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RESEARCH ARTICLE

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Identification of plant semiochemicals and evaluation of their interactions with early spring insect pests of asparagus

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ABSTRACT

Information is lacking on the chemical ecology of asparagus, and knowledge about the effects of its volatile emissions on its associated early season pest species is completely absent. The current study aimed to (1) evaluate whether the asparagus miner responds to asparagus volatiles, (2) identify and compare the changes in asparagus host plant volatiles from mechanical and chewing damage by the black cutworm, a temporally co-occurring species with the asparagus miner, and (3) assess how asparagus volatiles affect asparagus miner populations in the field. Results indicated that asparagus miners were significantly attracted to healthy asparagus stems when compared to clean air. Damaged asparagus headspace volatiles were quantitatively and qualitatively different from healthy plants. Volatile baits elicited a range of responses, but their effects were inconsistent between sampling years and phenology-dependent. Overall, we demonstrated that the chemical ecology of asparagus may be altered by its pest community, and volatiles identified from asparagus may impact the behavior of the asparagus miner.

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Integrated pest management; asparagus miner; Agromyzidae; headspace sampling; Y-tube olfactometer

1. Introduction

There is increasing interest in understanding the chemical ecology of agricultural plants to enhance pest management (Turlings & Ton 2006; Khan et al. 2008; Åhman et al. 2010), through manipulating herbivore behavior (De Moraes et al. 2001; Bruce 2010). In the case of asparagus (*Asparagus officinalis* L.), there is a lack of knowledge about the plant volatiles it emits and their interactions with associated arthropods. Despite increasing demand (Huang & Huang 2007), asparagus production is in decline due to the increased prevalence of pests (Grogan & Kimble 1959; Morrison III et al. 2011). To date, only two published studies have investigated the chemicals emitted by asparagus, one using ground up whole spear preparations that were first frozen, then heated to 50°C to evaporate the volatiles onto a trap (Sun et al. 2001), and the other using two-hour cooked asparagus (Ulrich et al. 2001). The first study found the major plant volatiles to be hexanal, trans-2-hexenal and 1-octen-1-ol, depending on the asparagus cultivar (Sun et al. 2001). Other volatiles found in lower quantities included ketones, alkenes and terpenes. The second study found a total of 36 compounds, and analyzed their contribution to human odor perception of asparagus, but did not investigate their quantity (Ulrich et al. 2001). However, no study has examined asparagus headspace volatiles and their interaction with its pest insect complex.

Identifying the quality and quantity of asparagus headspace volatiles using currently available research tools and methods is the first necessary step to developing viable alternative management strategies for pests. This may be in the form of genetic manipulation of the asparagus resulting in cultivars with upregulated priming ability (e.g. Aharoni et al. 2006; Dudareva & Pichersky 2008), deployment of baits with insect herbivore-induced plant volatiles for attracting natural enemies to suppress pests (Rodriguez-Saona et al. 2011; Ali et al. 2012), or sprays that induce plants to become primed or to produce volatiles (Bruinsma et al. 2009) that attract biological control agents (Thaler 1999). However, this may also include using herbivore-induced volatiles as repellents to herbivores (De Moraes et al. 2001)

Asparagus is attacked by a suite of pests (Morrison III & Szendrei 2014), but its early colonizing species include black cutworm (*Agrotis ipsilon* (Hufnagel), Lepidoptera: Noctuidae), variegated cutworm (*Peridroma saucia* Hübner, Lepidoptera: Noctuidae) and the asparagus miner (*Ophiomyia simplex* Loew; Diptera: Agromyzidae) (Morrison III 2014). The cutworm larvae are generalists, chewing on vegetative asparagus tissue. These insects are problematic at the beginning of the asparagus growing season and are one of the first herbivores to start feeding on young plants. Asparagus miner adults appear around the same time as cutworms and start mating and laying eggs on plants (Ferro & Gilbertson 1982). Of the two species, the asparagus miner is the more serious of the pests, because it is closely associated with the spread of a pathogenic species of fungus that can cause early decline of fields (Barnes 1937; Morrison III et al. 2011). Asparagus miners have two generations in temperate regions and are present throughout the asparagus growing season (Lampert et al. 1984; Morrison III, Andresen et al. 2014). The larvae cause damage by feeding on the asparagus stem internally while the adults feed only on pollen, nectar and plant sap.

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Herbivore-induced plant volatiles can be herbivore-specific (Takabayashi et al. 1995), where asparagus plants may emit different volatiles depending on the specific pest. The elicitors in caterpillar saliva (Bonaventure et al. 2011, Turlings et al. 2000), for example, likely induce asparagus in a different way than induction by the internal feeding of asparagus miner larvae. Therefore, feeding by the cutworm in the beginning of the season may impact the behavioral responses and host-finding of the asparagus miner and other subsequent pests by altering the ambient volatile cues present in the field.

The asparagus miner is host-specific (Barnes 1937), and as a result, has likely evolved to recognize volatile cues from its only host plant species (Schoonhoven et al. 1998; Szendrei & Rodriguez-Saona 2010). The adult females seek out asparagus plants to oviposit under the epidermis of the stem near the base of the plant (Eichmann 1943), where larvae hatch and feed. Females may be important targets for manipulation with plant volatiles because they are often more responsive than males, as they frequently rely on constitutive plant volatile cues for recognizing appropriate oviposition sites (Szendrei & Rodriguez-Saona 2010). Insect pests may also be repelled by induced plant volatiles, because these may signal that plants are producing toxic secondary compounds, that potential competitors for food and oviposition may be present, or that the plant may now be attractive to predators and parasitoids (Bernasconi et al. 1998).

