15.57$; $P < 0.001$; Figure 3.3) and 59 DAP ($F_{2, 6} = 8.38$; $P = 0.018$; Figure 3.2b), but was much higher in NF plants until the end of the season, at which point the levels for all three treatments converged. There were no significant differences among the treatments in plant dry weight shortly before the aphid populations peaked ($F_{2, 18} = 1.14$; $P = 0.343$; Figure 3.2c). Yield between the two rhizobia-associated treatments (IR and CR) was not significantly different, but yield for NF plants was nearly

significantly higher than that for IR plants (seed mass: $F_{2, 65} = 5.68$, $p = 0.0053$; number of seeds: $F_{2, 66} = 3.13$; $p = 0.0594$; Figure 3. 4).

To better understand the phylogenetic relationships among the rhizobia strains infecting the CR and IR treatments and previously identified strains, the ITS region was sequenced and aligned with other known sequences. The resulting neighbor-joining and maximum parsimony phylogeny estimations produced similar results, with the latter providing better resolution (Figure 3.5). All field isolates appeared to be within the species *Bradyrhizobium japonicum* and clustered within one of three distinct groupings. Most isolates from the CR plants were clustered in a group which appeared to be most closely related to USDA 6. Isolates from the naturally inoculated plants fell into two separate groupings which were closely related to either USDA 122 or USDA 123 and USDA 127, all of which are frequently encountered in US soils. One isolate from a CR plant (CR-4) grouped with the IR isolates and USDA 122, suggesting that the commercially inoculated plants may have interacted to some degree with the indigenous rhizobia.

Discussion

In our study, plants inoculated by indigenous strains of rhizobia hosted reduced populations of a specialized aphid herbivore compared to plants inoculated by a commercial rhizobial preparation or given a nitrogen fertilizer. Plant size, total leaf nitrogen content, and general intensity of nodulation were similar in both rhizobia-associated treatments (IR and CR), suggesting that these factors do not explain the observed differences in aphid abundance. Genetic analyses confirmed that the commercial rhizobia strains were distinct from indigenous strains. These results suggest that the plant-rhizobia interaction can influence plant resistance to herbivores and that particular rhizobia strains can confer greater resistance to their mutualist partners than others. The greater herbivore resistance conferred by indigenous rhizobia strains in our experiments may reflect adaptation to local ecological conditions. Alternatively, commercial strains may have lost traits that confer herbivore-resistance during the course of artificial selection favoring other traits.

Rhizobia genotype has been shown to significantly influence plant size (Burdon et al., 1999; Laguerre et al., 2007; Thrall et al., 2007; Wilkinson et al., 1996), which could affect herbivore abundance per plant. If all other traits are equal, we would expect larger plants to support more herbivores by supplying increased resources and more surface area on which transient insects can land. The efficacy of natural enemies may also be affected by plant size, and size differences induced by different species of mycorrhizal fungi appeared to affect parasitism rates in *Leucanthemum vulgare* (Gange et al., 2003). However, we found no difference in overall plant size between the soybeans associating with different rhizobia strains in our study.

Differential effects on the nutritional quality of inoculated plants is another possible mechanism that could potentially explain differences in aphid abundance on plants associating with different rhizobial genotypes. In our study, total plant nitrogen levels were the same for soybeans regardless of rhizobial source. Herbivore performance could also be influenced by differing ratios of nitrogen forms present in plant tissues, which are influenced by rhizobial genotype (Mattson, 1980). Inoculated legumes transport nitrogen to the shoots in the form of amides or ureides, as opposed to the inorganic forms, such as nitrate, that are transported in non-inoculated legumes. Allantoin, a type of ureide found in soybeans, has been shown to reduce the performance of lepidopteran larvae when added to artificial diet (Wilson and Stinner, 1984). While increased levels of ureides could negatively affect herbivores in a strain-dependent manner, this is unlikely to be the basis for the different aphid population levels observed in our study because our rhizobia-associated plants had similar total nitrogen contents and nodule intensity, both of which are strongly correlated to ureide concentration in soybeans (van Berkum et al., 1985). However, we cannot rule out the possibility that variation in rhizobia strains influences some other aspect of plant nutrition, such as the amino acid content of the phloem.

Another possible explanation for the observed differences in aphid performance involves differential activation of defense-related pathways in the plant by different rhizobia strains. Some phytohormones that have well-established roles in the activation of defenses against herbivores, such as jasmonic acid (JA) and salicylic

acid (SA) (Walling, 2000), also have putative roles in rhizobia-plant interactions, as demonstrated by the nodule suppression observed in response to increased levels of JA (Sun et al., 2006) and SA (Sato et al., 2002) and the inhibition of SA production during the initial stages of infection (Martinez-Abarca et al., 1998). This sharing of hormonal pathways could augment or suppress plant responses to herbivores in the presence of rhizobia. While it is unknown whether levels of these phytohormones are influenced by the genotype of the rhizobial partner, such strain-specific effects could lead to differential production of defensive compounds in the host plant.

Differential translocation of rhizobia-produced compounds throughout the plant is still another possible explanation for the observed strain-specific differences in plant resistence. For example, some strains of rhizobia produce an ethylene-inhibiting enol-ether amino acid, referred to as rhizobitoxine (RT), which can cause chlorosis and fitness consequences in some legumes (Owens and Wright, 1965; Parker and Peters, 2001; Teaney and Fuhrmann, 1992; Vasilas and Fuhrmann, 1993). Ethylene induces a cascade of defense-related events, suggesting that the inhibition of this hormone by RT could influence plant-herbivore interactions, although the effects of RT on herbivores have not been explored (Harfouche et al., 2006; Kahl et al., 2000; Stotz et al., 2000).

Whatever the mechanism, our results suggest that benefits to the plant other than nitrogen fixation may play an important role in the ecology and evolution of the mutualism. Nitrogen-fixing efficiency is strongly influenced by rhizobia strain, and ranges from very high (allowing the host plant to meet most of its nitrogen needs through the rhizobia) to very low (with little or no nitrogen being fixed). Kiers et al. (2003) demonstrated that host plants can regulate nodulation according to how much nitrogen is fixed by imposing sanctions on individual nodules that are not sufficiently beneficial to the plant. Yet, less efficient nitrogen fixers are nonetheless abundant in many soils, and frequently outcompete more efficient strains (Fuhrmann, 1990; Streeter, 1994; Thies et al., 1991). Thus, it is possible that traits other than nitrogen fixing ability may influence the plant's degree of association with rhizobia. Enhanced protection against herbivores could be as important to plant fitness as efficient nitrogen acquisition, especially under intense herbivory. Herbivory may also

impose negative fitness consequences on nodulated rhizobia directly, as plants often abort nodules following aboveground damage (Layton and Boethel, 1987; Sirur and Barlow, 1984).

We examined the phylogenetic relationships of *Bradyrhizobium* strains by sequence comparisons of the ITS region between the 16S and 23S rRNA, as this region more accurately reflects divergence within the genus than the 16S gene commonly used to study bacterial diversity (Kwon et al., 2005; van Berkum and Fuhrmann, 2000; Willems et al., 2001). We determined that the isolates cultured from our naturally inoculated plants to be most closely related to USDA 122 or 123 and USDA 127, each of which has serologically related strains commonly encountered in US soils (Fuhrmann, 1989; Keyser et al., 1984; Weber et al., 1989). USDA 122 is considered a superior nitrogen-fixer due to its hydrogenase uptake system (Albrecht et al., 1979), while strains related to USDA 123 and USDA 127 are considered to be less efficient nitrogen fixers, but extremely competitive nodulators (Fuhrmann, 1990; Streeter, 1994). The majority of isolates from the commercially inoculated plants clustered together in a group most closely related to USDA 6, which is the type strain of *B. japonicum* originally isolated from Japan. Although one isolate of the CR group clustered with the USDA 122 group, we believe that most CR plants interacted with strains distinct from the IR treatment, suggesting that the commercial inoculant we used was a good competitor against the indigenous strains. Some of the observed host plant effects within the IR treatment could also be a result of multiple rhizobia strains inoculating a single plant. In another microbe-plant mutualism, Gange et al. (2003) found that inoculations by single species and combinations of different species of mycorrhizal fungi caused variations in plant height, herbivory, and parasitism.

Nitrogen fertilization effectively blocked nodulation by rhizobia in our study, but significantly increased total leaf nitrogen content over that of the inoculated treatments.

Studies investigating the correlation between nitrogen levels and aphid fitness demonstrate a wide range of positive and negative effects (Jansson and Ekbom, 2002; Mattson, 1980). Since nitrogen levels were higher in the fertilized plants, we must

also consider this as a possible explanation for increased aphid populations compared to those feeding on rhizobia-associated (IR and CR) plants.

In summary, our study suggests that different sources of rhizobia can confer different levels of protection against herbivores to their host plant. Specifically, we found that plants inoculated with strains indigenous to our study site supported fewer aphids than plants associating with a commercial rhizobial mixture. This difference might reflect adaptation to local soil conditions by the indigenous rhizobia or compromised defense attributes of commercial strains that have been selected for other traits. However, interactions with additional commercial rhizobial preparations will need to be investigated in a similar manner before we can draw general conclusions about the effects of inoculation by artificially selected versus natural strains. Further research is also needed to determine the mechanisms underlying the observed differences in host plant resistance and whether they extend to other herbivores with different feeding modes. The demonstration that different rhizobia strains can differentially impact plant resistance to herbivory suggests that this and other previously overlooked features of the plant-rhizobia mutualism should be considered when selecting strains for use in agricultural inoculations.

