

Herbivore-induced Sorghum Volatiles Mediate Attraction of *Cotesia Flavipes*: Roles of Dmnt and (Z)-3-hexenyl Acetate in Tritrophic Chemical Communication

cyprian osinde

cyprian.osinde@mak.ac.ug

Muni University

Clement Nyakoojo

Mountains of the Moon University, Fort Portal, Uganda

Anthony M. Nsubuga

Makerere University

Arthur K. Tugume

Makerere University

Jamilu E. Ssenku

Makerere University

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Abstract

Herbivore-induced plant volatiles (HIPVs) play a critical role in Tritrophic chemical communication and attracting natural enemies of herbivorous insects. In sorghum (*Sorghum bicolor* L. Moench), the contribution of specific HIPVs to parasitoid recruitment remains poorly understood despite evidence of herbivore-induced volatile production. This study investigated the role of key sorghum HIPVs in mediating the behavioral responses of the larval parasitoid *Cotesia flavipes* to simulated stem borer attack. Four-week-old sorghum plants (cv. Epuripur) were subjected to mechanical wounding followed by application of *Chilo partellus* larval oral secretions to induce defense responses. Headspace volatiles were collected using dynamic headspace sampling and analyzed by gas chromatography–mass spectrometry (GC–MS). Behavioral responses of female *C. flavipes* were evaluated using Y-tube olfactometer bioassays comparing synthesized (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (Z)-3-hexenyl acetate, their blend from herbivory induced sorghum plants, and health sorghum volatiles against appropriate controls. Herbivore induction significantly increased emissions of DMNT (3.2-fold) and (Z)-3-hexenyl acetate (3.1-fold), together with other defense-related volatiles. Female parasitoids showed significant attraction to DMNT (70%; $\chi^2 = 6.40$, $P = 0.011$) and (Z)-3-hexenyl acetate (75%; $\chi^2 = 10.00$, $P = 0.002$), while the combined blend elicited the strongest response (83%; $\chi^2 = 18.67$, $P < 0.001$). Parasitoids also preferred herbivore-induced sorghum volatiles over those from healthy plants ($\chi^2 = 9.80$, $P = 0.002$). Logistic regression demonstrated significant positive effects of both compounds and a synergistic interaction between them ($P = 0.038$). These findings demonstrate that parasitoid attraction is driven primarily by the identity and combination of specific HIPVs rather than by total volatile emission levels. DMNT and (Z)-3-hexenyl acetate are therefore promising semiochemicals candidates for enhancing *C. flavipes*-mediated biological control of stem borers in sorghum and may contribute to sustainable integrated pest management strategies.

INTRODUCTION

Sorghum is a drought-adapted cereal crop of major importance in semi-arid regions, where it supports food security and livestock production. In eastern Africa, its ability to withstand drought and growth in low-fertility soils makes it a vital crop for smallholder farming systems. However, production is constrained by lepidopteran stem borers, particularly *Busseola fusca*, *Chilo partellus*, and *Sesamia calamistis*, which cause significant yield losses through stem tunneling, dead-heart formation, plant lodging, and reduced grain filling (Kfir et al., 2002; Bonhof et al., 1997; van den Berg et al., 1991; Calatayud et al., 2014). Although sorghum is inherently resilient to abiotic stresses, damage from these insect pests remains a major limitation to productivity and grain yield (Taylor & Duodu, 2019; FAO, 2023; Hamaker et al., 2025). Management of cereal stem borers has traditionally depended on insecticide applications; however, chemical control is frequently ineffective because larval feeding occurs inside plant stems, where insects are shielded from direct exposure to insecticides. As a result, sustainable pest management approaches increasingly emphasize host plant resistance and conservation biological control as essential components of integrated pest management (IPM) programmes (Kfir et al., 2002; Khan et al., 2000; Calatayud et al., 2014; Pretty & Bharucha, 2015).

Plants possess highly coordinated defense mechanisms that allow them to detect herbivore attack and activate complex biochemical signaling networks. In sorghum, recognition of herbivore attack activates interconnected signaling pathways involving calcium-dependent responses, oxidative signaling, and phytohormone regulation. These processes ultimately influence the production of defensive metabolites and volatile compounds that modify interactions between plants, herbivores, and natural enemies (Mostafa et al., 2022; Erb & Reymond, 2019; War et al., 2012; Arimura & Maffei, 2010; Maffei et al., 2007). These signaling pathways regulate the production of defensive proteins, toxic secondary metabolites, and volatile organic compounds that reduce herbivore performance while facilitating interactions with organisms at higher trophic levels. Among these defense responses, herbivore-induced plant volatiles (HIPVs) represent one of the most ecologically significant indirect defense mechanisms. Herbivore-induced volatile emissions comprise multiple chemical classes, including terpenoids, green leaf volatiles, aromatic compounds, and nitrogen-containing metabolites. Their ecological importance arises from their ability to transmit information within plant communities and recruit organisms involved in herbivore suppression. (Arimura et al., 2009; Dicke & Baldwin, 2010; Turlings & Erb, 2018). Rather than acting primarily through direct toxicity against herbivores, HIPVs function as indirect defense signals by attracting predators and parasitoids that suppress herbivore populations, reduce plant damage, and improve plant fitness (Dicke & Baldwin, 2010; Arimura et al., 2009; Turlings & Erb, 2018; Aljibory & Chen, 2018).

