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C_6-Green leaf volatiles trigger local and systemic VOC emissions in tomato

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Abstract

In response to insect feeding, tomato plants (*Lycopersicon esculentum*) release elevated levels of volatile organic compounds; that is, monoterpenes and sesquiterpenes are released both locally and systemically with wounding while C_6 green leaf volatiles are released only from damaged leaves. With the exogenous application (100 nmol) of the C_6-tomato-volatile (*E*)-2-hexenal, an increase in the release of local and systemic terpenes was observed, while an equimolar amount of methyl jasmonate triggered only local emissions of terpenes. Labeling studies with $^{13}CO_2$ showed that de novo synthesis was not required for monoterpene or sesquiterpene release immediately following chemical treatment or insect feeding. Comparative measurements were made between aldehyde doses applied to the plant and levels naturally released from leaves with insect damage.

Keywords: *Lycopersicon esculentum*; Solanaceae; Herbivores; Monoterpenes; Sesquiterpenes; C_6-Volatiles

1. Introduction

Plants have multiple mechanisms to protect themselves against either mechanical wounding, herbivore damage, dessication, or pathogen attack. Jasmonic acid (JA), its methyl ester, certain amino acid conjugates, Glc esters, and various hydroxylated forms, which are collectively termed jasmonates, occur ubiquitously in all plant species and constitute a major signal in stress-induced gene expression (Wasternak and Parthier, 1997). The role of jasmonates in stress-related signaling has been best characterized with respect to the wound-induced expression of proteinase inhibitor (*pin*) genes, which protect the plant against digestive Ser proteinases of herbivorous insects (Farmer and Ryan, 1992; Koiwa et al., 1997). In both *Arabidopsis* and tomato plants, JA-deficient mutants have suppressed levels of proteinase inhibitor (PI) proteins and show greater susceptibility to herbivore damage than wild type parental lines (Howe et al., 1996; McConn et al., 1997).

In addition to the elevation of PI or polyphenol oxidase that directly target herbivore pests, plants can also defend themselves against insect damage indirectly by emitting herbivore-induced volatiles that attract natural enemies of the herbivores. These volatile cues operate in several agricultural species, including cotton (Loughrin et al., 1994; Paré and Tumlinson, 1999), lima bean (Dicke et al., 1990; Takabayashi and Dicke, 1996), and tomato (Dicke et al., 1998). Recent studies have indicated that the octadecanoid pathway with jasmonic acid (JA) as the central component is involved in this ancillary defense response. In cotton, lima bean and tomato plants, exposure to jasmonates results in the production of volatiles that mimic those emitted with spider mite damage (Hopke et al., 1994; Boland et al., 1995; Thaler et al., 1996). Airborne-volatiles released upon herbivore damage may also function as signals for neighboring uninfested plants by activating defense related genes (Farmer and Ryan, 1990; Bruin et al., 1995; Miksch and Boland, 1996; Shulaev et al., 1997; Arimura et al., 2000).

While the emissions of terpenoids, nitrogen containing compounds and salicylic acid derivatives vary among plant species, C_6-volatiles produced from the catalytic activity of hydroperoxide lyase (HPL) can be

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generated in all green tissues (Hatanaka et al., 1987) and are among the earliest components to be released from damaged leaves (Turlings et al., 1995). The biosynthesis of C_6-volatiles from an 18 carbon fatty acid precursor involves two enzymatic steps catalyzed by lipoxygenase (LOX) and HPL. Depending upon the degree of saturation of the substrate, HPL produces either (*Z*)-3-hexenal or hexanal. Alcohol dehydrogenase (ADH) and an isomerization factor (IF) catalyze the synthesis of the other C_6-volatiles including (*E*)-2-hexenal, (*E*)-2-hexenol, (*Z*)-3-hexenol and hexenol (Hatanaka, 1993).

When released from the plant, these compounds can trigger responses in neighboring plants, including phytoalexin accumulation in cotton (Zeringue, 1992), lower insect feeding rates in tomato (Hildebrand et al., 1993) and reduced germination frequency in soybean (Gardener et al., 1990). Several of these C_6- compounds can also act as antimicrobial agents on their own (Croft et al., 1990). As a signal molecule, exogenous application of (*E*)-2-hexenal to *Arabidopsis* seedlings induces a group of genes that closely mimics methyl jasmonate (MeJA) induction as well as triggering the up- regulation of LOX pathway and PAL genes (Arimura et al., 2001). Interestingly (*E*)-2-hexenal treatment does not induce HMGR-1, the gene that encodes a key regulatory step enzyme involved in isoprenoid biosynthesis (Nicholas and Steven, 1998). The role of (*E*)-2-hexenal as well as other C_6- volatile components in triggering VOC emissions is unclear and the broader biological significance of these compounds is under current investigation (Farmer, 2001).

