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Cotton Plant, *Gossypium hirsutum* L., Defense in Response to Nitrogen Fertilization

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Abstract Plants respond to insect herbivory by producing dynamic changes in an array of defense-related volatile and nonvolatile secondary metabolites. A scaled response relative to herbivory levels and nutrient availability would be adaptive, particularly under nutrient-limited conditions, in minimizing the costs of expressed defensive pathways and synthesis. In this study, we investigated effects of varying nitrogen (N) fertilization (42, 112, 196, and 280 ppm N) on levels of cotton plant (*Gossypium hirsutum*) phytohormones [jasmonic acid (JA) and salicylic acid (SA)], terpenoid aldehydes (hemigossypolone, heliocides H_1, H_2, H_3, and H_4), and volatile production in response to beet armyworm (*Spodoptera exigua*) herbivory. Additional bioassays assessed parasitoid (*Cotesia marginiventris*) host-searching success in response to cotton plants grown under various N fertilizer regimes. At low N input (42 ppm N), herbivore damage resulted in significant increases in local leaf tissue concentrations of JA and volatiles and in systemic accumulation of terpenoid aldehydes. However, increased N fertilization of cotton plants suppressed *S. exigua*-induced plant hormones and led to reduced production of various terpenoid aldehydes in damaged mature leaves and undamaged young leaves. While increased N fertilization significantly diminished herbivore-induced leaf volatile concentrations, the parasitism of *S. exigua* larvae by the parasitoid *C. marginiventris* in field cages did not differ among N treatments. This suggests that, despite significant N fertilization effects on herbivore-induced plant defenses, at short range, the parasitoids were unable to differentiate between *S. exigua* larvae feeding on physiologically different cotton plants that share large constitutive volatile pools releasable when damaged by herbivores.

Keywords Plant–herbivore interactions · Tritrophic interactions · Plant resistance · Direct defense · Indirect defense · *Spodoptera exigua* · Malvaceae · Hymenoptera · Lepidoptera · Noctuidae · Braconidae · *Cotesia marginiventris* · Optimal defense (OD) theory

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Introduction

Plant secondary metabolites are known to have diverse ecological and physiological roles in modulating biotic and abiotic interactions, but there remains no unifying theory explaining how and why plants produce, transport, and store such a diverse array of chemicals (see Peñuelas and Llusià 2004; Firn and Jones 2006; Pichersky et al. 2006 for discussion). Many of these compounds adversely affect herbivore colonization and life histories (Berenbaum 1995; Duffey and Stout 1996) and/or attract natural enemies of herbivores (Dicke et al. 1990; Röse et al. 1998; Choh et al. 2004; Wäckers and Bonifay 2004). These two broad

functions have been categorized as direct and indirect resistance mechanisms, respectively.

Nitrogen (N) application is an important practice in crop production. It can exert a variety of bottom-up effects and may significantly alter tritrophic interactions through qualitative and quantitative alteration of plant direct and indirect defensive compounds (McNeil and Southwood 1978; Stiling and Moon 2005). Soil nitrogen availability affects the expression of constitutive and induced plant defenses in a wide range of plant species (Stout et al. 1998; Cipollini and Bergelson 2001; Coviella et al. 2002). The level of expression of secondary metabolites may correlate positively (Cipollini and Bergelson 2001; Lou and Baldwin 2004), negatively (Stout et al. 1998; Hemming and Lindroth 1999), or not at all (Dudt and Shure 1994) with nitrogen availability among systems examined, but surprisingly few crop species have been examined in detail.

Nitrogen levels affect the release of plant volatile organic compounds (VOCs), which serve as cues for natural enemies to locate potential hosts/prey (Dicke et al. 1990; Choh et al. 2004). For example, in maize (*Zea mays* var Delprim), the maximal-induced volatile emission was detected after both mechanical wounding and addition of an elicitor from oral secretions of the noctuid *Spodoptera exigua* (Schmelz et al. 2003a) when N fertilization was the lowest. Similarly, celery with additional N had a lower quantity of constitutively emitted volatiles (Van Wassenhove et al. 1990). In contrast, Gouinguené and Turlings (2002) found that unfertilized maize plants (*Z. mays* var Delprim) emitted less volatiles following application of crude *Spodoptera littoralis* oral secretions when compared with those that had received a complete nutrient solution. Unfortunately, the role of N alone was unclear in this study because all the nutrient amounts were varied. In tobacco (*Nicotiana attenuata*), volatile emission levels were not affected by N, although low N availability attenuated the jasmonate and salicylate levels and reduced two N-containing antiherbivore defensive compounds, namely nicotine and trypsin proteinase inhibitors (Lou and Baldwin 2004). Based on this documented variability, the responses of any given plant species or crop cannot yet be readily predicted.

Changes in insect-induced phytohormone production or sensitivity mediated by nutrient availability may provide a mechanism for regulating the magnitude of plant defense responses. With jasmonates established as key regulators of plant responses to insect herbivores (Browse and Howe 2008), jasmonic acid (JA) serves as a useful marker in probing interactions between nutrients and induced defenses. In maize, direct positive relationships have been established between *S. exigua*-induced JA accumulation in the leaves and subsequent induced volatile emission (Schmelz et al. 2003b). In this system, low N availability resulted in higher levels of sustained JA accumulation and subsequent induced volatile emission, following treatment with insect-derived elicitors, than identically treated plants grown under medium N availability (Schmelz et al. 2003a). Despite some advances, few studies have considered the interactions between N fertilization, herbivore-induced JA, and defense in agronomically significant crops (Schmelz et al. 2003a; Lou and Baldwin 2004).

In this study, we investigated the defense response of cotton plants in relation to nitrogen fertilization. Specifically, we tested three hypotheses: (1) increased N fertilization will result in a reduced production of carbon-rich herbivore-induced defense responses, specifically JA and terpenoid aldehydes, in cotton leaves; (2) production of volatile organic compounds will be affected by N fertilization; and (3) the parasitoid *Cotesia marginiventris* will inflict higher mortality on sentinel beet armyworm larvae feeding on plants emitting higher VOC levels.