The plant volatile blends that are emitted at different time points of the year may be more or less attractive to the pest in question, depending on whether the primary concern of an individual is foraging, mating or oviposition. For example, when codling moth (*Cydia pomonella*; Lepidoptera: Tortricidae) females are searching for places to oviposit, they seek out hosts having a characteristic blend of volatiles, including a series of eight compounds present during the peak flight of codling moth in apple (Bengtsson et al. 2001; Witzgall et al. 2005).

The primary aims of the current study were to (1) evaluate whether the asparagus miner responds to asparagus volatiles in an olfactometer, (2) identify and compare the changes in asparagus host plant volatiles from mechanical and chewing damage by the black cutworm, and (3) assess whether asparagus volatiles affect asparagus miner populations in the field in a phenology-dependent manner.

2. Methods

2.1. Insect collection

Asparagus stems were collected weekly from 1 to 5 commercial asparagus fields in Oceana Co., MI from 2011 to 2013 (Morrison III, Andresen et al. 2014). Stems were cut 5 cm below the soil surface and again at the height of the longest mine (visible externally), transported to the laboratory in a cooler, and placed at 5°C until dissection. Asparagus miner pupae were dissected out of the stems within 1–14 d of collection, and placed individually in plastic portion cups or petri dishes where they were allowed to develop at 23 ± 0.3°C under a 16:8 L:D cycle. Pupae were checked daily, and emerged adults were sexed and used for the Y-tube olfactometer assays.

2.2. Y-tube assay

In order to assess the asparagus miners' preference for healthy asparagus headspace a Y-tube olfactometer (two 7 cm and one 13 cm arms; 1.5 cm diameter, ground glass joints; Michigan State University, East Lansing, MI) was employed. Air was first filtered through activated charcoal, then humidified, and was subsequently split into two 1 L/min air streams, regulated by flow meters. Afterwards, each air stream was delivered to a 250 mL glass jar (16.5 cm × 8.5 cm H:D) with a cap containing an inlet and outlet for air, which was connected to the olfactometer by inert Teflon™ tubing. The odor source placed in the jar included healthy asparagus stems (153.8 ± 3.9 g) compared with empty jars serving as the negative control. Upon addition of the plant material, deionized water was filled to 3 cm level within the jars to keep the stems hydrated. All plant material came from five commercial fields (all var. Millennium) in Oceana Co., Michigan and was collected between May and August from 2012 to 2013. Before the beginning of the assays, plant material was stored in a refrigerator at 5°C in a sealable bag for 1–4 d. Prior to placement in the volatile chamber, the asparagus was carefully rinsed with deionized water to remove any sand, dirt or other foreign substrate from the surface.

Each adult asparagus miner was used in the Y-tube olfactometer on the same day that it emerged from the pupa to ensure uniformity of age among tested adults. In addition, a 1:1 sex ratio of adults was used for the assay. At the beginning of the assays, unmated asparagus miners were placed individually at the bottom of the Y and were observed until a choice was recorded, or for a maximum of 15 min. A choice was recorded when the insect moved past the mid-point of one of the arms containing a volatile source. Non-responding individuals were marked as such and excluded from the subsequent statistical analysis. After every second run, the position of the odor sources was randomized to eliminate the possibility of positional bias. After every trial, the Y-tube was rinsed with methanol, then hexane and heated in a drying oven for at least 10 min at 60°C to dry. Glassware was allowed to cool down for 3 min before being used again in assays. A total of 38 adults were used from 4 May 2012 to 12 August 2013. All experiments were performed between 0800–1600 h at 22.8 ± 0.3°C and 600–700 lx, under laboratory conditions.

2.3. Plants for headspace collection

Asparagus used in the headspace collection was grown in the greenhouse from var. Millennium one-year-old crowns from a nursery in Oceana Co., MI. Crowns were stored at 5°C in a cold room with complete darkness until needed for planting in the greenhouse. Plants were potted in 15.1 l pots (27.9 cm × 29.2 cm H:D) with a mixture of 75% washed play sand (Kolorscape, Oldcastle, Inc., Atlanta, GA, USA) with 25% potting soil (SureMix Perlite, Michigan Grower Products, Inc., Galesburg, MI, USA). Sand was used to simulate the sandy soils that asparagus prefers. Plants were watered in the greenhouse once daily during the warm season, once weekly during the cool season, and kept on a 16:8 L:D cycle throughout the year. Fertilizer (20–20–20 N:P:K with micronutrients, J.R. Peters, Allentown, PA, USA) was delivered to plants in liquid form (1–2% v/v) as plants were watered.

2.4. Headspace collection

In order to evaluate how asparagus headspace changes with plant damage, plant volatiles were collected from asparagus

plants grown in the greenhouse. Plants were either intact (healthy), mechanically damaged or black cutworm-damaged. The cutworm was chosen as the herbivore because they co-occur with the asparagus miner and precede its arrival in the field, likely affecting host-finding by the asparagus miner. In addition, the complexity of the asparagus miner's life cycle makes it currently impossible to establish a lab-reared colony (W.R.M, unpublished data), so directly assessing changes in the headspace of asparagus by this pest is challenging. The mechanical damage consisted of grinding three separate 5 cm segments of cladophylls between gloved fingers with about 5 g silicon carbide powder (120 grit, Alfa Aesar, Lancs, UK) for ca. 10 s, and was performed immediately before headspace collection. Black cutworm eggs were reared on an artificial diet (Benzon Research, Inc., Carlisle, PA, USA) in plastic containers (12 × 7 × 5 cm L:W:H) until the third instar, then they were transferred to asparagus plants to feed. Five black cutworms were placed on an individual asparagus plant, and were allowed to feed overnight prior to headspace collection. The caterpillars were removed 10–30 min prior to headspace collection. The treatments were replicated over time, and each treatment was represented at least once in each replication. In addition to the three plant treatments, a negative control consisting of an empty glass chamber was also included. At least 6 asparagus crowns were planted at a time in the greenhouse for each replication to ensure uniformity of soil and abiotic conditions for the plants.