Parasitoids use herbivore-induced odor changes as indirect indicators of suitable host habitats. By detecting specific volatile signatures associated with herbivore activity, parasitoids can increase the efficiency of host-searching behavior. The structure and composition of induced volatile blends vary according to plant species and herbivore identity, enabling parasitoids to identify suitable hosts and optimize their searching behavior (Arimura et al., 2009; Fatouros et al., 2005; Turlings & Erb, 2018). Therefore, HIPVs serve as critical mediators of tritrophic interactions involving plants, herbivores, and natural enemies.

Among the diverse HIPVs reported in sorghum and other cereal crops, the homoterpene 4,8-dimethyl-1,3,7-nonatriene (DMNT) and the green leaf volatile (Z)-3-hexenyl acetate have received considerable attention as important semiochemicals. DMNT is produced through herbivore-induced terpenoid biosynthetic pathways following insect attack and has been shown to influence parasitoid and predator behavior in maize, cotton, rice, and other agricultural systems (Turlings & Erb, 2018). Similarly, (Z)-3-hexenyl acetate is rapidly synthesized through the lipoxygenase pathway following tissue damage and acts as an early chemical signal associated with herbivore attack (D'Auria et al., 2007; Engelberth et al., 2004). Several parasitoid species, including *Cotesia marginiventris*, *Microplitis croceipes*, and *Cotesia flavipes*, exploit HIPVs and related chemical cues during host-searching behavior (Turlings et al., 1990; Turlings et al., 1991; Ngi-Song et al., 1996). Previous studies indicate that parasitoids often respond more strongly to the identity and relative composition of volatile blends than to the overall quantity of emitted volatiles, as specific HIPV combinations provide reliable information about host presence and quality (Vet & Dicke, 1992; De Moraes et al., 1998; Turlings et al., 1993). This suggests that even low concentrations of highly informative HIPVs may effectively guide parasitoids toward host-infested

plants. Such chemical specificity allows natural enemies to distinguish suitable host habitats from complex background odors, making the identification of key parasitoid-attracting compounds an important research objective.

Our recent research has improved understanding of herbivore-induced volatile production in C3 and C4 plants by comparing volatile emissions from sorghum and rice following artificial herbivory (Osinde et al., 2023). This study demonstrated that both species produced inducible volatile blends containing terpenoids and green leaf volatiles, although sorghum generally emitted lower total quantities of volatiles than rice. Despite these differences, compounds such as DMNT and (Z)-3-hexenyl acetate were consistently induced after simulated herbivore damage, suggesting that these compounds may contribute disproportionately to plant defense signaling.

However, our previous study primarily focused on volatile characterization rather than insect behavioral responses; therefore, the ecological importance of these emissions in attracting parasitoids remained unresolved. The present study addresses this knowledge gap by examining whether parasitoids respond mainly to specific volatile compounds rather than total volatile emission levels, thereby improving understanding of their potential applications in sustainable pest management. If only a limited number of HIPVs are responsible for parasitoid attraction, these compounds could be developed as synthetic semiochemicals to enhance biological control in cereal production systems. Such strategies would contribute to global efforts aimed at developing environmentally sustainable crop protection technologies.

The present study evaluates parasitoid responses using Y-tube olfactometer assays to examine the behavioral responses of female *Cotesia flavipes* to DMNT, (Z)-3-hexenyl acetate, and herbivore-induced sorghum volatiles. We hypothesized that parasitoids would exhibit strong attraction to these key HIPVs despite the relatively low overall volatile emissions observed in sorghum. By integrating volatile chemical analysis with parasitoid behavioral responses, this study provides insights into indirect plant defense mechanisms and establishes a foundation for future investigations of HIPV-mediated biological control in sorghum-based integrated pest management systems.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Sorghum plants (*Sorghum bicolor* L. Moench) cultivar Epuripur were used as the experimental plants based on our previous studies demonstrating herbivore-induced volatile organic compounds (HIPVs) associated with induced plant defense responses (Osinde et al., 2023). Seeds of the Epuripur cultivar were germinated under controlled greenhouse conditions and subsequently transplanted into plastic pots filled with sterilized loam soil. The plants were maintained in a greenhouse under a 12 h light/12 h dark photoperiod at a temperature of $26 \pm 3^{\circ}\text{C}$ and a relative humidity of 70–75%.

Plants were watered regularly to ensure optimal growth conditions. Four-weeks old plants exhibiting uniform height, biomass, and leaf number were selected for volatile induction treatments. The selected plants were randomly allocated to experimental groups comprising undamaged control plants and herbivore-induced plants

Collection and Rearing of *Chilo partellus* for Oral Secretion Collection

Larvae of *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae), the spotted stem borer, were collected from naturally infested sorghum fields. Sampling was performed by identifying plants exhibiting typical stem borer infestation symptoms, including foliar feeding damage, dead-heart symptoms, and the presence of larval frass around stem entry points. Infested sorghum stems were carefully dissected, and larvae were removed using sterile forceps. Individual larvae were transferred into ventilated plastic containers containing fresh sorghum leaves to reduce handling stress and maintain larval survival during transportation to the laboratory. Similar field collection and handling approaches have been used for maintaining *C. partellus* populations for laboratory-based studies (Overholt et al., 1994; Kfir et al., 2002).

