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Comparison of ex situ volatile emissions from intact and mechanically damaged walnuts

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ABSTRACT

The codling moth (*Cydia pomonella*) and navel orangeworm (*Amyelois transitella*) are insect pests that inflict serious economic damage to California walnuts. Feeding by these larvae causes physical damage to the nut and can lead to contamination by aflatoxigenic fungi. Over the years volatile natural products have played a critical role in efforts to control or monitor these and other insect pest moths.

The ex situ volatile emissions from intact and mechanically damaged Howard variety walnuts from the California Central Valley were evaluated over the course of a typical growing season. The volatile profiles were compared and differences in emission considered as a means to identify candidate volatiles for use in host plant-based attractants or in conjunction with pheromone blends to enhance attractancy.

Walnut volatiles were extracted by headspace solid phase microextraction (HS-SPME) in a semi-closed system and analyzed by gas chromatography mass spectrometry (GC–MS). Ninety two volatiles were identified, including monoterpenes as the predominant class of compounds. A multivariate analysis of the data highlighted two sampling periods (late July–late August) where intact walnuts and mechanically damaged walnuts can be distinguished due to their volatile profile composition.

The results of this study provide relevant information regarding potential host plant-based semiochemicals of two insect pests, valuable data regarding the ambient odors these insects encounter in walnut orchards and add some potentially interesting volatile compounds to the existing literature.

1. Introduction

Almonds, pistachios and walnuts are food commodities affected by food safety and trade issues associated with aflatoxin contamination. Insect feeding damage can lead to contamination by aflatoxin, which is produced by the ubiquitous orchard fungi *Aspergillus flavus* and *Aspergillus parasiticus*. However, hulls of walnuts are most highly resistant to *Aspergillus* growth in comparison with other tree nuts such as pistachios and almonds (Campbell, Molyneux, & Schatzki, 2003). Two principal insect pests of tree nuts are larvae of the codling moth, *Cydia pomonella* (L.) (Lepidoptera, Tortricidae), infesting husks and kernels of walnuts and the navel orangeworm, *Amyelois transitella* Walker (Lepidoptera, Pyralidae), infesting kernels of almonds, walnuts and pistachios. Because navel orangeworm cannot infest sound, uninjured nuts, the principal strategies of its management are orchard sanitation to reduce overwintering populations, prompt harvest and protection of the crop from in-season hull damage, including walnut blight, sunburn and codling moth infestation. The in-season codling moth control program is especially critical to effectively managing navel orangeworm in walnut orchards (UC IPM, 2014).

Environmental concerns and the development of insecticide-resistant populations have promoted the use of more environmentally safe techniques for pest control and monitoring in agriculture and food production. Pheromones of different types (sex, aggregation) are the most important class of attractants used in pest control, and have been used globally in numerous crops for mating disruption, lure and kill, or mass trapping (Beck & Higbee, 2013). For example, the navel orangeworm sex pheromone has for long been known and documented by many researchers (Coffelt, Vick, Sonnet, & Doolittle, 1979). However, host-plant volatiles are growing in importance in the control of different insect pests (Beck, Higbee, et al., 2012; Beck, Mahoney, Cook, & Gee, 2012; Beck & Higbee, 2013; James, 2003; Light et al., 2001; Reddy, Cruz, Bamba, & Muniappan, 2005). Additionally, fungal spore-associated volatiles have been recently recognized as attractants for lepidopteran insects, suggesting that fungal spores posses an important role as signaling when plant may be vulnerable to insect pests (Beck, Baig, Cook, Mahoney, & Marsico, 2014).

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In recent years, some many studies have been carried out on the behavioral and electrophysiological responses of navel orangeworm to host volatile emissions (Beck, Higbee, et al., 2012; Beck, Mahoney, et al., 2012; Beck, Baig, et al., 2014; Beck & Light, 2014; Phelan, Roelofs, Youngman, & Baker, 1991), to find alternative attractants to the sex pheromone for pest monitoring. A blend of host plant volatiles, based on various almond emissions, has demonstrated effective attractancy of both male and female navel orangeworms. However, studies carried out suggested that either an orchard specificity of the moth or perhaps a temporal component expressed as a change in background odors of the orchards (Beck, Mahoney, Higbee, et al., 2014). Although many advances have been made with respect to navel orangeworm response to host volatiles, with most of the effort focused on almond and pistachio (Beck, Higbee, Merrill, & Roitman, 2008; Beck, Mahoney, Cook, et al., 2014; Mahoney, Gee, Higbee, & Beck, 2014) little progress has been made on navel orangeworm responses to walnut volatiles.

The aims of the present study were to characterize and compare the volatile profiles of mechanically damaged walnuts from the Howard variety with that of intact walnuts to ascertain if any volatiles were unique to damaged walnuts. For this purpose, walnut volatiles were collected ex-situ at five different phenological stages of the tree. The volatiles were extracted by headspace solid phase microextraction (HS-SPME) and then identified by GC–MS. From these experiments, the basic background volatile profile of walnut was also obtained, which could provide useful information regarding the ambient volatile bouquet that insect pests encounter in walnut orchards. Finally, those compounds were compared to the extensive database of electroantennographic (EAG) assay responses of navel orangeworm antennae to almond and pistachio volatiles from previous studies (Beck, Light, & Gee, 2014) to determine likely semiochemicals produced by walnuts.

