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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.

from analyses. Logarithm transformation of the raw data was calculated in order to assure a normal distribution, and then the data was scaled to assure a common variance. All models were built by full cross-validation. In PCA, uncorrelated principal components (PCs) were extracted by linear transformations of the original variables so that the first few PCs contained most of the variations in the original dataset (Esbensen, 2001).

3. Results and discussion

3.1. HS-SPME procedure

To set the best procedure for the qualitative analysis of walnut volatile emissions the influence of type of fiber and extraction time was studied in order to extract the greatest number of compounds. Based on the normalized chromatographic peak areas, the results revealed that the less effective fiber was PDMS, followed by PDMS/DVB. Since higher chromatographic signals were obtained using the DVB/CAR/PDMS fiber for most of the compounds, it was used in all remaining experiments. Accordingly different extraction times (1, 10, 20, 30 and 40 min) were then studied. The extraction time profiles obtained showed that the minimum extraction time required to reach maximum extraction was estimated to be about 20–30 min for all the compounds. During prolonged extraction time, a slight decrease of the chromatographic peak area could be observed for some of the major compounds. Extraction time was thus fixed at 30 min as a compromise between sufficient sensitivity and reasonable extraction time.

Table 1
Volatile compounds detected from ex-situ headspace analysis of walnut fruits over a four-month seasonal period. Compounds were verified by comparison with an authentic standard when possible and compared to internally generated data base values. Volatiles are listed in ascending order regarding the abundances obtained together with their calculated retention indexes (RI). When standards were not available, retention indexes and/or mass spectra were only compared with internally generated data base values and/or with NIST (NIST02) and Wiley (7th) fragmentation pattern databases ([a,b,c,d]).

	Compound	RI sample	RI stand.		Compound	RI sample	RI stand.
1	β-Pinene	973	975	47	β-Cubebene	1389	1390
2	α-Pinene	934	936	48	Phellandral[a,*]	1180	–
3	1,8-Cineole	1022	1023	49	Calarene[*]	1430	1431
4	Sabinene	969	969	50	Carvone	1215	1215
5	Limonene	1023	1025	51	Allo-ocimene	1133	1133
6	*Trans*-β-ocimene	1040	1041	52	β-Selinene[*]	1485	1485
7	β-Myrcene	984	985	53	γ-Cadinene[*]	1509	1509
8	Germacrene D	1479	1480	54	Cyclofenchene[a,*]	1150	–
9	β-Caryophyllene	1421	1423	55	*p*-Cymenene[a,c]	1075	–
10	Pinocarvone	1141	1142	56	*Trans*-p-mentha-1(7),8-dien-2-ol[a,*]	1117	–
11	*Trans*-pinocarveol	1125	1127	57	(*E*)-4-decen-6-yne[a,*]	878	–
12	Myrtenal	1171	1171	58	δ-Guaiene[*]	1502	1502
13	Myrtenol	1180	1181	59	Neryl acetate	1344	1344
14	*p*-Cymene	1013	1014	60	unk3 (69, 67, 81, 138, 41,)[d]	1078	–
15	*Trans*-verbenol	1130	1127	61	*Cis*-3-hexenyl acetate	988	989
16	*Trans*-β-farnesene	1449	1449	62	unk4 (79, 91, 107, 77, 105)[d]	1201	–
17	Bornyl acetate	1272	1272	63	Camphor[b,*]	1121	1118[b]
18	Camphene[*]	947	950	64	1,4-Cyclohexadiene,1-methyl[a,c,*]	758	–
19	α-Campholenic aldehyde	1105	1105	65	unk5 (79, 77, 94, 91, 43)[d]	981	–
20	Isopinocamphone	1153	1154	66	Butanoic acid, 2-methyl,ethyl ester[*]	838	838
21	Nopinone[a]	1106	–	67	α-Cubebene	1352	1353
22	γ-Terpinene	1053	1053	68	Tricyclene[*]	923	922
23	unk1 (91, 92, 119, 134)[d]	1091	1127	69	Sabinaketone[a]	1127	–
24	Linalool	1085	1086	70	Hexanoic acid, 2-methylpropyl ester[*]	1137	1136
25	α-Thujene	926	928	71	Calarene[*]	1434	1436
26	unk monoterp (121, 93, 136, 44, 41)[d]	1084	–	72	*Cis*-p-mentha-1(7),8-dien-2-ol[a,*]	1155	–
27	α-Terpinolene	1081	1081	73	Myrtenyl acetate[a,*]	1224	–
28	Verbenene[a,c,*]	951	–	74	Tridecane	1299	1300
29	Sabinene hydrate	1056	1059	75	(E)-4,8-dimethyl-1,3,7-nonatriene	1106	1107
30	Chrysanthenone	1099	1100	76	1,5,8-p-Menthatriene[a,*]	1276	–
31	α-Phellandrene	997	998	77	Decanal	1186	1186
32	*Cis*-ocimene	1029	1030	78	Cyclosativene	1373	1373
33	β-Bourbonene	1386	1386	79	Valencene	1491	1492
34	α-Terpinene	1010	1012	80	3-Hydroxy-2-butanone	679	680
35	α-Humulene	1454	1454	81	Perillaldehyde	1247	1248
36	Zingiberene[b]	1489	1486[b]	82	Squalene[a]	1570	–
37	Terpinen-4-ol	1164	1164	83	unk sesq (161, 105, 119, 41, 204)[d]	1396	–
38	δ-Cadinene	1516	1518	84	Borneol	1153	1152
39	α-Terpineol	1174	1175	85	Isopiperitenone[a]	1240	–
40	γ-Amorphene[b]	1474	1469[b]	86	*p*-Mentha-1,8-dien-6-ol[a]	1281	–
41	*p*-Cymen-8-ol	1161	1162	87	6-Methyl-5-hepten-2-ona	964	965
42	*Trans*-carveol[b,c]	1197	1196[b]	88	Octanal	981	983
43	Bicyclogermacrene[b,c]	1494	1489[b]	89	unk6 (97, 69, 43, 112, 41,)[d]	907	–
44	Epi-bicycloses-quiphellandrene[a,c]	1462	–	90	α-Eudesmol[b,c]	1644	1639[b]
45	unk2 (69, 67, 138, 41, 66)[d]	1060	–	91	Hinesol[a]	1628	–
46	Caryophyllene oxide	1574	1577	92	Agaruspirol[a]	1618	–

