FRIEND OR FOE? THE CONDITIONALITY OF MYCORRHIZAE-CONFERRED RESISTANCE TO INSECT HERBIVORES

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Zoe Getman-Pickering

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FRIEND OR FOE? THE CONDITIONALITY OF MYCORRHIZAE CONFERED RESITANCE TO INSECT HERBIVORES

Zoe Getman-Pickering, PhD
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Plants face a constant struggle to acquire nutrients and defend themselves against herbivores. Association with soil microbes like mycorrhizal fungi can increase plant growth, and alter resistance to herbivores. Mycorrhizae are traditionally seen as mutualists that increase plant growth, and as such are used in agriculture. However, the effects of mycorrhizae on resistance to herbivores are variable. The conditions that drive either mycorrhizae-conferred resistance or mycorrhizae-conferred susceptibility to herbivores are not well understood. To determine the conditions under which mycorrhizae confer resistance, I conducted a series of greenhouse experiments testing the effects of different abiotic and biotic conditions on mycorrhizae-conferred resistance, specifically manipulating intraspecific plant competition, fertilization, plant domestication, and plant species identity. For each of these experiments, I measured resistance traits within the plant to identify potential mechanisms by which mycorrhizae might change resistance.

In my first experiment, mycorrhizae increased susceptibility to herbivores when the plants were not in competition, but had no effect in competition. I also showed that mycorrhizae induced jasmonic acid-mediated decreases in foliar nitrogen, a novel mechanism by which mycorrhizae affect resistance to herbivores. In my second experiment, I investigated mycorrhizae-conferred resistance along a gradient of fertilization treatments. I found that mycorrhizae only conferred resistance to herbivores at medium levels of fertilization. Increased resistance was again correlated with changes in the plant's foliar nitrogen content. In my third experiment looking at

the effects of domestication on mycorrhizae-conferred resistance to three different herbivores, I found that mycorrhizae changed the growth and resistance of undomesticated plants to a larger degree than domesticated plants. The change in mycorrhizae-conferred resistance in undomesticated, mycorrhizal plants corresponded with an increase in protease inhibitors, a class of chemical defenses.

By changing the defensive chemistry and nutrient content of their host plants, mycorrhizae can shift plant resistance to herbivores. While mycorrhizae are traditionally seen as mutualists, under many conditions, and when viewed in a tri-trophic context, they can act parasitically. My research demonstrates the limitation of mycorrhizae as an agricultural tool and provides insights into ways that mycorrhizae can manipulate aboveground herbivore community composition.

BIOGRAPHICAL SKETCH

Zoe grew up in upstate New York, where she loved hiking in the forest and turning over rocks and logs to search for bugs. She loved learning about all the ways organisms interacted and constantly bugged her parents with questions about the different plants and animals she saw in the woods. When her parents ran out of answers, she set off to find her own through ecological research.

Before Cornell Zoe attended Hampshire College, an experimental liberal arts college that allowed her to develop and peruse her passion for ecology. While there, she worked with Lynn Adler at the nearby University of Massachusetts to study pharmacophagy in honeybees. She was selected for an REU at the Kellogg Biological Station to work with Dr. Jen Lau. While there, she conducted and published an experiment testing the evolution of increased competitive ability in invasive Medicago plants.

During her PhD at Cornell, Zoe researched tri-trophic interactions between plants, herbivorous insects, and arbuscular mycorrhizal fungi. She didn't like studying mycorrhizal fungi at first, but they grew on her. Specifically, she worked on the conditionality of this plant-fungus mutualism, and the biotic and abiotic factors that shape the plant's susceptibility and resistance strategies to herbivory. In addition, she developed LeafByte, an award-winning app for entomological and ecological research which has been downloaded over 2600 times. She has been passionate about outreach, which she has accomplished through bug zoos, classroom visits, YouTube videos, and murals.

I dedicate my thesis to my family, biological and otherwise, who supported my love of science.

ACKNOWLEDGMENTS

I am deeply grateful to my advisor Dr. Jennifer Thaler for her incredible mentorship and support. You made me the scientist I am. You taught me how to ask good questions, design sound experiments, and say no so that I could say yes to the things I wanted to do. Thank you to my committee Anurag Agrawal and Janice Thies for their thoughtful advice on experimental design and writing.

It has been a pleasure and a privilege to work with a number of wonderful undergraduate students during my time at Cornell. Thank you to Marina Mann, Deidra Wirakusumah, Danielle Rutkowski, George Stack, Sheyla Finkner, José Rangel, Christina Zhao, Nishat Tasneem Peuly, and Matthew Sorge for all of your hard work and insight on my research projects.

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PREFACE

This dissertation is an original intellectual product of the author, Zoe Getman-Pickering.

For CHAPTER 1 Zoe Getman-Pickering conceived of and designed the study with help from and Jennifer Thaler and Danielle Rutkowski. Zoe Getman-Pickering designed the methods and carried out the experiment and assays with assistance from Danielle Rutkowski. Zoe Getman-Pickering wrote the manuscript with input from Jennifer Thaler and Danielle Rutkowski. Jennifer Thaler and Danielle Rutkowski are co-authors on the paper.

For CHAPTER 2 Zoe Getman-Pickering conceived of and designed the study with help from and Jennifer Thaler. Zoe Getman-Pickering and George Stack carried out the study and analyzed results. Zoe Getman-Pickering wrote the paper with input from George Stack, and Jennifer Thaler. Jennifer Thaler and Zoe Getman-Pickering provided funding for the study. Jennifer Thaler and George Stack are co-authors on the paper.

For CHAPTER 3 Zoe Getman-Pickering conceived of and designed the study with help from and Jennifer Thaler. Sheyla Finkner designed and carried out assessments of trichomes and *Podisus maculiventris* growth. Zoe Getman-Pickering carried out all other components of the study. Zoe Getman-Pickering analyzed the results with input from Anurag Agrawal. Zoe Getman-Pickering wrote the manuscript with input from Jennifer Thaler. Jennifer Thaler and Sheyla Finkner are co-authors on the paper.

INTRODUCTION

Mycorrhizae are fungal symbionts that grow into plant roots and spread out into the soil. They create a secondary root system for their plant hosts, taking up macro- and micro-nutrients and water which they provide to the plant in exchange for photosynthate. Mycorrhizae are ubiquitous in the soil and associate with over 80% of land plants. Those plants that associate with mycorrhizae often have increased growth, survival, and tolerance to stresses like drought and heavy metals. Association with mycorrhizae also alters plants interactions with antagonists such as herbivores, and other mutualists such as predatory insects. By differentially benefiting different plants and by altering plant interactions with herbivores and beneficial insects, mycorrhizae can influence multitrophic interactions and community composition. Additionally, mycorrhizae and other mutualistic microbes have the potential reduce the need for fertilizer and biocide inputs in agricultural systems. However, the effect of mycorrhizae on herbivores is highly variable, making it difficult to use them to control herbivores in agricultural settings. My work aims to disentangle some of that variation to determine when and how mycorrhizae confer resistance to herbivores.

Mycorrhizal fungi alter both their host plant's resistance to herbivores and their competitive ability. However, most studies on how mycorrhizae alter resistance have been conducted in single plant studies, and so the interacting effects of mycorrhizae and competition on constitutive and induced plant resistance is largely unexplored. In **Chapter 1** I tested whether mycorrhizal colonization with *Rhizophagus intraradice* would alter herbivore performance and the expression of chemical resistance traits in tomato plants with and without intraspecific competition. Mycorrhizae decreased resistance (increased leaf consumption) to herbivores when

the plants were not in competition, but had no effect in competition. This was driven by changes in the C:N ratio. I also show that mycorrhizae induced decreases in plant nutritional quality; a novel mechanism by which mycorrhizae affect resistance to herbivores.

Mycorrhizae are nutritional mutualists that provide macro and micronutrients to their hosts in exchange for photosynthate. The addition of fertilizer can disrupt the mutualism, but there is limited evidence for how fertilizer application alters mycorrhizae conferred resistance to herbivores. In **Chapter 2**, I test how different quantities and types of fertilizer alter mycorrhizae conferred resistance to herbivores. Using local mycorrhizae, I show that mycorrhizae increased resistance to herbivores most effectively at medium levels of fertilization and when plants were fertilized with a high phosphorus, organically derived fertilizer. Increased resistance was correlated with changes in the plant's foliar nitrogen content. My work shows that the growth and defensive benefits of mycorrhizal fungi can be altered depending on the type and amount of fertilizer applied.

During domestication, changing selection pressures has increased plant susceptibility to herbivores and led to less beneficial relationships with microbial mutualists. Domestication has disrupted the symbiosis between plants and mycorrhizal fungi, a symbiont that has been shown to increase plant growth and alter plant resistance to herbivores. In **Chapter 3** I tested whether reduction in the plant-mycorrhizal symbiosis from domestication has led to change in mycorrhizae-conferred resistance to herbivores. I found that domestication reduced mycorrhizae-conferred susceptibility to a generalist but not specialist herbivore. In undomesticated plants, mycorrhizae significantly increased feeding from the generalist herbivore *Trichoplusia ni* and

decreased feeding from a generalist omnivore *Podisus maculiventris*, but mycorrhizae had no effect on herbivore performance in domesticated plants. The increased feeding in undomesticated, mycorrhizal plants corresponded with an increase in digestibility reducers. Specialist *Manduca sexta* were unaffected by mycorrhizae or domestication. My results suggest that domestication has disrupted the plant-mycorrhizal symbiosis and altered mycorrhizae-conferred susceptibility to herbivores.

In conducting research on herbivory for years, I noticed that the techniques for measuring leaf area and herbivores were either painfully slow, prohibitively expensive, or imprecise. To vastly increase the speed and accuracy of measuring leaf area consumed I collaborated with a computer scientist to develop LeafByte, an award-winning iOS app for measuring leaf area and herbivory quickly and accurately. In **Appendix 1** published in Methods in Ecology and Evolution, I detail how the app works, and compare it to four common methods for analyzing leaf are and herbivory. LeafByte was equally accurate to and much faster than ImageJ, the field standard for free leaf area and herbivory quantification. LeafByte has been downloaded over 2,700 times and is being used all around the world for research and education.

In summary:

My studies on mycorrhizae have shown
what scientists should surely have known.
Plant-microbe interactions are complex
with conditionally varying effects
and are mediated by many hormones.

CHAPTER 1

INTRASPECIFIC COMPETITION REDUCES MYCORRHIZAE CONFERRED SUSCEPTIBILITY TO HERBIVORES

Authors: Zoe Getman-Pickering[1], Danielle Rutkowski[2], and Jennifer S. Thaler[1]

1. Department of Entomology, Cornell University, Ithaca, NY 14853
2. Department of Entomology and Nematology, UC Davis, Davis, CA 95616

Abstract

Mycorrhizal fungi alter their host plant's resistance to herbivores and their competitive ability. However, most studies on how mycorrhizae alter resistance have been conducted in single plant studies, and so the interacting effects of mycorrhizae and competition on constitutive and induced plant resistance is largely unexplored. We tested whether mycorrhizal colonization with *Rhizophagus intraradice* would alter herbivore performance and the expression of chemical resistance traits in tomato plants with and without intraspecific competition. We treated the plants with jasmonic acid to measure their induced chemical resistance traits which we evaluated by measuring leaf consumption by *Trichoplusia ni* caterpillars and two traits that affect herbivore performance: protease inhibitors, an antinutritive protein, and carbon/nitrogen ratio, a metric of plant nutritional quality. Mycorrhizae decreased resistance (increased leaf consumption) to herbivores when the plants were not in competition but had no effect in competition. While mycorrhizae reduced protease inhibitors, independent of competition or treatment with jasmonic acid, this did not increase caterpillar feeding. However, mycorrhizae, competition and induction with jasmonic acid interacted to decrease plant nutrition, measured as C:N ratio, which was correlated with caterpillar feeding. Here, we show that mycorrhizae induced decreases in plant nutritional quality; a novel mechanism by which mycorrhizae affect resistance to herbivores.

Mycorrhizae and competition interact to decrease plant nutritional quality and alter resistance to herbivores.

Introduction

Arbuscular mycorrhizal fungi (AMF), shape ecosystem community assembly and function by altering plant survival and growth as well as their resistance to antagonists, Arbuscular mycorrhizae are fungal endosymbionts that provide plants with micro and macro nutrients and water in exchange for photosynthate. This can increase plant nutritional quality, growth, and fitness, as well as alter investment in plant defense, thus altering the outcome of their host plant's competitive interactions (Allen & Allen, 1984; Crowell *et al.*, 1988; Shi *et al.*, 2016) and their ability to defend themselves against herbivores (Mohr *et al.*, 1998; Vannette & Hunter, 2009; Gan *et al.*, 2017).

Arbuscular mycorrhizal fungi have a wide range of effects on the defensive abilities of their plant partners. There is ample evidence in a wide range of systems of both arbuscular mycorrhizae-conferred resistance with mycorrhizae changing traits that affect herbivore performance or preference (Gange & Nice, 1997; Gange, 2001; Wooley & Paine, 2007), and mycorrhizae-conferred susceptibility to herbivores and diseases (Gange *et al.*, 1999, 2005; Babikova *et al.*, 2013a). Mycorrhizae can affect not only the constitutive defenses, those that exist regardless of herbivory (Hause *et al.*, 2002; Bennett *et al.*, 2009; Fontana *et al.*, 2009) but also the induced defenses, the production of defensive compounds in response to herbivory (Mohr et al. 1998; Riedel, Groten, and Baldwin 2008). The two main mechanisms by which AMF affect plant resistance to herbivores are by 1) altering expression of hormonally regulated secondary metabolites, and 2) altering plant nutritional composition. Mycorrhizae change their host plant's levels of resistance related hormones (Hause *et al.*, 2002, 2007; Khaosaad *et al.*, 2007) as well as defensive compounds such as β -1,3-glucanase and phenylalanine (Mohr *et al.*, 1998), aucubin and catalpol (Bennett *et al.*, 2009), and polyphenol oxidase (Minton *et al.*, 2016).

Mycorrhizae can alter plant nutritional quality(Gange & Nice, 1997) and palatability to herbivores (Gange & West, 1994a). While plants can reduce nutrient levels in specific tissue in response to an herbivore (Newingham *et al.*, 2007; Gómez *et al.*, 2010), it is not known whether this response is affected by mycorrhizae.

The few studies that have tested how arbuscular mycorrhizae-induced changes in both nutrients and chemical resistance traits find conflicting results. For example, Wurst et al. (2004) found that arbuscular mycorrhizae in plantago increased phosphorus and carbon in leaves as well as nitrogen in the roots but did not affect resistance compounds. This increase in foliar phosphorus caused accelerated development in *Myzus persicae* aphids. Gange and West (1994a) also found that arbuscular mycorrhizal fungi increased leaf C:N ratios and carbon and nitrogen based chemical defenses in their plant hosts, and subsequently reduced herbivory by the chewing lepidopteran *Arctia caja*. As most herbivores are nitrogen limited, a plant's total nitrogen and C:N ratio can be an important determinants of herbivore preference and performance.

However, the fact that most plants grow in competitive environments further complicates the relationships between plants and herbivores and plants and mycorrhizae. The effects of competition on defense are traditionally viewed as a corollary of the growth defense trade-off hypothesis (Stamp, 2003). Limited resources can either be allocated to growth or defense depending on the perceived levels of competition and herbivory. For instance, the ratio of red to far red light, a cue that a plant is being overshadowed by a competitor, leads to decreased sensitivity to jasmonate and thus a reduction of induced defenses and increased levels of herbivory (Moreno *et al.*, 2009). Using predictions from the growth defense trade-off, one might assume that arbuscular mycorrhizae which bring nutrients to their hosts could create a 'high nutrient environment' and thus lead to lower defense levels. However, this is complicated by the

fact that mycorrhizae are highly generalist, with single individuals often forming associations with multiple plant individuals and species in a common mycorrhizae network (CMN) (Smith and Read 2008). This often creates a complex and asymmetrical trading network with mycorrhizae distributing resources unequally among its connected hosts and can shift the outcome of plant-plant competitive interactions. Mycorrhizae also shifts the outcome of interspecific competition and thus community assembly (Watkinson and Freckleton 1997 and citations there in; Marler et al. 1999a; Danieli-Silva et al. 2010; Daisog et al. 2012), with mycorrhizal plants gaining a large competitive advantage over less mycorrhizal species (Hartnett *et al.*, 1993). However, mycorrhizae have also been shown to reduce competition and competitive dominance to increase evenness and diversity (Wagg *et al.*, 2011; Stanescu & Maherali, 2017). In intraspecific competitive environments, mycorrhizae can preferentially allocate resources to the larger or older plants, (Moora & Zobel, 1996, 1998; Weremijewicz & Janos, 2013; Weremijewicz *et al.*, 2016).

Previous work has highlighted the conditional nature of the interactions between plants and mycorrhizae. Competition is likely to alter the outcome of plant-mycorrhizae interactions by increasing stress. While competition is nearly ubiquitous in natural and agricultural systems, its role in mycorrhizae conferred resistance to herbivores has not yet been investigated. To address this gap, we employed tomato as a model plant system to investigate: 1) Do mycorrhizae confer resistance to herbivores in competitive environments? and 2) Are the effects of mycorrhizae and competition on plant resistance to herbivores due to constitutive or induced changes in nutritional quality or defensive secondary metabolites?

Materials and Methods

Study system

We conducted this experiment on tomato plants var. Castlemart (*Solanum lycopersicum*). Tomatoes are a valuable field and greenhouse crop which associate with mycorrhizal fungi and have a range of chemical defenses against herbivores. Protease inhibitors are common defenses in tomato leaf tissue (Broadway 1986), which are induced through the jasmonic acid pathway. Since herbivores are commonly nitrogen limited, the inability to digest proteins can significantly retard growth and result in starvation and death.

The efficacy of this defense strategy has been shown in Castlemart tomatoes (Felton et al. 1989, Farmer and Ryan 1990, Rodriguez-Saona et al. 2010, Shrivastava et al. 2015). We chose to grow the tomatoes in intraspecific competition, as tomato plants grown in agricultural systems will most likely be planted with conspecifics.

Rhizophagus intraradices is a generalist arbuscular mycorrhizal fungus in the sub-phylum Glomeromycotina. It is commercially available and used in organic agricultural systems to increase nutrient uptake and decrease fertilizer use. It has been shown to colonize tomato plants (Caron et al. 1986, Fierro-Coronado et al. 2013, Shrivastava et al. 2015). The mycorrhizal inoculum containing *R. intraradices* spores, and non-mycorrhizal inoculum for controls were obtained from the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) at West Virginia University. Both the mycorrhizal inoculum and non-mycorrhizal control were produced in leek trap pots in sand-turface media. The inoculum itself was comprised of sand, turface, leek root fragments, and in the treatment: mycorrhizal spores and hyphal fragments. While the control medium did not contain mycorrhizal propagules neither inoculum was sterile.

We used first instar *Trichoplusia ni* caterpillars in a bioassay to measure leaf quality. These larvae were obtained from a colony maintained on artificial diet at Cornell University for many years. *Trichoplusia ni* are generalist Noctuid caterpillars that feed on a wide variety of crop plants including Solanaceous and cruciferous vegetables.

Experimental design

We ran a 2x2x2 fully factorial randomized design with plants grown singly or in competition, with or without mycorrhizae, and with or without induction of jasmonate defenses (n=~24 per treatment, n=191 total).

Castlemart tomato seeds were surface sterilized using a solution of 30% of household bleach in distilled water for 30 minutes. They were then rinsed for 1 minute under running water. We sterilized 2.5x3 cm peat pots, 10 cm plastic pots, and 1:1 sand and turface medium (by volume) in an autoclave at 121℃ at 1 bar of pressure for 90 minutes. This was repeated 3 times with a minimum of 24 hours between each autoclaving. Due to uncertain germination rates, we planted 2-4 tomato seeds into each peat pots filled with either sand-turface mixture and mycorrhizal inoculum or control inoculum in a **3:1** ratio. Following germination, we established our competition and no competition treatments by thinning all treatments so that each pot had either one or two seedlings.

