CHEMICAL DEFENSES OF COTTON PLANTS AND FACULTATIVE FUNGAL ENDOPHYTES: INDUCTION BY CATERPILLAR HERBIVORY

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2020

Major Subject: Entomology

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ABSTRACT

Facultative fungal endophytes (FFEs) are unspecialized plant-associated fungi capable of colonizing plant tissues without causing symptoms of disease. Studies of cotton (*Gossypium hirsutum* L.) treated with FFEs suggest that they may enhance plant defense against insect herbivores. Chemical defenses are typically classified as direct or indirect, with direct defenses toxic or repellant to herbivores, and indirect defenses attractive to natural enemies of herbivores. I examined volatile organic compound (VOC) emissions and extrafloral (EF) nectar production in the presence and absence of herbivores to determine whether indirect defenses were affected by FFE treatments. Cotton produces a class of chemicals known as terpenoid aldehydes that are responsible for much of this plant's direct defense against chewing pests such as caterpillars, and I examined those chemicals similarly. I found clear evidence that changes in these chemical defenses are involved in cotton's induced response to caterpillar herbivory, which corroborates many other studies in this field. I did not find overarching evidence that the FFE treatments influenced plant production of these chemical defenses, but this is likely the result of low colonization rates throughout my experiments. Furthermore, despite low colonization rates, I found that emissions of the acyclic monoterpene ocimene appeared to be enhanced by FFE treatments, and this finding warrants further investigation. I conclude that the various techniques used throughout these chapters should be combined into a single comprehensive design that might provide a clearer examination of FFE-mediated herbivore resistance in cotton.

ACKNOWLEDGEMENTS

I thank Dr. Anjel Helms, Dr. Heath Blackmon, and Dr. Giri Athrey for their guidance and support in this research. I also thank Dr. Hojun Song, without whom I may not have come this far. I thank Pierre Lesne for his mentorship. I thank Cesar Valencia for going above and beyond to be a great lab manager and lab mate. I thank my undergraduate students Zoey Kramer, Christine Madamba, and Caroline Wilson for their diligent contributions. I owe thanks to many members of the Entomology Department, USDA Southern Plains Agricultural Research Center, and High Performance Research Computing for all of their assistance. Lastly, I could not have achieved this without the love and support of Marina and our family.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Professors Greg Sword, Micky Eubanks, and Raul Medina of the Department of Entomology, Professor Mike Kolomiets of the Department of Plant Pathology, and Dr. Charles Suh of the USDA Southern Plains Agricultural Research Center.

The chemical analyses in Chapter 4 were conducted by Lorraine S Puckhaber of the USDA Southern Plains Agricultural Research Center (SPARC), and Jose Perez of SPARC assisted greatly in the analysis of volatile chemistry in Chapter 2. The majority of the bioinformatics performed in Chapter 3 was accomplished with the assistance of Michael Dickens of the High Performance Research Computing Center. All other work conducted for the dissertation was completed by the student independently.

Funding Sources

This work was made possible by the United States Department of Agriculture-National Institute of Food and Agriculture Award Number 2016-76013-24758. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the United States Department of Agriculture-National Institute of Food and Agriculture.

NOMENCLATURE

FFE	Facultative Fungal Endophyte
ISR	Induced Systemic Resistance
VOC	Volatile Organic Compound
EF	Extrafloral
SPME	Solid-Phase Micro-Extraction
ANOVA	Analysis of Variance
PERMANOVA	Permutational Analysis of Variance
PCA	Principal Component Analysis
GLV	Green Leaf Volatile
GLMM	Generalized Linear Mixed Model
JA	Jasmonic Acid
G	Gossypol
HGQ	Hemigossypolone
ODT	Optimal Defense Theory

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1. INTRODUCTION TO THE STUDY SYSTEM

1.1. Facultative Fungal Endophytes

Plant-associated microbes are regarded as critical determinants of plant health, especially when plants are challenged by abiotic stressors, but they can also mediate plant responses to biotic stressors such as insect herbivores (Pineda et al. 2010; Pineda et al. 2013; Martin et al. 2017; Gange et al. 2019; Harman & Uphoff 2019). While a growing body of knowledge demonstrates how various members of the plant microbiome have powerful influence on plant physiological processes such as water retention (Waller et al. 2005; Dimkpa et al. 2009; Yang et al. 2009; Redman et al. 2011; Aroca et al. 2013), mechanistic knowledge is lacking of how certain fungal endophytes may shape plant-insect interactions.

Mycorrhizal fungi associate with plants through the root-soil interface of the rhizosphere, whereas other fungi, known as endophytes, are capable of colonizing aboveground tissues and develop within the plant without sign of infection. Importantly, both types of fungi are known to influence plant-herbivore interactions (Hartley and Gange 2009; Gange et al. 2019). Fungi that form arbuscular or ectomycorrhizal associations with plant roots directly enhance nutrient acquisition and as a result they can indirectly affect herbivores by changing the quality of host plants (Manninen et al. 1998, Bennett et al. 2006; Koricheva et al. 2009; Meier et al. 2018). The mechanisms by which most fungal endophytes affect herbivores are still poorly understood because of variation in the herbivore responses to different plant-endophyte combinations, with

dependencies on whether the herbivores and endophytes are generalists or specialists, and the manner in which the experiments are conducted (Rodriguez et al. 2009; Pineda et al. 2010; Pineda et al. 2013; Gange et al. 2019).

The fungal endophytes that are best known and functionally understood are fungi in the genus *Epichloë,* obligate endosymbionts of grasses that are vertically transmitted across generations within seeds (Clay 1988; Clay 1990; Cheplick & Faeth 2009; Leuchtmann et al. 2014). In this system, the fungi extensively colonize the blades of grass and produce alkaloids that are toxic to many herbivores. As such, the symbiosis is considered a defensive mutualism because both the plant and the endophyte benefit from reduced herbivore damage (Brem & Leuchtmann 2001; Faeth 2002; Faeth & Saari 2011). In contrast, the overwhelming majority of fungal endophytes are unspecialized, capable of surviving outside the plant as well as colonizing a range of unrelated host species, and are horizontally transmitted to other plants mainly through spore dispersal (Rodriguez et al. 2009). These facultative fungal endophytes (FFEs) are ubiquitous in terrestrial ecosystems and may play multiple functional roles in plants (Vega et al. 2008; Rodriguez et al. 2009; Hartley & Gange 2009; Porras-Alfaro & Bayman 2011). The ecological significance of FFEs has been a topic of debate (*e.g.,* Faeth 2002; Faeth & Fagan 2002) and some have hypothesized that many FFEs could be dormant pathogens or saprobes which colonize the plant and essentially wait for the plant to senesce or be weakened by another attacker (Porras-Alfaro & Bayman 2011). On the other hand, the simple fact that FFEs colonize plant tissues implies that the fungi must interact with

plant immunity, thus the symbiosis could be maintained due to defensive mutualism wherein the endophytes act as plant protectants (Jung et al. 2012; Pineda et al. 2013).

Entomopathogenic FFEs such as *Beauveria bassiana*, *Metarhizium spp.*, and *Lecanicillium spp.*, are capable of utilizing both insects and plants as hosts, and have received the most attention in studies investigating the capacity for FFEs to protect plants against herbivores (Vega et al. 2008; Barelli et al. 2016; Jaber & Ownley 2018; Gange et al. 2019). Remarkably, there is very little evidence of insects being directly infected when feeding on plants colonized by these fungi as endophytes (*e.g.,* Quesada-Moraga et al. 2009; Akello & Sikora 2012; Akutse et al. 2013; Batta 2013; Lopez et al. 2014; Lopez & Sword 2015; Vianna et al. 2018). Although endophytic entomopathogens have received the majority of attention, FFEs without an insect parasitic life stage have also been investigated for their effects on plant-insect interactions. Comparisons of the effects that entomopathogenic versus non-entomopathogenic FFEs have on plant-insect interactions is the topic of a recent comprehensive review by Gange et al. (2019).

1.2. The Premise of Priming and Induced Systemic Resistance

The mechanisms by which FFEs mediate plant-insect herbivore interactions are not fully understood, but are generally believed to be the result of changes in plant chemistry (Ownley et al. 2010; Menjivar et al. 2012; Kusari et al. 2012; Gange et al. 2012). A key challenge in understanding FFE-mediated changes in plant chemistry is distinguishing whether the compounds are fungal products or if the plant-fungus interaction triggers plant-mediated changes in defensive chemistry via priming. Priming

is the modulation of phytohormones that regulate the plant immune response, which can result from an interaction with beneficial microbes (Conrath et al. 2006; Van Wees et al. 2008; Jung et al. 2012; Mauch-Mani et al. 2017). As a result of priming, plants are capable of launching a defensive response more efficiently than plants that did not interact with those microbes. This phenomenon, as a whole, is known as induced systemic resistance (ISR) (Pieterse et al. 2009; Zamioudis & Pieterse 2012; Pineda et al. 2013; Nguvo & Gao 2019).

1.3. Volatile Organic Compound Emissions

Volatile organic compounds (VOCs) are odors emitted by plants as a fundamental part of their secondary metabolism (Pichersky & Gershenzon 2002; Pichersky et al. 2006; Dudareva et al. 2013). Constitutive VOC emissions are the blends of compounds emitted from a healthy plant in the absence of herbivory, and can be used as olfactory cues by herbivorous insects in the search for a host plant (Holopainen 2004; Dudareva et al. 2006). On the other hand, plants respond to herbivory with an induced VOC response wherein a blend of compounds that is typically quantitatively and qualitatively distinct from the constitutive emissions is released. These induced VOC emissions can serve as both direct and indirect defenses, either being toxic or repellent to the herbivore, or serving as olfactory cues attracting predators and parasitoids to their herbivorous prey (Bezemer & Van Dam 2005; Dicke & Baldwin 2010; McCormick et al. 2012; Aljbory & Chen 2016; Turlings & Erb 2018).

A recent study examined the behavior of sucking-bug pests towards flower buds and developing fruits on cotton (*Gossypium hirsutum*) plants treated with the entomopathogenic FFE *Beauveria bassiana* and the non-entomopathogenic FFE *Phialemonium inflatum* (Sword et al. 2017). In choice assays, the sucking bugs preferred reproductive structures of non-treated controls over FFE-treated plants. In no-choice assays, they exhibited a stronger hesitancy to feed, or a longer latency to first contact, with the reproductive structures of FFE-treated plants compared to controls. This behavior indicates that the herbivores could be reacting to VOC olfactory cues which influence their behavior prior to initiating feeding, rather than reacting to gustatory cues that they encounter during feeding.

1.4. Extrafloral Nectar Production

Extrafloral (EF) nectar, like VOCs, is an indirect plant defense, but is not as ubiquitous as VOCs. EF nectar functions as an indirect defense by recruiting parasitoids and predatory insects, especially ants, to forage on the plant for carbohydrates. This recruitment increases the probability that any herbivores that are present on the plant will be preyed upon or parasitized (reviewed in Heil 2015).

In the genus *Gossypium*, at least 36 species are known to bear EF nectaries while only a single species lacks them, *G. tomentosum* (Weber & Keeler 2012, Wäckers & Bonifay 2004, Meyer & Meyer 1961). *G. tomentosum* is native to Hawaii where there are no native ant species. The lack of EF nectaries on this species lends support to the hypothesis that their maintenance comes with an allocation cost to the plant, but many

Gossypium species have maintained EF nectaries through a facultative mutualism with ants (Wäckers & Bonifay 2004, Heil & McKey 2003, Agrawal & Rutter 1998, Meyer & Meyer 1961).

1.5. Glandular Terpenoids, Direct Chemical Defenses

Cotton produces a suite of terpenoid aldehydes that are responsible for much of the plant's direct defense against chewing herbivores, and FFE treatments may enhance the production of these chemicals. Several studies of cotton treated with FFEs have demonstrated negative effects on both above- and below-ground herbivory. Isolates of the non-entomopathogenic FFEs, *Chaetomium globosum* and *Phialemonium inflatum*, found naturally occurring in cultivated cotton (Ek-Ramos et al. 2013), have been shown to negatively affect cotton aphids, beet armyworm caterpillars (*Spodoptera exigua*), and root-knot nematodes (*Meloidogyne incognita*) (Zhou et al. 2016, 2018). Commercially available *B. bassiana* was found to reduce cotton aphid (*Aphis gossypii*) reproduction under greenhouse and field conditions when inoculated to plants via seed treatment (Lopez et al. 2014). This treatment was also found to enhance plant growth and reduce the survivorship of cotton boll worm (*Helicoverpa zea*) (Lopez & Sword 2015).

1.6. A Brief Summary of the Experiments

In Chapter 2, I describe experiments designed to test whether ISR occurs in cotton plants treated with *B. bassiana* or *P. inflatum* by assessing constitutive and induced VOC emissions of live intact leaves. ISR resulting from arbuscular mycorrhizal

colonization has been shown to enhance emission of volatile organic compounds (Guerrieri et al. 2004; Leitner et al. 2010; Schausberger et al. 2012). Priming due to FFE colonization may be similar to priming by mycorrhizal fungi, and VOC emissions from FFE-treated plants could be altered compared to controls.

In Chapter 3, I describe a study designed to test whether the experimental FFE treatments influenced the production of extrafloral nectar. The inducibility of indirect defenses, both VOC emissions and EF nectar production, are known to be JA-dependent (reviewed in Schuman & Baldwin 2016, Heil 2015), and EF nectar production has been found to be influenced by fungal endophytes (Navarro-Melendez & Heil 2014, Jaber & Vidal 2009). Thus, an analysis of EF nectar complimentary to the analysis of VOCs was warranted. FFE-treated and non-treated control plants were grown in the greenhouse and EF nectar was collected from herbivore-free and herbivore-infested plants.

FFE treatments negatively affected a range of above- and belowground pests in previous studies. Pest species and feeding mode (*e.g.*, chewing versus piercing-sucking) can determine the defensive chemical response to attack in cotton plants (Eisenring et al. 2018). Induction of direct chemical defenses aboveground is well documented in response to caterpillar herbivory. In Chapter 4, I describe an experiment designed to test the hypothesis that the reduced survivorship of *H. zea* on FFE-treated cotton (Lopez et al. 2014) resulted from relatively higher levels of terpenoid aldehydes in the leaves. I test constitutive (herbivore-free) and induced (herbivore-present) levels of glandular terpenoids in greenhouse-grown cotton.

Overall, I test whether multiple forms of inducible chemical defenses in cotton, in response to caterpillar herbivory, are affected by FFE treatments. I find that the chemical defenses assessed in these studies are involved in cotton's induced response to caterpillar herbivory, which corroborates a large body of literature on the subject. Many of the chemical responses reported in these studies are well documented, but a few are unique.

FFE treatments did not influence the defensive chemical responses to herbivory in a statistically significant manner, but this is likely the result of relatively low levels of FFE colonization compared to previous studies. In the study of VOCs, despite the low levels of detectable FFE colonization, there were trends in individual compounds that suggest FFE treatments may prime plants as suspected. In the study of EF nectar production, I found a novel systemic response to foliar herbivory in the form of increased sucrose concentration at bracteal EF nectaries. In the study of glandular terpenoid aldehydes I found clear signals of the induction of a particular class of compounds in response to caterpillar herbivory, despite significant variation across experiments. In general, these studies contribute to a growing body of literature that details the various ways that FFE-plant interactions may influence plant-insect interactions.

1.7. References

Agrawal AA, Rutter MT (1998) Dynamic anti-herbivore defense in ant-plants: the role of induced responses. Oikos 83: 227-236.

Akello J, Sikora R (2012) Systemic acropedal influence of endophyte seed treatment on *Acyrthosiphon pisum* and *Aphis fabae* offspring development and reproductive fitness. Biol Control 61:215-221.

Akutse KS, Maniania NK, Fiaboe KKM, Van Der Berg J, Ekesi S (2013) Endophytic colonization of *Vicia faba* and *Phaseolus vulgaris* (Fabaceae) by fungal pathogens and their effects on the life-history parameters of *Liriomyza huidobrensis* (Diptera: Agromyzidae) Fungal Ecol 6:293-301.

Alijbory Z, Chen M-S (2016) Indirect plant defense against insect herbivores: a review. Insect Sci doi:10.1111/1744-7917.12436.

Aroca R, Ruiz-Lozano JM, Zamarreño AM, Paz JA, García-Mina JM et al. (2013) Arbuscular mycorrhizal symbiosis influences strigolactone production under salinity and alleviates salt stress in lettuce plants. J Plant Physiol 170:47-55.

Barelli L, Moonjely S, Behie SW, Bidochka MJ (2016) Fungi with multifunctional lifestyles: endophytic insect pathogenic fungi. Plant Mol Biol 90:657-664.

Batta, YA (2013) Efficacy of endophytic and applied *Metarhizium anisopliae* (Metch.) Sorokin (Ascomycota: Hypocreales) against larvae of *Plutella xylostella* L. (Yponomeutidae: Lepidoptera) infesting *Brassica napus* plants. Crop Protect 44: 128-134.

Bennett AE, Alers-Garcia J, Bever JD (2006) Three-way interactions among mutualistic mycorrhizal fungi, plants, and plant enemies: hypothesis and synthesis. Am Nat 167:141-152.

Bezemer TM, Van Dam NM (2005) Linking aboveground and belowground interactions via induced plant defenses. Trends Ecol Evol 20:617-624.

Brem D, Leuchtmann A (2001) *Epichloë* grass endophytes increase herbivore resistance in the woodland grass *Brachypodium sylvaticum*. Oecologia 126:522-530.

Cheplick GP, Faeth SH (2009) Ecology and evolution of the grass endophyte symbiosis. Oxford University Press, Oxford, UK.

Clay K (1988) Fungal endophytes of grasses: a defensive mutualism between plants and fungi. Ecol 69:10-16.

Clay K (1990) Fungal endophytes of grasses. Annu Rev Entomol 21:275-297.

Conrath U, Beckers GJ, Flors V et al. (2006) Priming: getting ready for battle. Mol Plant Microbe In 19:1062-1071.

Dicke M, Baldwin IT (2010) The evolutionary context for herbivore-induced plant volatiles beyond the 'cry for help'. Trends Plant Sci 15:167-175.

Dimkpa C, Weinand T, Asch F (2009) Plant-rhizobacteria interactions alleviate abiotic stress conditions. Plant Cell Environ 32:1682-1694.

Dudareva N, Klempien A, Muhlemann JK, Kaplan I (2013) Biosynthesis, function and metabolic engineering of plant volatile organic compounds. New Phytol 198:16-32.

Dudareva N, Negre F, Nagegowda DA, Orlova I (2006) Plant volatiles: recent advanced and future perspectives. CRC Cr Rev Plant Sci 25:417-440.

Eisenring M, Glauser G, Meissle M, Romeis J (2018) Differential impacts of herbivores from three feeding guilds on systemic secondary metabolite induction, phytohormone levels, and plant-mediated herbivore interactions. J Chem Ecol https://doi.org/10.1007/s10886-018-1015-4

Ek-Ramos MJ, Zhou W, Valencia C, Antwi JB, Kalns LL, Morgan GD, Kerns DL, Sword GA (2013) Spatial and temporal variation in fungal endophyte communities isolated from cultivated cotton (*Gossypium hirsutum*). PLoS ONE doi: https://doi.org/10.1371/journal.pone.0066049

Faeth SH (2002) Are endophytic fungi defensive plant mutualists? OIKOS 98:25-36.

Faeth SH, Fagan WF (2002) Fungal endophytes: common host plant symbionts but uncommon mutualists. Integr Comp Biol 42:360-368.

Faeth SH, Saari S (2011) Fungal grass endophytes and arthropod communities: lessons from plant defense theory and multitrophic interactions. Fungal Ecol doi:10.1016/j.funeco.2011.09.003

Gange AC, Eschen R, Wearn JA, Thawer A, Sutton BC (2012) Differential effects of foliar endophytic fungi on insect herbivores attacking a herbaceous plant. Oecologia 168:1023-1031.

Gange AC, Koricheva J, Currie AF, Jaber LR, Vidal S (2019) Meta-analysis of the role of entomopathogenic unspecialized fungal endophytes as plant bodyguards. New Phytol doi:10.1111/nph.15859

Guerrieri E, Lingua G, Digilio MC, Massa N, Berta G (2004) Do interactions between plant roots and the rhizosphere affect parasitoid behaviour? Ecol Entomol 29:753-756.

Harman GE, Uphoff N (2019) Symbiotic root-endophytic soil microbes improve crop productivity and provide environmental benefits. Scientifica https://doi.org/10.1155/2019/9106395

Hartley SE, Gange AC (2009) Impacts of plant symbiotic fungi on insect herbivores: mutualism in a multitrophic context. Annu Rev Entomol 54:323-342.

Heil M (2015) Extrafloral nectar at the plant-insect interface: a spotlight on chemical ecology, phenotypic plasticity, and food webs. Annu Rev Entomol 60: 213-232.

Heil M, McKey D (2003) Protective ant-plant interactions as model systems in ecological and evolutionary research. Annu Rev Ecol Evol Syst 34: 425-453.

Jaber LR, Ownley BH (2018) Can we use entomopathogenic fungi as endophytes for dual biological control of insect pests and plant pathogens? Biol Control 116:36-45.

Jaber L, Vidal S (2009) Interactions between an endophytic fungus, aphids, and extrafloral nectaries: do endophytes induce extrafloral-mediated defences in *Vicia faba*? Funct Ecol 23: 707-714.

Jung SC, Martinez-Medina A, Lopez-Raez JA, Pozo MJ (2012) Mycorrhiza-induced resistance and priming of plant defenses. J Chem Ecol 38:651-664.

Koricheva J, Gange AC, Jones T (2009) Effects of mycorrhizal fungi on insect herbivores: a meta-analysis. Ecol 90:2088-2097.

Kusari S, Hertweck C, Spiteller M (2012) Chemical ecology of endophytic fungi: Origins of secondary metabolites. Chem Biol 19:797-798.

Leitner M, Kaiser R, Hause B, Boland W, Mithöfer A (2010) Does mycorrhization influence herbivore-induced volatile emission in *Medicago truncatula*? Mycorrhiza 20:89-101.

Leuchtmann A, Bacon CW, Schardl CL, White Jr JF, Tadych M (2014) Nomenclature realignment of *Neotyphodium* species with genus *Epichloë*. Mycologia 106:202-215. doi: https://doi.org/10.3852/13-251

Lopez DC, Sword GA (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 boll worm (*Helicoverpa zea*). Biol Control 89:53-60.

Lopez DC, Zhu-Salzman K, Ek-Ramos MJ, Sword GA (2014) The entomopathogenic fungal endophytes *Purpureocillium lilacinum* (Formerly *Paecilomyces lilacinus*) and *Beauveria bassiana* negatively affect cotton aphid reproduction under both greenhouse and field conditions. PLoS ONE 9 e103891.

Manninen AM, Holopainen T, Holopainen JK (1998) Susceptibility of ectomycorrhizal and non-mycorrhizal Scots pine (*Pinus sylvestris*) seedlings to a generalist herbivore, *Lygus rugulepennis*, at two nitrogen availability levels. New Phytol 140:55-63.

Martin FM, Uroz S, Barker DG (2017) Ancestral alliances: Plant mutualistic symbioses with fungi and bacteria. Science 356:1-9.

Mauch-Mani B, Baccelli I, Luna E, Flors V (2017) Defense priming: An adaptive part of induced resistance. Annu Rev Plant Biol 68:485-512.

McCormick AC, Unsicker SB, Gershenzon J (2012) The specificity of herbivore-induced plant volatiles in attracting herbivore enemies. Trends Plant Sci 17:1360-1385.

Meier AR, Hunter MD (2018) Arbuscular mycorrhizal fungi mediate herbivore-induction of plant defenses differently above and belowground. Oikos 127:1759-1775.

Menjivar RD, Cabrera JA, Kranz J, Sikora RA (2012) Induction of metabolite organic compounds by mutualistic endophytic fungi to reduce the greenhouse whitefly *Trialeurodes vaporariorum* (Westwood) infection on tomato. Plant Soil 352:233-241.

Meyer FR, Meyer VG (1961) Origin and inheritance of nectariless cotton. Crop Sci 1: 167-169.

Navarro-Meléndez AL, Heil M (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.

Nguvo KJ, Gao X (2019) Weapons hidden underneath: bio-control agents and their potential to activate plant induced systemic resistance in controlling crop *Fusarium* diseases. J Plant Dis Protect doi:10.1007/s41348-019-00222-y

Ownley BH, Gwinn KD, Vega FE (2010) Endophytic fungal entomopathogens with activity against plant pathogens: ecology and evolution. Biol Control 55:113-128.

Pichersky E, Gershenzon J (2002) The formation and function of plant volatiles: perfumes for pollinator attraction and defense. Curr Opin Plant Biol 5:237-243.

Pichersky E, Noel JP, Dudareva N (2006) Biosynthesis of plant volatiles: nature's diversity and ingenuity. Science 311:808-811.

Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SC (2009) Networking by small-molecule hormones in plant immunity. Nat Chem Biol 5:308-316.

Pineda A, Dicke M, Pieterse CM, Pozo MJ (2013) Beneficial microbes in a changing environment: are they always helping plants to deal with insects? Funct Ecol 27:574-586.

Pineda A, Zheng SJ, Van Loon JJA, Pieterse CM, Dicke M (2010) Helping plants to deal with insects: the role of beneficial soil-borne microbes. Trends Plant Sci 15:507-514.

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

Quesada-Moraga E, Muñoz-Ledesma FJ, Santiago-Álvarez C (2009) Systemic protection of *Papaver somniferum* L. against *Iraella luteipes* (Hymenoptera: Cynipidae) by an endophytic strain of *Beauveria bassiana* (Ascomycota: Hypocreales). Biol Control 38:723-730.

Redman RS, Kim YO, Woodwards CJ et al (2011) Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. PLoS ONE doi:10.1371/journal.pone.0014823

Rodriguez RJ, White Jr JF, Arnold AE, Redman RS (2009) Fungal endophytes: diversity and functional roles. New Phytol 182:314-330. doi:10.1111/j.1469-8137.2009.02773.x

Schausberger P, Peneder S, Jürschik S, Hoffmann D (2012) Mycorrhiza changes plant volatiles to attract spider mite enemies. Funct Ecol 26:441-449.

Schuman MC, Baldwin IT (2016) The layers of plant responses to insect herbivores. Annu Rev Entomol 61: 373-394.

Sword GA, Tessnow A, Ek-Ramos MJ (2017) Endophytic fungi alter sucking bug responses to cotton reproductive structures. Insect Sci 24:1003-1014. doi:10.1111/1744-7917.12461

Turlings TC, Erb M (2018) Tritrophic interactions mediated by herbivore-induced plant volatiles: mechanisms, ecological relevance, and application potential. Annu Rev Entomol 63:433-452.

Van Wees SC, Van der Ent S, Pieterse CM (2008) Plant immune response triggered by beneficial microbes. Curr Opin Plant Biol 11:443-448.

Vega FE, Posada F, Aime MC et al. (2008) Entomopathogenic fungal endophytes. Biol Control 46:72-82.

Vianna F, Pelizza S, Russo L, Allegrucci N, Scorsetti A (2018) Endophytic *Beauveria bassiana* (Ascomycota: Hypocreales) alters *Helicoverpa gelotopoeon's* (D.)

(Lepidoptera: Noctuidae) life cycle and reproductive parameters. J Plant Protect Res. doi:10.24425/jppr.2018.124643

Wäckers FL, Bonifay C (2004) How to be sweet? Extrafloral nectar allocation by *Gossypium hirsutum* fits optimal defense theory predictions. Ecol 85: 1512-1518.

Waller F, Achatz B, Baltruschat H et al. (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. PNAS 102:13386-13391.

Weber MG, Keeler KH (2013) The phylogenetic distribution of extrafloral nectaries in plants. Ann Bot 111: 1251-1261.

Yang J, Kloepper JW, Ryu C-M (2009) Rhizosphere bacteria help plants tolerate abiotic stress. Trends Plant Sci 14:1-4.

Zamioudis C, Pieterse CM (2012) Modulation of host immunity by beneficial microbes. Mol Plant Microbe In 25:139-150.

Zhou W, Starr JL, Krumm JL, Sword GA (2016) The fungal endophyte *Chaetomium globosum* negatively affects both above- and belowground herbivores in cotton. FEMS Microbiol Ecol 92. doi:10.1093/femsec/fiw158

Zhou W, Wheeler TA, Starr JL, Valencia CU, Sword GA (2018) A fungal endophyte defensive symbiosis affects plant-nematode interactions in cotton. Plant Soil 422:251-266. doi:10.1007/s11104-016-3147-z

2. SAMPLING VOLATILE ORGANIC COMPOUNDS FROM INDIVIDUAL COTTON LEAVES TO TEST FOR EFFECTS OF FUNGAL ENDOPHYTE TREATMENTS

2.1. Introduction

Many species of fungi are known to be endophytic, capable of colonizing plant tissues and developing entirely within the plant without eliciting any sign of infection. The majority of fungal endophytes are not obligately endophytic or specialized; they can develop outside of plant tissues and colonize a range of unrelated host species or be transmitted horizontally to other plants mainly through spore dispersal. These facultative fungal endophytes (FFEs) are ubiquitous in terrestrial ecosystems and may play multiple functional roles in plants (Porras-Alfaro and Bayman 2011). Studies of cotton (*Gossypium hirsutum*) inoculated with FFEs *Phialemonium inflatum* (strain TAMU 490) or *Beauveria bassiana* (strain GHA) via seed treatment showed reduced infestation by cotton aphid (*Aphis gossypii*) and reduced survivorship of bollworm (*Helicoverpa zea*) (Lopez and Sword 2015; Lopez et al. 2014).