[a] Tentative assignment by comparison with NIST and Wiley fragmentation pattern databases, compound not available for authentication.
[b] RI calculated relative to *n*-alkanes on DB-1 and compared to internally generated data base values, compounds not available for authentication.
[c] Detected in two columns DB-1 and DB-wax, compounds not available for authentication.
[d] Five highest fragmentation peaks (m/z) provided for unknown monoterpene (unk monoterp) unknown sesquiterpene (unk sesq) or unknown class of compounds (unk1-6).
[*] Compounds not detected in other works dealing with the analysis of walnut volatile emissions (Buttery et al., 2000; Casado et al., 2008; Elmore et al., 2005; Lee et al., 2011).

3.2. Ex-situ emission of walnut volatiles

Table 1 lists the volatiles detected from the headspace analyses of the walnut samples during the 2014 growing season. Compound identification was confirmed by injection of synthetic compounds when possible. When standards were not available, retention indexes (RIs) and/or mass spectra were compared with those in internally generated databases. National Institute of Standards and Technology (NIST) and Wiley databases were used for fragmentation pattern identification. RIs were calculated using a homologous series of *n*-alkanes on a DB-1MS and a DB-Wax. Volatiles included in Table 1 fulfilled the following assumptions: Volatiles were detected in at least two of the three replicates at any sampling period and their relative abundances (peak areas) were greater than 0.5% of the largest volatile organic compound peak present in each run. Note that intact and control samples were statistically comparable in all the cases (F and t-tests, $P < 0.05$).

Using the criteria noted above, a total of 92 volatile compounds, common to all walnut samples (intact, control and mechanically damaged walnuts), were identified and listed in Table 1. Compounds that did not match the RIs of known volatile compounds from our database and/or did not provide sufficient mass fragmentation pattern matches were assigned as unknown. Eight compounds could not be identified. Based on common mass fragments, one unknown was deemed to be a monoterpene, and second was deemed a sesquiterpene, and the remaining non-identifiable compounds were termed "unknown 1" to "unknown 6". All of the volatiles presented in Table 1 were apparent at two or more sampling dates with the exception of (E)-4,8-dimethyl-1,3,7-nonatriene, borneol and valencene, which were present only in the first monitoring. The compound octane was present only in the second monitoring. Fifty two compounds out of 92 (Table 1) were detected consistently throughout the entire sampling period.

The vast majority of the volatiles detected were terpenoids including 39 monoterpenes, 24 sesquiterpenes and 1 triterpene. In addition 21 detected compounds comprised alcohols, ketones, aldehydes, esters and aliphatic hydrocarbons. None of the compounds listed in Table 1 are confirmed semiochemicals of navel orangeworm and codling moth taking into account current literature (Beck, Light, et al., 2014; Beck, Mahoney, Cook, et al., 2014; Vallat & Dorn, 2005). However, some of the compounds identified in this study have been shown to be either electrophysiologically or behaviorally active in codling moth and navel orangeworm bioassays. They include for example, β-ocimene, α-humulene, β-caryophyllene, linalool, sabinene hydrate, *cis*-ocimene, α-pinene, terpinen-4-ol and α-terpinolene (Beck, Higbee, et al., 2012; Beck, Mahoney, et al., 2012; Beck, Light, et al., 2014; Casado, Gemeno, Avilla, & Riba, 2008). Of the sesquiterpenes detected in the present study, valencene is among this class of compounds that elicited the strongest antennal response from male and female navel orangeworms (Beck, Light, et al., 2014). These will be discussed in-depth presently.

As a summary of the quantitative releases, the total mean peak area at a given sampling date was summed over all compounds (Fig. 2) and divided into two groups (intact and mechanically damaged walnut). A total of two maxima of emissions were reached at distinct times in the season: the first maximum was reached in late June (third monitoring, M3) and the second one late in July (fourth monitoring, M4). Quantities of volatiles were lowest at the end of May (first monitoring, M1), early in June (second monitoring, M2), and in the mid of August (fifth monitoring, M5). Significant differences in total volatile release (sum of all peak areas) were found between intact and mechanically damaged walnut samples.

The predominant compound in all collection periods was the monoterpene β-pinene. The compounds, α-pinene, 1,8-cineole, sabinene, limonene and *trans*-β-ocimene were other highly emitted monoterpenes. These six monoterpenes represented on average the 80% ± 4% of the walnut volatile composition. The rest of compounds (Table 1, from 7 to 92) constituted on average the 20% ± 4% of the walnut volatile

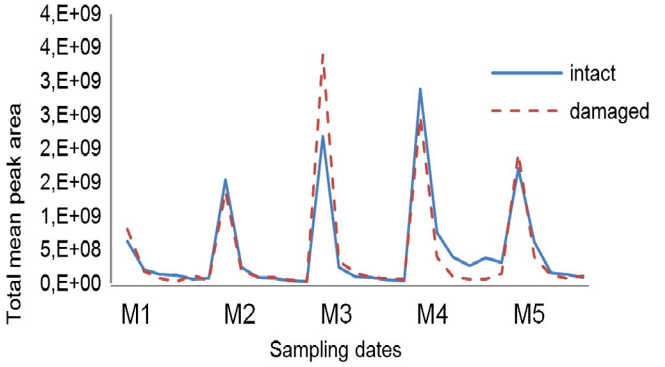

Fig. 2. Total mean peak area (n = 6 samples for each sampling date) of the sum of all compounds detected over the growing season of 2014, from May 19 to August 26.

profile. With regard to the compounds emitted in lower amounts, there were a number of compounds not detected in other works dealing with walnut analyses (indicated in Table 1 with an asterisk *) (Buttery et al., 2000; Casado et al., 2008; Elmore, Nisyrios, & Mottram, 2005; Lee, Vázquez-Araújo, Adhikari, Warmund, & Elmore, 2011). Since minor volatiles have shown in several instances to be responsible for insect biological activity (Clavijo-McCormick, Gershenzon, & Unsicker, 2014) they should be also considered as possible sensory cues.

