AM fungal colonization minimizes disease damage on tomato during later life stages while delaying fruit development

Research Thesis

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By

Jimmy Bingman

The Ohio State University

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Project Advisor: Professor Alison Bennett, Department of Evolution and Ecology

Introduction

Just like animal immune systems, plants have a variety of defense mechanisms against their foes, both pathogenic and herbivorous. For example, when plants are eaten by chewing herbivores, the damage can induce chemical defense mechanisms in the plant that reduce herbivory and prevent excessive further damage (Stamp 2003). Given the presence of such defense mechanisms, it is also well understood that beneficial soil microbes such as arbuscular mycorrhizal (AM) fungi can help ready plants for defense through a mechanism known as priming (Martínez-Medina et al 2016, Jung et al. 2012). Priming alters the plant immune system such that the delay between the initiation of plant defense and the full activation of defenses is reduced (Jung et al 2012). Practically speaking, priming quickens and strengthens the plant defense system, making it crudely analogous to a vaccine in humans and other animals.

AM fungi are a group of soil microbes that live in and around plant roots, providing plants with nutrients in exchange for carbon (Smith and Read 2008). While this exchange of nutrients is the primary driver of the relationship between AM fungi and plants, defense priming is one of its most interesting consequences. Yet, despite its potentially vast agricultural and ecological implications, the dynamics of AM fungal-induced changes in plant defense remain relatively unexplored.

One of the most open questions in the field of AM fungal-mediated plant defense, and the topic of this thesis, is whether the effectiveness of AM fungal-induced priming remains consistent throughout the plant life cycle. Many studies have shown that AM fungal colonization provides a defensive benefit to plants against microbial pathogens such as *Alterniaria solani* on tomato and *Blumeria graminis* on wheat (Fritz et al. 2006, Mustafa et al. 2017). Numerous studies have also shown that AM fungal colonization primes plants for defense against

herbivorous insects (Hilker and Schmulling 2019). However, almost all of these studies examine only the immediate AM fungal-mediated defense response during early, vegetative plant life stages. This leaves open the question of whether priming during early plant life stages (such as what occurs with AM fungal colonization) can affect plant defenses during later life stages as well. Additionally, studies that examine only the immediate AM fungal-mediated defense response of plants to attackers do not address the degree to which that response actually benefits the plant in the long term, or whether the timing and severity of the attack influences the AM fungal benefit to plants.

This brings up the question of whether AM fungal-mediated defense responses and their effects on plant fitness are context-dependent. It has been shown that cost of resistance to a pathogenic bacterium is context-dependent for plant hosts, however the degree to which AM fungal colonization plays a part in that context is not well understood (Meaden et al. 2015, Borowicz 2001). Conversely, numerous studies have demonstrated that plant responses to AM fungi are context-dependent, but this has not been explored thoroughly under the context of a plant pathogen (Pozo et al. 2007, Hoeksema et al. 2010). While mycorrhizal association may improve a host plant's disease systems for certain pathogens, how that improvement may vary under different contexts remains an open question.

To address some of these issues, we initially fed *Manduca sexta* (tobacco hornworm) larvae on tomato (*Solanum lycopersicum*) that were treated with either live or sterile AM fungal inocula during the first two life stages of the plant. While the original plan for this study was to examine AM fungal-mediated response to herbivory in tomato across the plant's main life stages, an unexpected fungal pathogen arose between the flowering and fruiting stages of the experiment. After this occurred, the study transitioned into an examination of the effect of AM

fungal colonization on later growth-stage resistance to disease. Concurrently, fruiting and flowering data were collected that allude to potential context-dependent effects of AM fungal inoculation on overall plant fitness.

Methods

Study system

We used tomato (*S. lycopersicum*) as our plant host. S. lycopersicum is widely considered a model for studying plant defenses against both herbivores and microbial pathogens, and is an important crop and garden plant (Arie et al., 2007). This study used the Moneymaker cultivar, and seeds were acquired from Urban Farmer in Westfield, IN, USA.

