VOLATILE ORGANIC COMPOUND EMISSIONS FROM FLOWER BUDS AND FRUITS OF COTTON PLANTS TREATED WITH BENEFICIAL FUNGI

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

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The purpose of this research is to analyze volatile organic compounds (VOCs) of developing flower buds (squares) and immature fruits (bolls) of cotton plants (*Gossypium hirsutum* L.) when treated with plant associated fungi (*Beauveria bassiana* and *Phialemonium inflatum*). These compounds will be analyzed by gas chromatography to mass spectrometry (GC-MS). Experiments will be conducted using excised squares and bolls from both greenhouse and field plants.

The results will be evaluated to indicate if plant associated fungi impact the ability of the plant to produce chemicals that could potentially deter insects from consuming the plant. VOCs are known to influence herbivorous insects' ability to locate plants (Dudareva et al. 2006, Holopainen 2004). Beneficial fungi were found to affect herbivorous insects' behavior, potentially from the influence of VOC emissions (Sword et al 2017). Through this research it can be expected that there will be a difference in volatile emissions due to treatment, tissue type, and locations, but the positive or negative effects may not be as clear.

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NOMENCLATURE

VOC	Volatile Organic Compound
GC-MS	Gas Chromatography to Mass Spectroscopy
SPME	Solid Phase Micro Extraction

CHAPTER I

INTRODUCTION

Fungal endophytes are microorganisms that live in plant tissues without causing any sign of infection (Porras-Alfaro and Bayman 2011; Bamisile et al. 2018). Unlike mycorrhizal fungi, which are restricted to roots, fungal endophytes can colonize the above-ground plant tissues as well. Endophytic fungi have been reported as important in helping plants deal with abiotic stresses like drought or heat (Backman and Sikora 2008). Endophytic fungi have also been shown to enhance plant resistance against biotic stressors as well such as insect herbivores and pathogens (Gao, Dai, and Liu 2010; Lopez et al. 2014).

Integrating the use of fungal endophytes in cropping systems is becoming more common as evidence suggests that the plant microbiome could promote a healthier crop. The presence of these endophytes colonizing plants can have a variety of effects on insect behavior and performance such as deterring herbivory from piercing and sucking insects such as *Lygus hesperus, Nezara viridula*, and *Aphis gossypii* (Lopez et al. 2014, Sword et al. 2017), as well as chewing insects like cotton bollworm *Helicoverpa zea* (Lopez and Sword 2015). The presence of endophytes has also been shown to influence oviposition on host plants, although in this case the colonized plants were found to have a higher oviposition preference. (Jallow et al. 2008).

In the Sword et al. (2017) study, feeding preferences of the southern green stink bug (*Nezara viridula*) were tested with reproductive tissues of cotton (*Gossypium hirsutum* L.) treated with endophytes compared to controls. The endophytes used in this experiment were *Beauveria bassiana* and *Phialemonium inflatum*. Reproductive tissues, flower buds referred to as squares and immature fruits referred to as bolls, were taken from both greenhouse and field plants. Data were analyzed separately depending on the location of the tissue. As previously alluded to, this study found the insects took significantly longer to interact with tissues from treated plants compared to untreated controls. They also demonstrated with choice tests that these insects preferred to feed on tissues from control plants. Sword et al. (2017) hypothesized that this behavior could be a result of a change in the volatile organic compound composition of the plants due to interaction with the endophyte treatment.

Volatile organic compounds (VOCs) function as direct defenses emitted by various plant tissues. VOCs are known to have a variety of ecologically important roles such as impacting the ability of herbivorous insects to locate host plants (Dudareva et al. 2006, Holopainen 2004). In a previous study of lima beans treated with fungal endophytes it was found that there was a detectable increase in the number of VOCs emitted from intact leaves (Navarro-Meléndez and Heil 2014). This suggests that there could be a direct effect on VOC composition as a result of treatment of plants by endophytic fungi and this could correlate with the differences in insect behavior noted in previous studies.

Research in various plant systems has also demonstrated differences in VOC composition depending on the location where plants are grown. For example, studies done with red clover and tomatoes showed that there was a detectable increase in the number of volatiles produced by the plants in the field versus in the greenhouse (Dalal et al. 1967, Kigathi et al. 2009). This could be attributed to various abiotic and biotic conditions that the plants are exposed to in the field versus the controlled conditions of being reared in a greenhouse.