29 compounds not included in Table 1, but verified by comparison with an authentic standard, were inconsistent, in that they only appeared in one of the three samples and/or represented less than the 0.5% of the largest volatile organic compound peak, β-pinene. They include ethyl propionate (696), 3-methyl-1-butanol (719), 2-methyl-1-butanol (722), *cis*-3-hexenal (773), 1-octene (789), octane, (*E*)-2-hexenal (828), butanoic acid 3-methyl ethyl ester (840),1-hexanol (850), nonane (900), benzaldehyde (932), 1-octen-3-ol (966), *trans*-linalool oxide (1075), nonanal (1083), 2-phenylethanol (1084), 1-undecene (1090), *cis*- and *trans*-limonene oxide (1117, 1122), fenchyl acetate (1208), *p*-mentha-1,8-dien-7-al (1247), α-longipinene (1356), geranyl acetate (1361), α-copaene (1379), β-ylanglene (1414), α-bergamotene (1433), β-bisabolone (1500), *cis*-calamenene (1512), and other unknown compounds, among them two sesquiterpenes (1416, 1422). Another compound detected in this study, 2-ethyl-1-hexanol (433), was reported in a previous work (Beck, Baig, et al., 2014) to be a possible contaminant. This was confirmed in present study by running several blank jars not used previously in these walnut analyses. The chromatographic signal corresponding to 2-ethyl-1-hexanol appeared constant in the different replicates carried out with different SPME fibers. Among the inconsistently emitted compounds listed above, nonanal and 1-octen-3-ol have been shown to have navel orangeworm semiochemical activity (Beck, Higbee, et al., 2012; Beck, Mahoney, et al., 2012; Beck, Mahoney, Higbee, et al., 2014). The compound 1-octen-3-ol was present in the first monitoring and only in one of the intact walnuts. This compound has been associated with fungal emissions and is a semiochemical for several insects including navel orangeworm (Beck, Mahoney, Higbee, et al., 2014). Other volatile compounds noted to occur during fungal growth (particularly *Aspergillus* species) were the aforementioned 2-methyl- and 3-methyl-1-butanol. Additionally, 2-phenylethanol is also related to *Aspergillus* fungi. Fungi on host plant can play an important role in the communication between plants and insects, and therefore fungal spores-associated volatiles like these three compounds have been studied in previous studies (Beck, Baig, et al., 2014; Beck et al., 2008). For instance, 1-octen-3-ol elicited high electrophysiological responses from both male and female navel orangeworm antennae, with 2-phenylethanol eliciting a very strong response from male navel orangeworm antennae (Beck, Light, et al., 2014). Because the study sought to investigate whether or not fungi would play a role in emitting known semiochemicals in response to the damaged walnut tissue, it was anticipated that the spiroketal

chalcogran or other common fungal volatiles such as 2-alkanones, 8-carbon alcohols, 2-pentylfuran, among others, would be detected. Walnuts are known to be rich in linolenic acid, which under certain conditions is a known precursor for the spiroketals chalcogran (Beck, Mahoney, Cook, et al., 2014). In the present study and unlike a similar study on ex-situ almonds (Beck et al., 2008), no findings with regard to fungal growth were discovered. The absence of compounds indicative of fungal growth could possibly be due to the high content of monoterpenes present in walnut samples, which have been implicated in antifungal activity (Magwa, Gundidza, Gwerua, & Humphrey, 2006). This observation was also purported regarding the lack of fungal volatiles detected in pistachios (Beck, Mahoney, Higbee, et al., 2014), which are also high in monoterpene content. In addition, walnuts have a lower potential for exposure to aflatoxin, and therefore to fungal-spore associated compounds, because walnut kernels are relatively free of aflatoxin when compared to other tree nuts (Campbell et al., 2003). As walnuts started to dry towards the end of the season, the content of monoterpenoids was expected to decrease and therefore, increase fungal volatile emission. On the other hand, increases of some monoterpenes (for example α-pinene, β-pinene and β-ocimene), some sesquiterpenes and linalool have been reported from apples, corn and cotton plant parts following insect herbivore attack, which may indicate a response by the plant to the adverse situation (Buttery et al., 2000).

Lastly, the compounds unique to the damaged walnuts, albeit in trace amounts, were 3-hydroxy-2-butanone, ethyl propionate, 1-octene and the two unknown sesquiterpenes (1416, 1422). The compounds 3-hydroxy-2-butanone and 1-octene have been reported as volatiles from bacteria and fungi, respectively (Kiviranta et al., 1998). 3-Hydroxy-2-butanone has also been reported from yeast-inoculated banana (Phelan & Hengchen, 1991). Though reported as a semiochemical of other insect species, 3-hydroxy-2-butanone elicited very weak responses from both male and female navel orangeworm antennae (Beck, Light, et al., 2014). The detection of 1-octene was interesting given that its 8-carbon alcohol and ketone relatives are more commonly reported as fungal volatiles and semiochemicals (Davis, Crippen, Hofstetter, & Tomberlin, 2013) but rarely is 1-octene reported as a fungal volatile. Finally, the short-chain ester ethyl propionate, a component of oil palm, is known to enhance the response of palm weevil to the weevil aggregation pheromone (Gries et al., 1994). Ethyl propionate and 1-octene have not been subjected to navel orangeworm bioassay studies.