For headspace collection, five plants at a time were individually covered by 4 L glass chambers (36 × 20 cm H:D, tapered at top), an empty chamber was used as a negative control. Using a push–pull system, air was pumped through a flow meter and a charcoal filter for purification. Purified air entered through a valve near the top of each chamber at 1.2 L/min, and volatiles were collected in Alltech SuperQ adsorbent traps (30 mg/trap; Analytical Research Systems, Gainesville, FL) by pulling air from the chambers at a rate of 1 L/min (Szendrei et al. 2009). The positive pressure assured that ambient air did not enter the headspace equipment during collection.

Plants were spaced ca. 60 cm apart, and metal guillotines (23 × 31 cm W:L) were clamped around the stems of the plants at the base of the glass chambers, with the opening of the guillotine blocked with cotton balls around the stem of the plant. The guillotines and glass chambers were elevated above the pots of the asparagus plants. A round of headspace collection lasted on average 6 h, typically from 0900 to 1500 h, coinciding with the photosynthetically active stage of asparagus. After headspace collection, the above ground plant biomass was weighed, and all glassware, SuperQ traps, and surfaces were washed and wiped down with methanol, then hexane.

2.5. Gas chromatography-mass spectrometry headspace analysis

The collected volatiles from the SuperQ traps were eluted with dichloromethane (150 µL), and 400 ng of tetradecane (Sigma-Aldrich, St. Louis, MO) was added as an internal standard. The volatile extracts were analyzed on an Agilent 6890 N gas chromatograph (GC) equipped with an Agilent Durabond DB-5 column (10 m length, 100 µm diameter and 0.34 µm film thickness, He as the carrier gas at constant 5 mL/min flow and 39 cm/s velocity) coupled with an Agilent 5975B inert XL mass spectrometer (MS). Compounds were separated by injecting 1.0 µL of sample into the GC/MS. The program consisted of 40°C for 1 min followed by 14°C/min increase to 180°C for 8 min, and 40°C/min increase to 300°C for 2 min. After a solvent delay of 3 min, mass ranges between 50 and 550 atomic mass units were scanned. Compounds were identified by comparison of spectral data with those from the NIST library and by GC retention index (Adams 2009) and confirmed by comparing their retention times and mass spectra with those of commercially available compounds run on the same column.

2.6. Baiting field experiment

During 2011, 2012, and 2013, a mix of plant volatiles were deployed in baits in 3–5 commercial asparagus fields in Oceana Co., MI. Volatiles were selected from the headspace analysis if preliminary data indicated a specific volatile had a library match of > 90% during the GC-MS analysis. We also tested methyl salicylate in all years on the basis of the finding that it serves as a common attractant in many systems and taxa, including Diptera (Rodriguez-Saona et al 2011). Volatiles varied in different years, and were only kept between years if they initially showed promise in attracting asparagus

Table 1. Summary of volatiles used in baited yellow sticky traps in commercial asparagus fields from 2011 to 2013 in Oceana Co., MI.

		Concentration (µL volatile/µL MO[a])		Years tested				
Compound	Identified from or role	2011 & 2012	2013	2011	2012	2013	Purity[b]	Supplier
Mineral oil	Negative control	–	–	X	X	X	light	Sigma-Aldrich[c]
(*Z*)-3-Hexen-1-ol	Healthy plants	0.5	0.35	X	X	X	>98	Sigma-Aldrich
Methyl salicylate	Common attractant	0.5	0.35	X	X	X	>95	Ag Bio[d]/Sigma-Aldrich
6-Methyl-5-hepten-1-ol	Other systems	0.5	–	X			>98	Sigma-Aldrich
β-Caryophyllene	Other systems	0.5	–	X			>80	Sigma-Aldrich
(*E*)-3-Hexenyl acetate	Herbivore damage	0.5	–	X			natural	Sigma-Aldrich
Nonanal	Other systems	0.5	–	X			>95	Sigma-Aldrich
(*Z*)-β-Ocimene	Mechanical damage	0.5	–		X		>90	Sigma-Aldrich
1-Hexadecene	Herbivore damage	0.5	–		X		>94	Alfa Aesar[e]
Pentadecane	Herbivore damage	0.5	0.35		X	X	>98	Sigma-Aldrich
Decanal	Constitutive volatile	0.5	0.35		X	X	>96	Alfa Aesar
Hexanoic acid	Constitutive volatile	–	0.35			X	>98	Sigma-Aldrich

[a]MO – mineral oil.
[b]Initial stock concentration of volatiles, v/v%.
[c]Location: Milwaukee, WI, USA.
[d]Location: Westminster, CO, USA.
[e]Location: Ward Hill, MA, USA.

miners in the field trial performed in 2011 (see Table 1 for suppliers). The volatiles added included additional candidates from headspace that had a library match of >90% and had either not been initially tested or placed in as substitutions for compounds shown not to elicit a response. All trials included a negative control that consisted of mineral oil (light oil, Sigma-Aldrich, Milwaukee, WI, USA) only. In 2011 and 2012, six volatiles were tested in addition to the mineral oil, while five were tested in 2013 (Table 1). The average ± SEM release rate across baits was 10.7 ± 2.7 mg/day (see Table S1 for individual release rates, and the supplemental methods for their calculation). Because previous literature has shown that combinations of volatiles may be important in generating attraction to a lure (Szendrei & Rodriguez-Saona 2010), combinations of 2–3 volatiles were used in 2013 (for details see Table 1). Volatiles were added to 1.7 mL (2011 and 2012) or 2.0 mL (2013) plastic centrifuge tubes with snap caps, and were punctured once on the side near the top of the tube with a dissecting needle to allow diffusion of the volatiles into the surrounding air. In 2011, 500 µL of a volatile was added to 1000 µL of mineral oil, while in 2012, 350 µL of a volatile was added to 700 µL of mineral oil. Finally, in 2013, the total volume of liquid in a tube was kept constant at 1000 µL, and 350 µL of each volatile was added while the remaining volume was filled with mineral oil.