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Figure 3.1

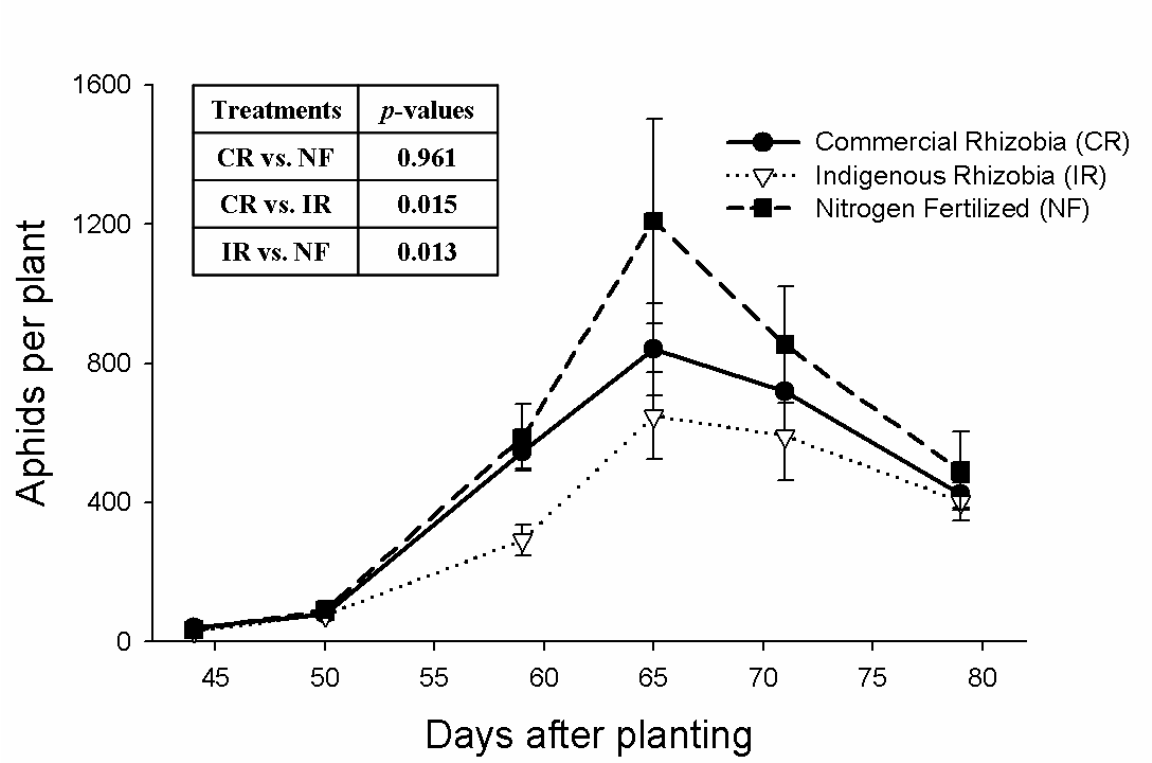

Figure 3.1. Abundances of soybean aphid throughout the field season on soybean plants either treated with a commercial rhizobial inoculant at the time of planting (circles), associating solely with indigenous rhizobia (triangles), or given a nitrogen fertilizer to suppress nodulation (squares). Log transformed data were analyzed with block, date, and treatment as variables with repeated measures, with means of the untransformed data ± 1 standard error shown. Results of treatment comparisons (orthogonal contrasts) are shown in the inset table.

Figure 3.2

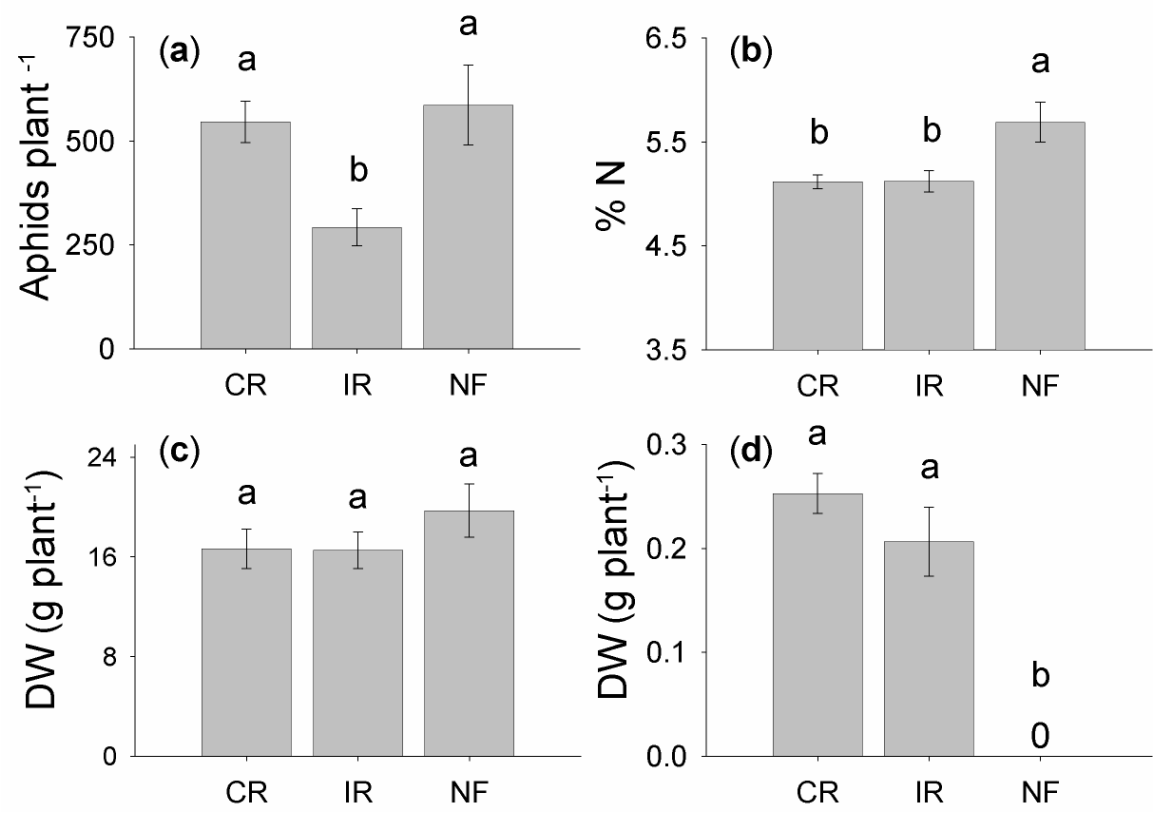

Figure 3.2. Aphid abundance (a), percent leaf nitrogen content (b), shoot dry weight (c), and nodule dry weight (d) of soybean plants shortly before the aphid population peak at the middle of the summer. Data shown as means ± 1 standard error. Different letters within each graph represent significantly different treatment means for that characteristic. CR, commercial inoculation of seeds at planting; IR, association with indigenous rhizobia; NF, nitrogen fertilized.