3.3. Multivariate analysis

Though no significant differences were observed between intact and damaged walnuts using F-test and t-test ($P < 0.05$), a multivariate analysis was carried out. In this instance, instead of evaluating one variable at a time all variables were evaluated simultaneously.

Initially, correlation analysis was applied to evaluate the relationships among the volatile compounds. Taking into account the abundances obtained over the entire sampling period, the first 34 compounds listed in Table 1 (the most abundant and consistent compounds) were considered for PCA. Based on log-transformed values, cross correlations were very strong for the most abundant compounds (1–34, Table 1). Fifty two percent of the variables showed Pearson correlation coefficients higher than 0.90, and the 87% higher than 0.80. Conversely, the less abundant compounds (35–92, Table 1) exhibited much lower correlation coefficients.

Both data sets (intact and damaged) were statistically analyzed by PCA in order to determine sample or variable clustering. A prior criterion has been set to select the number of principal components (PC) that explain a maximum amount of variance. In all cases, only the first three PCs were considered to interpret the projection plots since those three components explained more than 90% of the total variance.

When all samples were considered, no differences between intact and mechanically damaged walnut samples were found over the five sampling periods (Fig. 3). Samples of the first day monitoring (non 0 and dmg 0, Fig. 3) are exceptional, likely due to their total volatile concentration, and in this case, they were considered outliers in order to simplify the resulting model. In this way, the first component (PC1) was easily identified as volatile content, while the contribution on PC2 seemed to be more representative for the characterization on samples and variables.

Once those samples were eliminated, the seasonal variation of the samples was more noticeable in the PC2/PC3 projection (20% of the total variance explained), as shown in Fig. 4a. The large separation of M1 seen in Fig. 4a may be due to incomplete nut development at the beginning of the season (the end of April and beginning of May, M1), and thus a different volatile fingerprint. Walnuts are known to reach their final size in mid June (Bezemer & Mills, 2001) depending on the weather. In this sense, from M2 on, the samples were mostly grouped. If this rationalization is valid, it would explain the isolation of M1 and the possible clustering of the remaining monitoring periods. Also, M1 could be

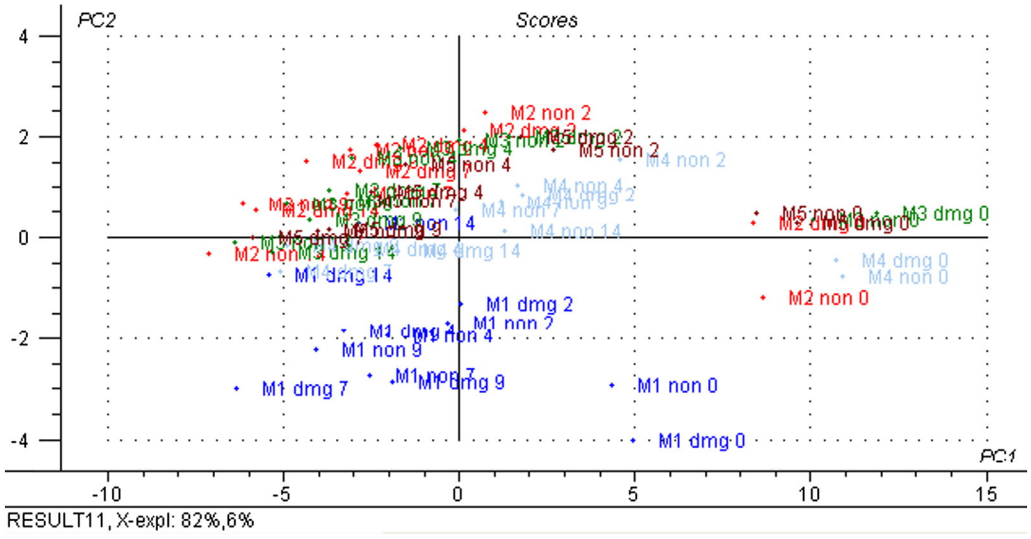

Fig. 3. Principal Component scores of the first two canonical variables of the volatiles emitted by walnut samples. M1 = 1st monitoring, M2 = 2nd monitoring, M3 = 3rd monitoring, M4 = 4th monitoring, M5 = 5th monitoring, non = non-damaged walnuts (intact walnuts), dmg = damaged walnuts, 0 = day 0, 2 = day 2, 4 = day 4, 7 = day 8, 9 = day 9, and 14 = day 14.

linked to an initial maturation stage, which was positively associated with the compounds *trans*-β-farnesene, germacrene D, *trans*-β-ocimene and β-caryophyllene and negatively associated with verbenene, isopinocamphone, camphene, *trans*-verbenol and α-campholenic aldehyde. From the obtained data it can be observed that the relative abundances of some of these compounds decreased in subsequent stages. For example, *trans*-β-ocimene, which is in the top 6 (Table 1) represented between 9 and 10% of the walnut volatile profile during M1 and then, its contribution decreased to a 2–5% of the total composition, which remains constant until the last sampling period (from M2 to M5). The converse was noted for the unknown monoterpene (1084), which was found from M3, the third monitoring period, and on through M4 and M5.

To demonstrate more definitively the differences between intact and mechanically damaged walnuts, an in-depth study of the volatile profiles was performed using PCA for each sampling period. While M1, M2 and M3 did not show any clear distinction between intact and damaged walnuts (data not shown), the samples corresponding to M4 and M5 showed differences between both groups (see Figs. 5 and 6).