After the tomato plants had germinated and had been thinned, we transferred the peat pots into 10 cm pots that were filled with a 1:1 mixture of sand and turface. Each individual pot was placed in a petri dish to prevent mycorrhizal contamination between plants. We randomized the position of the plants on the greenhouse bench. Plants were watered as needed using tap water and fertilized with 21-5-20 NPK fertilizer diluted to 6ppm every 10-15 days. When the tomato

plants were 50 days old, half of them were sprayed with approximately 0.3 g of a 0.5 mM jasmonic acid solution dissolved in a 4% ethanol solution to simulate insect herbivory and induce defenses. Control plants were sprayed with 0.3 g of a 4% ethanol solution. Three days after the application of jasmonic acid, we measured plant height as a metric of plant vigor and harvested leaf tissue for bio and chemical assays. Biomass was not collected as most plant tissue was harvested for assays.

To determine the effect of competition and mycorrhizae on resistance to herbivores, we harvested the terminal leaflet from the second fully expanded leaf using a clean razor blade and placed it in petri dishes on moist filter paper for a bioassay. First-instar cabbage looper caterpillars (*Trichoplusia ni*) were placed on the leaves and allowed to feed. After 3 days, caterpillars were weighed to determine changes in herbivore mass in each of the eight treatments (Thaler and Bostock 2004). We also measured plant damage by measuring leaf area consumed using the grid method (Coley, 1983).

To test the potential mechanisms of altered resistance we measured the constitutive and induced resistance traits: Protease inhibitors and C:N ratio. Protease inhibitors are a class of chemical defenses that reduce the digestibility of leaf tissue by breaking down the herbivore's digestive enzymes. Protease inhibitors are produced through the jasmonic acid pathway and can be used to measure expression of this pathway. The C:N ratio on the other hand provides information on both the health of a plant, with a low ratio correlated to healthier, plants as well as its attractiveness to herbivores. Most herbivores are nitrogen limited (White 1984), so plant tissue with a high C:N ratio can be less attractive and nutritious (Behmer 2009). Additionally, plants can lower nitrogen levels in tissue in response to herbivory to deter herbivores and protect valuable resources (Newingham *et al.*, 2007; Gómez *et al.*, 2010).

We took the terminal leaflet from the first fully expanded leaf from each plant for analysis using colorimetric protease inhibitor assays (Orians *et al.*, 2000). The remaining leaf tissue was dried and homogenized using 2.3mm zircon beads (RPI) in a ball grinder. Then, 0.8 mg of ground leaf tissue was balled into 4x6mm tin capsules and analyzed using an Elementar analyzer (CHNS) to determine carbon and nitrogen levels.

The soil was dried prior to harvesting roots from each plant to measure levels of mycorrhizal colonization. Roots were stored in ethanol until they could be stained following the ink and vinegar method (Vierheilig *et al.*, 1998). Following staining, samples were stored in a 50% glycerol, 45% water, 5% 1.65 M HCl solution until mycorrhizal colonization was confirmed using microscopy.

Statistics

For plants grown in competition, we averaged the data for both plants before analysis. Caterpillar weight was Ln+1 transformed to fit a normal distribution. Protease inhibitor data were square root transformed to fit a normal distribution and analyzed using a linear model. We ran a series of linear testing the interacting effects of competition, mycorrhizae and induction on each of the response variables: height, protease inhibitor activity, C:N ratio, caterpillar mass, and leaf area consumed. Each of these models were analyzed using a linear model in R using the nlme package (Pinheiro et al. 2018). We also analyzed the effects of the potential mechanisms: protease inhibitor activity and C:N ratio on caterpillar weight and leaf area consumed. For each of these analyses, we ran a full factorial model and dropped non-significant terms in a backwards stepwise fashion. Significant and non-significant results are shown in Table 1.1. To measure the effect of mycorrhizae on size dimorphism, the data were subset to select only plants grown in

competition and we took the difference between plant A and plant B. The absolute value of the difference was log transformed to meet assumptions of normality and analyzed using a linear model with mycorrhizae as a predictor variable. Data were analyzed using R version 3.3.3.

Results

The effect of mycorrhizae and competition on plant damage and herbivore performance

There was an interaction between competition and mycorrhizae such that cabbage looper caterpillars consumed more plant tissue from mycorrhizal associated plants than non-mycorrhizal plants, but only when the plant was not experiencing competition. When the plants were grown in competition, mycorrhizae had no effect on herbivore consumption (Fig. 1.1, $F_{1,31}$=6.28, p=0.018). Cabbage looper caterpillars on mycorrhizal plants were marginally heavier than those reared on non- mycorrhizal plants ($F_{1,46}$=2.9620, p=0.091), but there was no effect of competition on caterpillar mass ($F_{1,46}$<0.001, p=0.996). While induction increased defensive protease inhibitors ($F_{1,148}$=9.55, p=0.002), it had no effect on amount of leaf tissue caterpillars consumed ($F_{1,42}$=0.262, p=0.611), or their mass ($F_{1,42}$=0.324, p=0.572).

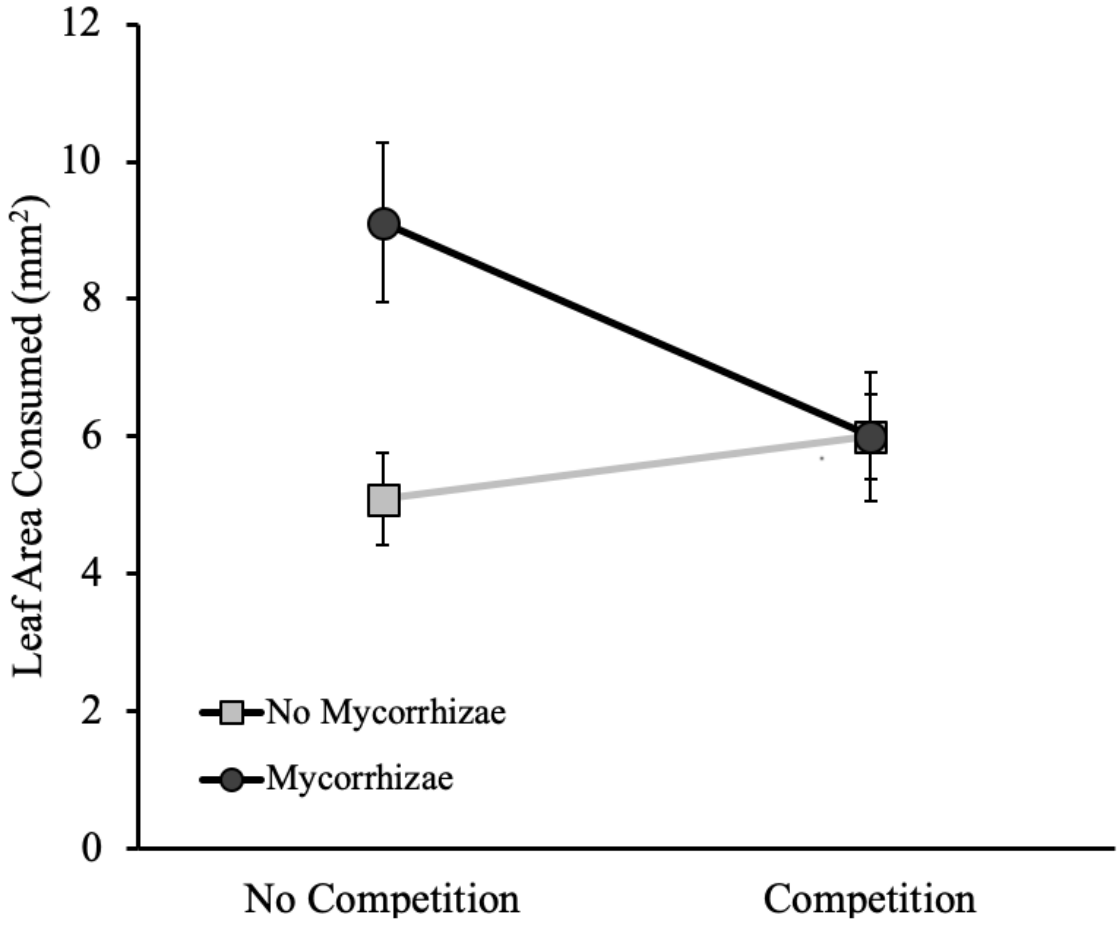

Fig. 1.1 When competition was not present, cabbage looper caterpillars consumed almost twice as much of leaf tissue from mycorrhizal plants compared to non- mycorrhizal plants. When plants experienced competition, mycorrhizae did not influence leaf area consumed. Symbols represent mean +/- SE.

Mechanisms by which mycorrhizae and competition altered herbivory

Overall, mycorrhizae decreased plant nutritional quality by 19% (Fig. 1.2, $F_{1,78}$=6.40 $p < 0.001$). Plants inoculated with mycorrhizae and experiencing competition had 13% lower nutritional quality (elevated C:N ratios) in response to jasmonic acid induction (three- way

interaction between competition, mycorrhizae and jasmonic acid induction (Fig. 1.2, $F_{1,78}$=5.35, p=0.024). In contrast, plants grown without mycorrhizae did not respond to JA induction by altering nutritional quality. The % carbon in the leaf tissue remained constant across all treatments (Mycorrhizae $F_{1,45}$=0.229 p=0.634, Competition $F_{1,45}$=1.063 p=0.307, Induction $F_{1,45}$= 0.017 p=0.898) so the changes in the C:N Ratio was driven by changes to the percent Nitrogen (df=53, R^2=0.930, p>0.001).

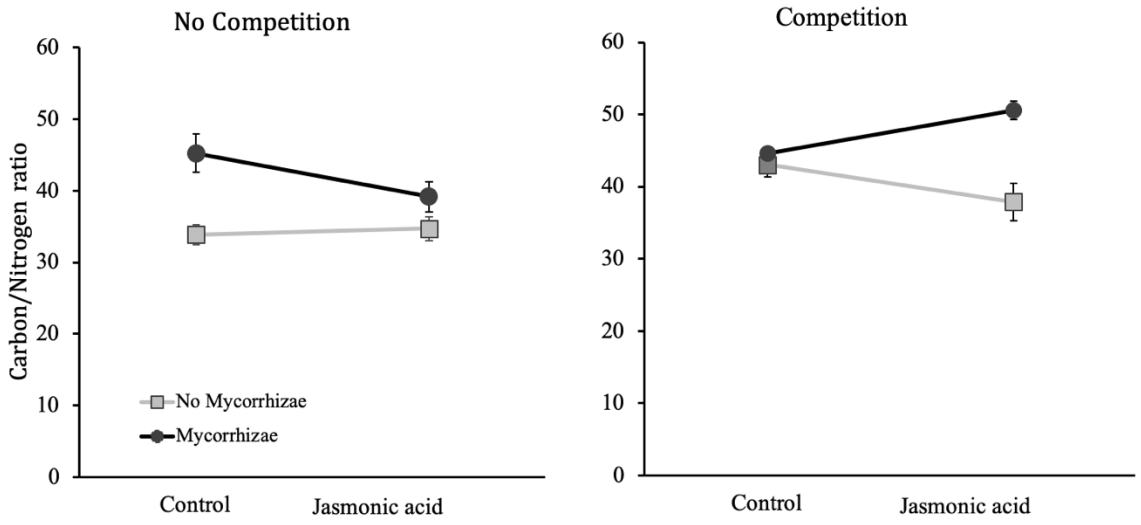

Fig. 1.2 Tomato plants grown in competition (A) and tomato plants grown with mycorrhizae had higher C:N ratios compared to tomato plants grown without competition (B), or without mycorrhizae. Tomato plants grown with competition and mycorrhizae induced an increase in carbon nitrogen ratio and thus a decrease in nutritional quality for herbivores. A higher C:N ratio means a lower plant nutritional quality. Symbols represent mean +/- SE.

Overall, plants treated with jasmonic acid had almost double the level of protease inhibitor activity (Fig. 1.3, $F_{1,148}$=9.55, p=0.002), confirming that the treatment was effective at inducing

the plants. Mycorrhizae decreased protease inhibitor levels by 30% regardless of whether the plant was treated with jasmonic acid (Fig. 1.3, $F_{1,148}$=4.35, p=0.039). Neither competition ($F_{1,148}$=0.057, p=0.451) nor interaction between competition and mycorrhizae ($F_{1,148}$=0.986, p=0. 0.323) impacted protease inhibitors.

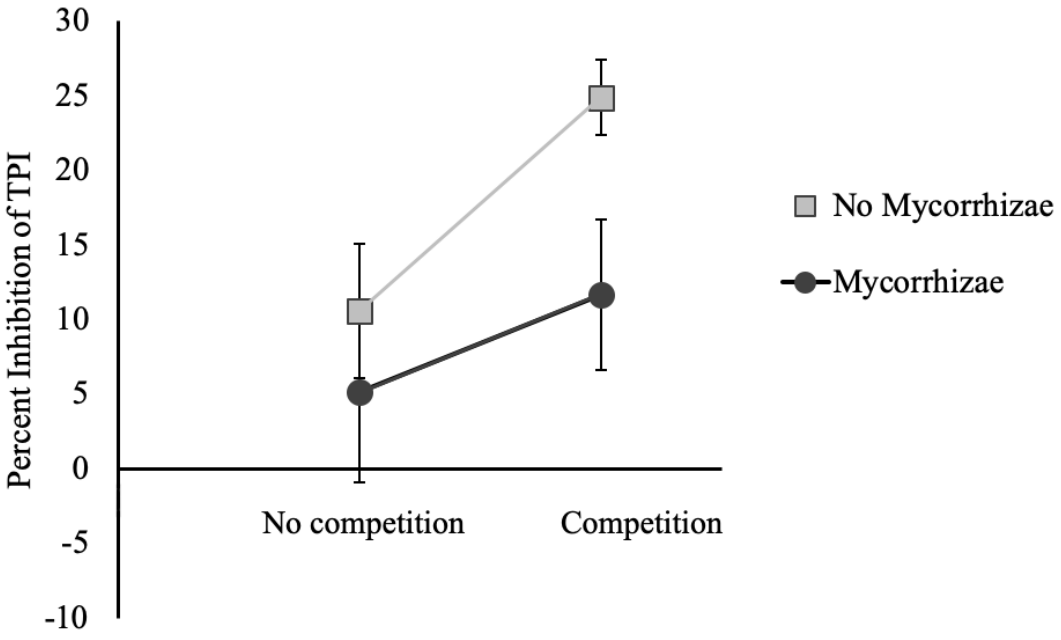

Fig. 1.3 Percent inhibition is a measure of trypsin protease inhibitor activity, with high inhibition indicating high levels of protease inhibitor activity. Tomato plants grown without mycorrhizae induced protease inhibitors strongly, while plants grown with mycorrhizae did not show significant induction. Symbols represent mean +/- SE.

Since mycorrhizae and competition interacted to affect leaf consumption and affected both plant nutritional content and resistance traits, we tested which traits quantitatively correlated with leaf damage. Cabbage loopers consumed less leaf tissue when C:N ratios were high ($F_{1,31}$=6.99, p=0.013). While we saw a similar effect with nitrogen alone ($F_{1,35}$=5.50, p=0.025), the C:N ratio explained a higher proportion of the variation (R^2=0.16). While treatments with

high protease inhibitors also had low cabbage looper leaf consumption, neither constitutive or induced protease inhibitor levels correlated with either cabbage looper feeding or mass gain ($F_{1,29}$=0.781, p=0.383, $F_{1,34}$=0.365, p=0.550).

The effect of mycorrhizae and competition on plant growth

Plants grown with a conspecific were 25% shorter than those grown individually (Fig. 1.4, $F_{1,174}$=105, p<0.001), confirming that the plants were competing with each other. Mycorrhizae did not alter height (Fig. 1.4, $F_{1,174}$=1.54, p=0.216). We did not find that mycorrhizae promoted size dimorphism when the plants were grown in competition ($F_{1,100}$=2.30, p= 0.133).

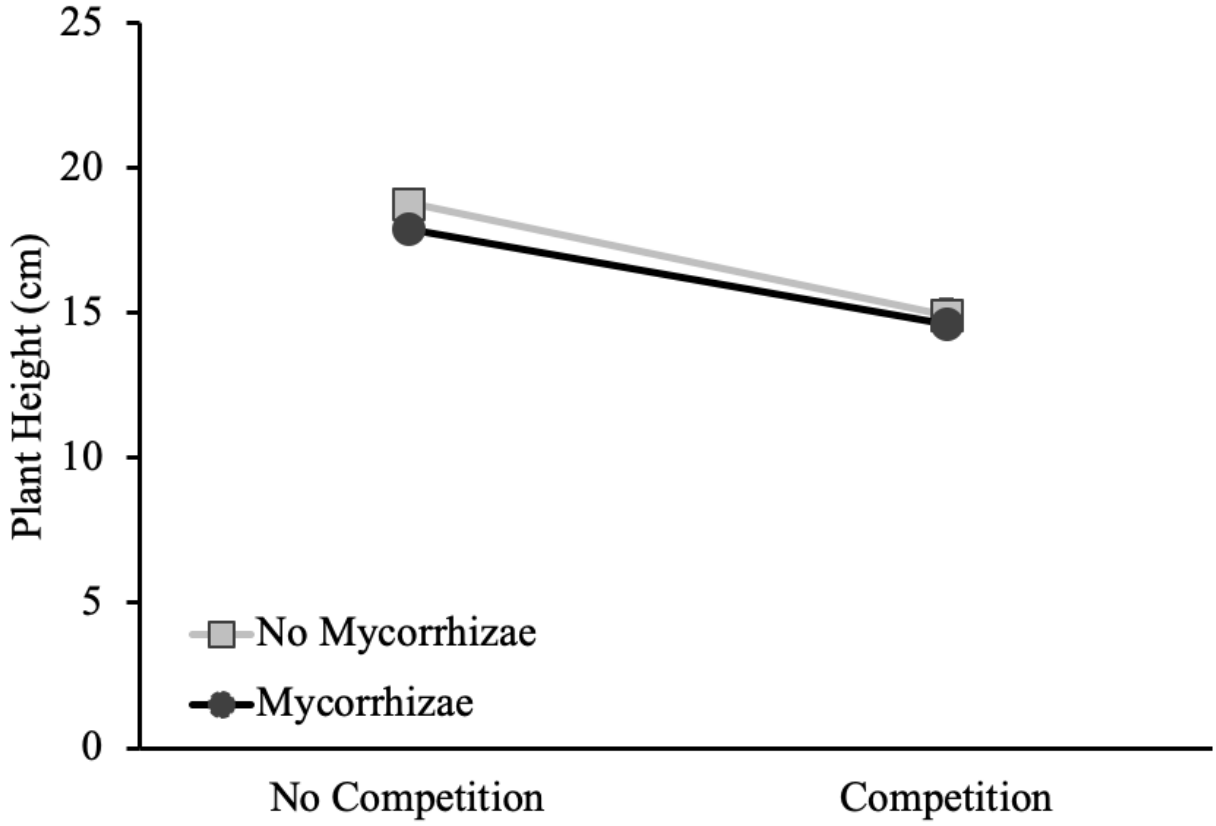

Fig. 1.4 Tomato plants grown in competition were smaller than those grown singly, while the presence or absence of *R. intraradices* mycorrhizae did not affect height. Symbols represent mean +/- SE.

Discussion

Our results demonstrate that an intraspecific competitive environment alters the effect of the ubiquitous plant-fungi mutualism on plant-herbivore interactions. Arbuscular mycorrhizae decreased plant resistance to herbivores when the plants were grown without competition, but not when they were grown in competition. We found that arbuscular mycorrhizae decreased

plant nutritional quality when grown in competition and induced with jasmonic and this correlated with increased resistance to herbivores. While most studies on mycorrhizae find positive or neutral effects on plant quality, mycorrhizae conferred benefits are highly conditional. Our results are in agreement with the work of Gange and West (1998) and Wurst et al. (2004) who found that, under certain conditions, mycorrhizae can reduce plant resistance to herbivores. Furthermore, we found that mycorrhizae suppress induction of jasmonic acid pathway based defensive protease inhibitors. This supports a number of studies which have found that mycorrhizal do not always benefit plant growth (Ryan & Angus, 2003; Bennett & Bever, 2007) but can still have effects on plant resistance to herbivores.

