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In Situ Seasonal Study of the Volatile Production of Almonds (*Prunus dulcis*) Var. 'Nonpareil' and Relationship to Navel Orangeworm

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Nonpareil almonds, *Prunus dulcis*, account for the largest percentage of almond varieties grown in the Central Valley of California. Several studies have investigated the various nonvolatile and volatile components of various plant parts; however, the volatile organic compound (VOC) emission of almonds from a single cultivar has not been studied over the course of a growing season. This aspect is particularly relevant to research concerning the navel orangeworm (NOW), a major insect pest of almonds and other tree nuts. Despite the continued presence of NOW, the identification of particular VOCs and their relationship to NOW have not been addressed. The VOC emission of Nonpareil almonds was collected in situ over the course of a growing season by solid-phase microextraction (SPME). The VOCs (*Z*)-hex-3-enyl acetate, (*Z*)-hex-3-enyl butyrate, undecan-2-ol, β-bourbonene, and tetradecane were present for the majority of the days investigated. Several VOCs exhibited positive electroantennographic signals from male and/or female NOW moths.

KEYWORDS: Almond; electroantennogram; in situ; navel orangeworm; Nonpareil; SPME; VOCs

INTRODUCTION

The Nonpareil almond (*Prunus dulcis*) variety represents the most widely planted almond variety in the Sacramento and San Joaquin Valleys of California and comprises ca. 37% of the total acres of varieties grown (*1*, *2*). California is the top producer of almonds, supplying 80% of the world's needs and 100% of the U.S. market's supply. Approximately 5% of California's cropland is committed to almond production (*3*). Moreover, the California almond industry is approaching $2 billion/year (*2*, *4*).

The navel orangeworm (NOW), *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), is an insect pest of California tree nuts. Its feeding damage lowers nut kernel quality, resulting in extensive monetary loss to growers, producers, and shippers. Moreover, NOW feeding damage contributes to aflatoxin contamination (*2*).

There are numerous reports in the literature on both the volatile and nonvolatile composition of various tree parts of almond cultivars: e.g., proteins (*5*) and fatty acids (*6*) from kernels; fatty acids (*7*) and triterpenoids (*8*) from hulls; volatile organic compounds (VOCs) extracted via steam distillation of dried almond hulls (*9*, *10*)); almond oil (*11*); and ex situ VOCs of whole damaged and undamaged almonds (*12*). Nonetheless, the VOC emission of almonds of any one single cultivar has *not* been studied over the course of an entire growing season. This aspect is particularly relevant to research concerning NOW. Despite the presence of NOW throughout the almond growth period, the identification of particular VOCs, or their potential role in NOW behavior, has not been addressed.

The discovery of an efficacious attractant for NOW monitoring/trapping has remained elusive despite breakthroughs with the female sex pheromone (*13*), the pheromone blend (*14*), long-chain fatty acids (*15*), or the use of almond press cake or trapped virgin female NOW. The ability of an insect to locate the desired host plant is in part dependent upon its ability to detect a specific VOC (semiochemical). As with the complex blend of NOW pheromone noted by Leal et al. (*14*), a complex mixture of ubiquitous plant VOCs may be necessary to elicit an appropriate response from the insect to the host plant (*16*, *17*).

This study investigated the VOC emission of Nonpareil almonds in a single growing season from bloom to hull split. The collected VOCs were subjected to electroantennogram (EAG) bioassays with both male and female NOW to determine the responses.

MATERIALS AND METHODS

Plant Material. The VOCs of the fruits of *P. dulcis* (P. Mill) D. A. Webb var. Nonpareil, common name sweet almond, were collected in situ via an inert collection system similar to the reported method (*18*). A representative VOC collection experiment is illustrated in **Figure 1**. All VOC collections were performed within the

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Figure 1. In situ VOC collection system showing the inert bag with enclosed almonds and SPME cartridge in place.

same row of Nonpareil almonds between the hours of 8:00 and 11:00 a.m., at S&J Ranch, Madera, CA.