Five, three, and four commercial asparagus fields were used in 2011, 2012, and 2013, respectively, for testing the baits. Baited centrifuge tubes were affixed with floral wire to the base of yellow sticky traps (7.6 cm × 12.7 cm W:L, Great Lakes IPM, Vestaburg, MI, USA), attached to metal stakes, and the traps were set at the top of 1 m long pieces of metal conduit at each sampling point. One (2011) or three (2012 and 2013) transects of baited traps were placed on three randomly chosen edges of an asparagus field, with seven traps (2011 and 2012) or eight traps (2013) spaced 10 m apart within the transect at the asparagus crop edge, since this is where the highest abundance of asparagus miner adults are located (Morrison III & Szendrei 2013). Traps were changed every 6–10 days during the growing season, from 6 July to 3 October 2011, 11 April to 1 October 2012 and 7 May to 5 September 2013. Traps were brought to the laboratory and the abundance of asparagus miner adults was recorded (2011, 2012 and 2013).

2.7. Statistical analyses

A G-test for goodness of fit coupled with William's correction for the *p*-value (Sokal & Rohlf 1995) was performed to assess the significance of asparagus miner preference for volatiles in the Y-tube, with the null hypothesis that asparagus miners would choose both sides of the olfactometer with equal probability.

For analyzing the asparagus volatiles in different treatments, raw peak areas were extracted from the gas chromatograms using MSD ChemStation v.2.00 software (Agilent Technologies, Inc., Santa Clara, CA). These were transformed into units of ng of volatiles per gram of fresh plant biomass per hour, using the ratio of the given volatile's peak area to that of the tetradecane internal standard, the weight of fresh plant biomass the volatiles originated from and the sampling duration of the headspace collection. Background compounds found in the control (no plant) that were also present in the asparagus plant samples were discarded from the analysis. In addition, compounds that were only found in two or fewer samples were also discarded, since these likely represent transient background volatiles or idiosyncratic emissions from individual genetic variation, and are thus not of interest in making generalizations about asparagus headspace among plants. Using ng per gram fresh tissue per hour values for individual volatiles, pairwise Bray–Curtis similarities were calculated between treatments, and non-metric multi-dimensional scaling (NMDS) was used to visualize the differences. Stress values for NMDS procedure were <0.1, indicating that good interpretation was possible. To test significance of differences, an analysis of similarity (ANOSIM) with 1000 permutations was employed. The 95% confidence ellipses based on the centroid of each treatment was calculated. These statistical tests and all others, except where otherwise noted, were carried out in R Software (Team RDC 2013) with $\alpha = 0.05$.

In order to understand the relative contribution of each volatile compound to the overall similarity or dissimilarity of the headspace from each treatment (healthy, mechanically damaged or black cutworm-damaged), a similarity percent (SIMPER) procedure was used (Primer E v.6.1.6), where each compound's contribution to the similarity (within a treatment) or dissimilarity (between treatments) was calculated. The cutoff for inclusion of compounds was when the overall cumulative average similarity within a group or dissimilarity between groups reached at least 90%. In addition, Tukey's Honestly Significant Differences (HSD) were performed on compounds contained within the headspace between the different treatments to assess differences in quantities.

Because different volatiles were assayed for asparagus miners in different years and at different concentrations with different release rates, single mixed model, analyses of variance (ANOVAs) were performed separately for each year between 2011 and 2013, using the asparagus miner abundance as the response variable, field as a random variable, and volatile and phenology (non-peak or peak flight) as the independent variables, along with their interaction (volatile × phenology). Peak flight was defined according to the degree-day model developed for the asparagus miner as well as the predicted occurrences of the first and second generation for the species (Morrison III, Andresen et al. 2014). Degree-days were calculated for each year at the field sites using the Hart, MI Enviroweather station maintained by Michigan State University (Andresen et al. 2011). In particular, data from the week of peak flight as well as the two sampling intervals bracketing the peak flight event for the first generation and second generation (if present) were grouped, while all other dates were designated as 'non-peak flight'. This was performed to evaluate whether the cues that the asparagus miner uses varies over time in the growing season. The parameters for the statistical models were estimated using restricted maximum likelihood to account for unequal sample sizes. Asparagus miner abundance did not conform to the expectations of a normal distribution in any of the years, therefore the residuals in each year were log transformed. The resulting residuals were inspected and found to conform to the assumptions of a normal distribution, and log-transformed data was used for all subsequent analyses. When a significant result was found with the

ANOVA, pairwise Tukey's HSD comparisons were performed to separate treatment means.

3. Results

3.1. Y-tube assay

The asparagus miner was significantly attracted to healthy asparagus stems over air ($G_{adj.} = 4.19$, df = 1, $p < .05$). Of the 38 adults tested, twice as many chose the healthy asparagus (20) compared with those that chose the purified air (9), while 9 adults were unresponsive. On average, it took an asparagus miner 3.9 ± 1.0 min to make a decision.