Methods and Material

Plants Cotton (*Gossypium hirsutum* cv. FiberMax 989) plants were grown using methods and nutrient solutions described by Chen et al. (2008), except as otherwise noted. Plants were fertilized with 100 ml of 112 ppm N nutrient solution daily for approximately 2 weeks, at which time they were of the same height and with leaves of similar size at the same leaf position were randomly assigned to different N treatments. Cotton plants were subsequently fertilized with respective N nutrient treatment solutions (42, 112, 196, and 280 ppm N) for approximately 2 weeks until experimentation. Leaching (watering without nutrients) followed every fourth N nutrient solution application to limit soil salinity. All experimental plants had three–five mature true leaves.

Insects Beet armyworm (*S. exigua*) larvae (reared on modified Pinto bean diet; Chen et al. 2008) and their parasitoids (*C. marginiventris*) were from laboratory colonies maintained in the Biological Control Laboratory at the University of Georgia in Tifton, GA, USA.

Plant Hormone and Volatile Production in Relation to N Fertilization To examine the effects of N fertilization on production of phytohormones and volatiles, a 2 (leaf position—local and systemic)×3 (N fertilization—42, 112, and 196 ppm N)×2 (herbivore infestation—control and 20 *S. exigua* larvae/plant) factorial experiment was designed. For the herbivore damage treatments, 20 3-day-old *S. exigua* larvae were caged on the third true leaf on the main stem for 48 h (see Chen et al. 2006 for cage description). In control treatments, a cage with no larvae was placed on the third true leaf to account for possible physiological changes caused by cages. The cages were checked twice daily for

larval escape and availability of leaf tissue for caterpillars. If all the leaf tissue within the cages were eaten, then the cages were moved to an undamaged location on the same leaf. Immediately following continuous feeding for 48 h, the insect-damaged leaves of all treatments were briefly and gently cleaned with a fine brush to remove larvae and debris. Damaged leaves were photographed digitally for herbivory assessment immediately following removal of larvae and debris, and approximately 150–200 mg fresh leaf tissue from each sample leaf were collected, weighed, stored in FastPrep® tubes containing approximately 1 g Zirmil beads (1.1 mm; SEPR Ceramic Beads and Powders, Mountainside, NJ, USA), and frozen in liquid N as described in Schmelz et al. (2004). Leaf damage was quantified by using the digital images as described in Chen et al. (2006). Samples were collected from leaf 3 (local, damaged, mature leaf) and leaf 6 (systemic; expanding at the time of experiment) and stored at −80°C until analysis. Each treatment was replicated four times.

Terpenoid Aldehyde Production in Relation to N Fertilization To examine the effects of N fertilization on terpenoid aldehyde production, the same 2 (leaf position)×3 (N fertilization)×2 (herbivore infestation) factorial experiment as phytohormone and volatile assessment was designed. At the beginning of the experiment, 20 3-day-old *S. exigua* larvae were caged on the third true leaf of the treatment plants to induce production of the terpenoid aldehydes (see Chen et al. 2006 for cage description). A cage with no larvae was placed on the third true leaf of control plants (zero larvae) to account for possible physiological changes caused by cages. Similarly, cages were checked twice daily for escape of larvae and availability of leaf tissue. Cages and *S. exigua* larvae were removed after 48 h of continuous feeding. The insect-damaged leaves of all treatments were briefly and gently cleaned with a fine brush to remove larvae and debris. A photo of the damaged leaves was taken immediately following removal of larvae and debris and excised at the distal end of the petiole and stored at −80°C until lyophilization. The leaf damage was quantified as in the previous experiment. The sixth true leaves (young and expanding leaves) of all treatment plants were also collected to quantify the effects of N fertilization on systemic production of terpenoid aldehydes. Each treatment was replicated four times.

C. marginiventris Foraging Tests To examine the effects of N fertilization on short-range host location by parasitoids, three cotton plants of one N treatment were placed in one end of 2×2×2 m cages covered with fine mesh. The mesh has square holes with 1 mm openings. Three plants of the other N treatment were placed in the opposite side of the cage. Plants were so arranged that the same N treatments were touching each other but were separated from the other treatment by at least 50 cm. N pairings tested were 42 vs. 196 and 112 vs. 280 ppm N. Forty (42 vs. 196 ppm N trial) or 30 (112 vs. 280 ppm N trial) neonate *S. exigua* larvae were placed on the top leaves of each cotton plant (a total of 120 and 90 larvae/treatment for 42 vs. 196 and 112 vs. 280 ppm N trials, respectively) and allowed to feed for 24 h before five *C. marginiventris* females were released into the center of the cage. *C. marginiventris* females were prepared as follows: 1-day-old male and female wasps were placed together in a plastic cage and 2-day-old *S. exigua* larvae were provided for 24 h for oviposition experience of females; the 2-day-old male and female wasps were then transferred to a new cage without *S. exigua* larvae but supplied with a cotton ball soaked in 5% honey solution for 24 h before experimentation. Remaining *S. exigua* larvae were recovered 24 h after parasitoid release and placed in groups of five in 5-ml plastic cups filled with 3 ml of modified Pinto bean diet. Parasitoid offspring emergence was checked daily. Recovery rate was calculated as the total number of *S. exigua* larvae recovered divided by 120 (42 vs. 196 ppm N trial) or 90 (112 vs. 280 ppm N trial). Parasitism rate was calculated as the number of *C. marginiventris* offspring that emerged from hosts, regardless of cocoon construction, divided by the total number of *S. exigua* larvae recovered. Total mortality was calculated as the total number of dead *S. exigua* larvae divided by the total number of larvae recovered.

Chemical Analyses The high performance liquid chromatography (HPLC) procedure outlined by Stipanovic et al (1988) was used to analyze terpenoid concentrations. Samples (100 mg of freeze-dried ground plant material) were shaken for 30 min in a capped 125-ml Erlenmeyer flask with 15 ml of glass beads, 10 ml of 3:1 hexane:ethyl acetate (HEA), and 100 μl of 10% HCL. The solution was filtered over a glass-fritted filter funnel into a 50-ml pear-shaped flask, and the beads and residue were rinsed three times with 3 ml of HEA. The solvent was left to evaporate in a 90°C water bath, and the residue in the flask was redissolved with four 150-μl HEA washes. Each wash was transferred to a Maxi-clean silica cartridge (Alltech, Breda, The Netherlands). The silica cartridges were dried with compressed air, and terpenoid compounds were eluted with 5 ml of isopropyl alcohol, acetonitrile, water, and ethyl acetate (35:21:39:5). The eluent was filtered through a 45-μm nylon filter and transferred to a crimp top vial. Twenty microliters of each sample were analyzed by an HPLC system (DIONEX Corp., Sunnyvale, CA, USA), using a single wavelength UV absorbance detector (λ= 272 nm) and a 250-mm long, 4.6-mm (*id*) Alltima C-18 column (Alltech, Breda, The Netherlands). The column was eluted with ethanol/methanol/isopropyl/alcohol/acetonitrile/water/ethylacetate/dimethylformamide/phosphoric acid

(16.7:4.6:12.1:20.2:37.4:3.8:5.1:0.1) at a flow rate of 1.25 ml per min and kept at 55°C during analysis (Stipanovic et al. 1988). Standard of gossypol (G) was purchased from Sigma Aldrich (UK), and standards of hemigossypolone (HGQ), heliocides 1 and 4 (H_1 + H_4), and heliocides 2 (H_2) and 3 (H_3) were kindly provided by Dr. R.D. Stipanovic. These standards were used to assess retention times of the individual terpenoids. Terpenoids were calculated as microgram per gram dried plant material.