Volatile Collection. Three to five almonds were enclosed in a customized Teflon bag (manufactured by SKC West, Inc., Fullerton, CA), 11 × 21 cm, thermally sealed on three sides, with two aluminum portals for SPME (Supelco, Bellefonte, PA; 100 μm, polydimethylsiloxane fiber) analysis. The bag was gently sealed onto the stem/branch of the plant by the use of a twist tie, and one SPME was inserted and attached to a portal by means of a large clip (**Figure 1**). VOC collections were kept consistent by applying the following PEST (permeation; exposure; storage; thermal desorbtion) parameters: P = 2 min; E = 30 min; S = 3−6 h; and T = 15 min (*18*). The long VOC storage times on the fiber (S = 3−6 h) were due to long transportation times and multiples (up to eight samples) being run on two GC instruments. The SPME cartridges were sealed with Teflon tape, covered with a septum, sealed in a plastic bag, and transported in an ice chest at 0 °C. Background analyses were performed on an unexposed SPME fiber and ambient bag/air volatiles.

Volatile Analyses. After transportation from the field, all experiments utilized transfer of adsorbed volatiles onto either a J&W Scientific (Folsom, CA) DB-Wax column (60 m × 0.32 mm i.d. × 0.25 μm) or a J&W Scientific DB-1 column (60 m × 0.32 mm i.d. × 0.25 μm) installed on one of two HP-6890 gas chromatographs (GC) coupled to HP-5973 mass selective detectors (MS; Palo Alto, CA). Desorbed volatiles were analyzed with the following methods. For DB-Wax: injector temperature, 200 °C; splitless mode; inlet temperature, 200 °C; constant flow, 3.0 mL/min; oven settings, initial temperature, 40 °C; hold time, 0.0 min; ramp 1, 4 °C/min; final temperature, 200 °C; hold time, 40 min. For DB-1: injector temperature, 200 °C; splitless mode; inlet temperature, 200 °C; constant flow, 2.0 mL/min; oven settings, initial temperature, 40 °C; hold time, 0.0 min; ramp 1, 4 °C/min; final temperature, 250 °C; hold time, 30 min. MSD parameters: source temperature, 230 °C; MS source temperature, 150 °C; EI mode, 70 eV; solvent delay, 1 min; scan group 1, 40−300 amu; scan group 2 at 20 min, 40−450 amu. NIST, Wiley, and internally generated databases were used for fragmentation pattern identification. The retention indices (RIs) were calculated using a homologous series of *n*-alkanes on the DB-Wax and DB-1 columns. Volatile identifications were verified by injection of authentic samples and comparison to retention times of an internally generated list of volatiles on identical columns. Each experiment was performed in duplicate, but injected onto separate columns for RI comparison purposes.

Calculated RIs were used to assist in compound identification and to perform comparison of DB-1 to DB-Wax column results. Due to the equilibrium of adsorbed VOCs on the SPME fibers in conjunction with the long VOC storage times, a strict quantitative interpretation of the peak areas was untenable. The relative abundances (peak areas) were noted, and the inclusion of a VOC into **Table 1** was based upon presence in both GC analyses, as well as a minimum peak area based on a percentage of the largest VOC peak present in each run.

Electroantennogram Bioassays. The antennae of laboratory-reared, sexed navel orangeworm moths, *A. transitella* (Walker) (Lepidoptera: Pyralidae), were excised, positioned on a fork electrode using electrode gel, and connected to an IDAC-4 acquisition controller electroantennogram using Syntech's PC-based software (Syntech, Kirchzarten, Germany). The antennae were humidified with a stream of purified air bubbled through distilled water at a flow rate of 200 mL/min. The individual compounds for EAG analysis (50 μg; 10 μL of a 5 μg/μL solution in pentane) were loaded onto oven-dried 0.25 in. assay disks, allowed to air-dry for 5 min, and inserted into 5.75 in. Pasteur pipets, and the ends were temporarily capped with parafilm. The sources of the VOCs for EAG analyses are noted in **Table 1**. Negative control (NegCtrl) disks were prepared using a similar method, but with 10 μL of pentane. Positive control (PosCtrl) disks were prepared using the major pheromone component (*Z*,*Z*)-11,13-hexadecadienal (50 μg; Suterra, Bend, OR). The pipets loaded with the individual compounds were attached via tubing to a stimulus controller unit (Syntech). The antennae were exposed to each compound by a 2 s puff of air, and the resulting response was recorded. The antennal response was duplicated for each VOC with a 1 min delay between puffs. Each antenna pair was exposed to seven duplicated puffs in the order PosCtrl, compound A, NegCtrl, compound B, compound C, compound D, and PosCtrl, with each run lasting no longer than 30 min from excision to completion of run on the antenna pair.