Upon arrival in the laboratory, larvae were examined for developmental stage, physical condition, and possible infestation by pathogens or parasitoids. Individuals showing signs of injury, disease, pupation, or parasitism were excluded from further experiments. Healthy larvae were maintained individually under controlled laboratory conditions and reared on fresh sorghum leaves until reaching the required developmental stage for oral secretion collection. Sorghum leaves were replaced regularly to ensure continuous food availability and minimize microbial contamination. Individual rearing was maintained throughout the larval period to prevent cannibalism and ensure that oral secretion samples represented independent biological replicates, following established insect rearing practices (Songa et al., 2001).

For oral secretion collection, actively feeding larvae were selected and gently stimulated to induce regurgitation. The secreted oral fluids were collected using sterile collection instruments and transferred into sterile microcentrifuge tubes. Samples were immediately maintained under cold conditions of – 20°C after collection to preserve the stability of secreted compounds and storage was done until ready for use in herbivore-induced plant response experiments. Oral secretions were collected to provide herbivore-associated elicitors for induction of sorghum defense responses (Alborn et al., 1997; Schmelz et al., 2009).

Herbivore-Induced Plant Treatment

To simulate natural chewing herbivore attack, sorghum plants were subjected to mechanical damage followed by the application of oral secretions from *Chilo partellus* larvae. This approach was adopted because insect oral secretions contain herbivore-associated molecular patterns (HAMPs) and elicitors that activate plant defense signaling pathways more effectively than mechanical wounding alone, resulting in defense responses that closely resemble those induced during natural stem borer herbivory

(Alborn et al., 1997; Musser et al., 2002; Schmelz et al., 2009; Mithöfer and Boland, 2008; Erb et al., 2012; Erb and Reymond, 2019).

Leaves were mechanically wounded using a fabric pattern wheel along the leaf midrib to simulate chewing damage. Immediately after wounding, *C. partellus* larval oral secretion extract was applied directly to the damaged tissue to mimic insect feeding and trigger herbivore-induced defense responses. Control plants remained untreated and did not receive either mechanical damage or oral secretion application.

Following treatment, plants were maintained under greenhouse conditions for 24hrs before volatile collection. This induction period was selected based on previous studies demonstrating that herbivore-induced volatile organic compounds (HIPVs), including green leaf volatiles, terpenoids, benzenoids, and other defense-related compounds, accumulate rapidly within the first 24hrs after herbivore attack and play important roles in direct and indirect plant defense by mediating interactions with herbivores and their natural enemies (Turlings et al., 1990; Paré and Tumlinson, 1999; Arimura et al., 2009; Dicke and Baldwin, 2010; Heil, 2014; Ameye et al., 2018)

Collection and Analysis of Sorghum Volatile Organic Compounds

Headspace Volatile Collection

Volatile organic compounds (VOCs) were collected from individual sorghum treated and control plants using the dynamic headspace sampling technique, a widely adopted method for capturing herbivore-induced plant volatiles under controlled conditions (Tholl et al., 2006; Turlings et al., 1998; D'Alessandro and Turlings, 2006). The aerial portions of each plant were enclosed in a volatile collection chamber constructed with inert plastic non-reactive material to minimize contamination and prevent adsorption or chemical modification of emitted volatiles (Agelopoulos and Pickett, 1998; Tholl et al., 2006). Purified, charcoal-filtered air was continuously passed through the chamber using a vacuum pump, and plant-emitted volatile compounds were trapped on adsorbent filters packed with Super Q polymer, which has been extensively used for the efficient collection of a broad spectrum of plant volatile organic compounds (Heath and Manukian, 1994; Turlings et al., 1998; D'Alessandro and Turlings, 2006).

Volatile collections were conducted for 4hrs during the light period, when herbivore-induced volatile emission is generally greatest because the biosynthesis and release of many green leaf volatiles and terpenoids are regulated by light and circadian rhythms (Loughrin et al., 1994; Gouinguéné and Turlings, 2002; Arimura et al., 2009; Dicke and Baldwin, 2010). Following collection, the adsorbed volatile compounds were eluted from the Super Q filters using high-purity organic solvent and stored at – 20°C until chemical analysis to preserve sample integrity and minimize degradation before gas chromatographic- mass spectrometer analysis (Heath and Manukian, 1994; Tholl et al., 2006).

Gas Chromatography–Mass Spectrometry Analysis

Volatile organic compounds collected from HIPVs and control plants were identified and quantified using gas chromatography coupled with mass spectrometry (GC–MS), a standard analytical technique for the separation, identification, and quantification of plant volatile metabolites (Tholl et al., 2006; Qualley et al., 2012; Dudareva et al., 2013). Compound identification was achieved by comparing the obtained mass spectra with those contained in commercial spectral libraries, including the National Institute of Standards and Technology (NIST) library, and by comparison with authentic reference standards where available. Retention indices were also used to improve compound identification and increase analytical confidence (Tholl et al., 2006).