Sword et al. (2017) found that seed treatments of cotton with *P. inflatum* or *B. bassiana* reduced the feeding preference of sucking-bug pests in choice assays. These treatments also increased the latency to first contact, *i.e.*, hesitancy to begin feeding, on FFE-treated plants in no-choice assays. Before feeding, several herbivorous insects perceive plant odors through volatile organic compounds (VOCs), which helps them determine a suitable host plant (Turlings and Erb 2018). The behavior observed by

Sword et al. (2017) indicated that *Lygus hesperus* and *Nezara viridula* could be reacting to VOC olfactory cues prior to initiating feeding. On the other hand, plants respond to herbivory by releasing a blend of compounds that may be qualitatively or quantitatively distinct from the constitutive emissions. These induced VOC emissions can serve as both direct and indirect defenses, either being toxic or repellent to the herbivore, or serving as olfactory cues attracting predators and parasitoids to their herbivorous prey (Turlings and Erb 2018).

I designed this study to assess the constitutive and induced VOC emissions of cotton plants treated with the FFEs *P. inflatum* or *B. bassiana* and tested for differences against non-treated plants. I adapted a solid-phase micro-extraction (SPME) method described in Park et al. (2020) and designed a chamber to sample VOCs from individual cotton leaves. I used beet army worm larvae (*Spodoptera exigua*) as herbivores (Paré and Tumlinson 1997) and quantified the amount of leaf tissue consumed so that differences in VOC emissions due to variations in leaf consumption were not erroneously attributed to FFE treatment effects.

2.2. Materials and Methods

2.2.1 Fungal Material

The fungal culture for *P. inflatum* (strain TAMU 490) originated from a field survey of FFEs of cultivated cotton grown in Texas (Ek-Ramos et al. 2013). The fungal culture for *B. bassiana* (strain GHA) was cultured from the commercially available biological control product BotaniGard (BioWorks, Inc., Victor, NY). Fungi were

cultured on potato dextrose agar (PDA) (VWR, Radnor, PA) amended with penicillin and streptomycin (Mediatech, Inc., Manassas, VA), using the manufacturer's recommended concentration, to prevent bacterial growth. Cultures of *B. bassiana* were incubated for 2 weeks and *P. inflatum* for 3 weeks before spore harvest. FFE spore solutions were prepared by adding 5 mL of autoclaved 0.1% Triton-X100 solution to the PDA plate and spores were put into suspension by gently scraping the surface with a spatula. The solutions were passed through a No. 500 (25 µm) USA standard test sieve and the spore concentration was calculated with a hemocytometer. Spore solutions were then adjusted to 10^7 spores/mL with nanopore water.

2.2.2 Plant Material

We used PhytoGen (PHY-367-WRF, PhytoGen Cottonseed, Dow Agrosciences LLC, Indianapolis, IN) cotton germplasm for two similar experiments. Approximately 120 acid-delinted and chemical treatment-free seeds were surface sterilized by soaking in 3% sodium hypochlorite solution for 3 minutes followed by 70% EtOH for 2 min. After air drying under a laminar flow hood, seeds were split into groups of 40 and soaked overnight in 6 mL spore solution. Seeds were planted in 150 cc 6-cell starter pots filled with MetroMix900 for the first experiment, which I refer to as the 8-hour experiment because the plants were exposed to herbivory for 8 hours. Seeds were planted in 400 cc pots for the second experiment, which I refer to as the 32-hour experiment becau. Plants were provided with fertilizer at planting by adding 1 L of water to the tray with 10 mL of CNS17 GROW 3-1-2 (Botanicare, Vancouver, WA). Plants were grown in Percival environmental chambers (Percival Scientific, Perry, IA) on a

14:10 h (L:D) cycle and a 29°C day: 22°C night cycle. These conditions were chosen after a discussion with Dr. Alois Bell at the USDA Southern Plains Agricultural Research Center. Plants were grown until the 3rd true leaf was fully expanded for the 8-hour experiment and the 7th fully expanded leaf for the 32-hour experiment. Larger plants were used in the 32-hour experiment so that larger leaves could be used for the extended period of herbivory.

2.2.3 Insect Material

Eggs of *S. exigua* were obtained from Benzon Research Inc. (Carlisle, PA) and reared individually in 4 cm diameter by 4 cm deep plastic cups on artificial diet (Southland Products Inc., Lake Village, AR). The rearing room was kept at 28°C with a 14:10 h (L:D) cycle. Once larvae reached the third instar, they were transferred from the diet cups into glass Petri dishes containing excised leaves of conventional (*i.e.,* non-transgenic, lacking *Bt* toxins) cotton (variety LA122) so that they could acclimate to feeding on leaf tissue prior to the start of the experiment. In the 8-hour experiment, we placed two third-instar larvae on either the first or second true leaf and in the 32-hour experiment we placed two fourth-instar larvae on the fifth or sixth true leaf. The number of larvae to use for each experiment was determined by preliminary trials such that herbivory would be sufficient for quantification by image analysis but not so extensive that the leaf would be consumed entirely.

2.2.4 Chemicals

Volatiles were assigned tentative identities by comparison of mass spectra to the Wiley Registry of Mass Spectral Data 10th Edition and the 2011 NIST/EPA/NIH Mass

Spectral Libraries. Standards were purchased based on tentative identities and identities were verified by comparison of retention times. Standards purchased from Sigma-Aldrich (St. Louis, MO): α-pinene, β-pinene, (*Z*)-3-hexenal, (*Z*)-3-hexen-1-ol, isomeric butyrates, 6-methyl-5-hepten-2-one, 1-hexyl acetate, 1-hexanol, 2-hexenal, 2-hexyl acetate, 3-hexyl acetate, 3-hexyl butyrate, limonene, humulene, caryophyllene, 2-hexen-1-ol, hexyl-2-methyl butyrate, linalool, (*E*)-β-farnesene, sabinene, phellandrene, β-myrcene, 3-methyl-butyl acetate, (*Z*)-β-ocimene. Standards purchased from Toronto Research Chemicals, Inc., (Ontario, CA): 3,8-dimethyl-1,4,7-nonatriene, (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene, and (*E*)-β-ocimene.

2.2.5 Assessment of Endophyte Colonization by PDA plating

A total of 50 plants treated with either *B. bassiana* (n=25) or *P. inflatum* (n=25) were used for FFE colonization confirmation. These were planted in sterilized sand because the intertwining of roots with mulch in the MetroMix900 soil inhibited consistent collections of root samples. Additionally, the roots of plants grown in the MetroMix900 soil in preliminary trials were found to have a high incidence of fast-growing non-target FFEs that would typically occupy the plate before enough time had passed for the target fungi to potentially grow out from other tissues on the same plate.

Seeds were treated as described above, with surface sterilization followed by overnight soak in spore solution of 10^7 spores/mL. Seeds were planted in autoclaved sand and grown in environmental chambers under the same conditions as the plants used in the experiments. At the first true leaf stage, the entire plant was removed from the pot and the roots were gently rinsed with tap water. Plants were surface sterilized in a

laminar flow hood in 0.5% sodium hypochlorite solution for 2 min followed by 70% EtOH solution for 2 min. The above- and below-ground portions were plated separately on PDA plates prepared as described above. The taproot and attached lateral roots were sectioned into approximately 1 cm fragments. Leaves were cut into approximately 1 cm^2 fragments while stems were not plated. Any fungal growth from fragments were sub-cultured onto a new PDA plate and target FFEs were identified by morphological comparisons of the conidia to conidia of inoculum cultures. Conidia were stained with cotton lactophenol blue and viewed under the microscope at 400x magnification.

2.2.6 Sampling Chambers

I used solid-phase micro-extraction (SPME) to sample static headspace of intact individual leaves enclosed in custom-made chambers. SPME allows for relative quantification of VOCs by comparison of peak areas for the same compounds across samples granted that sampling conditions are identical throughout. The chambers were constructed by modifying mason jars with two-part lids. We modified the disc part of the lid by drilling a slot from near the center of the disk to the edge for the petiole of the cotton leaf and a hole covered with a septum through which the SPME fiber would be inserted for sampling (Figure 2.1). The importance of using intact plant tissue in FFE studies is reviewed in Gange et al. (2019), and in sampling VOCs, the use of excised tissues has been found to produce significantly different results than intact tissues (Schmelz et al. 2001).

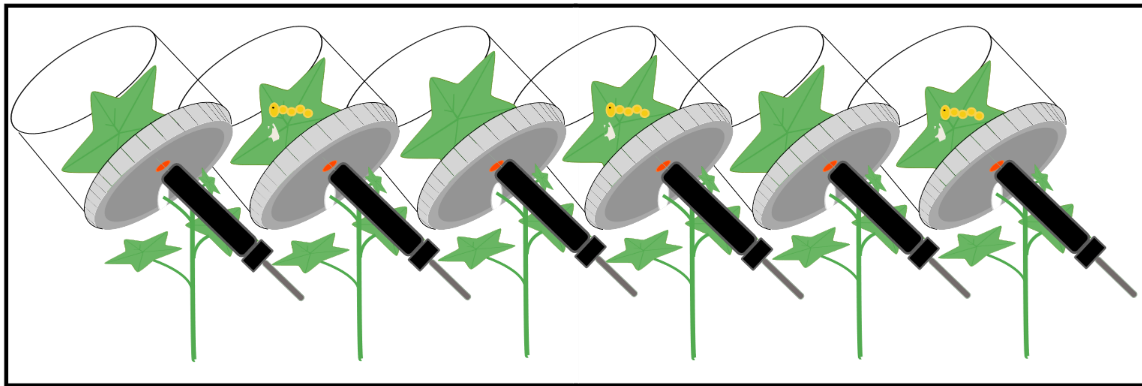


Figure 2.1 Depiction of the sampling design. Lids of common mason jars were modified with a slot for the leaf petiole and a hole covered by a septum to insert the SPME fiber. The leaf was held in place inside the jars by tightly packing the area around the petiole in the slot with clean cotton, which is also how air movement was prevented. Six plants were sampled simultaneously: 2 plants per treatment (Controls, *B. bassiana*, *P. inflatum*), of which 1 is without *S. exigua* larvae (constitutive VOCs) and 1 with *S. exigua* larvae (induced VOCs). At the end of the herbivory period (either 8 or 32 hours) the retracted SPME fiber was pushed through the septum and then exposed to the static headspace around the leaf, without the fiber contacting the leaf.

2.2.7 Experimental Procedure

Six plants were included in each round of sampling to accommodate the fully factorial design such that one technical replicate from all groups was collected in one round of sampling (Figure 2.1). For each treatment (*B. bassiana*, *P. inflatum*, control) two plants were sampled in each round: one without *S. exigua* larvae to collect the constitutive profile, and one with *S. exigua* larvae to collect the induced profile. For the 8-hour experiment, I carried out seven rounds of sampling on seven consecutive days (n=7 for all groups). For the 32-hour experiment, I carried out ten rounds of sampling. Two samples of induced emissions from *B. bassiana*-treated plants were lost because the

larvae chewed through the petiole, excising the leaf. Thus, n=8 for induced emissions of *B. bassiana*-treated plants and n=10 for all other groups.

2.2.8 Analytical Procedures

Leaves were enclosed in the sampling chambers at 0900 h and sampling began at 1700 h of the same day in the 8-hour experiment and of the following day in the 32-hour experiment. SPME fibers were exposed to the static headspace for 30 minutes and stored in an airtight bag at -20°C until the sample could be injected to the GC, with fibers given 5 minutes to reach room temperature before injection. I used PDMS-DVB fibers in the 8-hour experiment and PDMS-DVB-CAR fibers in the 32-hour experiment (Sigma-Aldrich, St. Louis, MO).

The samples from the 8-hour experiment were analyzed on a Shimadzu GC-2010 equipped with a flame ionization detector and a ZebronWAX ZB-5 column (Phenomenex, Torrance, CA). The splitless injection port was set to 220°C and the detector to 300°C. Helium was used as the carrier gas at a column flow of 3.7 mL/min and a purge flow of 3.0 mL/min. Temperature program: held at 35°C 4 min, ramped at 8°C/min to 75°C, 5°C/min to 120°C, 9°C/min to 200°C, and then ramped to 250°C and held at this temperature for 4 min to flush the column . The samples from the 32-hour experiment were analyzed on a Shimadzu GCMS-QP2010 (Kyoto, Japan) equipped with the same column used in the 8-hour experiment. Injection port and detector temperatures were set to 230°C. Helium was used as the carrier gas at column flow of 0.5 mL/min and purge flow of 1 mL/min. Temperature program: held at 40°C for 3 min, ramp at 4°C/min to 180°C, then at 50°C/min to 250°C and held for 4 min to flush the column. Peaks were

integrated using Shimadzu GCsolutions software with a minimum peak integration value of 1,000 units.

2.2.9 Herbivory Quantification

Prior to the start of the experiments, the individual leaf on which the larvae from which the VOCs would be sampled was photographed against white paper beside a ruler with a camera from a fixed overhead position. After sampling, the leaves fed on by *S. exigua* larvae were photographed again, and the difference in leaf area between the before- and after-photos was calculated using ImageJ software (github.com/imagej).

2.2.10 Statistical Analyses

All statistical analyses were performed in R version 3.6.3. Relative quantities were compared using fourth-root transformed peak integration values following the recommendations of Hervé et al. (2018). Comparisons were performed by PERMANOVA using the "adonis" function of "vegan: Community Ecology Package" version 2.5-6. The 8 and 32-hour experiments were analyzed separately. All VOCs for each respective experiment were included as the dependent variables. The independent variables were herbivore status, FFE treatment, and their interaction term. Differences in herbivory were assessed by ANOVA on the square-root transformed number of square centimeters consumed.

2.3. Results

Constitutive and induced VOC emissions were distinct after 8 hours of herbivory (PERMANOVA: $F_{1,36}$=23.85, $P < 0.001$) (Figure 2.2). FFE treatments did not

significantly impact emissions overall (PERMANOVA: $F_{2,36}$=0.61, P = 0.619) and the interaction of herbivory and FFE treatment was also non-significant (PERMANOVA: $F_{2,36}$=1.551, P = 0.194).

VOC emissions of constitutive and induced plants were much more distinct after 32 hours of herbivory (PERMANOVA, $F_{1,51}$=108.82, P < 0.001) (Figure 2.3), but the effects of FFE treatment (PERMANOVA: $F_{2,51}$=1.98, P = 0.113) and interaction of FFE treatment and herbivory were again non-significant (PERMANOVA: $F_{2,51}$=1.44, P = 0.212).

A total of 32 compounds were detected in the 32-hour experiment, of which 3 did not have identities confirmed. These unknown compounds are labeled as "unknown monoterpene", "unknown sesquiterpene 1" and "unknown sesquiterpene 2" based on their molecular formulas of $C_{10}H_{16}$ and $C_{15}H_{24}$, which are typical of monoterpenes and sesquiterpenes, respectively.

Although no significant differences were detected among FFE treatments in the multivariate analyses, each compound has been plotted independently as a function of FFE treatment and herbivory, and tested with Kruskal-Wallis, to examine individual trends that may be biologically informative (Appendix).

The *S. exigua* larvae consumed leaf area similarly across FFE-treatments in both the 8-hour (ANOVA: $F_{2,18}$=1.03, P = 0.377) and 32-hour (ANOVA, $F_{2,26}$=0.25, P = 0.784) experiments. The amount of tissue consumed in the 8-hour versus 32-hour experiment was very different, as expected, with mean consumption in the 8-hour

experiment at 2.6 ± 0.51 cm^2 and mean consumption in the 32-hour experiment at 23.3 ± 2.78 cm^2.

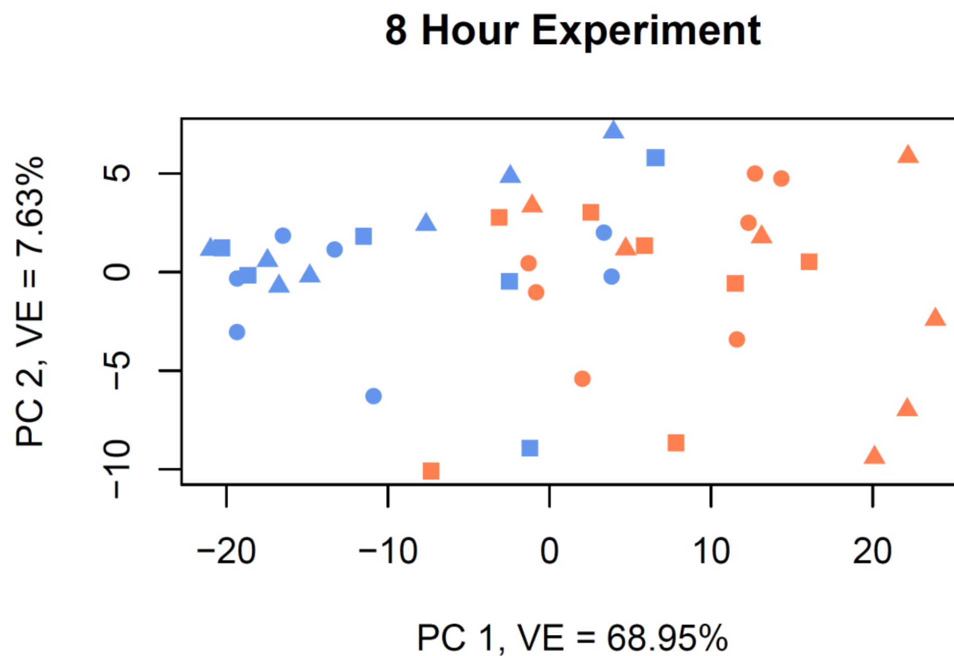


Figure 2.2 Principal component (PC) analysis showing separation of the constitutive (blue shapes) and induced (orange shapes) profiles after 8-hours. Circles are control plants, squares are *B. bassiana*-treated, triangles are *P. inflatum*-treated. VE, variance explained.

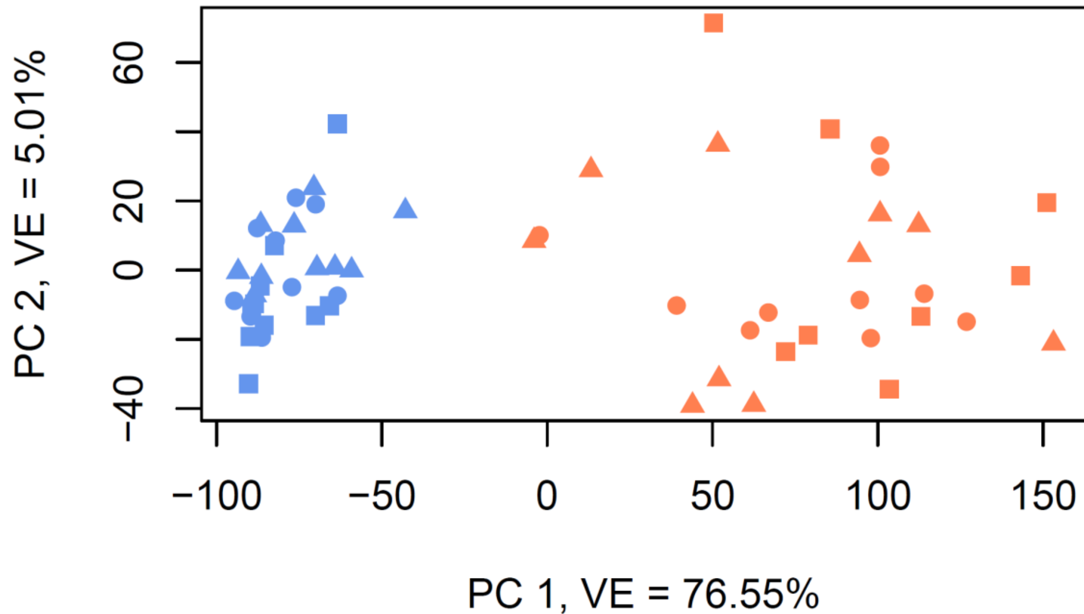


Figure 2.3 Principal component (PC) analysis showing separation of the constitutive (blue shapes) and induced (orange shapes) profiles after 32-hours. Circles are control plants, squares are *B. bassiana*-treated, triangles are *P. inflatum*-treated. VE, variance explained.

Endophyte colonization was confirmed by detection of the target FFE growing from at least one plant fragment. Colonization was detected in 3 of 25 plants treated with *B. bassiana*, with 2 of 539 total leaf fragments and 2 of 735 total root fragments revealing colonization. Colonization was detected in only below-ground tissues in 3 of 24 plants treated with *P. inflatum*, with 13 of 641 total root fragments revealing colonization. Colonization was lower than expected for both treatments.

2.4. Discussion

I set out to test the hypothesis that the FFEs *B. bassiana* and *P. inflatum* could influence cotton plant VOC emissions. Colonization by FFEs in this study was lower than expected and may have contributed to a lack of signal for FFE effects on VOC emissions. The exact reason for the low colonization rates is unclear, but could be due to the use of a different genotype of cotton than the Sword et al. (2017) study in which the pest deterrence was originally observed.

The variety of cotton used in my study was transgenic (PHY-367-WRF) whereas the variety used in the Sword et al. (2017) study was non-transgenic (LA122, All-Tex Seed, Inc., Levelland, TX). A study by Vieira et al. (2011) found no difference in the capacity for FFEs to colonize transgenic and non-transgenic varieties of cotton, but the varieties of cotton and the FFEs investigated were different than those used here.

Variation in FFE colonization due to host plant genotype, with variable effects on plant phenotype, have been reported in cultivated plant species such as chickpea (*Cicer arietinum*) (Bazghaleh et al. 2018), carrot (*Daucus carota* subsp. “sativus”) (Abdelrazek et al. 2019, 2020), and orchids (*Dendrobium catenatum*) (Wu et al. 2020). Given the lack of evidence for FFE-mediated changes to VOC emissions from PHY367 in this study, but the strong evidence of effects on VOC emissions in LA122 in Sword et al. (2017), a comprehensive study that examines FFE colonization and resultant changes to VOC emissions across these genotypes is warranted.

This study, to the best of my knowledge, provides the first description of a VOC sampling chamber for static headspace sampling of live, intact leaves. Agelopoulos et al.

(1999) describe a design for dynamic headspace sampling of live, intact leaves which employs specialized equipment to collect VOC samples. The sampling chambers designed for my study are constructed from common mason jars which makes this design highly accessible and relatively inexpensive.

The novel sampling design employed in this study allowed for the detection of significant differences in constitutive and herbivore-induced VOC emissions after 8 hours of VOC accumulation in the chambers, despite the fact that an average of only a few square centimeters were consumed by *S. exigua* in that time. The results demonstrate that this design can be used for the comparison of constitutive and herbivore-induced VOC emissions, and the chambers can be constructed for use with a variety of plants other than cotton. There are some drawbacks to the design because it only allows for the examination of the response from the site of attack rather than the entire plant, and it may interfere with intra-plant signaling.

Herbivore-induced VOCs can serve as intra-plant signals for triggering the defensive response (Farag & Paré 2002; Heil & Bueno 2007; Heil & Ton 2008). The chambers described in this study trap all VOCs in static headspace, so VOC signals emitted from the site of herbivory may not be transmitted to the rest of the plant, which may alter observations of the defensive response. On the other hand, the fact that emissions were trapped within the sampling chambers allowed us to simultaneously collect constitutive and induced emissions across all treatments without the herbivore-induced VOCs triggering a response in neighboring herbivore-free plants (Farag & Paré 2002; Heil & Bueno 2007; Heil & Ton 2008).

The multivariate analyses did not support the hypothesis that FFE treatments altered VOC emissions, but there are some trends in individual compounds that suggest differences. Overall, there is a trend for herbivory by treatment effects. In other words, the effects of FFE-treatments are detectable when the plants are induced by herbivores, but not constitutively. In the 8-hour experiment, *B. bassiana*-treated plants released, on average, approx. 3x more (*E*)-β-ocimene than control plants, and *P. inflatum*-treated plants released approx. 6x more of this same compound than controls (Appendix, Kruskal-Wallis uncorrected P = 0.022). Levels of herbivory were similar across treatments, thus the elevated levels of (*E*)-β-ocimene cannot be directly attributed to differences in leaf consumption by *S. exigua*.

Paré & Tumlinson (1997) performed a study of cotton VOCs released in response to *S. exigua* larvae by using isotopically labeled carbon dioxide to determine which terpenes are released from storage and which are synthesized *de novo* during the induced response. They found that the acyclic monoterpene (*E*)-β-ocimene was primarily synthesized *de novo* in response to herbivory. The observed higher levels of (*E*)-β-ocimene from FFE-treated plants in the 8-hour experiment may indicate that plants are "primed" by FFE treatments.

Primed plants have enhanced defensive response capacities compared to plants that are not primed (Pieterse et al. 2009; Zamioudis & Pieterse 2012; Pineda et al. 2013; Nguvo & Gao 2019) and this primed state can result from an interaction with beneficial microbes (Conrath et al. 2006; Van Wees et al. 2008; Jung et al. 2012; Mauch-Mani et al. 2017). Thus, considering how cotton plants primarily synthesize (*E*)-β-ocimene *de*

novo in response to herbivory (Paré & Tumlinson 1997) and how an effect of the primed state can be a relatively more rapid defensive response (Pineda et al. 2013), the observed higher levels of (*E*)-β-ocimene from FFE-treated plants compared to controls indicates that the FFE treatments may have enhanced the capacity of the plant to synthesize this acyclic monoterpene in response to herbivory.

While the trend in (*E*)-β-ocimene emissions are the clearest for both FFE treatments, there were other monoterpenes that appear to have been affected. For example, α-pinene ($P = 0.066$) and myrcene ($P = 0.078$) both show marginally-significant increased emissions from *P. inflatum*-treated plants (Appendix). Furthermore, sesquiterpenes emissions appear to be specifically enhanced by *P. inflatum* in the 8-hour experiment, with both sesquiterpenes, caryophyllene ($P = 0.068$) and humulene ($P = 0.038$), at elevated levels (Appendix). Paré & Tumlinson (1997) found that, in contrast to (*E*)-β-ocimene, caryophyllene and humulene were released from storage, rather than synthesized *de novo*. Considering the findings of Paré & Tumlinson (1997) in conjunction with our own, the potential priming effects triggered by the FFEs in our study appears to have led to changes in both VOC emission routes mentioned above: the constitutive production of additional VOCs which are then stored and released in response to herbivory, and enhanced *de novo* biosynthesis of compounds in response to herbivory.

Our findings of herbivore-induced differences, but not constitutive differences, are in contrast to results of other studies examining FFE effects on VOCs. For example, Jallow et al. (2008) found that the constitutive emissions of tomato plants were altered

by the root-inhabiting FFE *Acremonium strictum*. Specifically, Jallow et al. (2008) found that FFE-colonized plants released lower quantities of VOCs and this led to increased attractiveness to the polyphagous moth *Helicoverpa armigera*, suggesting previous findings that the same plant-FFE interaction led to increased attractiveness to whiteflies was also a result of changes in the constitutive VOC emissions (Vidal 1996). Our results were similar to those of Jallow et al. (2008) in the aspect that the VOCs which differed due to FFE treatment were all terpenes, suggesting that they were of plant rather than fungal origin. Notably, the sesquiterpene humulene contributed to differences between controls and *B. bassiana*-treated plants in our study, and *B. bassiana* is capable of producing sesquiterpenes (Crespo et al. 2008), but the low levels of colonization that we detected suggest that the humulene was likely not of fungal origin.

There were also trends for differences in green leaf volatile (GLV) emissions due to FFE treatment in response to herbivory. GLVs are derived from fatty acids that are released from chloroplasts in large quantities in response to caterpillar herbivory, making GLVs the volatiles most often associated with caterpillar herbivory (Matsui 2006; Matsui et al. 2012; Allman and Baldwin 2010). One of the GLVs examined in the 8-hour experiment, (Z)-3-hexenal, also appeared to be enhanced by *P. inflatum*, and this trend was corroborated in the 32-hour experiment (P = 0.058) (Appendix).

When plants produce GLVs, lipoxygenase acts on linolenic acid to produce linolenic acid 13-hydroperoxide which is subsequently cleaved by hydroperoxide lyase, and a primary product is (*Z*)-3-hexenal (Matsui et al. 2012). Thus, the results of both experiments suggest that FFE treatment may trigger changes in plant regulation of the

GLV pathway, and this provides a potential route for further investigating the molecular mechanisms by which FFEs influence plant responses.