3.2. Headspace collection

Twenty-five compounds were identified on the GC-MS from intact asparagus plants, 15 from mechanically damaged plants and 20 from herbivore-damaged plants (Table 2). The volatile headspace emitted by asparagus was significantly different among treatments, depending on whether it was intact, mechanically damaged or damaged by cutworm (ANOSIM: $R = 0.308$, $n = 33$, $p < .001$; Figure 1). Asparagus headspace compounds include five- and six-chained carbon compounds, such as alcohols, alkenes and aldehydes, as well as higher order carbon compounds, such as 1-octadecene (Table 2). The intact plants emitted significantly greater amounts of (*Z*)-3-hexen-1-ol when compared to mechanically or cutworm-damaged plants (Table 2, $F_{2,30} = 3.83$, $p < .05$). The headspace of intact plants contained numerically about half the amount of (*E*)-3-hexenyl acetate ($F_{2,30} = 0.61$, $p = .55$) and 1-hexadecene ($F_{2,30} = 1.89$, $p = .17$), 13 times more (*Z*)-3-hexen-1-ol, and 50% less pentadecane ($F_{2,30} = 11.33$, $p < .001$) when compared to the headspace of cutworm-damaged plants. By contrast, the headspace of black cutworm-damaged plants, on average, contained numerically about 50% more (*E*)-3-hexenyl acetate, hexanoic acid, and decanal, and four times more pentadecane ($F_{2,30} = 11.33$, $p < .001$) when compared with mechanically damaged asparagus headspace. Additionally, mechanically damaged headspaces had significantly more neryl acetone ($F_{2,30} = 5.90$, $p < .01$) and pentadecanol ($F_{2,30} = 5.50$, $p < .01$) than the other two treatments, while herbivore-damaged headspaces had significantly more (*Z*)-threo-davanafuran than the mechanically damaged treatment ($F_{2,30} = 5.46$, $p < .05$). Intact headspace had significantly more of an unidentified compound (unknown1: $F_{2,30} = 4.81$, $p < .05$) compared to the mechanically damaged treatment, though the quantity was less than 2% of the total headspace emission.

Between 5 and 7 compounds accounted for over 90% of the similarity in asparagus headspace within a treatment, but only 2–4 of these compounds were commonly found in each sample (Table 2, % cumulative similarity), as indicated above. The amounts of the volatile compounds present in the headspace were reliable discriminators between the treatment groups, for example, (*E*)-3-hexenyl acetate (Table 2, % dissimilarity).

3.3. Baiting field experiment

In 2011, there were 352 asparagus miner adults caught on traps over 12 weeks. The lure type significantly impacted the abundance of asparagus miners caught (ANOVA: $F_{6,385} = 35.11$, $p < .001$). All the volatile lures except nonanal caught about 2.75–5 times significantly more asparagus miner adults than the control. In addition, there were significantly fewer adults during non-peak flight times (ANOVA: $F_{6,385} = 7.27$, $p < .001$). Over 20% more adults were found on sticky traps during the peak flight than during non-peak flight weeks. Finally, the effect of the volatiles on attraction of asparagus miner adults varied over the course of the season (ANOVA, volatile × phenology interaction: $F_{6,385} = 3.88$, $p < .001$; Figure 2(a)), with methyl salicylate and (*Z*)-3-hexen-1-ol exhibiting increased and nonanal with decreased captures of adults during the peak flight compared to non-peak flight times (Figure 2(a), Tukey's HSD).

In 2012, the total number of adults caught was 16,620 during the 25-week sampling period. Similar to 2011, the volatile lure significantly affected the number of asparagus miner adults captured (ANOVA: $F_{6,1546} = 6.79$, $p < .001$). However, while there was a significant difference between the most and least attractive volatile, none of the tested volatiles were significantly different from the control (mineral oil only) within a flight period (Figure 2(b), Tukey's HSD). The point in asparagus miner phenology also significantly affected the abundance of miners caught (ANOVA: $F_{1,1546} = 208.5$, $p < .001$). The baits caught about three times more asparagus miner adults during peak periods of activity than between peaks. There was also an interaction between the volatile cues and phenology of the asparagus miner (ANOVA, volatile × phenology interaction: $F_{6,1546} = 4.23$, $p < .001$; Figure 2(b)), with decanal being significantly more repellent during non-peak flight periods.

The total number of asparagus miner adults caught during 2013 was 20,408 during the 17-week sampling period. Like in the two prior years, the lure volatile significantly altered the abundance of adults caught between the most and least attractive volatile (ANOVA: $F_{7,1583} = 9.53$, $p < .001$). However, as in 2012, none of the volatiles tested were significantly different from the control within the season or the peak flight period. In addition, the number of adults caught significantly depended on the phenology of the asparagus miner (ANOVA: $F_{1,1583} = 78.6$, $p < .001$). In particular, there were over two times as many adults caught on average during the peak flight compared with the non-peak times. Finally, adults differed in the number caught on a given lure depending on the time in the season (ANOVA, volatile × phenology interaction: $F_{7,1583} = 4.81$, $p < .001$; Figure 2(c))

4. Discussion

This study is the first to describe, in detail, the headspace volatiles emitted by asparagus and how it affects part of its associated pest community, especially those pests that arrive early in the season. The asparagus miner oriented to asparagus using the chemical cues emitted by the plant. Mechanically damaged and black cutworm-induced asparagus produced a qualitatively and quantitatively different volatile headspace than intact plants. The number of asparagus miners caught on yellow sticky traps in the field was significantly affected when black cutworm-induced and healthy asparagus volatiles were deployed in the field in baits, though the results were dependent on the phenology of the insect and sampling year.

Table 2. Volatiles identified by GC-MS from asparagus plants (var. Millenium) that were either left intact (healthy), mechanically damaged by rubbing silicon carbide along the stem, or herbivore-damaged by five black cutworm larave.