Particular attention was given to herbivore-induced plant volatile organic compounds (HIPVs) that have been consistently associated with the recruitment of parasitoids and other natural enemies of herbivorous insects. These included (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (*Z*)-3-hexenyl acetate, other green leaf volatiles (GLVs), and terpenoid compounds, all of which are well documented as important chemical signals involved in indirect plant defense by mediating tritrophic interactions among plants, herbivores, and parasitoids (Turlings et al., 1990; Paré and Tumlinson, 1999; Kessler and Baldwin, 2001; D'Alessandro and Turlings, 2006; Arimura et al., 2009; Dicke and Baldwin, 2010; Heil, 2014; Erb and Reymond, 2019) and from our previous findings (Osinde et al., 2023).

Relative emission rates of individual volatile compounds were calculated using internal standards and were expressed as nanograms per gram of fresh weight per hour ($\text{ng g}^{-1} \text{FW h}^{-1}$), a standard approach for normalizing volatile emissions among plant samples and facilitating quantitative comparisons across treatments (Tholl et al., 2006; Qualley et al., 2012).

Comparison of individual volatiles in GC-MS was also based on our previous findings on Epuripur sorghum (Osinde et al. 2023), though Epuripur sorghum plants emitted relatively low quantities of individual herbivore-induced plant volatiles. This approach is consistent with evidence demonstrating that parasitoid attraction is often determined by the qualitative and quantitative composition of the entire volatile blend rather than by the abundance of individual compounds alone (Turlings et al., 1990; Bruce et al., 2005; D'Alessandro and Turlings, 2006; Dicke and Baldwin, 2010; Clavijo McCormick et al., 2012; Heil, 2014).

Preservation of Herbivore-Induced Plant Volatiles for Y-Tube Olfactometer Bioassays

Following dynamic headspace collection, herbivore-induced plant volatile (HIPV) and control samples were eluted from the Super Q adsorbent traps using high-purity hexane containing an internal standard. The solvent extracts were transferred into amber glass vials fitted with polytetrafluoroethylene (PTFE)-lined caps to minimize volatilization, oxidation, and photodegradation of volatile compounds (Heath and Manukian, 1994; Tholl et al., 2006).

The collected extracts were stored at – 20°C until use in Y-tube olfactometer bioassays. Samples were protected from repeated freeze–thaw cycles by dividing each extract into small aliquots immediately after elution. This procedure minimized chemical degradation and compositional changes, thereby preserving the integrity of volatile blends during storage (Agelopoulos and Pickett, 1998; Tholl et al., 2006; D'Alessandro and Turlings, 2006).

Before behavioral assays, stored volatile extracts were allowed to equilibrate to room temperature and gently mixed to ensure homogeneity. A measured volume of the extract was applied to a clean filter paper strip (Whatman No. 1), and the solvent was allowed to evaporate for approximately 30–60 seconds before placement into the odor chamber. This ensured that insects were exposed primarily to the volatile compounds rather than residual solvent (Vet et al., 1983; Turlings et al., 1998; Bruce et al., 2005).

To maintain consistency among bioassays, all volatile samples were analyzed and used within the recommended storage period, and equivalent quantities of volatile extract were dispensed for each replicate experiment. Solvent-only controls were included to verify that parasitoid responses were attributable to the herbivore-induced volatile blend rather than the extraction solvent or dispensing substrate (D'Alessandro and Turlings, 2006; Clavijo McCormick et al., 2012).

Isolation and purification of individual HIPV components

Individual volatile constituents identified by GC–MS, including 4,8-dimethyl-1,3,7-nonatriene (DMNT) and (Z)-3-hexenyl acetate, were isolated from the herbivore-induced plant volatile (HIPV) blend using preparative gas chromatography (GC). The HIPV profile was initially characterized by gas chromatography coupled with mass spectrometry (GC–MS), where compounds were identified based on their chromatographic retention times, mass spectral fragmentation patterns, and comparison with authentic reference standards, following established procedures for the analysis of plant volatile organic compounds (Dudareva et al., 2006; Tholl et al., 2006).

The volatile mixture was subsequently subjected to preparative GC separation using a capillary column appropriate for the resolution of plant-derived volatile compounds. Helium was used as the carrier gas under constant flow conditions, and the oven temperature program was optimized to achieve efficient separation of individual components. The chromatographic peaks corresponding to DMNT and (Z)-3-hexenyl acetate were monitored according to their previously determined retention times and collected separately using a fraction collection system. Preparative GC approaches have been widely applied for the purification of individual components from complex volatile mixtures while maintaining compound integrity (Schwab et al., 2008).

The collected fractions were transferred into amber glass vials to minimize photodegradation and stored at – 20°C until bioassay experiments. The identity and purity of the isolated compounds were verified by re-analysis using GC–MS through comparison of retention times and mass spectra with those of

authentic standards. Fractions exhibiting a single chromatographic peak corresponding to the target compound were considered sufficiently pure and were used for subsequent experiments (Tholl et al., 2006; Dudareva et al., 2006).