Multiple trends in individual compounds that support the hypothesis that FFEs can trigger ISR and lead to enhanced VOC emissions in response to herbivory. When I compare my results to those of similar studies, I find broad similarity in that the microbial treatments led to changes in VOC emissions, but to my knowledge, this is the first to quantify the herbivory associated with the induced emissions in an experimental design with both microbial and herbivore treatments. For example, in the Jallow et al. (2008) study of the root-restricted FFE *Acremonium strictum,* they detected significant differences in constitutive emissions due to FFE treatment, but induced emissions were not measured. Guerrieri et al. (2004) found insect behavior indicative of VOC changes in constitutive emissions due to arbuscular mycorrhizal fungi (AMF) colonization, but the design was not fully factorial and only insect-infested controls were compared to uninfested AMF plants. Leitner et al. (2010) did use a fully factorial design and found that AMF influenced VOC emissions, but they did not quantify herbivory.

Most notably, Schausberger et al. (2012) found that colonization of bean plants by AMF specifically increased emissions of (*E*)-β-ocimene and caryophyllene which were synthesized *de novo* in response to herbivory. The similarities in our results indicate that the genes responsible for the biosynthesis of (*E*)-β-ocimene may be important indicators of defensive priming by beneficial microbes.

Considering the multiple trends in individual compounds that indicate differences due to FFE treatment, I suspect that low statistical power due to low sample number,

coupled with my use of a non-parametric PERMANOVA which considered all compounds equally, likely contributed to the lack of a signal for FFE effects overall.

VOC emissions from cotton plants varies with plant genotype (Loughrin et al. 1994) and the lack of evidence for FFE-mediated effects in this study could also be the result of the fact that I used a different variety of cotton than the Sword et al. (2017) study in which the altered plant-insect interactions were observed. As a follow-up to this study, a more comprehensive study, one that targets the VOCs for which I found biologically relevant trends, and assesses emissions from whole PHY367 and LA122 plants treated with *B. bassiana* and *P. inflatum,* would be highly informative.

2.5. References

Abdelrazek S, Choudhari S, Thimmapuram J, Simon P, Colley M, Mengiste T, Hoagland L (2020) Changes in the core endophytic mycobiome of carrot taproots in response to crop management and genotype. Sci Rep https://doi.org/10.1038/s41598-020-70683-x

Abdelrazek S, Simon P, Colley M, Mengiste T, Hoagland L (2020) Crop management system and carrot genotype affect endophyte composition and *Alternaria dauci* suppression. PLoS ONE 15(6): e0233783

Agelopoulos NG, Hooper AM, Maniar SP, Pickett JA, Wadhams LJ (1999) A novel approach for isolation of volatile chemicals released by individual leaves of a plant in situ. J Chem Ecol 25:1411-1425.

Allmann S, Baldwin IT (2010) Insects betray themselves in nature to predators by rapid isomerization of green leaf volatiles. Science 329:1075-1077.

Bazghaleh N, Hamel C, Gan Y, Tar'an B, Knight JD (2018) Genotypic variation in the response of chickpea to arbuscular mycorrhizal fungi and non-mycorrhizal fungal endophytes. Can J Microbiol https://doi.org/10.1139/cjm-2017-0521

Conrath U, Beckers GJ, Flors V et al. (2006) Priming: getting ready for battle. Mol Plant Microbe In 19:1062-1071.

Crespo R, Pedrini N, Juárez MP, Bello GMD (2008) Volatile organic compounds released by the entomopathogenic fungus *Beauveria bassiana*. Microbiol Res 163:148-151.

Ek-Ramos MJ, Zhou W, Valencia C et al. (2013) Spatial and temporal variation in fungal endophyte communities isolated from cultivated cotton (*Gossypium hirsutum*). PLoS ONE https://doi.org/10.1371/journal.pone.0066049

Farag MA, Paré PW (2002) C6-Green leaf volatiles trigger local and systemic VOC emissions in tomato. Phytochem 61:545-554.

Gange AC, Koricheva J, Currie AF, Jaber LR, Vidal S (2019) Meta-analysis of the role of entomopathogenic unspecialized fungal endophytes as plant bodyguards. New Phytol https://doi.org/10.1111/nph.15859

Guerrieri E, Lingua G, Digilio MC, Massa N, Berta G (2004) Do interactions between plant roots and the rhizosphere affect parasitoid behaviour? Ecol Entomol 29:753-756.

Heil M, Bueno JCS (2007) Within-plant signaling by volatiles leads to induction and priming of an indirect plant defense in nature. PNAS 104:5467-5472.

Heil M, Ton J (2008) Long-distance signaling in plant defense. Trends Plant Sci 13:264-272.

Hervé MR, Nicolè F, Cao K-AL (2018) Multivariate analysis of multiple datasets: a practical guide for chemical ecology. J Chem Ecol https://doi.org/10.1007/s10886-018-0932-6

Jallow MFA, Dugassa-Gobena D, Vidal S (2008) Influence of an endophytic fungus on host plant selection by a polyphagous moth via volatile spectrum changes. Arthropod-Plant Inte 2:53-62.

Jung SC, Martinez-Medina A, Lopez-Raez JA, Pozo MJ (2012) Mycorrhiza-induced resistance and priming of plant defenses. J Chem Ecol 38:651-664.

Leitner M, Kaiser R, Hause B, Boland W, Mithöfer A (2010) Does mycorrhization influence herbivore-induced volatile emission in *Medicago truncatula*? Mycorrhiza 20:89-101. doi:DOI 10.1007/s00572-009-0264-z

Lopez DC, Sword GA (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 boll worm (Helicoverpa zea). Biol Control 89:53-60. https://doi.org/10.1016/j.biocontrol.2015.03.010

Lopez DC, Zhu-Salzman K, Ek-Ramos MJ, Sword GA (2014) The entomopathogenic fungal endophytes *Purpureocillium lilacinum* (Formerly *Paecilomyces lilacinus*) and *Beauveria bassiana* negatively affect cotton aphid reproduction under both greenhouse and field conditions. PLoS ONE 9 e103891. https://doi.org/10.1371/journal.pone.0103891

Loughrin JH, Manukian A, Heath RR, Turlings TCJ, Tumlinson JH (1994) Dinural cycle of emission of induced volatile terpenoids by herbivore-injured cotton plants. PNAS Plant Biol 91:11836-11840.

Matsui K (2006) Green leaf volatiles: hydroperoxide lyase pathway of oxylipin metabolism. Curr Opin Plant Biol 9:274-280.

Matsui K, Sugimoto K, Mano Ji, Ozawa R, Takabayashi J (2012) Differential metabolisms of green leaf volatiles in injured and intact parts of a wounded leaf meet distinct eco-physiological requirements. PLoS ONE 7:1-10.

Mauch-Mani B, Baccelli I, Luna E, Flors V (2017) Defense priming: An adaptive part of induced resistance. Annu Rev Plant Biol 68:485-512.

Nguvo KJ, Gao X (2019) Weapons hidden underneath: bio-control agents and their potential to activate plant induced systemic resistance in controlling crop *Fusarium* diseases. J Plant Dis Protect doi:10.1007/s41348-019-00222-y

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

Park J, Thomasson JA, Gale CC, Sword GA, Lee KM, Herrman TJ, Suh CPC (2020) Adsorbent-SERS technique for determination of plant VOCs from live cotton plants and dried teas. ACS Omega 5:2779-90.

Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SC (2009) Networking by small-molecule hormones in plant immunity. Nat Chem Biol 5:308-316.

Pineda A, Dicke M, Pieterse CM, Pozo MJ (2013) Beneficial microbes in a changing environment: are they always helping plants to deal with insects? Funct Ecol 27:574-586.

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

Schausberger P, Peneder S, Jürschik S, Hoffmann D (2012) Mycorrhiza changes plant volatiles to attract spider mite enemies. Funct Ecol 26:441-449.

Schmelz EA, Alborn HT, Tumlinson JH (2001) The influence of intact-plant and excised-leaf bioassay designs on volicitin- and jasmonic acid-induced sesquiterpine volatile release in *Zea mays*. Planta 214:171-179.

Sword GA, Tessnow A, Ek-Ramos MJ (2017) Endophytic fungi alter sucking bug responses to cotton reproductive structures. Insect Sci 24:1003-1014. https://doi.org/10.1111/1744-7917.12461

Turlings TC, Erb M (2018) Tritrophic interactions mediated by herbivore-induced plant volatiles: mechanisms, ecological relevance, and application potential. Annu Rev Entomol 63:433-452.

Van Wees SC, Van der Ent S, Pieterse CM (2008) Plant immune response triggered by beneficial microbes. Curr Opin Plant Biol 11:443-448.

Vidal S (1996) Changes in suitability of tomato for whiteflies mediated by a non-pathogenic endophytic fungus. Entomol Exp Appl 80:272-274.

Vieira PDS, Motta CMS, Lima D, Torres JB, Quecine MC, Azevedo JL, Oliveira NT (2011) Mycol https://doi.org/10.1080/21501203.2011.584390

Wu LS, Dong WG, Si JP, Liu JJ, Zhu YQ (2020) Endophytic fungi, host genotype, and their interaction influence the growth and production of key chemical components of *Dendrobium catenatum* Fun Biol 124:864-876.

Zamioudis C, Pieterse CM (2012) Modulation of host immunity by beneficial microbes. Mol Plant Microbe In 25:139-150.

3. FOLIAR HERBIVORY INDUCES INCREASED SUCROSE CONCENTRATION IN BRACTEAL EXTRAFLORAL NECTAR OF COTTON

3.1. Introduction

Extrafloral (EF) nectar is an indirect plant defense that functions by encouraging ants, parasitoids, and other predatory insects to forage for carbohydrates on the plant (reviewed in Heil 2015). This improves the chance of an encounter between the predators/parasitoids and herbivores such as caterpillars that may be damaging the plant. Indirect defenses are defined by their function to recruit predators and/or parasitoids to defend the plant, as opposed to direct defenses such as toxins or trichomes that act on the herbivores themselves.

The most common indirect plant defenses are volatile organic compounds (VOCs), which are odor compounds emitted by plants to attract predators and parasitoid wasps (Turlings and Erb 2018). Cotton plants, *Gossypium spp.*, produce both forms of these indirect defenses, bearing EF nectaries on leaves and leaf-like bracts that enclose reproductive structures (Wäckers et al. 2001, Wäckers and Bonifay 2004, Llandres et al. 2019), and by emitting a bouquet of VOCs in response to herbivory (Loughrin et al. 1994, Rose et al. 1996, Paré and Tumlinson 1997a, Paré and Tumlinson 1997b).

VOC emissions and EF nectar production are modulated by jasmonic acid (JA) and JA-related plant hormones which respond to insect herbivory (reviewed in Arimura et al. 2005, Turlings and Erb 2018). The induction of VOCs from cotton by herbivory is well documented (*e.g.*, Loughrin et al. 1994, Rose et al. 1996, Paré and Tumlinson

1997a, Paré and Tumlinson 1997b), but reports of EF nectar induction are rare (Wäckers et al. 2001, Wäckers and Bonifay 2004).

EF nectar production has been found to be influenced by the presence of fungal endophytes (Navarro-Melendez & Heil 2014, Jaber & Vidal 2009). Many fungi and bacteria are capable of, but not limited to, the colonization of plant tissues with various effects on plant-insect interactions (reviewed in Gange et al. 2019). These so-called facultative fungal endophytes (FFEs) have been reported to influence plant-herbivore interactions when inoculated to cotton plants via seed treatments (Sword et al. 2017, Lopez & Sword 2015, Lopez et al. 2014).

Specifically, Sword et al. (2017) found anecdotal evidence that the VOC emissions of cotton plants might be altered by the FFE treatments. Considering the similar role that EF nectar and VOCs play in indirect plant defense, in conjunction with the shared regulation of these indirect defenses by JA, I designed this study to test whether FFE seed treatments might alter EF nectar produced by cotton plants. In studies that have demonstrated an induction of cotton EF nectar in response to herbivory (Wäckers et al. 2001, Wäckers and Bonifay 2004), the volume of EF nectar was reported to increase whereas the carbohydrate composition did not. I collected standardized volumes of EF nectar from FFE-treated plants and analyzed carbohydrate composition in the presence and absence of herbivores.

3.2. Methods

3.2.1 Seed Treatments

The fungal treatments consisted of 2 strains: the commercially available *Beauveria bassiana* strain GHA (Botanigard, Bioworks, Inc., Victor, NY), and a strain of *Phialemonium inflatum* (TAMU490*)* that originated from a field survey of fungal endophytes collected from cotton plants grown in Texas (Ek-Ramos et al. 2013).

Fungi were cultured on potato dextrose agar (PDA) (VWR, Radnor, PA) amended with penicillin and streptomycin (Mediatech, Inc., Manassas, VA), using the manufacturer's recommended concentration, to prevent bacterial contamination. Cultures of *B. bassiana* were incubated for 2 weeks and *P. inflatum* for 3 weeks before spore harvest. Spore solutions were prepared by adding 5 mL of autoclaved 0.1% Triton-X100 solution to the PDA plate and gently scraping with a spatula. The solutions were passed through a No. 500 (25 µm) USA standard test sieve and the spore concentration was calculated with a hemocytometer. Spore solutions were then adjusted to 10^7 spores/mL with nanopore water.

Lint-free, chemical-free cotton seeds (PHY367) were surface sterilized in 3% sodium hypochlorite solution for 2 mins followed by 70% ethanol solution for 3 mins and then air dried under laminar flow. Seeds were soaked overnight in spore solutions of a single fungal strain at a ratio of 7 mL per 40 seeds. Sterile water was used for controls at the same ratio.

3.2.2 Plant Growth Conditions

Seeds were planted in 150 cc 6-cell seed-starter trays filled with Jolly Gardener Pro-Line C/25 growing mix (Oldcastle Lawn & Garden, Poland Spring, ME). Plants were grown in environmental chambers (Percival Scientific, Inc., Perry, IA) on a 16:8 h (L:D) cycle and 28:22°C for approximately 4 weeks. Seven plants per treatment were set aside for genomic DNA extraction to test for the presence of the target endophytes. Experimental plants were transplanted in the greenhouse into 2-gallon plastic pots filled with the same soil and fertilized with 1 L of 1% v/v CNS Grow 3-1-2 (Botanicare, Vancouver, WA) liquid fertilizer every 4 weeks. Experiments took place in a greenhouse between the months of April-June, 2019. Plantings were performed regularly, every 1-2 weeks, so that a steady supply of plants bearing their first reproductive structures would be available. The greenhouse was kept insect-free for approximately the first 8 weeks until plants began to flower.

3.2.3 Insects

The beet armyworm, *Spodoptera exigua,* was chosen for this experiment because the larvae are strong foliar feeders, and the late-instar larvae can withstand consuming a considerable amount of *Bt*-toxin containing leaf tissue. I used the *Bt*-containing variety PHY367 in this experiment as I did for the VOC analyses in Chapter 2 for consistency in examining the indirect chemical defenses. Eggs of *S. exigua* were obtained from Benzon Research Inc. (Carlisle, PA). Upon hatching, larvae were reared individually in 4-cm diameter by 4-cm deep plastic cups on artificial diet (Southland Products Inc., Lake Village, AR) in a rearing room kept at ~28°C on a 14:10 h L:D cycle. Once larvae

reached the third instar, they were transferred from the diet cups into glass Petri dishes containing conventional (non-*Bt*) cotton leaves so that they could acclimate to feeding on leaf tissue, and were kept in the rearing room until the start of the experiment.

3.2.4 Experimental Procedures

The experiment was designed to test whether foliar herbivory could induce qualitative changes in the carbohydrate composition of bracteal EF nectar, and if seed treatments with facultative fungal endophytes might influence this induction.

Cotton flowering follows a predictable developmental pattern, with the first flowers blooming individually, about 3 days apart, at the same fruiting position of successional branches. I used this predictable time interval to systematize the amount of herbivory to which individual plants were exposed. Wäckers & Bonifay (2004) found peak EF production at cotton bracteal nectaries on the day of anthesis, the day a flower blooms. I performed preliminary collections to determine a suitable standardized collection volume, and found that in the absence of herbivory, 5 µL of nectar could be consistently collected on the day of anthesis for each of the first few flowers.

At 1000 h on the day of anthesis for the first flower (Figure 3.1 a), 5 µL EF nectar was collected from the bracteal nectaries, using graduated micropipettes with a metal plunger (Drummond Scientific, Broomall, PA), and dispensed into 45 µL HPLC-grade water. The samples from the first flowers, in the absence of herbivory, are referred to as the constitutive samples. Immediately following, I exposed plants to herbivory by enclosing the terminal leaf of the blooming branch with two 4th- or 5th-instar *S. exigua* larvae using a draw-string Organza bag. I did the same to the terminal leaf of the

succeeding branch, the branch that would bear an open bloom in approximately 3 days. On the day of anthesis for the second flower (Figure 3.1 b), another 5 µL EF nectar was collected in the same way. These samples from the second flowers are referred to as the induced samples.

A set of control plants (no fungal seed treatment) were used to test for qualitative differences in the bracteal nectar of the first and second flower in the absence of herbivory. These plants were sampled as described above, except that no *S. exigua* larvae were placed on the plants.

Samples were frozen at -20°C until the experiments were complete. Immediately before chemical analysis, the samples were allowed to thaw at room temperature. The thawed samples were sonicated for 2 minutes to ensure total dissolution of sugars and then passed through 0.4 µm filters.

3.2.5 Chromatographic Procedures

Samples were analyzed by high performance liquid chromatography to refractive index detection (HPLC-RID). Sucrose, glucose, and fructose were quantified using standard curves, with pure standards purchased from Sigma-Aldrich (St. Louis, MO). Three µL injections were performed with an autosampler on an Agilent 1260 Infinity II HPLC system equipped with a Hi-Plex Calcium ion exchange column 300 mm in length with 7.7 mm ID. The column compartment was maintained at 80°C and the RID at 55°C. The chromatographic method was isocratic with 100% HPLC-grade water at a flow rate of 0.4 mL/min for 30 min.

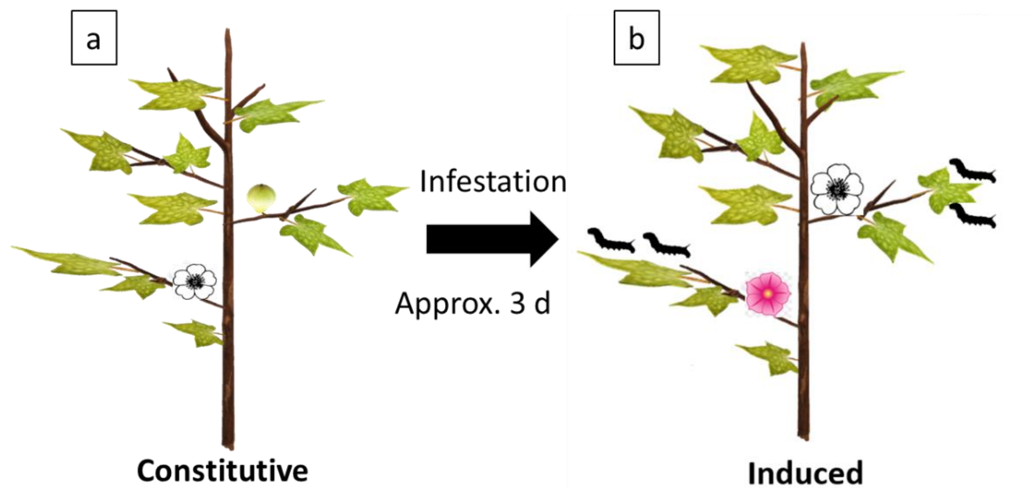


Figure 3.1 a) Plants on the day constitutive (herbivore-absent) samples are collected. The first flower blooms and 5 µL of EF nectar is collected from the bracteal nectaries. A flower bud develops on the succeeding branch. The terminal leaves of both branches are infested with *S. exigua* larvae. b) Plants on the day the induced (herbivore-present) samples are collected. The second flower blooms approximately 3 days after the first, and 5 µL EF nectar are collected from the bracteal nectaries. The first flower has turned pink as part of the natural development.

3.2.6 Ant Recruitment Field Tests

Given the results of the carbohydrate analysis, field experiments were carried out to assess whether ant recruitment to vials of nectar varied with nectar formulation. Two artificial stock solutions were made to mimic the average carbohydrate composition of constitutive and induced EF nectar samples using high-purity sucrose, glucose, and fructose (Sigma-Aldrich, St. Louis, MO). Five hundred tubes of nectar were prepared by

stuffing 1.5 mL microcentrifuge tubes with 100 mg of cotton fiber saturated with 1 mL of either nectar formulation and snapping the lid shut. Tubes were combined in batches of 50, 25 of each nectar formula, and mixed in plastic bags to randomize.

At 2 sites located near the Texas A&M campus 5 linear transects were laid out, each 50 m in length and separated from other transects by 25 m. The test at site 1, a managed grass lawn, took place on June 15, 2020. The test at site 2, the edge of a small agricultural field, took place on August 1, 2020. One hour before sunset, a tube was randomly selected from the bag, opened, and placed every 1 m along the transects. After 1 hr, tubes were collected and quickly snapped shut, enclosing ants recruited to the nectar (Kaspari et al. 2008). Ants were identified to genus and counted in the laboratory.

3.2.7 Assessment of Endophyte Colonization

A metabarcoding approach was used determine whether the seed inoculations led to successful colonization of the plants by the target endophytes. Treated plants were grown for 4 weeks as described above. Plants were gently removed from the plastic pots and the roots were washed to remove all potting soil. Above-ground and below-ground tissues were separately placed in 50-mL centrifuge tubes with 0.1% Triton solution. Tissues were gently vortexed in the Triton solution to remove soil particles and fungal spores from the surfaces of the plant. Tissues were then surfaced sterilized by soaking for 2 min in 70% ethanol, followed by 3 mins in 0.5% sodium hypochlorite and then washed twice in sterile water. Genomic DNA was extracted using the CTAB protocol (Doyle and Doyle 1987). Concentrations of DNA were standardized across samples and sequencing of the internal transcribed spacer (ITS) region was performed on the Illumina

MiSeq platform to obtain 300 bp paired-end reads using the primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3', Gardes and Bruns 1993) and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3', White et al.1990) (Pauvert et al. 2019).

The goal of the analysis pipeline was to detect the target endophytes with maximum sensitivity. Pauvert et al. (2019) performed a study to determine which combinations of popular pipeline tools produced the most taxonomically sensitive results in fungal community analyses. My pipeline was based on "Se1", a combination of USEARCH v11.0.667 (Edgar 2010) and VSEARCH v2.14.1 (Rognes et al. 2016) tools found by Pauvert et al. (2019), to produce the most accurate operational taxonomic units (OTUs). The pipeline was implemented using Texas A&M High Performance Research Computing instance of Galaxy (http://hprcgalaxy.tamu.edu) (Afgan et al. 2018).

Specifically, forward (R1) and reverse (R2) reads were merged with a minimum final length of 40 bp; minimum overlap length of 10 bp and maximum of 10 mismatches. Not-merged R1 reads were concatenated to the respected merged fastq files and sequences with more than 1 expected error per base were removed (for an explanation of expected errors see https://drive5.com/usearch/manual/exp_errs.html). All samples were concatenated into a single fasta file for dereplication and singleton reads were discarded. Reads were then clustered at 98% similarity to generate the OTU table. Taxonomy was assigned by global pairwise alignment of the centroid sequences for each cluster to the UNITE ITS 2019 UTAX reference database at 97% similarity. Appended to the database sequences were ITS sequences of the fungal endophyte strains used in this study that originated from Ek-Ramos et al. (2013), which would allow us to identify

our strain of inoculum in case the same fungal species incidentally colonized experimental plants.

3.3. Statistical Analyses

3.3.1 EF Nectar Carbohydrate Composition

All analyses were performed in RStudio with R version 3.6.3 (R Core Team 2020). The overall distribution of the data was bimodal, with carbohydrate composition dominated by glucose and fructose at similar levels, and sucrose a much more minor component. The monosaccharides (hexoses), glucose and fructose, in EF nectar are the result of post-secretory hydrolysis of the disaccharide sucrose by invertase enzymes (Heil et al. 2005). Due to the bimodality of the data as a whole and shared origin of glucose and fructose (i.e. non-independent measurements), the variables are analyzed separately as sucrose, the sum of the hexose concentrations (glucose + fructose), and total sugar content. Quantities were square-root transformed to meet assumptions of normality.

To test for the effects of seed treatments (Controls N = 14, *B. bassiana* N = 14, *P. inflatum* N = 18), herbivory (N = 46), and their interaction, repeated measures factorial ANOVA was carried out using base R. To test for positional differences between the first and second flowers of non-treated plants in the absence of herbivory (N = 19), one-way repeated measures ANOVA was performed. Residuals were tested for normality using the Shapiro-Wilk test and homogeneity of variance tested with Levene's test from the "car" package (version 3.0-7).

3.3.2 Ant Recruitment

Ant recruitment to constitutive and induced EF nectar formulations were compared two ways: the proportion of vials in each transect to which ants were successfully recruited (hit percent) and the average number of ants recruited to hit vials. Each transect represents a data point, and N=10 for both groups.

To test for differences in hit percent, a generalized linear mixed effect model (GLMM) for the binomial distribution was carried out in R using the "lme4" package (version 1.1-23). Hits were treated as a Bernouilli response variable, formulation (constitutive or induced) as the fixed effect, and transect nested within site as the random effect.

To test for differences in the number of ants recruited, a GLMM for the zero-truncated negative binomial distribution (from "glmmTMB" package version 1.0.2.1) was used because no zeroes (*i.e.* only vials containing ants) were included in the analysis. This model took the same form, with count as the response variable, formulation (constitutive or induced) as the fixed effect, and transect nested within site as the random effect. Model diagnostics were performed with the "DHARMa" package (version 0.3.3).

3.4. Results

3.4.1 EF Nectar Carbohydrate Composition

The carbohydrate composition of the EF nectar in our study was similar to that previously reported for cotton (Wäckers & Bonifay 2004), with the monosaccharides (glucose and fructose) as the dominant components at similar concentrations and sucrose as the minor component. Overall, the nectar in our study contained an average of 827 ± 31 mg/mL total sugar (mean ± 1 SE). The factorial repeated measures ANOVA revealed that sucrose concentration was significantly altered by herbivory whereas hexose (total glucose and fructose) concentration was not. Sucrose concentration increased from an average of 69 mg/mL in constitutive samples to 83 mg/mL in induced samples (Figure 3.2). This change in sucrose concentration alone, as it is the least abundant sugar, was not sufficient to significantly change total sugar content (Table 3.1). Seed treatments and the interaction of seed treatment and herbivory had no significant effects (Table 3.1).

The one-way repeated measures ANOVA showed no significant differences in the sucrose, hexose, or total sugar concentration due to positional differences between the first and second flower in the absence of herbivory (Table 3.2).

3.4.2 Endophyte Detection by Metabarcoding

Sequence number across samples was rather low, with 15 samples averaging approx. 16,000 reads and the remaining 27 samples averaging approx. 2,000 reads. A total of 134 OTUs were produced, 51 of which could be identified to the species level. The target endophyte *B. bassiana* was detected in 1 *B. bassiana*-treated root tissue sample. A strain of the target endophyte *P. inflatum* was detected in the roots of 1

control plant, but this strain aligned more closely to the sequence of *P. inflatum* from the UNITE database than the consensus sequence for our lab strain TAMU490.

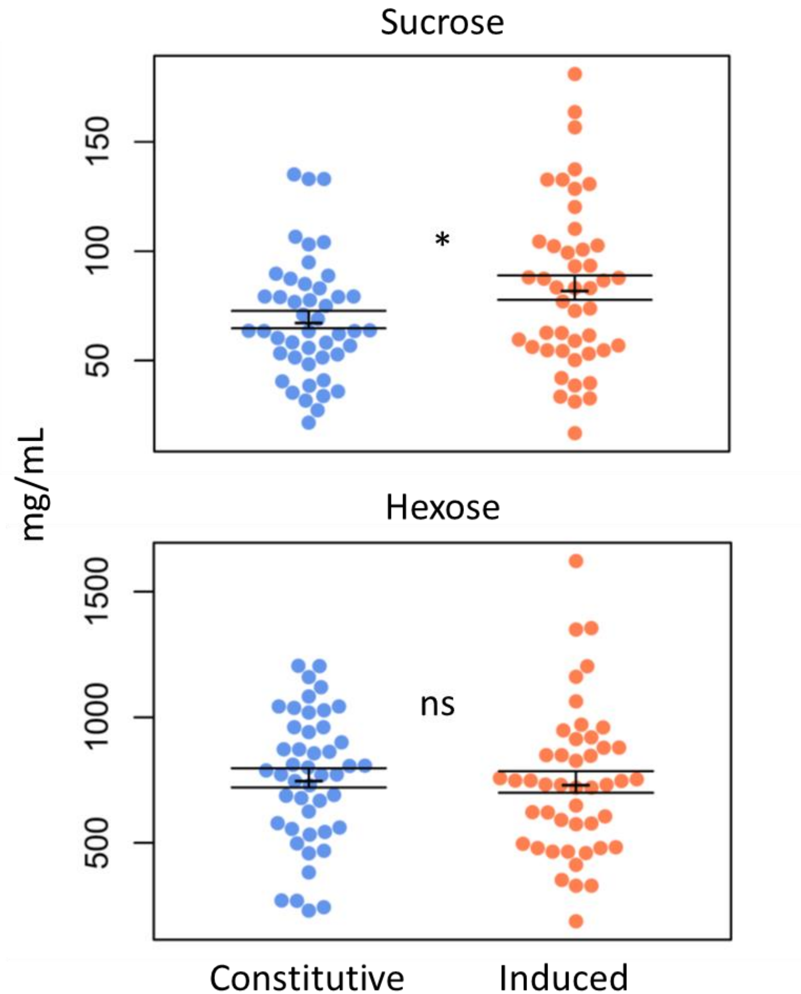


Figure 3.2 Sucrose and hexose (total glucose and fructose) concentrations in bracteal EF nectar when herbivores are absent (constitutive) or present (induced). Means ± 1 SE shown with black brackets. * = significantly different, ns = not significantly different, as determined by repeated measures factorial ANOVA at α=0.05.

