FUNGAL ENDOPHYTES AFFECT STINKBUG OLFACTORY RESPONSES TO COTTON BOLLS

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Abstract

Piercing-sucking bugs have become a primary pests in cotton agriculture in the wake of decreased broad-spectrum insecticide sprays. These insects are not typically affected by *Bt* traits and so require other methods of control. The plant microbiome is an important determinant of plant health and can help protect the plant from insect herbivores and other stressors. Fungal endophytes, asymptomatic endosymbionts of plants, have particularly been recognized for their ability to influence herbivore host-plant selection. Recently published research demonstrated that cotton seeds which were treated with one of two fungal endophytes, *Beauveria bassiana* and *Phialemonium inflatum,* caused a significant deterrence to *Nezara viridula* feeding on fruits (bolls) excised from the treated plants. In this study, we expand on these findings by testing stink bug olfactory preference for endophyte-treated or untreated plants using live intact bolls in a Y-tube olfactometer. We found that *N. viridula* exhibited strong preference for the headspace odors of bolls from untreated plants over plants treated with *B. bassiana*. We did not observe a similar trend in *N. viridula* behavior when given a choice between untreated plants and plants treated with *P. inflatum*. In tests with another stink bug, *Euschistus servus*, the insects showed no preference for untreated plants over *B. bassiana*-treated plants. Although *E. servus* tended to choose headspace odors from untreated plants over *P. inflatum*-treated plants, this trend was not statistically significant. Differences in volatile organic compound emissions from damaged versus undamaged plant tissues might explain why we observed different herbivore behavior in this study using live intact tissues compared to the study which used excised tissues. We are continuing this research by directly investigating changes in volatile profiles due to endophyte-treatment and how these changes might influence herbivore behavior.

Introduction

Stink bugs such as *Nezara viridula*, the southern green stink bug, and *Euschistus servus,* the brown stink bug, are pests of cotton. These insects feed on the fruits (bolls), transmit boll rot pathogens, and can cause the plant to abscise the bolls which ultimately result in yield loss (Medrano et al. 2009). The widespread success of *Bt* traits in combination with the Boll Weevil Eradication Program has led to decreased use of broad-spectrum insecticides and pests such as stink bugs, which were once secondary, are now a primary concern (Medrano et al. 2009). The piercing-sucking mode of feeding for stink bugs prevents them from being affected by the insecticidal traits of *Bt* cotton which typically target chewing insects (Medrano et al. 2009). This combination of factors necessitates the development of alternative methods of control for these insects. Specifically, we designed these experiments to test if manipulating the microbiome of the cotton plants could affect olfactory preference of stink bugs.

The plant microbiome is increasingly recognized as an important determinant of plant health and potential source of protection for the plant against abiotic and biotic stressors (Sword et al. 2017). Fungal endophytes are microscopic fungi that live within plant tissues and have been shown to influence host plant selection behavior by herbivores (Jallow et al. 2008). In a study by Sword et al. (2017), *N. viridula* was deterred from feeding on bolls from cotton plants which were treated as seeds with the fungal endophytes *Beauveria bassiana* and *Phialemonium inflatum.* Specifically, *N. viridula* demonstrated a preference for bolls from untreated plants compared to endophyte-treated plants in choice-tests, and a significant proportion refused to feed on bolls from endophyte-treated plants in no-choice-tests. In this study, we used a Y-tube olfactometer to examine the olfactory preferences of *N. viridula* and *E. servus* for the live intact bolls of plants treated with either *B. bassiana* or *P. inflatum* compared to untreated control plants.

Methods

We collected *N. viridula* from cowpea grown at the A&M Field Station in Burleson Co., TX in June 2018. Adults and juveniles were maintained in 33cm^3 screen cages maintained at 30°C on a 16L:8D photoperiod and fed fresh corn, string beans and supplemental water provided in a plastic 15ml centrifuge tube stopped with cotton gauze. We collected *E. servus* from weedy *Guara spp.* along roadsides in College Station, TX in May 2018 and maintained them in similar fashion to *N. viridula* in a separate cage.

We used a spore-soak method to inoculate the cotton seeds (Phytogen 367) with the fungi of interest. The fungus *B. bassiana* was obtained as the commercially available BotaniGard (BioWorks). The fungus *P. inflatum* (strain TAMU490) was isolated as an endophyte from field-grown cotton in Snook, TX as part of earlier research on fungal endophytes of cotton (Ek-Ramos et al. 2013). To create the spore suspension for the seed soaks, spores were harvested from 3-week old cultures on potato dextrose agar by scraping the surface with a sterile scalpel and rinsing with autoclaved water. The concentration of spores in the solution was determined using a haemocytometer and the titer was adjusted to 10^7 spores/mL. We surface sterilized seeds prior to the spore soak by immersing them in 1% hypochlorate solution for 3 minutes and 70% ethanol for 2 minutes followed by rinses in sterile water. We then soaked the seeds overnight in sterile dishes with the spore solution at a ratio of approximately 1 mL/4 seeds. We verified the viability of spores on the seed after treatment by vortexing individual seeds in a 1 mL centrifuge tube with 1 mL of .01% Triton X-100 and plating a dilution of this wash on PDA.