Vapor phase extraction was used to estimate acidic phytohormones and herbivore-induced VOCs simultaneously, with analyses following those of Schmelz et al. (2004). Briefly, the plant metabolites were extracted with 300 µl of H_2O/1-propanol/HCl (1:2:0.005) and 1 ml dichloromethane ($MeCl_2$). The $MeCl_2$/1-propanol layer into which plant metabolites were extracted was then transferred to a glass vial, and 2 µl of 2.0 M trimethylsilyldiazomethane solution were added to form methyl esters of carboxylic acid containing analytes. Residual trimethylsilyldiazomethane was neutralized with excess acetic acid. Plant volatiles were trapped on filters containing 30 mg Super Q (Alltech Associates, Inc., Deerfield, IL, USA) at 200°C and eluted with $MeCl_2$. Samples were later analyzed with chemical ionization-gas chromatography/mass spectrometry (CI-GC/MS) profiling method. The settings of the CI-GC/MS profiling method are described elsewhere (Engelberth et al. 2003; Schmelz et al. 2004). Estimates of total JA and salicylic acid (SA) represent combined pools of endogenous methyl esters and free acids. Plant volatiles analyzed were (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate, indole, α-pinene, β-pinene, myrcene, (*E*)-β-ocimene, β-caryophyllene, (*E*)-β-farnesene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-trideca-tetraene (TMTT), α-bergamotene, α-humulene, γ-bisabolene, bisabolol, and limonene. Unless otherwise noted, quantification was based on the slopes of external standard calibration curves derived from the peak areas of $[M + H]^+$ parent molecular ions generated for each compound. Authentic standards for α-bergamotene and bisabolol were not available; thus, tentative identifications based on electron ionization spectra were made by using a National Institute of Standards and Technology database. To estimate quantities, the slopes generated for β-caryophyllene and γ-bisabolene were applied to the tentative α-bergamotene and bisabolol peak areas, respectively. All reagents and solvents used in the experiments were acquired from Sigma-Aldrich (St Louis, MO, USA) or previously established research standards.

Statistical Analyses The experimental design for phytohormone and volatile production assessment was a 2 (leaf position—young and mature)×3 (nitrogen levels—42, 112, and 196 ppm N)×2 (herbivore infestation—0 and 20 *S. exigua* larvae) factorial, so the amounts of chemical compounds were analyzed by a three-way analysis of variance (ANOVA). The data were square root (x+3/8) transformed for heteroscedasticity before analysis. The experimental design and analysis for terpenoid aldehyde production was the same as for phytohormone and volatile production assessment, except that untransformed data were analyzed. Leaf damage inflicted by *S. exigua* was analyzed by one-way ANOVA with data untransformed. Recovery rate, parasitism, and total mortality of *S. exigua* larvae were arcsine (square-root) transformed before being subjected to one-way ANOVA. All ANOVAs were conducted with PROC GLM in SAS (SAS Institute 1999), and means were separated by Bonferroni's t tests if the null hypothesis was rejected.

Results

Increased N Fertilization Decreased Herbivore-Induced JA Accumulation and Volatile Pools Nitrogen fertilization did not significantly influence feeding damage inflicted by inducing *S. exigua* larvae (P=0.38). The average leaf areas consumed on damaged leaves receiving 42, 112, and 196 ppm N were 13.93±1.30, 15.62±0.87, and 15.64±0.50 cm^2, respectively.

Leaf position marginally affected JA concentration (P=0.057). As expected, all herbivore-damaged leaves (local leaves) had higher JA content than undamaged leaves in the same position (P<0.001); however, the magnitude of herbivore-induced JA accumulation sharply and significantly decreased with increasing N levels (Fig. 1 (1)). After 48 h of herbivory, herbivore-damaged local leaves from 42 ppm N plants displayed 11-fold increases in JA while those from 196 ppm N plants were increased by only 1.5-fold. JA levels in the systemic leaves did not exhibit the sharp positive response to herbivory observed in the local leaves, but like the local leaves, the mean JA concentrations of the systemic leaves were highest in plants receiving the least N (Fig. 1 (2)).

Unlike JA, SA was either not significantly different by leaf position or was significantly reduced in herbivore-damaged local leaves compared to controls (Fig. 1 (3)). Herbivory resulted in a significant decrease in SA levels in the 112 ppm N group, while the lowest average concentrations of constitutive SA in local leaves were observed in the highest fertilization treatment (196 ppm N) (Fig. 1 (3)). Increasing plant N levels tended to decrease SA concentrations in systemic leaves of damaged and undamaged plants, although statistically significant differences were observed only in the damaged plants (P<0.05 or 0.001; Fig. 1 (4)).