Fig. 5 shows the plot obtained in the fourth sampling period for intact and damaged walnuts. The PC2/PC3 biplot (10% of the variance explained) for the fourth monitoring samples showed two groups of loadings (black circles, Fig. 5a). One group included isopinochampone, γ-terpinene, α-terpinene, camphene, β-pinene, α-pinene, α-thujene, p-cymene, 1,8-cineole, α-terpinolene, *trans*-β-ocimene, verbenene, nopinone, myrcene and sabinene (in the right side of PC2), and the other group showed pinocarvone, α-campholenic aldehyde, unknown monoterpene, linalool, sabinene hydrate, chrysanthenone, bornyl acetate, *trans*-pinocarveol, *trans*-β-farnesene, *trans*-vervenol, β-bourbonene, myrtenol, unknown 1, β-caryophyllene, *cis*-ocimene, α-phellandrene, germacrene D and myrtenal (in the left side of the PC2). Regarding walnut samples they were clearly divided into two groups (except one of the damage sample, M4 dmg2) (see Fig. 5b). Intact walnuts were located up to the right side of the PC2, while damaged

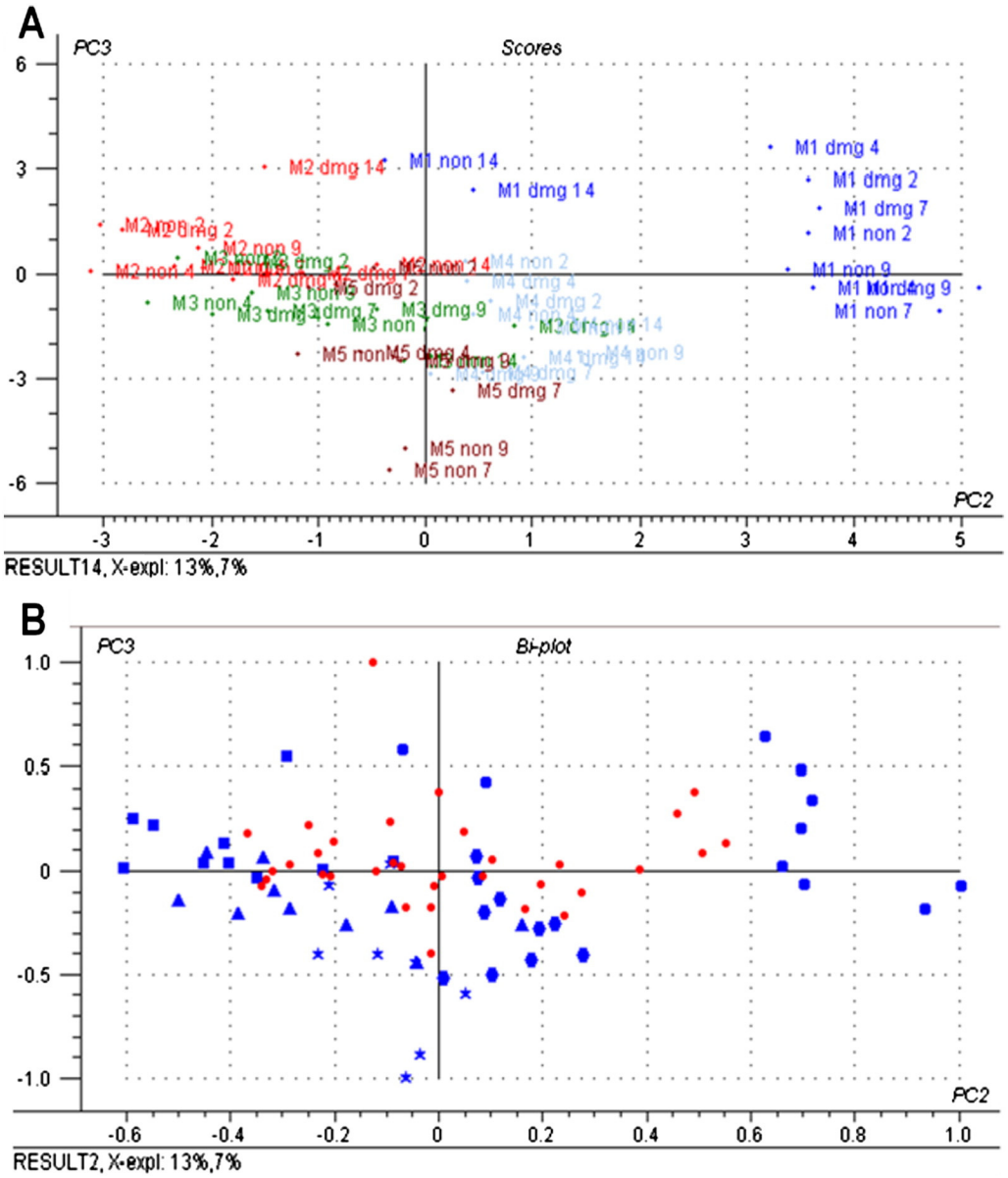

Fig. 4. Principal component analysis of the second and the third canonical variables of the volatiles emitted by walnut samples. M1 = 1st monitoring, M2 = 2nd monitoring, M3 = 3rd monitoring, M4 = 4th monitoring, M5 = 5th monitoring, non = non-damaged walnuts (intact walnuts), dmg = damaged walnuts, 2 = day 2, 4 = day 4, 7 = day 8, 9 = day 9, and 14 = day 14. (A) Principal Component scores (B) Factor loading and principal scores extracted from the five sampling periods.

samples were located down to the left side of the PC2. Following the direction of the arrow depicted in Fig. 5b the intact walnuts were better characterized by the variables of the right side group, being isopinochampone, γ-terpinene and *cis*-ocimene the most contributing. Mechanically damaged walnuts would be defined by the variables of the left side group where pinocarvone, α-campholenic aldehyde, and unknown monoterpene were the most representative.

Fig. 6 shows the plot obtained in the fifth monitoring for intact and damaged walnuts. In this case the PC2/PC3 loadings (17% of the variance explained) variables were mostly located in the central axis of the plot and no groups were notable (Fig. 6a). However, PC2/PC3 scores in Fig. 6b showed that the samples were primarily divided again into two groups, intact (mainly on the positive side of the PC2) and damaged (mainly on the negative side of the PC2). Thus in M5, the mechanically damaged walnuts were represented by α-terpinene, α-terpinolene, chrysanthenone, α-campholenic aldehyde, chrysanthenone, *trans*-β-farnesene and *trans*-verbenol. Conversely, intact walnuts in M5 were represented by pinocarvone, sabinene, myrtenol, *trans*-β-ocimene, *trans*-pinocarveol and limonene.