Our results support the vast body of literature ((Zimdahl 1980; Weiner 1990; Casper and Jackson 1997; Schwinning and Weiner 1998; Getman-Pickering et al. 2018 among many) showing that competition reduces plant growth. However, our results show no effect of mycorrhizae on size dimorphism in competing plants contradicting the findings by Weremijewicz and Janos (2012), and Ayers, Gange and Aplin who found that mycorrhizae increased size dimorphism in Andropogon gerardii and decreased size dimorphism in *Plantago lanceolate* **r**espectively. However, both studies do not use entangled root systems, which may account for the difference.

Mycorrhizae suppressed induction of defensive protease inhibitors. These results support the growing body of work that shows that the relationship between arbuscular mycorrhizae and plants can be antagonistic, especially in tri-trophic frameworks. Arbuscular mycorrhizae have been shown to suppress defensive compounds and increase susceptibility of their hosts to a variety of insect herbivores, including garden tiger moths, chrysanthemum leaf-miners and garden buckeyes (Gernns *et al.*, 2001; Gange *et al.*, 2003; Bennett & Bever, 2007; Hartley &

Gange, 2009; Gehring & Bennett, 2009). Similar effects have been shown in mycorrhizae-plant-pathogen studies (Volpin *et al.*, 1995; Shaul *et al.*, 1999; Gernns *et al.*, 2001). In our study and others, a suppression of defenses did not necessarily mean a decrease in overall growth. The decrease in induction and the fact that mycorrhizae did not increase growth suggest a more parasitic relationship between the plant and fungus in this experiment.

The fact that arbuscular mycorrhizae did not alter the plant's constitutive defenses but did suppress induced defenses supports previous research that finds that the formation of the association between plants and arbuscular mycorrhizae alters defensive hormone signaling in the plant. As protease inhibitors are known to be regulated by the jasmonate pathway in tomato plants, it is not surprising that protease inhibitor induction was altered by arbuscular mycorrhizae. However, while overall trends in protease inhibitor activity matched the treatment effects on cabbage looper caterpillars, protease inhibitor activity in each individual plant was not correlated with caterpillar weight or the leaf area they consumed. This result implies that while arbuscular mycorrhizae are altering expression of the jasmonic acid pathway, the protease inhibitors are not the primary chemicals responsible for the effect on cabbage loopers.

The presence of mycorrhizae and competition alter the way that plants induce changes in nitrogen levels and subsequently the C:N ratio. While carbon levels independently didn't impact herbivory, high ratios of carbon to nitrogen decreased cabbage looper feeding. As herbivores are frequently nitrogen limited, increased C:N ratios in foliage can be an effective defense strategy for deterring herbivory. Plants associated with mycorrhizae may have lower nutritional levels due to competition between the two organisms for limited nutrients in the soil medium (Kaye and Hart 1997). This novel demonstration that mycorrhizae can suppress leaf nutrient content following herbivory provides a new mechanism for the effects of mycorrhizae on plant nutrition

and resistance. It is particularly interesting that mycorrhizae can affect constitutive and induced defenses quite differently.

In our study, competition had no effect on induction of protease inhibitors or leaf area consumed. This supports the growing body of literature that fails to find support for the competition-defense tradeoff (Viola *et al.*, 2010). While our study only addressed intraspecific competition, chosen because it is common in agricultural settings, weeds and intercropping systems can result in interspecific competition for the target crop. We predict that in interspecific competitive situations, the effects of arbuscular mycorrhizae on herbivory might not be suppressed for both partners as we found in this study. Rather, the stronger partner might continue to see a strong effect of arbuscular mycorrhizae on herbivory, while the weaker partner will not, depending on the strength of the interaction as mycorrhizae often preferentially give nutrients to one partner over another (Marler *et al.*, 1999b).

Our results, along with the many papers cited above, show that tri-trophic effects of arbuscular mycorrhizae are context dependent, and while previous studies have shown that the species of insect and arbuscular mycorrhizae can alter the outcome of interactions, our work shows that the presence of competition can change the direction of the interaction between plant and mutualist. Due to the challenges of working with mycorrhizae, the vast majority of studies on mycorrhizae and herbivory have been conducted in greenhouse experiments where the plants are grown individually (Riedel *et al.*, 2008; Bennett *et al.*, 2009; Tomczak *et al.*, 2016), although a few have studied this phenomenon in field settings (Gehring & Whitham, 1991; Gange & West, 1994; Gange *et al.*, 2005). As plants mostly exist in competition, either with conspecifics or other species, it is important that future tests of the effect of mycorrhizae-conferred resistance to herbivores account for the role of competition.

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Data Management

Data will be archived with Dryad.

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Table 1.1: The effect of mycorrhizae, competition, and the jasmonic acid induction treatment on plant defenses (protease inhibitors), nutritional quality (C:N ratio), herbivory (leaf area eaten) and height. Caterpillar mass and Protease inhibitor data were Ln+1 transformed, and square root transformed, respectively, to fit assumptions of normality.

Response variable	Predictor Variable	df	F	p
Leaf area eaten	Mycorrhizae	1,31	2.51	0.123
	Competition	1,31	0.816	0.373
	Jasmonic acid induction	1,31	0.7386	0.3967
	Mycorrhizae x Competition	1,31	6.2835	**0.0176**
	C:N Ratio	1,31	6.9904	**0.0127**
C:N Ratio	Mycorrhizae	1,78	6.4023	**0.0001**
	Competition	1,78	**7.3427**	**0.0084**
	Jasmonic acid induction	1,78	0.4229	0.5175
	Mycorrhizae x competition x jasmonic acid induction	1,78	5.3446	**0.0235**
Protease Inhibitor Activity	Mycorrhizae	1,148	4.3542	**0.0386**
	Competition	1,148	0.5717	0.4508
	Jasmonic acid induction	1,148	9.9495	**0.0024**
	Mycorrhizae x competition	1,148	0.9855	0.3225
	Mycorrhizae x Jasmonic acid induction	1,148	0.3405	0.56041
Plant height	Mycorrhizae	1,174	1.5413	0.2161
	Competition	1,174	105.7047	<0.0001

CHAPTER 2

THE ROLE OF NUTRIENT AVAILABILITY IN MYCORRHIZAE-CONFERRED RESISTANCE TO HERBIVORES

Authors: Zoe L Getman-Pickering[1*], George M Stack[2], and Jennifer S Thaler[1].

[1] Department of Entomology, Cornell University, Ithaca, New York 14853, United States
[2] Section of Plant Breeding and Genetics, Cornell University, Ithaca, New York 14853, United States

ABSTRACT

1. Plants face a constant struggle to acquire nutrients and defend themselves against herbivores. Mycorrhizae, fungal mutualists, can provide nutrients, in particular phosphorus, that can increase plant growth and alter resistance to herbivores. The beneficial effects of mycorrhizae for nutrient acquisition can depend on the quantity and type of soil nutrients available, with plants usually benefiting more in terms of growth from mycorrhizae when nutrients are limited. However, it is unclear how the addition of different nutrients might shift mycorrhizal conferred resistance to herbivores by changing defensive secondary chemistry and nutrient availability.
2. We conducted two concurrent greenhouse experiments: one to test how three levels of fertilizer (low, medium, and high) and the other comparing three types of fertilizer (organic, organically derived, and inorganic) altered mycorrhizae conferred resistance to herbivores in tomato plant. In addition, we looked at whether changing resistance was driven by plant secondary metabolites or through the plant's nutrient content.
3. As expected, mycorrhizae increased plant biomass at low to moderate levels of fertilization, but decreased biomass at high levels of fertilization. Interestingly, mycorrhizae increased resistance to herbivores as evidenced by a 45% reduction in leaf area consumed by caterpillars, with the effect being strongest at medium levels of fertilization. Mycorrhizae

suppressed herbivory most strongly when plants were fertilized with a high phosphorus, organically derived fertilizer. In both experiments, increased resistance was correlated with changes in the plant's foliar nitrogen content.

4. Our study supports the conditional nature of the plant-mycorrhizae mutualism and shows growth and defensive benefits of mycorrhizal fungi can be altered the type and amount of fertilizer applied.

INTRODUCTION

Mycorrhizae, fungal symbionts that associate with plant roots, shape the survival and success of their host plants (Hartnett & Wilson, 1999; Smith & Read, 2008; van der Heijden *et al.*, 2008). Mycorrhizae have traditionally been recognized for their ability to supply their host plant with macro and micro-nutrients as well as water resulting in increased plant survival and growth. However, in the last two decades, it has become clear that the effects of mycorrhizae extend beyond nutrient acquisition to multitrophic interactions. Plants associated with mycorrhizae showed enhanced resistance to a wide range of diseases (Cordier *et al.*, 1998; Zhu & Yao, 2004; Khaosaad *et al.*, 2007; Frew *et al.*, 2018), and herbivores (Gange and Nice, 1997; Wooley and Paine, 2007; Koricheva et al., 2009; Jung et al., 2012), although these effects are highly variable across the literature (Gange et al., 2005; Bennett and Bever, 2007; Koricheva et al., 2009 and citations there in). Depending on environmental conditions, mycorrhizae can have no effect (Vicari *et al.*, 2002) or even in some cases harm the plant by reducing growth (Bethlenfalvay *et al.*, 1983) or resistance (Gange *et al.*, 2005). While fertilizer addition has been shown to decrease mycorrhizal benefits to growth (Grant *et al.*, 2005), less research has delved into how fertilizer addition alters mycorrhizae conferred resistance to herbivores.

As mycorrhizae are nutritional mutualists, their relationship with plants is determined in part by the nutrients available in the soil. While mycorrhizae can deliver water, macronutrients, and micronutrients to the plant, the most beneficial service is the delivery of phosphorus (Clark and Zeto, 2000 and citations there in). Mycorrhizae tend to be most beneficial when the plant is nutrient limited, increasing plant biomass substantially (Grant *et al.*, 2005). In some cases, moderate phosphorus addition can increase the growth benefits of mycorrhizal plants (Kapoor *et al.*, 2004; Ortas, 2019). However the addition of high levels of nitrogen or phosphorus fertilizers

can decrease mycorrhizal association and lead to variable effects on the growth and productivity of mycorrhizae associated plants (Jensen & Jakobsen, 1980; Joner *et al.*, 2000; Gryndler *et al.*, 2006; Cheeke *et al.*, 2011). In agroecosystems, mycorrhizae are more beneficial when farmers use complex fertilizers such as animal manure, bone char, and compost compared to mineral fertilizers, as mycorrhizae can help free nutrients bound in complex organic molecules and aggregates (Kabir *et al.*, 1997; Sáinz *et al.*, 1998; Zwetsloot *et al.*, 2016).

Fertilization and nutrient availability don't just interact to alter plant growth but can also alter the ways that mycorrhizae affect a plant's susceptibility to herbivores. For example, nitrogen fertilizer increased the performance of the gall fly *Urophora cardui* on mycorrhizal thistle plants but not on non-mycorrhizal thistle plants (Alan C. Gange & Nice, 1997). Low and medium levels of phosphorus fertilizer increased the weight and development time of two aphid species on mycorrhizal *Plantago* plants; whereas high levels of phosphorus had no effect (Gange *et al.*, 1999). Mycorrhizae reduced *Phlogophora meticulosa* caterpillar survivorship on ryegrass most effectively at low phosphorus fertilizer levels compared to those that received adequate phosphorus (Vicari *et al.*, 2002). These studies show that addition of fertilizer in different amounts and types can shift the effects of mycorrhizae on resistance to herbivores. However, little work has identified the mechanisms by which mycorrhizae and fertilizer interact to shift secondary metabolites and resistance (but see Gange and Nice, 1997; Vicari et al., 2002).

Mycorrhizae can alter plant resistance to herbivores through changes in both defensive secondary metabolites and by altering the plant's nutritional quality for herbivores. Mycorrhizae can have a similar effect on plants as fertilizer addition. By increasing N and P in the plant, nutrients often closely tied to herbivore performance, mycorrhizae often increase insect herbivore survival and growth (Gange & Nice, 1997; Wurst *et al.*, 2004). A plant can also

respond to mycorrhizal colonization by inducing hormonal signaling pathways (Barazani *et al.*, 2005; Cameron *et al.*, 2013) and thus changing the expression of defensive secondary metabolites such as trypsin protease inhibitors (Song *et al.*, 2013, 2014),and altering herbivore performance (Meixner *et al.*, 2005; Barazani *et al.*, 2005; Babikova *et al.*, 2013b). Few studies have examined how mycorrhizal changes in plant nutrients and defensive compound alter resistance to herbivores. To address how fertilizer quantity and type alter mycorrhizae conferred benefits to plant growth and resistance, we conducted two experiments in the presence and absence of mycorrhizae and measured herbivore performance as well as changes in plant nutritional content and secondary chemicals.

METHODS

Study system

We conducted this experiment on tomato plants var. Castlemart (*Solanum lycopersicum*). Tomatoes are a valuable field and greenhouse crop that associate with mycorrhizal fungi and have a range of chemical defenses against herbivores. The defensive chemistry, including protease inhibitors, of tomatoes has been well characterized (Felton *et al.*, 1989; Rodriguez-Saona *et al.*, 2010; Shrivastava *et al.*, 2015).

We used first instar cabbage looper (*Trichoplusia ni)* caterpillars in a bioassay to measure plant resistance. First-instar caterpillars were used because this stage is most sensitive to plant defenses (Thaler unpublished data), and because using later instar caterpillars fed on leaves may develop resistance to plant defenses (Lee & Berenbaum, 1989) . These larvae were obtained from a colony maintained on artificial Cabbage Looper diet (Southland Products Inc.) at Cornell University for many years. Cabbage loopers are generalist Noctuid caterpillars that feed on a

wide variety of crop plants including Solanaceous and cruciferous vegetables. These caterpillars were chosen because they are sensitive to changes in host plant quality.

In both experiments, we used mycorrhizae extracted from soil collected at the Dilmun Hill student organic farm at Cornell University (Ithaca, NY). Diverse mixtures have been found to be more beneficial to plants than monocultures (Rúa *et al.*, 2016), and are more representative of the conditions crop plants experience in the field. To isolate mycorrhizal spores, the soil was wet sieved to remove large rocks and debris, and then blended for 20 seconds using a Cuisinart™ immersion blender. The resulting liquid was passed through a series of sieves with the smallest having a pore size of 600um. We then used a 20-micron nylon mesh to remove excess water. The soil slurry was divided into 5 ml aliquots and resuspended in 40 ml of a 30% sucrose solution. This was centrifuged in a bucket attachment at 2200 rpm for 2 minutes. The supernatant was decanted through a 30-micron mesh set over a funnel. The spores on the mesh were washed with 20 ml of DI water into a beaker.

Spores were filtered through 30-micron mesh, surface sterilized with a solution of 4% chloramine T, 0.05% tween 20, 0.02% Gentamicin and 0.01% Streptomycin using the methods outlined by Mukerji et al., 2002 (page 305). We used microscopy to determine that extracted spores were clean and contain a diverse range of morphologies. The spores were resuspended in DI water such that 10 ul of water contained between 10-12 viable spores. The solution was kept suspended using a vortex. Two weeks after the plants germinated, half were inoculated using 100 ml of the spore solution pipetted at the base of the plant and watered down. The control plants were treated with 100 ml of DI water and also watered down. To recover soil microbes, which could be have an impact on both plant health (Berendsen et al. 2012 and references there in), mycorrhizal fungi (Desirò et al 2014), and the interaction between the two, we filtered a mixture

of Lambert LM-AP potting soil and water through a 1 micron sieve, and added 20 mL of the resulting solution to each pot. We used potting soil to reduce the risk of introducing pathogenic species.

Experimental conditions

We grew 220 Castlemart tomato plants in individual 10 cm pots filled with a 1:1 sand, calcined clay media. The medium was autoclaved for one hour 3 times, 24 hours apart at 121 °C to sterilize it before use. The tomato seeds were surface sterilized for 15 minutes in a 15% household bleach solution and then rinsed under running water for 1 minute. The plants were grown at 34 °C and watered with 60 ml of water every 2-4 days.

We divided plants into two concurrent experiments. In the first, we tested the effects of mycorrhizae and different amounts of inorganic fertilizer. Plants were treated with a low, medium or high dose of inorganic 21-5-20 NPK fertilizer (Table 2.1). Sixty plants were given a low dose (20 ml) of a 21-5-20 fertilizer diluted to 150 mg/L. Another 60 plants were given a medium dose (30 ml) of the same fertilizer. A third set of 60 plants were given a high dose (40 ml) of the same fertilizer. Each plant was given supplemental water such that each plant received an equal quantity of liquid. We chose this low phosphorus fertilizer to encourage association with mycorrhizal fungi.

To test the effect of fertilizer type, we compared three types of fertilizer: an organic (n=20), organically derived (n=20), and inorganic commercially available fertilizer (n=60). In the organically derived treatment 20 plants were fertilized using a higher phosphorus fertilizer: Foxfarm Grow Big liquid plant food 6:4:4 diluted to 4 ml/L of fertilizer. In the organic treatment, 20 plants were fertilized with the organic, carbon rich Alaska brand fish fertilizer 5:1:1 diluted to

14.3 ml/L water as recommended. For the inorganic treatment, we used the same plants that were given a high dose (40 ml) of the 21-5-20 fertilizer from experiment 1. We fertilized the plants grown in the 21-5-20 fertilizer once every two weeks, while the other two fertilizers were applied once every 4 weeks, to maintain a more comparable total nutrient addition.

Table 2.1. Fertilizer application regimes and total NPK applied per dose.

Treatment	**Brand**	**Quantity applied**	**Water**	**Application frequency**	**Total N (mg)/dose**	**Total P (mg)/dose**	**Total K (mg)/dose**
Inorganic	Jack's Professional	20 ml	20 ml	Every 2 weeks	0.630	0.150	0.600
Inorganic	Jack's Professional	30 ml	10 ml	Every 2 weeks	0.945	0.225	0.900
Inorganic	Jack's Professional	40 ml	0 ml	Every 2 weeks	1.260	0.300	1.200
Organically Derived	FoxFarm	10 ml	30 ml	Every 4 weeks	2.400	1.600	1.600
Organic	Alaska	10 ml	30 ml	Every 4 weeks	7.150	1.430	1.430

Measurements

To confirm mycorrhizal colonization, we bleached the roots using potassium hydroxide and stained the roots using Schiffer black ink (Vierheilig et al. 1998). Using microscopy, we assessed the roots to confirm that plants in the mycorrhizal treatment were colonized and those in the control were not. We had no accidental colonization in the control treatment. Three without colonization in the mycorrhizal treatment were moved to the control treatment.

Plant Growth

The plants were grown for two months after germination before we harvested them. We excised the terminal leaflet from the first and second most recently fully extended leaves for

protease inhibitor analysis and the bioassay, respectively. We harvested and dried the remaining leaf and stem tissue for 1 week to measure dry biomass and to analyze for C:N ratio, a measure of the nutritional content of plants.

Resistance to herbivores

To measure herbivore performance, we excised the first leaflet from the third, fully extended leaf, and placed it in a 9 cm petri dish lined with damp filter paper for the bioassay. We placed 2 neonate *Trichoplusia ni* caterpillars on each leaf, closed the petri dish and sealed it with parafilm. After 6 days, we measured mortality and the mass of each caterpillar. We observed that many caterpillars left the leaf and died. We recorded the number of caterpillars that left the leaf and died as a metric of repellence. We also measured levels of herbivory (mm^2) using a $4mm^2$ grid to assess the quantity of leaf consumed (Coley 1982). Leaf area consumed data represents total damage to each leaf independent of mortality.

Resistance Traits

We measured plant nutritive quality using C:N ratio. The C:N ratio is indicative of both the health of a plant, with a low ratio correlated to healthier, more fertilized plants, and its attractiveness to herbivores. Most herbivores are N limited (White, 1984), so plants with a low C:N ratio can be more attractive and nutritious (Behmer, 2009). To test the role of mycorrhizae and fertilizer on leaf nutrient quality, and the effect of leaf nutrient quality on herbivory one leaf from each plant was analyzed to determine the C:N ratio. Each leaf was ground into a powder using 2.3mm zircon bead using an Mp Biomedical Fastprep 24. Then 5 ± 0.1 mg leaf tissue from each leaf was balled into 4x6mm tin capsules (Costech Analytical Technologies Inc) and

analyzed using a Costech 4010 CHNS-O Analytical Combustion System. As herbivorous insects are nitrogen limited, diet choices can be strongly tied to nitrogen availability. High nitrogen levels can also change plant defense strategies, perhaps allowing them to synthesize nitrogenous defenses such as alkaloids in the case of tomatoes.