2. Materials and methods

2.1. Plant material

Fruits of *Juglans regia L.*, variety Howard, were collected every 3 weeks from mid May to late August 2014 from the commercial orchards of D & D Farms, Yuba City, CA, USA. Each batch was analyzed in triplicate over different days. Collections of six walnut fruits were made every three weeks over five different periods in the season to provide a representative profile of fruit emissions at varying developmental stages: May 19 (monitoring 1), June 9 (monitoring 2), June 30 (monitoring 3), July 21 (monitoring 4), and August 11 (monitoring 5). Collections were performed in the morning and sample trees were chosen randomly from three different trees each period.

Batch 1 consisted of control walnuts that were not injured, removed from the tree and placed in lunch paper bags (a bag for each tree). Batch 2 consisted of walnuts that had been injured after detached from the tree, and then placed in lunch paper bags (a bag for each tree). The injury/damage consisted of hull penetration (10 times) with a sterilized nail (3 mm diameter). Batches 1 and 2 were collected during concurrent time frames and transported immediately to the USDA-ARS facility in Albany, CA, USA for headspace analysis.

2.2. Collection of volatiles

To obtain the highest recovery of the analytes, different extraction times and fiber types were studied. For the selection of the fiber type (PDMS, DVB/CAR/PDMS and PDMS/DVB), three-similar-weight walnuts were used. Initially, the extractions were carried out at 30 °C in a closed system and, after 5 min of pre-heating time, each fiber was exposed (E) for 2 min to the sample headspace. Once the fiber was selected, the influence of the extraction time was studied (E = 1, 10, 20, 30 and 40 min) by extracting, this time, five-similar-weight walnuts with the optimum fiber. Extraction temperature (30 °C), storage time (S) of the adsorbed volatiles on the fiber (S < 1 min) and desorption time (T = 6 min) were set according to previous works (Beck, Mahoney, et al., 2012; Beck, Mahoney, Cook, et al., 2014).

For HS-SPME volatile collection walnuts (ca 6 per experiment 1 per each container) were placed in 250 mL modified vessels with special adapters (see Fig. 1). Modified vessels were fitted with an inlet for HS-SPME extraction and a venting port. After HS-SPME adsorption of the volatiles, the headspace of the jars was gently vented with 250 mL of air via a glass 250 mL syringe and through a sterile Millipore Millex-GP 0.22 μm filter. Headspace volatiles of the triplicates of both batches were monitored on days 0, 2, 4, 7, 9 and 15 in the semi-closed system (Fig. 1). The collection chambers were maintained at 30 °C during storage and SPME volatile collections. Mechanically damaged walnuts were analyzed before (control) and after hull injury with the aim to compare intact with control walnuts.

2.3. Gas chromatography/mass spectrometry (GC/MS) analysis

Collected volatiles were desorbed onto a DB-1MS column (30 m × 0.25 mm i.d. × 0.25 μm; J&W Scientific, Folsom, CA, USA) installed on an HP 6890 gas chromatograph (GC) coupled to an HP 5973 mass selective detector (MSD) (Hewlett Packard, Palo Alto, CA, USA). Extracts were analyzed with the following method: injections by SPME; injector temperature, 200 °C; splitless mode; He constant flow, 1.2 mL min^{-1}; oven settings: initial temperature, 40 °C; ramp, 10 °C min^{-1}; and final temperature, 260 °C. The MSD parameters were as follows: source temperature, 230 °C; MS quadrupole temperature, 150 °C; electron impact (EI) mode, 70 eV; and solvent delay, 2 min.

To obtain retention times for additional analysis of RI values, some supplemental samples were also injected onto a DB-Wax column (60 m × 0.32 mm i.d. × 0.25 μm; J&W Scientific, Folsom, CA, USA). These extracts were analyzed with the following method: injector temperature, 200 °C; splitless mode; He constant flow, 3 mL min^{-1}; oven settings: initial temperature, 40 °C; hold time; ramp, 4 °C min^{-1}; and final temperature, 240 °C.

2.4. Statistical analysis

Univariate data analysis was carried out between intact and control walnuts as well as between intact and mechanically damaged walnuts by means of F-test and t-test (Excel). The F-test compared the variances of two distributions, while the t-test (*unequal variance* or *equal variance t-test*) compared their means. Differences between sample groups and treatments were considered statistically significant at $P < 0.05$.

Multivariate analysis of the data was carried out by Principal Component Analysis (PCA) using The Unscrambler® program (v.7.6, Camo, Trondheim, Norway). The compounds not detected in some samples were assumed as missing values. Compounds not detected in at least two of the three replicates within any sampling period were omitted

Fig. 1. Volatile collection system (semi-closed system) used to collect ex-situ walnut volatiles by HS-SPME and the venting set up used for headspace exchange after each HS analysis.