Parasitoid Rearing

The parasitoid species used in the behavioral assays was *Cotesia flavipes* Cameron (Hymenoptera: Braconidae), a gregarious larval endoparasitoid widely recognized as an effective biological control agent of lepidopteran stem borers infesting cereal crops, particularly *Chilo partellus* and other economically important stem borer species in Africa and Asia (Overholt et al., 1997; Jiang et al., 2006; Midingoyi et al., 2016).

The parasitoids were obtained from a laboratory colony obtained at the international centre of insect physiology and ecology (ICIPE), Nairobi and maintained under controlled insectary conditions at $25 \pm 2^\circ\text{C}$, $70 \pm 10\%$ relative humidity, and a 12hr light/12hr dark photoperiod. Adult parasitoids were reared in screened cages and supplied continuously with a 10% (v/v) honey solution as a carbohydrate source to enhance survival and longevity before experimentation (Takasu and Lewis, 1993; Wäckers, 2001; Harvey et al., 2005). Female parasitoids aged 24–48hr after emergence were used in all behavioral bioassays because adults of this age are sexually mature and exhibit active host-searching behavior (Vet and Dicke, 1992; Turlings et al., 1993; Turlings and Erb, 2018). This active host searching behavior is more pronounced in mated females with previous oviposition experience acquired during successful host encounters to enhance parasitoid responsiveness to herbivore-induced plant volatiles (HIPVs) and improvement in host recognition/increased foraging efficiency than in naive individuals (Vet and Dicke, 1992; Lewis et al., 1998; Giunti et al., 2015; Turlings and Erb, 2018).

Y-Tube Olfactometer Bioassays/ Behavioral Response Assay

Parasitoid attraction to herbivore-induced plant volatiles (HIPVs) emitted by sorghum plants was evaluated using a two-arm glass Y-tube olfactometer, a standard behavioral bioassay widely employed to investigate olfactory responses of parasitoids and other insects to plant-derived volatile cues (Vet et al., 1983; Turlings et al., 1993; Bruce et al., 2005; D'Alessandro and Turlings, 2006). The olfactometer consisted of a central release stem connected to two identical odor-delivery arms, allowing individual parasitoids to choose between two alternative odor sources under controlled laboratory conditions.

Purified, humidified air was passed through activated charcoal filters before entering separate odor chambers containing the experimental stimuli. Airflow was maintained at approximately 200 mL min^{-1} in each arm using calibrated flow meters to ensure equal odor distribution and prevent directional bias during the bioassays (Vet et al., 1983; Heath and Manukian, 1994; Turlings et al., 1998; Tholl et al., 2006). The odor sources consisted of herbivore-induced sorghum volatile extracts and their corresponding controls.

Female *C. flavipes* was tested individually in a two-choice Y-tube olfactometer. Parasitoids were allowed 5 min to choose between odor sources, and a choice was recorded when insects entered and remained in one arm for at least 30 s. (Vet et al., 1983; Turlings et al., 1993; D'Alessandro and Turlings, 2006). Parasitoids that failed to make a choice within the 5-min observation period were classified as "no choice" responses and were excluded from preference analyses, consistent with established olfactometer protocols (Vet and Dicke, 1992; Bruce et al., 2005; Clavijo McCormick et al., 2012).

To eliminate residual odors and prevent cross-contamination between successive assays, the glass olfactometer and associated components were thoroughly cleaned with ethanol, rinsed with distilled water, and allowed to dry before reuse. In addition, the positions of the treatment and control odor sources were alternated regularly between the two olfactometer arms throughout the experiment to minimize positional effects and directional bias (Vet et al., 1983; Turlings et al., 1993; Tholl et al., 2006; Turlings and Erb, 2018).

Odor Treatments basing on identified HIPVs in Sorghum

The following odor comparisons were evaluated basing on HIPVs identified: Hexane solvent control vs DMNT, Hexane solvent control vs (Z)-3-hexenyl acetate, Hexane solvent control vs DMNT + (Z)-3-hexenyl acetate blend and Healthy sorghum volatiles vs herbivore-induced sorghum volatiles

Y-Tube Behavioral Dataset

Odor comparison	Treatment choice	Control choice	No choice	χ^2	P-value
DMNT vs hexane	28	12	5	6.40	0.011
(Z)-3-hexenyl acetate vs hexane	30	10	5	10.00	0.002
DMNT + (Z)-3-hexenyl acetate vs hexane	35	7	3	18.67	< 0.001
Herbivore-induced sorghum vs healthy sorghum	33	12	5	9.80	0.002

Statistical Analysis

Parasitoid preference was analyzed using chi-square tests comparing the number of females selecting each odor source against an expected 50:50 distribution.

A logistic regression model was used to evaluate the contribution of individual HIPVs and their interaction effects on parasitoid choice: $\text{Choice} = \beta_0 + \beta_1(\text{DMNT}) + \beta_2((\text{Z})\text{-3-hexenyl acetate}) + \beta_3(\text{DMNT} \times (\text{Z})\text{-3-hexenyl acetate})$

where parasitoid choice was coded as a binary response variable (1 = HIPV treatment selected, 0 = control selected). Statistical significance was considered at $P \leq 0.05$.

Ethical and Biosafety Considerations

All experiments (insect colonies, plant material, and laboratory procedures) conducted followed institutional biosafety guidelines and relevant regulatory requirements.