We measured plant chemical defense by measuring protease inhibitor activity. Protease inhibitors are a class of chemical defenses that reduce the digestibility of leaf tissue by breaking down the herbivore's digestive enzymes (Chen *et al.*, 2005). In tomatoes it plays a strong role in the resistance to herbivores including *T. ni* (Scott *et al.*). Protease inhibitors are produced through the jasmonic acid pathway and can be used to measure expression of this pathway. Additionally, protease inhibitors are

Mycorrhizae have been shown to alter protease inhibitor levels under different conditions (Barazani 2004, Getman-Pickering et al). We excised the terminal leaflet from the first fully extended leaf and immediately froze it on dry ice. We analyzed 100mg of tissue using a colorimetric assay to calculate the activity of defensive Trypsin Protease Inhibitors using a method adapted from Hegedus et al. (2003) (Appendix 2).

Statistics

All results were analyzed using R version 3.5.2. Protease inhibitor levels and leaf area consumed data were log transformed, and C:N ratio data were square root transformed to meet assumptions of normality. For both experiments we ran a series of linear models to look at the effects of mycorrhizae and fertilizer on biomass, leaf area eaten by caterpillars, C:N ratio, and protease inhibitors. We analyzed biomass using a linear model with mycorrhizae and fertilizer as predictor variables. We analyzed leaf area consumed by caterpillars using two linear models. The

first used mycorrhizae and fertilizer as predictor variables, and the other used C:N ratio and protease inhibitors as predictor variables. We ran two linear models with C:N ratio and protease inhibitors as response variables and mycorrhizae and fertilizer as predictor variables.

We used a generalized linear model with a binomial distribution and logit link to analyze caterpillar mortality before and after feeding. Non-significant terms were recorded and then removed from the models using backwards stepwise regressions. For leaf area consumed, we used pre-planned pairwise comparisons to compare mycorrhizal and non-mycorrhizal treatments for each fertilizer level. We applied a Bonferroni adjustment so the threshold for significance was 0.016 for linear comparisons.

RESULTS

Comparing Fertilizer Levels

Plant growth

The benefits of mycorrhizae to plant biomass depended on inorganic fertilizer levels (mycorrhizae x fertilizer interaction $F_{1, 160} = 5.395$, $p>0.001$, Fig 2.1 A). At low levels of fertilization, mycorrhizae increased biomass by 65% compared to plants without mycorrhizae, while at higher levels of fertilization, they decreased biomass by almost 25%.

Resistance to herbivores

The interaction between mycorrhizae and fertilizer was marginally significant ($F_{1, 46}=2.48$ $p=0.094$ Fig 2.1 B). Mycorrhizae increased plant resistance, measured as the suppression of leaf area consumed by cabbage looper caterpillars ($F_{1, 89}=6.36$ $p=0.013$). This was strongest at the medium fertilizer level where mycorrhizae reduced consumption by 70% (Pairwise- $F_{1, 27}=7.65$

p=0.010). At low and high levels of fertilization, the differences were only 26% and 32% respectively, although these differences were not significant in pairwise comparisons. Cabbage looper caterpillars that fed on leaves from mycorrhizal plants were 30% more likely to die before feeding compared to cabbage looper caterpillars fed on-non mycorrhizal plants ($Z_{1, 165}$=2.519, p=0.012) regardless of fertilizer levels. There was no effect of mycorrhizal addition or fertilizer levels on the weight of surviving *T. ni*.

Resistance traits

Given the effect of mycorrhizae on plant resistance to *T. ni* caterpillars, we tested two potential resistance traits: nutritional quality (estimated as foliar C:N) and defensive protease inhibitors. Plants that received higher levels of fertilizer had a lower C:N than plants that received medium or lower doses of inorganic fertilizer ($F_{1, 160}$ = 18.3, p <0.001, Fig 2.1 C). Mycorrhizae marginally raised their host plant's C:N ratio ($F_{1, 160}$ =3.23, p=0.074, but there was no interaction with fertilizer, $F_{2, 160}$ = 0.803, p=0.45).

While neither mycorrhizae nor fertilizer level affected protease inhibitor levels alone, there was a marginally significant interaction between fertilizer quantity and mycorrhizae ($F_{2,106}$=2.54, p=0.084), with mycorrhizae increasing protease inhibitor levels by 60% but only at medium levels of fertilization (Fig 2.1 D).

Caterpillars consumed marginally more leaf tissue when C:N ratios were low, meaning the plant was more nitrogen rich ($F_{1,59}$=3.817, p=0.055). There was no correlation between protease inhibitor level and the amount of leaf tissue the caterpillars consumed ($F_{1,59}$=0.637, p=0.413) at the individual level, but treatments with higher levels of protease inhibitors had lower levels of herbivory (Fig 2.1 A and B).

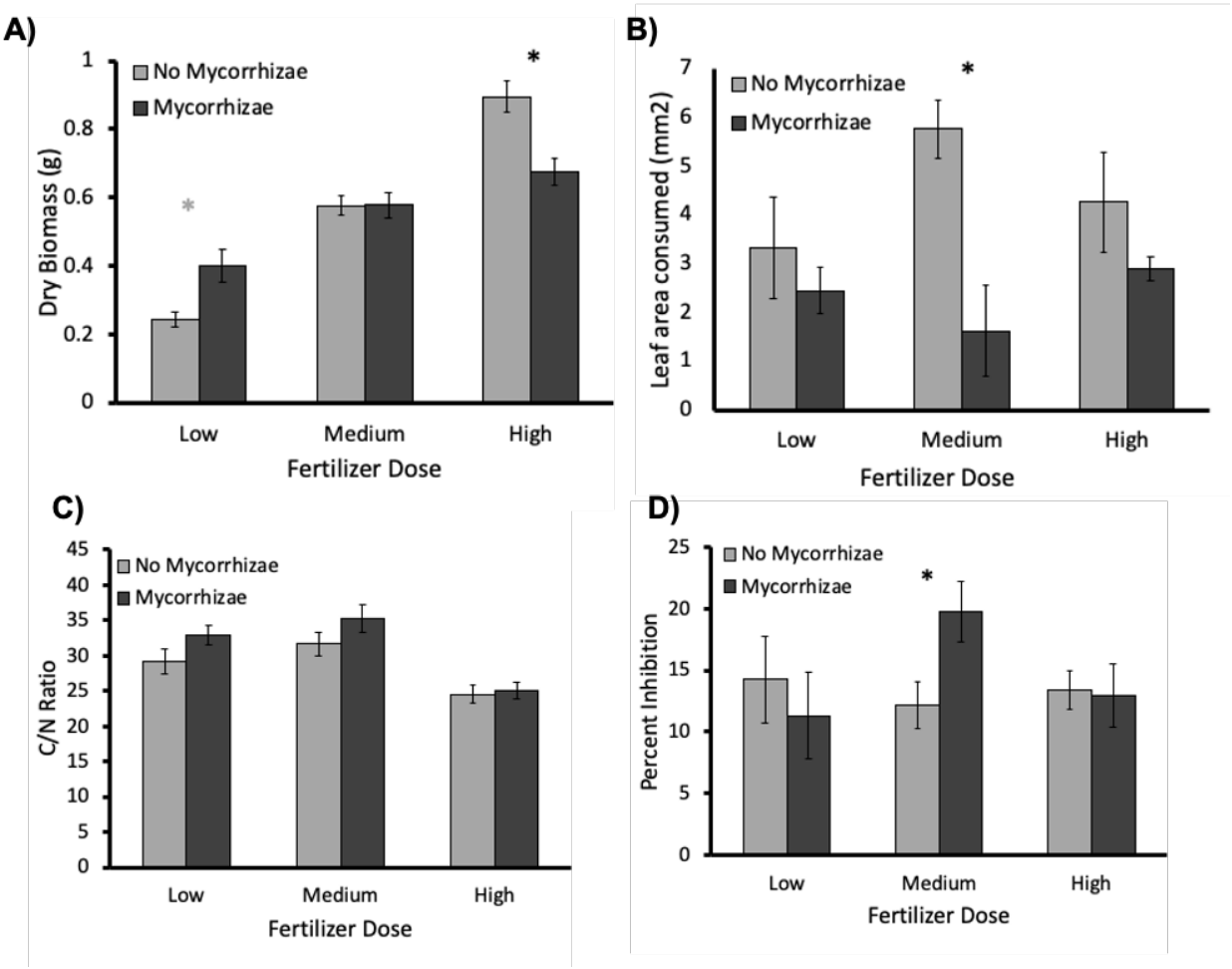

Fig 2.1. The effect of mycorrhizae and fertilizer quantity on A) biomass, B) leaf area consumed by *T. ni* caterpillars in a no choice experiment, C) Carbon Nitrogen Ratio, and D) Protease inhibitor levels as measured by percent inhibition. Bars represent mean +/- SE. Asterisks denote significance determined through a TukeyHSD post-hoc test.

Experiment 2: Comparing Fertilizer Types

Plant Growth

Mycorrhizae decreased plant biomass by an average of 20% ($F_{1,89}$=9.59, p<0.001, Fig 2.2 A) regardless of fertilizer type. Plants grown with high levels of mineral inorganic fertilizer grew larger than those grown with either organic or organically derived fertilizer ($F_{2,89}$=8.20, p=0.003,

Fig 2.2 A).

Resistance to herbivores

The effect of mycorrhizae on leaf area consumed was marginally dependent on the fertilizer treatment ($F_{2,46}$=2.49, p=0.094), which was driven mostly by mycorrhizae suppressing herbivory in the organically derived fertilizer treatment (Pairwise comparison $F_{1,8}$=6.495 p=0.034, Fig 2.2 B). Without mycorrhizae, plants with organically derived fertilizer received three times more damage than the plants fertilized with organic or high levels of inorganic fertilizer ($F_{2, 48}$=6.25, p=0.004, Fig 2.2 B). Mycorrhizae did not suppress herbivory in either the organic or inorganic fertilizer treatments.

Resistance traits

Mycorrhizae increased the C:N ratio (lowered the nutritional quality) in the plants grown with the organically derived fertilizer, but not the organic fertilizer or high levels of inorganic fertilizer (interaction-$F_{1,89}$=6.869, p=0.002, Fig 2.2 C). *T. ni* fed more on plants that had higher nutritional quality (a lower C:N ratio) ($F_{1,35}$=6.593, p=0.015). Fertilizer type had a strong effect on the level of defensive protease inhibitors ($F_{2,66}$=9.990, p<0.001), with the plants fertilized with the organic fertilizer having much higher levels of protease inhibitors, compared to plants grown with organically derived or inorganic fertilizer. Mycorrhizae marginally decreased protease inhibitor levels ($F_{1,66}$=3.319, p=0.073, Fig 2.2 D). Feeding was not correlated with protease inhibitor activity ($F_{1,35}$=1.921, p=0.175).

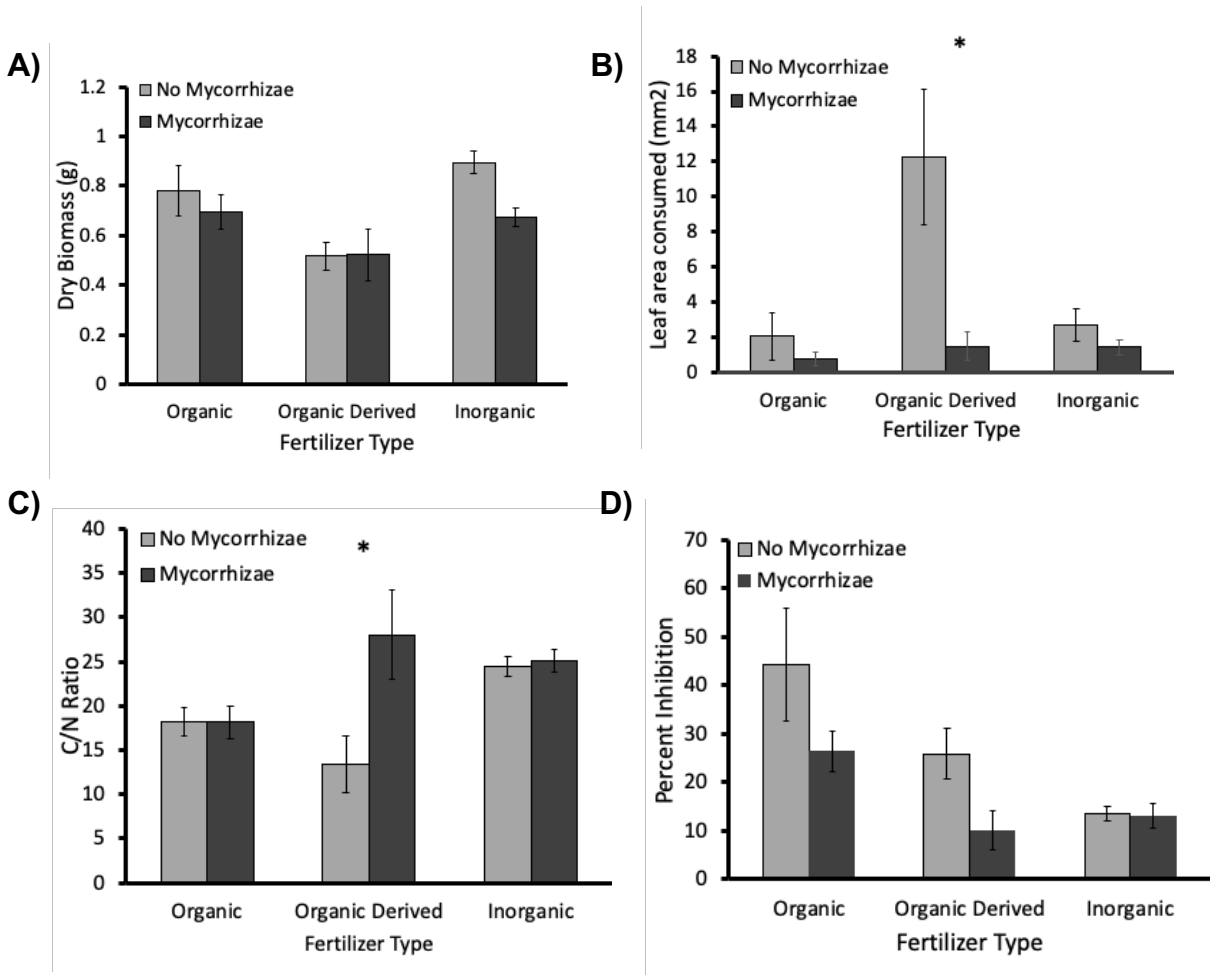

Fig. 2.2: The effect of mycorrhizae and fertilizer type on A) Biomass, B) leaf area consumed by *T. ni* caterpillars in a no choice experiment, C) Carbon Nitrogen ratio, and D) Protease inhibitor levels as measured by percent inhibition. Bars represent mean +/- SE. Asterisks denote significance determined through an lsmeans post-hoc test.

Discussion

Our results support the growing literature that mycorrhizae are most beneficial to plant growth at lower levels of fertilization and tend to have no or negative effects at higher levels of fertilization (Jensen & Jakobsen, 1980; Joner *et al.*, 2000; Gryndler *et al.*, 2006; Cheeke *et al.*, 2011). Our results show that mycorrhizae conferred resistance to herbivores is equally

conditional and strongest at medium levels of fertilization and with an organically derived fertilizer. This enhanced resistance to herbivore damage was correlated to changes in the plant's C:N ratio at the individual level and changes in trypsin protease inhibitors; defensive secondary metabolites. While mycorrhizal fungi are often presented as simple mutualists, our research, along with a wealth of studies, show that they can act more commensalistic or even parasitic depending on the environmental conditions (Borowicz & Fitter, 1990; Gange *et al.*, 1999; Shaul *et al.*, 1999; Gernns *et al.*, 2001; Riedel *et al.*, 2008).

Different types of fertilizer altered mycorrhizae conferred changes in growth and resistance. Fertilizing plants with nitrogen rich organic fish emulsion fertilizer did not change growth regardless of mycorrhizal presence. Past studies have found that amendments high in organic matter can lead to more positive effects of mycorrhizae on growth (Celik *et al.*, 2004) although that was not evident in our study. Non-mycorrhizal plants that received the organically derived fertilizer, a fertilizer with a much higher ratio of phosphorus, had much higher levels of herbivory. However, the presence of mycorrhizae suppressed herbivory dramatically. This effect was related to altered C:N ratio. The plants fertilized by the organically derived fertilizers had a very low C:N ratio when grown without mycorrhizae. When grown with mycorrhizae, these plants had a much higher C:N ratio, making the plant less nutritious and less appealing to herbivores, despite having no difference in growth.

Overall, mycorrhizae tended to increase resistance to herbivores in treatments where the plant received no growth benefits, and increased growth in treatments where the plant received no resistance benefits. In plants grown in organically derived fertilizer or at medium levels of mineral fertilization, mycorrhizae had no effect on growth but dramatically reduced resistance to herbivores. In the low level of fertilization, plants with mycorrhizae grew larger but got no

resistance benefits. The growth benefits of mycorrhizae have largely been attributed to nutrients they provide, although there is evidence that they can produce plant growth hormones such as gibberellins (Barea & Azcón-Aguilar, 1982). In previous studies the effects of mycorrhizae on defense are attributed both to the aforementioned nutrients (Gange & Nice, 1997; Wurst *et al.*, 2004), and changes in the jasmonic (Hause *et al.*, 2002; Song *et al.*, 2013) and salicylic acid (Jung et al., 2012 and citations there in) pathway expression, particularly during the initial colonization period (Jung et al 2012 and citations there in). The disconnect between effects on growth and defenses suggest that mycorrhizal benefits are due to a complex and conditional mix of each of these factors.

We employed a no choice bioassay using excised leaves to test the effects of mycorrhizae on resistance. This common approach (Stout *et al.*, 1994; Thaler *et al.*, 1996, 2010; Kumar *et al.*, 2016) has a number of benefits and challenges. By excising the leaves we were able to collect tissue for the bioassay, protease inhibitor assay, and nutrient analysis simultaneously, reducing confounding temporal variation (Karban, 2011). However, removing the leaves from the plant for the bioassay may differentially induce resistance in the mycorrhizal and non-mycorrhizal plants (Pozo and Azcón-Aguilar, 2007 and citations therein). Mycorrhizal priming may exacerbate the plant's response to excision. We cannot exclude the interaction between mycorrhizal priming and excision as mechanism by which mycorrhizae affect resistance to herbivores.

Plants tend to form stronger or more mutualistic associations with mycorrhizae when that plant is stressed (Hobbie & Colpaert, 2003). For this reason, many greenhouse studies that examine mycorrhizae-plant-pest interactions use very low levels of fertilizer. These results suggest that studies that use very low levels of fertilizer to ensure strong colonization risk

missing potential negative interactions. Furthermore, many of these studies use common crop plants such as tomatoes, barley, and corn. While greenhouse studies are always unrealistic to some extent, this low level of nutrient availability is additionally unrealistic for crops that are heavily fertilized in some agricultural systems (Hills *et al.*, 1983; Bierman *et al.*, 2012). This low-level fertilization strategy may be more appropriate for studies looking to test the role of mycorrhizae in plant defense in smallholder systems, where mineral fertilizer may be prohibitively expensive or in un-managed systems where soil nutrition is limited. If the results we found hold true in situ, it would suggest that mycorrhizae will be more likely to protect plants against chewing herbivores in agricultural systems where fertilization is the norm. However, in systems without human inputs, macronutrients are scarce (Chapin *et al.*, 1986). It is likely that in these systems, mycorrhizae will offer little to no protection, but will increase growth.

