

N O T E

Analysis of Inducible Terpenoids in Cotton Leaves to Test for Indirect Plant–Endophyte–Herbivore Interactions¹

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J. Entomol. Sci. 57(1): 114–118 (January 2022)

Key Words cotton, *Gossypium hirsutum*, gossypol, heliocide, fungal endophyte

Many species of fungi are capable of endophytic lifestyles, herein referred to as facultative fungal endophytes (FFE), in which they can arrive as spores on a host plant (horizontal transmission) and establish an asymptomatic infection. Our research in recent years has focused on using cotton, *Gossypium hirsutum* L. (Malvales: Malvaceae), as a model system to examine how plant–insect interactions are influenced by experimental FFE treatments. Lopez and Sword (2015, Biol. Control 89: 53–60) found that the treatment of cotton seeds with spores of the FFEs *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin strain GHA (Hypocreales: Cordycipitaceae) or *Phialemonium inflatum* (Burnside) strain TAMU490 (Sordariales: Cephalothecaceae) led to negative effects on *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) survivorship and performance when larvae fed on those plants.

A collection of biosynthetically related chemicals known as glandular terpenoids are produced by cotton and stored in the dark glands visible throughout the plant. These compounds are toxic to many caterpillars (heliocides are named for their lethal effects on *Heliothis* larvae; Nazarova et al. 1981, Chem. Nat. Compd. 17: 87–102) and are the best characterized inducible chemical defenses in cotton (Hagenbucher et al. 2013, Crit. Rev. Plant Sci. 32: 458–482). The reduced caterpillar survivorship previously observed on FFE-treated plants may be the result of plant-mediated changes to plant chemistry (Gange et al. 2019, New Phytol. 223: 2002–2010). We tested the hypothesis that FFE-treated cotton plants may produce more glandular terpenoids than nontreated plants.

¹Received 2 February 2021; accepted for publication 11 February 2021.

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We conducted six independent trials, three to test constitutive (herbivore-free) effects and three to test herbivore-induced effects of FFE treatments on the glandular chemistry of preflowering cotton. Natural, untreated cotton seeds variety LA122 (All-Tex Seed, Inc., Levelland, TX) were surface sterilized and treated with FFE spore solution prepared using the methods of Lopez et al. (2014, PLoS ONE 9: e103891). 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. Plants were grown in a greenhouse on a 14:10 light:dark (L:D) cycle at 29:25°C in 7.6-L (2-gallon) pots filled with JollyGardener C-25 (Oldcastle Lawn and Garden, Inc., Atlanta, GA) soil and fertilized with 30 ml (2 tablespoons) of Osmocote 15-9-12 (Scotts Miracle-Gro, Marysville, OH).

Plants were grown until they began to develop flower buds, typically between the 8th and 10th nodes. Eggs of *H. zea* were purchased from Benzon Research, Inc. (Carlisle, PA) and reared in the lab on artificial diet (Southland Products, Inc., Lake Village, AR) at 29°C on a 14:10 L:D cycle.

To obtain herbivore-induced samples, the three 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 d, the developing terminal leaf was excised, placed 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 18 April, 9 May, and 17 October 2018. Tissue collection dates for the three induced trials were 24 June, 2 September, and 1 October 2019. All trials included 15 plants/treatment.

The glandular terpenoid aldehydes known as gossypol (G), methylG, hemigossypolone (HGQ), methylHGQ, and the heliocides H1, H2, H3, and H4 (H1–H4) were quantitated by high-performance liquid chromatography following the methods of Wagner et al. (2015, Phytochem. 115: 59–69). All samples were found to contain quantifiable amounts of G, HGQ, and the heliocides. MethylHGQ and methylG were detected rarely and at very low levels, thus 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). Quantities of the glandular terpenoid aldehydes (micrograms compound/milligrams leaf tissue) were analyzed by linear mixed effect models using the “lmer” function of the “lme4” package version 1.1.23 (Bates et al. 2015, J. Stat. Softw. 76: 1–48). Data were square-root transformed to meet normality assumptions. Herbivory, FFE treatment, and the interaction term were included as fixed effects and trial nested within herbivory as the random effect. Significance of the effects was calculated by the Wald χ^2 test using the “Anova” function in the “car” package version 3.0.7 (Fox and Weisberg 2019, R Companion Appl. Regres., 3rd ed.).