This experiment was designed to test whether treatment of cotton plants with *Beauveria bassiana* and *Phialemonium inflatum* fungi would influence the profiles of VOCs produced as well as the quantity of individual compounds. Based on previous research, it can be expected that the treated plants have different qualitative and quantitative levels of VOCs emitted. The second part of this experiment tested the differences between VOCs emitted from the reproductive structures of the plant, the squares and bolls. The third objective of this experiment was to observe if there were differences in VOC emissions between greenhouse and field trials. Overall, the VOCs were analyzed using gas chromatography to mass spectrometry (GC-MS) and analyzed using statistical tests in RStudio comparing the effects of treatment, location, and reproductive structure.

CHAPTER II

METHODS

Seed Treatments and Plant Growth

PhytoGen (PHY367) cotton seeds were surface sterilized in a 3% sodium hypocholrate solution for 3 minutes and then a 70% ethanol solution for 2 minutes. Next, they were air dried in a laminar flow hood. The seeds were soaked in 7mL of a 10^7 spores/ml of either *Beauveria bassiana* or *Phialemonium inflatum* for every 40 seeds overnight. For the controls, autoclaved water was used, and there were two treatments, *Beauveria bassiana* and *Phialemonium inflatum.*

The *Phialemonium inflatum* spores were harvested originally from surface-sterilized cotton leaves as part of a survey of foliar fungal endophytes in Texas, USA (Ek-Ramos et al. 2013). The *Beauveria bassiana* isolate was obtained from a commercially available strain (Botanigard®). Both fungi were cultured on potato dextrose agar media (PDA) in Petri dishes. The stock spore concentration solutions used to treat the seeds was made by adding 10mL of sterile 0.1% Triton X-100 solution to the fungal plates and scraping the spores free from the agar with a sterile scalpel. This solution obtained was filtered through a cheese cloth with sterile water into a sterile beaker and a Neubauer hemocytometer was used to calculate spore concentrations which were then diluted to 10^7 spores/ml.

Every trial sampled 10 structures, either squares or bolls, per treatment per location, for a total of 30 samples per experiment. The plants were grown in both the field and the greenhouse until reproductive structures, squares and bolls, were present. Squares were sampled when they were the size of a matchhead and bolls were sampled when they were quarter dollar sized in diameter.

Collection and Analysis of Volatile Organic Compounds (VOCs)

Reproductive structures were excised and sealed in a VOC sampling chamber. These chambers were constructed out of mason jars modified so there was a small hole the size of a syringe to allow for the solid phase micro extraction fiber (SPME) to pass through. Four squares or three bolls per plant were excised and incubated in the VOC chamber for one hour before sampling. The SPME fiber was exposed for thirty minutes.

Three bolls versus four squares were used so that there would be a similar biomass and since bolls tend to be heavier than squares, fewer were needed to constitute a similar biomass.

Samples were analyzed by using gas chromatography to mass spectrometry (GC-MS) on a GCMS-QP 2010 Ultra (Shimadzu Scientific Instruments (Oceania) Pty Ltd, Henderson, New Zealand) equipped with a Zebron ZB-WAX plus column (30 m length × 0.25 mm I.D. × 0.25 µm film thickness; Phenomenex, Torrence CA, USA). Helium was used as the carrier gas at a constant starting flow rate of 1.5 mL min^{-1}. The injection port temperature was 250 °C while the initial column temperature was set at 40 °C and maintained at that temperature for 3 min. Then the temperature was increased to 240 °C at a rate of 5 °C min^{-1} and held at the temperature for 3 min. Lastly, the temperature was increased to 250 °C at a rate of 40 °C min^{-1} ramp. Mass spectrometry used electron impact ionization at an interface temperature of 250 °C and an ion source temperature of 200 °C.

The identities of the VOC's were established tentatively using the Wiley Registry of Mass Spectral Data 10th Edition as well as the NIST/EPA/NIH Mass Spectral Libraries. The identities of the compounds were confirmed using authentic standards purchased from Sigma-Aldrich. The quantitative analysis of the compounds was performed with automatic peak integration. Figure 1 shows an example of typical chromatogram.