M. sexta is a common pest of tomatoes (Lange and Bronson 1981). Hornworms were acquired as eggs from Great Lakes Hornworm and fed a wheat germ mix until added to plants at second instar. For the first stage, hornworms were reared at a residential home in Bowling Green, Ohio, where lower temperatures (approximately 17 ºC, with temporal variation) resulted in these worms having a significantly lower mass than those added in the flowering stage, which were reared in an office at Ohio State University (approximately 21ºC).

Background soil was prepared by mixing two parts silicate construction sand and one part soil (Scioto River dredge from Jones Topsoil, Columbus, OH, USA) which was then sterilized in a steam cart for 8-12 hours, cooled overnight, and sterilized again for 8-12 hours.

We added either a live or sterile commercial mix of 4 species of AM fungi (*Glomus intraradices, Glomus mosseae, Glomus aggregatum, Glomus etunicatum*; Mycorrhizal Applications, Grants Pass, OR, USA). AM fungal inocula was either added live or autoclaved twice for 30 minutes at 121ºC.

Identification of the disease that spread across the plants midway through the first experiment is pending, and the disease is referred to in this thesis simply as a "putative fungal pathogen." Symptoms began around day 70 after plants sprouted as small dots of dryness and discoloration in the leaflets that gradually spread outward in concentric, irregular shapes until drying out entire leaves. Infection of new growth and main stems occurred last and usually only occurred after all older leaves had fully dried and fallen. Even in heavily infected plants, fruits remained mostly healthy in appearance until the experiment was abandoned due to COVID19 restrictions (On March 16, 120 days after sprouting).

Experimental Set-up

Originally, we planned to conduct a 2x2x4 factorial experiment in which the presence or absence of AM fungi and the presence or absence of an herbivorous insect was manipulated across four plant life stages: vegetative growth, flowering, fruit development, and fruit maturation. Leaf chemistry was going to be analyzed for this experiment along with herbivory, but this did not happen for two reasons: (1) it was difficult to get the hornworms to eat on command, making it difficult to collect adequate leaf samples for chemical analysis, and (2) the unexpected disease that rapidly spread across the plants made it impractical to continue adding insects during the last two life stages. Disease damage was quantified beginning with the onset of the pathogen, and throughout the remainder of the experiment. We therefore analyzed the two parts of the experiment (herbivory and pathogen infection) as two independent experiments, because plants exposed to the pathogen were not exposed to herbivores, and vice versa. Additionally, plants were exposed to herbivores during the vegetative growth and flowering stages, while the disease occurred during the later flowering and fruiting stages. The first experiment, which is not discussed as deeply in this thesis, examined the effect of AM fungal

colonization on herbivory during the first two stages (vegetative growth and flowering) of tomato. The second experiment examined the effect of AM fungal colonization on disease resistance during the last two growth stages (fruit growth and fruit maturation) of the plants, along with differences in growth, flowering, and fruiting.

Each of the original 16 treatments contained 8 replicates, resulting in a total of 128 plants for the experiment. After the first two stages were harvested, the plants designated for the last two stages were compiled into a single group of two treatments (live or sterile AM fungal inocula), with 32 replicates in each treatment. In this study comparisons are only made between AM fungal colonized plants and non-colonized plants infected with the pathogen.

We established an AM fungal network in the pots to better simulate the type of interaction plants have with AM fungi in the field. To accomplish this, we grew two rounds of plants in the same pot- a preliminary round of plants to establish an AM fungal network and then the experimental plants, which were able to tap into the previously established AM fungal hyphae as seedlings. To set up the preliminary plants, two-gallon pots were filled with soil in three layers: a 1.2-liter bottom layer of sterilized background soil, a 5-liter middle layer of 3 parts live or sterile inocula mixed with 17 parts sterilized background soil, and a top layer of another 1.2 liters of sterilized background soil. This produced a pot filled with sterile soil plus inocula consisting of 10% total pot volume, mixed in the interior of the pot to ensure maximum contact with roots.