The role of LOX products in stress-related signaling is best characterized with respect to the wound-induced expression of *pin* genes in tomato. In tomato, an updated model for defense gene activation proposes that C_6-volatiles produced by HPL upon wounding is the first step in the octadecanoid-signaling cascade; these C_6-volatiles lead to the production of systemin, a systemic intercellular polypeptide signal, that activates the LOX pathway with JA accumulation known to be involved in the induction of *pin* genes (Sivasankar et al., 2000).

The aim of this research was to examine the role of C_6-aldehydes/alcohols in the emission of plant volatiles and compare this response with activated VOC emissions triggered by insect damage. Owing to the wealth of knowledge of plant insect interactions in tomato, this system is likely to provide a good model for assessing the role of oxylipins in the induction of volatile chemicals. Here we report the activation of volatile emissions from the monoterpene and sesquiterpene pathways by a series of C_6-volatiles. The release of volatiles from leaves distal to C_6-aldehyde treatment supports the theory that green leaf volatiles activate a systemic signaling cascade triggering volatile emissions in untreated leaves.

2. Results and discussion

2.1. Whole plant volatile analysis

To determine how tomato plants exposed to C_6-aldehyde compare with herbivore damage in triggering VOC emissions, the more abundantly released terpenes and C_6-components (Fig. 1A–C) were analyzed and quantified; C_6-aldehyde treatment (*E*-2-hexenal) was also compared with the known signal defense molecule JA by application of its methyl ester MeJA . Accordingly, the major components which overlap with those previously identified in wounded tomato plants (Andersson et al., 1980; Buttery et al., 1987) are grouped by their biosynthetic origin and include the C_6-components (*Z*)-3-hexenal [1], and (*E*)-2-hexenal [2], the sesquiterpenes, β-caryophyllene [3], α-humulene [4], and δ-elemene [5] and the monoterpenes, α-pinene [6], β-pinene [7], 2-carene [8], and β-phellandrene [9]. It was thus found that continuous tobacco hornworm (THW) damaged-tomato leaves released significantly higher levels of monitored VOCs (Fig. 2A) than undamaged control plants (Fig. 2D) within the first 3 h of insect damage (Duncans, $P<0.05$). Treatment with either 100 nmol MeJA or (*E*)-2-hexenal (Fig. 2B and C) also activated the release of monoterpenes and sesquiterpenes to significantly higher emission levels than with untreated control plants (Fig. 2D) (Duncans, $P<0.05$) within the first 3 h collection interval. However, MeJA triggered release of significantly higher levels of monoterpenes and sesquiterpenes than (*E*)-2-hexenal (Duncans, $P<0.05$), whereas MeJA treatment did not activate C_6-volatile emissions. Additionally, that is, the sum of terpenes for MeJA treatment was 210 ± 28 μg, whereas that for (*E*)-2-hexenal and solvent (control) treatments were 29 ± 3.5 and 3.3 ± 0.7 μg, respectively. Plant volatile emissions then returned to untreated control levels with either MeJA or (*E*)-2-hexenal treatment over the next 3 h collection period: that is in this case, the sum of terpenes for MeJA treatment was 4.5 ± 1.1 μg, whereas (*E*)-2-hexenal and solvent treatments were 3.8 ± 0.4 and 3.8 ± 1.1 μg, respectively. These data are thus consistent with reports that in *Arabidopsis* the HPL gene is induced by wounding but not by MeJA treatment (Bate et al., 1998), as well as in cotton since exogenous MeJA did not trigger C_6-volatile release (Saona et al., 2001). With tobacco, on the other hand, MeJA triggered terpene and C_6-volatile emissions, while (*Z*)-3-hexene-1-ol did not trigger release of any VOCs (Kessler and Baldwin, 2001); for lima bean plants treated with MeJA, C_6 volatiles were elevated along with a blend of terpenes (Dicke et al., 1999). These data suggest that there may be plant specific differences in VOC emissions with either MeJA or C_6-volatile treatment.