Table 3.1 Analysis of variance table for the repeated measures factorial ANOVA test for the effects of herbivory and FFE seed treatments. * = statistically significant at α = 0.05.

Response Source of Variation	df	SS	MS	*F*	P-value
Sucrose					
Between plant					
Treatment	2	9.86	4.931	1.02	0.369
Error	43	207.95	4.836		
Within plant					
Herbivory	1	13.26	13.258	6.025	0.0182*
Herbivory:Treatment	2	2.52	1.259	0.572	0.5685
Error	43	94.63	2.201		
Hexose					
Between plant					
Treatment	2	8.1	4.06	0.107	0.899
Error	43	1629.9	37.9		
Within plant					
Herbivory	1	2.8	2.84	0.166	0.686
Herbivory:Treatment	2	73.2	36.6	2.143	0.13
Error	43	734.4	17.08		

Table 3.1 Continued

Response Source of Variation	df	SS	MS	*F*	P-value
Total Sugars					
Between plant					
Treatment	2	9.5	4.75	0.119	0.888
Error	43	1716.3	39.91		
Within plant					
Herbivory	1	0.1	0.1	0.005	0.942
Herbivory:Treatment	2	72.4	36.18	1.99	0.149
Error	43	781.7	18.18		

Table 3.2 Analysis of variance table for one-way ANOVA to test for positional differences between the first and second flower (flower pos.) on non-treated control plants in the absence of herbivory.

Response Source of Variation	df	SS	MS	*F*	P-value
Sucrose					
Between plant					
Error	1	4.246	4.246		
Within plant					
Flower pos.	1	1.68	1.682	1.294	0.263
Error	35	45.5	1.3		
Hexose					
Between plant					
Error	1	0.5918	0.5918		
Within plant					
Flower pos.	1	16.9	16.85	1.322	0.258
Error	35	446.3	12.75		
Total Sugars					
Between plant					
Error	1	0.05643	0.05643		
Within plant					
Flower pos.	1	18.5	18.48	1.416	0.242
Error	35	456.8	13.05		

3.4.3 Ant Recruitment Field Tests

Constitutive and induced mock EF nectar solutions were formulated from the results of the carbohydrate analyses. Only sucrose concentration was allowed to vary. Constitutive and induced formulations both contained 367 mg/mL fructose and 384 mg/mL glucose, while the constitutive formula contained 69 mg/mL sucrose and the induced formula contained 83 mg/mL sucrose.

The proportion of tubes with and without ants (hit percent) were not significantly different (GLMM comparison of the full and null models by Type II Wald Chi-Square test; $\chi^2 = 0.352$, df = 1, P = 0.552) (Figure 3.3) and neither were the differences in the number of ants recruited to hit tubes ($\chi^2 = 1.66$, df = 1, P = 0.198) (Figure 3.4).

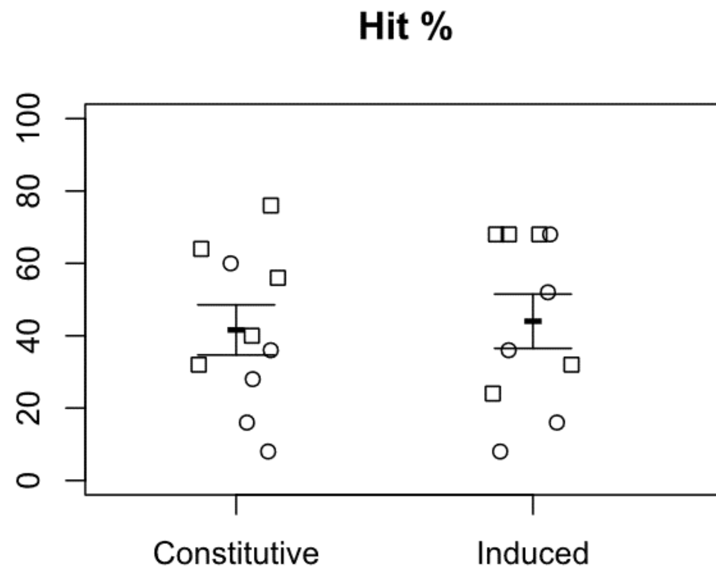


Figure 3.3 Proportion of tubes to which ants were successfully recruited in each transect. Squares are transects at site 1, circles are transects at site 2. Means ± 1 standard error shown with black brackets. No differences were detected in analysis by generalized linear mixed model.

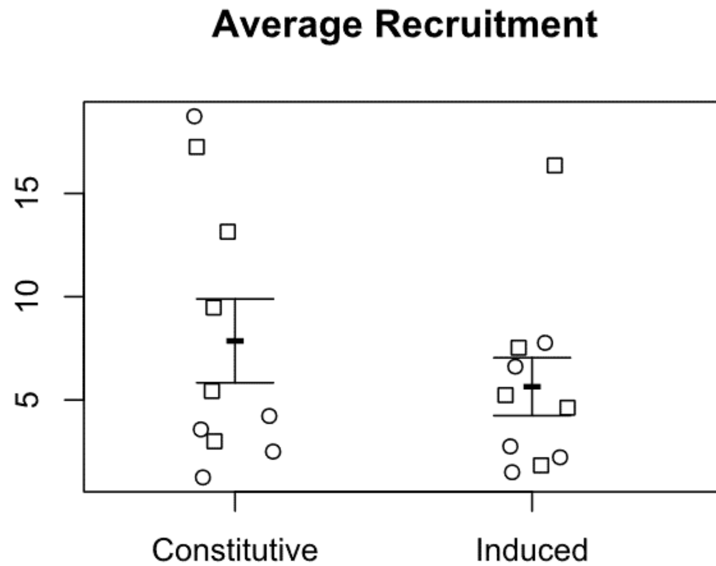


Figure 3.4 The mean number of ants per transect in tubes containing at least one ant. Squares are transects at site 1, circles are transects at site 2. Means ± 1 standard error shown with black brackets. No differences were detected in analysis by generalized linear mixed model.

3.5. Discussion

In this study, I tested whether FFE treatments altered the carbohydrate composition of EF nectar produced by cotton plants. The carbohydrate composition of EF nectar collected at bracteal EF nectaries was similar across FFE treatments both in the absence and presence of caterpillar herbivory. The lack of a signal for endophyte-mediated changes to EF nectar may be the result of a lack of colonization by FFEs across FFE-treated plants. FFEs have been reported to influence EF nectar production in other plant species (Navarro-Melendez & Heil 2014, Jaber & Vidal 2009), so further study is needed to understand whether EF nectar production in cotton might also be influenced by FFEs when successfully inoculated to plants. Although I did not find

results to support my hypothesis of FFE-mediated changes to EF nectar, I did detect changes in the carbohydrate composition due to herbivory.

The sucrose concentration of EF nectar is a determining factor in the foraging behavior of generalist ants (Heil et al. 2005). Invertase enzymes are responsible for the transfer of sucrose from phloem to EF nectar during secretion, and are also responsible for post-secretory hydrolysis of sucrose into glucose and fructose (reviewed in Heil 2011). Generalist ants typically prefer a higher concentration of sucrose, and in specialized ant-plant symbioses of *Acacia* trees and *Pseudomyrmex* ants, generalists are deterred by a lack of sucrose in the EF nectar due to high invertase activity (Heil et al. 2005).

My study indicates a response to foliar herbivory in the carbohydrate composition of bracteal EF nectar of cotton. When fed on by caterpillars, the sucrose concentration increased significantly in the bracteal EF nectar produced on the day of anthesis, but hexose concentration was unaffected (Figure 3.2). To the best of my knowledge, this is the first recorded observation of a systemic inducible response to herbivory in EF nectar carbohydrate composition.

Most studies have examined how EF nectar production changes at the site of herbivory, and inducible increases in EF nectar volume are seemingly quite common (see Heil 2015 and Agrawal & Rutter 1998 for lists of responses across plant species to different types of damage). I hypothesized that the increased sucrose concentration we observed could be a defensive response of the plant to produce EF nectar that is qualitatively more preferable to generalist ants when faced with herbivory.

The results of my ant recruitment field study do not agree with this inference, but in the end, this is not very surprising. The shift in sucrose concentration observed in my study was not sufficient to significantly alter the total sugar content of the EF nectar (Table 3.1). Total sugar content is typically reported to be a determining factor in changes to ant foraging preferences to different EF nectars. For example, a study by Fagundes et al. (2017) examined the EF nectar foraging behaviors, and subsequent herbivore-removal, of 23 ant species and found a strong positive correlation between the total sugar content of EF nectar and ant recruitment. A study by Alves-Silva and Del-Claro (2013) found similar results, showing that plants bearing the most concentrated EF nectar sustained the highest levels of ant visitation.

Other studies that have examined carbohydrate composition of EF nectar in response to herbivory found no changes (Wäckers et al. 2001, Wäckers and Bonifay 2004). A more common response to herbivory in EF nectar chemistry may be an increase in amino acid content. For example, Smith et al. (1990) found amino acid concentrations in EF nectar from *Impatiens* plants increased dramatically in response to foliar herbivory and this was coupled with no change in sugar concentrations. In a follow-up study to the finding of amino acid induction, Lanza et al. (1993) found that amino acid concentration strongly influenced the feeding preference of fire ants (*Solenopsis spp.*) and, contrary to studies mentioned above, they also found that these ants preferred EF nectar that was the least-viscous with the least-concentrated sugars.

In cotton specifically, a study by Llandres et al. (2019) tested for differences in EF nectar production between wild and domesticated varieties of cotton. They found that

domesticated varieties produced significantly less EF nectar, but that the carbohydrate composition was similar across varieties. I find this result surprising because EF nectar in my study contained a seemingly distinct sucrose:hexose ratio compared to the results of Wäckers and Bonifay (2004) reporting a ratio approximately 50% greater. Information on the variety of cotton used in their study is unavailable.

Considering the differences in results between my experiment and others that have examined carbohydrate composition in EF nectar in response to herbivory, further study is needed to understand if the increased sucrose levels I observed is ecologically relevant to the relationship that generalist ants have with cotton EF nectar. Furthermore, my study only examined carbohydrate composition and did not examine amino acid content which is an important factor that shapes ant foraging behavior. These limitations likely contributed to the lack of signal in the ant preference tests.

Another potential explanation for the observed increase in sucrose concentrations is unrelated to the relationship between EF nectar and ants. Sucrose is the primary form of carbohydrate transport in many plants as it is synthesized in leaves (sources) and reallocated to other tissues such as roots and reproductive organs (sinks) (Tauzin and Giardina 2014). Sucrose acts as a carbon source that the plant can use to synthesize defensive secondary metabolites, and previous studies of cotton's many responses to herbivory have reported systemic induction of sucrose in leaves (Schmidt et al. 2009, Eisenring et al. 2018). Cotton reproductive structures represent a rather strong sucrose sink (Wullschleger and Oostherhuis 1990). It's possible that the vasculature in cotton reproductive structures releases additional sucrose into EF nectar when systemic

induction of sucrose occurs in response to caterpillar herbivory (Orians et al. 2000, Wäckers and Bonifay 2004), but this should be tested directly.

As a whole, my study examined whether the EF nectar production of *G. hirsutum* was affected by seed treatments with the FFEs *B. bassiana* and *P. inflatum*. While I did not detect any differences due to FFE treatment, this is likely because I did not detect very much colonization by the target FFEs in treated plants. In other studies that have examine the effects of FFEs on EF nectar production (Navarro-Melendez & Heil 2014, Jaber and Vidal 2009) the endophytes were inoculated as much higher densities, and EF nectar volume, rather than carbohydrate composition, was investigated. For example, Jaber and Vidal (2009) performed a soil drench with 50 mL of 10^6 spores/mL and Navarro-Melendez and Heil (2014) sprayed leaves with 2 mL of spore suspension at the same titer. Preliminary results from concurrent work in Dr. Sword's lab with foliar applications of FFEs suggest that foliar applications to cotton can modulate phytohormone levels in leaves. Similar results were presented in Navarro-Melendez and Heil (2014), and I predict foliar application methods may be better suited than seed treatments for studies of FFE-mediated changes to EF nectar moving forward.

3.6. References

Afgan E, Baker D, Batut B, Van den Beek M, Bouvier D, Čech M, Chilton J, Clements D, Coraor N, Grüning B, Guerler A, Hillman-Jackson J, Jalili V, Rasche H, Soranzo N, Goecks J, Taylor J, Nekrutenko A, Blankenberg D (2018) The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. Nucleic Acids Res 46: 537–544.

Agrawal AA, Rutter MT (1998) Dynamic anti-herbivore defense in ant-plants: the role of induced responses. Oikos 83: 227-236.

Alves-Silva E, Del-Claro K (2013) Effect of post-fire resprouting on leaf fluctuating asymmetry, extrafloral nectar quality, and ant-plant-herbivore interactions. Naturwissenschaften 100:525-532.

Arimura G, Kost C, Boland W (2005) Herbivore-induced, indirect plant defenses. Biochimica et Biophysica Acta 1734:91-111.

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19: 11-15.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. Bioinform 26: 2460–2461.

Eisenring M, Glausser G, Meissle M, Romeis J (2018) Differential impact of herbivores from three feeding guilds on systemic secondary metabolite induction, phytohormone levels and plant-mediated herbivore interactions. J Chem Ecol 44:1178-1189.

Ek-Ramos MJ, Zhou W, Valencia C, Antwi JB, Kalns LL, Morgan GD, Kerns DL, Sword GA (2013) Spatial and temporal variation in fungal endophyte communities isolated from cultivated cotton (*Gossypium hirsutum*). PLoS ONE doi: https://doi.org/10.1371/journal.pone.0066049

Fagundes R, Dattilo W, Ribeiro SP, Rico-Gray V, Jordano P, Del-Claro K (2017) Differences among ant species in plant protection are related to production of extrafloral nectar and the degree of leaf herbivory. Biol J Linn Soc 122:71-83.

Gange AC, Koricheva J, Currie AF, Jaber LR, Vidal S (2019) Meta-analysis of the role of entomopathogenic and unspecialized fungal endophytes as plant bodyguards. New Phytol 223:2002-2010.

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol Ecol 2: 113-118.

Heil M (2011) Nectar: generation, regulation and ecological functions. Trends Plant Sci 16:191-200.

Heil M (2015) Extrafloral nectar at the plant-insect interface: a spotlight on chemical ecology, phenotypic plasticity, and food webs. Annu Rev Entomol 60: 213-232.

Heil M, Rattke J, Boland W (2005) Postsecretory Hydrolysis of Nectar Sucrose and Specialization in Ant/Plant Mutualism. Sci 308: 560-563.

Jaber L, Vidal S (2009) Interactions between an endophytic fungus, aphids, and extrafloral nectaries: do endophytes induce extrafloral-mediated defences in *Vicia faba*? Funct Ecol 23: 707-714.

Kaspari M, Yanoviak SP, Dudley R (2008) On the biogeography of salt limitation: A study of ant communities. PNAS 105: 17848-17851.

Lanza J, Vargo EL, Pulmin S, Chang YZ (1993) Preferences of the fire ants *Solenopsis invicta* and *S. geminata* (Hymenoptera: Formicidae) for amino acid and sugar components of extrafloral nectars. Environ Entomol 22:411-417.

Llandres AL, Verdeny-Vilalta O, Jean J, Goebel FR, Seydi O, Brevault T (2019) Cotton extrafloral nectaries as indirect defense against insect pests. Basic App Ecol 37:24-34.

Lopez DC, Sword GA (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 boll worm (*Helicoverpa zea*). Biol Control 89:53-60.

Lopez, D. C., Zhu-Salzman, K., Ek-Ramos, M. J., & Sword, G. A. (2014) The entomopathogenic fungal endophytes *Purpureocillium lilacinum* (Formerly *Paecilomyces lilacinus*) and *Beauveria bassiana* negatively affect cotton aphid reproduction under both greenhouse and field conditions. PLoS ONE 9: e103891.

Loughrin JH, Manukian A, Heath RR, Tumlinson JH (1995) Volatiles emitted by different cotton varieties damaged by feeding beet army worm larvae. J Chem Ecol 21:1217-1227.

Navarro-Meléndez AL, Heil M (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.

Orians CM, Pomerleau J, Ricco R (2000) Vascular architecture generates fine scale variation in systemic induction of proteinase inhibitors in tomato. J Chem Ecol 26:471-485.

Paré PW, Tumlinson JH (1997a) Induced synthesis of plant volatiles. Nature 385:30-31.

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

Pauvert C, Buée M, Laval V, Edel-Hermann V, Fauchery L, Gautier A, Lesur I, Vallance J, Vacher C (2019) Bioinformatics matters: The accuracy of plant and soil fungal community data is highly dependent on the metabarcoding pipeline. Fung Ecol 41: 23-33.

R Core Team (2020) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rognes T, Flouri T, Nichols B, Quince C, Mahé F (2016) VSEARCH: a versatile open source tool for metagenomics. PeerJ 4 e2584.

Röse USR, Manukian A, Heath RR, Tumlinson JH (1996) Volatile semiochemicals released from undamaged cotton leaves. Plant Physiol 111:487-495.

Schmidt L, Schurr U, Roese US (2009) Local and systemic effects of two herbivores with different feeding mechanisms on primary metabolism of cotton leaves. Plant Cell Environ 32:893-903.

Smith LL, Lanza J, Smith GC (1990) Amino acid concentrations in extrafloral nectar of *Impatiens sultanii* increase after simulated herbivory. Ecol 71:107-115.

Sword GA, Tessnow A, Ek-Ramos MJ (2017) Endophytic fungi alter sucking bug responses to cotton reproductive structures. Insect Sci 24:1003-1014.

Tauzin AS, Giardina T (2014) Sucrose and invertases, a part of the plant defense response to biotic stresses. Front Plant Sci 5:293.

Turlings TC, Erb M (2018) Tritrophic interactions mediated by herbivore-induced plant volatiles: mechanisms, ecological relevance, and application potential. Annu Rev Entomol 63:433-452.

Wäckers FL, Bonifay C (2004) How to be sweet? Extrafloral nectar allocation by *Gossypium hirsutum* fits optimal defense theory predictions. Ecol 85:1512-1518.

Wäckers FL, Zuber D, Wunderlin R, Keller F (2001) The effect of herbivory on spatial and temporal dynamics of foliar nectar production in cotton and castor. Ann Bot 87:365-370.

White TJ, Bruns T, Lee S, Taylor J, (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protoc: Guide Meth Appl 315-322.

Wullschleger SD, Oosterhuis (1990) Photosynthetic carbon production and use by developing cotton leaves and bolls. Crop Sci 30:1259-1264.

4. ANALYSIS OF INDUCIBLE TERPENOID ALDEHYDES IN COTTON LEAVES TO TEST FOR INDIRECT PLANT-ENDOPHYTE-HERBIVORE INTERACTIONS

4.1. Introduction

Many species of fungi are capable of endophytic lifestyles, herein referred to as facultative fungal endophytes (FFEs) in which they can arrive as spores on a host plant (horizontal transmission) and establish an asymptomatic infection. FFEs may colonize above- or belowground tissues and may not remain endophytes for the entirety of their lifecycle (Porras-Alfaro and Bayman 2011). FFEs are highly diverse and may include latent pathogens or latent saprobes, and the relationship between the FFE and hostplant may be mutualistic or commensalistic (Porras-Alfaro and Bayman 2011). In a mutualistic relationship between FFE and hostplant, the fitness of both organisms is increased by the symbiosis. A growing body of literature supports the hypothesis of defensive mutualism in FFE-plant interactions with observations of reduced insect herbivore performance when the insects feed on endophyte-inoculated plants (e.g., Jaber and Vidal 2010; Gange et al. 2012; Akutse et al. 2013; Lopez and Sword 2015; Vianna et al. 2018; reviewed in Gange et al. 2019).

A detail that is often absent in research on FFE-plant-insect interactions is whether the negative effects on herbivores are due to direct or indirect interactions with the endophytes. Hypotheses for either direct or indirect mechanisms are often based on the effects of toxic metabolites, with direct effects being the result of fungal metabolites and indirect effects the result of changes in plant metabolites (reviewed in Gange et al.

2019). The reason this detail is lacking in many studies is because direct and indirect interactions are difficult to distinguish in practice and could be acting together. The present study is designed to specifically test the hypothesis of indirect effects. I examine levels of herbivore-deterrent plant metabolites in FFE-treated plants compared to non-treated controls.

Research in Dr. Sword's laboratory in recent years has focused on using cotton (*Gossypium hirsutum*) as a model system to examine plant-insect interactions and how those are influenced by experimental FFE treatments. Lopez and Sword (2015) found that the treatment of cotton seeds with spores of the FFEs *Beauveria bassiana* or *Phialemonium inflatum* (TAMU490) (formerly misidentified as *Purpureocillium lilacinum*) led to negative effects on *Helicoverpa zea* (Boddie) survivorship and performance when larvae fed on those plants. While *B. bassiana* is entomopathogenic, the observed negative effects on *H. zea* in the study by Lopez and Sword (2015) were not due to mycosis. Similar negative effects on herbivores are reported in Akutse et al. (2013) and Vianna et al. (2018) with multiple strains of *B. bassiana* in different host plants.

Direct contact between the endophyte, entomopathogenic or otherwise, and the herbivore is not necessary for negative effects on the herbivore to occur (Jaber and Vidal 2010). Furthermore, colonization of different plant tissues by experimentally inoculated fungi is highly variable (Gange et al. 2012; Akutse et al. 2013; Lopez and Sword 2015; Vianna et al. 2018), leading to the hypothesis that many cases of negative effects on

insect herbivores may be due to changes in plant defensive chemistry as a result of the plant-fungus interaction rather than accumulation of toxins produced by the fungi.

Many cotton varieties are speckled with glands containing secondary metabolites that are toxic to chewing herbivores and it is common knowledge among cotton growers that destructive insect pests such as caterpillars perform and survive better on glandless cotton varieties compared to varieties with these glands. A collection of biosynthetically related chemicals known as gossypol, hemigossypolone, and heliocides are produced by cotton and stored in the dark glands visible throughout the plant. These compounds are referred to as glandular terpenoid aldehydes and are the best characterized inducible chemical defenses in cotton (Hagenbucher et al. 2013).

In response to caterpillar herbivory, glandular terpenoids are systemically induced (Bezemer et al. 2004, Eisenring et al. 2017, McAuslane et al. 1997, Opitz et al. 2008). Considering that Lopez and Sword (2015) found reduced caterpillar survivorship on FFE-treated plants, and that FFE-mediated plant-insect interactions are likely the result of plant-mediated changes to plant chemistry (Gange et al. 2019), I developed the hypothesis that FFE-treated cotton plants may produce more glandular terpenoids than non-treated plants.

I used high-performance liquid chromatography (HPLC) to quantitatively assess amounts of these compounds in terminal leaves of FFE-treated plants compared to non-treated controls. I tested whether the FFE treatments affected glandular terpenoids constitutively, in the absence of herbivores, as well as in the presence of herbivores as part of the induced defensive response.

4.2. Methods

4.2.1 Fungal Material

The fungal spores used for treatments were harvested from cultures maintained in the lab on potato dextrose agar (PDA) in 10 cm diameter Petri dishes. The original inoculum for *B. bassiana* (strain GHA) was obtained as the commercially available biocontrol agent in Botanigard (BioWorks Inc., Victor, NY). The fungus *P. inflatum* (strain TAMU490) was originally isolated as a naturally occurring endophyte of field-grown cotton (Ek-Ramos et al. 2013). Spores were harvested by pouring 10 mL 0.1% Triton X-100 solution over the Petri dish culture and gently scraping the conidia free with a sterile spatula. Spore solutions were filtered through a No. 500 (25 µm) American Standard Sieve and spore concentrations calculated with a hemocytometer. Spore titers were adjusted to 10^7 spores/mL by diluting with pure sterile water.

4.2.2 Plant Materials

Lint-free, non-treated cotton seeds, variety LA122 (All-Tex Seed, Inc.), were surface sterilized by soaking in 3% sodium hypochlorite solution for 3 minutes followed by 70% ethanol for 2 minutes, and then rinsed twice in sterile water. Endophyte-treated seeds were soaked in covered dishes overnight in spore solutions of either *B. bassiana* or *P. inflatum* with 6 mL spore solution per 40 seeds, whereas control seeds were soaked in sterile water. Seeds were planted in 6-cell seed starter pots filled with JollyGardener C-25 soil and 1 L of water added to the tray. Plants were reared in a Percival environmental chamber (Perry, IA) on a 14:10 L:D cycle at 29:25°C until the second true-leaf was fully expanded. Plants were then transplanted in the greenhouse into 2 gallon pots filled with

JollyGardener C-25 soil and fertilized with 2 tablespoons of Osmocote 15-9-12 (ScottsMiracle-Gro, Marysville, OH).

4.2.3 Insect Materials

Eggs of *H. zea* were purchased from Benzon Research and reared in the lab on artificial diet (Southland Products Inc.) at 29°C on a 14:10 (L:D) cycle.

4.2.4 Experimental Procedures

We performed 6 independent trials, 3 to test constitutive and 3 to test herbivore-induced effects on the glandular chemistry of pre-flowering cotton. All trials included approximately 15 plants/treatment, final replicates for each treatment are as follows: Control n=93, *B. bassiana* n=95, *P. inflatum* n=96. Plants were maintained in an insect-free greenhouse until they began to develop flower buds, typically around the 8th to 10th node. To obtain herbivore-induced samples, the 3 uppermost fully expanded leaves were bagged in Organza, with a single 4th-instar *H. zea* larva in each bag, and the terminal meristem left free. After 3 days, the developing terminal leaf was excised, stowed in a microcentrifuge tube, and frozen in liquid nitrogen. Plant material was then completely freeze-dried and finely ground prior to extraction. The same procedure was followed to collect the constitutive samples, but in those trials no *H. zea* larvae were placed in the Organza bags. Tissue collection dates for the three constitutive trials were April 18, May 9, and October 17, 2018. Tissue collection dates for the three induced trials were June 24, September 2, and October 1, 2019.

4.2.5 Chemical Analysis

Chemical extraction and chromatographic analysis methods followed Wagner et al. (2015). Specifically, samples were extracted in acetonitrile/water/phosphoric acid (80:20:0.1) for 3 minutes with ultrasonification at a ratio of 1 mL solvent per 20 mg tissue. Samples were then centrifuged at 2800G for 3 min and the supernatant transferred to autosampler vials for analysis. Gossypol (G), methylG, hemigossypolone (HGQ), methylHGQ, and the heliocides H1-H4 were quantitated via external calibration curves.

4.2.6 Assessment of FFE Colonization by PDA Plating

Surface-sterilized plant fragments were plated on potato dextrose agar (PDA) to culture out target FFEs. Seeds were treated as described above, planted in Jolly Gardener C-25 growing mix, and grown in the greenhouse. At the second true leaf stage, 10 plants per treatment (*B. bassiana* and *P. inflatum*) were removed from their pots and the roots gently rinsed. Plants were surface sterilized in a laminar flow hood in 0.5% sodium hypochlorate solution for 3 min followed by 70% EtOH solution for 2 min. The above- and below-ground portions were plated separately on PDA plates prepared as described above. The taproot and attached lateral roots were sectioned into approximately 1 cm fragments. Leaves were cut into approximately 1 square cm fragments while stems were not plated. Any fungal growth from fragments were sub-cultured onto a new PDA plate and target FFEs were identified by morphological comparisons of conidia to inoculum cultures.

4.2.7 Statistical Analyses

All samples were found to contain quantifiable amounts of G, HGQ, and the heliocides. MethylHGQ and methylG were inconsistently detected and were not included in the statistical analysis. All statistical analyses were performed in R version 3.6.3 (R Core Team 2020, R foundation for Statistical Computing, Vienna, Austria). First, a multivariate approach was taken to assess absolute quantities (µg compound / mg dried leaf tissue) of all compounds as a function of FFE treatment, herbivory, and trial using permutation multivariate analysis of variance (PERMANOVA). The parametric MANOVA showed high heteroscedasticity and non-normal residuals.

Data were also analyzed by linear mixed effect models using G, HGQ, and total heliocides individually as the response to the fixed effects of FFE treatment and herbivory, and trial as the random effect, with the "lme4" package (version 1.1-23).

Data were centered, scaled, and fourth-root transformed for ordination by principal components analysis (PCA) (Hervé et al. 2018).

4.3. Results

Herbivory significantly impacted terpenoid aldehyde concentrations, as expected, according to the PERMANOVA analysis (Table 4.1). The analysis also revealed that trial, or the date on which leaf samples were collected, significantly impacted terpenoid levels as well (Table 4.1). FFE treatment effects were marginally significant while the interaction of treatment and herbivory were not significant (Table 4.1).