We planted the seeds in 6-cell seed-starter pots in SunGro Professional Growing Mix soil and transplanted into 1.5-gallon pots after the development of the 3rd true-leaf. Plants were maintained in a greenhouse and provided water and fertilizer as needed until bolls developed (approximately 3 months). We kept the plants pest-free by spraying abamectin on a biweekly basis. Y-tube tests were only performed after 1 week minimum had passed since the previous spray to minimize the effects of residues in our tests.

Y-tubes were constructed using clear acrylic tubes with a 3.5 cm inner diameter x 17 cm long main tube which bifurcates into two 13 cm long arms of equal inner diameter. Each arm is fitted with airtight Teflon tubing which carries air from the plant headspace chambers into the Y-tube. Each arm receives air from an individual plant headspace chamber for either an endophyte-treated or untreated plant. We constructed headspace chambers from half-gallon mason jars fitted with air-tight inlet/outlet valves and the two-part lids modified. Briefly, a slot was cut into the disc part of the lid to fit a petiole of a boll and the spaces on either side of the petiole in the slot were plugged with clean cotton. This allowed excess air to escape through the cotton plugs without allowing outside air to enter the system. 2 L/min of filtered air were pumped into each headspace chamber and a total of 2 L/min passed through the Y-tube.

Naïve adult insects were food-deprived for 8 hours prior to the experiment. The insect was placed in an assimilation chamber at the entrance of the Y-tube for 1 minute prior to the experiment. Insects were given up to 20 minutes to choose an olfactory preference. An insect was declared as having made a choice when they passed fully into one arm of the Y-tube. Insects that failed to demonstrate a preference were recorded as “no choice” and were omitted from statistical analysis. Each insect was only used for one test and for each test a new set of plants was used. Jars were washed in 1% Alcanox solution after each experiment. We used JMP to perform a 1-tailed binomial distribution test to determine if the insects chose untreated plants more frequently than what would be observed by random chance.

Results

N. viridula exhibited a significant olfactory preference for the headspace odors from bolls of untreated plants when given a choice between *B. bassiana* treated- and untreated plants (Figure 1). Interestingly, we did not observe the same trend when *N. viridula* was given a choice between the headspace odors of *P. inflatum* treated- and untreated plants (Figure 1).

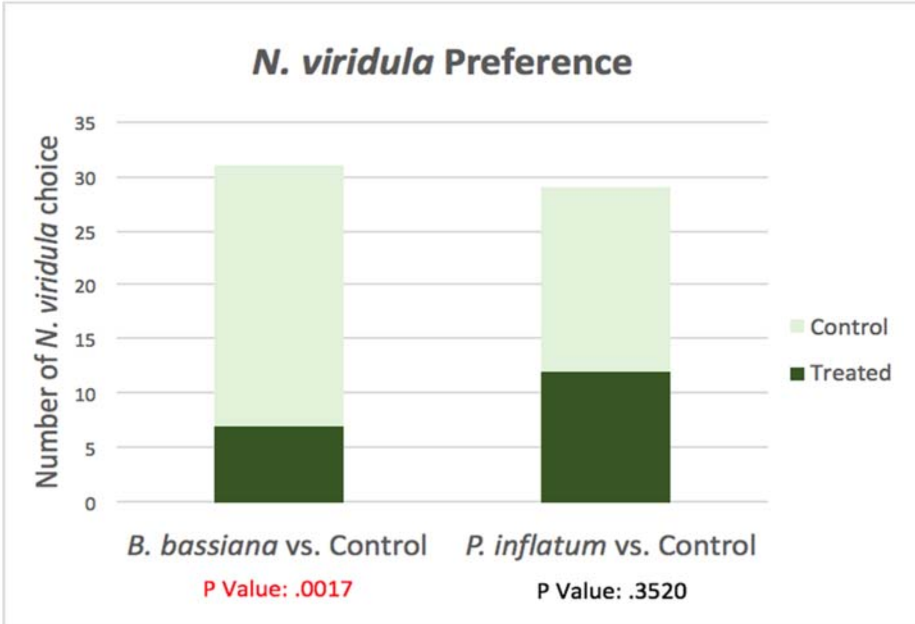

Figure 1. *N. viridula* olfactory preference for cotton plants treated with either *B. bassiana* or *P. inflatum* versus untreated plants.