Figure 3.3

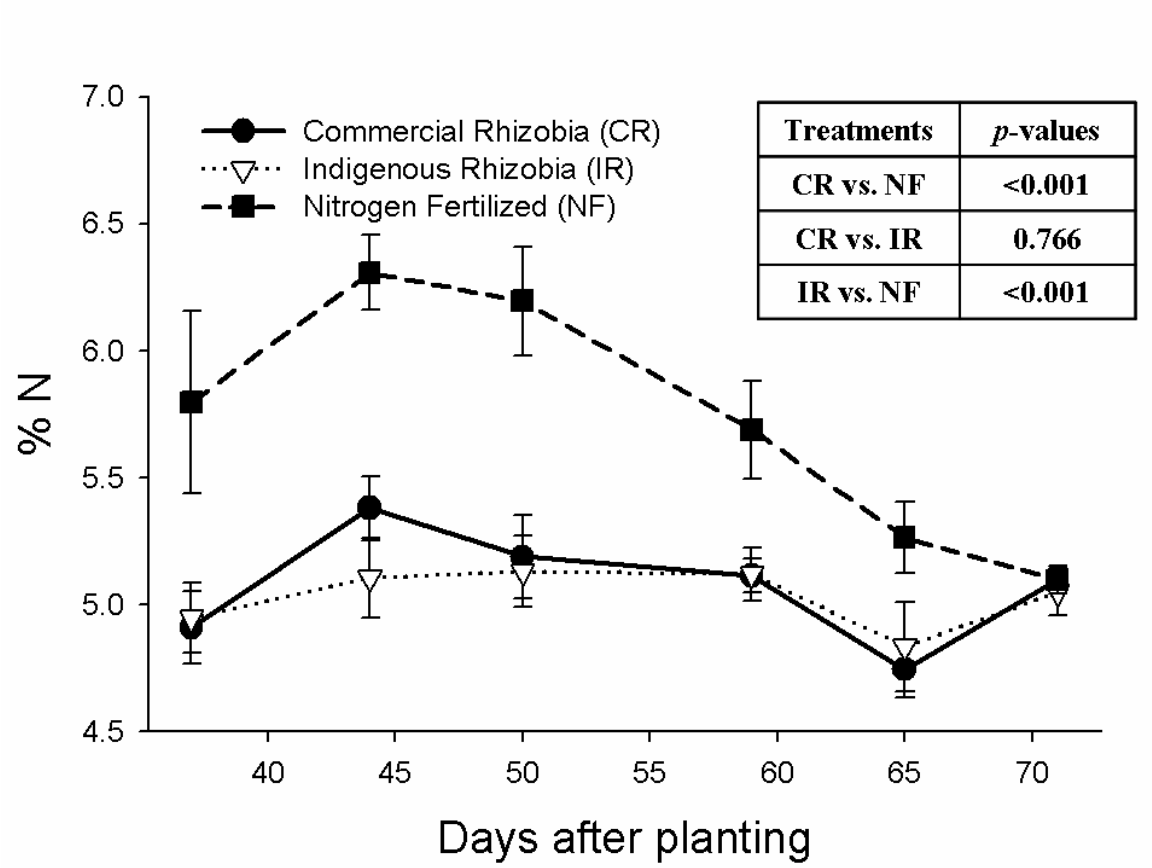

Figure 3.3. Leaf nitrogen content (% N) throughout the field season for soybean plants either treated with a commercial rhizobial inoculant at the time of planting (circles), associating solely with indigenous rhizobia (triangles), or given a nitrogen fertilizer to suppress nodulation (squares). Data with means ± 1 standard error shown. Results of treatment comparisons (orthogonal contrasts) are shown in the inset table.

Figure 3.4

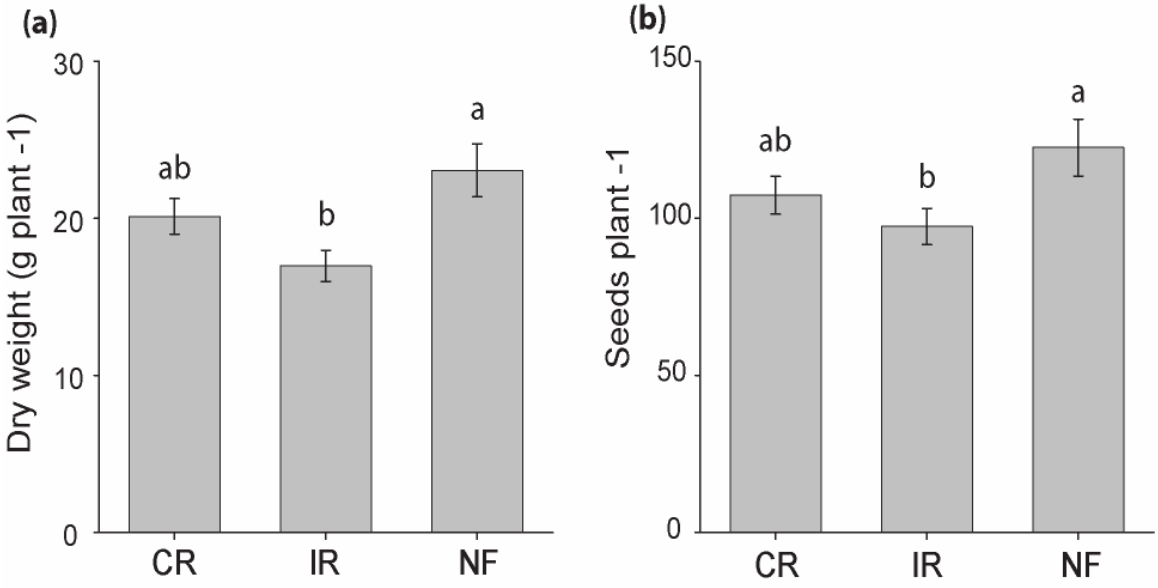

Figure 3.4. Soybean yield in terms of (a) seed biomass per plant and (b) number of seeds per plant, shown as mean ± 1 standard error. Data analyzed with ANOVA using block and treatment as variables on log-transformed values, with the untransformed data shown. Different letters within each graph represent significantly different treatment means. CR, commercial inoculation of seeds at planting; IR, association with indigenous rhizobia; NF, nitrogen fertilized.

Figure 3.5

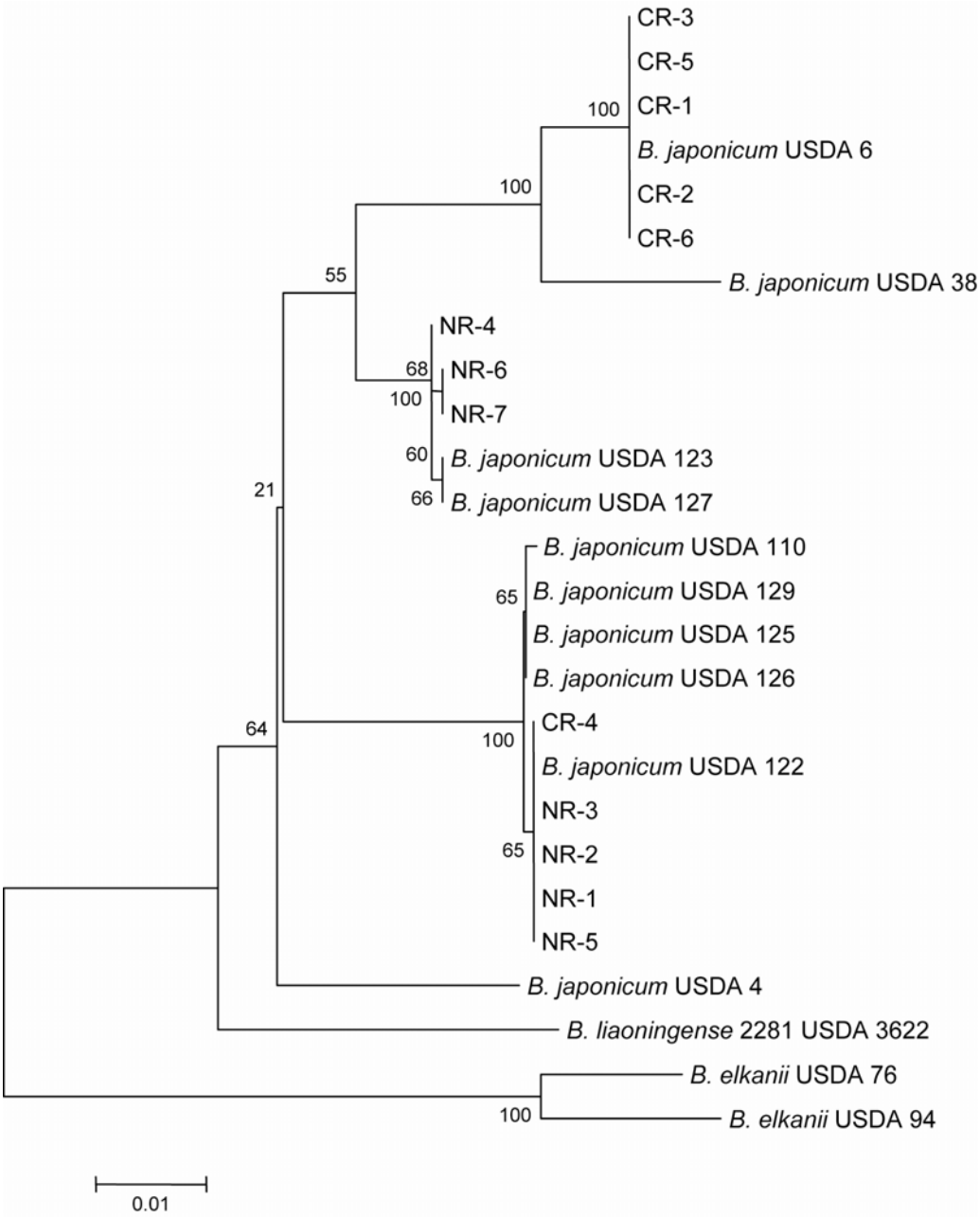

Figure 3.5. Relationships (neighbor-joining) of rhizobia isolates from field plants treated with a commercial rhizobial inoculant at the time of planting (CR) or associating solely with indigenous rhizobia (IR) and known strains (*B.* (*Bradyrhizobium*) *japonicum* or *B. elkanii* USDA plus strain number, accessions from van Berkum and Fuhrmann, 2000) based on alignment of ITS region sequences using MEGA 3.1. Bootstrap values resulted from 500 permutations of the data.

Chapter 4

Preliminary assessment of the effects of rhizobitoxine production on feeding by *Helicoverpa zea*

Abstract

Some strains of rhizobia produce compounds which are subsequently translocated from the nodules to the shoots. Rhizobitoxine (Rtx), an ethylene-inhibiting enol-ether amino acid, is one such compound that is produced by many soybean-nodulating strains of rhizobia found around the world. The role of Rtx in the rhizobia-legume mutualism is unclear, especially since it can cause a reduction in plant fitness in some cases. If the presence of Rtx results in less herbivore damage, it could be advantageous for the rhizobia to produce Rtx, since herbivores are competing with rhizobia for plant resources, and advantageous for the plant to associate with producing strains if fitness is protected under herbivory. We assessed the growth rate of podworm larvae given either leaves from soybeans inoculated with an Rtx-producing rhizobia strain (USDA 61) or from plants inoculated with a non-Rtx producing mutant of this strain in no-choice tests. Larvae fed RT containing leaves grew less and created less damage than larvae fed leaves without RT over 4 days. The efficiency in which larvae utilized consumed food was the same between the treatments. Our results suggest that RT-producing strains can confer some type of resistance to the host plant by acting as a feeding deterrent or toxin. The need for further studies and implications for pest management are discussed.