			Intact[a]						Mechanically damaged						Herbivore-damaged						Average % Cum. Dissimilarity[e]		
	Compound	RT	Mean[b]	±	SE	% Cum. Sim.[c]	% Total		Mean[b]	±	SE	% Cum. Sim.	% Total		Mean[b]	±	SE	% Cum. Sim.	% total		IN vs. MD[f]	IN vs. HD	MD vs. HD
1	Hexanoic acid	6.84	16.19	±	9.3	4.65	6.3		19.17	±	3.9	22.21*	9.7		28.30	±	10.6	17.66	8.8		8.22	8.87	8.52*
2	Mesitylene	7.61	0.95	±	0.6	–	0.4		0.00	±	0.0	–	0.0		0.00	±	0.0	–	0.0		–	–	
3	(*Z*)-3-Hexen-1-ol	7.72	33.64	±	14.3	13.36	13.1	*a*[d]	0.00	±	0.0	–	0.0	*b*	0.00	±	0.0	–	0.0	*b*	18.05*	17.61*	
4	(*E*)-3-Hexenyl acetate	7.84	50.11	±	10.4	51.02	19.5		59.12	±	15.2	60.66*	29.9		112.08	±	38.9	48.39	34.9		33.77*	40.61*	36.57*
5	Limonene	8.69	2.17	±	1.3	–	0.8		0.00	±	0.0	–	0.0		1.02	±	0.7	–	0.3		–	–	
6	(*Z*)-β-Ocimene	8.96	7.11	±	4.9	–	2.8		25.19	±	25.2	–	12.8		0.00	±	0.0	–	0.0		39.89	44.66	40.86
7	5-Methylhexanoic acid	9.03	6.10	±	6.3	–	2.4		12.25	±	5.1	67.27	6.2		2.00	±	1.3	–	0.6		45.68	47.03	45.92
8	Methyl salicylate	15.35	4.27	±	2.6	–	1.7		0.00	±	0.0	–	0.0		0.00	±	0.0	–	0.0		47.37	48.56	
9	Decanal	15.83	26.54	±	5.0	75.86*	10.3		18.93	±	3.0	85.7*	9.6		33.10	±	11.1	62.27*	10.3		52.91*	56.57	53.93*
10	Undecanal	20.45	1.12	±	1.0	–	0.4		2.16	±	0.9	–	1.1		2.61	±	2.3	–	0.8		–	–	55.18
11	Unknown1	21.7	4.07	±	1.2	–	1.6	*a*	0.00	±	0.0	–	0.0	*b*	2.68	±	2.0	–	0.8	*ab*	54.06	57.75	
12	Decanoic acid	23.02	2.00	±	2.1	–	0.8		0.71	±	0.5	–	0.4		1.14	±	0.8	–	0.4		55.11	59.05	
13	1-Tetradecene	24.1	2.01	±	1.6	–	0.8		0.00	±	0.0	–	0.0		4.71	±	2.7	–	1.5		–	–	
14	(*Z*)-threo-Davanafuran	25.23	11.37	±	8.5	–	4.4	*ab*	0.44	±	0.4	–	0.2	*b*	17.67	±	10.2	–	5.5	*a*	57.23	63.51	58.84
15	β-Caryophyllene	25.36	1.42	±	1.2	–	0.6		1.07	±	1.1	–	0.5		2.70	±	2.6	–	0.8		–	–	
16	Neryl acetone	26.06	5.15	±	3.4	–	2.0	*b*	6.45	±	2.0	–	3.3	*a*	1.50	+	0.01	–	0.0	*b*	60.05*	–	61.31
17	Unknown2	27.2	4.66	±	3.5	–	1.8		0.00	±	0.0	–	0.0		3.27	±	3.2	–	1.0		61.57	65.13	
18	Pentadecane	28.82	18.33	±	5.8	86.18*	7.1	*b*	5.97	±	1.9	90.64*	3.0	*b*	45.54	±	14.6	87.72*	14.2	*a*	65.90*	73.15*	70.26
19	Unknown3	30.3	4.31	±	2.6	–	1.7		0.00	±	0.0	–	0.0		15.30	+	0.01	–	0.0		–	–	
20	1-Hexadecene	32.44	17.48	±	8.5	89.61	6.8		0.00	±	0.0	–	0.0		39.54	±	16.4	94.44	12.3		70.28	81.16*	77.31
21	Unknown4	34.9	0.00	±	0.0	–	0.0		0.79	±	0.6	–	0.4		4.83	±	1.0	–	1.5		–	–	78.86
22	Unknown5	37.3	6.04	±	4.0	–	2.3		0.00	±	0.0	–	0.0		0.00	±	0.0	–	0.0		71.46	–	
23	n-Pentadecanol	39.42	0.00	±	0.0	–	0.0	*b*	3.50	±	2.1	–	1.8	*a*	0.00	±	0.0	–	0.0	*b*	72.64	–	80.11
24	Unknown6	39.8	14.05	±	6.9	91.96	5.5		0.00	±	0.0	–	0.0		0.00	±	0.0	–	0.0		76.22	84.37	
25	1-Octadecene	40.02	10.06	±	7.0	–	3.9		0.00	±	0.0	–	0.0		8.78	±	6.8	–	2.7		78.09	86.87	
26	Unknown 7	47.45	0.00	±	0.0	–	0.0		37.66	±	35.6	–	19.1		0.00	±	0.0	–	0.0		84.86	–	86.96
27	Unknown 8	48.2	3.71	±	2.6	–	1.4		4.01	±	1.5	–	2.0		9.27	±	5.0	–	2.9		89.50	89.17	90.18
28	Unknown 9	48.8	4.31	±	3.3	–	1.7		0.00	±	0.0	–	0.0		2.20	±	1.2	–	0.7		90.71	90.51	
	Total		257	±	104		100		197	±	99		100		338	±	130		100				

Note: Compounds marked with an asterisk reliably typify (or act as reliable discriminating compounds between) the treatment group(s) (e.g. the average contribution to the overall similarity (or dissimilarity) is much greater than the standard deviation for that compound: avg. contribution/SD $\geq$ 1.0).