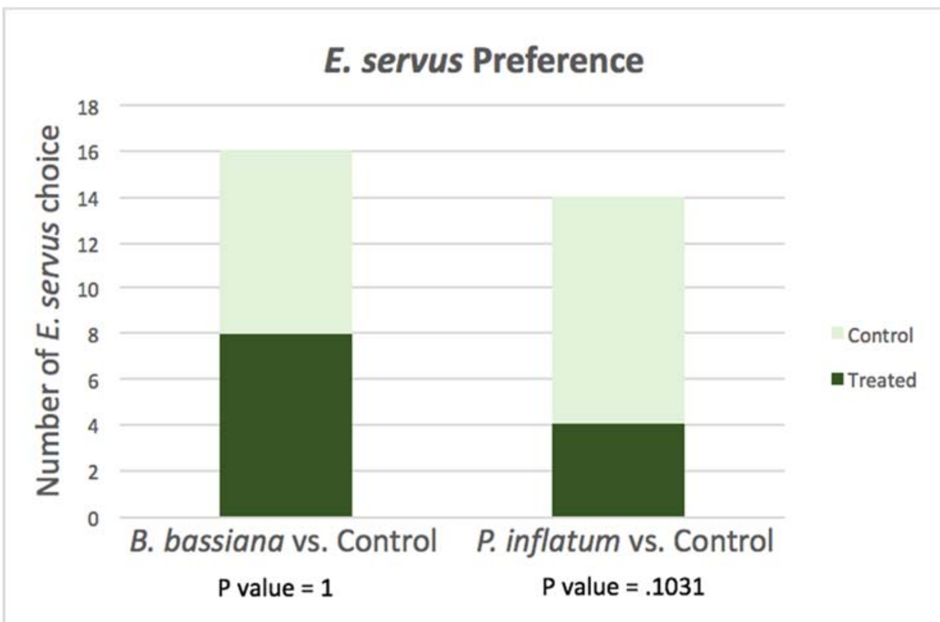

Figure 2. *E. servus* olfactory preference for cotton plants treated with either *B. bassiana* or *P. inflatum* versus untreated plants.

E. servus did not exhibit a significant olfactory preference for the headspaces odors from bolls of either *B. bassiana*-treated or untreated plants (Figure 2). When given a choice between untreated and *P. inflatum*-treated plants, *E. servus* chose the headspace odors of untreated plants more frequently, but this trend was not statistically significant.

Summary

Our results corroborate findings from Sword et al. (2017) which suggest that *N. viridula* prefers untreated plants over plants treated with *B. bassiana,* but plants treated with *P. inflatum* did not invoke the same response. In contrast, *E. servus* did not demonstrate any preference for the headspace odors of untreated plants over those from *B. bassiana*-treated plants. Although *E. servus* showed some olfactory preference for untreated bolls compared to bolls of *P. inflatum*-treated plants, this trend was not statistically significant. Our findings suggest that *B. bassiana*-treatment might influence herbivore behavior in a species-specific manner.

Another factor which influences herbivore behavior and might explain differences between our findings and the Sword et al. (2017) study is the use of damaged or undamaged plant tissues in these types of studies. We utilized specially designed headspace chambers to allow us to use live intact bolls on greenhouse-grown plants in these Y-tube olfactometer tests. In contrast, the Sword et al. (2017) assessed insect preference using choice and no-choice assays with excised plant tissues from plants grown in the greenhouse and field. These differences in experimental design are an important consideration because the odors emitted from plant tissues in these experiments can change dramatically in response to excision. Williams et al. (2005) found that volatile emissions from excised plant tissues can be up to 60% different than emissions from intact tissues in terms of the abundance of certain compounds. We are currently performing experiments to directly assess if endophyte treatment influences volatile emissions from both damaged and undamaged cotton plants. We plan to use the findings from these experiments to further investigate how endophyte-treatment might alter the host-plant seeking behavior of cotton pests. Understanding the mechanisms underlying endophyte-mediated volatile production and their effects on insect olfactory responses can potentially play an important role in the management of a broad range of problematic piercing-sucking bugs.

Acknowledgments

We extend our gratitude to Janice Krumm for help constructing the Y-tube olfactometers. We also thank Cesar Valencia and Jason Wulff for help with fungal cultures and identification. We appreciate the help received from Bill Ree, Polly Harding, Wenqing Zhou who helped collect and maintain insects.

References

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.

Jallow, M.F.A, 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.

Sword, G.A., Tessnow, A., and Ek-Ramos, M.J. (2017) Endophytic Fungi Alter Sucking Bug Responses to Cotton Reproductive Structures. Insect Science 24(6):1003-14.

Medrano, E. G., Esquivel, J.F., Nichols, R.L., and Bell, A.A. (2009) Temporal Analysis of Cotton Boll Symptoms Resulting from Southern Green Stink Bug Feeding and Transmission of a Bacterial Pathogen. Journal of Economic Entomology 102(1):36-42.

Williams III, L., Rodriguea-Saona, C., Pare, P.W., and Crafts-Brandner, S.J. (2005) The Piercing-Sucking Herbivores *Lygus hesperus* and *Nezara viridula* Induce Volatile Emissions in Plants. Archives of Insect Biochemistry and Physiology 58:84-96.