Mycorrhizae have the potential to reduce the need for fertilizers and biocides in agricultural systems, especially organic ones that often maintain higher natural levels of mycorrhizae and use more varied fertilizer types (Gosling *et al.*, 2006). When crop plants associate with naturally occurring or supplemented mycorrhizae, they are buffered against stress from abiotic factors such as salt (Al-Karaki, 2000; Giri *et al.*, 2007), heavy metals, and drought (Al-Karaki & Clark, 1998; Al-Karaki *et al.*, 2004), as well as biotic factors including disease (Pozo and Azcón-Aguilar, 2007 and citations therein), nematodes (Hol & Cook, 2005) and some herbivores(Koricheva et al., 2009c and citations therein). It is possible that careful selection of fertilizer types and regimes might encourage the defensive benefits of mycorrhizae. Further research is needed to determine conditions that encourage mycorrhizae to provide both growth and defensive benefits to their host plants.

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Author Contributions

ZGP and JT conceived of and designed the study. ZGP and GS carried out the study and analyzed results. ZGP, GS, and JT all contributed to writing the paper. JT and ZGP provided funding for the study.

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CHAPTER 3

HOW DOMESTICATION HAS ALTERED MYCORRHIZAE-CONFERRED RESISTANCE TO HERBIVORES

Authors: Zoe Getman-Pickering[1], Sheyla Finkner[2], Jennifer Thaler[1]

[1] Department of Entomology, Cornell University, Ithaca, New York 14853, United States
[2] MilliporeSigma, Burlington, MA

Abstract

During domestication, selection typically increased plant susceptibility to herbivores and reduced beneficial relationships with microbial mutualists such as mycorrhizae. Nonetheless, it is unclear whether reductions in plant-mycorrhizal symbioses during domestication altered mycorrhizae-conferred resistance to herbivores.

Accordingly, here we conducted a factorial experiment using 6 pairs of domesticated and wild solanaceous crop species, with and without mycorrhizae, and measured resistance to three species of insects.

Our results show that, seperate from the effects of mycorrhizae, domesticated plants grew larger, had lower chemical defenses, and had lower resistance to herbivores. This adds further evidence to the long-held hypothesis that domestication has decreased plant defences and resistance to herbivores. We found that mycorrhizae more strongly affected undomesticated plants than domesticated plants. Mycorrhizae suppressed growth in undomesticated but not domesticated plants. In undomesticated plants, mycorrhizae increased feeding by generalist *Trichoplusia ni* and decreased feeding by omnivorous *Podisus maculiventris*, but these effects were absent for domesticated plants. The increased feeding in undomesticated, mycorrhizal plants corresponded with an increase in digestibility reducing protease inhibitors. In undomesticated plants

mycorrhizae decreased weight gain by specialist *Manduca sexta,* but in domesticated plants mycorrhizae increased *Manduca sexta* weight gain.

This suggests that domestication has disrupted the plant-mycorrhizal symbiosis and altered mycorrhizae-conferred resistance to herbivores.

Introduction

During the process of crop domestication, plants face novel changes in selective forces (Meyer *et al.,* 2012, Turcotte *et al.,* 2017). Human-mediated selection typically results in increased plant growth (Pickersgill 2007) and nutritional content, decreased defensive chemistry (Lindig-Cisneros, Roberto *et al.,* 1997, Whitehead *et al.,* 2017, Luna-Ruiz *et al.,* 2018), and altered interactions with mutualists (Turcott *et al.,* 2014, Pérez-Jaramillo 2016 and citations therein) and herbivores (Turcott *et al.,* 2014, Chen *et al.,* 2015, Whitehead *et al.,* 2017 and citations therein). Domesticated plants tend to be more susceptible to herbivores (Whitehead *et al.,* 2017 and citations therein). Increased susceptibility to herbivores has been attributed to changes in different selective forces including selection for increased growth, selection for increased nutrient quality, and selection for more palatable plants. First, selection for faster growing and larger plants might lead to reduced resistance to herbivores as plants funnel resources away from defense and into producing edible tissue (Huot *et al.*, 2014), assuming a growth-defense tradeoff (Rosenthal & Dirzo 1997). Secondly, humans have selected for more nutritious plants, which may make the plants more attractive to herbivores (Manuel Delgado-Baquerizo *et al.,* 2016). Finally, humans have selected for lower levels of bitter and unpalatable defense compounds, making the plants more palatable for both humans and insects (Benrey *et al.,* 1998, Whitehead *et al.,* 2017, Chomicki *et al*., 2019). Beyond that, human pest control efforts can alleviate selective pressure by herbivores, further reducing the need for defense (Macfadyen and Bohan 2002). In sum, these effects result in lower defenses and lower resistance to herbivores in domesticated plants compared to their wild relatives (Whitehead *et al.,* 2017).

Through the domestication process, farming practices such as the use of organic and mineral fertilizers in agriculture have altered many crop species' relationships to microbial

symbionts (Mutch *et al.,* 2004, Zachow *et al.,* 2014, Coleman-Derr *et al.,* 2015). A ubiquitous microbial symbiont, mycorrhizae are endophytic fungi that provide nutrients in exchange for photosynthate, increasing their host plant's growth and survival. As seen with symbionts more broadly, domesticated plants tend to form less robust associations with mycorrhizae. In a manipulative greenhouse study Turrini *et al.,* (2016) found that domesticated sunflower (*Helianthus annuus*) plants had lower instances of mycorrhizal colonization than their wild counterparts. However, the results were variable, and it was not tested whether the sunflower plants benefited from the association. In a field survey, assessment of over 50 breadfruit (*Artocarpus altilis*) cultivars found that again, domesticated plants had lower colonization and lower diversity of colonizing fungi compared to wild and landrace varieties. A comprehensive greenhouse study of 27 crop species found that wild progenitors with mycorrhizae had higher colonization and growth while domesticated species with mycorrhizae only had increased higher colonization and growth at low phosphorus availability but not at high phosphorus availability (Martín-Robles *et al.,* 2016). This and other studies (Aghili et al., 2014) indicate that selection during domestication might alter mycorrhizal response to phosphorus fertilization. While reduced association with mycorrhizae appears common, it is unclear how the reduction in symbioses in domesticated plants might affect mycorrhizae-conferred resistance to insects.

Mycorrhizae can alter plant resistance and susceptibility to herbivores, although the effects are highly variable depending on abiotic conditions and the identities of the organisms involved (Gange and West 1994, Pozo *et al.,* 2001, Koricheva *et al.,* 2009). There is ample evidence in a wide range of systems of both mycorrhizae-conferred resistance (Gange and Nice 1997, Gange 2001, Wooley and Paine 2007), and mycorrhizae-conferred susceptibility to herbivores and diseases (Gange *et al.,* 1999, Gange *et al.,* 2005, Babikova 2013). Abiotic

conditions like phosphorus availability can alter mycorrhizae-conferred resistance (Gange *et al.,* 1999, Treseder 2004, Grant *et al.,* 2015). For example, Gange *et al.,* (1999) found that mycorrhizae-conferred resistance at low, but not high, levels of phosphorus fertilization. Mycorrhizae most frequently confer resistance to generalist herbivores and mesophyll-feeding, piercing-sucking insects, while mycorrhizae most frequently confer susceptibility to specialist herbivores and phloem feeders (Koricheva *et al.,* 2009). Furthermore, mycorrhizae confer resistance differently in different plant species. A study of two undomesticated solanum species found that mycorrhizae altered defenses against herbivores in only one of the two species (Minton *et al.,* 2016). It is unclear whether related domesticated and non-domesticated plants show increased susceptibility or resistance to herbivores when colonized by mycorrhizae.

Mycorrhizae alter their host plant's resistance to herbivores through mechanisms including altering expression of defensive compounds, changing host plant nutrient quality, and by making plants more or less attractive to natural enemies. A plant can respond to mycorrhizal colonization by inducing jasmonate and salicylate signalling pathways (Pozo and Azcón-Aguilar 2007, Jung *et al.,* 2012) and thus upregulating the expression of plant defenses controlled by those pathways, such as trypsin protease inhibitors (Song *et al.,* 2013a, Song *et al.,* 2013b) and trichomes (Malik *et al.,* 2018). By increasing the absorption of macro- and micronutrients, mycorrhizae can increase nitrogen and decrease the carbon:nitrogen ratio in their host, making the plant more attractive to herbivores. Finally, mycorrhizal colonization can increase or decrease its host plant's attractiveness to natural enemies, altering predation and parasitism of herbivorous pests (Gange *et al.,* 2003). These changes can alter herbivore growth and reproduction (Babikova 2013, Barazani 2016).

Domestication and association with mycorrhizae can each independently change plant resistance to herbivores. We predicted that domestication has reduced mycorrhizae-conferred resistance to herbivores. Our key goal in this study was to evaluate the generality of mycorrhizae-conferred resistance on 6 pairs of domesticated and undomesticated solanaceous plants. Specifically, we asked the following questions: 1) Has domestication altered the degree to which plants benefit from mycorrhizae? 2) Do domestication and mycorrhizae interact to alter the host plant's physical and chemical resistance traits? 3) How has domestication altered mycorrhizae-conferred resistance to herbivores in the host plants? 4) Because the relationship between mycorrhizae and plants is strongly influenced by phosphorus, we tested if the effects of mycorrhizae on resistance altered based on the availability of phosphorus?

Methods

Design

To test whether domestication has changed mycorrhizae-conferred resistance to herbivores, we used a paired approach with domesticated and undomesticated progenitors of 6 common solanaceous crops. This paired crop design (Martın-Robles 2017) allowed us to reduce noise from interspecific variation in our statistical analyses. Using a fully factorial design, the paired domesticated and undomesticated crops were grown with and without mycorrhizae. Each of these plants was grown with either low, medium, or high phosphorus fertilizer for a total of 72 treatments (6 crops x 2 domestication levels x with or without mycorrhizae x 3 fertilizer treatments) (n=11-12 plants per treatment, 835 total). This experiment was conducted in a single experimental run from July-November 2018.

Plants

We selected 6 agronomically important solanaceous crops: tomato, potato, eggplant, chili, tomatillo, and tobacco. For each solanaceous crop, we obtained seeds of two accessions: one representative of a domesticated genotype, and one of its recognized undomesticated progenitors. For tomatillo (*Physalis philadelphica*), potato (*Solanum tuberosum*), and chili (*Capsicum annum*) we used an undomesticated accession of the same species. For tomato (*S. lycopersicum*), eggplant (*S. melongena*) and tobacco (*Nicotiana tabacum*) we used closest known available undomesticated relatives from a different species *S. pimpinellifolium*, *S. linnaeanum*, and *N. sylvestris* respectively. Detailed information about seed accessions are in Appendix 1.

Sterilization and Germination

We grew the plants in a 1:1 sand and calcined clay media (Turface™). To ensure there were no naturally occurring mycorrhizal communities in the media, we autoclaved the media for one hour 3 times, 24 hours apart at 121°C. To recover soil microbes, which could be have an impact on both plant health (Berendsen *et al.,* 2012 and references therein), mycorrhizal fungi (Desirò *et al.,* 2014), and the interaction between the two, we filtered a mixture of Lambert LM-AP potting soil and water through a 1 micron sieve, and added 20 mL of the resulting solution to each pot. We used potting soil to reduce the risk of introducing pathogenic species.

Seeds were surface sterilized 50:50 water bleach solution with 0.05% Triton-X. Undomesticated and domesticated tobacco seeds were surface sterilized for 4 minutes while all other seeds were surface sterilized for 30 minutes. They were then rinsed and allowed to germinate in petri dishes with moist filter paper and kept in the dark at 21 °C. After germination, the seedlings were transferred to a 10 cm pot filled with autoclaved media.

Mycorrhizal inoculation

Rhizophagus irregularis (previously *Glomus intraradices*) is a commercially available species of mycorrhizal fungi used in agriculture and home gardens. It is highly generalist and will colonize pepper (Aissa *et al.,* 2016, Balog *et al.,* 2017), tomato (Formenti and Rasmann 2000, Pozo *et al.,* 2001 Calvo-Polanco *et al.,* 2014), tobacco (Shaul *et al.,* 1999, Groten *et al.,* 2015, Davis *et al.,* 2019, Song 2019), eggplant (Douds Jr *et al.,* 2017), tomatillo (Gómez and Margarita 2014) and potato (Hijri 2015, Alaux *et al.,* 2018), and has been shown to alter its host's resistance to herbivores in tomato and tobacco (cite). Mycorrhizal inoculum was sourced from Mycorrhizae Premier Tech P-501. After growing for 2 weeks, seedlings were randomly assigned mycorrhizae treatments (~400 plants/treatment) and inoculated with either 0.75 g of 500 spores/g inoculum or 0.75 g triple autoclave sterilized inoculum. The inoculum was suspended in water and pipetted to the base of the seedling and watered down with X vol water.

Fertilization and Harvest

After transplanting, the plants were allowed to grow for 12 weeks at 27 °C in a greenhouse. All pots were kept on top of ½ a petri dish to reduce contamination between pots and to improve water retention. All plants were kept at least 10 cm apart so they were never overshadowing each other.

Plants from each species were randomly assigned to one of three fertilizer treatments: low phosphorus (13-1.3-13), medium phosphorus (13-13-13), and high phosphorus (13-26-13). Plants were fertilized with 40ml of fertilizer diluted to 5 ppm weekly and watered as needed.

At the end of the experiment before harvesting, we recorded the presence and number of buds, flowers, and fruit both to monitor development time and because budding and flowering plants have different defensive strategies such as reduced induction of defenses. The remaining above and below ground biomass was harvested and dried for 3 days at X degrees before being weighed.

Defense traits

Trichomes

Trichomes are hair like structures that form on the surface of leaves and contribute to plant resistance to herbivores in many plants including many solanaceous species. To determine how mycorrhizae and domestication affected trichome expression, we counted trichomes on one leaf from all plants that were fertilized with medium fertilizer (n=172). We used a dissecting microscope at 25x magnification to count the trichomes. We placed an index card with a (6+/-0.5mm) hole punched out of it on top of the leaf, avoiding the midrib and major veins. We recorded the amount of trichomes present in the 6 mm disk. Trichomes were counted on three consistent spots per leaf and the results were averaged.

Protease Inhibitors

We evaluated the effect of domestication and mycorrhizae on plant chemical defense by measuring trypsin protease inhibitor activity. Protease inhibitors are a class of chemical defenses that reduce the digestibility of leaf tissue and can have strong negative effects on chewing herbivores (Mithöfer and Boland 2012). Mycorrhizae have been shown to alter protease inhibitor levels in solanaceous plants (Barazani 2004, Getman-Pickering *et al.,* Getman-Pickering *et al*.).

We excised leaves or leaflets from a consistent place on each crop type in each treatment and immediately froze it on dry ice. We analyzed 100 mg of tissue using a colorimetric assay to calculate the activity of defensive Trypsin Protease Inhibitors using a method adapted from Hegedus *et al.,* (2003).

Protein

As many herbivores are nitrogen limited, their host choice and performance can be dependent on the availability of nitrogen and protein in their host plant's tissue (Mattson 1980, Felton 1996). We excised leaves or leaflets from a consistent place on each crop type from every plant and immediately froze it on dry ice. We analyzed the total leaf protein using one leaf for each plant (mg/g) with a modified version of the ThermoFisher Pierce™ BCA Protein Colorimetric Assay following the manufacturer's instructions.

Herbivore assays

T. ni

We used the second-instar cabbage looper *Trichoplusia ni* (hereafter *T. ni)* caterpillars in a bioassay to measure plant resistance. *T. ni* are generalist Noctuid caterpillars that feed on a wide variety of plant species including Solanaceous and cruciferous crops (ref). These caterpillars were chosen because they can feed on the range of plants used in this study and because they are sensitive to changes in host plant quality. We obtained *T. ni* eggs from a colony maintained on artificial diet at Cornell University for many years. The eggs were hatched, and the larvae were allowed to feed on artificial diet (ref) for three days. The second-instar larvae were weighed and placed in a petri dish on an excised leaf from one of the 6 plant species pairs

on moist filter paper. We placed a single larva on one leaf from every surviving plant in the experiment (n=790). The petri dishes were wrapped in parafilm to prevent desiccation and maintained in a growth chamber at 27°C with an 18/8 light cycle for three days. After three days, we noted survival and weighed the surviving larvae. We measured the leaf area consumed by each *T. ni* larvae using LeafByte (Getman-Pickering *et al.,* 2020).

M. sexta

Tobacco hornworm caterpillars, *Manduca sexta* (hereafter *M. sexta)* are specialist sphingid herbivores that feed on solanaceous plants. They are a common pest of tomato and tobacco in southern parts of the United States and have demonstrated resistance to solanaceous defenses (). The eggs were obtained from a colony maintained on artificial diet at Cornell University for many years. After hatching, the caterpillars were immediately placed onto the excised leaf in a petri dish on moist filter paper. As with the *T. ni*, the petri dishes were wrapped in parafilm to prevent desiccation and maintained in a growth chamber at 27°C with an 18/8 light cycle for three days. After three days, we noted survival and weighed the surviving larvae. We measured the leaf area consumed by each *M. sexta* using LeafByte (Getman-Pickering *et al.,* 2020).

P. maculiventris

Spined soldier bug *Podisus maculiventris* (hereafter *P. maculiventris)* are omnivorous pentatomidae stink bugs. They are highly generalist and feed on a wide variety of both plants and insects. While they will drink phloem and mesophyll from leaves and stem, they do very little damage to their host plant and get most of their calories from consuming insects. Because of this,

they are commonly used as a biocontrol. *P. maculiventris* spends much of its time walking on and feeding on its host plants and is therefore affected by plant defenses more than many other natural enemies (Thaler *et al.,* 2015). Early instars in particular can be harmed and killed by plant trichomes (tiny hairs on the leaf surface). Previous work has found that trichomes can limit, incapacitate or kill early instar *P. maculiventris* nymphs (Lambert 2007). However, it is unknown how mycorrhizae and domestication have altered the host plant quality for *P. maculiventris* growth.

We conducted a bioassay to determine the effect of mycorrhizae and domestication on the omnivorous natural enemy *P. maculiventris*. Due to time and funding constraints, we chose a single fertilizer level to analyze the effects of domestication and mycorrhizae on trichomes and *P. maculiventris* nymphs. This assay was only conducted on plants that were fertilized with medium phosphorus fertilizer. When the plants were ready to be harvested, we removed a leaf from the middle of a plant sample, placed it inside of a labeled 9 cm petri dish, on top of moist filter paper. Twelve hours before beginning a bioassay, we placed second-instar *P. maculiventris* nymphs in deli cups with a moist cotton ball for water so they were ready to plant-feed the next day.

After about 12 h of starving, *P. maculiventris* nymphs were weighed. We then used a paint brush to carefully place a single nymph on the center of each leaf, near the stem. Every 2 h for twelve hours we checked the location and recorded if they were on the top or bottom of the leaf or if they were on the petri dish to determine if plant defenses might deter the *P. maculiventris* nymphs from being on the plant. After 12 h we weighed the nymph and stored each leaf in the -80°C freezer for trichome counting.

Mycorrhizal Quantification

Mycorrhizal colonization can be variable, and the extent of colonization may impact herbivore performance (CITE). Samples of root tissue were cut and placed in micromesh biopsy cassettes. To clear the roots, they were bleached in a boiling 10% KOH solution for three minutes, rinsed under running water for one minute (ref). To dye the bleached roots, the micromesh biopsy cassettes were then boiled for three minutes in a 5% ink-vinegar solution (Sheaffer Skrip Bottled Ink, Black). For each plant, ten 1cm segments of root were randomly selected and mounted on slides for quantification with microscopy.

Statistics

All statistics were conducted using R version 3.5.2. Linear models and generalized linear models were performed using lme4 (Bates *et al.,* 2004). In all models testing the effects of mycorrhizae and domestication on plant growth and resistance to herbivores we included mycorrhizae, domestication, fertilizer, and crop as interacting fixed effects. Four way interactions were removed from all models. Post-hoc pairwise comparisons were conducted using lsmeans (Lenth 2019), and pairwise comparisons of crops were corrected using FDR.