The PCA results described in this report concluded that the volatiles α-terpinene, α-terpinolene, pinocarvone, α-campholenic aldehyde, chrysanthenone, *trans*-pinocarveol, *trans*-β-farnesene and *trans*-verbenol appeared to be discriminant variables between intact and mechanically damaged samples in M4 and M5. The compounds pinocarvone, α-campholenic aldehyde, chrysanthenone, *trans*-pinocarveol, *trans*-β-farnesene, *trans*-β-verbenol were noted to be in larger amount in mechanically damaged walnuts in both monitoring. However, as previously mentioned they did not show statistical differences by univariant analysis (F and t-tests, $P < 0.05$). On the contrary, α-terpinene and α-terpinolene appeared in relatively equal amount in both matrices. The main correlations obtained for these compounds are collected in Table 2.

3.4. Walnut-produced semiochemicals?

Of the 92 volatiles detected in this work, 30 were evaluated in a previous study by means of electrophysiological responses of male and female navel orangeworm antennae (Beck, Light, et al., 2014). Among

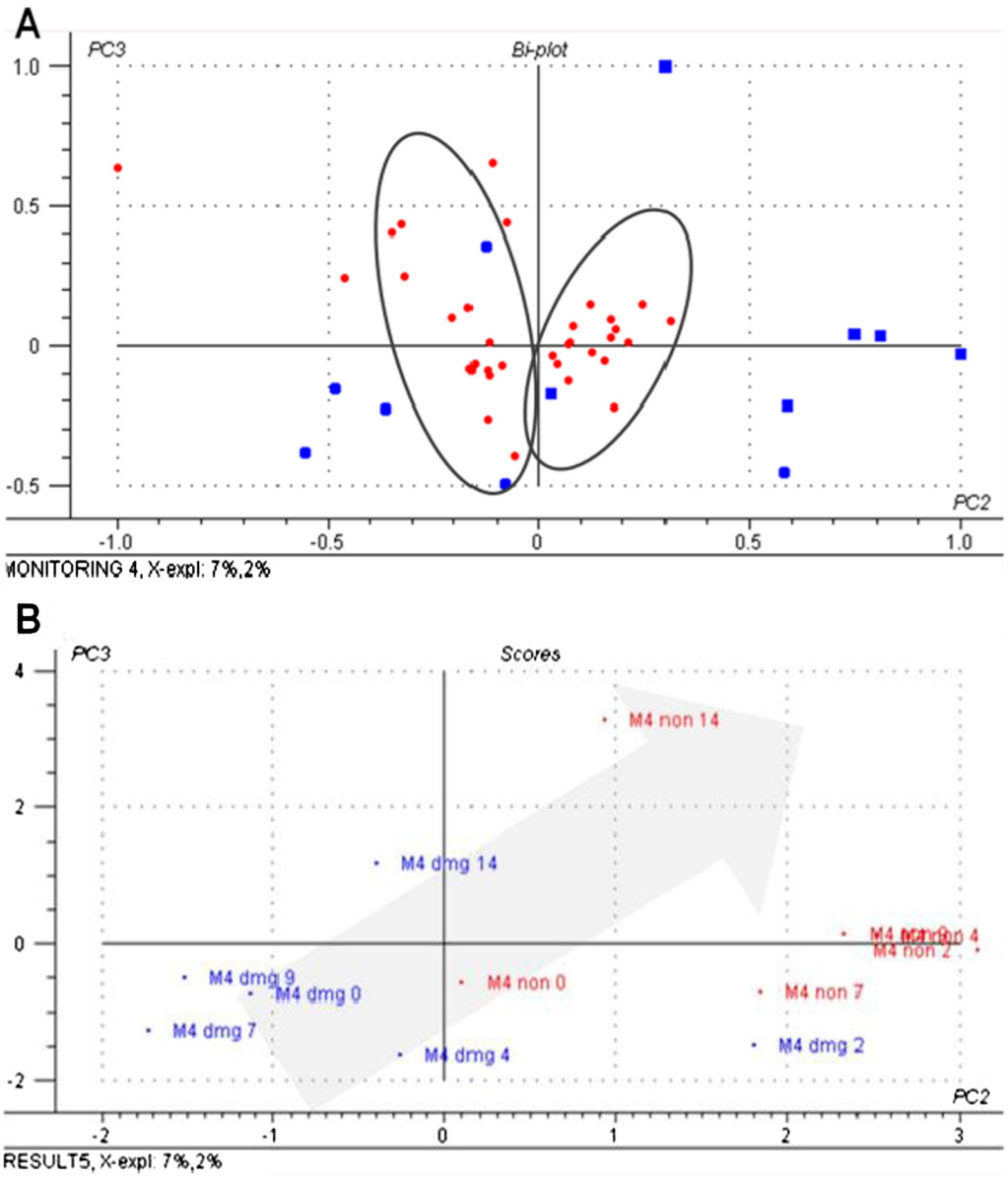

Fig. 5. (A) PC2/PC3 scores and loadings biplot of the volatiles emitted by walnut fruits in M4, non = non-damaged walnuts (intact walnuts), dmg = damaged walnuts, 0 = day 0, 2 = day 2, 4 = day 4, 7 = day 8, 9 = day 9, and 14 = day 14. (B) PC2/PC3 score plot for samples belongs to fourth monitoring stage.