Introduction

The evolution of mutualism between legumes and nitrogen-fixing bacteria (rhizobia) has resulted in a variety of complex interactions between the two organisms. The primary interaction that defines this mutualism involves the exchange of fixed

nitrogen from rhizobia for reduced carbon from the host plant, which occurs inside protective root nodules. Less obvious are the signaling events involving specific isoflavonoids produced by the plant and the subsequent release of lipochitin oligosaccharides by rhizobia to induce the plant to prepare for the interaction (Lee and Hirsch, 2006). Many studies have also examined the regulation of this mutualism, which appears to be mostly controlled by the plant, as defense mechanisms can be initiated and nodules aborted when the interaction ceases to be beneficial to the plant (Kiers *et al.*, 2003). However, very little is known about substances other than fixed-N products produced by the bacteria which can influence plant physiology.

In the 1950s, an unknown chlorotic disease was linked to certain strains of rhizobia (Erdman *et al.*, 1956). An enol-ether amino acid, referred to as rhizobitoxine (Rtx), was identified as the causative agent of chlorosis of newly formed leaves of some legumes (Owens and Wright, 1965). Rhizobitoxine has been shown to block ethylene production by inhibiting β-cysthathionase and 1-aminocyclopropane-1-carboxylate synthase (Figure 4.1), two important enzymes in the methionine and ethylene biosynthetic pathways (Giovanelli et al., 1972; Owens et al., 1971; Yasuta et al., 1999). Ethylene is an important regulator of nodule formation in many legumes, and in most cases suppresses nodule formation (reviewed in (Okazaki et al., 2004). By utilizing bacteria mutants with the production of Rtx eliminated, researchers have shown a positive role for Rtx in nodulation success through the inhibition of ethylene for some legumes (Duodu et al., 1999; Parker and Peters, 2001; Yuhashi et al., 2000). However, nodulation in soybeans (*Glycine max*) is insensitive to ethylene, and an association between nodulation success and Rtx in soybeans could not be established (Hunter, 1993; Ruan and Peters, 1992).

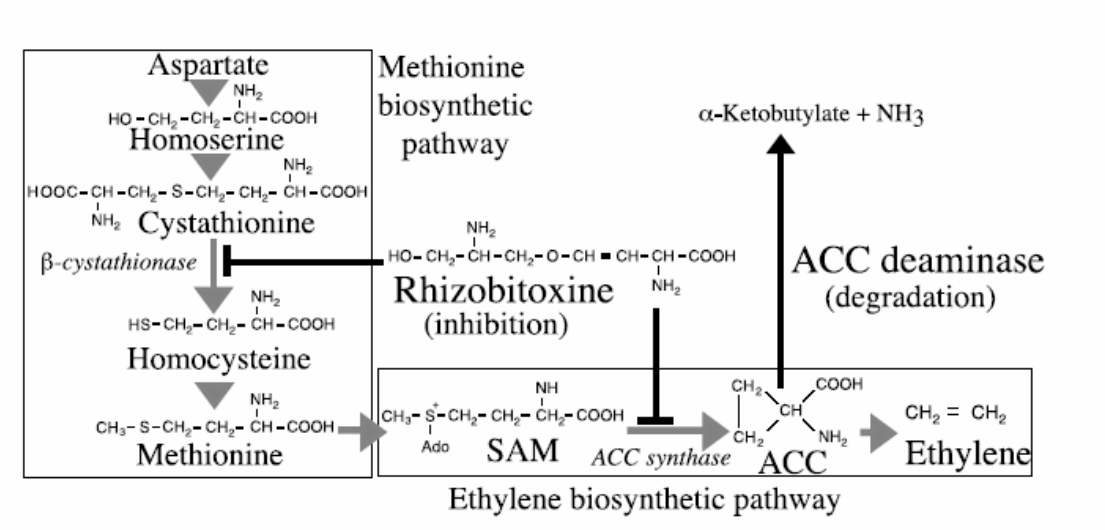

Figure 4.1. Target reactions of rhizobitoixine. SAM, S-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylate. From Okazaki et al. 2004.

The severity of symptoms of Rtx on legumes is very dependent on the combination of bacteria strain and plant cultivar, somewhat resembling the plant-pathogen compatibility model. In combinations resulting in extreme chlorosis, plants often exhibit reductions in chlorophyll content, leaf nitrogen, and protein content, and even yield (Teaney and Fuhrmann, 1992; Vasilas and Fuhrmann, 1993; Xiong and Fuhrmann, 1996). These symptoms are often enhanced in nitrogen-limited greenhouse experiments when compared to plants grown in the field, which is possibly explained by the finding that low levels of supplemental nitrate can enhance nodule formation and reduce Rtx symptoms (Teaney and Fuhrmann, 1993). The severity of chlorosis of leaves was most strongly correlated to Rtx concentration of the shoots in soybean (Xiong and Fuhrmann, 1996).

The effects induced by Rtx producing strains are alleviated in plants when isogenic Rtx^- mutants are used, but the degree of change between wildtype and mutant depends on the level of susceptibility in the cultivar (Xiong and Fuhrmann, 1996). Assays of plants and nodules revealed Rtx accumulation is highest in the nodules, followed by the shoots, and lowest in the roots (Xiong and Fuhrmann, 1996). "Resistant" cultivars of legumes inoculated with Rtx producing strains can still

exhibit some symptoms, depending on the "virulence" of the bacterial strain, but to a much lesser degree than symptoms in "susceptible" plants inoculated by the same strain (Xiong and Fuhrmann, 1996). The mechanism behind "resistance" in some plants is unknown, and may involve less Rtx production by the bacteria or detoxification by the plant.

It is now known that all strains within *Bradyrhizobium elkanii*, formerly Group II of *Bradyrhizobium japonicum*, can produce Rtx, but not all strain-cultivar combinations result in chlorosis (Okazaki *et al.*, 2004). Strains of *B. elkanii* that have been shown to produce Rtx are prevalent in many parts of the world, as shown by surveys in the United States (Fuhrmann, 1990; Johnson and Means, 1960; Lafavre and Eaglesham, 1986), east Asia (Minamisawa, 1990; Parker and Peters, 2001), and Central America and South America (Lafavre and Eaglesham, 1986). And while some strains of *Bradyrhizobium japonicum* contain the *rtx* gene required for Rtx synthesis, no known strains in this group can produce the toxin (Okazaki *et al.*, 2004). However, one other bacteria has been shown to produce Rtx: *Burkholderia andropogonis*, a plant pathogen, utilizes Rtx as a means to invade its hosts (Mitchell and Frey, 1988).

The production of a compound by a presumed mutualist that can harm its corresponding partner under certain conditions is puzzling, and deserves attention. Is it possible that Rtx production by rhizobia is able to confer a degree of protection to the host plant (and thus to itself) against plant enemies? If so, the costs of associating with Rtx+ strains of rhizobia may be offset by the benefits to the host plant under intense herbivory. Rtx has been shown to have anti-fungal properties, but other effects on other organisms have not been explored (Chakraborty and Purkayastha, 1984). Here we provide a preliminary assessment of the effects of Rtx production on a foliage-feeding generalist, the soybean podworm (*Helicoverpa zea*). We examined growth, efficiency of food usage, and leaf damage caused by podworm larvae feeding on soybeans inoculated with an Rtx-producing rhizobia strain and its non-producing mutant. With this study, we hope to establish the groundwork for further studies within this system.

Methods

Rhizobia

We used the rhizobia isolates obtained from M. Parker (State University of New York, Binghamtom, NY): USDA 61 (a moderate Rtx producer) and its corresponding RT-negative mutant (Rx17e) which was originally isolated by K. Peters through transposon mutagenesis (Hunter, 1993; Ruan and Peters, 1992). Isolates were stored in a glycerol/broth solution at -80° C. Prior to use, cultures were reinitiated under aseptic conditions by adding to a yeast-mannitol nutrient broth as recommended for rhizobial growth (Vincent 1970). Cultures were then incubated and shaken at room temperature for 7 days.

Plants

Soybean seeds (Williams 82) were rinsed with a 10% sodium hypochlorite solution, followed by copious amounts of water, and then placed on wet perlite to germinate for 2 to 3 days. Sprouted seeds were planted in an autoclaved sand/ perlite mixture in plastic pots lined with coffee filters. Plants were inoculated by adding 1 mL of the rhizobia broth to the sand/ perlite growing medium as the sprouted seeds were planted. Plants were maintained in climate-controlled, pest-free growth chambers on a 16:8 light:dark cycle and watered regularly with a dilute, N-free nutrient solution. The risk of cross-inoculation between treatments was minimized by slowly watering each plant from the sand surface to minimize splashing and containing any excess water from pots in trays. Plants were 8 weeks old when used for the experiment.

Insects

Soybean podworm (*H. zea*) eggs were obtained from the USDA/ARS facility in Tifton, GA, and larvae were reared on an artificial casein-based diet. For the experiment, first-instar larvae which were showing signs of molting were removed from diet the day before the experiment and placed in empty diet cups with sufficient

moisture. By the day of the experiment, all larvae had freshly molted into the second-instar with empty guts.