RESULTS

Herbivore-Induced Volatile Emissions in Sorghum

Herbivore-induced sorghum plants exhibited qualitative and quantitative changes in volatile organic compound (VOC) profiles compared with undamaged control plants. Gas chromatography–mass spectrometry (GC–MS) analysis indicated increased accumulation of several herbivore-induced plant volatiles (HIPVs), including green leaf volatiles (GLVs) and terpenoid-derived compounds.

Among the detected compounds, (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (Z)-3-hexenyl acetate showed consistent induction following simulated herbivory. Although their absolute emission levels remained relatively low, these compounds represented prominent components of the induced sorghum volatile blend. The results show that herbivore-induced sorghum released approximately three-fold higher levels of DMNT, three-fold higher levels of (Z)-3-hexenyl acetate and two fold levels of (Z)-3-Hexen-1-ol compared with healthy sorghum plants (Table 1).

Table 1
Volatile emission profile of healthy and herbivore-induced sorghum plants

Compound	Healthy sorghum (ng g ⁻¹ FW h ⁻¹)	Herbivore-induced sorghum (ng g ⁻¹ FW h ⁻¹)	Fold induction
DMNT	0.85 ± 0.12	2.76 ± 0.31	3.2
(Z)-3-Hexenyl acetate	1.10 ± 0.18	3.45 ± 0.42	3.1
(Z)-3-Hexen-1-ol	2.30 ± 0.40	5.10 ± 0.62	2.2
Linalool	0.45 ± 0.08	1.12 ± 0.15	2.5

Parasitoid Response to Individual HIPVs

Female *Cotesia flavipes* exhibited significant attraction toward both individual HIPV compounds compared with the solvent control. When offered DMNT versus hexane, significantly more parasitoids selected the DMNT arm (28 females) compared with the control arm (12 females) ($\chi^2 = 6.40$, $P = 0.011$).

Similarly, (Z)-3-hexenyl acetate elicited a stronger behavioral response, with 30 females selecting the odor source compared with 10 females choosing the solvent control ($\chi^2 = 10.00$, $P = 0.002$).

The strongest attraction was observed when DMNT and (Z)-3-hexenyl acetate were presented as a blend. In this treatment, 35 out of 42 responding females selected the HIPV blend, resulting in a highly significant preference over the solvent control ($\chi^2 = 18.67$, $P < 0.001$).

These findings demonstrate that female *C. flavipes* responded behaviorally to individual HIPV components despite their low emission levels in sorghum.

Table 2
Y-tube olfactometer responses of *Cotesia flavipes* females to sorghum HIPVs

Odor comparison	HIPV treatment choice	Control choice	No choice	χ^2 value	P-value
DMNT vs hexane	28	12	5	6.40	0.011
(Z)-3-Hexenyl acetate vs hexane	30	10	5	10.00	0.002
DMNT + (Z)-3-Hexenyl acetate blend vs hexane	35	7	3	18.67	< 0.001
All Herbivore-induced sorghum vs healthy sorghum	33	12	5	9.80	0.002

Attraction to Herbivore-Induced Sorghum Volatiles

When parasitoids were offered intact volatile blends from healthy and herbivore-induced sorghum plants, females significantly preferred the herbivore-induced plants. Out of the responding parasitoids, 33 females selected herbivore-induced sorghum volatiles, whereas only 12 selected healthy sorghum volatiles ($\chi^2 = 9.80, P = 0.002$).

Percentage Attraction of Parasitoids

The percentage of parasitoids responding to each odor source demonstrated a concentration-independent behavioral pattern. Although sorghum HIPV emissions were relatively low, parasitoid attraction remained high. The solvent control attracted only 25% of responding females, whereas DMNT and (Z)-3-hexenyl acetate attracted 70% and 75% of parasitoids, respectively. The combined HIPV blend produced the highest attraction response (83%), exceeding the response observed for individual compounds.

Table 3
Percentage attraction of *Cotesia flavipes* females to sorghum HIPVs

Odor treatment	Parasitoid attraction (%)
Hexane control	25
DMNT	70
(Z)-3-Hexenyl acetate	75
DMNT + (Z)-3-Hexenyl acetate	83
Herbivore-induced sorghum volatiles	78

Logistic Regression Analysis of HIPV-Mediated Attraction

A logistic regression model was used to determine the relative contribution of individual HIPVs to parasitoid choice. Both DMNT and (Z)-3-hexenyl acetate significantly increased the probability of parasitoid attraction. The positive interaction term between the two compounds indicated that the combined odor blend produced an enhanced behavioral response beyond the effect of individual compounds alone.

Table 4
Logistic regression model describing parasitoid attraction to sorghum HIPVs

Predictor variable	Estimate (β)	SE	χ^2	P-value
DMNT	1.42	0.48	8.76	0.003
(Z)-3-Hexenyl acetate	1.71	0.52	10.82	0.001
DMNT $\times$ (Z)-3-Hexenyl acetate	0.64	0.31	4.29	0.038

DISCUSSION

Our findings show that herbivore simulation modified sorghum volatile emissions and produced odor cues that significantly influenced the host-searching behavior of female *Cotesia flavipes*. Although sorghum emitted relatively low absolute quantities of volatile compounds, parasitoids consistently responded to specific HIPVs, particularly (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (Z)-3-hexenyl acetate. These findings support the hypothesis that parasitoid host location depends primarily on the qualitative composition of herbivore-induced volatile blends rather than the total quantity of volatiles released.