Male NOW responses (μV) to the individual VOCs were normalized to the averaged EAG responses (μV) of the male to the PosCtrl and less the response to the NegCtrl. The lowest response to the PosCtrl of 500 μV was used as the normalization factor. The average male response to the PosCtrl was 1100 μV over the course of all VOCs tested. The crude male EAG responses (MaleEAG) were normalized using the equation MaleEAG × (500/PosCtrl) − NegCtrl. Therefore, any VOC demonstrating an EAG value of 500 μV or more would be judged as good as or better than the PosCtrl. Because there are no current "standards" to judge the female response, and females do not typically provide strong or consistent response to the pheromone, the female responses were stringently normalized by subtraction of response to both the PosCtrl and the NegCtrl. The crude female EAG responses (FemaleEAG) were normalized using the equation FemaleEAG − PosCtrl − NegCtrl. The values shown in **Table 1** are the average of the duplicate responses. Any normalized response of the female > 500 μV would be considered to be significant. Normalized responses of ca. 250 μV were considered to be moderate responses.

RESULTS AND DISCUSSION

The in situ collection and analysis of Nonpareil almonds provided 24 VOCs in various amounts and frequency. One interesting result was the small number, yet diverse class, of compounds emitted over the majority (> 55%) of the growing season: (*Z*)-hex-3-enyl acetate, **4**; (*Z*)-hex-3-enyl butyrate, **10**; undecan-2-ol, **12**; β-bourbonene, **16**; and, tetradecane, **18**. Of these, only β-bourbonene, **16**, and tetradecane, **18**, were present in the earlier months, both of which are common floral scent VOCs of numerous plants, including several genera from the family Rosaceae (*19*). Neither of these persistent VOCs has demonstrated semiochemical characteristics for Lepidoptera in the literature, and tetradecane, **18**, elicited only weak EAG activity from the female in this study (**Table 1**). β-Bourbonene, **16**, has been reported to demonstrate a slight increase in relative amount during a study of damaged ex situ almonds (*12*), however, was not available in isolable amounts for EAG bioassay.

The absence of the C_6 and C_9 compounds early in the almond growth stages supports the idea of the enzymatic breakdown of the fatty acids in almonds (*20*) to C_6 green leaf volatiles, in this case C_6 esterified derivatives, (*Z*)-hex-3-enyl acetate, **4**, and (*Z*)-hex-3-enyl butyrate, **10**, and the C_9

Table 1. Volatile Emission of Nonpareil Almonds from Bloom to Hull Split, March−August 2007, Madera, CA[a]