Plants for the preliminary round were germinated in trays of sterilized potting soil for 10 days before being transplanted into pots and organized into two randomized blocks. To control for the microbes introduced with the AM fungal inocula, we added a live microbial wash to pots with sterile inocula and a sterile microbial wash to pots with live inocula. We prepared the

microbial wash by collecting the filtrate from 100 mL of solid inocula passed through a 40 micrometer sieve and then vacuum filtered through 11µm Whatman Filter Paper. Half of the resulting filtrate was autoclaved to be added to the pots with live inocula. 10 mL of the sterile or live microbial wash was then added each day for 8 days until 80 total mL had been added to each pot. Plants were given a 16:8-hour light:dark cycle and were watered once every two days until flowering, after which they were watered once every 3 days. Plants were fertilized with 120 mL of half-strength Hoagland's solution once every two weeks.

After 5 weeks of preliminary plant growth we planted seeds for the experimental plants directly into the pots. After seeds had germinated the preliminary plants were cut at the base of their stems. Plants began to sprout between 1 and 2 weeks after seeds were planted, and "Day 1" since sprouting was counted as the first day all pots had a sprouted plant. The height, number of leaves, length of longest leaf, number of flowers, and number of fruits of the focal plants was recorded every two weeks beginning 30 days after "Day 1" since sprouting. The total of 128 pots were split into 32 pots for each stage, for a total of 8 plants per each treatment (Yes/no AM fungi and yes/no *M. sexta*) per stage.

Experimental plants were allowed to grow for 6 weeks before insects were added to 16 pots in the vegetative growth stage treatment. Two *M. sexta* larvae were added to separate leaves on each plant, each of which was then enclosed in a porous bread bag. Hornworms were placed on the 3rd and 4th leaves from the top of the plant with at least two leaflets of 3 cm in length. Leaves were photographed before and after herbivory in front of a white background so that surface area could be measured later using ImageJ software. Insects were allowed to feed for 48 hours, after which they were removed from the plants. This process was repeated again 30 days later for the flowering life stage, when insects were added to 16 more pre-designated plants. For

each stage, plants were harvested10 days after insects were removed. Shoots were immediately placed in a drying oven at 60 ºC for one week, and then weighed. Roots were washed before dried and weighed under the same conditions. Roots were then stained with 0.5% trypan blue solution to be scored for AM fungal colonization. Roots were scored for AM fungi at 60x magnification. Colonization was quantified as percent of root length colonized.

Between 78 and 92 days after sprouting, leaves that were at least moderately diseased were pruned in an effort to salvage the plants, but 92 days after sprouting only fallen leaves were removed from the pots. Plants were rated on a scale of 1-10 based on their disease symptoms from the onset of the pathogen, 1 being very few spots on one leaf, 5 being moderately sized dry spots on half of the leaves, and 10 being nearly dead, with the main stem heavily infected. As the disease began to take over, the disease status of new growth (the top three leaves of 3 cm or more) was also recorded. Data recording ended after day 120 due to the onset of the COVID19 restrictions.

Data analysis

Herbivory assays

All statistical analyses were done using R. For the herbivory assays, leaf surface area was analyzed using ImageJ software before and after herbivory to determine the surface area eaten per leaf. A three way ANCOVA was performed (“aov()” function in R) with surface area eaten per leaf ((surface area before)-(surface area after)) as the dependent variable, inocula treatment, stage, and block as independent variables, and insect size as a covariate.

Disease observation

For each date that disease was recorded, two-way ANCOVA's were performed with disease as the dependent variable, inocula treatment and block as independent variables, and fruiting as a covariate. Two-way ANCOVA's were also performed on each date for number of fruit, number of flowers, except with these measurements being the dependent variable and disease being the covariate. The independent variables remained the inocula treatment and block.

While a repeated measures ANOVA would have been ideal to test the disease and fruiting data over time, only within-date hypothesis tests were performed due to lack of coding experience and time.

For binomial data involving the infection status of new growth, Fisher's exact test was performed ("fisher.test()" function in R) with AM fungal inoculation as the independent variable and new growth infection as the dependent variable for each of the two dates this measure was collected.