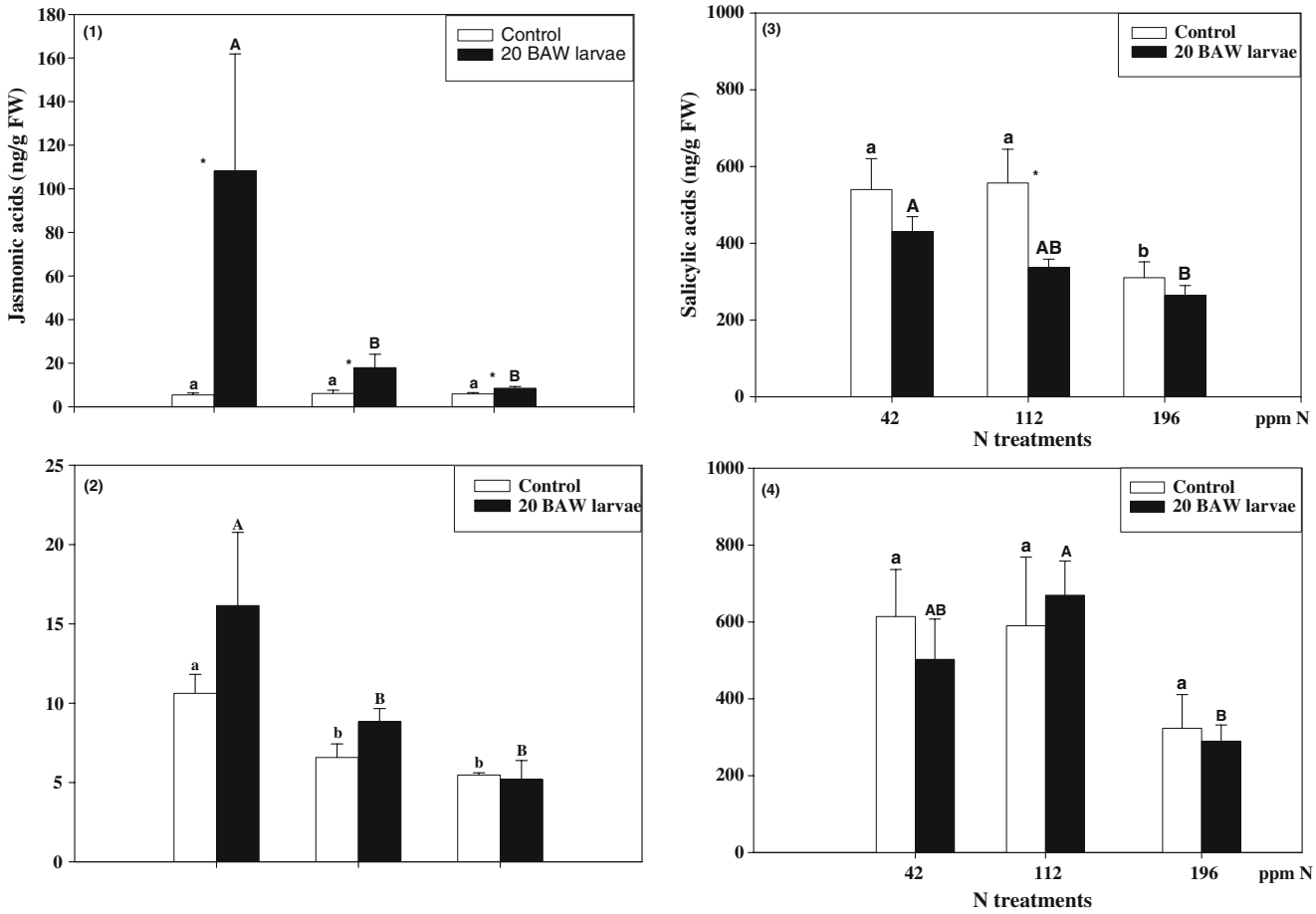

Fig. 1 Plant hormone production (mean ± SE ng g^{-1} fresh weight plant tissue) in response to nitrogen fertilization. *1* total jasmonic acid—local leaf (true leaf 3), *2* total JA—systemic leaf (true leaf 6), *3* total salicylic acid (SA)—local leaf, *4* total SA—systemic leaf. *Different lowercase letters above mean bars* denote significant difference between N treatments of control plants at α=0.05. *Different uppercase letters above mean bars* denote significant difference between N treatments of damaged plants at α=0.05; * Denotes significant difference between control and damaged plants of the same N treatment at α=0.05; damage induced by 20 3-day-old *S. exigua* (*BAW*) larvae

Effects of N on local production of volatiles are summarized in Table 1. Increasing N fertilization constrained leaf tissue concentrations of herbivore-induced volatiles. In plants grown under 42 ppm N, *S. exigua* herbivory significantly increased the local leaf tissue concentrations of seven volatile chemicals including (*Z*)-3-hexenal, (*E*)-2-hexenal, (*E*)-β-farnesene, DMNT, α-bergamotene, γ-bisabolene, and β-bisabolol (Table 1). As N fertilization increased to 112 ppm, only (*Z*)-3-hexenal and (*E*)-2-hexenal remained significantly different and increased in herbivore-attacked leaf tissues. At 196 ppm N, plants displayed an opposite pattern, with herbivory resulting in significantly lower levels of six predominant volatiles: β-caryophyllene, (*E*)-β-farnesene, α-bergamotene, α-humulene, γ-bisabolene, and β-bisabolol. DMNT, a widely occurring herbivore-induced plant volatile, exemplifies the above trend by exhibiting a significant 3.2-fold concentration increase in herbivore-damaged plants at the lowest N level, yet a steady decline in average fold induction under increasing N fertilization. Plant N addition increased constitutive levels of the lipoxygenase products (*Z*)-3-hexenal and (*E*)-2-hexenal (P<0.01 and P<0.001, respectively) in leaf extracts of undamaged control tissues (Table 1). Constitutive levels of (*Z*)-3-hexenyl acetate displayed the opposite trend, significantly decreasing in plants receiving 196 ppm N compared to those receiving 42 ppm N (P< 0.05). Aside from these lipoxygenase pathway products, N fertilization did not significantly influence the constitutive levels of volatiles in undamaged control leaves but instead demonstrated strong effects on the inducibility of volatiles in herbivore-attacked leaves.