	compound information[b]						VOC occurrence, m/dd 2007 (tree no.)[c]																				EAG response (μV)	
			DB-1 RI		DB-Wax RI																							
	compd library/ID	source[d]	lit.[e]	calcd[f]	lit.[e]	calcd[f]	3/21 (8)	3/28 (3)	4/04 (7)	4/12 (11)	4/18 (14)	4/25 (3)	5/02 (7)	5/07 (8)	5/14 (14)	5/18 (15)	5/21 (16)	6/01 (20)	6/04 (21)	6/11 (22)	6/18 (24)	6/25 (25)	7/02 (26)	7/09 (28)	7/16 (31)	7/23 (34)	7/30 (34)	8/06 (35)	8/13 (37)	M	F
1	(*Z*)-hex-3-en-1-ol	Iso	834	838	1381	1388	+					+			+		+		tr	+		+			+		+		+	0	0
2	butyrolactone	Ald	855	859	1623	1627	+	tr		tr			++		++							+					+	+		0	25
3	styrene	Ald	871	872	1252	1253										tr														0	0
4	(*Z*)-hex-3-enyl acetate	Bed	986	987	1312	1317					tr	+	+	+	++++	++++	++++	+	+	+++	+	+++	++	+	++	+	++		+	0	0
5	hexyl acetate	Ald	995	993	1268	1272									+	+	+	+	tr	+	tr	+	tr		tr		tr		tr	0	0
6	nonanal	Ald	1082	1081	1389	1392							+				+	+	+					+	tr		+	tr	tr	0	50
7	2-phenylethanol	Ald	1081	1082	1910	1912								tr																54	320
8	linalool	Iso	1083	1084	1546	1549								tr																58	200
9	non-2-enal	Ald	1134	1132	1532	1533																						tr		183	200
10	(*Z*)-hex-3-enyl butyrate	Bed	1167	1168	1458	1461						tr		+	+	+	+			+	+	+	+	+	+		+		tr	238	75
11	undecan-2-one	Iso	1273	1271	1596	1597																	+	+	+	+	+		tr	387	275
12	undecan-2-ol	Ald	1286	1284	1721	1724							+				+		+	+	tr	tr	+	+	+	+	+	+	+	0	25
13	methyl anthranilate	Iso	1302	1301	2238	2232							tr	tr																33	65
14	α-copaene	Iso	1374	1374	1490	1489	tr							+								tr								30	20
15	farnesane	Iso	1379	1378	1358	1354										+							tr	+		+				45	0
16	β-bourbonene	Tent	1382	1381	1516	1515	+	+	tr		tr	tr		+	+	+	+			+		+	+		+		+			-	-
17	decyl acetate	Iso	1393	1392	1679	1678				tr															+	+				264	75
18	tetradecane	Poly	1400	1398	1400	1397	+	+	+	+			tr	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	tr	0	25
19	caryophyllene	Iso	1415	1413	1594	1593						tr		+	+	+	+			++	+	+++	++		+				tr	0	190
20	geranylacetone	Ald	1429	1428	1853	1855									+			+	+	+	+	+	+	+	+	+	+	tr	tr	88	200
21	α-humulene	Iso	1449	1455	1666	1666								+	+	+	+			+	+	++	+		+					187	250
22	germacrene D	Iso	1474	1473	1707	1713	+	tr												tr		+								20	115
23	pentadecane	Poly	1500	1499	1500	1489	+	+	+	+	+																			0	150
24	hexadecane	Poly	1600	1596	1600	1597	+	tr			tr													tr						74	40

[a] Volatile relative peak area (× 10^6). tr, trace, <0.5; +, 0.5−20; ++, 20−50; +++, 50−150; ++++, 150−400. [b] Compound identification by RI relative to *n*-alkanes on DB-1 and DB-Wax columns, retention times, mass fragment libraries, and comparison to authentic samples. [c] Volatiles sampled on days noted between 8:00 a.m. and 11:00 a.m. Trees analyzed were from the same orchard. [d] Source of compound for authentication: Ald, Aldrich or Fluka; Bed, Bedoukian; Iso, previously isolated by a researcher in this laboratory (Buttery, Flath, or Binder); Poly, Polyscience Corp.; Tent, tentative. [e] Compound RI of literature value. [f] Calculated compound RI based upon retention time of respective column.

compounds, nonanal, **6**, and non-2-enal, **9** (*21*). The generation of the C_6 and C_9 compounds also correlates with the phenological production of fatty acid oils in the kernels, which generally increases at about 60 days after fertilization (*22*). The origin of undecan-2-ol, **12**, is speculative at this juncture because it has been detected from both plant and microbial sources. Undecan-2-ol, **12**, and undecan-2-one, **11**, have been reported from trees (*23*) and fruit (*24*), among others. Moreover, both of these compounds have been detected as volatiles from a myxobacterium (*25*), although one not found on almonds. Alternatively, it is possible that the undecan-2-ol, **12**, may ultimately be derived from a fatty acid in addition to the C_6 and C_9 compounds described above. The oxidized form of lauric acid is also formed during the enzymatic breakdown of fatty acids (*21*), and it known that undecan-2-one, **11**, can be generated from lauric acid (*26*). Therefore, it could be hypothesized that undecan-2-one, **11**, and undecan-2-ol, **12**, can be generated in this manner, thus explaining the similarities to the kernel phenological growth pattern. This hypothesis is being explored for merit.