Results

Herbivory assays

In the first part of this experiment, herbivory by *M. sexta* on tomato was quantified for two treatments (live AM fungal inoculated and sterile inoculated) across the first two life stages of tomato (vegetative growth and flowering). The results are shown in Figure 1, where the decrease in surface area eaten for the sterile inocula treatment appears substantial in the vegetative growth stage even though it is not significant based on one-way ANCOVA results (Figure 1. $F_{1,15} = 2.787$, $p = 0.1058$). The same trend is seen in the in flowering stage, although

there it is even less significant ($F_{1,15}$ = 5.63, p=0.459). The lack of significance in both cases is likely due to the high variance in surface area eaten relative to sample size.

Disease, fruit, and flower monitoring

Overall sampling shows the non-inoculated treatment experienced a higher disease rating than plants in the inoculated treatment beginning after 78 days since sprouting. (Figure 2; 92 days after sprouting: $F_{1,60}$= 16.931, p <0.00012 106 days after sprouting: $F_{1,60}$= 27.434,p <$2.2e^{-6}$; 120 days after sprouting: $F_{1,60}$= 32.189, p <$4.28\ e^{-7}$).

New growth infection was also significantly higher for non-colonized plants on both dates this measure was taken (p=$1.617e^{-5}$ for March 2nd and p=$9.458e^{-5}$ for March 16th). Plants that hosted AM fungi had significant lower pathogen infection rates (Figure 3).

Plants from the sterile treatment experienced increased number of fruit compared to the live treatment at all time points beginning at 92 days (Figure 4; 92 days: $F_{1,60}$ = 11.406, p =0.00129; 106 days: $F_{1,60}$ = 7.474, p =0.0082; 120 days: $F_{1,60}$ = 10.06, p =0.0024). There was a significantly larger number of flowers for sterile inocula treatment 78 days after sprouting (Figure 4; $F_{1,60}$ = 11.406, p =0.00129). This changed with the onset of the disease, and after 106 days there was a significantly higher number of flowers for the live inocula treatment (106 days: $F_{1,60}$ = 5.771, p =0.01941; 120 days: $F_{1,60}$ = 5.856, p =0.01857).

Shoot and root mass

Shoot and root dry mass was recorded for plants harvested after the vegetative growth and flowering stages. No significant difference was found in shoot or root mass between the live and sterile inocula treatments for either stage.

AM fungal colonization

After harvesting plants from the vegetative growth and fruiting stages, roots were stained scored for AM-fungal colonization. Roots given the live inocula had a significantly greater? percentage of root length colonized than plants given the sterile inocula ($F_{1,20}$=62.60, p=1.38e^{-7}).

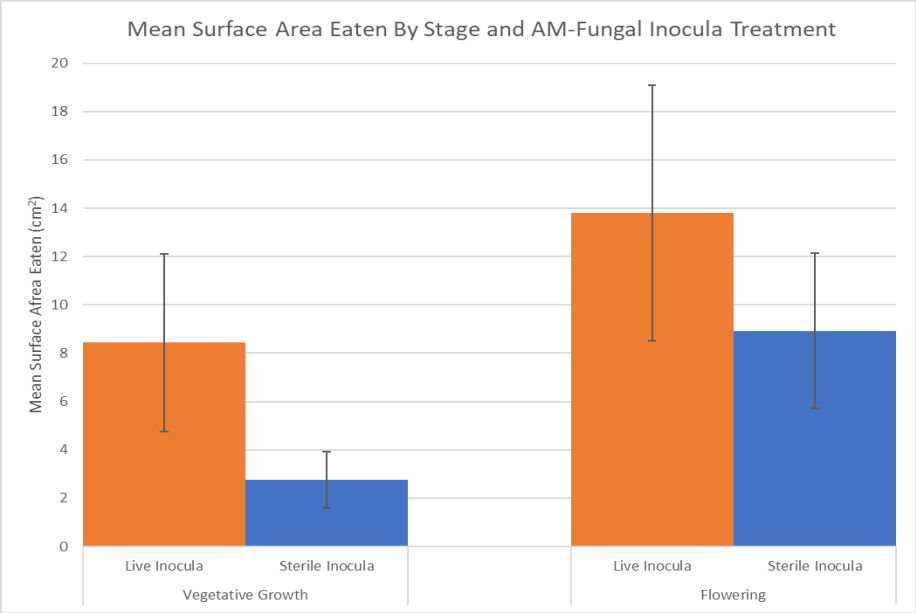


Figure 1: Mean leaf surface area eaten by *Manduca sexta*. Error bars show standard error.