No-choice growth rate assay

The growth rates of second-instar podwom larvae on soybean leaves from plants inoculated with different rhizobia strains were determined in no-choice assays. The largest expanding leaf (one node below the apex) was detached from each plant at the petiole and placed in diet cups containing 5 mL of 1% agar to retain moisture. Freshly molted individuals were weighed before placing in cups, allowed to feed for 4 days, and then weighed again after isolating them from the leaves for two hours. The bioassay was kept in an incubator with a 16:8 light:dark cycle. At the end of the test, the remaining leaves were scanned digitally to quantify leaf area consumed (SigmaScan Pro 5, SPSS Inc.). Specific leaf area (SLA) was determined as area of leaf per dry weight, and was used to establish the leaf mass consumed per larvae. Relative growth rate (RGR) was calculated as weight gain per initial weight per day (Farrar et al., 1989). The efficiency of conversion of ingested food (ECI) was calculated as biomass gained by larvae per mass of food consumed (Scriber and Slansky, 1981). Differences between the means of each measurement for RTX+ and RTX- treatments were analyzed with a two-sample t-test (n = 15).

Results

When fed leaves from soybeans inoculated with an RT-producing strain of rhizobia, podworm larvae grew less over four days than larvae fed leaves presumably lacking the rhizobial-produced toxin (Figure 4.2a; $t_{28} = 2.36$; $p = 0.026$). As the SLA for each treatment was similar ($t_4 = -0.11$; $p = 0.921$), we used the combined average SLA (36.83 $mm^2\ mg^{-1}$) for consumption calculations. Less leaf mass was consumed from Rtx+ plants as compared to Rtx- plants (Figure 4.2b; $t_{28} = 3.52$; $p = 0.002$). The consumption efficiency was similar between larvae fed leaves from plants inoculated

with either strain (Figure 4.2c; $t_{28} = -0.06$; $p = 0.951$). By the end of the experiment, all larvae had molted to the third instar.

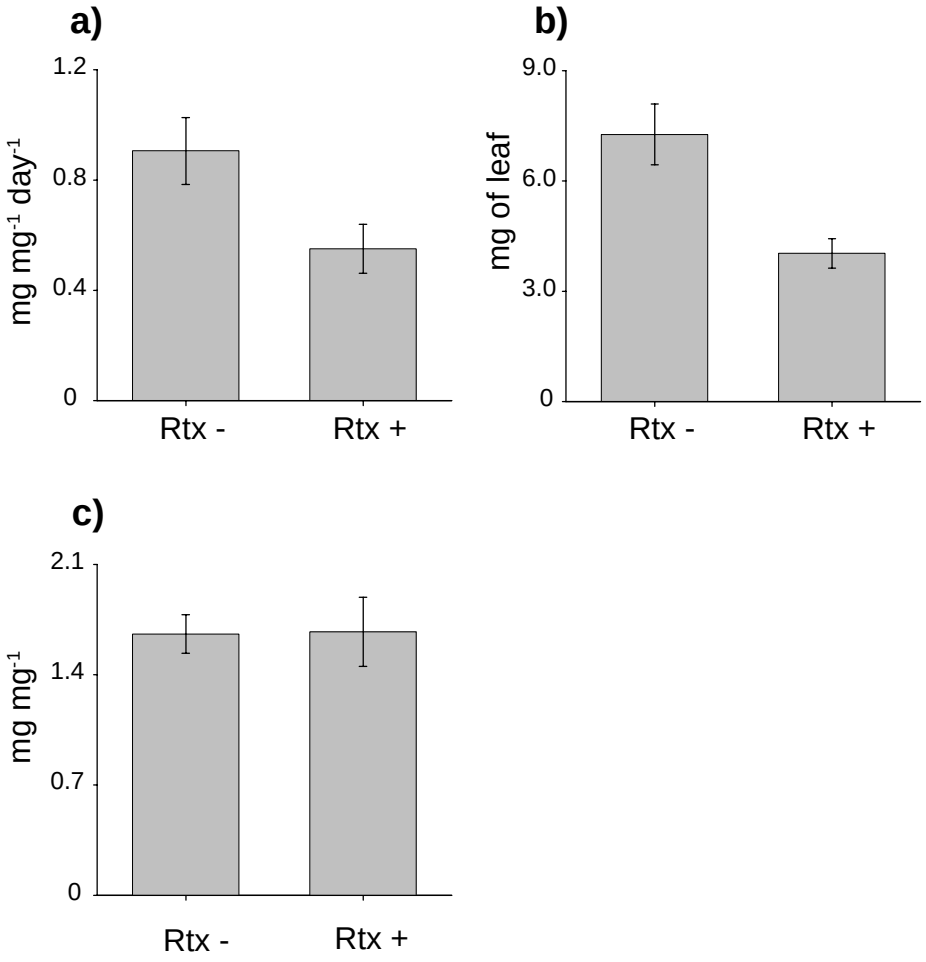

Figure 4.2. Feeding by *H. zea* in no-choice bioassay over 4 days. a) relative growth rate of larvae; b) weight of leaf material consumed by larvae; and c) utilization efficiency of food consumed. Results shown as means ± SE.

Discussion

Plants are known to produce a diversity of secondary compounds which are considered to have contributed to the even wider diversity of phytophagous insects. Effects of allelochemicals on herbivores range from feeding stimulants to feeding deterrents, in addition to direct toxicity. Other organisms interacting with plants are known to alter the chemistry of plant tissue consumed by insects. For example,

endophytic fungi of many grasses can produce alkaloids which often interfere with herbivory (Tintjer and Rudgers, 2006; Wilkinson et al., 2000). Here we investigated how inoculation with a strain of rhizobia known to translocate a bacterial compound through the host plant could effect herbivory.

Almost half of indigenous rhizobia strains nodulating soybeans in the U.S. produce rhizobitoxine, a compound shown to have herbicidal properties in some instances (Devine and Kuykendall, 1996). While the role of a phytotoxin in this mutualism remains unclear, the possibility exists that low levels of Rtx translocated to the shoots could provide some protection against plant enemies. Chakraborty and Purkayastha (1984) demonstrated anti-fungal properties of Rtx, suggesting a possible anti-pathogen function. Our results suggest that Rtx may also help protect the host plant against herbivores. Soybeans plants inoculated with a strain of rhizobia known to produce and translocate Rtx caused larval growth rate reductions when fed to a polyphagous foliar-feeder as compared to larvae fed plants inoculated with a mutant lacking the ability to produce Rtx. Damage was also significantly reduced on leaves presumably containing the toxin.

Allelochemicals can affect herbivore feeding through reduction in the efficiency in which consumed food is utilized, deterrence of feeding behavior, or interference with metabolic processes (Schoonhoven et al., 2005). Since growth efficiency was similar between larvae which were fed plants with or without Rtx-producing rhizobia, it is unlikely that the compound interferes with the insect's ability to utilize plant material, as do proteinase inhibitors. Also, this similarity in growth efficiency suggests that differences in *H. zea* feeding are not related to nutritional differences in the plants, since less nutritious plants typically stimulate herbivores to overcompensate by consuming more tissue (Simpson and Simpson, 1990). However, we can not eliminate the possibility that Rtx interaction with plant tissues causes the plant to produce compounds which are toxic or deterring to the herbivore. For example, as Rtx can inhibit ethylene, a compound which plays a vital role in plant responses to herbivore attack, the presence of Rtx in plant leaves could interfere with defense signaling pathways (von Dahl and Baldwin, 2007).

As the production of compounds such as Rtx are likely quite costly for rhizobia, we would expect some type of fitness advantage to exist for producers under certain conditions for this trait to have evolved. Herbivores and rhizobia are competing for similar host-plant resources and studies have shown that aboveground plant damage can reduce belowground nodulation (Johnson et al., 1987; Layton and Boethel, 1987; Quinn and Hower, 1986; Quinn and Hall, 1992; Sirur and Barlow, 1984). A rhizobia bacterium which participated in successful nodulation and N-fixation produces many magnitudes more offspring than if it remained in a saprophytic state in the soil (West et al., 2002). By conferring protection to the host plant against damage, rhizobia may be protecting its fitness under herbivore pressures.

For future studies, in order to show that Rtx production is an adaptive trait for rhizobia when the host plant is attacked, we must be able to demonstrate that Rtx+ strains have higher fitness levels under herbivore pressures than Rtx- strains. This can be accomplished experimentally by measuring the production of viable rhizobia cells from the different strains after prolonged herbivore attack on the host plant. As the macrosymbiont is considered to be the key regulator in the legume-rhizobia mutualism, we would also expect plants to be able to discriminate among rhizobia strains on the basis of Rtx production, and preferentially nodulate strains able to confer protection against herbivores. Further studies are also needed to elucidate the mechanism behind anti-herbivore properties of Rtx, which would be assisted by the ability to quantify levels of Rtx in the leaves and adding purified Rtx to artificial diet for the herbivore. The recognition of a host plant-rhizobia combination which protects the plant, and thus protects yield, during herbivore attack, could represent a novel, sustainable tool for pest management in legume crops.