Given the marginally significant effects of FFE treatment in the PERMANOVA, data were analyzed with linear mixed effect models as the compounds individually were suitable for parametric tests.

Table 4.1 PERMANOVA table for test of G, HGQ, and total heliocides as a function of herbivory, FFE treatment, trial, and the interaction of FFE treatment and herbivory. * = statistically significant at α = 0.05. Permutations = 999.

Source of Variation	df	SS	MS	Psuedo-*F*	P-value
Herbivory	1	6.971	6.971	188.844	>0.001*
Treatment	2	0.186	0.093	5.512	0.053
Trial	4	9.851	2.463	66.722	>0.001*
Treatment:Herbivory	2	0.030	0.0148	0.400	0.851
Residuals	275	10.151	0.0369		
Total	284	27.188			

When G, HGQ, and total heliocides were examined individually, I found that trial date significantly impacted the concentrations of all compounds (Table 4.2). Surprisingly, only total heliocides were changed by caterpillar herbivory in a statistically-significant manner (Table 4.3).

PDA plates of treated plant fragments did not reveal any successful colonization of the target FFEs. Of 558 total leaf tissue fragments plated, 24 produced non-target

endophyte cultures. Of 699 total root tissue fragments, 49 produced non-target endophyte cultures. Subcultures were taken of each non-target endophyte and they were identified if they produced conidia on PDA. Of the 73 total subcultures, 10 failed to be axenic cultures, 5 were identified as *Aspergillus flavus*, 9 as *Penicillium spp.*, 4 as *Trichoderma viridis*, 1 as *Culvuleria sp.*, and the remaining 44 isolates did not produce conidia.

Table 4.2 ANOVA-like table for the random effect of trial on gossypol (G), hemigossypolone (HGQ), and total heliocides (H1-H4). * = statistically significant at α = 0.05.

Response Source of Variation	npar	logLik	AIC	LRT	df	P-value
G						
none	8	-7.858	31.715			
Trial	7	-137.824	289.647	259.93	1	>0.001*
HGQ						
none	8	-206.1	428.19			
Trial	7	-333.46	680.92	254.73	1	>0.001*
H1-H4						
none	8	-216.25	448.5			
Trial	7	-266.59	547.18	100.68	1	>0.001*

Table 4.3 Analysis of deviance table for Type II Wald Chi-Square tests of the fixed effects for linear mixed-effect models of gossypol (G), hemigossypolone (HGQ), and total heliocides (H1-H4)

Response Source of Variation	χ^2	df	P-value
G			
Herbivory	0.088	1	0.7673
Treatment	4.532	2	0.1037
Status:Treament	2.154	2	0.3406
HGQ			
Herbivory	1.250	1	0.264
Treatment	3.152	2	0.207
Status:Treatment	1.331	2	0.514
H1-H4			
Herbivory	12.325	1	>0.001*
Treatment	4.322	2	0.115
Herbivory:Treatment	0.123	2	0.9409

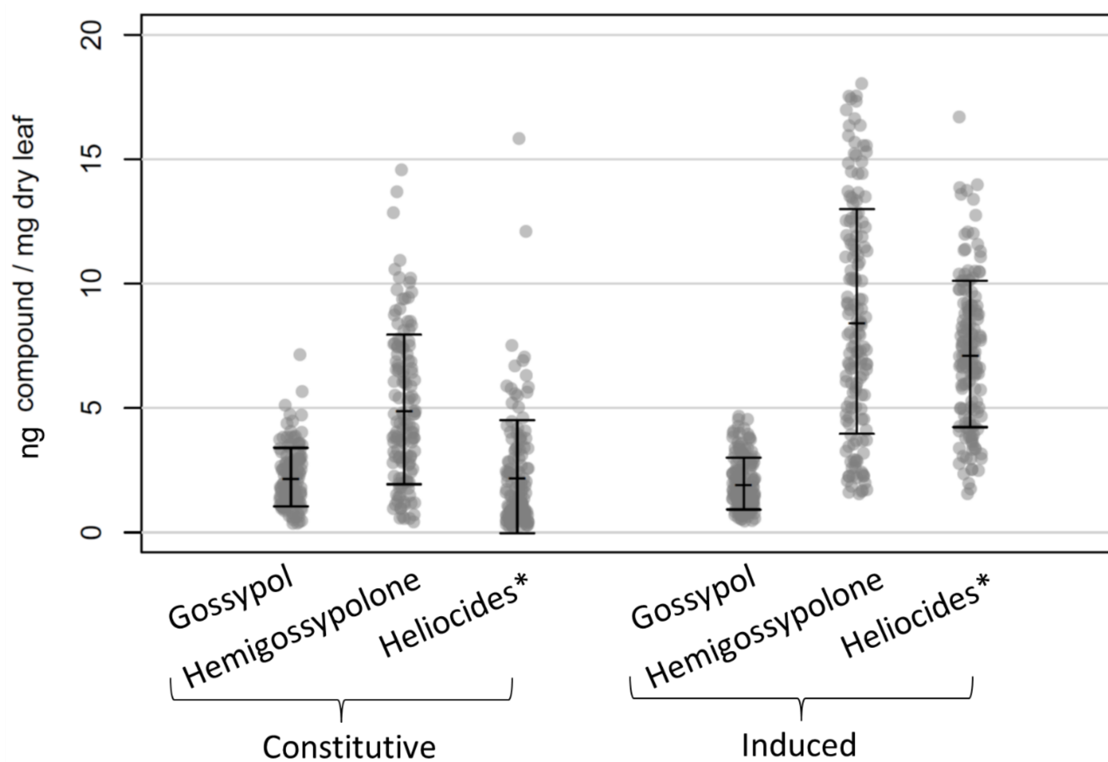


Figure 4.1 Concentrations of each compound across all treatments and trials. * = significantly different between constitutive and induced trials as determined by linear mixed effect models (α = 0.05). Means are shown with a horizontal black dash mark and error bars are constructed using 1 standard deviation from the mean.

4.4. Discussion

In this study, I investigated the defensive terpenoid aldehydes found in cotton plants to better elucidate potential mechanisms underlying negative effects on insect herbivores feeding on FFE-treated cotton plants. I specifically tested the hypothesis that the FFE treatments have indirect effects on herbivores by increasing terpenoid aldehyde concentrations in leaves. Previous observations of negative caterpillar survivorship on

endophyte-treated cotton (Lopez and Sword 2015) suggested that elevated levels of the compounds studied herein may have been responsible.

I did not detect colonization by the target endophytes by PDA plating. Considering the lack of target endophyte colonization, the lack of any significant effect of the FFE treatments on terpenoid aldehydes is not surprising. Further study is needed to better understand the consistency of target FFE colonization resulting from the seed treatments used in this study. DNA-based detection techniques such as diagnostic PCR or metabarcoding techniques may prove more reliable methods of target endophyte detection than PDA plating, especially considering the complications encountered with fast-growing non-target fungi.

Despite a lack of evidence for FFE-mediated changes, the results of this study have revealed experimentally and biologically informative patterns in the induction of these terpenoids by *H. zea* larvae in *G. hirsutum*. Gossypol levels were nearly identical across constitutive and induced trials, unaffected by *H. zea* herbivory (Figure 4.2). The result of non-induced gossypol levels in my study contrasts the results of several studies (McAuslane and Alborn 1998, Bezemer et al. 2004, Opitz et al. 2008, Eisenrig et al. 2017). Eisenring et al. (2018) found very similar results to mine, with the heliocides and HGQ induced but gossypol not significantly altered by *H. zea* feeding on *G. hirsutum*.

Heliocide levels in constitutive trials were consistently low, with many near-zero values. On the other hand, there were no near-zero values for heliocide concentrations in the induced trials (Figure 4.2). Heliocide induction by caterpillar herbivory is well documented across many studies (*e.g.*, McAuslane et al. 1997, McAuslane and Alborn

1998, Bezemer et al. 2004, Opitz et al. 2008, Eisenring et al. 2017, Eisenring et al. 2018) and heliocides are named as such because of their toxicity to *Heliothis* caterpillars (Nazarova et al. 1981).

I did not find a statistically significant increase in HGQ, but there is a clear trend of its induction (Figure 4.2). This study was limited in that the constitutive and induced trials were asynchronous, and the significant variation in these compounds due to trial date likely contributed to the lack of statistical significance for the increase in HGQ. In many studies, when the heliocides are induced, HGQ is also induced (McAuslane et al. 1997, McAuslane and Alborn 1998, Bezemer et al. 2004, Opitz et al. 2008, Eisenring et al. 2017, Eisenring et al. 2018).

An apparent reason for similarities and differences between my studies and others is the duration of herbivory before terpenoid quantification. In my study, 3 *H. zea* larvae were infested on the plant for 3 days before sampling. McAuslane et al. (1997) found that heliocides were induced after only 1 day of herbivory, and that induced gossypol levels were not detected until after 7 days. Eisenring et al. (2017) found that a single caterpillar was sufficient to induce heliocides after 2 days, but not HGQ or gossypol. Eisenring et al. (2017) also found that 3 caterpillars was sufficient to induce all terpenoids after only 2 days, indicating that not only does the duration of herbivory, but the intensity of herbivory can determine the rate of terpenoid induction in cotton.

From patterns in the literature, heliocides appear to be the first terpenoids that are induced by herbivory, and Eisenring et al. (2017) reported that the heliocides are also the terpenoids that remain at elevated levels for the longest period of time after herbivory

has ended. A study by Olsen et al. (2008) reported that 2 days of *H. zea* feeding was insufficient to induce any of the glandular terpenoids in *G. hirsutum*, but volatile terpenoids were induced. The findings of my study corroborate the findings of many others that clearly exhibit a role of heliocides in caterpillar-induced chemical defenses of cotton.

I set out to determine whether FFE treatments of cotton lead to increased levels of glandular terpenoids which could explain previously reported negative effects on caterpillars. While I did not detect FFE treatment effects on glandular terpenoids, I also did not detect the target endophytes in treated plants. For now, I can only speculate as to why that is, but perhaps FFE colonization levels have a threshold at which they then alter terpenoid levels, and in my study where no colonization was detectable that threshold was not met.

Although the clearest connection to explain previously reported negative effects on caterpillar survivorship on FFE-treated cotton is through the terpenoids, there are several other mechanisms by which cotton plants induce defense against chewing pests, and the FFE treatments may have enhanced these modes instead. For example, plants can respond to herbivory through induced resource sequestration wherein resources usable by the herbivore are reallocated from attacked tissues to other tissues (Orians et al. 2011, Bi et al. 1997). Additionally, an induced response to herbivory in cotton is a reduction in compounds that act as antioxidants (Eisenring et al. 2018), leading to a higher oxidative status in foliar tissues with potential negative effects on herbivores (Bi and Felton 1995, Bi et al. 1997). The FFE treatments reported by Lopez and Sword (2015) to reduce

caterpillar survivorship may have enhanced these mechanisms of herbivore resistance rather than terpenoids.

Abiotic factors such as nitrogen and water availability have been shown to impact cotton's chemical defenses (Olson et al. 2009). The plants in the Lopez and Sword (2015) study were not fertilized, whereas the plants in my study were, and this discrepancy could have also contributed to a lack of connection between the findings in our studies.

Overall, this study has corroborated a large body of literature that find heliocides to be highly inducible terpenoids in cotton in response to caterpillar herbivory. A pitfall of my study was the asynchronous constitutive and induced trials, which likely contributed to the lack of a statistically significant induction of HGQ, which is typically associated with induced heliocides. The lack of gossypol induction is not consistently reported in studies of caterpillar-induced cotton defenses, and the findings of this study may contribute to further understanding patterns of terpenoid induction across cotton variety-caterpillar combinations.

4.5. References

Akutse KS, Maniania NK, Fiaboe KKM, Van Den Berg J, Ekesi S (2013) Endophytic colonization of *Vica faba* and *Phaseolus vulgaris* (Fabaceae) by fungal pathogens and their effects on the life-history parameters of *Liriomyza huidobrensis* (Diptera: Agromyzidae). Fung Ecol 6:293-301.

Bezemer TM, Wagenaar R, van Dam NM, van Der Putten WH, Wäckers FL (2004) Above- and below-ground terpenoid aldehyde induction in cotton, *Gossypium herbaceum*, following root and leaf injury. J Chem Ecol 30:53–67.

Eisenring M, Meissle M, Haugenbucher S, Naranjo SE, Wettstein F, Romeis J (2017) Cotton defense induction patterns under spatially, temporally and quantitatively varying herbivory levels. Front Plant Sci 8:234.

Eisenring M, Glauser G, Meissle M, Romeis J (2018) Differential impact of herbivores from three feeding guilds on systemic secondary metabolite induction, phytohormone levels, and plant-mediated herbivore interactions. J Chem Ecol 44:1178-1189.

Ek-Ramos MJ, Zhou W, Valencia C, Antwi JB, Kalns LL, Morgan GD, Kerns DL, Sword GA (2013) Spatial and temporal variation in fungal endophyte communities isolated from cultivated cotton (*Gossypium hirsutum*). PLoS ONE https://doi.org/10.1371/journal.pone.0066049

Gange AC, Eschen R, Wearn JA, Thawer A, Sutton BC (2012) Differential effects of foliar endophytic fungi on insect herbivores attacking a herbaceous plant. Oecologia 168:1023-1031.

Gange AC, Koricheva J, Currie AF, Jaber LA, Vidal S (2019) Meta-analysis of the role of entomopathogenic and unspecialized fungal endophtyes as plant bodyguards. New Phytol 223:2002-2010.

Hervé MR, Nicolè F, Cao K-AL (2018) Multivariate analysis of multiple datasets: a practical guide for chemical ecology. J Chem Ecol https://doi.org/10.1007/s10886-018-0932-6

Jaber LR, Vidal S (2010) Fungal endophyte negative effects on herbivory are enhanced on intact plants and maintained in a subsequent generation. Ecol Ento 35:25-36.

Lopez DC, Sword GA (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*). Bio Con 89:53-60.

McAuslane HJ, Alborn HT (1998) Systemic induction of allelochemicals in glanded and glandless isogenic cotton by *Spodoptera exigua* feeding. J Chem Ecol 24:399-402.

McAuslane HJ, Alborn HT, Toth JP (1997) Systemic induction of terpenoid aldehydes in cotton pigment glands by feeding of larval *Spodoptera exigua*. J Chem Ecol 23:2861-2879.

Nazarova IP, Glushenkova AI, Umarov AU (1981) Gossypol-like compounds of the cotton plant: methods of determining gossypol. Chem Nat Compd 17:87-102.

Olson DM, Cortesero AM, Rains GC, Potter T, Joe Lewis W (2009) Nitrogen and water affect direct and indirect plant systemic induced defense in cotton. Biol Con 49:239-244.

Olson DM, Davis RF, Wäckers FL, Rains GC, Potter T (2008) Plant-herbivore-carnivore interactions in cotton, *Gossypium hirsutum*: linking belowground and aboveground. J Chem Ecol 34:1341-1348.

Opitz S, Kunert G, Gershenzon J (2008) Increased terpenoid accumulation in cotton (*Gossypium hirsutum*) foliage is a general wound response. J Chem Ecol 34:508-522.

Orians CM, Thorn A, Gómez S (2011) Herbivore-induced resource sequestration in plants: Why bother?. Oecologia 167:1-9.

Porras-Alfaro A, Bayman P (2011) Hidden fungi, emergent properties: endophtyes and microbiomes. Ann Rev Phytopath 49:291-315.

Veyrat N, Robert CAM, Turlings TCJ, Erb M (2015) Herbivore intoxication as a potential primary function of an inducible volatile plant signal. J Ecol https://doi.org/10.1111/1365-2745.12526

Vianna F, Pelizza S, Russo L, Allegrucci N, Scorsetti A (2018) Endophytic *Beauveria bassiana* (Ascomycota: Hypocreales) alters *Helicoverpa gelotopeon*'s (D.) (Lepidoptera: Noctuidae) life cycle and reproductive parameters. J Plant Prot Res https://doi.org/10.24425/jppr.2018.124643

Wagner TA, Liu J, Puckhaber LS, Bell AA, Williams H, Stipanovic RD (2015) RNAi construct of a cytochrome P450 gene *CYP82D109* blocks an early step in the biosynthesis of hemigossypolone and gossypol in transgenic cotton plants. Phytochem 115:59-69.

Wagner TA, Suh CPC, Liu J, Puckhaber LS (2017) Increased *Helicoverpa zea* (Boddie) larval feeding on cotton plants with RNAi contruct CYP82D109 that blocks gossypol-related terpenoid synthesis. Southwest Entomol 42:287-290.

5. CONCLUSIONS

The studies in this dissertation were designed to test how FFE seeds treatments interacted with chemical defenses of cotton plants. Overall, I did not detect statistically significant changes in the plant chemistry examined due to FFE treatments, and this appears to be related to a lack of FFE colonization in treated plants. Despite the lack of clear FFE-mediated changes, the results of my studies corroborate a large body of literature that exhibits the many chemical responses of cotton plants to caterpillar herbivory.

Each of my chapters highlights a way in which cotton plants dynamically invest defensive resources in response to caterpillar herbivory. The patterns in which plants allocate direct defenses to various tissues to combat herbivory have been found to fit the predictions of Optimal Defense Theory (ODT). ODT, originally put forward by McKey (1974, 1979), is so named because it predicts that individual plants allocate *limited defensive resources* based on the *value of the tissue* and the *likelihood of herbivore attack*, thus optimizing the cost-to-benefit ratio of the resource investment.

The successful development of reproductive structures is wholly linked to a plant's fitness, and although leaves are valuable sites of photosynthesis, they are typically more numerous and readily replaced compared to flowers or fruits. Based on these differences in relative value, Wäckers and Bonifay (2004) noted that ODT "makes two predictions with respect to defense allocation between vegetative and reproductive

structures: 1) Reproductive structures should receive a higher proportion of overall defensive investment; 2) Foliar defenses should be inducible, whereas reproductive tissues are predicted to have high levels of constitutive (*i.e.*, non-inducible) defense."

ODT has been tested on secondary metabolites that act as direct plant defenses in multiple plant species (*e.g.* Keith & Mitchell-Olds 2017, Godschalx et al. 2016, Pankoke et al. 2013, Moreira et al. 2012, Barto & Cipollini 2005, Pavia et al. 2002, Ohnmeiss & Baldwin 2000, Cronin & Hay 1996, Wilkens et al. 1996, Zangerl & Rutledge 1996), but studies of whether indirect defenses such as VOCs and EF nectar fit those predictions are far fewer (*e.g.* Delgado et al. 2017, Jones & Koptur 2015, Radhika et al. 2008, Wäckers & Bonifay 2004). I find support for ODT predictions in my assessment of indirect chemical defenses of cotton, corroborating a growing body of literature that suggests ODT predictions can be applied to indirect defenses in addition to direct defenses.

In my analysis of cotton leaf VOC emissions (Chapter 2), I find support for the predictions of ODT with regard to foliar indirect defenses. VOC emissions from herbivore-free leaves were low and showed clear induction by *S. exigua* larvae, even when only a few square centimeters of leaf tissue were consumed (Chapter 2, 8-hour experiment). In addition to the significant increase in quantities of VOCs, I also observed a qualitative induction, with many compounds in the induced emissions missing from the constitutive profiles. This is further exemplified in the 32-hour experiment, wherein plants exposed to an additional 24 hours of herbivory responded with more dramatic induction. The distinction between constitutive and induced profiles

is much clearer, and an additional 20 VOCs were identified as a result of the more extensive caterpillar herbivory.

In my analysis of EF nectar in Chapter 3, I again found support for ODT predictions applied to indirect defenses. The experiments that I conducted to assess EF nectar were designed in line with the others in this dissertation to test for the effects of FFE treatments and their interaction with herbivory. However, my experimental design in Chapter 3 was influenced by the study of Wäckers and Bonifay (2004) that directly tested whether EF nectar production fit predictions of ODT. So, although it had been established previously that EF nectar production in cotton does indeed match ODT predictions, my results compliment the results of Wäckers and Bonifay (2004) in a novel way.

According to ODT predictions, defenses of reproductive structures are typically considered to be non-inducible. This specific prediction holds well for direct chemical defenses, but is contradicted by induction patterns in indirect defenses. For VOC emissions, for example, herbivory induces systemic changes from all aboveground tissues including reproductive structures (reviewed in Turlings and Erb 2018). Thus, for VOCs at least, the ODT prediction of non-inducible defenses at reproductive structures is contradicted. My finding of increased sucrose concentrations in the EF nectar produced at bracteal EF nectaries in response to foliar herbivory may also challenge that prediction.

I originally hypothesized that the increased sucrose concentration could be an adaptive response to better attract generalist ants. As described in Chapter 3, the small

change I observed is unlikely to cause any ecologically relevant shift in predator behavior (and the ant preference tests I performed indicate this as well). Still, the fact that I observed a change in sucrose specifically could be interpreted as part of cotton's systemic defensive response to herbivory. I believe my report to be the first of sucrose induction in EF nectar in response to herbivory, but there have been reports of systemic sucrose induction in cotton leaves in response to herbivory (Schmidt et al. 2009, Eisenring et al. 2018). I deduce from the findings of these studies that the sucrose increase that I observed in EF nectar may be a product of systemically induced sucrose transport.

Unlike most of the other chemicals analyzed in this dissertation, sucrose is a primary, rather than secondary, metabolite (Tauzin and Giardina 2014). As such, it can be used by the plant as a carbon source to generate defensive secondary metabolites (Schmidt et al. 2009, Eisenring et al. 2018). In relation to the predictions of ODT, if the increased sucrose concentration I detected in EF nectar in response to herbivory is a precursor for defensive secondary metabolite production, then this could challenge the specific prediction of non-inducible defense in reproductive structures.

In Chapter 4, I investigated glandular terpenoids in a method consistent with experiments designed to test the predictions of ODT (Eisenring et al. 2017). Young developing leaves are crucial to future plant fitness, and as such ODT predicts that they will be highly defended. This prediction has been supported by numerous studies (reviewed in Hagenbucher et al. 2013) and the experiments in Chapter 4 further

corroborate the application of ODT to inducible terpenoids in cotton leaves, specifically highlighting the involvement of heliocides in cotton's induced response to herbivory.

Beyond this, the clear role that heliocides play in turn highlight the role of an induced acyclic monoterpene, ocimene. Ocimene is consistently detected as one of the most prominent monoterpenes induced by caterpillar herbivory on cotton (Loughrin et al. 1994, Loughrin et al. 1995, Paré and Tumlinson 1997ab, Opitz et al. 2008, Olson et al. 2008). Opitz et al. (2008) found that ocimene is a direct precursor of heliocide production, as it is reacted with hemigossypolone to produce heliocides H1 and H4. They also found that cotyledons of *G. hirsutum* which contained hemigossypolone, but not ocimene, contained no heliocides, implicating that production of ocimene could be a limiting step in the production of heliocides.

In my VOC analyses, I found a trend in the 8-hour experiment for greater ocimene emissions from FFE-treated plants (Appendix). From the results of Paré and Tumlinson (1997b), which found that ocimene was primarily synthesized *de novo* in response to caterpillar, I inferred that the elevated ocimene levels I detected might be due to priming effects of the FFE treatments. I did not find a similar trend in the 32-hour experiment, but this does not nullify the 8-hour data. In the 32-hour experiment, herbivory was often so extensive that little intact leaf tissue remained other than major veins. Terpenes like ocimene are emitted from intact plant cells by diffusion after synthesis or release from storage (Pichersky et al. 2006, Paré and Tumlinson 1997 ab, Bustos-Segura and Foley 2018). The fact that little intact tissue often remained at the end

of the 32-hour experiment implies that the levels detected in those samples may not reflect the extent of induction for volatile terpenes.

If enhanced ocimene emission from FFE-treated plants is a repeatable phenomenon, there are clear implications for the capacity of FFEs to enhance the defensive qualities of cotton plants through indirectly defensive terpene emissions and downstream directly defensive heliocide production. Another terpene that should be examined in future VOC assessments of FFE-treated plants is myrcene, which is reacted with hemigossypolone to yield heliocides H2 and H3 (Opitz et al. 2008). Myrcene emissions from *P. inflatum*-treated plants specifically showed a marginally significant increase compared to controls in induced emissions in the 8-hour experiment. These findings of enhanced terpene emissions from FFE-treated plants, with clear connections to heliocide production, warrant further investigation.

To move forward from the research presented in this dissertation, I present below a concept to combine nearly all of the techniques that I used throughout these chapters into a single comprehensive experimental design. An obvious issue throughout my chapters is the difficulty in detecting the target FFEs. Nonetheless, my PDA plating and metabarcoding data both support that plants of the variety PHY367 are colonized by *B. bassiana* at a rate of approx. 12%, and although only supported by PDA plating, *P. inflatum* appears to colonize at a similar rate. I only employed FFE detection techniques that were destructive, meaning that I did not test for colonization in the same plants that were to be used for chemical analyses. For a comprehensive test, FFE-treated and non-

treated control plants need to be non-destructively tested for endophyte colonization before proceeding with experimentation.

The sheer number of plant fragments that need to be plated on PDA to detect colonization (Chapter 2) suggests that it is too inefficient of a detection technique to employ non-destructively. On the other hand, the metabarcoding technique that I employed (Chapter 3) requires weeks or months to receive and process the sequence data, and thus is not conducive to a reasonable experimental timeline. As an alternative, I suggest a diagnostic PCR assay. I have tested diagnostic PCR primers designed by Landa et al. (2013) for *B.bassiana* and they yield a single product of approx. 450 bp with purified DNA of the *B. bassiana* strain GHA that I used in my studies. Additionally, I used the consensus sequence for *P. inflatum* strain TAMU 490 generated for detection by metabarcoding to design custom diagnostic PCR primers using the NCBI Primer-BLAST tool (https://ncbi.nlm.nih.gov/tools/primer-blast). The forward primer (FWD) 5'-CGACTCCCAAACCACTGTGA-3' and reverse primer (REV) 5'-TCCGCCACTGATTTTGAGGG-3', yield a single product of approx. 350 bp with purified DNA of *P. inflatum* TAMU 490. By using these primers for diagnostic PCR assays to detect the target FFEs, the comprehensive experiment may be achievable.

Imagine a cotton plant with 4 or 5 leaves. One or two leaves could be cut and DNA extracted for diagnostic PCR (after necessary washing and surface sterilization). Given that *P. inflatum* was only detected in roots, soil cores could be taken as well, and pieces of roots separated from the soil to be assayed similarly. As long as tissues are sampled evenly across all treatments, FFE-treated plants that reveal colonization, and

control plants that do not reveal inadvertent colonization, can be used for the chemical assays.

The sampling chambers I designed for the VOC analyses allow for VOC collection from a single intact leaf. Thus, one of the remaining leaves could be infested with a caterpillar and sampled for induced VOCs. Each leaf bears an EF nectary, so another leaf could be used to assess EF nectar composition. After sampling VOCs and EF nectar, the developing terminal leaf could be excised and glandular terpenoids quantified.

If employed in a manner similar to my tests of VOCs, with constitutive and induced chemical profiles assessed across all treatments simultaneously, this experiment would be a very comprehensive test of the effects of FFE colonization on the inducible chemical defenses of cotton. Perhaps with this procedure, the mechanisms underlying endophyte-mediated herbivore resistance in cotton may be elucidated.

5.1. References

Barto EK, Cipollini D (2005) Testing the optimal defense theory and the growth-differentiation balance hypothesis in *Arabidopsis thaliana*. Oecologia 146:169-178.

Bustos-Segura C, Foley WJ (2018) Foliar terpene chemotypes and herbivory determine variation in plant volatile emissions. J Chem Ecol 44:51-61. https://doi.org/10.1007/s10886-017-0919-8

Cronin G, Hay ME (1996) Within-plant variation in seaweed palatability and chemical defenses: optimal defense theory versus the growth-differentiation balance hypothesis Oecologia 105: 361-368.

Delgado MN, Somavilla NS, Báo SN, Rossatto DR (2017) Testing the optimal defense hypothesis in *Stryphnodendron adstringens* (Fabaceae, Mimosoideae) leaves: the role of

structure, number, position and nectar composition of extrafloral nectaries Plant Species Biology 32: 333-339.

Eisenring M, Meissle M, Haugenbucher S, Naranjo SE, Wettstein F, Romeis J (2017) Cotton defense induction patterns under spatially, temporally and quantitatively varying herbivory levels. Front Plant Sci 8:234.

Eisenring M, Glauser G, Meissle M, Romeis J (2018) Differential impact of herbivores from three feeding guilds on systemic secondary metabolite induction, phytohormone levels, and plant-mediated herbivore interactions. J Chem Ecol 44:1178-1189.

Godschalx AL, Stady L, Watzig B, Ballhorn DJ (2016) Is protection against florivory consistent with the optimal defense hypothesis? BMC Plant Biol 16:1-9.

Hagenbucher S, Olson DM, Ruberson JR, Wäckers FL, Romeis J (2013) Resistance mechanisms against arthropod herbivores in cotton and their interactions with natural enemies. CRC Cr Rev Plant Sci 32:458-482.

Jones IM, Koptur S (2015) Dynamic extrafloral nectar production: The timing of leaf damage affects the defensive response in *Senna mexicana* var. *chapmanii* (Fabaceae). Bot Soc Am 102: 58-66.