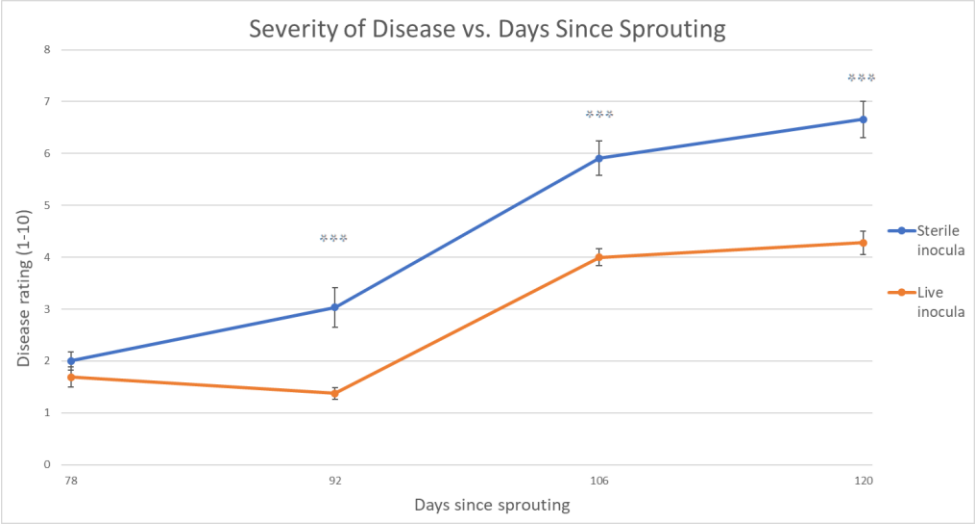


Figure 2: Severity of disease vs. days since sprouting, showing significantly more disease symptoms for the sterile inocula treatment after 92 days since sprouting. Three asterisks indicate p<0.001. Error bars show standard error.

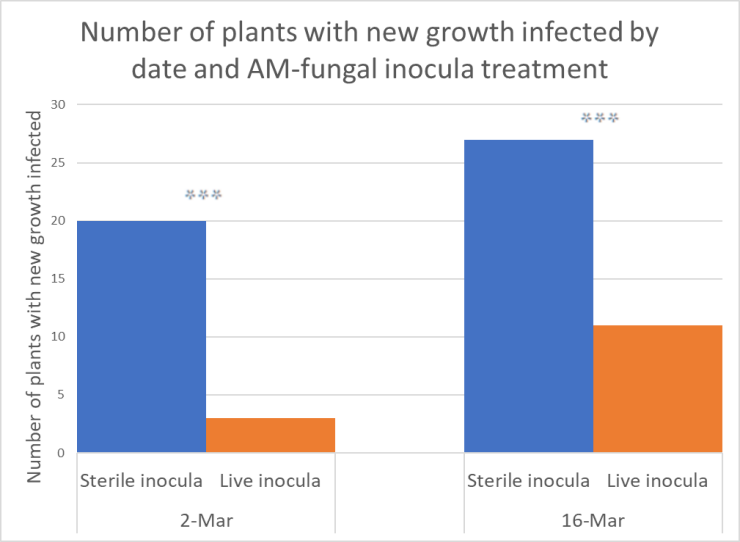
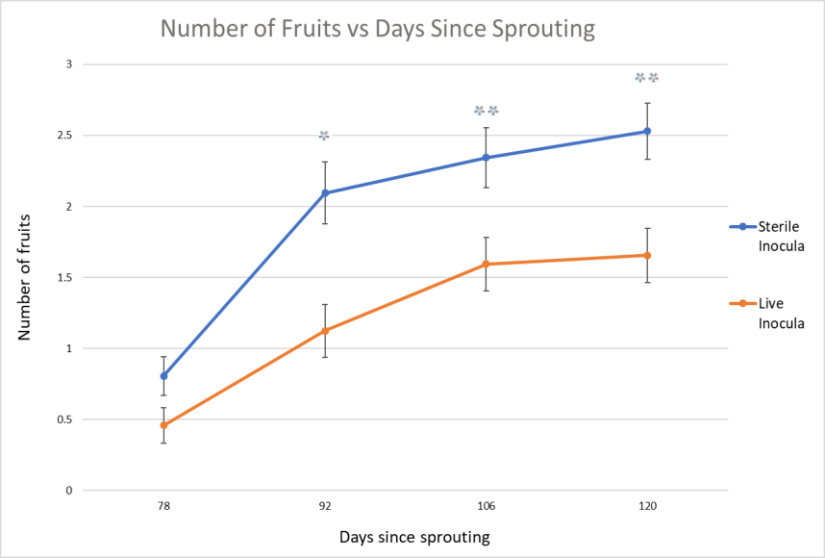