To determine the interacting effects of domestication, mycorrhizae, and fertilizer on plant growth, we analyzed total biomass, above ground biomass, below ground biomass, and probability of having flowered by the end of the study. Above and belowground biomass data was square root transformed to meet assumptions of homoscedasticity. We analyzed biomass using a linear model with the fixed effects outlined above.

To determine the interacting effects of domestication, mycorrhizae, and fertilizer on resistance traits, we analyzed protease inhibitor activity, trichomes and protein content. Protein

data were square root transformed to fit assumptions of normality. We analyzed protease inhibitor activity and protein using an LM. We analyzed abundance of trichomes using a GLM with a poisson distribution. In all models, we included the fixed effects outlined above.

To determine the interacting effects of domestication, mycorrhizae, and fertilizer on resistance to herbivores, we analyzed the leaf area consumed, survival, and weight change, for *T. ni and M. sexta* caterpillars and percent weight change of *P. maculiventris* as response variables. *T. ni* and *M. sexta* survival was extremely low on both and tomatillos, so both domesticated and undomesticated plants of these crops were excluded from herbivore performance analyses. *M. sexta* leaf area consumed and weight, and *T. ni* leaf area consumed data was square root transformed and *T. ni* weight change data was log transformed to meet assumptions of normality and homoscedasticity. Leaf area consumed and weight change for both *T. ni* and *M. sexta* were analyzed using an LM. Survival of *T. ni and M. sexta* caterpillars was analyzed using a GLM with a binomial distribution. *P. maculiventris* weight change data were log transformed to meet assumptions of homoscedasticity and normality and then analyzed using an LM. Because we only ran this bioassay on plants that received medium levels of phosphorus in their fertilizer, we did not include fertilizer in the model. We also excluded any data points from *P. maculiventris* that molted during the experiment as molting nymphs do not feed and the molting process will change their weight. Pairwise comparisons were analyzed using lsmeans.

To test the mechanisms by which domestication and mycorrhizae might alter resistance to herbivores, we analyzed the effect of plant defense traits on herbivore performance including *T. ni* and *M. sexta* survival, weight change, and leaf area consumed and *P. maculiventris* percent growth. In each of these models, unlike previous models, we used a linear mixed effects model and included protease inhibitors, protein levels, and the interaction between them as fixed effects

and crop as a random effect. For the models looking at *M. sexta* and *T. ni* weight change and leaf area consumed, the response variable was square root transformed to meet assumptions of homoscedasticity. The effects of these plant defense traits on caterpillar survival was analyzed using a GLMER with a binomial distribution.

Results

Plant growth traits

When undomesticated plants were grown with mycorrhizae, they were 13% smaller aboveground (t-ratio=2.69, df=546, p=0.008, Fig 3.1A) and 21% smaller belowground (t-ratio=2.63, df=542, p=0.0087, Fig 3.1B) than plants grown without mycorrhizae. On the other hand, domesticated plants grown with mycorrhizae were not substantially different aboveground (t-ratio=1.16, df=546, p=0.247, Fig 3.1A) or belowground (t-ratio=1.11, df=542, p=0.269, Fig 3.1B) compared to those grown without mycorrhizae. The effects of mycorrhizae and domestication were marginally dependent on fertilizer, with mycorrhizae suppressing growth most in undomesticated plants that were given low or high phosphorus fertilizer ($F_{2,503}$=2.63, p=0.073). Overall, domesticated plants were 29% larger than undomesticated plants ($F_{1,503}$=54.0, p<0.001), with domesticated eggplant, pepper, tobacco, and tomato growing larger than their undomesticated counterparts, while tomatillo and pepper grew to similar sizes to their wild counterparts ($F_{5,503}$= 3.70, p=0.002).

Domesticated pepper, tomatillo, and tomato plants were more likely to have flowered by the end of the experiment than their undomesticated versions (z-score=-6.168, p<0.001). Potato, chili and eggplant hadn't flowered at all by the end of the experiment regardless of domestication

status. In both domesticated and undomesticated plants, mycorrhizae and fertilizer interacted to affect flowering time such that mycorrhizal plants were less likely to flower by the end of the study if they were given high phosphorus fertilizer (z-score=-1.97, p=0.049).

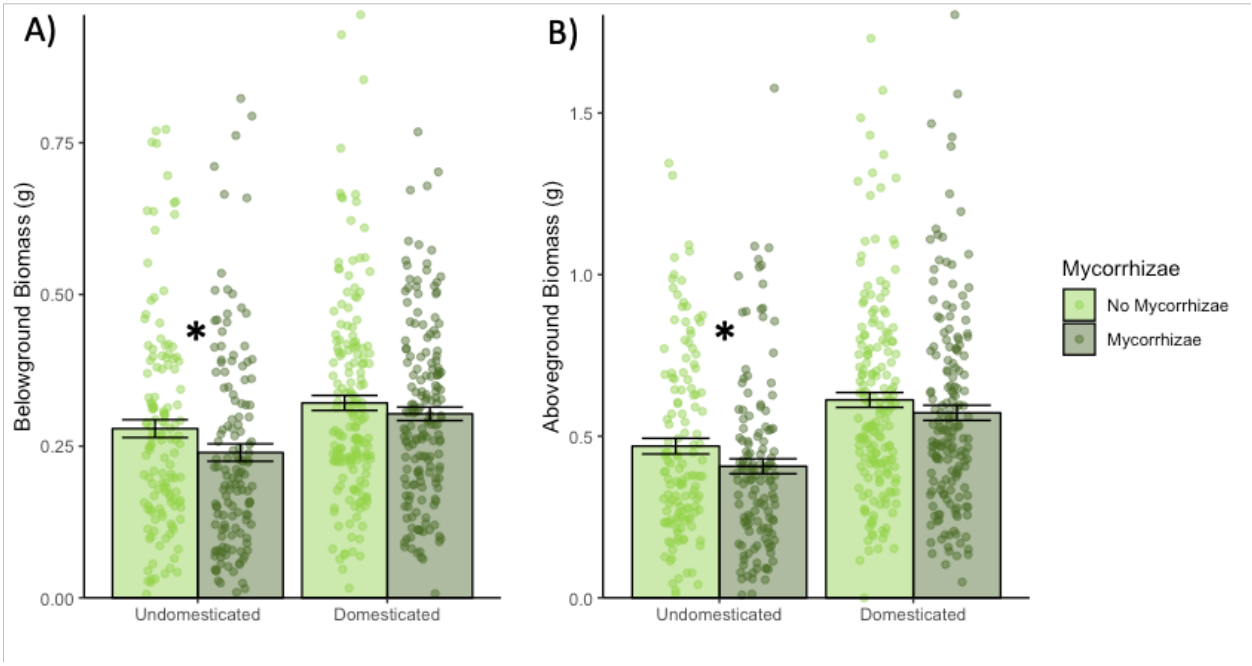

Fig 3.1. The effect of domestication and mycorrhizae on A) belowground biomass and B) aboveground biomass. Domesticated plants were significantly larger than undomesticated plants above- and belowground, and mycorrhizal plants were significantly smaller than non-mycorrhizal plants above- and belowground. Bars represent mean +/- SE. Dots represent the distribution of data points.

Plant resistance traits

Protease Inhibitors

Mycorrhizae increased protease inhibitor activity in undomesticated tobacco (z-score=4.01, $p<0.001$), tomato (z-score=-4.62, $p<0.001$), and pepper (z-score=8.25, $p<0.001$), but had no effect in any of the domesticated plants (z-score=-0.003, $p=0.997$). Overall, domesticated plants had 25% lower protease inhibitor activity than undomesticated plants ($F_{1,234}=11.8$, $p<0.001$, Fig. 3.2A).

Protein

We found no effect of mycorrhizae on leaf protein ($F_{1,393}=0.561$, $p=0.454$ Fig. 3.2B). The effects of domestication on leaf protein were dependent on crop, with domestication increasing leaf protein in tobacco (z-score=-3.542, df=393, $p=0.002$) and decreasing it in eggplant (z-score=-2.559, df=393, $p=0.033$). Crops differed in how fertilizer altered leaf protein ($F_{10,393}=2.102$, $p=0.023$) with fertilizer increasing protein in eggplant, pepper, tomatillo, and potato leaves. Fertilizer did not affect protein in tobacco and tomato leaves.

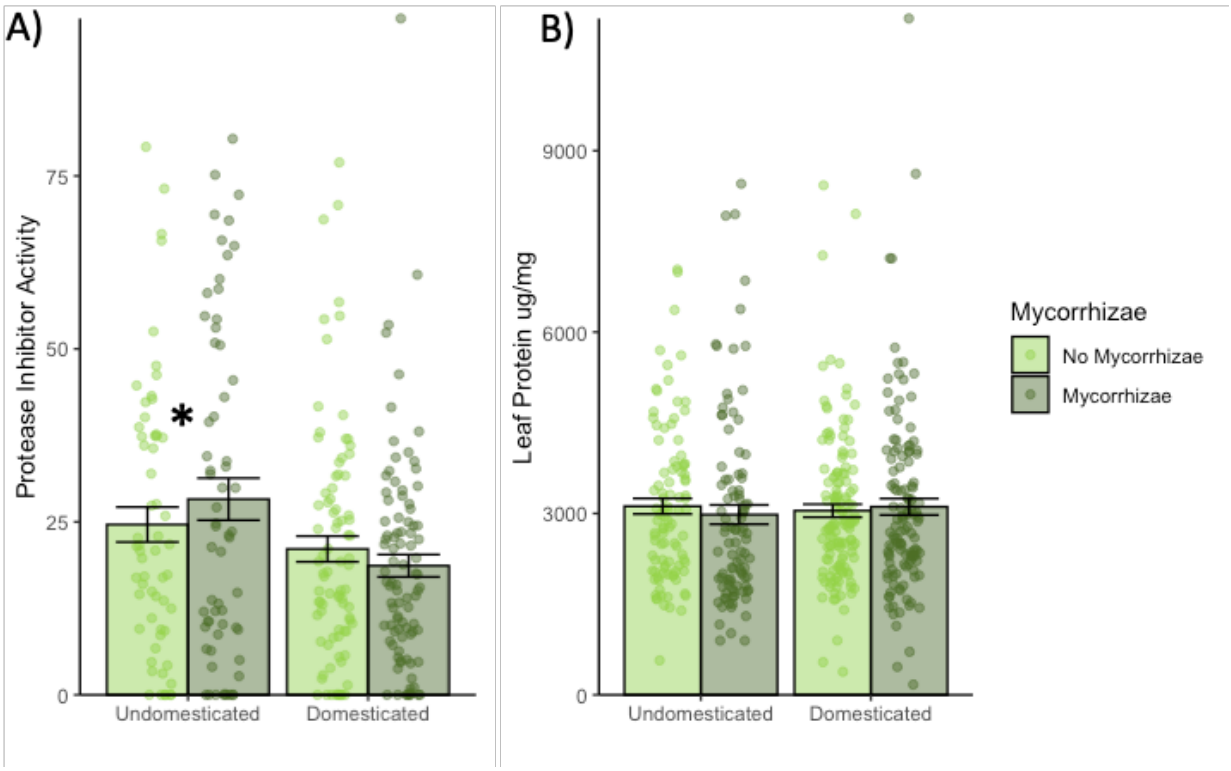

Fig 3.2 The effect of domestication and mycorrhizae on A) protease inhibitor activity as measured by percent inhibition and B) leaf protein. Bars represent mean +/- SE. Dots represent the distribution of data points. Asterisks denote significance.

Trichomes

The effect of domestication on mycorrhizae-conferred changes in trichome abundance was highly crop dependent, with mycorrhizae increasing total trichomes in domesticated tobacco (z-score=-4.15, $p<0.001$), domesticated potato (z-score=-5.00, $p<0.001$), and undomesticated potato (z-score=-7.01, $p<0.001$), and decreasing trichomes in domesticated eggplant (z-score=13.263, $p<0.001$).

Plant resistance to herbivores

T. ni

Mycorrhizae increased *T. ni* leaf area consumption in undomesticated plants (t-ratio=-2.49, df=126, p=0.014), but had no effect in domesticated plants (t-ratio=0.7587, df=126, p=0.56) (interaction $F_{1,03}$=8.42, p=0.005, Fig. 3.3A). In plants without mycorrhizae, *T. ni* that fed on domesticated plants ate twice as much as those that fed on undomesticated plants ($F_{1,103}$=8.25, p=0.004). There were also no effects or interacting effects of phosphorus fertilizer on *T. ni* leaf area consumed ($F_{2,103}$=1.05, p=0.352). *T. ni* fed more on tomato, tobacco and, potato compared to eggplant ($F_{1,103}$=8.25, p=0.005). While there was a marginal interaction between crop and fertilizer ($F_{4,103}$=2.31, p=0.063), there were no significant pairwise differences.

Domestication did not alter mycorrhizae-conferred resistance as measured by *T. ni* caterpillar weight gain ($F_{1,178}$=1.19, p=0.277, Fig. 3.3B). However, there was a significant interaction between mycorrhizae and crop on weight gain($F_{2,178}$=3.16, p=0.045), but there were no significant pairwise differences. Neither domestication (z-score=0.461, p=0.645) nor mycorrhizal status (z-score=-0.479, p=0.632) of the host plant altered *T. ni* caterpillar survival.

T. ni consumed more leaf area on plants that had higher levels of protein in the leaves ($F_{1,55}$= 4.48, p=0.039). *T. ni* had higher survival on plants that had higher levels of protein and lower levels of protease inhibitors (z-score=3.48 p<0.001). Neither protease inhibitors ($F_{1,86}$= 0.867, p=0.354), nor protein content of the leaves ($F_{1,86}$= 0.911, p=0.343) altered *T. ni* weight gain.

M. sexta

The effects of domestication on mycorrhizae-conferred resistance to *M. sexta* feeding varied marginally by crop ($F_{5,395}$=2.12, 0.062), although the effects of mycorrhizae were not

significant in pairwise comparisons. Overall, *M. sexta* that fed on domesticated plants ate 20% more than those that fed on undomesticated plants ($F_{1,395}$=7.29, 0.007).

The effects of domestication on mycorrhizae-conferred resistance to *M. sexta* weight gain were dependent on fertilizer. Mycorrhizae suppressed caterpillar weight gain on undomesticated plants given medium phosphorus fertilizer (t-ratio=-2.23, df=346, p=0.026, Fig. 3.3C) and marginally suppressed weight gain on plants given high phosphorus fertilizer (t-ratio=-1.83, df=346, p=0.068). In contrast, mycorrhizae increased caterpillars weight gain on domesticated plants at medium levels of phosphorus fertilizer (t-ratio=2.808, df=346, 0.005).

Neither protein nor protease inhibitor levels explained *M. sexta* leaf area consumed, growth, or survival.

P. maculiventris

Mycorrhizae suppressed *P. maculiventris* weight gain in undomesticated plants (t-ratio=2.12, df=139, p=0.036), but had no effect on *P. maculiventris* weight gain in domesticated plants (t-ratio=0.726, df=139, p=0.469, Fig. 3.3D). *P. maculiventris* weight change was also dependent on crop type with *P. maculiventris* gaining more weight on potato and tomatillo plants, and losing weight on eggplant, pepper, and tobacco ($F_{4,139}$=4.699, p=0.001).

P. maculiventris gained the most weight on plants that had high levels of protein and low levels of trichomes ($F_{1,13}$=6.31, p=0.027). There was a marginal interactive effect of ($F_{1,14}$=4.3730, p=0.060) between protein and protease inhibitors such that *P. maculiventris* gained the most weight on plants that had high levels of protein and lower levels of protease inhibitors.

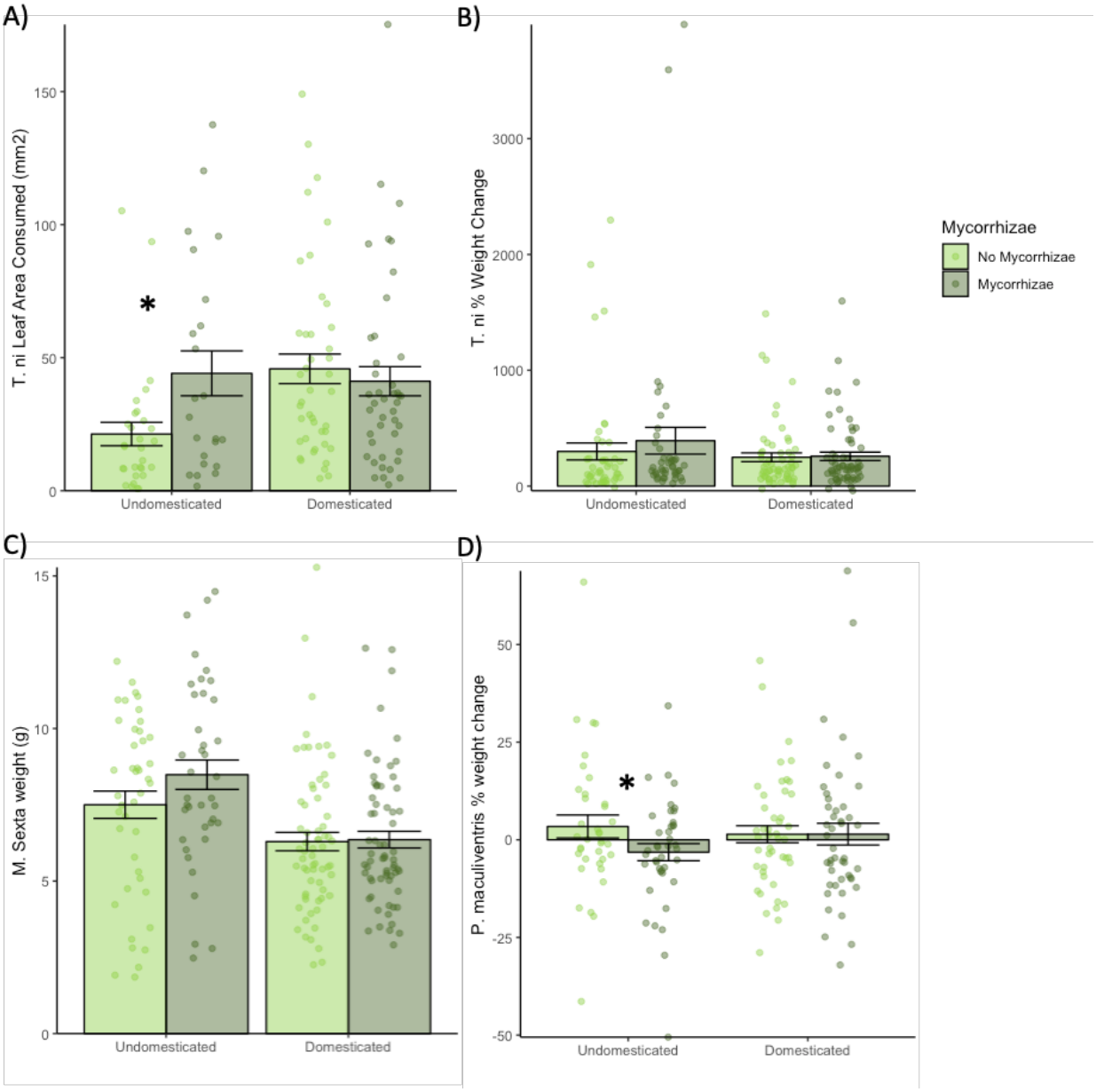

Fig 3.3. The effect of domestication and mycorrhizae on A) the amount of leaf tissue *T. ni* caterpillars consumed, B) *T. ni* percent weight gain C) *M. sexta* weight gain and D) *T. ni* percent weight change. Bars represent mean +/- SE. Dots represent the distribution of data points. Asterisks denote significance.

Discussion

Domestication can alter plant-mutualist associations. However, key knowledge gaps remain in how domestication can alter mycorrhizae-conferred resistance to herbivores. Here we showed that mycorrhizae changed the growth and resistance of their undomesticated host plants much more than domesticated host plants. We found that:

1) Mycorrhizae reduced plant biomass in undomesticated, but not domesticated, plants and reduced flowering in both.

2) Mycorrhizae increased the amount of defensive protease inhibitors in undomesticated plants but had no effect on domesticated plants. Mycorrhizae altered trichomes in both domesticated and undomesticated plants in a crop-specific manner.