[a]Numbers are based on headspace collected between 0800 and 1500 h for $N = 15$ intact plants, $N = 7$ mechanically damaged plants, and $N = 11$ herbivore-damaged plants. Compounds occurring in $\leq$2 samples were not included in this table. Compounds were identified by comparison of mass spectra decomposition patterns to those in reference literature for plant volatiles (Adams 2009), and confirming their identity with commercial standards.

[b]Mean ± SE units are ng volatile/gram plant tissue/hour.

[c]Compounds accounting for at least 90% cumulatively in the average Bray–Curtis similarity within each treatment. Only those compounds are included that contribute the greatest amount to this percentage.

[d]Letters denote significant differences among treatments for a compound; treatments with shared letters are not significantly different from one another Tukey's HSD ($\alpha = 0.05$).

[e]Compounds accounting for at least 90% cumulatively to the Bray–Curtis dissimilarity between each pair of treatments.

[f]IN: intact headspace; MD: mechanically damaged headspace; HD: herbivore-damaged headspace. Pairwise comparisons between the volatile treatments. Overall average dissimilarity between IN vs. MD, IN vs. HD and MD vs. HD are: 71.32, 74.39 and 70.35, respectively.

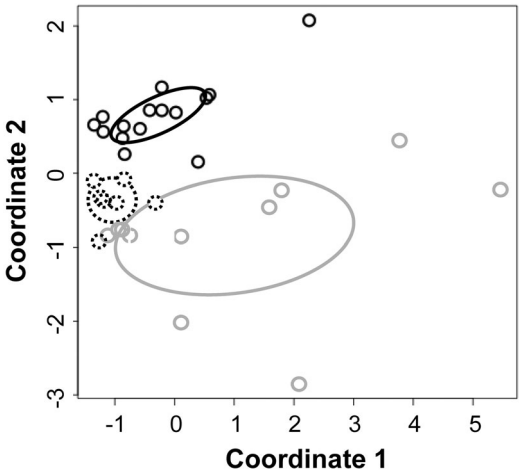

Figure 1. Non-metric multi-dimensional scaling plot of the differences in the headspace of asparagus plants assigned to one of three different treatments: intact, healthy (black, solid), mechanically damaged (black, dotted), or black cutworm-damaged (grey, solid). The ellipses of the treatment colors indicate the 95% confidence interval around the centroid of each group. Blends of volatiles were significantly different from one another among the treatments (ANOSIM: $R = 0.308$, $n = 32$, $p < .0001$).

From the olfactometer assays, we found that the asparagus miner was attracted to healthy asparagus stems over clean air. Asparagus miners seek out newly planted fields (Tuell 2003) that enter the fern stage (e.g. once harvesting of spears has ceased) early in the season, which are probably more attractive because of their relatively larger amount of biomass compared to fields that are actively being harvested. In a study on *Liriomyza sativae* Blanchard (Diptera: Agromyzidae), odor cues were found to be important for host-location of the crop, and these same cues also served as an aggregation cue for these leafmining flies (Zhao & Kang 2003).

Our headspace collection found aldehydes, alkenes, alkanes and alcohols characteristic of asparagus headspace. Sun et al. (2001) identified hexenal and 1-octene-3-ol as the most abundant compounds in ground-up asparagus samples. We identified green leaf volatiles as major components, including (*E*)-3-hexenyl acetate and (*Z*)-3-hexen-1-ol. We likely did not find the same main constituent of Sun et al. (2001), because we used a significantly different process of collecting the volatiles than they did; we collected naturally released volatiles from the plant, while they collected volatiles from flash frozen and heated-up asparagus pulp. The main compounds associated with the headspace from mechanically and herbivore-damaged asparagus plants in our study included: pentadecane, pentadecanol, (*Z*)-β-ocimene, (*E*)-3-hexenyl acetate and an unknown compound (#7). Common induced volatiles by caterpillars of various species in other systems include (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene and various terpenes in cotton (Röse et al. 1996) as well as (*Z*)-3-hexen-1-ol and β-caryophyllene in tobacco (De Moraes et al. 2001). In another study that examined the odor components of two-hour cooked asparagus, similar compounds were found as some of the components in our headspace collection, for example, many 5–10 carbon alkenes, alkanes, alcohols and aldehydes (Ulrich et al. 2001). Specifically, some of the volatiles included 1-pentanol, hexenal, 1-octen-3-ol, furan-containing and sulfur-containing compounds (Ulrich et al. 2001). In summary, many of the headspace compounds we found in asparagus are in alignment with findings for compounds identified earlier in the literature.