Effects of N on systemic volatile production are summarized in Table 2. Increasing N fertilization decreased systemic production of six volatiles in undamaged mature leaves, while decreasing systemic production of nine volatiles in upper leaves of damaged plants (Table 2). Under 42 ppm N, herbivory in local leaves significantly increased systemic production of the volatiles (*Z*)-3-

Table 1 Plant volatile production (mean ± SE μg g^{-1} fresh mass) in response to various N levels—local leaf

Volatile compounds	42 ppm N		112 ppm N		196 ppm N	
	Control[b]	20 larvae[c]	Control[b]	20 larvae[c]	Control[b]	20 larvae[c]
(*Z*)-3-Hexenal	2.8±0.3b	17.0±2[d]	7.4±2.5b	19.5±2.7[e]	17.4±3.3a	19.6±4.3
(*E*)-2-Hexenal	2.6±0.2c	12.4±2.6[e]	10.6±2.3b	19.8±2.6[e]	22.0±3.6a	16.8±2.8
(*Z*)-3-Hexenyl acetate	0.3±0.06ab	0.4±0.1A	0.3±0.1a	0.1±0.02B	$0.1\pm6.2\times10^{-3}$b	0.1±0.03B
Indole	$1.2\times10^{-3}\pm 3.4\times10^{-4}$	0.01±0.01	$1.1\times10^{-3}\pm 7.7\times10^{-4}$	$1.6\times10^{-3}\pm 7.3\times10^{-4}$	$4.2\times10^{-4}\pm 1.3\times10^{-4}$	$1.4\times10^{-3}\pm 1.2\times10^{-3}$
α-Pinene	29.6±17.2	7.5±7.3	8.8±8.7	11.7±6.7	17.1±10.3	17.2±8.3
β-Pinene	10.7±5.3	6.8±2.4	4.0±2.2	4.5±1.9	6.2±2.7	6.2±2.4
Myrcene	15.7±8.4	19.2±6.8	8.4±2.7	7.7±2.7	13.9±5.8	13.2±3.1
(*E*)-β-Ocimene	3.3±1.7	5.0±1.4A	2.3±0.4	1.7±0.2B	3.5±0.7	2.2±0.5B
β-Caryophyllene	177.2±24.2	222.3±33.6A	127.4±14.9	109.0±11.1B	123.0±9.9	90.9±5.4B[e]
(*E*)-β-Farnesene	11.2±1.0	19.6±2.7A[e]	10.3±1.5	9.4±1.1B	11.9±0.8	8.8±0.2B[e]
DMNT	$4.7\times10^{-4}\pm 9.9\times10^{-3}$	0.2±0.02[f]	0.05±0.01	0.1±0.02	$0.04\pm8.3\times10^{-3}$	0.05±0.01
TMTT	$0.02\pm3.9\times10^{-3}$	$0.02\pm5.2\times10^{-3}$A	$0.01\pm3.0\times10^{-3}$	$9.6\times10^{-3}\pm 1.9\times10^{-3}$B	$0.01\pm1.3\times10^{-3}$	$9.6\times10^{-3}\pm 9.1\times10^{-4}$B
α-Bergamotene[a]	8.4±0.8	14.2±1.9A[e]	7.7±1.2	7.0±0.8B	8.8±0.6	6.7±0.2B[e]
α-Humulene	59.1±8.0	78.2±12.0A	43.9±5.0	37.7±4.1B	43.2±3.2	31.2±1.7B[e]
γ-Bisabolene	80.5±6.5	145.2±18.3A[e]	77.4±10.2	72.1±8.9B	88.4±6.8	65.2±2.3B[e]
Bisabolol[a]	153.4±14.2	267.5±40.4A[e]	142.6±21.2	130.4±15.0B	161.4±12.4	119.2±3.7B[e]
Limonene	13.0±6.4	7.4±2.6	5.0±2.5	5.8±2.3	7.7±3.2	7.3±2.6
Total	635.6±75.7	893.6±107.3A	521.3±35.0	501.3±36.1B	580.7±40.1	461.8±24.5B

DMNT (*E*)-4,8-dimethyl-1,3,7-nonatriene, *TMTT* (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-trideca-tetraene

[a] Tentative identification due to lack of authentic standard, identification based on comparison of mass spectra with spectra from the National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health database

[b] Means followed by different lowercase letters denote significant difference among N treatments of control plants at $\alpha=0.05$

[c] Means followed by different uppercase letters denote significant difference among N treatments of damaged plants at $\alpha=0.05$

[d] Significant difference between control and damaged plants within the same N treatment at $\alpha=0.001$

[e] Significant difference between control and damaged plants within the same N treatment at $\alpha=0.05$

[f] Significant difference between control and damaged plants within the same N treatment at $\alpha=0.01$

hexenal, (*Z*)-3-hexenyl acetate, myrcene, and bisabolol (all $P<0.05$). Under 112 ppm N, systemic production of the volatiles (*Z*)-3-hexenal, (E)-2-hexenal, and DMNT was increased by herbivory (all $P<0.05$). However, at 196 ppm systemic production of none of the volatiles examined was affected by herbivory.

Constitutive and Herbivore-Induced Terpenoid Aldehyde Production Decreased Under Increased N Fertilization The effects of N fertilization and herbivory on production of nonvolatile terpenoid aldehydes of the local mature leaf (leaf 3) and the systemic young leaf (leaf 6) are summarized in Table 3. N fertilization did not significantly affect feeding damage caused by beet armyworm larvae ($P=0.50$).

The main terpenoids in the leaves were HGQ and heliocides (H_1, H_2, H_3, and H_4). Leaf position significantly affected production of HGQ, $H_1 + H_4$, H_2, H_3, and total terpenoids, and as expected, young leaves had greater terpenoids than mature leaves (HGQ: $P<0.001$; $H_1 + H_4$: $P<0.05$; H_2: $P<0.001$; H_3: $P<0.001$; total: $P<0.001$). Increased N fertilization decreased production of HGQ, H_2, H_3, and total terpenoid aldehydes (HGQ: $P<0.001$; H_2: $P<0.001$; H_3: $P<0.001$; total: $P<0.001$). *S. exigua* infestation increased HGQ and marginally elevated total terpenoid levels when all N treatments were pooled (HGQ: $P<0.05$; total: $P=0.06$). The interactions between leaf position and N were significant for HGQ, H_3, and total terpenoids (HGQ: $P<0.001$; H_3: $P<0.01$; and total: $P<0.05$, respectively). The interactions between leaf position and *S. exigua* infestation were significant for HGQ and total terpenoids (HGQ: $P<0.01$; total: $P<0.05$). The interactions between N and herbivore infestation were significant for HGQ ($P<0.01$). No other two-way and three-way interactions were observed ($P>0.05$).