3) Domestication has altered mycorrhizae-conferred resistance to herbivores. Mycorrhizae increased feed by *T. ni* and reduced weight gain by *P. maculiventris* in undomesticated, but not domesticated, plants. Mycorrhizae also suppressed weight gain by *M. sexta* in undomesticated plants, but increased it in domesticated plants.

4) The effect of mycorrhizae on growth, *M.sexta* weight gain, and protein content was dependent on phosphorus levels.

Mycorrhizae reduced plant biomass in undomesticated plants

Mycorrhizae reduced the above- and belowground biomass of their plant partners in undomesticated plants but not in domesticated plants. Our results provide further evidence against the mutualistic paradigm that mycorrhizae always improve plant growth. Mycorrhizae can reduce the biomass of their hosts (Stribley, Tinker and Rayner 1980, Wilson and Hartnet 1997, Treseder 2013, Stanescu and Maherali 2017), ostensibly through the carbon cost (Olson *et*

al., 2010). Plants can give up to 20% of their carbon-rich photosynthate to mycorrhizae (Hobbie and Hobbie 2016), and this cost may outweigh the nutritional benefits they get in return. There is also ample evidence that mycorrhizae can suppress belowground biomass (Wurst *et al.,* 2002) and decrease the root to shoot (Veresoglou *et al.,* and citations therein), although this is often attributed to the fact that mycorrhizae act as a secondary root system, reducing the need for the host plant to invest in belowground root tissue. In our system, both mechanisms may be at play as mycorrhizae suppressed growth belowground more dramatically than aboveground, but also significantly suppressed aboveground tissue.

Mycorrhizae increased physical and chemical resistance traits and resistance to herbivores

T. ni that fed on mycorrhizal undomesticated plants fed more but did not gain more weight. *P. maculiventris* that fed on these mycorrhizal undomesticated plants actually lost weight on average, while those that fed on non-mycorrhizaldomesticated plants gained weight (Fig 3.4). In undomesticated plants, mycorrhizae suppressed *M. sexta* weight gain, though only in plants that received medium or high fertilizer. We pose that, in undomesticated plants, mycorrhizae-conferred increases in protease inhibitors forced *T. ni* to feed more to maintain their growth, and protease inhibitors and trichomes deterred *P. maculiventris* from feeding. While mycorrhizae did not alter protein levels in either undomesticated or domesticated plants, both *T. ni* and *P. maculiventris* also fed more on plants with higher levels of protein. This conforms to previous research showing that herbivores feed more when there are higher levels of nitrogen-rich protein in their diet, but it suggests that this is not a mechanism by which mycorrhizae alter herbivore performance. Our study suggests that mycorrhizae confer resistance in undomesticated plants

through increasing chemical and physical defenses rather than through changing nutritional quality.

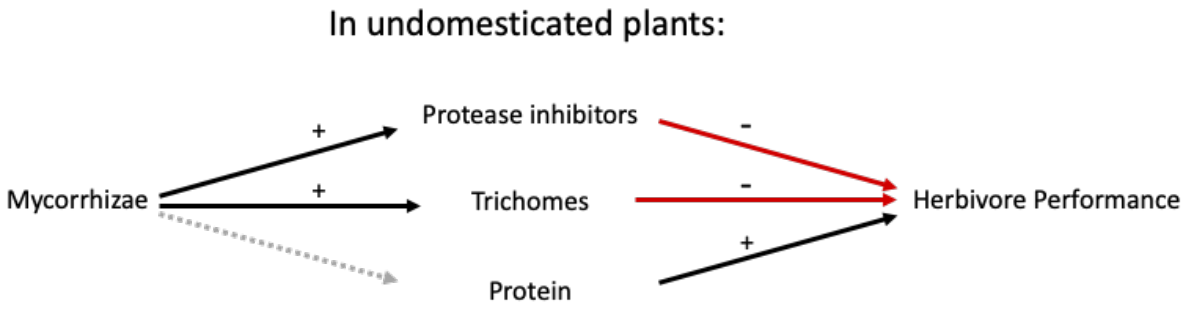

Fig 3.4. In undomesticated plants, mycorrhizae increase protease inhibitors and trichomes but not protein. These adversely affect herbivore performance. Solid arrows represent significance. Red arrows represent a negative effect and black arrows indicate a positive effect.

Our study builds on previous work by Martin Robles *et al.,* (2017) and Xing *et al.,* (2012) by demonstrating that, in addition to effects on growth and symbiosis, domestication has also led to changes in how mycorrhizae affect plant-herbivore interactions. Mycorrhizae differentially altered resistance to all three herbivores in domesticated and undomesticated plants. *T. ni* caterpillars fed more but didn't gain a commensurate amount of weight, while *M. sexta* and *P. maculiventris* gained less weight and lost weight respectively. We were surprised by the consistency of this negative effect. A review by Koricheva *et al.,* (2009) found that specialist (mono- and oligotrophic) chewing herbivores were more likely to benefit from mycorrhizae, while generalist chewing herbivores and piercing sucking herbivores were more likely to be negatively affected. Our results with *P. maculiventris* and *T. ni* support that mycorrhizae can negatively affect piercing sucking herbivores and generalist chewing herbivores respectively.

However, the specialist chewing herbivore *M. sexta* was also negatively affected on undomesticated plants, contradicting the prevailing patterns that they benefit, or at least are not affected.

Mycorrhizae have the potential to shape herbivore communities, not only through altering plant defenses, but also by changing their host plants' suitability to natural enemies of herbivorous pests (Gange *et al.,* 2003, Hempel *et al.,* 2009). *P. maculiventris* are a species of omnivorous stink bugs that are used for biocontrol of herbivores in agricultural systems. In our system, *P. maculiventris* nymphs lost mass on undomesticated plants with mycorrhizae but gained mass on undomesticated plants without mycorrhizae. However, this assay was performed in closed petri dishes with no prey, giving us limited ability to extrapolate to natural systems. More research is necessary to determine how mycorrhizae impact pest control by *P. maculiventris* and other predators that spend large amounts of time on the plant.

Previous work by Martin Robles *et al.,* (2017) found that domestication reduced the plant's ability to associate with mycorrhizae. The loss of symbiosis between mycorrhizae and domesticated plants has previously been attributed to fertilization regimes that might make associating with fungus unnecessary, as well as selective pressure to allocate carbon resources to growth instead of providing it to fungal symbionts. We suggest that the symbiosis has been further disrupted by changes in plant defensive chemistry and changes in pest pressure. Our results and previous studies found that domesticated plants have lower levels of defensive secondary metabolites and hormones (Whitehead *et al.,* 2017). We suggest that decreases in secondary metabolites and defensive chemistry may indirectly reduce the plant's ability to

associate with the fungus. It is well established that the jasmonate and salicylate pathways have a role in controlling both resistance traits and associations with fungal mutualists (Herrera Medina *et al.,* 2003, Tejeda-Sartoriu *et al.,* 2008). By increasing pest control and actively selecting for plants with lower levels of unpalatable defenses, it is possible that humans have selected plants that are less well defended and also less able to associate with belowground mutualists like mycorrhizae.

Domestication reduced resistance traits and resistance to herbivores

It is widely accepted that domesticated plants are larger and have less pest resistance than undomesticated plants (as reviewed by Whitehead *et al.,* 2017). Our results support this, with domesticated plants growing larger, having lower defensive protease inhibitor activity, and increased feeding from *T. ni* and *M. sexta*. There is much debate as to the selective forces that have driven this lowered resistance. Potentially, selection against unpalatable defenses and selection for increased growth and yield both may result in less defended plants. Alternately, selection for more nutritious plants may have resulted in plants that are more attractive to herbivores. Our results lend credence to the hypothesis that selection for larger, less well defended plants has reduced resistance to herbivores, as our domesticated plants were larger and had lower levels of protease inhibitors but no difference in protein content.

Phosphorus altered mycorrhizae-conferred growth and resistance

Application of phosphorus fertilizer can shift the mycorrhizae-plant mutualism (Gange 1999, Grant *et al.,* 2005). Mycorrhizae often confer benefits at low levels of phosphorus availability and confer no benefits or negative effects at high levels of phosphorus (Schroeder,

and Janos 2005, Hoeksema *et al.*, 2010, Sheng *et al.*, 2013). There is limited evidence that extremely low levels of phosphorus can also lead to negative effects of mycorrhizae (Carling *et al*., 1995). When comparing the effects of mycorrhizae and fertilizer on domesticated and undomesticated plants, Martin Robles *et al.,* (2017) found that mycorrhizae increased growth of undomesticated plants regardless of phosphorus fertilizer, but only increased the growth of domesticated plants at low and medium phosphorus fertilization. In our study, in undomesticated plants, mycorrhizae suppressed biomass more strongly at low and high levels of phosphorus and lowered *M. sexta* weight gain in the medium phosphorus fertilizer treatment. Conversely, in domesticated plants, mycorrhizae increase *M. sexta* weight gain in the medium phosphorus fertilizer treatment. This supports the results of our previous study (Getman-Pickering *et al.,* 2020), which show that there is a disconnect in the effects of mycorrhizae on growth and defense. Our previous study tested the effects of mycorrhizae at different levels of fertilization and found that similarly mycorrhizae affected plant growth in the low and high fertilizer treatments but had no effect on plants given a medium amount of fertilizer.

Conclusion

Our comparative approach of 6 domesticated and undomesticated solanaceous crop plants has shown that domestication has altered mycorrhizae-conferred resistance to herbivores and natural enemies. The lack of effect of mycorrhizae in domesticated plants supports previous studies that find a breakdown in the plant-mycorrhizae mutualism in domesticated plants. These results, in combination with the finding that mycorrhizae suppressed plant growth, demonstrate the limitation of mycorrhizae as an agricultural tool. Keeping in mind the multi-trophic effects of

mycorrhizae will be important in selecting for plants and fungi that form more mutualistic relationships.

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Chapter 3 Appendix: Plant varieties

Varieties-
Tobacco domesticated-Ontario light tobacco (nicotiana tabacum CT157) from Richters S6492-800g

Tobacco undomesticated-nicotiana sylvestris Woodland Tobacco 346 from Select Seeds-antique wildflowers
Tomatillo-undomesticated-physalis philadelphica
USDA ARS PGRU (NE9) Geneva NY Lot PI 51200697SD
Tomatillo-physalis philadelphica domesticated-Lot 60023 Johnnys selected seeds Toma Verde
Pimpinellifolium-USDA ARS PGRU (NE9) Geneva NY Lot-PI 126939 55AI
Tomatoes-Var Early cherry From Martha Muchler bulked up 7/18/18 closest bred relative to pimpinellifolium
Chili domesticated-cayenne pepper from nick
Chili wild-accession 50010 capsicum chacoense from New Mexico State University capsicum
Chili wild-C. Annum var glabriusculum from the chili project (originally from Oaxaca) New Mexico State University capsicum accession 10101
Eggplant wild-Solanum linnaeanum-PI 388846 01 SD from USDA ARS
Eggplant domesticated-Orient Express F1 hybrid Asian eggplant Solanum melongena (354.51 lot 59206) from Johnny's selected seeds.

APPENDIX 1

LEAFBYTE: A MOBILE APPLICATION THAT MEASURES LEAF AREA AND HERBIVORY QUICKLY AND ACCURATELY

Zoe L. Getman-Pickering†[1], Adam T. Campbell†[2], Nicholas Aflitto[1], Todd A. Ugine[1], Ari Grele[3], Julie Davis[1]

[1]Department of Entomology, Cornell University, Ithaca, New York 14853, United States
[2]The Blue Folder Project
[3]Department of Biology, University of Nevada, Reno, NV, 89557, United States
†These authors have contributed equally to this work

Abstract

1. In both basic and applied studies, quantification of herbivory on foliage is a key metric in characterizing plant-herbivore interactions, which underpin many ecological, evolutionary, and agricultural processes. Current methods of quantifying herbivory are slow or inaccurate. We present LeafByte, a free iOS application for measuring leaf area and herbivory. LeafByte can save data automatically, read and record barcodes, handle both light and dark colored plant tissue, and be used non-destructively.

2. We evaluate its accuracy and efficiency relative to existing herbivory assessment tools.

3. LeafByte has the same accuracy as ImageJ, the field standard, but is 50% faster. Other tools, such as BioLeaf and grid quantification, are quick and accurate, but limited in the information they can provide. Visual estimation is quickest, but it only provides a coarse measure of leaf damage and tends to overestimate herbivory.

4. LeafByte is a quick and accurate means of measuring leaf area and herbivory, making it a useful tool for research in fields such as ecology, entomology, agronomy, and plant science.

Introduction

The amount of leaf tissue consumed, hereafter "herbivory", is a fundamental metric used to understand plant-herbivore interactions in many disciplines spanning basic and applied science, including plant chemistry, plant-insect ecological and evolutionary dynamics, plant breeding, agronomy, and horticulture (Turcotte et al. 2014). Insect herbivores do billions of dollars of damage to crops every year (Bradshaw et al. 2016), often with devastating consequences, making pest control strategies a vital area of research. However, efficiently and accurately measuring damage remains challenging (Williams et al. 1991).

Herbivory from chewing insects is measured with software such as ImageJ (Abràmoff et al. 2004), mobile apps such as BioLeaf (Machado et al., 2016), and manual methods such as grid quantification (Coley 1983) or visual estimation (Johnson et al. 2016). While all of these methods have advantages, there is significant room for improvement. One of the most commonly used options, the image processing program ImageJ, is accurate but not optimized for measuring herbivory, and is therefore incredibly time-consuming. Images must be scanned or photographed, saved on a computer, and then uploaded, which is also slow. The mobile app BioLeaf (Machado et al., 2016) allows for quick and efficient measurements of herbivory. However, it only measures percent herbivory, not absolute measurements. Grid quantification entails placing a grid under a damaged leaf and counting the number of squares where an herbivore removed leaf tissue (Coley 1983). While measuring small amounts of herbivory is straightforward, measuring large amounts of herbivory or leaf area can be prohibitively slow. Finally, visual estimation of herbivory is quick but often sacrifices accuracy (Johnson et al. 2016).

We introduce LeafByte, a free and open source mobile app that solves common issues with the current tools and provides additional features. LeafByte can scan barcodes, measure

light colored petals or leaves, and save results (with the date, time, and GPS coordinates) to a spreadsheet on the phone or on Google Drive. LeafByte can be used non-destructively. We present a systematic comparison of the accuracy and efficiency of LeafByte and four of the most common herbivory measurement tools: ImageJ, BioLeaf, grid quantification, and visual quantification.

Methods

How LeafByte works

Users take or upload an image of a leaf surrounded by 4 dots in a square that act as a scale (see Supporting Information 1). LeafByte identifies the leaf and scale markings by separating the foreground of the image from the background in a process called "thresholding" (Otsu, 1979). Each pixel in the image is considered individually. If the luma of the pixel's color, a measure of perceived intensity (ITU-R, 1982-2015), is above a certain cutoff value (the "threshold"), that pixel will be considered foreground; otherwise, it becomes background. Because the leaf and scale markings are much darker than the background (typically a green leaf and black scale markings on white paper), they are marked as foreground, while the rest is marked as background. LeafByte also supports light tissue (such as white flowers) against dark backgrounds by simply reversing the process.

LeafByte separates foreground from background using an algorithm called Otsu's method (Otsu, 1979). Otsu's method considers a histogram of lumas in the image. This histogram is typically bimodal, with a mode of high luma, representing the leaf and scale markings, and a mode of low luma, representing the background. Otsu's method finds a luma that most clearly separates those two modes, effectively distinguishing foreground from background. This automatically-

determined threshold is generally effective, but LeafByte allows users to tweak as needed (Fig. 1A).

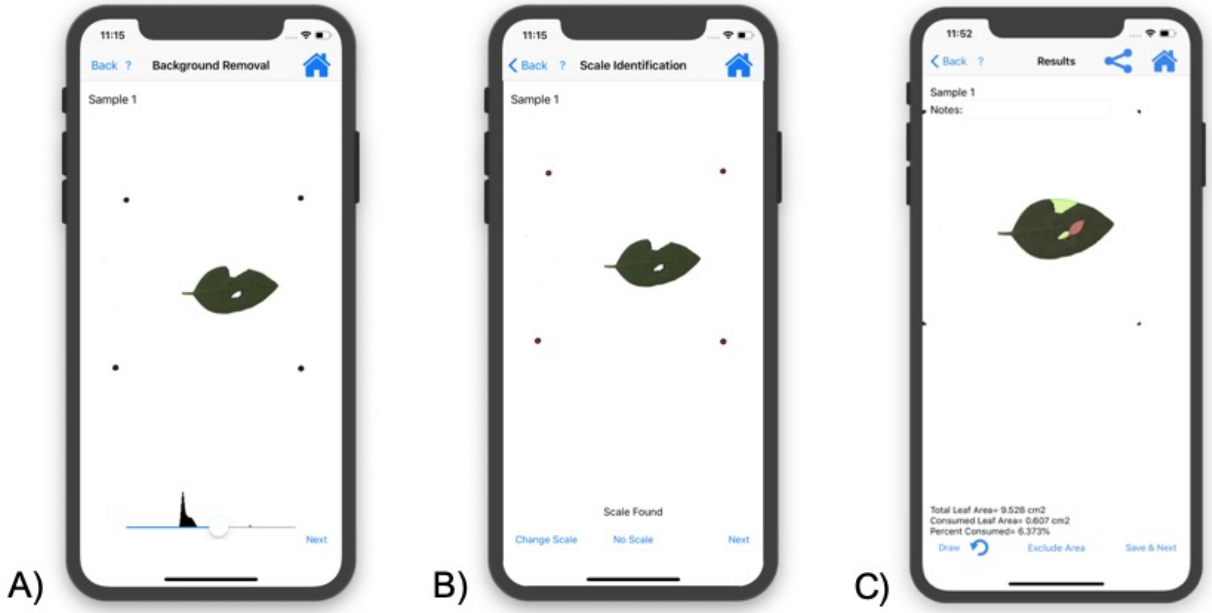

Fig 4.1. Screenshots of the main flow of the app. Once a picture is captured or selected, users (a) remove the background, (b) confirm that the scale is properly identified and adjust if needed, and (c) draw in missing leaf margins

Next, LeafByte determines what pixels represent the leaf and scale markings using an algorithm called connected-component labeling (Rosenfeld & Pfaltz, 1966) to separate pixels into groups representing different objects. LeafByte assumes that the largest group is the leaf, and the next four largest are the scale markings. This is right in most cases, and when it is not, the user can correct LeafByte's assumption by manually identifying scale markings (Fig. 4.1 B).

If the image was taken at an angle, the scale markings no longer form a square, and the leaf is distorted, causing error (Supporting Information 2). To correct this skew, LeafByte uses a technique called planar homography (Wang, Klette, & Rosenhahn, 2006) to re-distort the image

so that the scale markings once again form a square (Fig 2). LeafByte uses connected-components labeling again on background pixels to identify the holes within the leaf.