Figure 3: Number of plants with new growth infected by date and AM fungal inocula treatment, showing increased infection for the sterile inocula treatment. For both dates, almost all plants were in the fruit development or maturation stage. Three asterisks denote $p<0.001$.



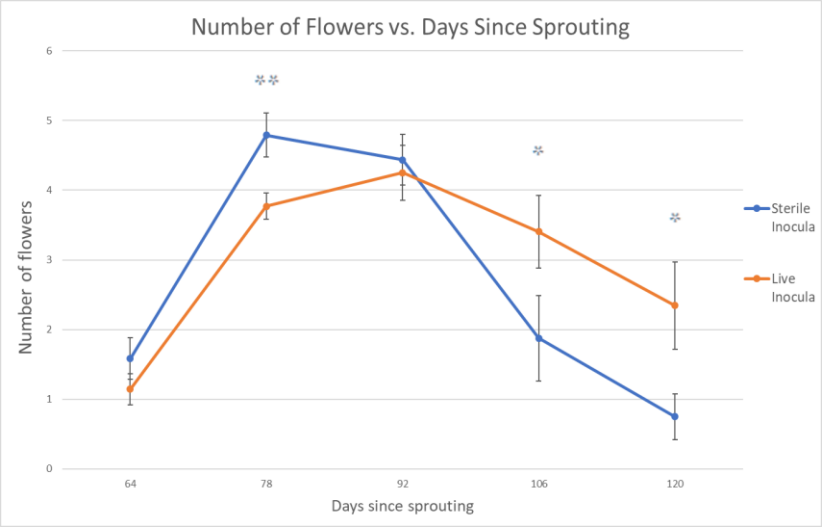


Figure 4: Number of fruits vs. days since sprouting showing increased fruit over time for the sterile treatment compared to the live treatment. Number of flowers vs. days since sprouting showing increased flowers over time for the live inocula treatment compared to the sterile treatment. One asterisk indicates $p<0.05$ Two asterisks indicate $p<0.01$. Three asterisks indicate $p< 0.001$.

	AM fungal colonization		
	df	F	*p*
Inocula	1	62.596	**1.38 e^{-7}**
Stage	1	1.861	0.188
Insects	1	0.430	0.0519
Error	20		

	Shoot mass			Root mass		
	df	F	*p*	df	F	*p*
Inocula	1	0.233	0.631	1	0.142	0.707
Stage	1	309.235	**<2e^{-16}**	1	182.601	**<2e^{-16}**
Block	1	2.552	0.115	1	0.298	0.587
Insects	1	2.701	0.105	1	0.747	0.391
Error	59			59		

	Surface area eaten		
	df	F	*p*
Inocula	1	2.078	0.155
Stage	1	2.483	0.121
Block	1	1.357	0.249
Insect mass	1	2.536	0.117
Inocula:Stage	1	0.138	0.711
Error	58		