N addition to control plants reduced constitutive expression of HGQ, H2, H3, and total terpenoid aldehydes in local leaves (all $P<0.01$) but did not affect terpenoid aldehydes of systemic leaves to the same degree. In contrast, increased N fertilization significantly decreased production of HGQ, H_2, H_3, and total terpenoid aldehydes

Table 2 Plant volatile production (mean ± SE μg g^{-1} fresh mass) in response to various N levels—systemic leaf

Volatile compounds	42 ppm N		112 ppm N		196 ppm N	
	Control[b]	20 larvae[c]	Control[b]	20 larvae[c]	Control[b]	20 larvae[c]
(*Z*)-3-Hexenal	1.8±0.6b	4.6±0.6B[d]	5.2±1.1ab	13.7±2.5A[d]	11.5±3.2a	16.3±3.5A
(*E*)-2-Hexenal	4.2±1.6c	8.4±3.3B	15.1±2.3b	38.6±6.7A[d]	38.7±7.9a	35.5±2.5A
(*Z*)-3-Hexenyl acetate	0.2±0.05	1.0±0.3A[d]	0.1±0.01	0.3±0.09B	0.1±0.02	0.1±0.03B
Indole	$1.7\times10^{-4}\pm 1.1\times10^{-4}$	$1.5\times10^{-3}\pm 7.6\times10^{-4}$	$1.1\times10^{-4}\pm 4.8\times10^{-5}$	$1.9\times10^{-4}\pm 9.1\times10^{-5}$	$7.0\times10^{-4}\pm 1.3\times10^{-4}$	$3.4\times10^{-4}\pm 1.8\times10^{-4}$
α-Pinene	61.6±35.9	23.9±23.5	19.6±19.4	52.4±30.2	35.1±20.9	45.5±18.2
β-Pinene	19.5±11.0	7.5±6.1	5.8±5.4	14.4±8.1	10.9±6.5	12.7±4.8
Myrcene	297.7±65.5a	549.5±75.7A[d]	162.8±20.7b	173.8±18.3B	186.9±23.8ab	177.8±32.7B
(*E*)-β-Ocimene	148.5±69.8	404.4±113.8A	60.2±22.0	64.4±8.0B	64.3±22.2	46.5±14.4B
β-Caryophyllene	447.8±105.6a	661.4±115.0A	231.9±31.1b	228.6±6.5B	239.4±8.8	194.7±24.4B
(*E*)-β-Farnesene	32.5±5.1a	64.3±12.6A	20.0±1.0b	20.4±1.2B	22.4±2.6ab	18.1±0.6B
DMNT	$0.02\pm1.1\times10^{-3}$a	0.1±0.07A	$0.01\pm5.0\times10^{-3}$b	$0.03\pm5.1\times10^{-3}$AB[d]	$0.01\pm9.8\times10^{-4}$ab	$0.01\pm9.0\times10^{-4}$B
TMTT	$0.06\pm9.5\times10^{-3}$a	0.1±0.03A	$0.02\pm5.4\times10^{-3}$b	$0.03\pm4.1\times10^{-3}$B	$0.03\pm1.4\times10^{-3}$b	$0.02\pm5.4\times10^{-3}$B
α-Bergamotene[a]	30.6±15.7	11.8±5.0A	12.9±0.5	13.0±0.7B	14.0±1.6	11.4±0.4B
α-Humulene	158.5±39.2a	246.8±39.1A	80.8±10.5b	79.5±2.3B	83.8±4.0b	66.8±7.6B
γ-Bisabolene	226.3±40.2a	400.0±77.6A	145.3±6.2b	147.0±9.7B	152.5±19.1ab	126.0±5.4B
Bisabolol[a]	386.2±56.1a	770.3±153.0[d]	238.5±12.1b	246.9±16.7	272.9±32.0ab	170.2±57.1
Limonene	31.5±14.1	26.1±3.8A	11.6±4.9	18.2±7.2B	16.4±6.2	16.8±4.4B
Total	1,925.0±421.4	3,268.8±559.6A	1,113.1±96.0	1,198.4±72.0B	1,234.4±105.6	1,016.4±138.0B

DMNT (*E*)-4,8-dimethyl-1,3,7-nonatriene, *TMTT* (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-trideca-tetraene

[a] Tentative identification due to lack of authentic standard, identification based on comparison of mass spectra with spectra from the National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health database

[b] Means followed by different lowercase letters denote significant difference among N treatments of control plants at $\alpha=0.05$

[c] Means followed by different uppercase letters denote significant difference among N treatments of damaged plants at $\alpha=0.05$

[d] Significant difference between control and damaged plants within the same N treatment at $\alpha=0.05$

in systemic leaves of damaged plants (HGQ: $P<0.001$; H_2: $P<0.01$; H_3: $P<0.001$; total: $P<0.0001$). Under low N fertilization (42 ppm) HGQ, the predominant leaf terpenoid aldehyde displayed a 2.1-fold increase in the systemic leaves of herbivore-damaged plants (Table 3). With the exception of HGQ under 112 ppm N where herbivory diminished the concentration, no significant herbivore-induced terpenoid aldehyde production was detected at N fertilization levels above 42 ppm.

C. marginiventris Short-Range Foraging Not Affected by Plant N Fertilization N treatment did not significantly affect the recovery rate, parasitism rate, or total mortality of sentinel *S. exigua* larvae in the caged preference studies (Table 4).

Discussion

In this commercial variety of cotton, increased N fertilization dramatically impaired the herbivore-induced accumulation of plant defense markers, including JA, volatile pools, and also terpenoid aldehydes in systemic leaves. Consistent with the established role of JA as a positive regulator of herbivore-induced defenses (Reinbothe et al. 1994; Schmelz et al. 2003a; Browse and Howe 2008), low N (42 ppm) plants that exhibited the highest induced JA levels also exhibited robust increases in leaf tissue concentrations of volatiles in local and systemic leaves and terpenoid aldehydes in systemic leaves. At N fertilization levels above 42 ppm, the induction of most biochemical defense markers examined greatly declined or disappeared altogether. However, despite the large differences in herbivore-induced leaf-tissue volatile concentrations among the N fertilization regimes, parasitism of *S. exigua* larvae by adult female *C. marginiventris* did not differ among N fertilization regimes.