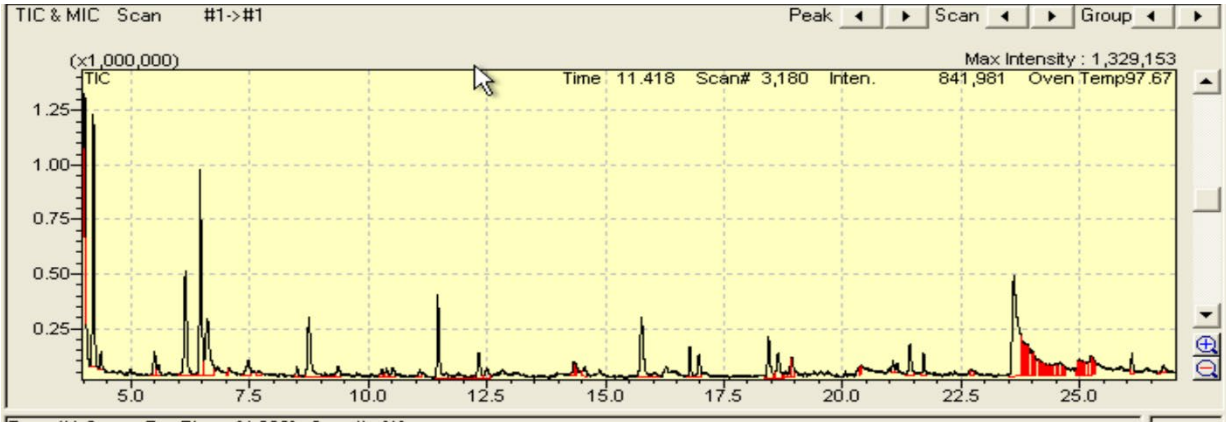

Figure 1. Typical chromatogram showing peaks that indicate the presence of certain volatile organic compounds with the areas under the peaks indicating the quantity of the compounds present.

The GC-MS and greenhouse were provided by Dr. Charles P.-C. Suh at the Southern Plains Agricultural Research Station (USDA-ARS) located in College Station, Texas. The cotton and endophytes were provided by the Sword Lab at Texas A&M University in College Station. The field cotton plants were collected from the Texas A&M AgriLife Research Farm in Snook, Texas.

Statistical Analysis

The statistical analyses were performed in RStudio. The data were transformed to the one fourth power. To test if the data were parametric, the Shapiro-Wilk's normality test was used. If the P-value for this test was below 0.05 the data were determined to be nonparametric. Since the data were nonparametric, PERMANOVAs were performed using the Adonis function of the vegan package in RStudio. This allowed us to test all VOCs simultaneously as a response to the endophyte treatment, tissue type, and location of origin, along with all their interaction terms. A redundancy analysis was used for a constrained ordination. The binary data was also compared using a principal coordination analysis.

CHAPTER III

RESULTS

A total of 11 compounds were identified, 9 terpenes and 2 green leaf volatiles. The PERMANOVA revealed that the structure, either squares of bolls, and the location, either greenhouse or field, were significant in impacting the volatile organic compound quantities emitted from the plant. Fungal treatments had no significant effect.

Table 1. PERMANOVA Results

	Df	**F. Model**	**Pr(>F)**
Location	1	27.9732	0.001
Structure	1	22.5554	0.001
Treatment	2	1.044	0.41
Location:Structure	1	22.8183	0.001
Locations:Treatment	2	0.1708	0.988
Structure:Treatment	2	-0.0667	0.999
Location:Structure:Treatment	2	1.057	0.413
Residuals	89		
Total	100		

As can be seen in Table 1, the p-values for location, structure, and the interaction between the two were all significant at 0.001. However, treatment and its interactions were found to not be significant. When the four trials were plotted together as a constrained ordination it exhibited clear grouping of field bolls, field squares, greenhouse bolls, and greenhouse squares.