Keith RA, Mitchell-Olds T (2017) Testing the optimal defense hypothesis in nature: Variation for glucosinolate profiles within plants PLoS ONE 12:1-17.

Landa BB, Lopez-Diaz C, Jimenez-Fernandez D, Montes-Borrego M, Muñoz-Ledesma FJ, Ortiz-Urquiza A, Quesada-Moraga E (2013) *In-planta* detection and monitorization of endophytic colonization by a *Beauveria bassiana* strain using a new-developed and nested quantitative PCR-based assay and confocal laser scanning microscopy. J Invert Path 114:128-138.

Loughrin JH, Manukian A, Heath RR, Turlings TCJ, Tumlinson JH (1994) Dinural cycle of emission of induced volatile terpenoids by herbivore-injured cotton plants. PNAS Plant Biol 91:11836-11840

Loughrin JH, Manukian A, Heath RR, Tumlinson JH (1995) Volatiles emitted by different cotton varieties damaged by feeding beet army worm larvae. J Chem Ecol 21:1217-1227.

McKey D (1974) Adaptive patterns in alkaloid physiology. Am Nat 108:305–320.

McKey D (1979) The distribution of secondary compounds within plants. *in* Rosenthal G, Janzen D, eds. Herbivores: their interaction with secondary plant metabolites. p56–133 Academic Press, New York, New York, USA.

Moreira X, Zas R, Sampedro L (2012) Differential allocation of constitutive and induced chemical defenses in pine tree juveniles: A test of the Optimal Defense Theory. PLoS ONE 7:1-8.

Ohnmeiss TE, Baldwin IT (2000) Optimal Defense Theory predicts the ontogeny of an induced nicotine defense. Ecol 81:1765-1783.

Olson DM, Davis RF, Wäckers FL, Rains GC, Potter T (2008) Plant-herbivore-carnivore interactions in cotton, *Gossypium hirsutum*: linking belowground and aboveground. J Chem Ecol 34:1341-1348.

Opitz S, Kunert G, Gershenzon J (2008) Increased terpenoid accumulation in cotton (*Gossypium hirsutum*) foliage is a general wound response. J Chem Ecol 34:508-522.

Pankoke H, Buschmann T, Müller C (2013) Role of plant β-glucosidases in the dual defense system of iridoid glycosides and their hydrolyzing enzymes in *Plantago lanceolata* and *Plantago major*. Phytochem 94:99-107.

Paré PW, Tumlinson JH (1997a) Induced synthesis of plant volatiles. Nature 385:30-31.

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

Pavia H, Toth GB, Åberg P (2002) Optimal Defense Theory: Elasticity analysis as a tool to predict intraplant variation in defenses. Ecol 83:891-897.

Pichersky E, Noel JP, Dudareva N (2006) Biosynthesis of plant volatiles: nature's diversity and ingenuity. Sci 311:808-811.

Radhika V, Kost C, Bartram S, Heil M, Boland W (2008) Testing the optimal defence hypothesis for two indirect defences: extrafloral nectar and volatile organic compounds. Planta 228:449-457.

Turlings TC, Erb M (2018) Tritrophic interactions mediated by herbivore-induced plant volatiles: mechanisms, ecological relevance, and application potential. Annu Rev Entomol 63:433-452.

Wäckers FL, Bonifay C (2004) How to be sweet? Extrafloral nectar allocation by *Gossypium hirsutum* fits optimal defense theory predictions. Ecol 85: 1512-1518.

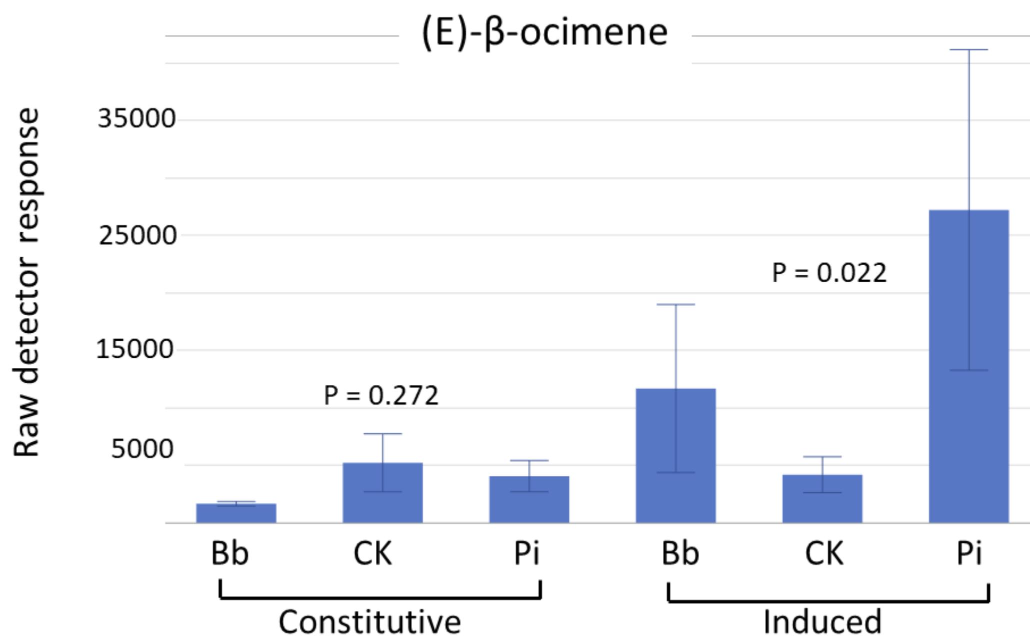
Wilkens RT, Spoerke JM, Stamp NE (1996) Differential responses of growth and two soluble phenolics of tomato to resource availability. Ecol 77:247-258.

Zangerl AR, Rutledge CE (1996) The probability of attack and patterns of constitutive and induced defense: A test of optimal defense theory. Am Nat 147:599-608.

APPENDIX

Levels of individual compounds detected in the VOC collection experiments in Chapter 2. Means ± 1 standard error are shown. Uncorrected P-values are presented from Kruskal-Wallis tests for differences between treatment groups (Bb, CK, Pi) within herbivory groups (Constitutive/Induced). The first 12 compounds are those in the 8-hour analysis. The compounds in the 32-hour analysis begin on pg 96.

Figure A.1



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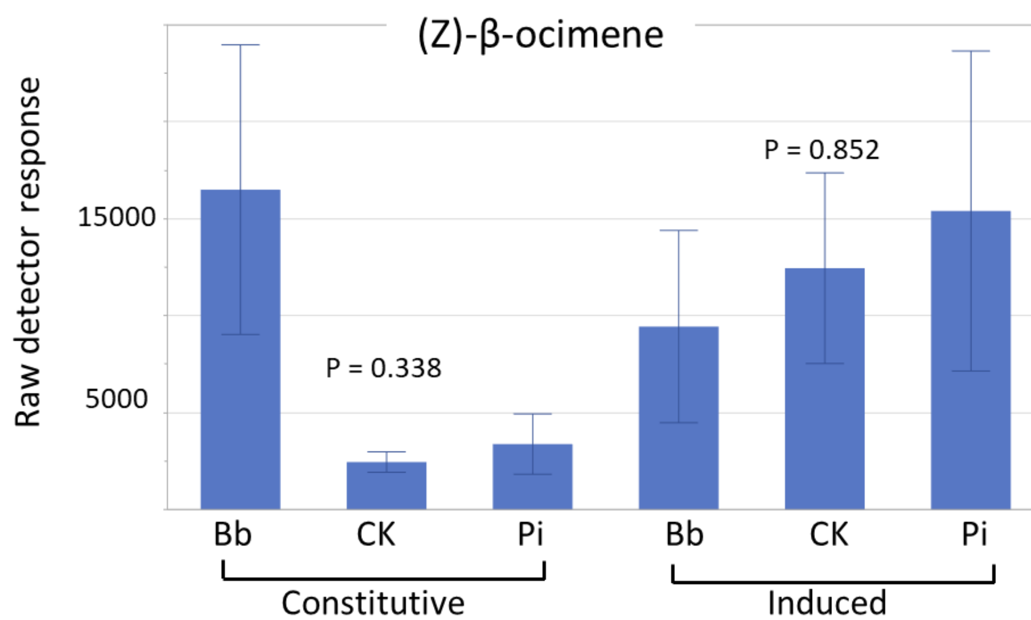

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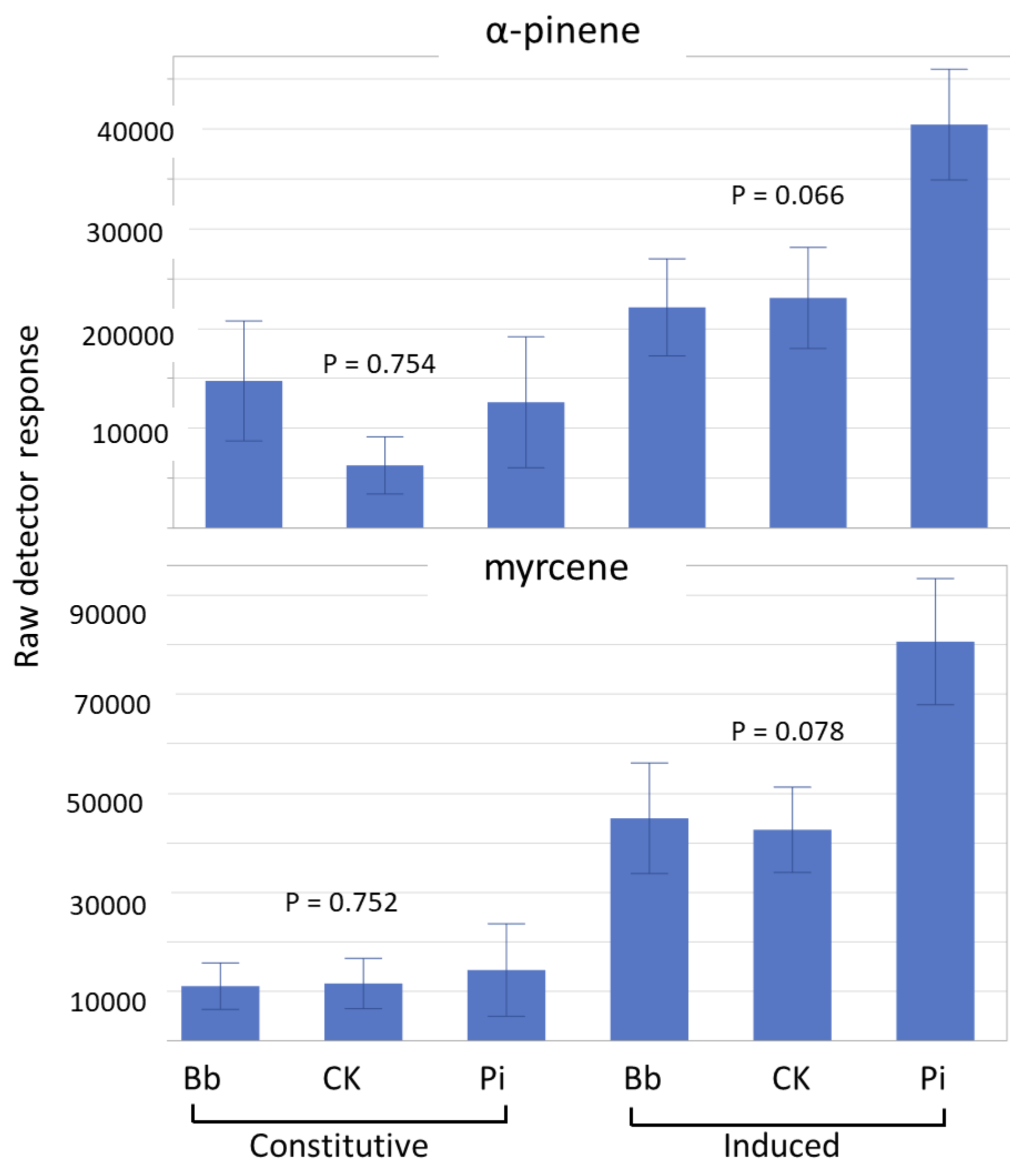

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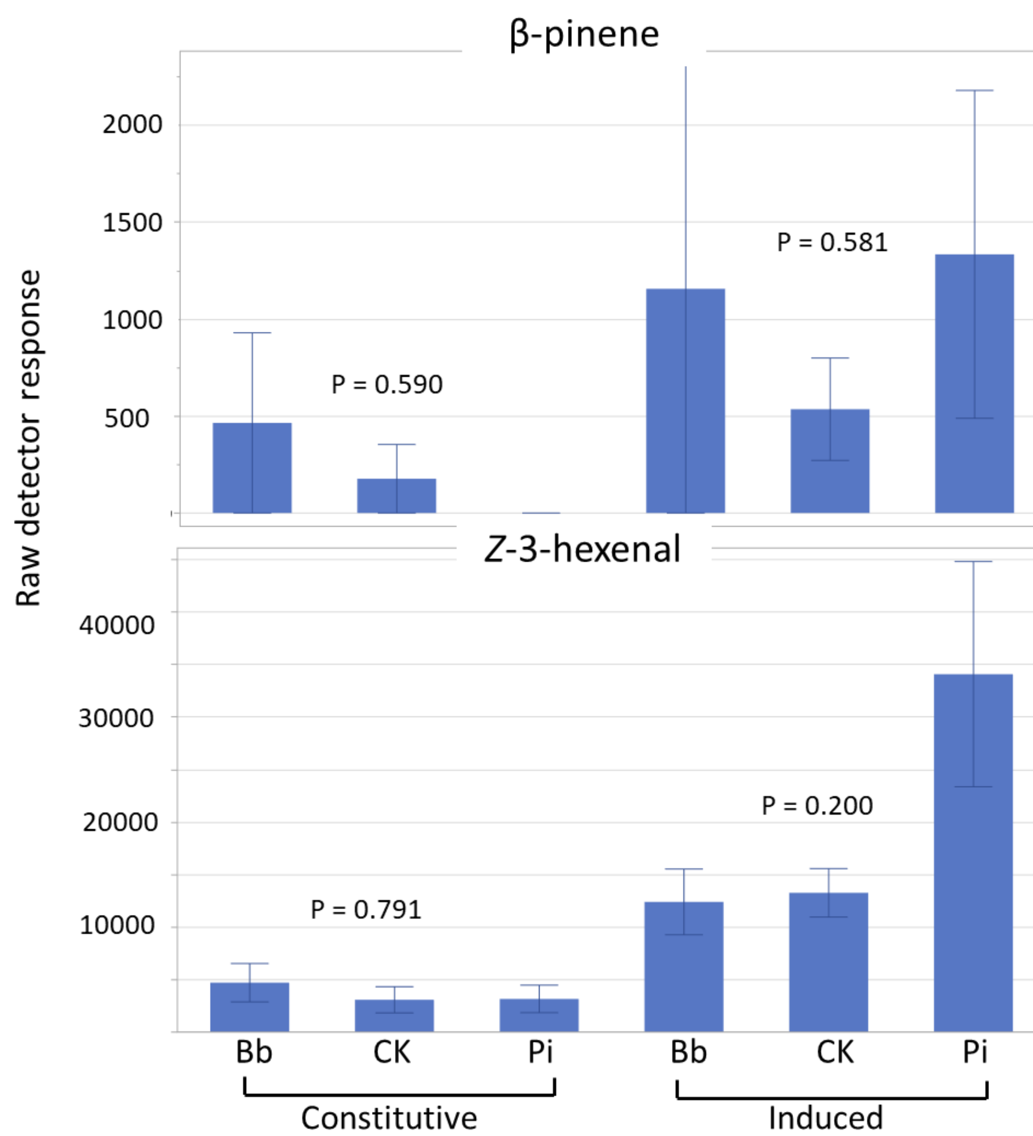

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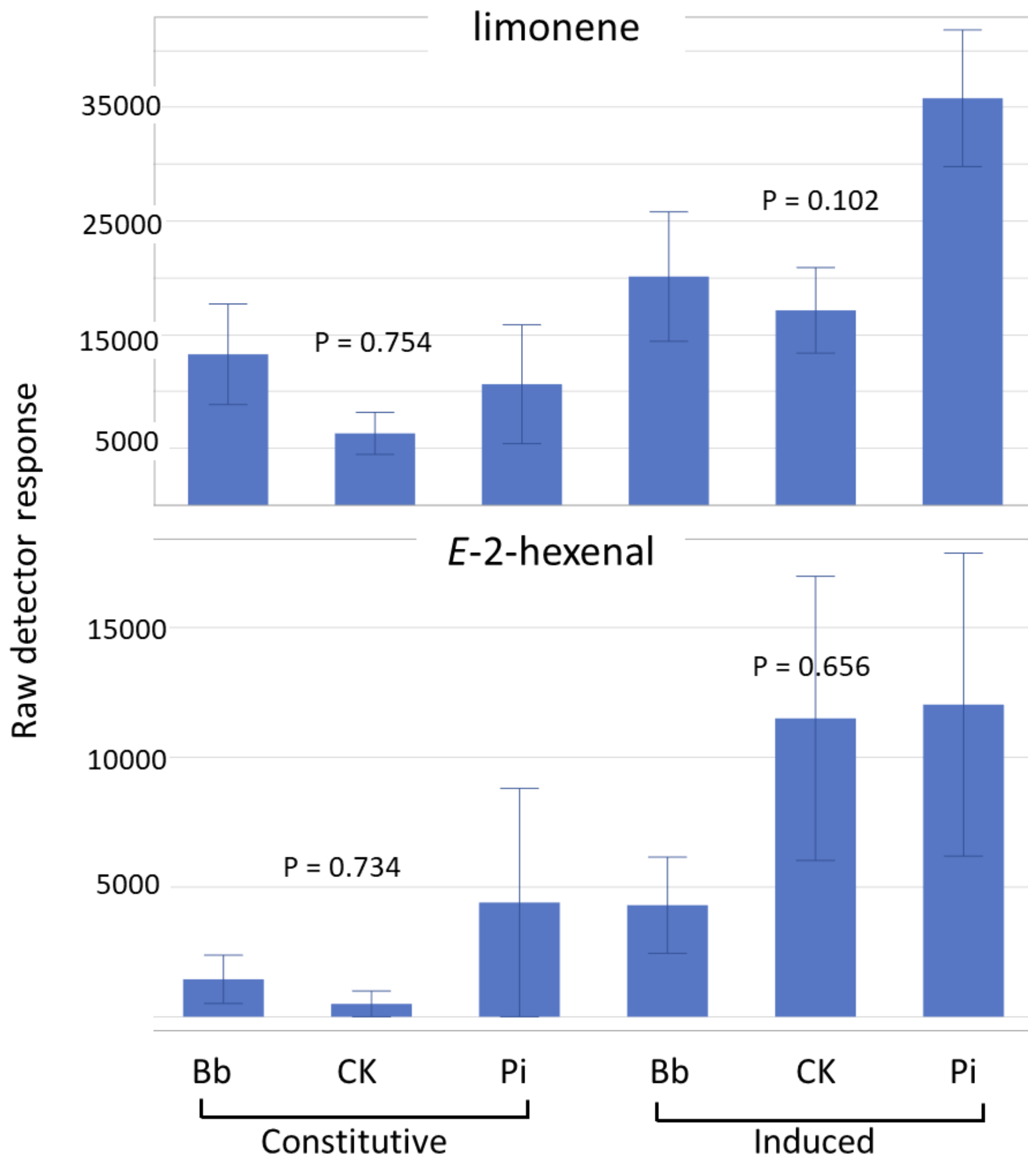

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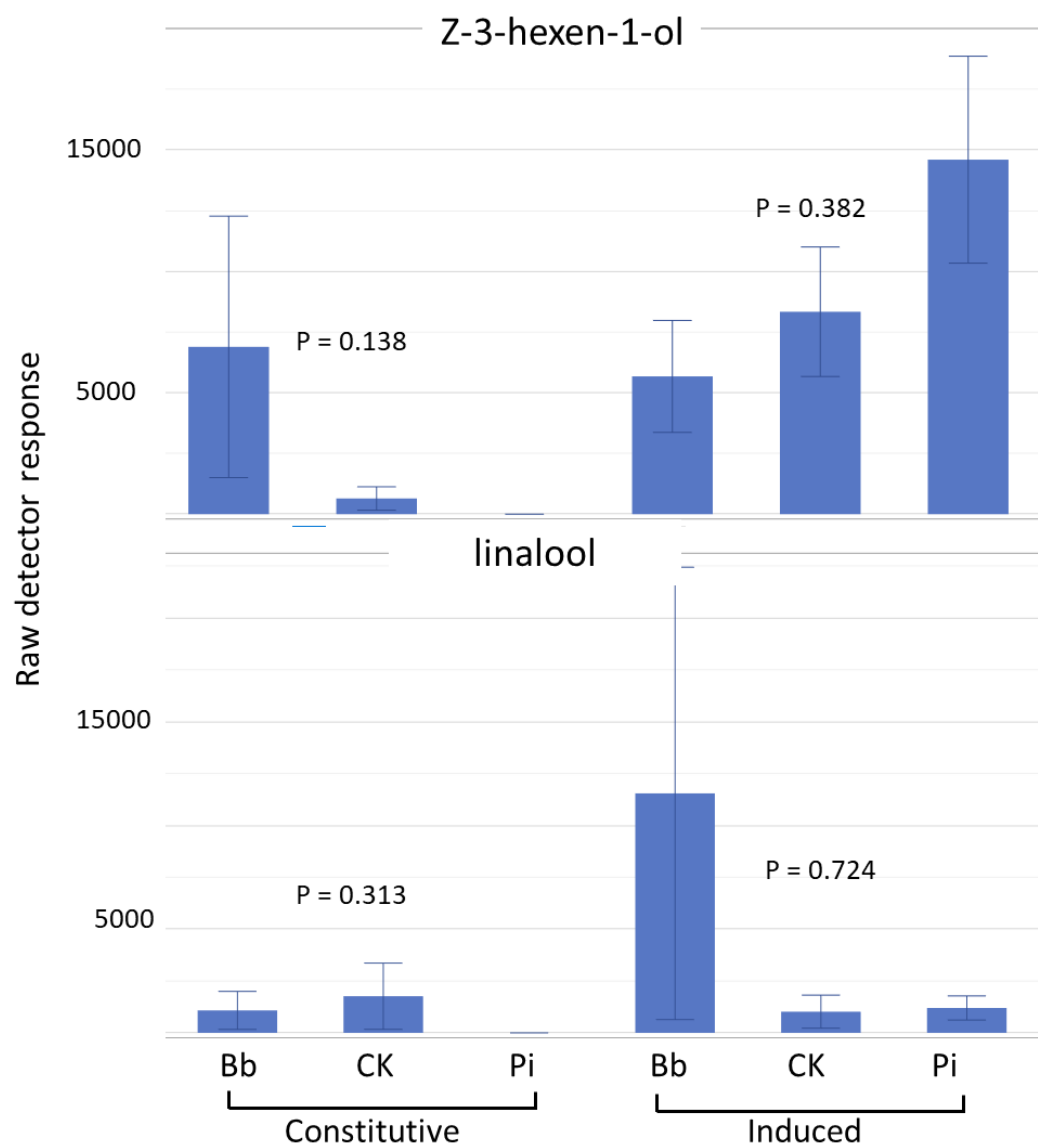

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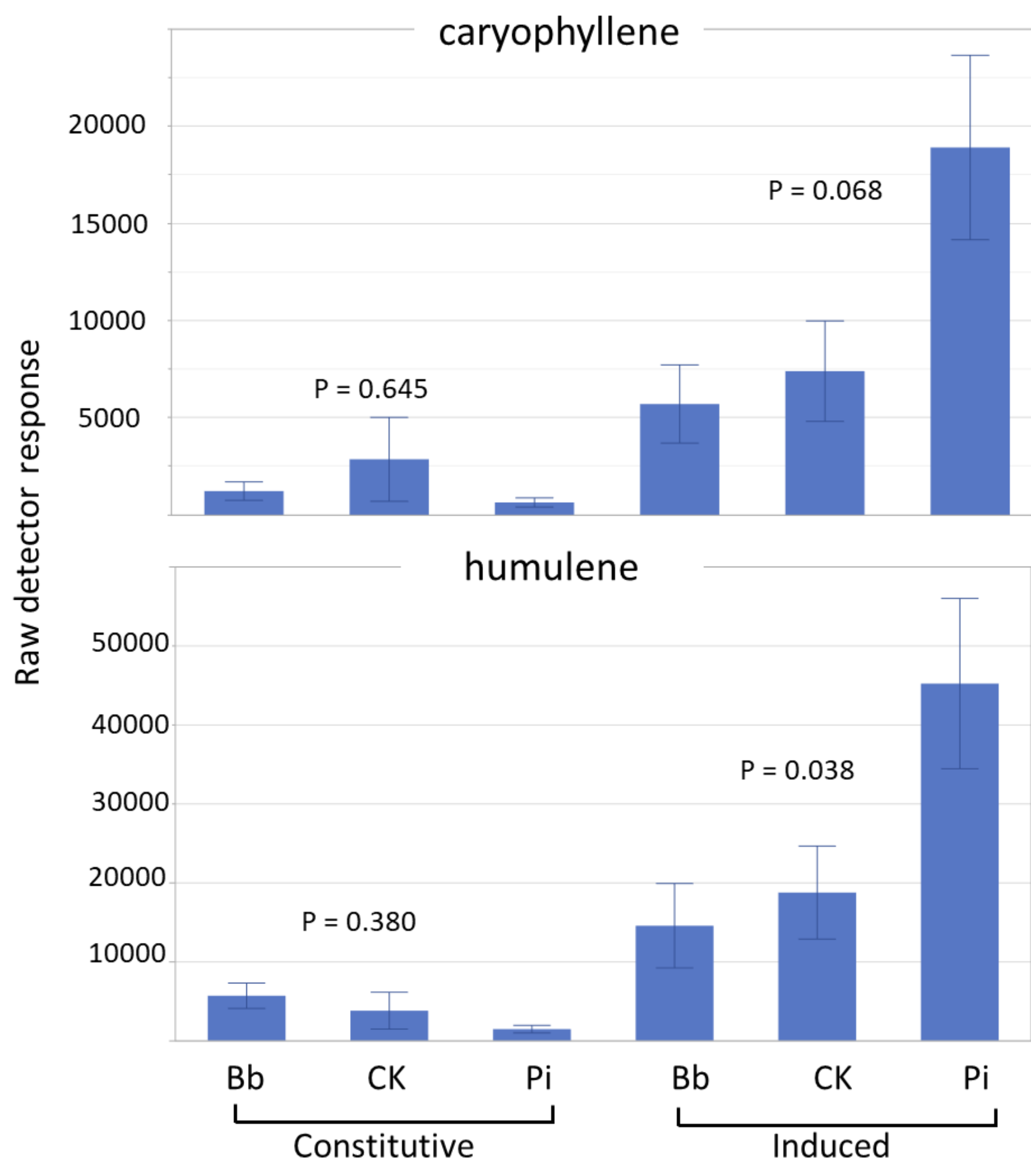


Compounds from the 32-hour experiment begin on this page. Means ± 1 standard error are shown. Uncorrected P-values are presented from Kruskal-Wallis tests for differences between treatment groups within herbivory groups.