In this study, we considered JA, terpenoid aldehydes, and concentrations of leaf volatiles as markers for herbivore-induced defenses. Significant induced biochemical responses were demonstrable only at low N fertilization levels. In the local herbivore-damaged leaves

Table 3 Cotton leaf terpenoid aldehyde (mean ± SE μg g^{-1} dry mass) production in relation to N fertilization

	42 ppm N		112 ppm N		196 ppm N	
	Control	20 larvae[c]	Control	20 larvae[c]	Control	20 larvae[c]
Local leaf[a]						
Damage (cm^2)	0	14.5±2.1	0	15.9±0.5	0	14.9±2.1
HGQ	1,334.3±229.2a	1,206.0±293.2	1,110.8±84.8a[d]	736.0±110.3	574.3±13.8b	623.8±31.0
G	27.8±27.8	0	0	19.0±19.0	0	0
H1 + H4	467.5±29.2	428.75±101.5	578.0±141.4	780.5±368.2	291.0±36.9	297.0±17.3
H3	322.3±30.5a	249.3±29.3	298.5±13.6a	314.0±44.9	205.5±14.2b	208.8±21.5
H2	1,025.5±80.4a	740.3±95.8	808.0±50.4b	828.5±138.8	551.5±20.8c	569.8±71.3
Total	3,177.3±260.5a	2,624.3±495.0	2,795.3±226.5a	2,678.0±660.5	1,622.3±49.3b	1,699.3±62.5
Systemic leaf[b]						
HGQ	2,309.8±516.6	4,806.3±265.7A[d]	1,787.3±225.8	1,577.3±127.8B	1,406.5±237.1	1,529.3±515.8B
G	41.8±41.7	149.5±88.4	0	30.0±30.0	30.5±30.5	0
H1 + H4	484.0±76.8	1,041.0±246.5	699.8±160.2	864.5±323.2	403.5±51.4	845.8±563.5
H3	434.8±48.6	624.3±90.7A	391.3±41.5	379.3±12.1B	290.3±22.8	290.8±20.3B
H2	1,007.0±120.8	1,364.8±274.1A	1,013.5±122.6	1,013.5±47.2AB	781.3±65.9	754.8±79.6B
Total	4,277.3±584.3	7,985.8±647.3A[d]	3,891.8±479.1	3,864.5±496.3B	2,912.0±304.3	3,420.5±1,148B

Means followed by different lowercase letters denote significant difference among N treatments of control plants at α=0.05. Means followed by different uppercase letters denote significant difference among N treatments of damaged plants at α=0.05

HGQ hemigossypolone, *G* gossypol, H_{14} heliocides 1–4

[a] True leaf 3

[b] True leaf 6

[c] Number of 3-day-old *S. exigua* larvae used to induce plant resistance in mature leaf (true leaf 3)

[d] Significant difference between control and herbivore-damaged leaves at α=0.05.

of 42 ppm N plants, concentrations of volatiles in induced tissue undoubtedly relate to actual volatile emission at the larval feeding sites. In corn (*Z. mays*) leaves, herbivore-induced volatiles are readily detected in leaf tissue extracts (Schmelz et al. 2003c). In contrast, under higher fertilization levels (196 ppm N), we detected significant decreases in the volatile concentrations of herbivore-attacked leaves. This result makes projections of volatile emission more complex. Actual emission of constitutive volatiles at the feeding sites still would likely be substantial. However, if low levels of herbivore-induced JA fail to stimulate new volatile synthesis, low volatile concentrations in damaged leaf tissue may be the result of increased emission and eventual depletion of leaf volatile pools. In support of this hypothesis, Loughrin et al. (1994) found decreased emission of many constitutive and herbivore-inducible volatiles after the third day of continuous *S. exigua* feeding on cotton plants. One anomaly is the presence of significant amounts of (*Z*)-3-hexenal and (*E*)-2-hexenal in undamaged control tissue. Production of these C6 volatiles from C18 fatty acids proceeds through the sequential activity of C13 lipoxygenase and hydroperoxide lyase enzymes (Matsui 2006). Artificially increased levels of C6 volatiles in control tissue may have occurred during leaf harvesting or initial sample extraction. Despite this potentially elevated background, clear differences in (*Z*)-3-hexenal and (*Z*)-2-hexenal at both 42 and 112 ppm are indicative of herbivore-induced enzyme activity.

Table 4 Influence of host plant N fertilization on survival (mean ± SE %) of *S. exigua* and parasitism (mean ± SE %) by *C. marginiventris* in outdoor-cage choice tests

Fate of *S. exigua* larvae	42 vs. 196 ppm N		112 vs. 280 ppm N	
	42 ppm	196 ppm	112 ppm	280 ppm
Recovery rate[a]	62.00±5.20	70.50±6.56	72.50±3.94	77.83±1.98
Parasitism rate[b]	17.39±6.27	13.21±4.68	26.71±1.69	23.35±2.95
Total mortality[c]	23.30±7.62	21.46±7.47	38.75±1.77	40.16±5.63

[a] 42 vs. 196 ppm N: P=0.32; 112 vs. 280 ppm N: P=0.28

[b] 42 vs. 196 ppm N: P=0.72; 112 vs. 280 ppm N: P=0.34

[c] 42 vs. 196 ppm N: P=0.88; 112 vs. 280 ppm N: P=0.83

S. exigua herbivory significantly increased JA production in local leaves. While increased N fertilization had no significant effects on the constitutive JA levels in local leaves, it was inversely related to the induced JA in herbivore-damaged local leaves. As with many insect-inducible defenses, applications of JA on cotton similarly promoted terpenoid aldehyde accumulation (Opitz et al. 2008). What has not been previously explored is the relationship between insect-induced endogenous JA levels and the systemic induction of terpenoid aldehydes. While precise N availability was not specifically examined in

previous cotton studies, a strong reduction in herbivore-induced JA and systemic terpenoid aldehyde accumulation at higher N fertilization levels was unexpected. However, this N-mediated reduction in herbivore-induced JA, terpenoid aldehydes, and volatiles in cotton is surprisingly consistent with short-term responses described in corn following application of the *S. exigua* elicitor, volicitin (Schmelz et al. 2003a). In hydroponically grown corn seedlings, volicitin-induced levels of JA, and sesquiterpene volatiles dramatically increase as N availability decreases. In contrast, low N availability in tobacco (*Nicotiana attenuata*) suppresses the levels of JA and SA induced by insect oral secretions and likewise results in lower induced accumulation of nonvolatile defenses (Lou and Baldwin 2004). SA is a plant hormone widely considered to be induced in response to pathogen attack or pathogen-like damage caused by phloem-feeding insects, such as whiteflies and aphids (Walling 2000). N addition to cotton plants significantly decreased SA content of both control and damaged local leaves. However, in contrast to JA, insect herbivory generally reduced SA levels. This result is consistent with antagonistic interactions of JA and SA observed in tomato (Pena-Cortés et al. 1993) and tobacco plants (Niki et al. 1998) with respect to wound- and pathogen-induced defenses.