FFE treatments had nonsignificant effects on the concentrations of G, HQG, or H1–H4 in developing cotton leaves, regardless of herbivory (Table 1). Three days of herbivory by *H. zea* larvae significantly increased the concentrations of H1–H4 in developing cotton leaves (Tables 1, 2). Herbivory also increased the concentration of HGQ (Table 2), but this difference was not statistically significant (Table 1).

Table 1. Analysis of deviance table for Type II Wald χ^2 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
	Herbivory:treatment	2.154	2	0.3406
HGQ	Herbivory	1.250	1	0.264
	Treatment	3.152	2	0.207
	Herbivory: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

* Statistically significant at $\alpha = 0.05$.

In this study, we investigated the defensive secondary metabolites found in FFE-treated cotton plants to better elucidate potential mechanisms underlying negative effects on insect herbivores. Despite a lack of evidence for FFE-mediated changes, the results of this study corroborate experimentally and biologically informative patterns in the induction of these terpenoids by *H. zea* larvae in *G. hirsutum*. Eisenring et al. (2018, *J. Chem. Ecol.* 44: 1178–1189) found very similar results to ours, with H1–H4 and HGQ induced but G unaltered by 7–8 d of *H. zea* feeding on *G. hirsutum*.

Our study highlights the clear role of heliocides in caterpillar-induced chemical defenses of cotton. While we did not find a statistically significant increase in HGQ, there is a clear trend of its induction (Table 2). Heliocide induction by caterpillar herbivory is well documented, and in many studies, when the heliocides are induced, HGQ is also induced (Bezemer et al. 2004, *J. Chem. Ecol.* 30: 53–67; Eisenring et al. 2017, *Front. Plant. Sci.* 8: 234; Eisenring et al. 2018; McAuslane et al. 1997, *J. Chem. Ecol.* 23: 2861–2879; McAuslane and Alborn 1998, *J. Chem. Ecol.* 24: 399–402; Opitz et al. 2008, *J. Chem. Ecol.* 34: 508–522).

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 that were not investigated in this study. 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 (Bi et al. 1997, *J. Chem. Ecol.* 23: 97–117; Orians et al. 2011, *Oecologia* 167: 1–9). 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, *J. Chem.*

Table 2. Mean \pm 1 SE concentrations of gossypol (G), hemigossypolone (HGQ), and total heliocides (H1–H4) within herbivory and facultative fungal endophyte (FFE) treatment groups. Concentrations are in micrograms compound/milligrams dry leaf weight.

<i>H. zea</i> Larvae	FFE Treatment	G	HGQ	H1–H4
Absent	Control	2.23 \pm 0.101	4.94 \pm 0.259	2.24 \pm 0.195
	<i>Beauveria bassiana</i>	2.18 \pm 0.171	4.68 \pm 0.44	2.02 \pm 0.254
	<i>Phialemonium inflatum</i>	2.20 \pm 0.195	5.06 \pm 0.50	2.62 \pm 0.456
Present	Control	1.97 \pm 0.09	8.48 \pm 0.389	7.17 \pm 0.253
	<i>Beauveria bassiana</i>	1.78 \pm 0.15	7.96 \pm 0.69	6.97 \pm 0.46
	<i>Phialemonium inflatum</i>	2.08 \pm 0.17	9.03 \pm 0.71	7.7 \pm 0.46

Ecol 21: 1511–1530; 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.

Acknowledgments. This study was supported by USDA–National Institute of Food and Agriculture Award No. 2016-67013-24758.