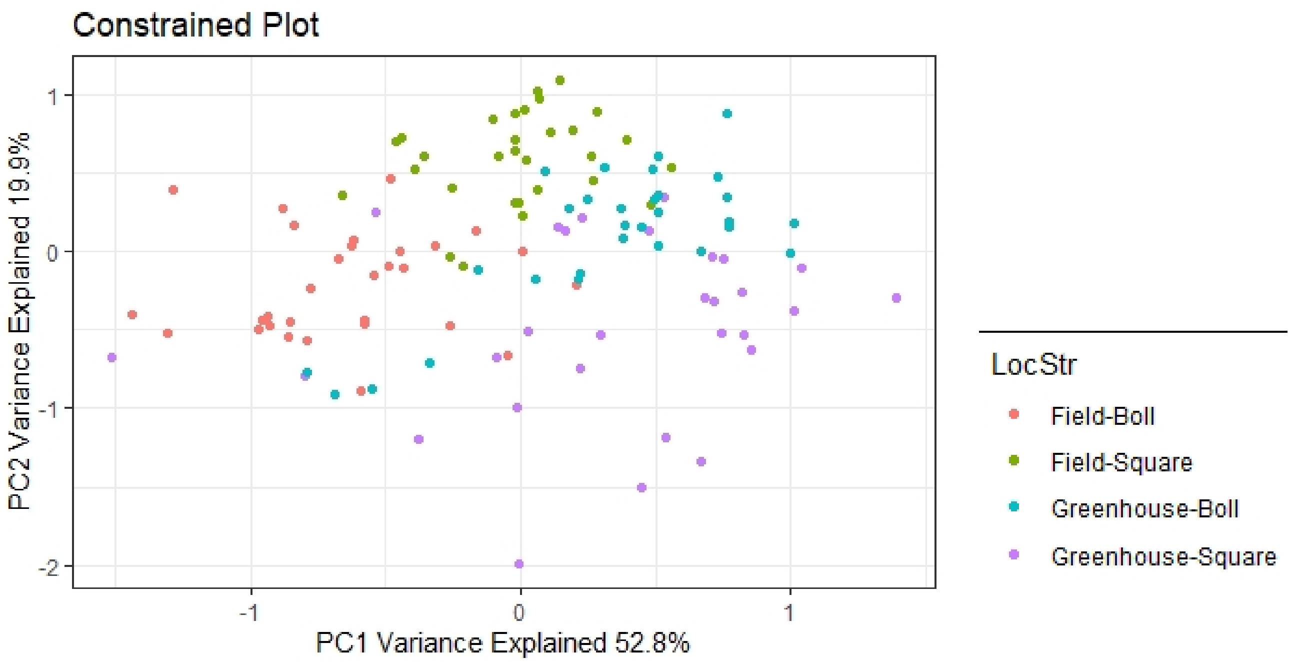

Figure 2. Constrained ordination showing the clustering of the four trials based on their variance.

Not only did the individual trials cluster as expected, the location data strongly clustered as well. A cluster of points representing the field plants was observed in the top left area of the plot and the greenhouse was in the bottom right area (Figure 2).

When observing binary data using a principle coordination analysis, there were significant differences between all locations and structures except for field squares versus greenhouse bolls. These interactions can be observed in Figure 3.