		Number of fruits			Number of flowers		
		df	F	*p*	df	F	*p*
Day 64	Inocula	1			1	1.472	0.228
	Block	1			1	10.100	**0.00201**
	Disease	*Disease onset was first recorded on day 78*					
Day 78	Inocula	1	1.833	0.179	1	7.711	**0.00665**
	Block	1	4.124	**0.0452**	1	0.389	0.536
	Disease	1	1.417	0.237	1	5.155	**0.0255**
Day 92	Inocula	1	11.41	**0.0129**	1	0.121	0.730
	Block	1	0.582	0.449	1	1.630	0.207
	Disease	1	3.389	0.0706	1	2.141	0.149
Day 106	Inocula	1	7.474	**0.0082**	1	5.771	**0.0194**
	Block	1	0.831	0.366	1	6.751	**0.0118**
	Disease	1	6.392	**0.0141**	1	7.270	**0.00909**
Day 120	Inocula	1	10.06	**0.0024**	1	5.856	**0.0186**
	Block	1	0.821	0.368	1	3.784	0.0564
	Disease	1	2.572	0.114	1	9.585	**0.00298**
	Error	60			60		

	Disease rating (1-10)			
		df	F	*p*
Day 78	Inocula	1	2.927	0.0905
	Block	1	10.940	**0.00134**
	Fruit #	1	1.417	0.237
Day 92	Inocula	1	16.931	**0.00012**
	Block	1	0.054	0.817
	Fruit #	1	3.389	0.0706
Day 106	Inocula	1	27.434	**2.2e^{-6}**
	Block	1	0.007	0.932
	Fruit #	1	6.392	**0.0141**
Day 120	Inocula	1	32.189	**4.28e^{-7}**
	Block	1	2.229	0.141
	Fruit #	1	2.572	0.114
	Error	60		

Table 1: Statistical tables for all analyses.

Discussion

Hornworm assays

We observed high variation in the surface area eaten by hornworms, which may have been driven by variation in the larval instar (future experiments will prioritize raising insects at a constant temperature). As a result, we cannot conclude whether tomato defense against *M. sexta* is promoted, hindered, or unchanged by AM fungal colonization, at least within the short-term context of a 48-hour herbivory assay. A previous study showed that AM fungi influenced chemical defense pathways but did not have any effect on *M. sexta* herbivory in *Solanum ptycanthum* and *Solanum dulcamara,* two relatives of the tomato (Minton et al. 2016).

Fungal pathogen

AM fungal colonization had an impact on late life stage tomato disease resistance. This was demonstrated in the plant's fruiting stage, when we observed significantly less pathogen-induced leaf damage as well as new-growth infection in plants hosting AM fungi compared to non-colonized plants. This corroborates past evidence of defense by AM-fungi against tomato pathogens (Fritz, et al. 2006, Song et al. 2015). However, in this experiment, AM fungal defense priming was shown to impact plant health at late life stages, not just during early, vegetative stages of growth.

We were not able to assess the degree to which AM fungal colonization offered long-term resistance to the disease. This presents one of the greatest avenues for future research on this subject: how colonized and non-colonized plants differ in symptoms, mortality, and reproductive output throughout the *entire* progression of the disease and lifespan of the plant.

Delayed onset of fruiting exhibits context dependent effects

Although incomplete, the data does hint that AM fungal colonized plants, could actually end up being less reproductively fit than non-inoculated plants given a certain timing of disease onset, even though they appear less impacted in terms of leaf damage. We observed that non-colonized plants produced a significantly higher number of fruits. Our data suggest two possible hypotheses. One, non-colonized plants have a higher fitness than colonized plants, or two, AM fungal colonized plants experience a delay in fruiting, after which they begin to fruit at a normal rate. Based on the data we collected, we are unable to distinguish between these two hypotheses. We also did not assess the quality of fruits or the number of seeds per fruit, which would also help determine the fitness of the plants.

The number of flowers per plant decreased for both treatments with the onset of the disease, but far more for non-inoculated plants, suggesting that investment in future reproductive output may have been minimized by lack of AM fungi or the disease. In either case, the changes in flower and fruit production brought on by AM fungal colonization and the disease suggests that the timing of disease onset may determine the impact of AM fungi on plant fitness. This presents opportunities for future studies on disease timing on the benefit of AM fungi in plant defense.

Conclusion

AM fungal colonization does not enhance tomato resistance to *M. sexta* herbivory in early life history stages. AM fungal colonization can promote plant disease resistance in late stages of the plant life cycle. However, the ultimate impact on plant fitness could depend on the timing of the disease.

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