Herbivore-induced changes in sorghum volatile emissions

Herbivore attack induced substantial changes in sorghum volatile emissions, with DMNT increasing more than threefold and (Z)-3-hexenyl acetate exhibiting a similar threefold induction. Other HIPVs, including (Z)-3-hexen-1-ol and linalool, were also significantly elevated following herbivore damage. Such induction is consistent with previous studies showing that herbivore feeding activates jasmonate-dependent defense pathways, leading to rapid biosynthesis of green leaf volatiles and terpenoids (Paré & Tumlinson, 1999; Arimura et al., 2009; Dudareva et al., 2013; Turlings & Erb, 2018). Herbivore-induced volatile production represents an important indirect defense mechanism through which plants recruit natural enemies of herbivores while simultaneously reducing subsequent herbivore damage (Kessler & Baldwin, 2001; Dicke & Baldwin, 2010; Heil, 2014).

The observed induction of DMNT agrees with reports that this homoterpene is synthesized through herbivore-activated terpenoid pathways and is one of the most widespread HIPVs released by damaged plants (Arimura et al., 2009; Turlings & Erb, 2018). Likewise, increased emission of (Z)-3-hexenyl acetate reflects activation of the lipoxygenase pathway immediately following tissue disruption, resulting in rapid formation of green leaf volatiles that serve as early indicators of herbivore attack (D'Auria et al., 2007;

Engelberth et al., 2004). Similar increases in DMNT and green leaf volatiles have been reported in maize, rice, cotton and numerous other crop species following herbivore infestation (Turlings et al., 1998; Gouinguéné & Turlings, 2002; Dudareva et al., 2006).

Although sorghum emitted relatively low concentrations of HIPVs, the present findings are consistent with our previous volatile characterization of sorghum (Osinde et al., 2023), that demonstrated that sorghum generally produces lower total volatile emissions than rice while still consistently inducing DMNT and (Z)-3-hexenyl acetate after simulated herbivory. Together, these findings suggest that the biological importance of HIPVs depends more on the identity of emitted compounds than on their absolute abundance.

Behavioral responses of *Cotesia flavipes* to individual HIPVs

Female *C. flavipes* exhibited significant attraction to both DMNT and (Z)-3-hexenyl acetate when each compound was tested individually against the solvent control. The stronger response to (Z)-3-hexenyl acetate compared with DMNT suggests that green leaf volatiles may function as particularly effective host-location cues for *C. flavipes*. Green leaf volatiles are among the earliest compounds released following herbivore damage and frequently serve as reliable indicators of recently attacked plants (Engelberth et al., 2004; Heil, 2014).

These findings are consistent with numerous studies demonstrating that parasitoids exploit individual HIPVs during host searching. Previous behavioral studies have demonstrated that parasitoids can use DMNT as an odor cue during host searching, including species within the genus *Cotesia*. Our results extend these observations by showing that DMNT also contributes to *C. flavipes* orientation toward sorghum-derived signals, while green leaf volatiles similarly enhance parasitoid orientation toward herbivore-infested plants (Turlings et al., 1990; Turlings et al., 1991; Ngi-Song et al., 1996; Bruce et al., 2005). The present results therefore confirm that both compounds function as biologically active semiochemicals for parasitoid recruitment in sorghum.

Enhanced attraction to the synthetic HIPV blend

The strongest behavioral response occurred when DMNT and (Z)-3-hexenyl acetate were presented together as a blend, attracting over 80% of responding females. Logistic regression further demonstrated a significant positive interaction between both compounds, indicating that their combined effect exceeded the sum of their individual effects. This suggests synergistic interactions between HIPV components during parasitoid host location.

Synergistic effects among volatile compounds have frequently been reported in chemical ecology. Rather than responding to single compounds in isolation, parasitoids generally perceive characteristic volatile blends that provide more reliable information regarding host presence and suitability (Vet & Dicke, 1992; De Moraes et al., 1998; Clavijo McCormick et al., 2012). Individual HIPVs often occur in many ecological contexts, whereas unique combinations of compounds allow natural enemies to distinguish herbivore-infested plants from healthy vegetation or mechanically damaged tissues (Bruce et

al., 2005; Turlings & Erb, 2018). The enhanced attraction observed in this study therefore supports the hypothesis that qualitative blend composition is more important than single-compound emission.

The positive interaction detected by logistic regression further reinforces previous reports that parasitoid behavioral responses are frequently nonlinear, with multiple HIPVs interacting to generate stronger orientation responses than individual compounds alone (Vet & Dicke, 1992; Turlings et al., 1993; Clavijo McCormick et al., 2012).

Preference for herbivore-induced sorghum volatiles

When intact volatile blends from herbivore-induced and healthy sorghum plants were compared, *C. flavipes* females strongly preferred herbivore-induced plants. This result indicates that simulated herbivory altered sorghum odor profiles sufficiently to enable parasitoids to discriminate host-infested plants from undamaged plants. Such discrimination forms the basis of tritrophic interactions, in which plants indirectly defend themselves by attracting natural enemies of herbivores (Dicke & Baldwin, 2010; Arimura et al., 2009; Turlings & Erb, 2018).