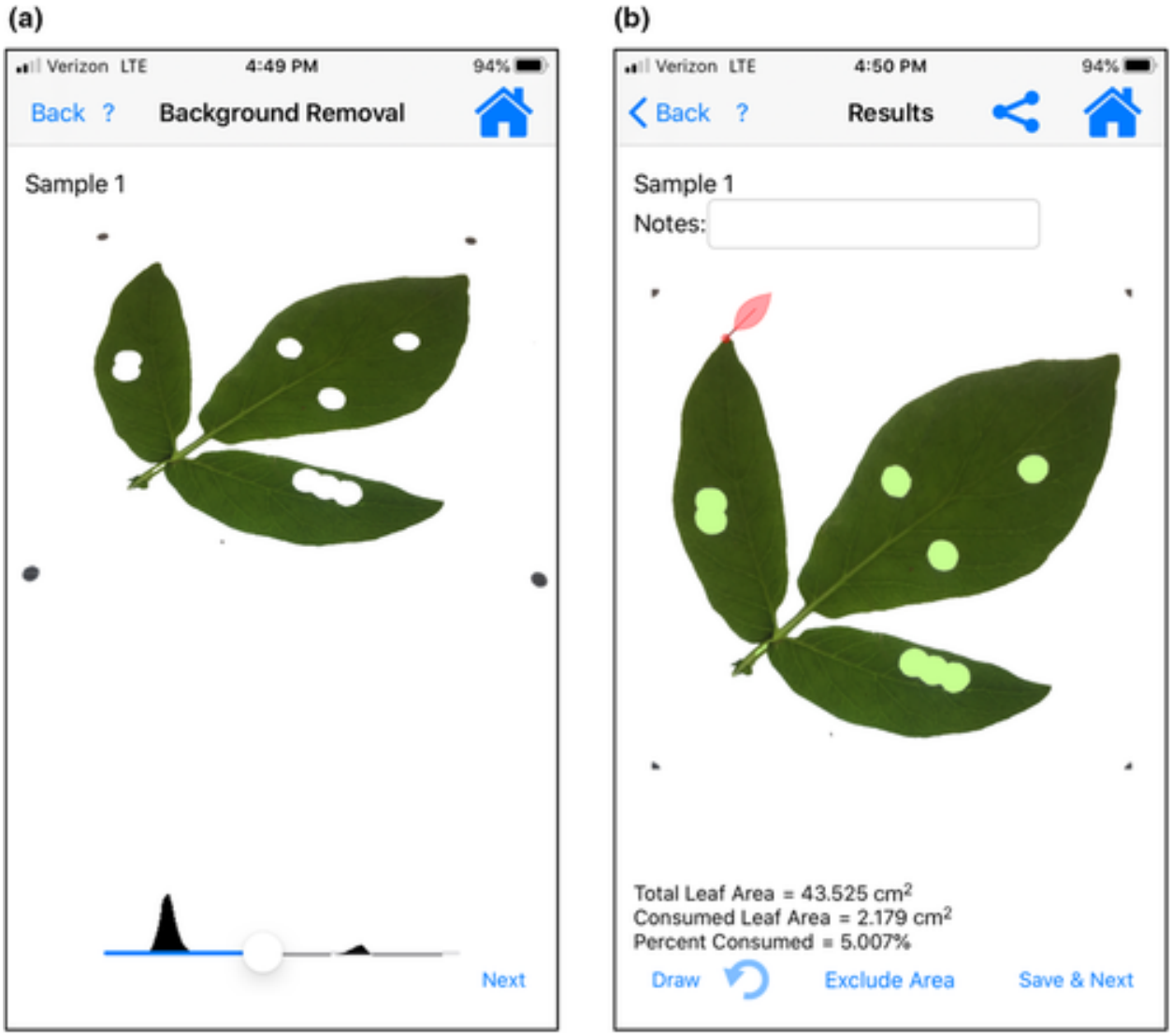

Fig 4.2. A leaf photographed at a 30° angle (a) before and (b) after skew is corrected by planar homography.

The user can draw missing margins onto the leaf image (Fig. 4.1C). Then, counting the number of pixels in the leaf and in the holes gives the relative amount of leaf eaten. Summing the number of pixels in the leaf and the holes gives the total size of the original leaf in pixels. Because there

is a known distance between each scale mark, LeafByte can convert numbers of pixels into real world units. The photo and results are saved in a CSV file to Google Drive or the phone.

Methods for Testing LeafByte

Accuracy

To confirm the accuracy of ImageJ and LeafByte, we used both methods to measure artificial "leaves" of known area. We printed out 16 black rectangles of known area with white "holes" of known size and analyzed them with both LeafByte and ImageJ, compared their results to the known area.

Precision

We tested if different researchers analyzing the same leaves got the same results. To create herbivory, we excised thirty *Solanum tuberosum* leaves and allowed a single first instar *Leptinotarsa decemlineata* larvaeto feed for 24 hours. Three independent researchers measured leaf area and herbivory using LeafByte.

Comparisons of different methods

We collected 67 leaves from 14 plant species (Supporting Information 3) from the Cornell Botanical Garden and grounds. Leaves were selected to represent a range of morphologies and were categorized by shape and margin type. If the leaf was undamaged, we created simple and complex artificial herbivory using hole punches and razor blades to remove 0-50% of the leaf. We recorded whether the leaf was damaged on the margin (n=36) or only internally (n=22). Herbivory was estimated visually and using grid quantification with $2mm^2$

grid paper (Coley 1983). For visual estimation, herbivory was estimated to the nearest 5%. Leaves with 0-2.5% herbivory were rounded to 5%. The leaves were then flattened between a sheet of printer paper with the scale printed on it and a Premium Matte Film Shield Screen Protector (J&D, Middleton, MA) and photographed. Each photograph was analyzed using LeafByte, BioLeaf, and ImageJ by at least two different researchers per method. LeafByte and ImageJ provided total leaf area, absolute herbivory, and percent herbivory. BioLeaf and visual quantification provided only percent herbivory, and the grid method provided only total herbivory. We also recorded the time it took to analyze each leaf and record the data. For ImageJ, we did not include the time it took to photograph and upload the pictures.

Statistics

All statistics were performed using R, Version 3.5.2 (R Core Team, 2018). We built global mixed effects models using the nlme package (Pinheiro et al., 2018). We dropped non-significant predictors from the models in a backwards stepwise fashion, assessed pairwise differences between the methods using lsmeans in emmeans (Lenth, R., 2019), and adjusted for multiple comparisons using false discovery rate.

Accuracy

To test for differences in measurement accuracy between ImageJ and LeafByte, we ran linear mixed effects models with area and herbivory as response variables. Method was included as a fixed effect, and the known size of each artificial leaf was set as the reference value. Additionally, we used an equivalency test (TOSTER, Lakens 2017) to evaluate whether the

methods produced the same results (as opposed to linear models that test for differences). We used ¼ of the standard deviation as upper and lower bounds of the model.

Precision

Because data were non-normally distributed, we used a Kruskul Wallis test to assess the effect of individual users on estimates of leaf area and leaf area consumed.

Comparisons of different methods

To analyze the effect of method on leaf area, we ran a linear mixed effects model with leaf area as the response variable and the interaction between method and leaf shape as predictor variables. Species and leaf ID were included as random effects in all models. Leaf areas were log transformed to meet assumptions of homoscedasticity.

To analyze the effect of method on herbivory, we ran a linear mixed effects model with herbivory as the response variable and the interaction between method and number of holes, and between method and presence of leaf margin herbivory as predictor variables. To analyze the effect of method on percent area consumed data, we ran a binomial generalized linear mixed effects model with herbivory as a response variable and the interaction between method and number of holes and the interaction between method and presence of leaf margin herbivory as fixed effects. Because low levels of herbivory (0-2.5%) were rounded to 5% rather than 0% when using visual quantification, we analyzed both the full data set and data where percent herbivory was greater than 5% to ensure that rounding did not skew our results.

Results

Accuracy

We found no difference between the known area and LeafByte for total area (t-ratio=0.126, df=36, p=0.991, Fig. 4.3A) or herbivory (t-ratio=1.11, df=36, p=0.512, Fig. 4.3B) or between the known area and ImageJ for total area (t-ratio=-1.53, df=36, p=0.285, Fig. 4.3C) or herbivory (t-ratio=0.793, df=36, p=0.710, Fig. 4.3D). On average, LeafByte differed from the known area by 1.3% while ImageJ differed from the known area by 3.2%. Based on the equivalence test comparing LeafByte to the known area, we can conclude that the difference between the treatments is equivalent to zero (t_{36}=20.4, p<0.001, t_{36}=-4.40, p<0.001) for both leaf area and hole area. Similarly, the difference between ImageJ and the known area is equivalent to zero for both leaf area and hole area (t_{36}=-20.2, p<0.001, t_{36}=-4.52, p<0.001).

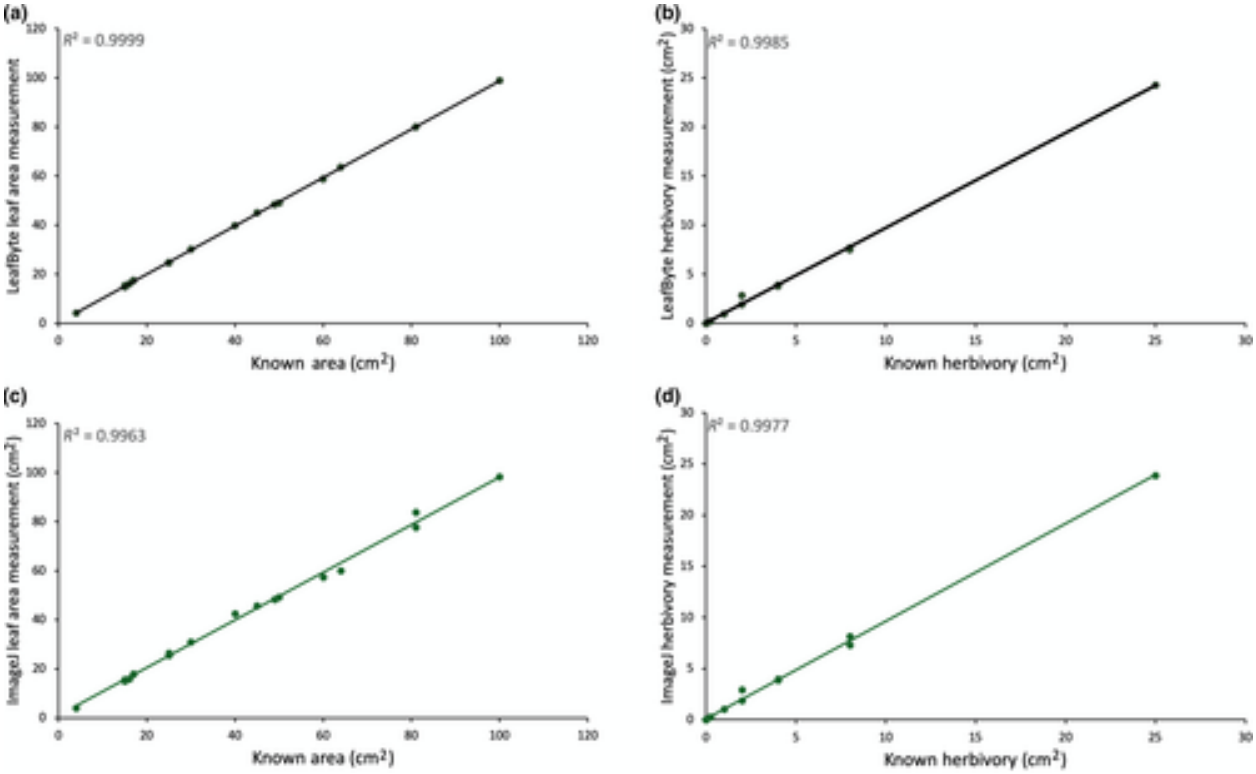

Fig 4.3. The measurements of LeafByte compared to the known size for a series of artificial leaves for (a) the total leaf area and (b) herbivory; and how ImageJ compared to the known size for (c) the total leaf area and (d) herbivory.

Precision

We found no effect of researcher on measurements of leaf area (X^2=0.065, df=2, p=0.968) or leaf area consumed (X^2=1.3612, df=2, p=0.506).

Comparisons of different methods

On average, leaf area measured by LeafByte was 2% lower than the leaf area measured by ImageJ (t_{248}=0.627, p=0.023, Fig. 4.4A). There was no effect of leaf shape on leaf area measurements using LeafByte or ImageJ (log likelihood=221 on 8 df, p=0.565).

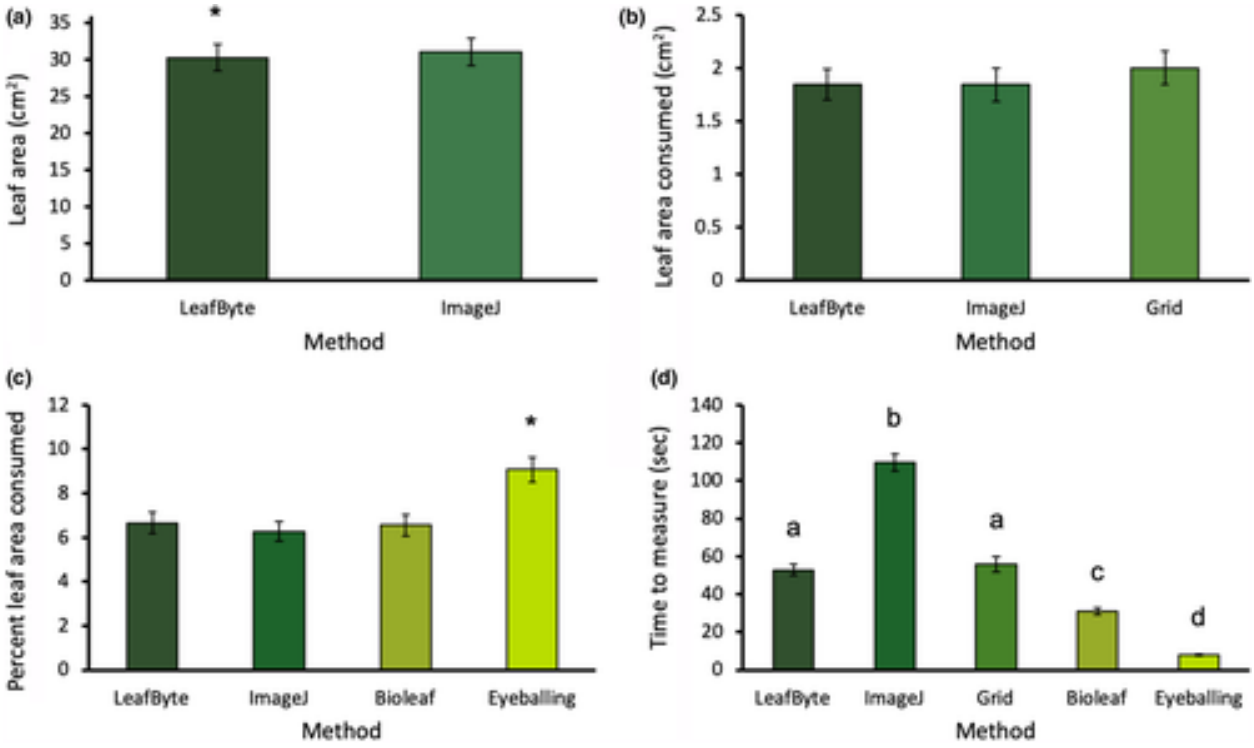

Fig 4.4. Comparison of methods for (a) leaf area, (b) absolute herbivory, (c) percent herbivory and (d) time to measure a leaf with each method. Bars represent $M \pm SE$. Asterisks denote significant difference from ImageJ ($p < .05$); letters denote pairwise differences.

There was a significant interaction between method and number of holes in a leaf on the area of herbivory measurements (log likelihood = 979 on 8 df, p=0.003), such that herbivory was underestimated when there were more holes using the grid method (t_{322}=-3.34, p=0.001), but not any of the other methods. When holding hole number constant, there was no significant difference in herbivory estimates between ImageJ and LeafByte (t-ratio=0.002, df= 322, p=1.0) or ImageJ and grid quantification (t-ratio=-2.02, df= 322, p=0.110, Fig. 4.44B).

There was a significant effect of method on percent herbivory ($F_{3,107}$= 35.8 p<0.001, Fig. 4.4C). Neither BioLeaf (z-ratio=-0.871, p=0.820) nor LeafByte (z-ratio= -0.955, p=0.775) were significantly different from ImageJ. Visual quantification overestimated percent herbivory compared to ImageJ (z-ratio= -5.12, p<0.001) or LeafByte (z-ratio=4.87, p<0.001). The accuracy of each method was not affected by the presence of margin damage (log likelihood= -767 on 14 df, p=0.102) or the number of holes (log likelihood = -770 on 10 df, p=0.912). The results were the same when analyzing the full data set or only the data >5%.

Different methods took different amounts of time to analyze a given leaf ($F_{4,549}$=202, p<0.001, Fig. 4.4D). ImageJ was by far the slowest option, taking twice as long as LeafByte (t-ratio=-15.0, df=549, p<0.001) on average. Grid quantification and LeafByte took a comparable length of time (t-ratio=-0.508 , df=549, p=0.612). BioLeaf was 40% faster than LeafByte (t-ratio=5.41, df=549, p<0.001) while visual quantification was 85% faster (t-ratio=11.7, df=546, p<0.001). The presence of margin herbivory slowed down leaf measurements for LeafByte (t-ratio=-3.14, df=52, p=0.003), ImageJ (t-ratio=-3.79, df=52, p<0.001), and BioLeaf (t-ratio=-2.67, df=52, p=0.0010), but not the grid method (t-ratio=-1.69, df=52, p=0.097) or visual quantification (t-ratio=0.655, df=52, p=0.515). The number of holes increased the time to

analyze for all methods ($F_{4,549}$=10.0, $p<0.001$), although it was drastically higher for ImageJ, which took ~8 seconds per additional hole, while all other methods were less than ½ a second per hole.

Discussion

LeafByte is a novel tool that combines and improves on the strengths of existing tools in a user-friendly application. LeafByte quickly, consistently, and accurately measures leaf area, herbivory from chewing herbivores, and percent herbivory. It is the first herbivory measurement app to automatically save measurements to a spreadsheet, reducing time and transcription errors. LeafByte can read and record barcodes, handle both light and dark colored plant tissue, and be used non-destructively. Our testing illustrates that while LeafByte produced average measurements 2% lower than ImageJ, both LeafByte and ImageJ were highly accurate when measuring "leaves" and "herbivory" of known sizes. LeafByte takes half as long as ImageJ to measure each leaf and can handle larger numbers of holes much more quickly. The time to measure leaves with ImageJ was highly variable and could be made faster with the use of macros when the leaves do not have margin damage.

We found that visual quantification led to overestimations. This was likely due to lack of training and the fact that most of our leaves had low levels of herbivory (Johnson et al. 2016). Tilting a phone/camera more than 15° caused high rates of error. Using a skew-correcting box as a scale rather than a line was an effective and necessary means of reducing error (Supporting Information 2). Researchers using methods that do not automatically correct for skew should take care to ensure that their photographs are not taken at an angle greater than 15%. Even with

skew correction, the error from shadow and leaves being three-dimensional means users should minimize the angle, perhaps going no further than 30%.

LeafByte has several limitations. Foremost, LeafByte can only measure herbivory that creates holes or clear changes in color, such as from chewing herbivores and some leaf miners. We do not recommend LeafByte for measuring damage from piercing-sucking or galling herbivores. Also, highly ruffled have more shadows and are difficult to flatten without overlap, leading to underestimates of leaf area and distorted measurements of herbivory. Poor quality photos or photos with extensive shadows make it difficult to cleanly remove the background, leading to less accurate measurements. This can be mitigated by using a lightbox (see website). It can also be difficult to analyze variegated leaves, though this can be mitigated through thoughtful choice of background color. All of the above limitations hold for other image processing software including ImageJ and BioLeaf. It is difficult to measure herbivory on highly complex, tripinnate leaves such as those from the Apiaceae family. Overlapping leaflets will create areas falsely identified as herbivory, and marking these areas for exclusion from calculations is slow. As with all methods of quantification, it is difficult to estimate herbivory and leaf size when the leaf is almost entirely consumed or the margin is hard to redraw due to complex leaf shapes. If researchers expect leaves to be mostly consumed, we recommend analyzing the leaves before and after and subtracting.

LeafByte allows for collection of more, and higher quality types of data. By reducing processing time, LeafByte makes it feasible to dramatically increase replication while reducing labor. This will make it easier to detect subtle trends in complex systems. Additionally, LeafByte

can be used to analyze herbaria specimens to answer questions related to global change (Meineke et al. 2018). Finally, as LeafByte is free and user friendly, it can be used as an educational tool or to facilitate citizen science based plant-herbivore interaction projects. While LeafByte was designed to measure leaf area and herbivory, it can also measure disparate things like damage on butterfly wings, and insect droppings on filter paper. LeafByte is a quick and accurate means of measuring leaf area and herbivory, making it a transformative tool for a wide variety of applications.

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Author Contributions

ZGP and AC designed the and created the app. ZGP, NA, TU, AG, and JD contributed to testing and improving the app. ZGP and JD collected and analyzed the data. ZGP, AC, NA, TU, JD, AG contributed to writing and editing the paper.

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