The electroantennogram (EAG) data are very interesting with respect to a potential relationship between almond VOCs and NOW. The acetylated green leaf volatile (*Z*)-hex-3-enyl acetate, **4**, and the alcohol undecan-2-ol, **12**, did not show any notable EAG response from either male or female; however, the other corresponding VOCs did demonstrate sufficient EAG responses to warrant interest as prospective background signaling volatiles (BSVs) (*17*). For the purpose of this study, BSVs are defined as ubiquitous volatiles from almonds that may act as obligatory cues to direct NOW toward key attractant(s). Hence, the BSVs need not demonstrate an EAG response greater than a specified attractant, such as the major aldehyde component of the female NOW pheromone (*13*), but rather a reasonable EAG response that suggests a basal interest in the individual VOC or bouquet.

The VOCs (*Z*)-hex-3-enyl butyrate, **10**, β-caryophyllene, **19**, geranylacetone, **20**, and α-humulene, **21**, elicited modest to moderate EAG responses from the male and/or female NOW and were present for $>40\%$ of the growing season. The VOC α-humulene, **21**, synonymous with α-caryophyllene, provided similar normalized responses for both the male and female antennae; it is a ubiquitous plant volatile and is known to possess semiochemical behavior, albeit none is reported for NOW (*27*). (*Z*)-Hex-3-enyl butyrate, **10**, β-caryophyllene, **19**, and geranylacetone, **20**, elicited preferential male/female EAG signals: 238/75, 0/190, and 88/200 μV responses, respectively. Geranylacetone, **20**, was not present for $>55\%$ of the evaluation period; however, it did show a modest yet persistent presence toward the later collections and tapered off toward hull-split. Geranylacetone, **20**, (*Z*)-hex-3-enyl butyrate, **10**, and β-caryophyllene, **19**, are ubiquitous plant VOCs; however, none demonstrate notable semiochemical behavior, although β-caryophyllene, **19**, has shown attractant behavior toward one Lepidoptera, Tortricidae (*28*). Finally, undecan-2-one, **11**, elicited the highest normalized combined male/female responses from NOW antennae, although present only during the later months of collection.

Other compounds, albeit infrequent and in trace amounts, that elicited notable EAG responses included 2-phenylethanol, **7**, linalool, **8**, non-2-enal, **9**, and decyl acetate, **17**. One observation can be made regarding these "transient" VOCs—they all have documented origins from Orchidaceae plant fatty acids (*29*). In fact, 12 of the 24 compounds (**5**−**9**, **11**, **17**−**21**, and **23**) listed in **Table 1** were listed as having plant fatty acid origins, and of those 12, 8 VOCs demonstrated modest to moderate responses from male and/or female NOW antennae. Moreover, it is interesting to note the presence of long-chain alkanes tetradecane, **18**, and pentadecane, **23**, at the early stages of almond growth.

The VOCs from a single cultivar of almonds were collected in situ over the 2007 San Joaquin Valley growing season. The cultivar Nonpareil was chosen primarily for its agronomic and organoleptic characteristics, which are compulsory to uniformity. The VOCs 2-phenylethanol, **7**, (*E*)-non-2-enal, **9**, undecan-2-one, **11**, and α-humulene, **21**, produced the highest combined EAG responses from the male and female antennae. In addition to the VOCs with notable NOW EAG activity, several VOCs demonstrated promising BSV characteristics. This is the first report of VOC emissions collected in situ from a single almond cultivar over the course of a growing season; however, the VOCs provided in Table 1 provide only a sampling of BSV candidates and/or VOC emissions. Further replications of tree VOCs are required to confidently assign a pattern between VOC emission and phenological development. Investigations into the possible fatty acid origin of several of the almond VOCs are ongoing.

ABBREVIATIONS USED

BSV, background signaling volatile; EAG, electroantennogram; GC-MS, gas chromatography−mass spectroscopy; NOW, navel orangeworm; PEST, permeation/exposure/storage/thermal desorbtion; RIs, retention indices; SPME, solid-phase microextraction; VOC, volatile organic compound.

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