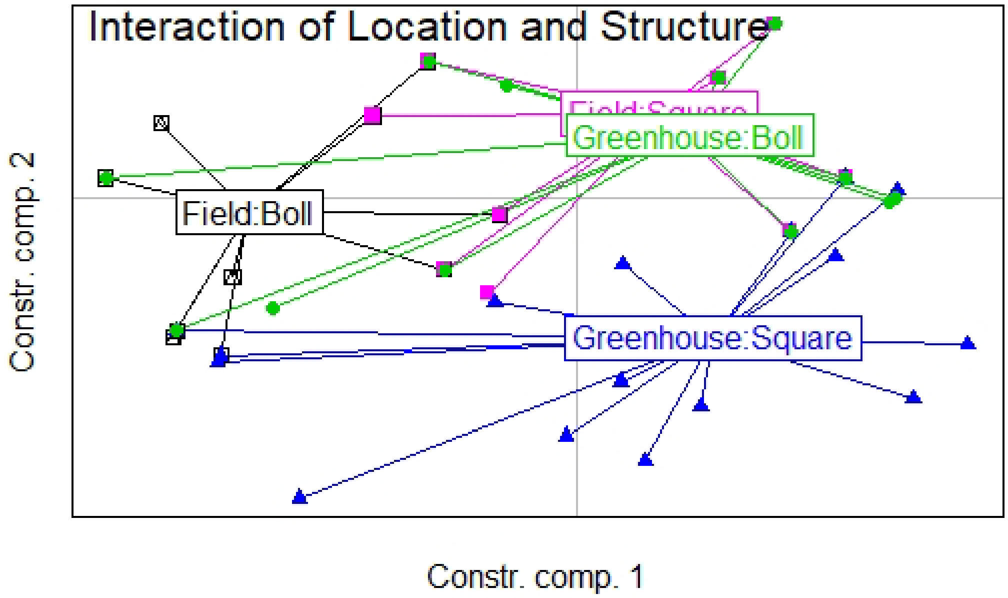

Figure 3. Plot of binary data representing the interactions between location and structure. The center boxes represent the trial. The individual samples are presented by circles, triangles, and squares of unique colors connected by a line to the center box according to the trial. These individual samples show the fungal treatment.

The differences between all the structures except greenhouse bolls and field squares all has a p-value of 0.0012 whereas the greenhouse bolls and field squares interaction had a p-value of 0.581, further indicating the insignificance of that interaction.

CHAPTER IV

DISCUSSION

Significant differences of the volatile organic compound quantities due to location that we observed corroborate studies done in various plant systems such as tomatoes and red clover as previously described (Dalal et al. 1967, Kigathi et al. 2009). These results show that it is important to consider where experiments are to take place as the location could influence the volatiles which in turn could potentially influence insect behavior. This difference is likely due to the increased levels of abiotic and biotic factors that the plant is exposed to in the field versus in the greenhouse. In the field there is no controlled temperature and the plants are exposed to herbivory which could influence how they utilize their defenses. Previous research has shown that when a plant is undergoing herbivory, this will activate induced defenses which can include increased volatile production in order to deter further herbivory (Paré and Tumlinson 1997).

The similarities in volatiles between the greenhouse bolls and field squares was an interesting and unexpected result. Considering how volatiles play a role in plant defense, it can be hypothesized that the younger reproductive tissues, the squares, when in the field have to develop more defensive compounds faster than a greenhouse square due to the presence of abiotic and biotic stressors in the field. It is possible that the older reproductive tissue, the bolls, in the greenhouse reach this level of defense at a slower rate because they are not influenced by abiotic and biotic factors as much as if they were in the field. This is something that could continue to be tested in the future in order to determine if this hypothesis is an accurate underlying mechanism for the pattern being described.

In the Sword et al (2017) study the differences in volatile organic compounds composition and quantity associated with location and tissue type of the plant were considered by separating the data analysis depending on the reproductive structures and the locations of the plant tissues that were used in the trials. They did this by doing different trials depending on whether the structure was a square or boll and if it was from the greenhouse or the field. However, the hypothesis that the insects were responding behaviorally due to differences in volatile organic compounds is not supported by the current study as the chemistry does not suggest that fungal endophyte treatment influences the composition and quantity of these volatiles.

This does not change the fact that the stink bugs behavior was significantly different between fungal endophyte treated tissues and there are some possible reasons that the behavior from the Sword et al (2017) study was observed. One hypothesis is that volatile organic compounds are not the factor that is influencing the behavior of the stink bugs in making their choices of approaching treated versus untreated reproductive structures.

Fungal endophytes have been shown to alter other aspects of plant tissue such as increasing the presence of toxic alkaloids in the tissue to deter feeding (Patchett et al 2008). It is possible the presence of these compounds in higher amounts in the tissue could have deterred stink bug feeding in the Sword et al (2017) study. There could also be other different interactions occurring that results in the lack of interest of the stink bugs in the treated tissues that have not yet been studied and finding these interactions could be another area to further research in the future.

Another reason that significant differences between volatile organic compounds due to fungal treatment were not found could be due to the fact that this experiment was conducted looking at 10 samples per treatment and this simply may not have been a large enough of a sample size to see a difference due to treatment. In the future if this experiment is repeated, utilizing a larger sample size could allow for more realistic interactions to be observed.

Another aspect of the current experiment that could be improved would be to weigh the squares and bolls to make sure that there was a more standard biomass across all the trials instead of just relying on approximate sizes of matchheads and quarter size diameters. It would also be ideal to have the same weights between square and boll trials because this would allow for more standardization of the data and could impact the results differently. It could be possible that a treatment effect could be observed if there was both a larger sample size and a more standardized measurement of the tissues tested. In this way the comparison between structures would be more accurate.