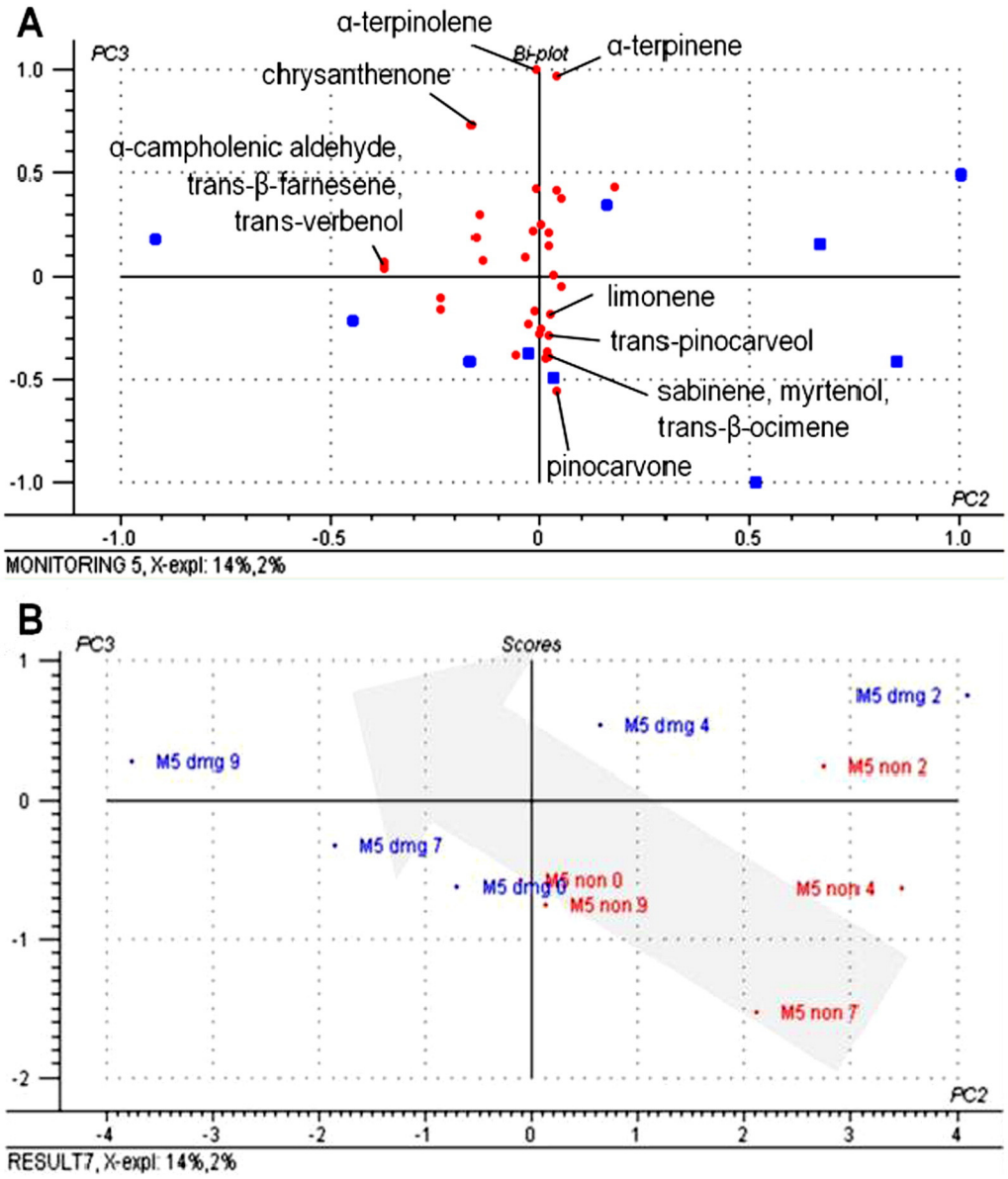

Fig. 6. Principal component analysis of the first two canonical variables of the volatiles emitted by walnut fruits in M5, non = non-damaged walnuts (intact walnuts), dmg = damaged walnuts, 0 = day 0, 2 = day 2, 4 = day 4, 7 = day 8, 9 = day 9, and 14 = day 14. (A) PC2/PC3 loading and score biplot extracted from the fifth sampling period. (B) PC2/PC3 score plot for the fifth monitoring.

the studied compounds were: *trans*-β-farnesene, α-terpinene and α-terpinolene. As reported in previous work (Beck, Light, et al., 2014) the corresponding mean EAG values (corrected to antennal responses of 1000 μV to acetophenone) from female and male navel orangeworms to these compounds were 400/472 μV, 352/390 μV and 1122/574 μV, respectively. The compound α-terpinolene was among the most stimulating volatiles from this EAG study. However, pinocarvone, α-campholenic aldehyde, chrysanthenone, *trans*-pinocarveol and *trans*-β-verbenol were not evaluated by EAG studies. It should be noted that pinocarvone and α-caryophyllene oxide have been reported as electrophysiological active compounds for codling moth (Casado et al., 2008), and therefore may be of interest as candidate

Table 2
Correlation matrix of walnut volatiles. Pearson correlation coefficients of discriminant volatiles.

		1141	1125	1130	1149	1105	1081	1099	1010
Pinocarvone	**1141**	**1.000**							
Trans-pinocarveol	**1125**	**0.980**	**1.000**						
Trans-verbenol	**1130**	**0.963**	**0.961**	**1.000**					
Trans-β-farnesene	**1149**	0.767	**0.803**	0.777	**1.000**				
α-Campholenic aldehyde	**1105**	**0.893**	**0.904**	**0.959**	0.730	**1.000**			
α-Terpinolene	**1081**	0.736	0.761	0.745	0.730	0.788	**1.000**		
Chrysanthenone	**1099**	**0.941**	**0.951**	**0.946**	**0.863**	**0.931**	**0.897**	**1.000**	
α-Terpinene	**1010**	**0.850**	**0.839**	**0.817**	0.646	0.776	**0.866**	**0.822**	**1.000**

Values higher than 0.8 in bold.

semiochemicals of navel orangeworm in ongoing studies. The investigation into their ability to elicit a response from navel orangeworm could provide useful information for future host plant-based attractants.