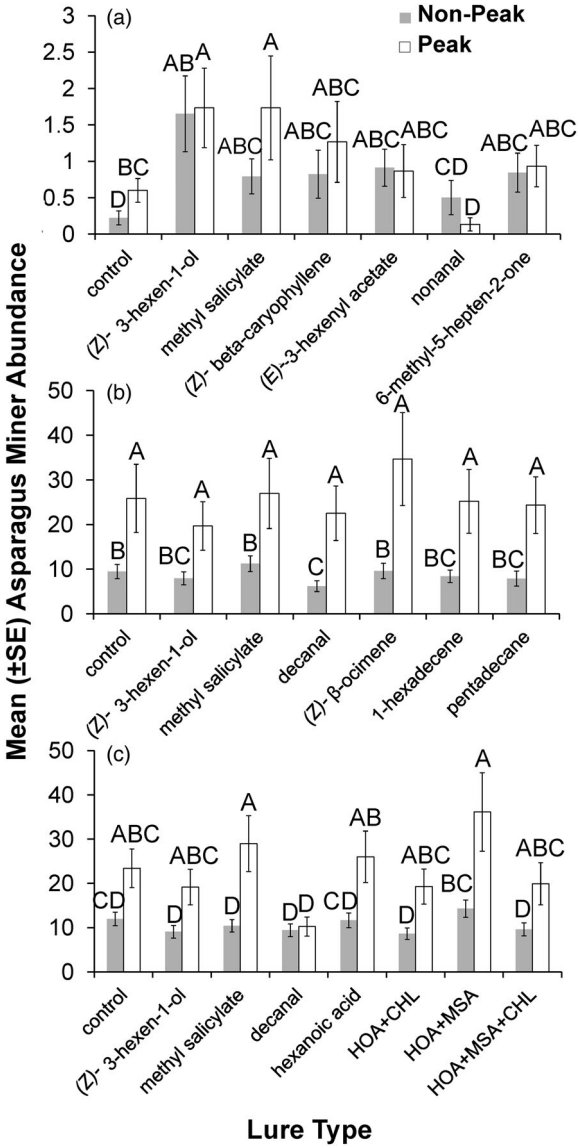

Figure 2. Mean (±SEM) weekly abundance of asparagus miner adults on yellow sticky traps baited with different volatiles (singly or in combination) during peak flight (white) or non-peak flight (grey) periods, which were deployed in commercial asparagus fields in Oceana Co., MI during (a) 2011: $N = 60$ per treatment, (b) 2012: $N = 222$, and (c) 2013: $N = 204$. CHL: (*Z*)-3-hexen-1-ol; HOA: hexanoic acid; MSA: methyl salicylate. Treatments with shared letters are not significantly different from one another (Tukey's HSD, $\alpha = 0.05$).

The asparagus miner showed a significant preference for methyl salicylate baited traps in 2011 compared to the control, but not in 2012 or 2013. When we paired methyl salicylate with green leaf volatiles such as hexanoic acid and (*Z*)-3-hexen-1-ol, we did not see significantly more miner adults on the traps. A review of the effect of methyl salicylate on various taxa in agricultural settings provided evidence that positive taxis towards methyl salicylate may be genetically conserved among Diptera (Rodriguez-Saona et al. 2011), but we found mixed support for this with the asparagus miner.

(*Z*)-3-hexen-1-ol, a general green leaf volatile (Paré & Tumlinson 1999), attracted the most asparagus miners in 2011 compared to all other treatments with the exception of methyl salicylate, but not in 2012 or 2013. This compound was only found in intact, healthy asparagus plants, which are attractive to the asparagus miner, suggesting that it may be a key compound affecting the behavior of this pest. Green leaf volatiles are widely attractive to insect taxa (Bruce et al. 2005), including Agromyzidae (James 2005). Another related species

of mining fly, for example, the serpentine leafminer (*L. sativae*), had the greatest antennal responses to a variety of green leaf volatiles, including (*Z*)-3-hexen-1-ol, and (*Z*)-3-Hexenyl acetate, in the headspace of lima beans (Zhao & Kang 2002).

From the field experiments we found that the behavioral response of the asparagus miner to the volatiles was phenology-dependent within a given year (i.e. peak flight or non-peak flight). It may be that different cues are relatively more important to the asparagus miner during different times in its life cycle. This fine-scale differentiation may be made possible by the miner's physiology because plant volatiles and pheromones are processed in two different nerve centers in insects (for a review, see Martin & Hildebrand 2010).

Though inconsistent, our results suggest that methyl salicylate and (*Z*)-3-hexen-1-ol may be involved in the attraction of the asparagus miner, depending on context and other related factors. The differences in asparagus miner responses among years to the various asparagus volatiles can be due to the fact that the community of organisms on asparagus varies from year to year. Each pest (disease or insect) is likely to induce the asparagus in a different way, eliciting some unique and some more general volatile cues. Because certain pests are found in higher abundance in certain years than others, some of the concentrations of these volatiles may differ between years, providing a different backdrop of volatiles from which the asparagus miner must find its host plant. Previous studies have suggested that the backdrop of volatiles in which an insect perceives a specific compound may modify its ecological role and the subsequent behavioral response (Webster et al. 2010). Future studies evaluating the chemical ecology of asparagus should investigate the role that other insects play in inducing asparagus, and how those volatiles may differ from the ones found here.

In a review of 34 published studies, including over 50 herbivores, Szendrei and Rodriguez-Saona (2010) found that plant volatile baits were most attractive when they contained multiple compounds. Our study employed a maximum combination of three volatiles within a single bait, but this may have been insufficient to elicit substantive attraction or repulsion by the asparagus miner. In fact, some studies have found that blends as complex as those containing five compounds are needed to maintain or increase attraction to a bait equivalent to that found in the natural headspace of a host plant (Piñero & Dorn 2007). For example, if a bait were to be used to monitor the asparagus miner, those volatiles showing attraction for this insect should be combined into a bait with multiple components in the ratios present in the plant (Szendrei & Rodriguez-Saona 2010).

Overall, our research is expected to contribute to basic and applied knowledge for an integrated pest management program for the asparagus miner. Outstanding topics that need to be investigated in the chemical ecology of asparagus include (1) understanding how the volatile blends of asparagus change with other specialist pests and their interactions with the mid-and late-season pest complex of asparagus; (2) using GC-EAD with the asparagus miner to screen the volatiles described in this study and additional synergistic ones for biological activity; and in the longer term; (3) understanding the role of pheromones in mediating the behavior of the asparagus miner among conspecifics and if these may be used for pest management; and finally (4) elucidating the influence of asparagus volatiles on potential conservation biological control candidates for the asparagus miner (e.g. *Thinodytes cephalon* and *Chorebus rondanii*: Morrison III, Gibson et al. 2014).

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Disclosure statement

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