(*Z*)-3-Hexenal was not detected with exogenous (*E*)-2-hexenal treatment, while plant-emitted (*E*)-2-hexenal could not be differentiated from applied (*E*)-2-hexenal,

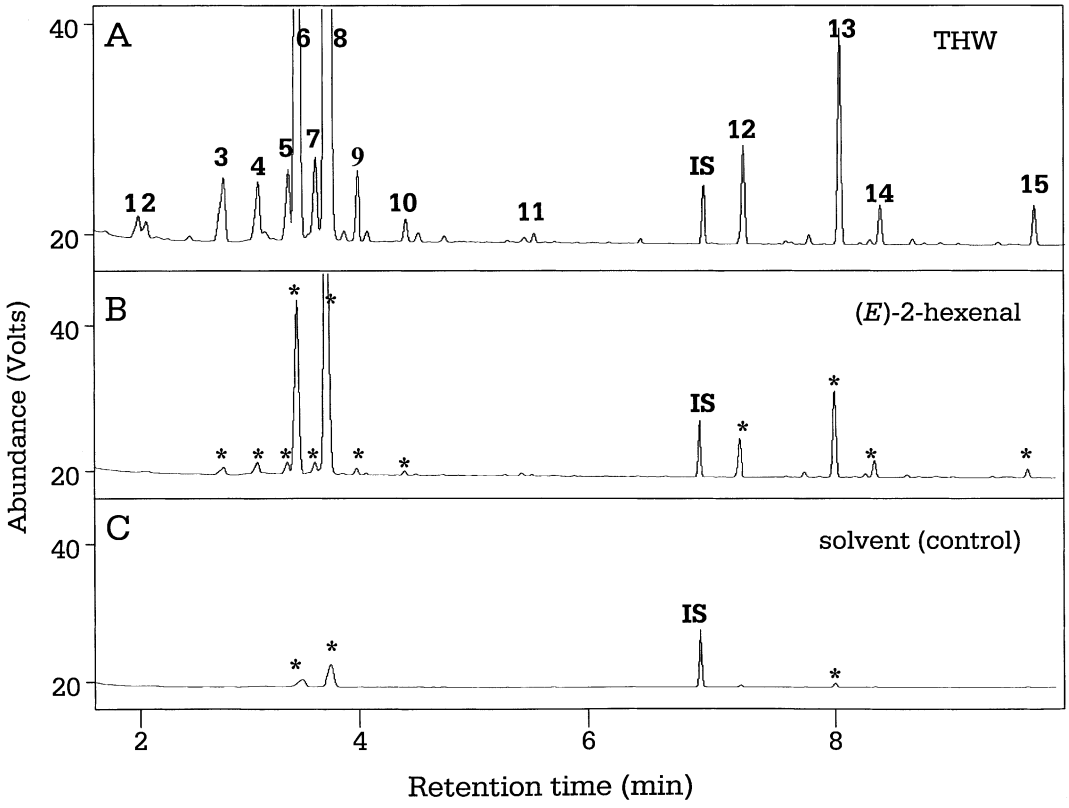

Fig. 1. Chromatographic profiles of VOCs collected from whole plants at 3-h intervals after THW feeding (A), 100 nmol (*E*)-2-hexenal treatment (B) and compared with a solvent control (C). Compounds include (*Z*)-3-hexenal [1], (*E*)-2-hexenal [2], α-pinene [3], β-pinene [4], unidentified monoterpene [5], 2-carene [6], limonene [7], β-phellandrene [8], γ-terpinolene [9], linalool [10], methyl salicylate [11], δ-elemene [12], β-caryophyllene [13], α-humulene [14], (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene [15]. An asterisk (*) designates that a compound aligns with the numbered peak in the corresponding chromatogram.

and thus was not measured for (*E*)-2-hexenal treatment of the whole plant (Fig. 2C). After the initial volatile burst with a single (*E*)-2-hexenal application, VOC emissions returned to, and remained at control levels when monitored over 5 days for 3 h intervals: that is, the sum of terpenes were 3.4±0.5 μg day 2 treated versus 3.8±1.1 μg control, and 4.1±0.9 μg day 4 treated versus 2.9±0.5 μg control, respectively. The maximum response observed with (*E*)-2-hexenal treatment was within a dose range of 10–100 nmol, with significantly higher VOC emission levels than untreated control plants; the dose of 5 nmol produced half the maximum VOC release response (Duncans, $P<0.05$) (Fig. 3). A dose of 1 nmol, however, did not produce a detectable increase in released VOCs, although earlier reports observed that this dose can still induce certain defense genes in *Arabidopsis* (Nicholas and Steven, 1998). Based on the maximum VOC response of 100 nmol determined for (*E*)-2-hexenal, a dose of 100 nmol of MeJA was used to compare their biological activities at an equimolar concentration.