Figure A.8

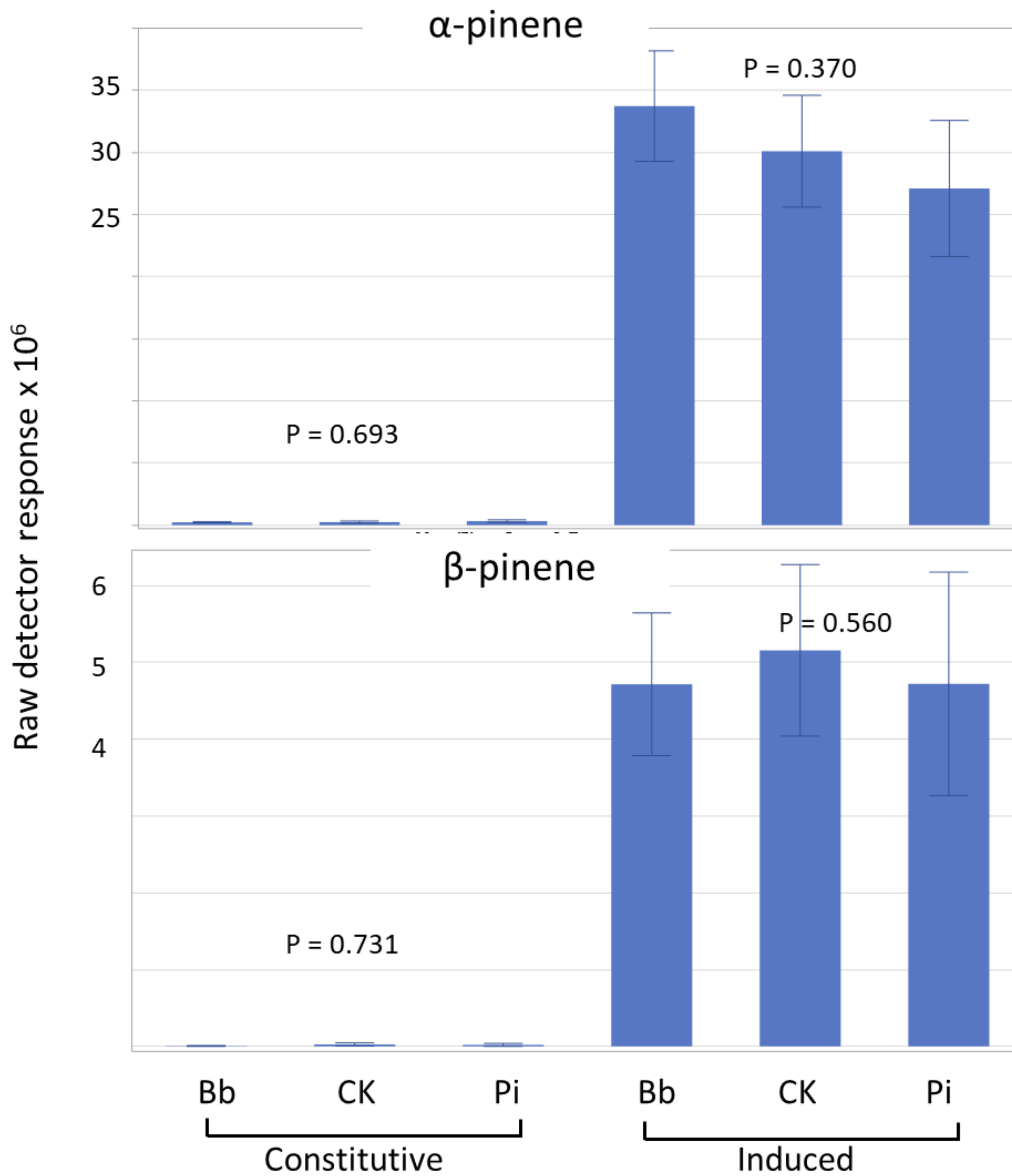
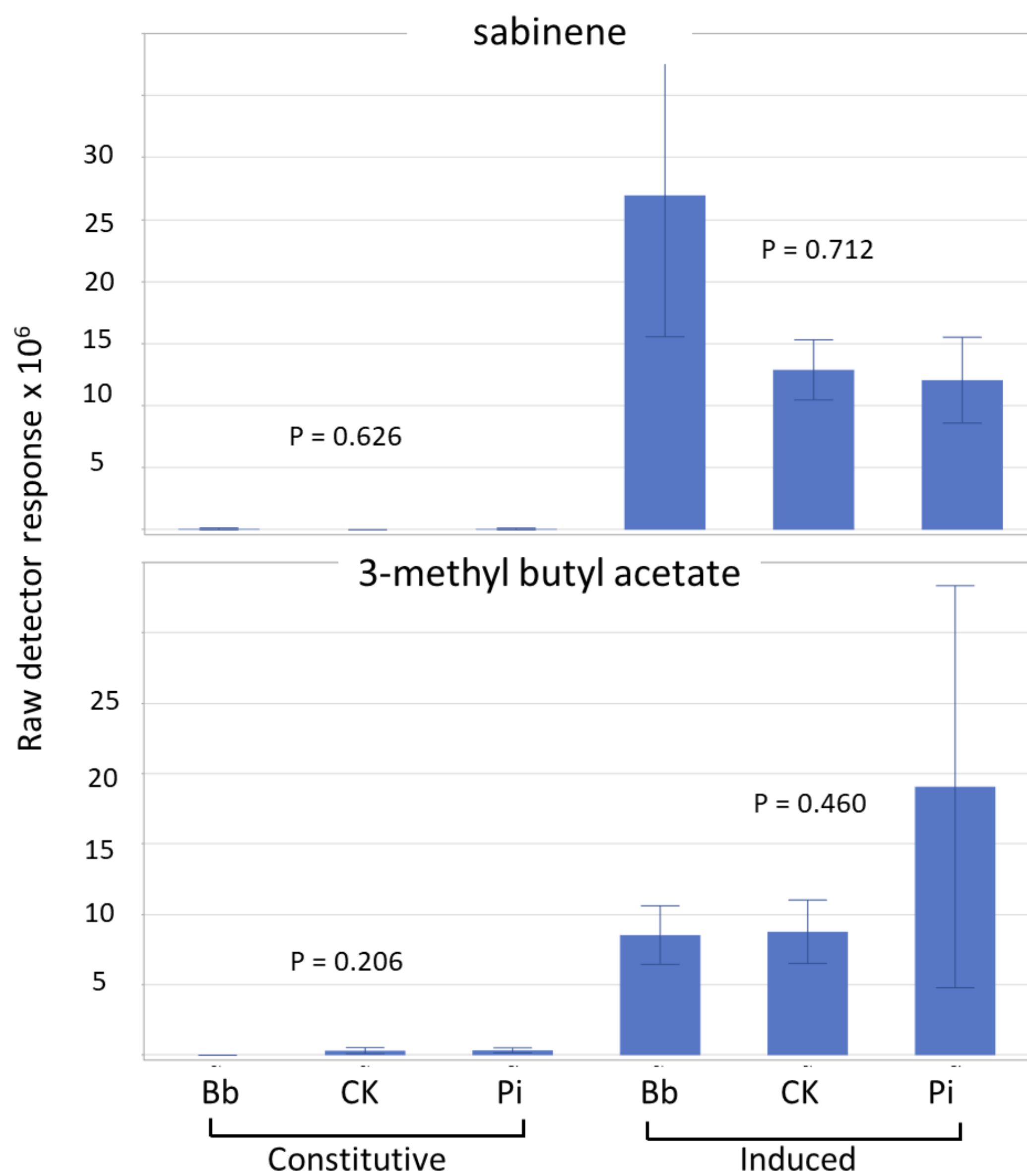


Figure A.9



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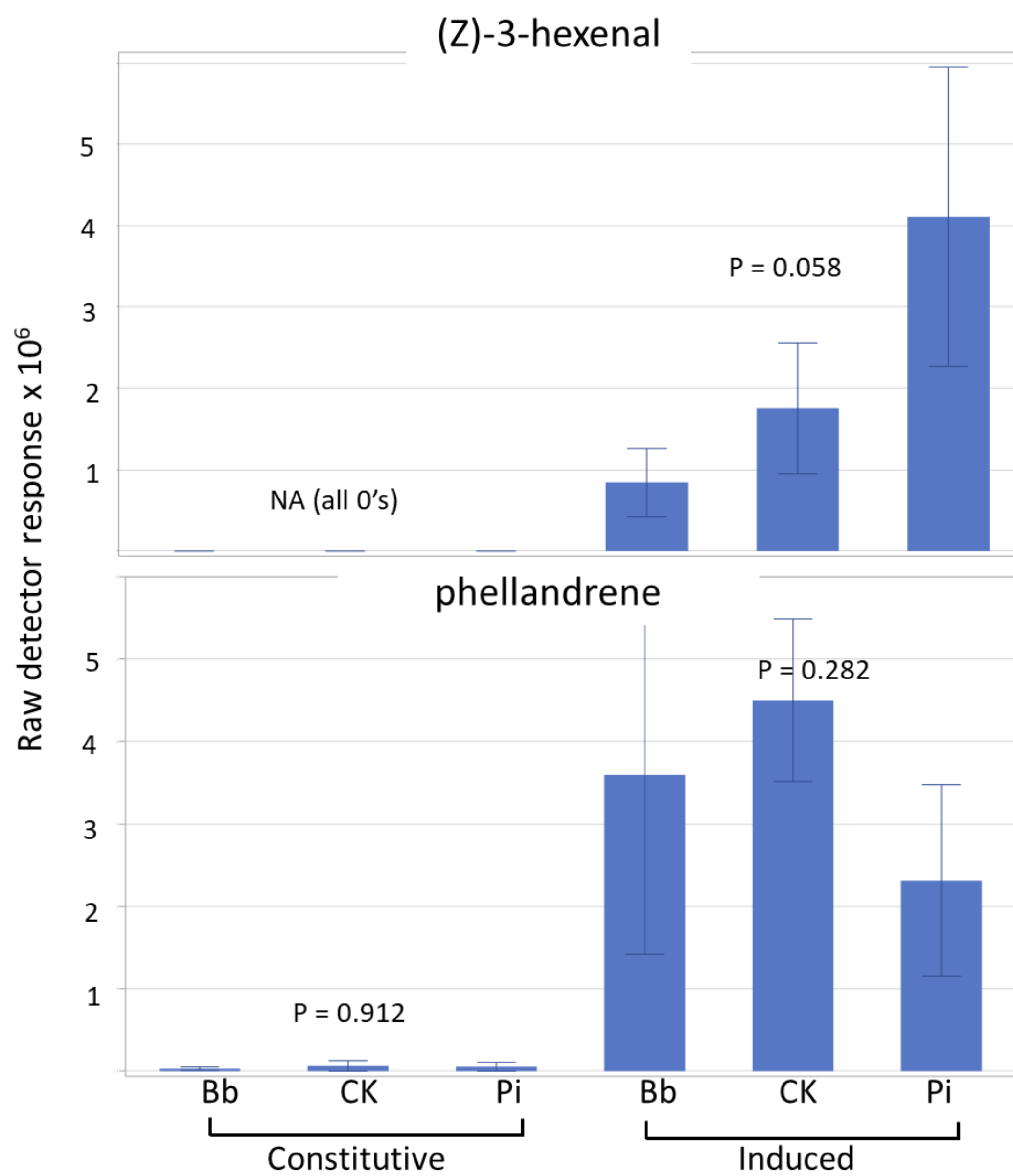

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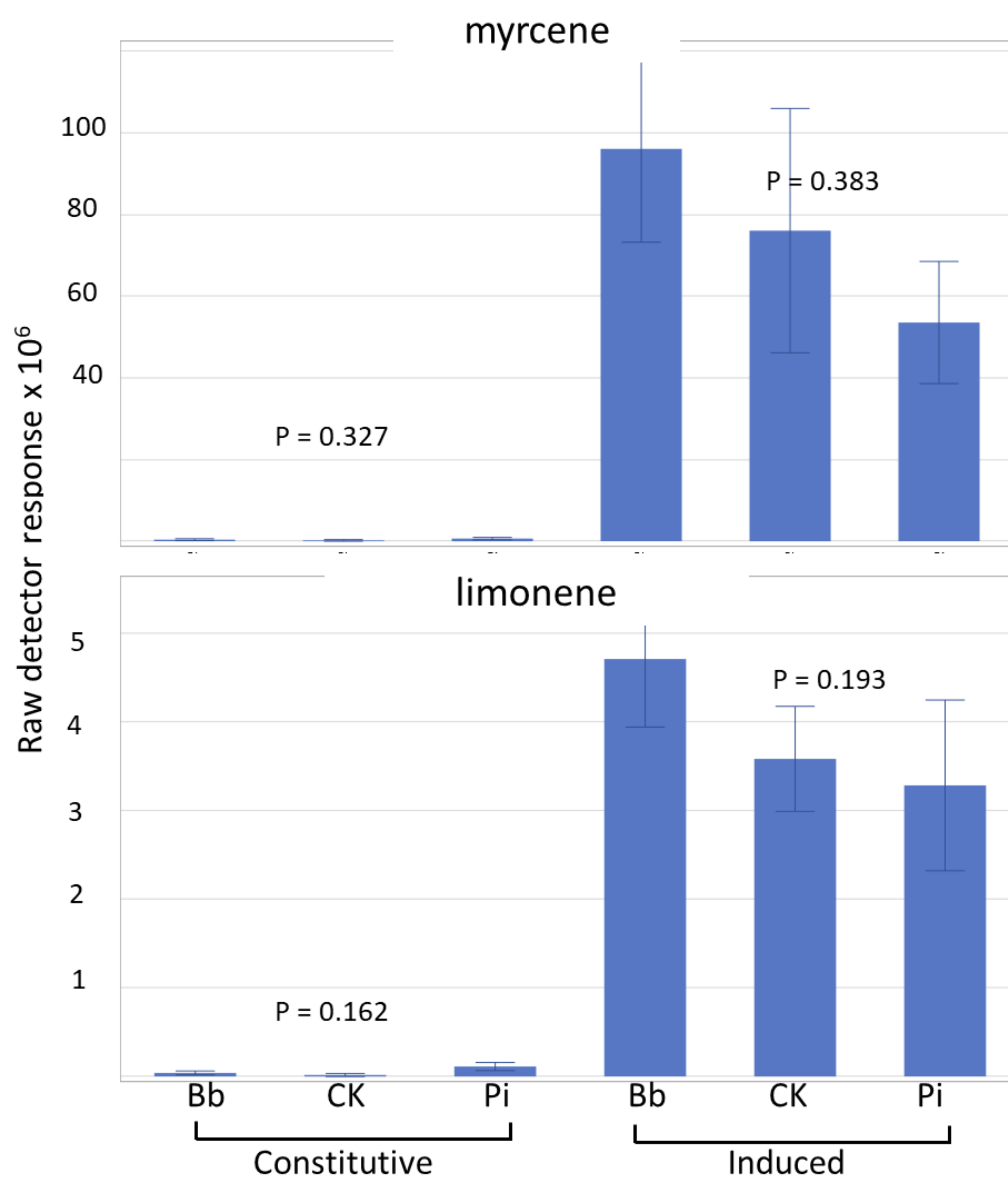

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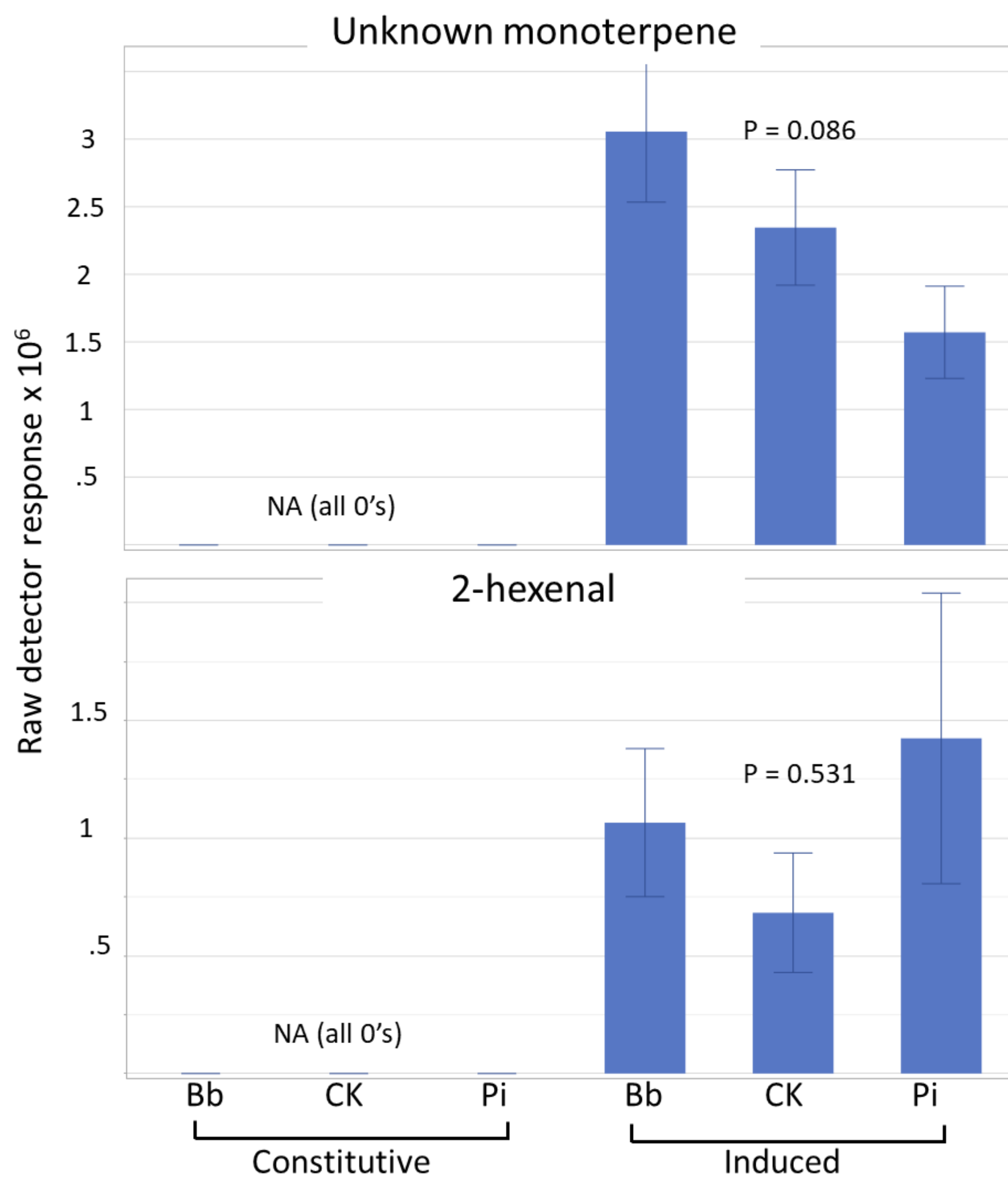

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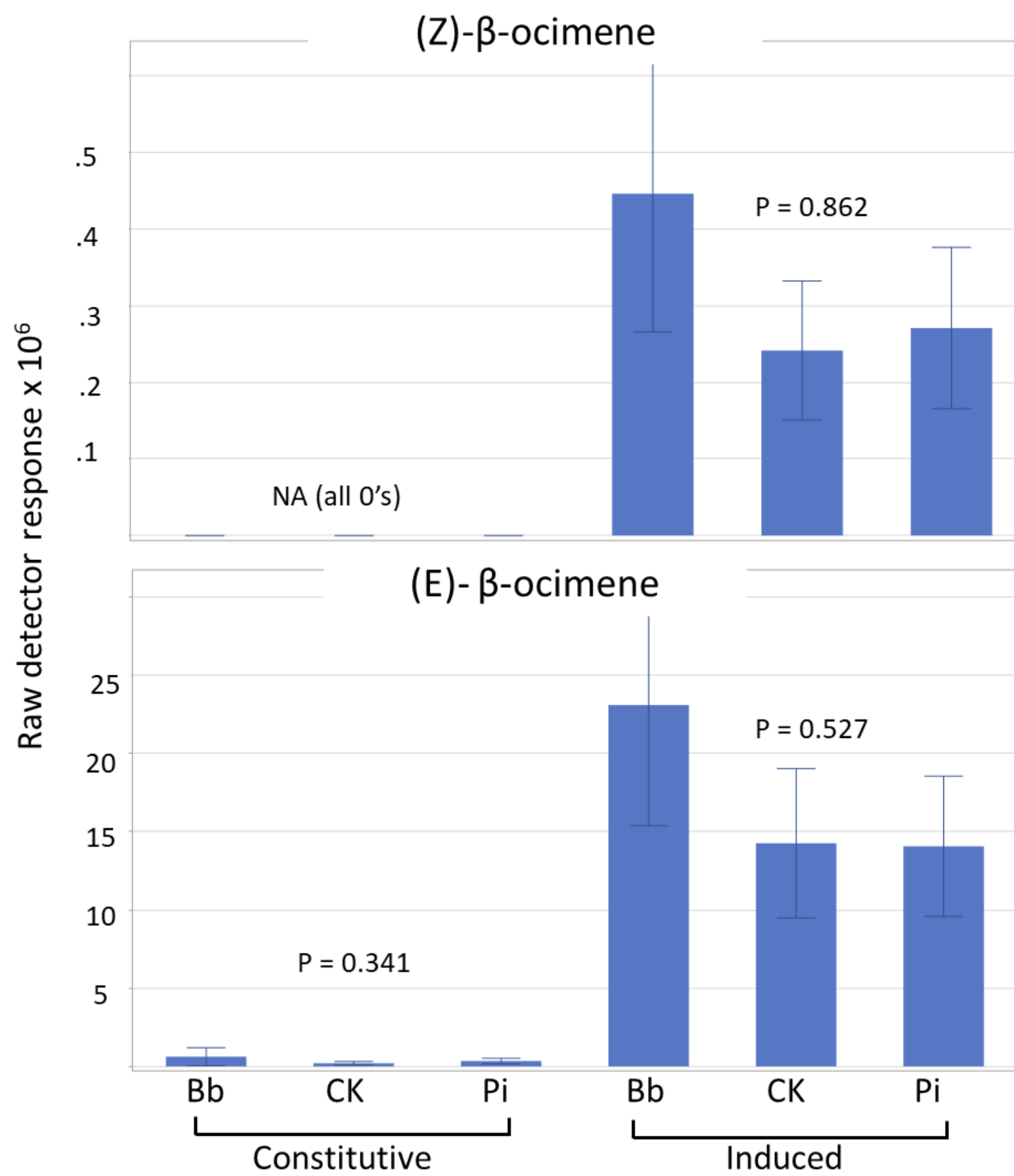

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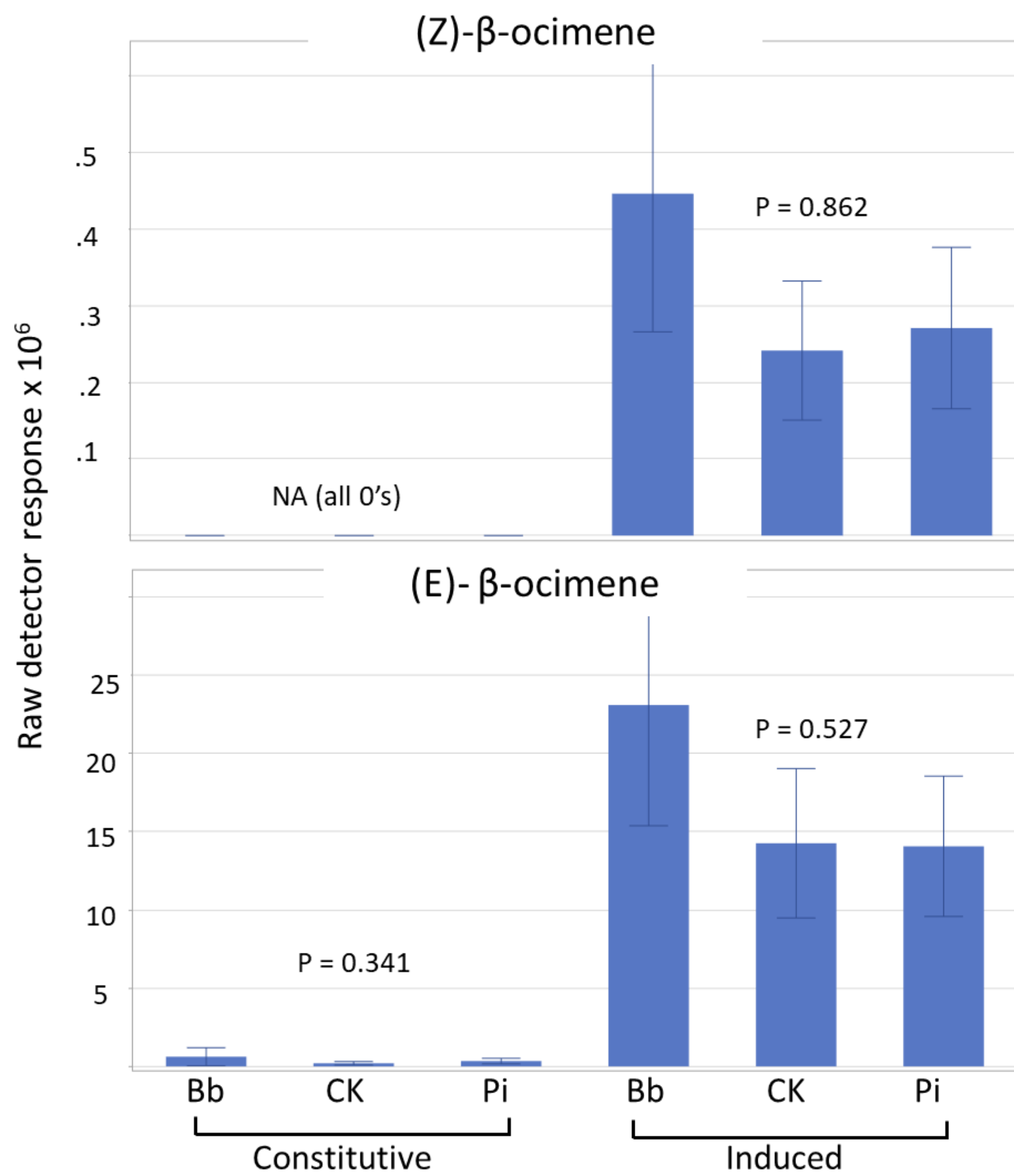

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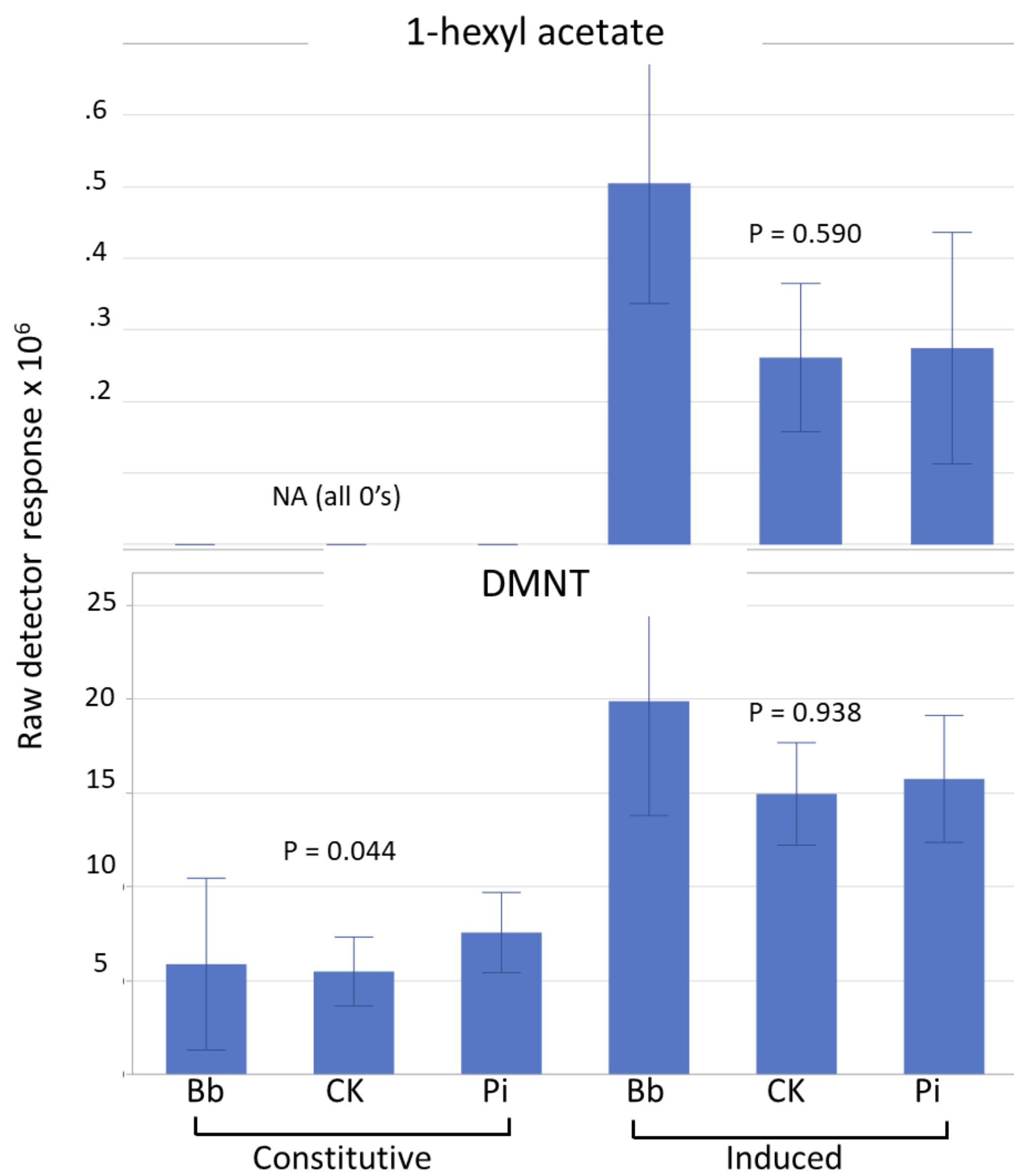