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Chapter 5

Effects of genetic modification on herbivore-induced volatiles from maize[1]

Abstract

Large-scale implementation of transgenic crop varieties raises concerns about possible non-target effects on other organisms. This study examines the effects of genetic modification on plant volatile production and its potential impacts on arthropod population dynamics. We compared herbivore-induced volatile emissions from Bt maize plants to those from a non-transformed isoline following exposure to various types of leaf damage. When equal numbers of *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) larvae fed on Bt and non-Bt maize, volatile emissions were significantly lower in the transgenic plants, which also exhibited less leaf damage. When damage levels were controlled by adding additional larvae to Bt plants, the plants' volatile emissions increased but displayed significant differences from those of non-transgenic plants: significantly higher amounts of linalool, myrcene, and geranyl acetate were released from transgenic maize than from non-Bt plants. Manipulating the duration of feeding by individual larvae to produce similar damage patterns resulted in similar volatile profiles for Bt and non-Bt plants. Controlling

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damage levels more precisely by mechanically wounding leaves and applying larval regurgitant likewise resulted in similar emission patterns for Bt and non-Bt maize. Overall, changes in the herbivore-induced volatile profiles of Bt maize appeared to be a consequence of altered larvae feeding behavior rather than of changes in biochemical plant defense pathways.

Introduction

Genetically modified (GM) crops designed to resist insect herbivores are rapidly being adopted in many parts of the world. Transgenic maize (*Zea mays* L.) engineered to express delta-endotoxins from the soil bacterium *Bacillus thuringiensis* Berliner (Bt) has been commercially available since 1996 and now accounts for 35% of all maize grown in the United States (National Agricultural Statistics Service, 2005). Large-scale implementation of transgenic crop technology has sparked substantial debate over the social, economic, and ecological implications of GM agriculture. Concerns about the ecology of insect-resistant transgenic crops have often focused on resistance development by pests and potential negative effects on natural enemies (Gould, 1998; Shelton et al., 2002). These issues are intricately linked to one another, because natural enemies can influence the rate that pest populations adapt to resistant plants (Gould et al., 1991). The mechanisms underlying possible unintended ecological effects of transgenic crops warrant increased research attention.

One way in which GM crops might affect pest and natural enemy population dynamics is by altering plant defense mechanisms, including herbivore-induced plant volatiles. Volatile compounds released by plants can influence the behavior of herbivores searching for oviposition sites (Anderson and Alborn, 1999; De Moraes et al., 2001) and foraging natural enemies (Dicke and van Loon, 2000; Dicke et al., 1990; Turlings et al., 1990). The ecological interactions mediated by these induced plant volatiles are often complex and can be quite sophisticated, for example, volatile blends can be keyed to particular herbivore species and attract species-specific

parasitoids (De Moraes et al., 1998). Thus, these interactions may be sensitive to any changes in plant biochemistry that alter plant responses.

Volatile responses might be altered in GM crops through unanticipated phenotypic changes in plant defense systems due to pleiotrophic effects or insertational mutations caused by incorporating a foreign gene (Schuler et al., 1999a). Significant phenotypic changes induced by genetic modification are not without precedent: the production of lignin, a major structural component of plants, increased in vascular tissues by 33-97% in Bt maize over non-Bt isolines (Saxena and Stotzky, 2001). Similar changes to complex biochemical pathways involved in plant defense might disrupt or alter the ability of plants to recognize and respond to herbivores, leading to significant differences between crop lines.

Induced volatile responses in transgenic crops might also be influenced by changes in interactions between herbivores and plants. The presence of Bt toxin changes the behavior of some insects. Lepidopteran larvae, for example, can detect and avoid plant parts or artificial diet containing varying forms of the toxin (Ashfaq et al., 2001; Gore et al., 2005; Gore et al., 2002). Such behavioral changes may alter herbivore feeding patterns, changing patterns and intensity of plant tissue damage and the exposure of the plant to oral secretions of herbivores. Plant volatile emissions are likely to be affected by such changes (Pare et al., 2005; Schmelz et al., 2003).

Whereas qualitative and quantitative changes in volatile emissions have been shown for undamaged Bt cotton and mechanically damaged GM tomatoes (Smith et al., 1996; Yan et al., 2004), the effects of genetic engineering on herbivore-induced plant volatiles remain largely unexplored. Further understanding of these effects will be accomplished most readily in plant species, such as maize, where the molecular and biochemical bases of induced volatile production are well-understood. In response to herbivore feeding on maize, specific blends of compounds from the lipoxygenase, terpenoid biosynthesis, and shikimic acid pathways are released both locally and systemically (Turlings and Tumlinson, 1992). While many of these compounds are not released in response to mechanical damage, the application of herbivore regurgitant induces a volatile response that closely resembles actual feeding (Turlings et al., 1990).

In this study, we investigated how induced volatiles varied between GM maize with the Bt *cry1A(b)* gene inserted and a non-transformed isoline. The endotoxin produced by the modified gene is targeted towards the European corn borer (*Ostrinia nubialis* Hübner), but is also effective at increasing developmental times and mortality rates in other lepidopteran larvae, including the corn earworm (*Helicoverpa zea* Boddie) (Wiseman et al., 1999). *Helicoverpa zea* is an economically important pest of maize kernels, but can also cause damage to whorl-stage maize plants by foliar feeding (Capinera, 2000). We first compared volatiles from undamaged plants to determine if Bt and non-Bt maize plants differed in their constitutive release of chemicals. Then we performed several experiments comparing volatile induction by *H. zea* feeding on Bt and non-Bt maize plants, manipulating larvae numbers, damage amounts, and feeding patterns. To further control damage levels and patterns, we mimicked herbivore feeding by mechanically wounding leaves and applying regurgitant collected from larvae fed either Bt or non-Bt maize.

Methods and materials

Plants and insects. Both Bt and non-Bt maize seeds were obtained from Dekalb (Monsanto Co., St. Louis, MO). The Bt maize, DKC61-25, contains the Monsanto Event 810, with the cry1A(b) gene. The isoline of the Bt hybrid, DKC61-24, was used for the non-Bt plants. Plants were grown in a growth chamber (14:10 L:D; 25:22˚C day:night; 65% RH) in pots (16-cm x 17-cm diameter) filled with a peat-based general purpose potting soil with micronutrients added.

Helicoverpa zea larvae were reared from eggs (USDA/ARS, Tifton, GA) on an artificial casein-based diet in plastic diet cups (30 mL, Solo Cup Co., Urbana, IL) in incubators (14:10 L:D; 25˚C; 60% RH). Larvae were starved for 24 hours before experiments.

Volatile collection and analysis. We collected volatiles in a greenhouse from potted, intact 2- to 3-week-old maize plants using a closed push/pull system (Analytical Research Systems, Gainesville, FL). About 3 inches above the soil line, a Teflon®

base consisting of two sliding plates (that when pushed together left a hole for stalk) was secured around the plant. A cotton ball was wrapped around the stalk to fill space between the base and plant, and to allow air to exit. A cylindrical glass chamber (46-cm tall, 8-cm diameter) was placed over the plant, resting on the Teflon® base. Air was pumped into the chamber (3.0 l/min) through Teflon® tubing and pulled out of the chamber (1.0 l/min) through side ports and across traps containing 40 mg SuperQ® (Alltech, Deerfield, IL). Two separate volatile collections were made daily: one during the light phase for 12 hr, and the other during the dark phase for 8 hr.

Traps were eluted with 150 µl of dichloromethane, and n-octane (80 ng) and nonyl acetate (400 ng) were added to each sample as internal standards. Volatiles were analyzed by gas chromatography with a Hewlett-Packard model 5890 GC. Samples were injected in 1-µl aliquots with a splitless injector held at 240ºC. The column (15m x 0.25 mm i.d., HP-1) was maintained at 60ºC for 2 minutes, and then increased 4ºC per minute to 180ºC, where it was held for 10 minutes. Quantifications of compounds were made relative to the internal standard using Enhanced ChemStation software (Agilent Technologies, Palo Alto, CA). Identifications were confirmed using mass spectrometry and by comparing retention times with those of with known standards.

Undamaged plants. Volatiles were collected from undamaged Bt and non-Bt maize to determine baseline emissions. Collections were made from four plants of each type. Means for Bt and non-Bt maize for this and all other experiments were compared with Student's t-test using Minitab v. 14.1 (State College, PA) (Sokal and Rohlf, 1995).

Equal number of larvae per plant. Models simulating the evolution of pest resistance often assume equal ovipositional preference for transgenic crops and non-transformed isolines (Caprio, 2001; Onstad and Gould, 1998), and preference tests support this assumption (Hellmich et al., 1999; Liu et al., 2002). To test induced responses of maize under these putative field conditions, we placed an equal number of larvae on

each plant type. Two larvae were added to each chamber containing individual Bt or non-Bt plants and allowed to feed freely on plants during four days of volatile collecting. Each day, dead or molting larvae were replaced with ones that had been starved for 24 hours. These comparisons were made on six Bt and six non-Bt plants.

To avoid disturbing the plants while larvae were feeding, we assessed herbivore damage while plants remained inside volatile collection chambers. Each morning during the four-day feeding experiment, we estimated the size of damage holes based on a guide composed of five different-sized circles of known area. From the number and estimated size of holes, we calculated the total leaf area removed.