The monoterpene β-pinene was the predominant compound emitted from the walnut samples and has been found in high amounts in other similar studies (Buttery et al., 2000). β-Pinene has been described to act as a repellent to mated females of codling moths (Vallat & Dorn, 2005). However, other studies reported no discernible results regarding the electrophysiological responses to β-pinene by either codling moths (Casado et al., 2008) or navel orangeworm (Beck et al., 2014). Taking into account the literature regarding navel orangeworm attractants, of the 92 volatiles included in Table 1, 20 were also associated with almond emissions and 35 with pistachio emissions (Beck et al., 2008; Beck, Light, et al., 2014; Beck, Mahoney, Higbee, et al., 2014). A notable difference between walnuts and almonds or pistachios was the large number of sesquiterpenes, which are known to be semiochemicals of other insects and also elicited high EAG responses from navel orangeworm. On the other hand, the emission profile of walnut differs widely from that of apple, which is the most studied codling moth host. However, some compounds common of both matrices have been shown to be attractive to codling moth, for instance *trans*-β-farnesene, linalool and β-caryophyllene. Some of these compounds are also in common with the volatiles of almond and pistachio samples such as for example germacrene D, β-caryophyllene, nonanal and β-farnesene (Beck, Mahoney, Cook, et al., 2014; Vallat & Dorn, 2005).

As described by Beck et al. in a previous work (Beck, Light, et al., 2014), monoterpenes such as β-pinene, α-pinene, limonene and myrcene, were found in the top seven volatiles (Table 1) and represented approximately 50% of walnut volatile emissions. These compounds have been shown to elicit ovipositional behavior from other Pyralidae. Literature also reports that seven of the monoterpenoids found in the top 34 volatiles (Table 1) include the most stimulating volatile for female antennae of navel orangeworm, and include sabinene hydrate, *cis*-ocimene, α-pinene, linalool, α-terpinolene, limonene and *trans*-β-ocimene (Beck, Light, et al., 2014). The compounds *trans*-β-farnesene, nonanal, germacrene D, β-caryophyllene and linalool have been reported to elicit strong electrophysiological responses from codling moth antennae (Beck, Mahoney, Cook, et al., 2014; Bäckman, Bengtsson, Borg-Karlson, Liblikas, & Witzgall, 2001).

One final thought regarding the results of this work is consideration of a preceding report that purports an apparent orchard specificity of navel orangeworm to the host plant-based blend (Beck, Mahoney, Higbee, et al., 2014). The navel orangeworm, a primary pest of almonds and pistachios and a secondary pest of walnuts (although in some years it causes more damage than any other insect) has several and diverse host plants, which span across several agricultural commodities (Siegel, 2008). Each of these commodities likely emits a distinctive background odor, which could cause orchard specificity when applying the synthesized host blend. This suggests that research on the varying compositions of host plant volatiles is required for better monitoring efficacy and applicability to different crops. Although there are numerous reports indicating progress towards attractants using semiochemicals, background volatiles appear to have an important role in the attractiveness of the blend. This research of the volatile emission pattern of walnuts may provide the volatile bouquet that insect pests encounter in walnut orchards. In this study, the volatile profile of walnuts demonstrated a consistent collection of monoterpenes that may significantly add to the overall ambient odors of the orchard, which also includes leaf odors. Finally, the obtained data obtained agrees with existing literature regarding fungal-associated volatiles and walnuts, which report that while navel orangeworm larvae frequently inhabit environments highly contaminated with fungi (aflatoxin), this is not the case for codling moth which inhabit hosts with low amounts, if any, of this mycotoxin (Campbell et al., 2003).

4. Conclusions

HS-SPME–GC/MS has proved to be an effective tool for the extraction and evaluation of walnut volatile emissions. By a proper selection of the SPME fiber certain selectivity and different sensitivities can be achieved.

The collection and characterization of volatile emissions from ex-situ intact and mechanically damaged walnuts revealed that the observed high level of emission of terpenoids agrees with the existing literature. It is important to note that the monoterpene fraction present in walnut emissions represented more than the 80% of the volatile profiles, even in the later monitoring periods when walnut hulls should have started to dry. Many monoterpenes have been reported to possess antifungal activity and therefore, the levels of monoterpenes may play an important role in the apparent decreased activity of fungi relative to other tree nuts. The large monoterpene content also appeared to contribute to the absence of other semiochemicals detected in preceding studies.

The major volatiles from the intact and damaged walnuts were compared and contrasted by PCA. The volatile profiles contained a large number of compounds that do not appear to be walnut specific and are also emitted by commodities affected by codling moth and navel orangeworm such as apple, almond or pistachio. The comparison of the volatile emissions among different sampling periods demonstrated that the volatile profile of the mid-May sampling differed significantly from the subsequent collections (early June to late August). Additionally, although volatile organic compounds unique to damaged walnuts were inconsistent and detected only in trace amounts, these experiments have highlighted two sampling periods from late July to late August, where intact walnuts differed from their mechanically damaged counterparts. The compounds pinocarvone, α-campholenic aldehyde, chrysanthenone, *trans*-pinocarveol, *trans*-β-farnesene, *trans*-β-verbenol, α-terpinene and α-terpinolene were noted as discriminant variables between the two treatments. These compounds should be considered as good candidates in ongoing experiments utilizing electrophysiological and behavioral assays. The detection of the volatiles noted above offers evidence that further investigation into their ability to elicit a response from navel orangeworm is needed. Although researchers usually focus upon the most abundant compounds, minor volatiles have shown importance regarding insect biological activity. Accordingly, further attention to these compounds, specifically to the compounds not previously reported from walnuts, is warranted.

Considering that walnut analyses were carried out over five different periods in the season this study will help delineate phenological differences and identify potentially important background volatiles. This research provides the identification of electrophysiologically active host plant volatiles for an insect pest of walnuts, valuable data regarding the ambient odors insect pests encounter, and potentially important information for future host plant-based attractants in other tree nut orchards.

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