CHAPTER V

CONCLUSION

Even though there is an impact on insect behavior and performance when interacting with endophyte treated plants, testing for differences in volatile organic compound composition does not support the hypothesis of differences in insect behavior being due to volatile organic compounds. However, plant structure and location where plants are grown can both significantly impact the composition and quantity of volatile organic compounds released from the tissues. This finding demonstrates the importance of utilizing consistent tissues and locations when experiments are conducted, especially regarding plant-insect interactions.

REFERENCES

Backman P, R Sikora. 2008. Endophytes: an emerging tool for biological control. Biol. Control. 46: 1-3.

Bamisile B, C Dash, K Akutse, K Ravindran. 2018. Prospects of endophytic fungal entomopathogens as biocontrol and plant growth promoting agents: an insight on how artificial inoculation methods affect endophytic colonization of host plants. Microbiol. Res. 217: 34-50.

Dalal K, Olson L, Yu M, Salunkhe D. 1967. Gas chromatography of the field-, glass-Greenhouse-grown, and artificially ripened tomatoes.: *Lycopersicon esculentum* mill Phytochem 6:155-157 doi:10.1016/0031-9422(67)85025-8

Dudareva N, F Negre, D Nagegowda, and I Orlova. 2006. Plant Volatiles: recent advances and future perspectives. Critical Reviews in Plant Sciences. 25: 417-40.

Ek-Ramos, M.J., Zhou, W., Valencia, C.U., Antwi, J.B., Kalns, L.L., Morgan, G.D., Kerns, D.L. and Sword, G.A. 2013. Spatial and temporal variation in fungal endophyte communities isolated from cultivated cotton (*Gossypium hirsutum*). PLoS ONE, 8(6), e66049.

Gao F, C Dai, X Liu. 2010. Mechanisms of fungal endophytes in plant protection against pathogens. Afr. J. Microbiol. Res. 4(13): 1346-1352

Holopainen, Jarmo K. "Multiple functions of inducible plant volatiles." Trends in plant science 9.11 (2004): 529-533.

Jallow M, Dugassa-Gobena D and Vidal S. 2008. Influence of an endophytic fungus on host plant selection by a polyphagous moth via volatile spectrum changes Arthropod-Plant Interactions 2:53-62 doi:10.1007/s11829-008-9033-

Kigathi R, S Unsicker, M Reichelt, J Kesselmeier, J Gershenzon, W Weisser. 2009. Emission of volatile organic compounds after herbivory from *Trifolium pratense* (L.) under laboratory and field conditions J Chem Ecol 35 doi:10.1007/s10886-009-9716-3

Lopez D, G Sword. 2015. The endophytic fungal entomopathogens *Beauveria bassiana* and *Purpureocillium lilacinum* enhance the growth of cultivated cotton (*Gossypium hirsutum*) and negatively affect survival of the cotton bollworm (*Helicoverpa zea).* Biol. Control. 89: 53-60.

Lopez D, K Zhu-Salzman, MJ Ek-Ramos, G Sword. 2014. The entomopathogenic fungal endophytes *Pupureocillium lilacinum* (Formly *Paecilomyces lilacinus*) and *Beauveria bassiana* negatively affect cotton aphid reproduction under both greenhouse and field conditions. PLOS One. DOI: 10.1371/journal.pone.0103891

Navarro-Meléndez A, M Heil. 2014. Symptomless endophytic fungi suppress endogenous levels of salicylic acid and interact with the jasmonate-dependent indirect defense traits of their host, Lima bean (*Phaseolus lunatus*). J Chem Ecol 40:816–825

Patchett B Chapman R Fletcher Let al. 2008. Root loline concentration in endophyte-infected meadow fescue (*Festuca pratensis*) is increased by grass grub (*Costelytra zealandica*) attack N Z Plant Prot 2008 61 2104

Paré PW, JH Tumlinson. 1997. De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. Plant Physiol 114:1161–1167

Porras-Alfaro A, P Bayman. 2011. Hidden fungi, emergent properties: endophytes and microbiomes. Annu Rev Phytopathol. 49: 291-315.

Sword G, A Tessnow, and MJ Ek-Ramos. 2017. Endophytic fungi alter sucking bug responses to cotton reproductive structures. Insect Science. DOI: 10.1111/1744-7917.12461