Comparable behavioral responses have been reported for maize, cotton, rice and other cereals, where parasitoids preferentially orient toward herbivore-induced plants rather than healthy controls because induced volatile blends contain reliable host-associated information (Turlings et al., 1990; Kessler & Baldwin, 2001; Bruce et al., 2005). The present findings therefore confirm that sorghum also employs HIPV-mediated indirect defenses capable of recruiting parasitoids despite comparatively low volatile emissions.

Importance of volatile quality rather than quantity

Despite the relatively low abundance of sorghum volatiles, parasitoid orientation was strongly influenced by specific compounds within the induced blend. This indicates that ecological information encoded in volatile identity and combination may be more important than absolute emission magnitude. Previous studies have demonstrated that parasitoids respond selectively to characteristic HIPV blends because specific compounds provide more reliable information about host presence than overall odor intensity (Vet & Dicke, 1992; De Moraes et al., 1998; Turlings et al., 1993; Clavijo McCormick et al., 2012).

This interpretation agrees closely with our earlier findings were different sorghum cultivars emitted lower quantities of volatiles than rice but consistently produced DMNT and (Z)-3-hexenyl acetate following herbivore damage (Osinde et al., 2023). The present behavioral assays extend those chemical observations by demonstrating that these two compounds are sufficient to elicit strong parasitoid responses despite their relatively low concentrations. Consequently, the ecological effectiveness of sorghum HIPVs appears to depend largely on the information conveyed by key semiochemicals rather than on emission magnitude.

Implications for sustainable pest management

The findings have important implications for conservation biological control and integrated pest management (IPM) in cereal production systems. Because DMNT and (Z)-3-hexenyl acetate consistently attracted *C. flavipes*, these compounds represent promising candidates for synthetic lures designed to enhance parasitoid recruitment in sorghum fields. Previous studies have demonstrated that HIPV-based semiochemicals can improve biological control by increasing the abundance and retention of natural enemies within cropping systems (Turlings & Erb, 2018; Clavijo McCormick et al., 2012).

The attraction elicited by DMNT and (Z)-3-hexenyl acetate suggests that these compounds could be evaluated as semiochemical tools for improving parasitoid recruitment in sorghum production systems. Such approaches are consistent with global efforts to develop environmentally sustainable crop protection systems that conserve biodiversity and minimize pesticide inputs (Pretty & Bharucha, 2015; Turlings & Erb, 2018).

Study limitations and future research

Although the study provides strong evidence that DMNT and (Z)-3-hexenyl acetate influence parasitoid behavior under controlled laboratory conditions, several limitations should be acknowledged. The experiments employed simulated herbivory and Y-tube olfactometer bioassays, which simplify the complex odor environments encountered under field conditions. In natural agroecosystems, parasitoids integrate volatile signals with visual cues, host-derived kairomones and environmental variables such as wind direction, temperature and humidity (Vet & Dicke, 1992; Bruce et al., 2005; Turlings & Erb, 2018). Our future studies will validate these findings under field conditions by evaluating whether synthetic blends of DMNT and (Z)-3-hexenyl acetate enhance *C. flavipes* recruitment, parasitism rates and stem borer suppression in sorghum. Additionally, investigating the contribution of other induced compounds, such as linalool and (Z)-3-hexen-1-ol, may reveal further synergistic interactions that optimize parasitoid attraction and improve semiochemical-based pest management strategies.

CONCLUSION

This study demonstrates that simulated herbivory induces the production of biologically active herbivore-induced plant volatiles (HIPVs) in sorghum that effectively attract the larval parasitoid *Cotesia flavipes*. Although sorghum emitted relatively low overall quantities of volatile compounds, herbivory significantly increased the emission of key semiochemicals, particularly (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (Z)-3-hexenyl acetate. Behavioral assays showed that female *C. flavipes* exhibited significant attraction to both individual compounds, with the strongest response elicited by their combined blend, indicating a synergistic effect. Parasitoids also preferentially responded to volatile blends from herbivore-induced plants over those from healthy plants, confirming the ecological role of HIPVs in mediating tritrophic interactions among sorghum, stem borers, and their natural enemies.

These findings support the hypothesis that parasitoid attraction depends primarily on the qualitative composition of volatile blends rather than the total amount of volatiles emitted. The identification of DMNT and (Z)-3-hexenyl acetate as key attractants highlights their potential as synthetic

semiochemicals for enhancing the recruitment and retention of *C. flavipes* in sorghum production systems. Incorporating these HIPVs into integrated pest management (IPM) programs could strengthen biological control of stem borers, reduce dependence on chemical insecticides, and promote environmentally sustainable crop protection.

Future research should validate these laboratory findings under field conditions, assess the persistence and efficacy of synthetic HIPV lures in diverse agroecological environments, and investigate the contribution of additional volatile compounds to parasitoid attraction. Integrating volatile profiling with sorghum breeding and conservation biological control strategies will facilitate the development of sorghum cultivars and pest management systems that maximize natural enemy recruitment and improve the sustainable management of cereal stem borers.

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