Equal amount of herbivore damage. To determine if the amount of larval feeding affects induced responses of Bt and non-Bt maize, we added *H. zea* larvae to Bt plants daily to achieve equivalent damage on both plant types. On the first day of the experiment, 8 to 10 third-instar larvae were added to each Bt maize plant and one or two larvae were added to each non-Bt plant. Each day, more larvae (~ 5) were added to Bt maize through the tops of chambers to replace ones that had died. By the end of the four-day sampling period, most larvae were fourth instars. As a control, the tops of the non Bt chambers were vented for an equal time. Each day we assessed and estimated the amount of plant damage as above. Three plants of each type were used for comparisons.

Equal pattern and amount of herbivore damage. Individual larvae on non-Bt plants feed longer and cause greater localized damage. Because the feeding pattern (i.e., the number and size of damage holes) may influence induced responses, we manipulated the duration of larval feeding, resulting in the consumption of an equal amount of leaf tissue in a similar pattern on each plant type. Larvae placed on Bt and non-Bt maize plants were monitored closely as they fed, and individual larvae were removed once they consumed an area of leaf tissue equivalent in size to the damage a Bt plant would typically incur from one larva (~ 5 mm^2). The number of larvae on each plant was manipulated to achieve similar amounts of damage across plants (~ 60 mm^2). After all larvae were removed, we collected volatiles in a growth chamber for the next three

days using a collection system similar to the one used in the other experiments (Analytical Research Systems, Gainesville, FL). Air was pumped into the chambers at 2 l/min and pulled out through traps at 0.8 l/min. A total of five Bt plants and four non-Bt plants were used as replications in this experiment. When volatile collections were finished, we cut plants at the base and digitally scanned leaves to quantify leaf area removed (SigmaScan Pro v. 5.0, SPSS Inc., Chicago, IL).

Mechanical damage and regurgitant application. To control the pattern and amount of damage more precisely than was possible using natural herbivory, we artificially simulated herbivory by mechanically damaging leaves and applying larval regurgitant. We collected regurgitant from fourth-instar *H. zea* by probing the pre-oral cavity with a pipette tip and drawing in the fluid released from the mouth. Larvae in diet cups had either been fed foliage from non-Bt maize for 24 hr or foliage from Bt maize for 6 hours after having been starved for 18 hours prior to collecting. The shorter feeding time was necessary for those fed Bt maize since the larvae would become inactive soon after eating the transgenic leaves, at which point regurgitant could not be obtained. Regurgitant was kept on ice while being collected, and boiled for three minutes to stop enzyme activity (Mori et al., 2001). Samples were stored at –80ºC until needed. Preliminary experiments showed no clear differences between the volatile profiles of Bt or non Bt plants induced by regurgitant collected from larvae fed either Bt leaves or non-Bt leaves (data not shown). Due to this similarity in responses and the difficulties involved in collecting regurgitant from larvae exposed to the Bt toxin, regurgitant collected from non-Bt fed larvae was used to compare volatiles from Bt and non-Bt plants with artificial herbivory.

Volatiles were collected from mechanically damaged plants with and without regurgitant. Plants were damaged by scraping leaf tissue from the upper side of leaves with a razor blade in a 1-cm x 2-cm rectangle. Each day, the glass volatile collection chambers were temporarily removed and three leaves on each plant were damaged, with regurgitant (10 µl) applied to each damage spot. The chambers were replaced and volatiles collected. Three Bt and three non-Bt plants were used for each experiment (with and without regurgitant).

Results

Undamaged plants. We found no differences in the total amount of volatiles released or in quantities of individual compounds in the emissions from undamaged Bt and non-Bt maize (Table 5.1).

Equal numbers of larvae per plant. When equal numbers of *H. zea* larvae fed on both maize isolines, non-Bt plants suffered four to 22 times more damage by the end of the experiment (Student's *t*-test: $t_6 = -3.53$, $P = 0.012$). By the third day, the amount of damage as well as the amount of volatiles released was significantly greater on non-Bt plants (Table 5.1). Damage to Bt maize typically occurred only on the first day larvae were placed on the plants since after initial feeding, larvae often would be inactive but still alive for one or two more days. On non-Bt plants, larvae would continue to feed for the four days of the experiment, causing extensive damage.

Equal amount of herbivore damage. When we equalized the amount of herbivory each day, there was a significant difference in the total amount of volatiles released by day three (Table 5.1). Four compounds constituted a major proportion of this difference: (Z)-3-hexenyl acetate, linalool, myrcene, and geranyl acetate (Table 2). The pattern of damage inflicted by each larvae differed between plant lines, however, with larvae on Bt plants feeding in short bouts and leaving many small, scattered holes, whereas larvae on non-Bt plants fed more or less continuously, creating large areas devoid of leaf tissue (Figure 5.1).

Equal pattern and amount of herbivore damage. Manipulating the amount of time larvae were allowed to feed resulted in plants with similar numbers of damage holes, leaf area removed, and perimeters of damaged areas (Table 5.3). In this case the two maize isolines emitted similar blends in terms of total volatiles released and proportions of individual compounds (Table 5.1).

Mechanical damage and regurgitant application. The volatiles released by Bt and non-Bt plants after applying larval regurgitant did not differ substantially from one another quantitatively or qualitatively (Table 5.1). The volatile profile of maize plants with artificial herbivory closely approximated that of plants exposed to actual larvae damage (Figure 5.2). However, regurgitant-treated plants did not release some "green leafy volatiles" (Turlings et al., 1998) and had a weaker induction of β-farnesene and bergamotene (Figure 5.2). Plants that were wounded with a razor blade appeared to release fewer volatile compounds and smaller quantities of the ones that were emitted relative to those damaged by herbivores.

Discussion

Genetic modification does not appear to alter the volatile profile of undamaged maize (Table 1). This is in contrast to the finding that undamaged Bt cotton plants emit unique compounds and different proportions of typical compounds when compared to non-Bt cotton (Yan et al., 2004), illustrating that genetic modification can have contrasting effects in different plants.

Undamaged Bt maize plants do not appear to influence the ovipositional preferences of some Lepidopteran pests (Hellmich et al., 1999; Liu et al., 2002), suggesting that an equal number of larvae would initially be expected on each plant type. When we allowed equal numbers of larvae to feed on plants continuously for four days, non-Bt maize received up to 22 times more damage, and emitted significantly more volatiles, than Bt maize (Table 1). In the field, similar reductions in induced volatile emissions could cause areas planted with Bt maize to be less attractive to natural enemies that rely on herbivore-induced plant cues to find suitable hosts. Sked (2003) showed that fields sown with Bt maize had one-third the numbers of *Macrocentrus cingulum,* a parasitoid specialist on the European corn borer, as non-Bt plots. Such reductions in population size could conceivably lead to local extirpation of natural enemies. At the same time, reduced volatile emissions could attract herbivores seeking a suitable oviposition site. For example, *O. nubialis* oviposits in the field preferentially on undamaged maize plants compared to those

infested with conspecific larvae (Harmon et al., 2003). More pest egg masses laid on Bt plants emitting fewer volatiles than severely damaged non-Bt maize could increase the possibility of resistance emerging within pest populations (Hellmich et al., 1999).

If an ovipositional preference for Bt plants did arise in the field, the greater numbers of larvae might produce an amount of damage closer to that incurred by non-Bt plants. Our results indicate that even with an equal amount of damage, volatile emissions still varied between Bt and non-Bt plants (Table 2). Linalool, myrcene, and geranyl acetate were released in significantly greater quantities from Bt maize. These compounds have been implicated in natural enemy attraction through behavioral studies and antennal electrophysiological responses (Gouinguene et al., 2005; Rose et al., 1998; Turlings et al., 1991).

Although damage amounts were equalized by increasing the number of larvae on Bt plants, these larvae exhibited a different feeding behavior, and thus created a different pattern of damage, than larvae feeding on non-Bt plants. Damage holes were more numerous but smaller on Bt maize, increasing the perimeter of damaged areas and maximizing the contact zone of oral secretions and exposed plant tissue. When the feeding of individual larvae was manipulated to achieve similar damage patterns, differences in volatile emissions between Bt and non-Bt maize were no longer evident, suggesting these differences resulted from the altered feeding behavior of larvae on Bt plants, rather than physiological changes in plant response resulting from genetic modification.

When the damage pattern and amount were standardized, either by manipulating the feeding duration of larvae or by applying larval regurgitant to mechanically damaged leaves, the induced volatile emissions of Bt and non-Bt maize were similar. These results suggest that Bt and non-Bt fields subjected to similar amounts and patterns of feeding damage, as might occur if pest populations developed resistance to the Bt maize, would be equally attractive to natural enemies. Similarly, the parasitic wasp *Cotesia plutellae* was found to be equally attracted to Bt oilseed rape plants fed upon by Bt-resistant hosts (*Plutella xylostella*) and wild-type plants equally damaged by the same herbivore, suggesting no change in the composition of the induced volatiles (Schuler et al., 1999b).

In summary, observed differences in induced volatile profiles between Bt and non-Bt maize appear to result from different amounts and patterns of feeding damage inflicted. Individual larvae on transgenic plants fed in short bouts, causing less damage and leaving distinctive damage patterns relative to conventional plants. When we controlled for these differences in the amount and pattern of feeding damage inflicted, differences in the volatile profiles of Bt and non-Bt plants disappeared, suggesting that these differences are a consequence of altered larvae feeding behavior on Bt plants rather than of changes in biochemical plant defense pathways. While reduced herbivory is the goal of insect-resistant transgenic crops, the consequent reductions in volatile emissions may have important implications for sustainable pest management.

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Figure 5.1.