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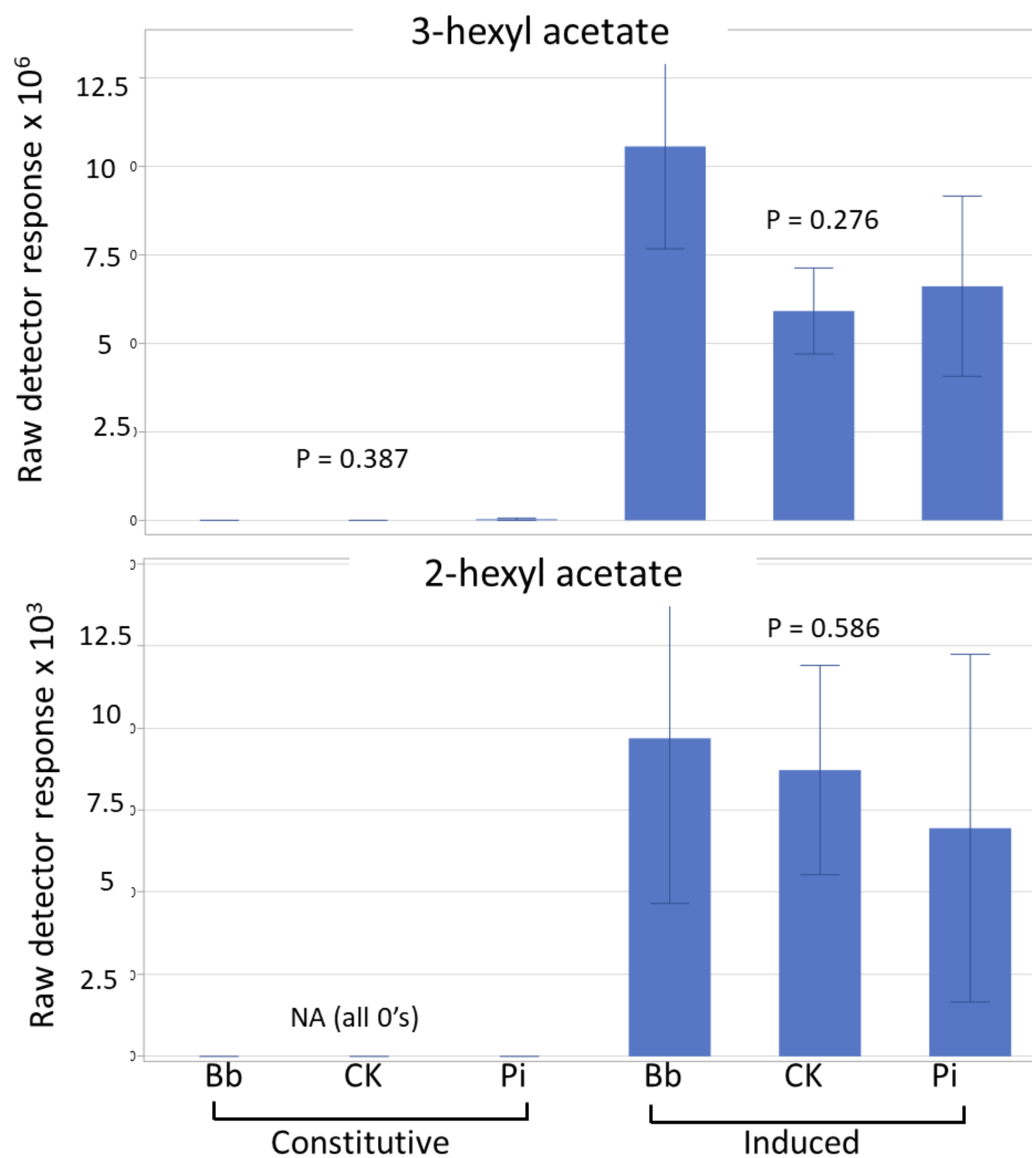

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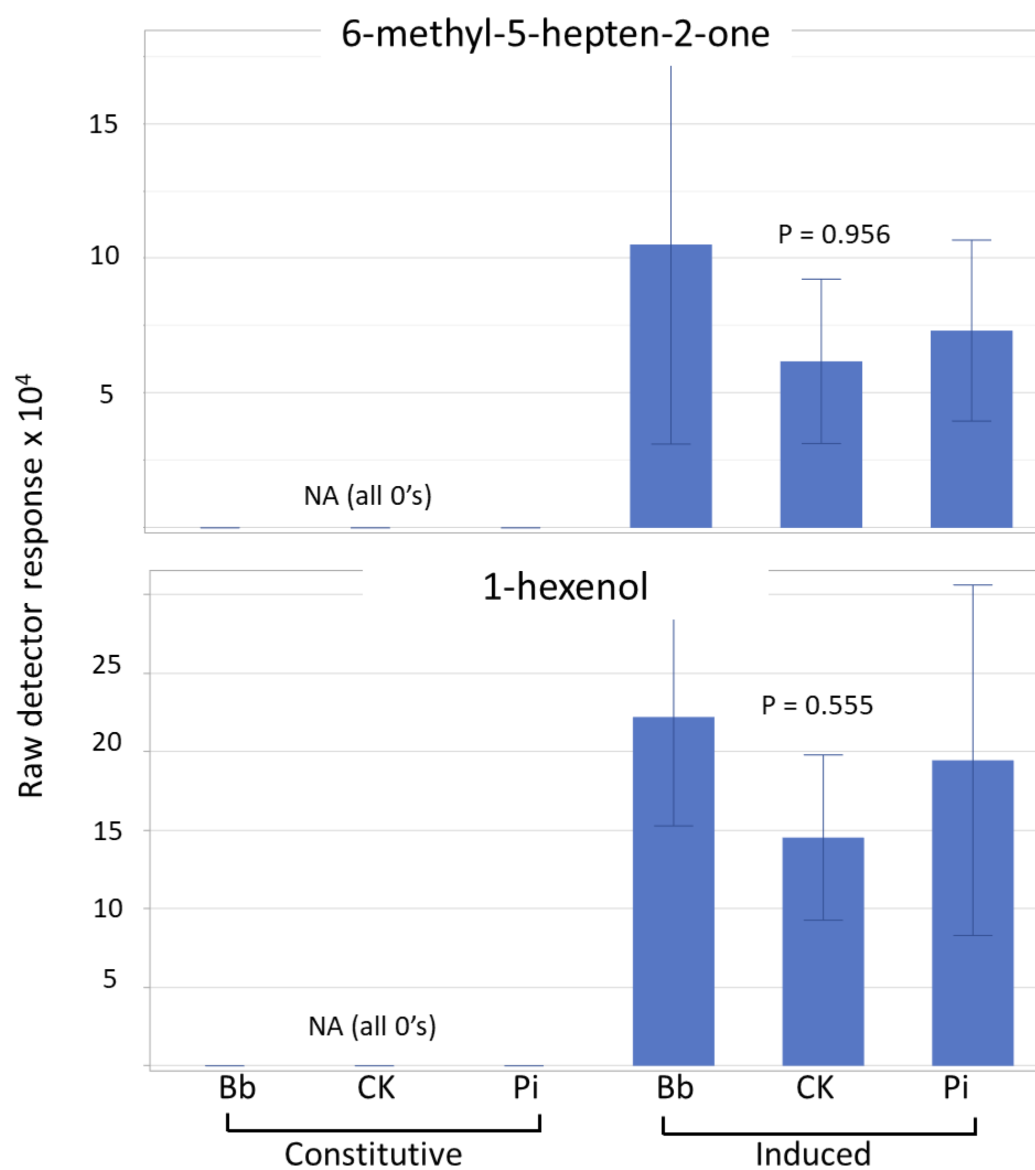

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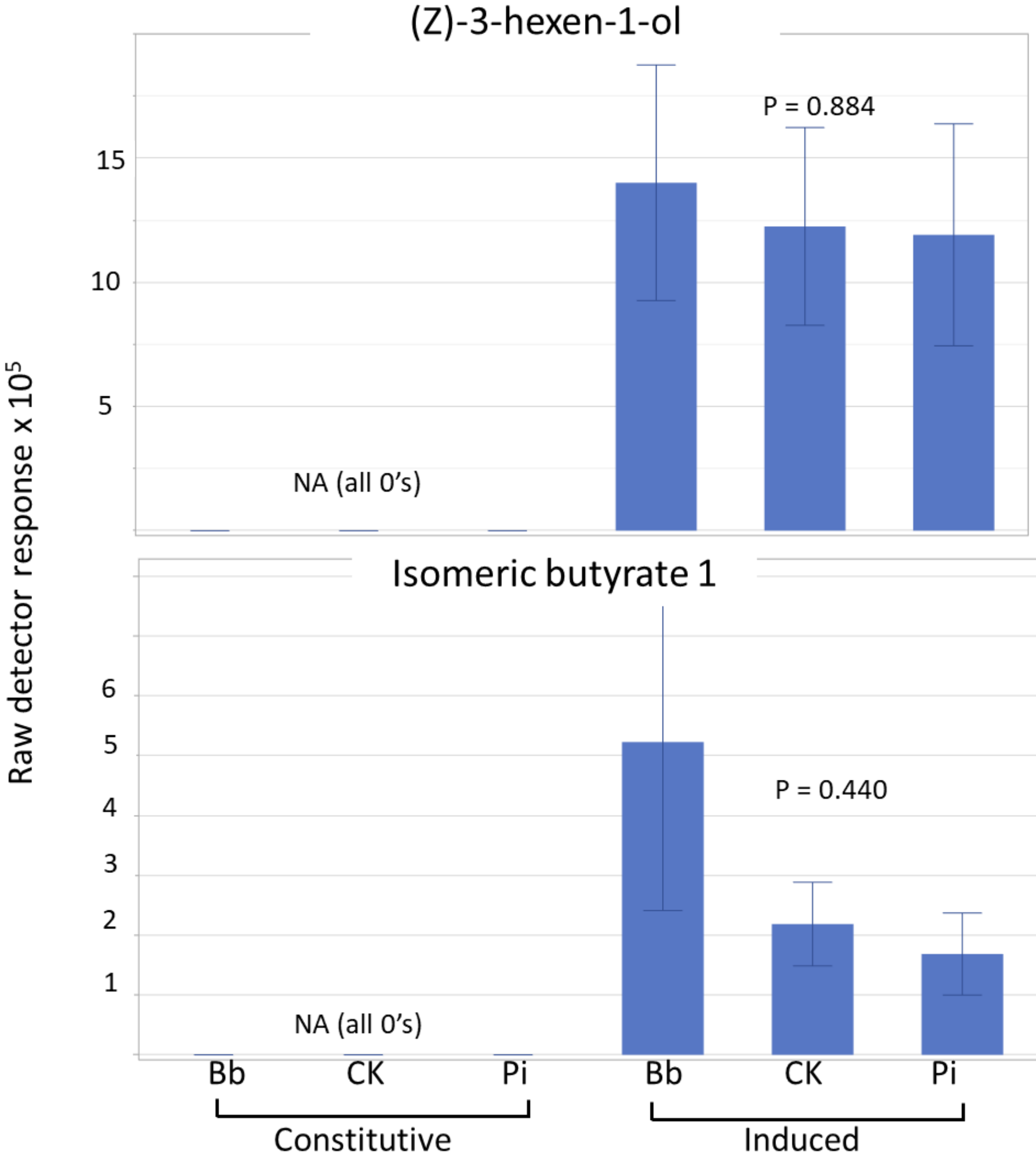

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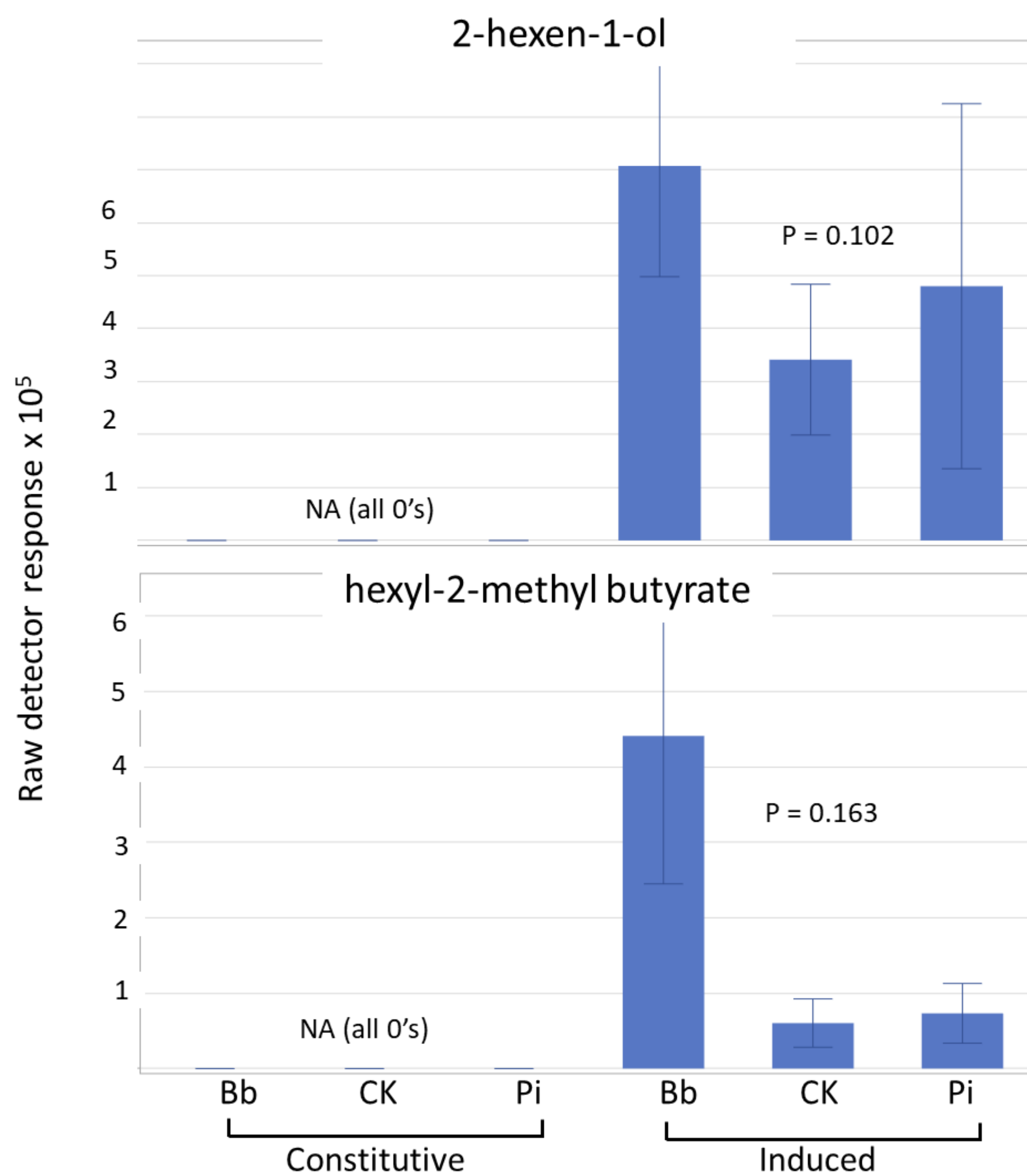

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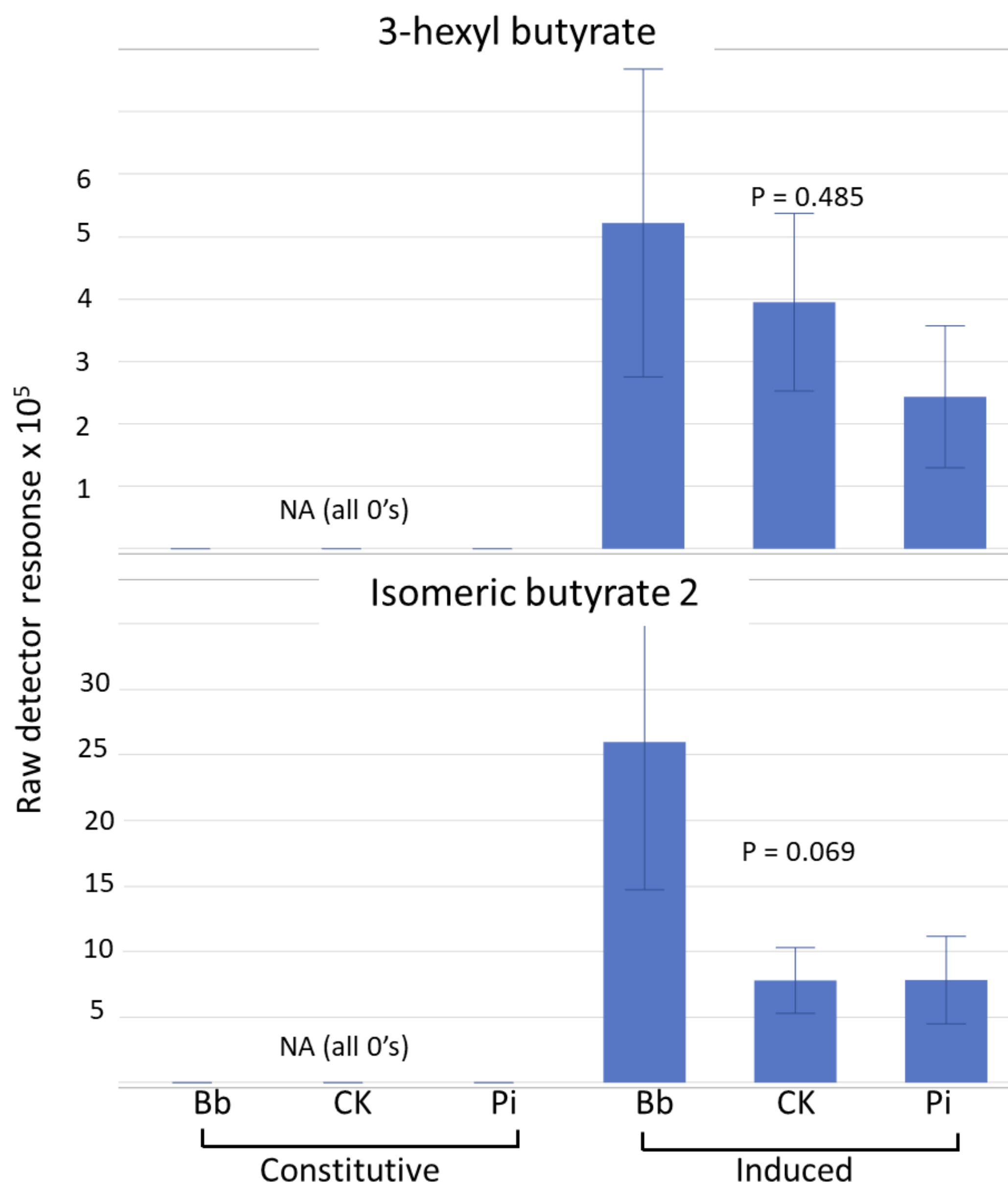

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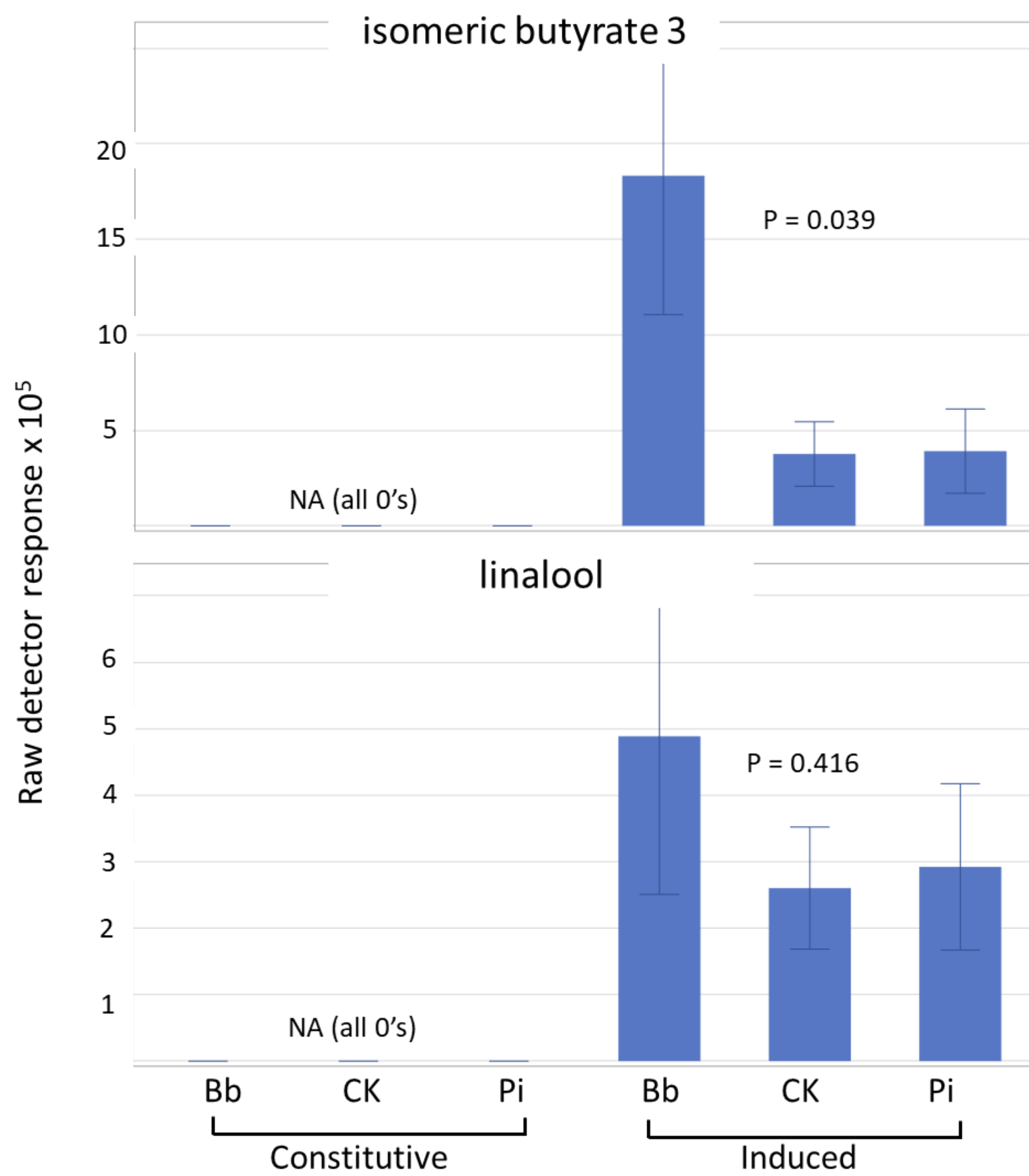

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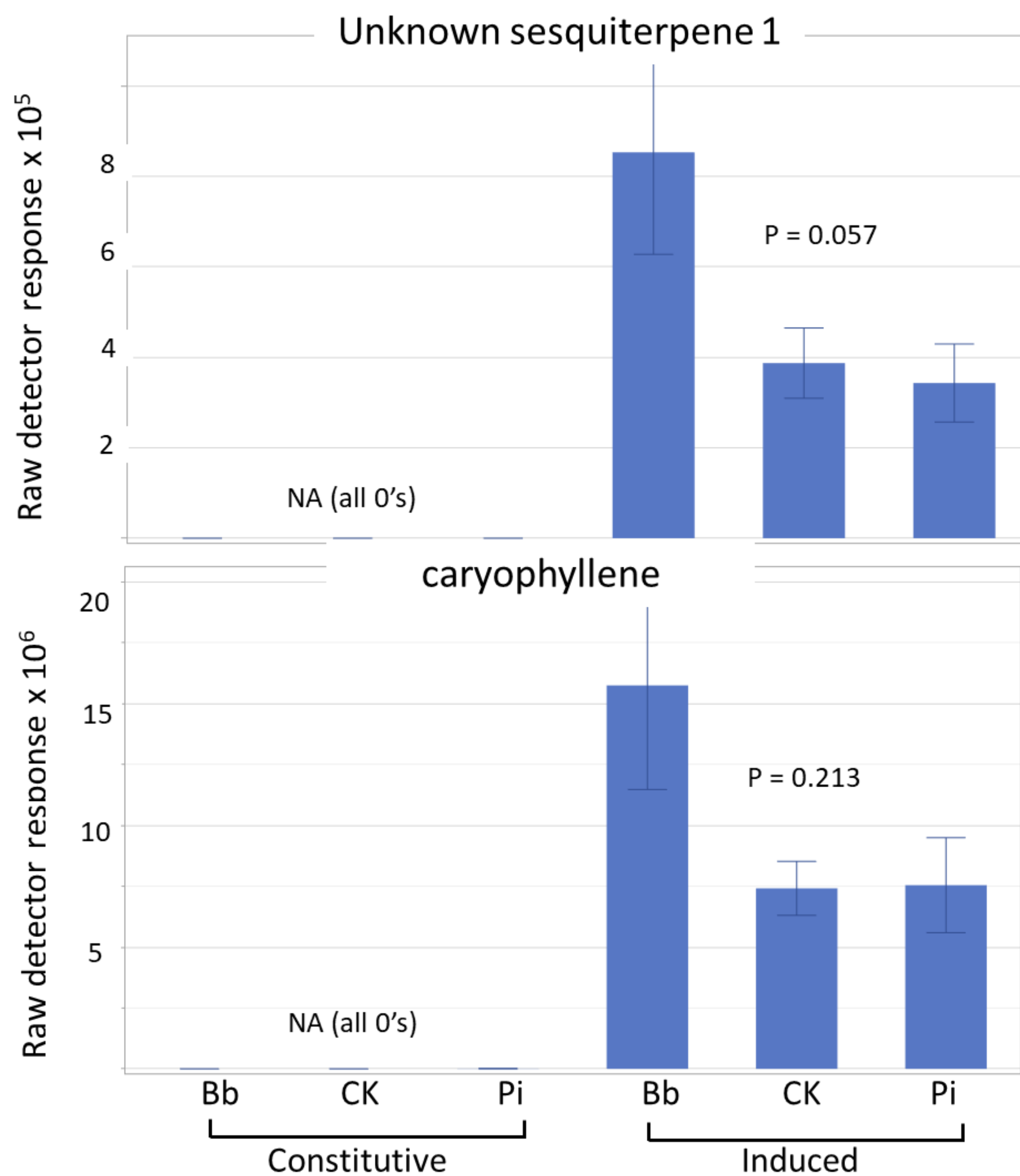

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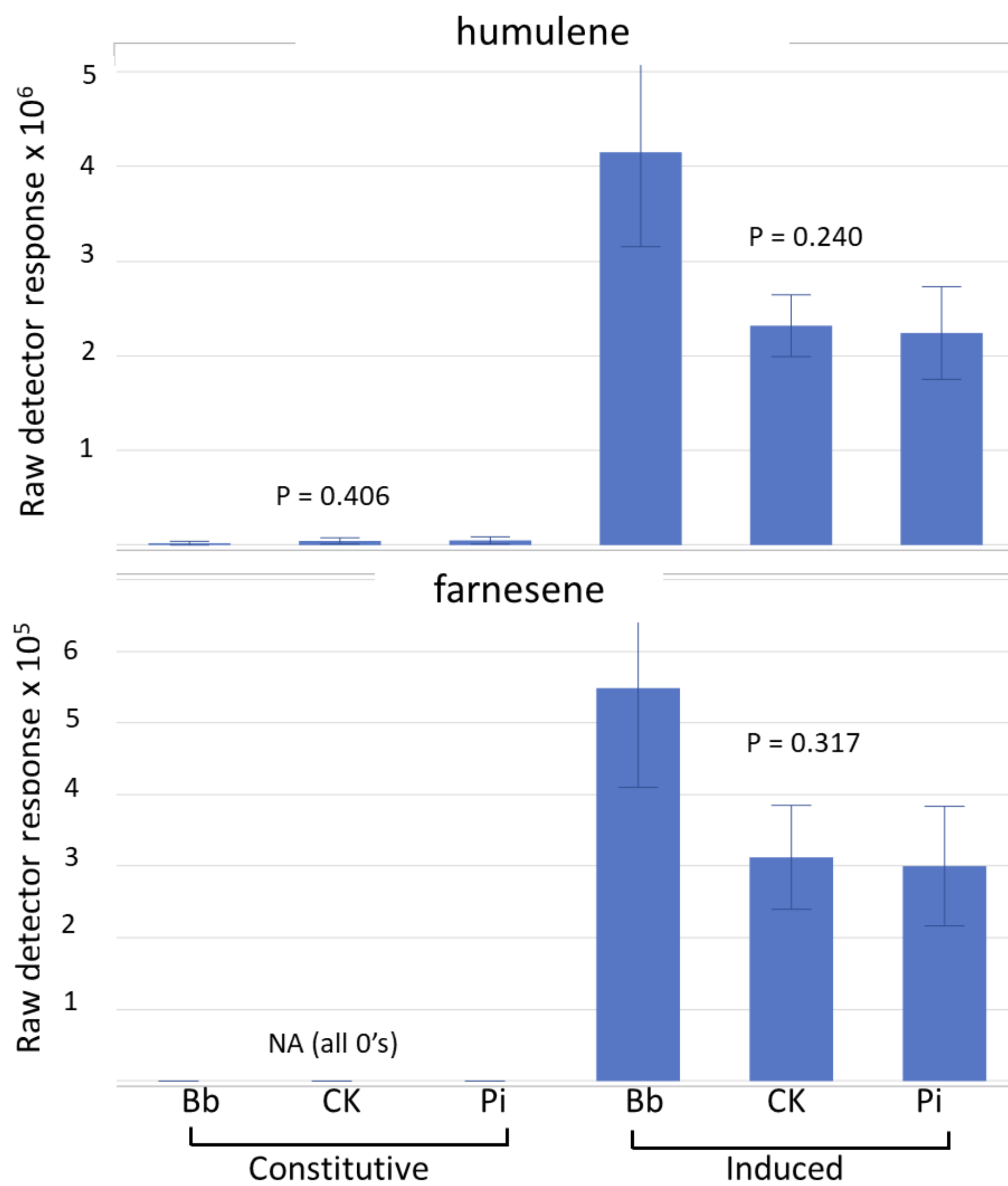

Figure A.24