Herbivory by *S. exigua* larvae had no significant effects on the induction of terpenoid aldehydes under high (112 and 196 ppm) N conditions in this study but significantly increased induced terpenoid aldehydes of young leaves under low (42 ppm) N conditions. The lack of induction of terpenoid aldehydes by insect feeding in mature cotton leaves has been shown in another cotton variety (cv. Deltapine 90; McAuslane et al. 1997); however, the lack of systemic terpenoid aldehyde production under high N conditions contrasts with previous research (Alborn et al. 1996; McAuslane et al. 1997; McAuslane and Alborn 1998; Bezemer et al. 2003). We do not yet know if this is a specific response to N fertilization in *G. hirsutum* cv. FiberMax 989 or a more generalized pattern common to cotton cultivars. Many plants, including corn, tobacco, and cotton, exhibit significant cultivar or genetic variation in the production of both direct and indirect herbivore-induced defenses (Loughrin et al. 1995; Gouinguené and Turlings 2002; Wu et al. 2008).

Foliar terpenoid aldehydes in cotton function as dietary toxins for numerous lepidopteran insects and are typically dominated by HGQ, while heliocides (H_1, H_2, H_3, H_4) also occur at significant levels (Elliger et al. 1978; Stipanovic et al. 1988; Bezemer et al. 2003). N fertilization significantly reduced the constitutive (local mature leaves) and induced (systemic young leaves) accumulation of HGQ and most heliocides in this study. Plants with higher N may be more capable of compensating for biomass loss and therefore need not invest as much in defense as is the case for lower N plants. As shown by Chen et al. (2008), both *S. exigua* larvae and adult females preferred cotton leaves receiving high N fertilization for feeding and oviposition, respectively. Higher N content or weaker constitutive and inducible defenses of plants grown with high N fertilization or a combination of the two may contribute to this preference.

Young expanding leaves of plants with no previous herbivore damage had higher terpenoid aldehyde contents than mature leaves, which is consistent with predictions of the optimal defense (OD) theory (McKey 1974). The significance of defending the young leaves is underscored by our observation that terpenoid aldehyde titers in young leaves of control plants were not significantly affected by changing N levels, whereas increasing N led to significantly reduced terpenoid aldehyde content of mature leaves. OD theory predicts that plant parts having higher fitness value should be better defended. The greater fitness value of young expanding leaves over older leaves has been experimentally demonstrated in some plants (McKey 1979; Krischik and Denno 1983), as has the elevated accumulation of chemical defenses in these leaves (Ohnmeiss and Baldwin 2000; Bezemer et al. 2004). The general applicability of our results must be tempered by the extensive domestication of the cotton variety used in the present study. Nevertheless, the patterns observed tend to conform to adaptive theory and suggest that defensive responses may be intensified under nutrient deficiency.

For herbivore-induced indirect defenses, large differences in leaf-tissue volatile concentrations were found among the N fertilization regimes; however, parasitism of *S. exigua* larvae by adult female *C. marginiventris* was not different in caged choice tests with neonate larvae feeding on host plants grown under 42 and 196 ppm N. *C. marginiventris* females are known to exploit insect-induced volatiles for long-range attraction to hosts (Turlings et al. 1991a; Loughrin et al. 1995; Hoballah et al. 2002; Gouinguené et al. 2005), and both qualitative and quantitative differences in VOC blend are suggested to affect this attraction (Pickett 1999; Hoballah et al. 2002). At first glance, the lack of dose-dependent responses of the parasitoid to overall VOC levels is surprising, since attractiveness has been shown to increase at higher VOC release rates in some systems (Turlings et al. 1991b; Weissbecker et al. 1999; Hoballah et al. 2002). Unlike the commonly studied corn model, cotton foliage contains large preexisting pools of volatiles which are released immediately upon feeding damage (Elzen et al. 1985; Loughrin et al. 1994). In a short-range attraction assay, rankings for the total number of flights completed by female *C. marginiventris* suggested wasps preferred cotton plants systemically releasing herbivore-induced volatiles, followed by artificially damaged cotton plants, and lastly undamaged

control plants (Röse et al. 1998). While these results indicate that *C. marginiventris* has a strong attraction to herbivore-induced cotton volatiles, it also demonstrates an innate attraction to the volatiles from wounded cotton plants.

The lack of N effects on observed parasitism rates may be a result of the experimental arena. As parasitoids were exposed to plants in a closed field cage, they were unable to leave the plant patch. Under open-field conditions, they might have been more likely to ignore or abandon patches where plants release lower levels of VOCs. Parasitoid success is hierarchically divided into host habitat location, host location, host acceptance, and host suitability (Nordlund et al. 1981). In cages, the cues involved in host habitat location might be obscured, mixed, or even concentrated, thereby possibly rendering the results of limited ecological significance. Once parasitoids locate the plant–herbivore complex, additional cues including those associated with host frass can also be significant (Eller et al. 1988). Thus, the degree of difference in host searching cues between the treatments may have been insufficient to significantly modify parasitoid behaviors over the 24-h assay period. In the field, qualitative and quantitative differences of individual VOCs in the herbivore-induced blend are believed to significantly affect host searching behavior of parasitoids (De Moraes et al. 1998). The apparent disparity of VOC levels and parasitoid host finding also might be attributable to limitations of chemical analytical methods. VOCs of leaf extracts analyzed in the study may not necessarily reflect the parasitoid-orienting VOCs released into the air. Nevertheless, it is possible that the relatively large preexisting pools of plant volatiles (known to be emitted during larval herbivory) across all N fertilization treatments overshadow weak vs. strong herbivore-induced biochemical defenses and impair the ability of *C. marginiventris* to discriminate, at short range, host *S. exigua* larvae on these physiologically different host plants.

In summary, N fertilization rates and herbivory exerted variable effects on cotton plant chemistry, with low N input (42 ppm N) and herbivore damage inducing significant increases in JA, volatiles, and in systemic accumulation of terpenoid aldehydes relative to higher N rates. However, increased N fertilization of cotton plants suppressed *S. exigua*-induced plant hormones and led to reduced production of various terpenoid aldehydes in undamaged mature leaves and systemic young leaves above mature leaves that had been fed on by *S. exigua* larvae. However, although increased N fertilization significantly diminished herbivore-induced leaf volatile concentrations, parasitism of *S. exigua* larvae by *C. marginiventris* in field cages did not differ among N treatments. This suggests that parasitoids were unable to differentiate in the field cages among the varying VOC titers induced by different N treatments.

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