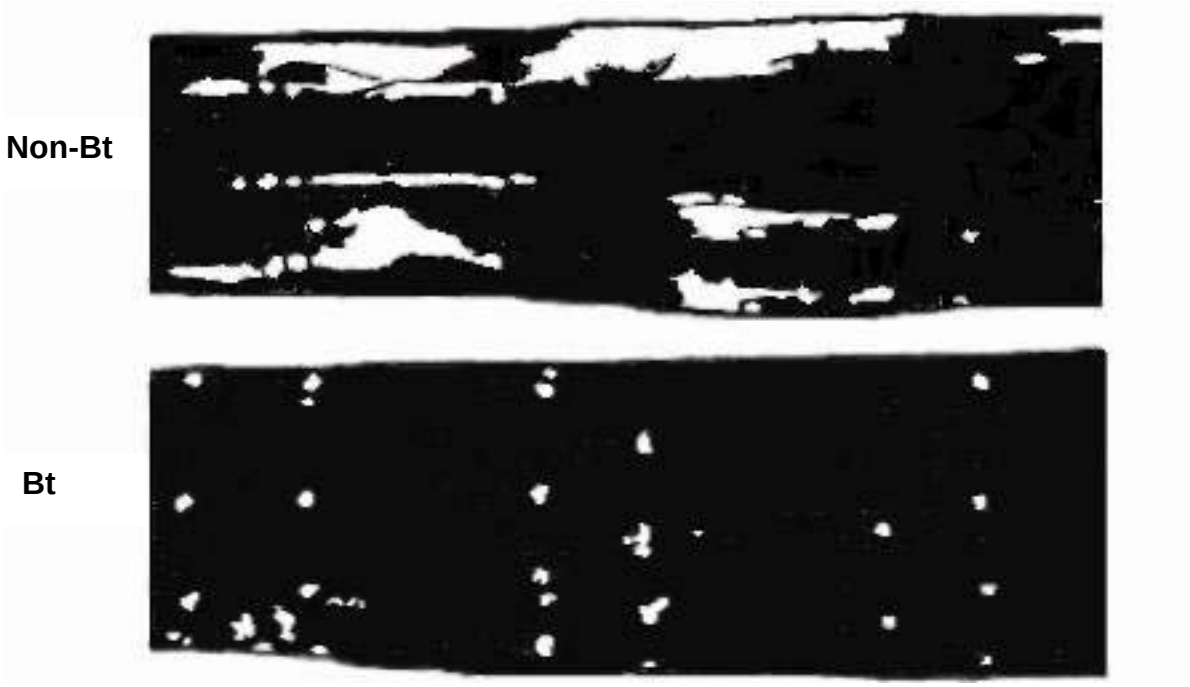

Figure 5.1. Sample of feeding patterns created by *H. zea* larvae on Bt and non-Bt maize leaves.

Figure 5.2.

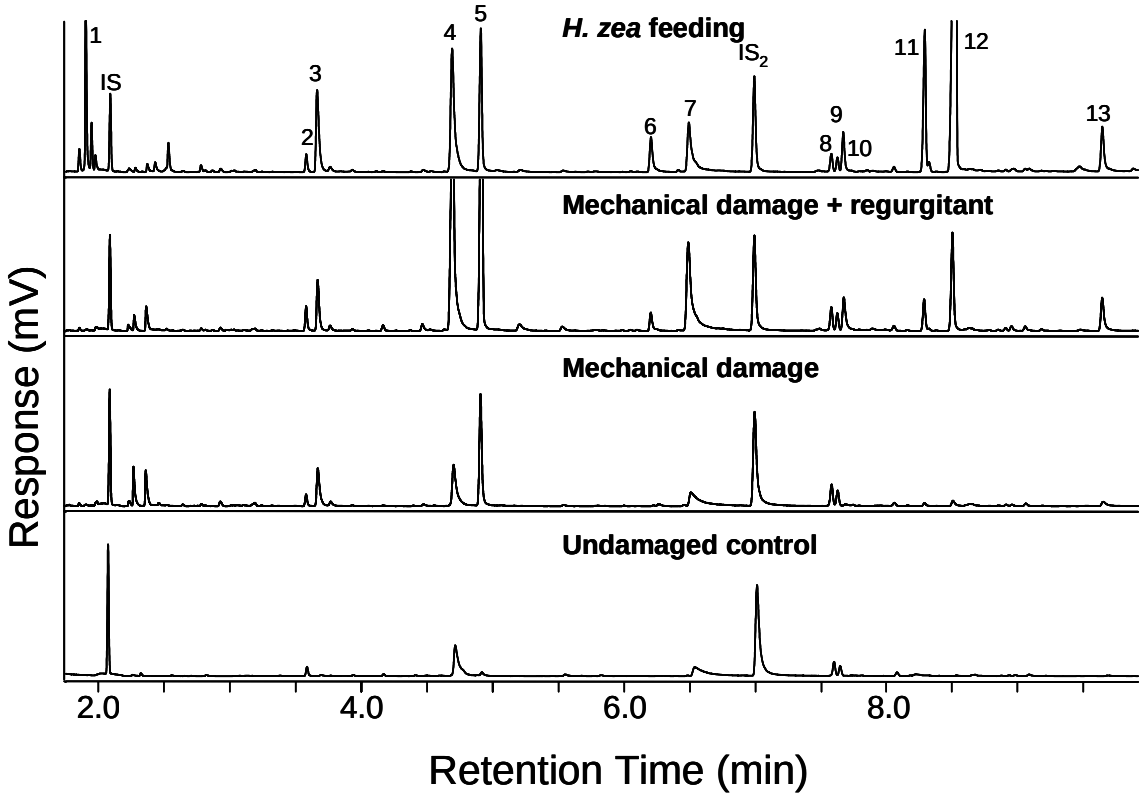

Figure 5.2. Chromatograms of volatile compounds emitted on Day 3 from non-Bt maize plants subject to feeding by *H. zea* larvae, mechanical damage, mechanical damage with regurgitant applied, and no damage. Major compounds (with a threshold emission of 2 ng/h) are labeled as followed: 1, unknown 1; 2, myrcene; 3, (Z)-3-hexenyl acetate; 4, linalool; 5, nonatriene; 6, unknown 2; 7, indole; 8, unknown 3; 9, unknown 4; 10, geranyl acetate; 11, bergamotene; 12, β-farnesene; 13, tridecatetraene; IS and IS_2, internal standards (n-octane and nonyl acetate).

Table 5.1. Total volatiles (mean ± SE) released during day 3 from Bt and non-Bt maize

Treatment	*Type*	*Total volatiles (ng/h)*	P	*Total damage (mm^2)*	P
Undamaged control	Bt	180.7 ± 41.6	0.834	0	n/a
	Non-Bt	193.5 ± 41.7		0	
Equal number of larvae per plant	Bt	652.7 ± 236.7	**0.003**	39.4 ± 23.3	**0.006**
	Non-Bt	3118.6 ± 482.9		302.4 ± 59.8	
Equal amount of herbivore damage	Bt	3869.3 ± 413.5	**0.049**	159.7 ± 25.3	0.519
	Non-Bt	2275.7 ± 274.8		139.3 ± 6.8	
Equal pattern and amount of herbivore damage	Bt	1192.0 ± 65.3	0.419	62.1± 6.2	0.913
	Non-Bt	1300.0 ± 115.5		61.0 ± 7.3	
Mechanical Damage	Bt	949.6 ± 293.1	0.959	600.0	n/a
	Non-Bt	925.0 ± 330.5		600.0	
Mechanical Damage + Regurgitant Application	Bt	1521.2 ± 460.1	0.908	600.0	n/a
	Non-Bt	1614.7 ± 587.2		600.0	

Table 5.2. Major compounds released from Bt and non-Bt maize receiving an equal amount of herbivore damage on day 3[a]

Compound	Bt	NonBt	*P*
β-farnesene	867.4 ± 148	632.6 ± 150	0.347
Bergamotene	177.6 ± 31	124.8 ± 25	0.275
Caryophyllene	23.3 ± 2	16.6 ± 5	0.308
Geranyl acetate	205.2 ± 31	75.9 ± 17	**0.034**
Indole	257.6 ± 20	293.4 ± 154	0.839
Linalool	1000.9 ± 89	443.4 ± 94	**0.023**
Myrcene	46.6 ± 5	22.58 ± 4	**0.035**
Nonatriene	606.7 ± 112	318.6 ± 89	0.138
Tridecatetraene	151.3 ± 27	82.2 ± 19	0.131
Unknown 1	23.1 ± 9	5.5 ± 1	0.182
Unknown 2	57.1 ± 2	40.5 ± 22	0.530
Unknown 3	45.0 ± 8	29.7 ± 7	0.244
Unknown 4	35.4 ± 6	23.2 ± 5	0.231
(Z)-3-hexenyl acetate	83.7 ± 19	21.6 ± 10	0.066

[a] Values represent means (± SE) in ng/h.

Table 5.3. Characteristics of damage holes created by H. zea larvae while controlling duration of feeding to achieve an equal amount and pattern of damage

	Bt	*Non-Bt*	P
Number of holes	15.5 ± 3.6	17.0 ± 3.7	0.789
Area (mm^2)	62.1 ± 6.2	61.0 ± 7.3	0.913
Perimeter (mm)	122.6 ± 11.0	126.9 ± 9.4	0.793

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