2.2. Systemic emissions of VOCs

The rapid transient release of terpenes observed with THW damage suggested that physical damage to the leaf glands might be responsible for the increase in VOC emissions. By collecting volatiles from the upper untreated portion of THW damaged plants, volatile emissions triggered by simple mechanical damage to leaf glands could be differentiated from volatile emission responses triggered by endogenous plant signals. Lower leaves placed outside the volatile collection chamber were thus treated with either MeJA, (*E*)-2-hexenal or were subjected to herbivore damage, with headspace volatiles being collected from the upper untreated portion of the plant. (A positive pressure in the chamber insured that room volatiles did not enter into the chamber.) This collection of systemically released volatiles allowed for measurement of plant produced C_6-volatiles released with exogenous (*E*)-2-hexenal treatment. The emission levels of monoterpenes and sesquiterpenes from undamaged portions of THW damaged plants (Fig. 4A) were significantly higher than with control plants (Fig. 4D) (Duncans, $P<0.05$), but consistently less than the amounts observed from THW damaged whole plants (Fig. 2A). The emissions of systemic aldehydes did not increase with THW, MeJA or (*E*)-2-hexenal treatment (Fig. 4A–C), being at undetectable levels similar to that of control plants (Fig. 4D). MeJA did not activate the emissions of mono- and

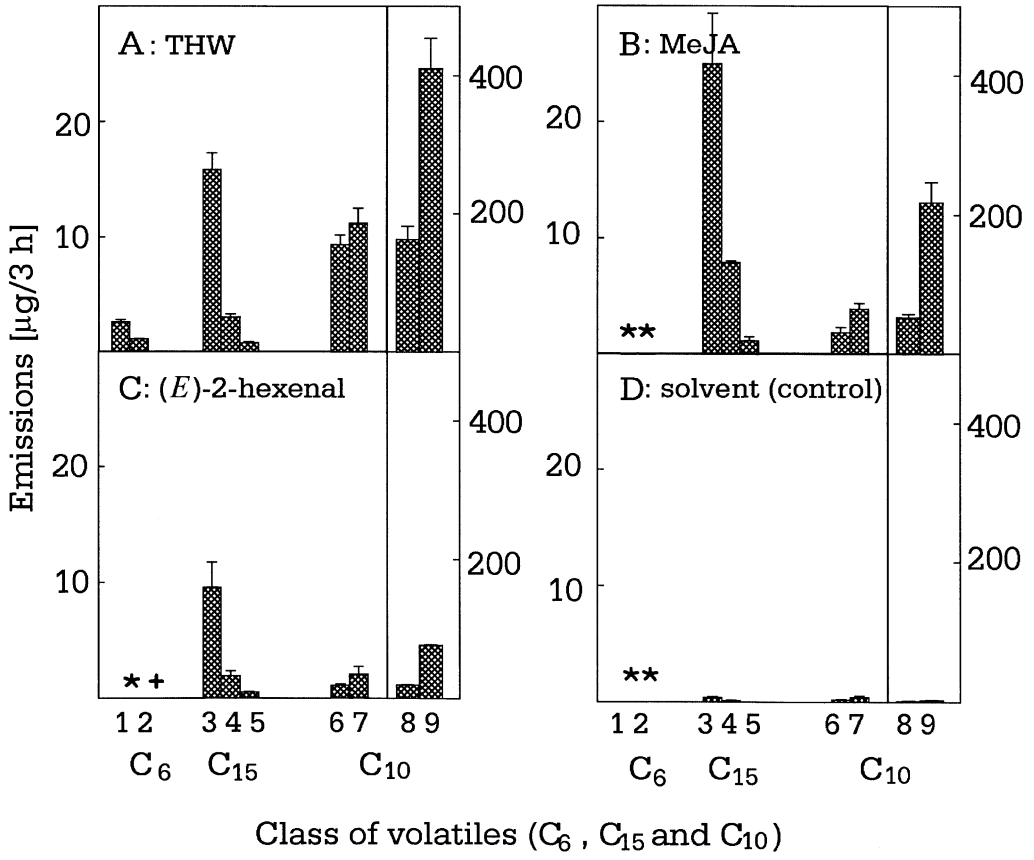

Fig. 2. VOCs collected from whole plants at 3-h intervals with THW damage (A), 100 nmol MeJA (B), 100 nmol (*E*)-2-hexenal (C), and solvent control (D). Compounds are grouped as C_6-volatiles (C_6), sesquiterpenes (C_{15}) and monoterpenes (C_{10}) and include (*Z*)-3-hexenal [1], (*E*)-2-hexenal [2], β-caryophyllene [3], α-humulene [4], δ-elemene [5], α-pinene [6], β-pinene [7]. The scale for 2-carene [8] and β-phellandrene [9] is different that other components as labeled; (*) not detected, (+) not measured. Error bars represent standard error ($n=3$).

sesquiterpenes (Fig. 4B), with volatile emission levels being indistinguishable from control plants (Fig. 4D). (*E*)-2-hexenal treatment triggered the release of sesquiterpenes at levels not significantly different than that caused by THW damage (Duncans, $P<0.05$) while monoterpenes were released at 30% of THW activated levels (Fig. 4A and C) . These data suggest that MeJA, as a mimic of the signal molecule JA, is involved locally in release of volatiles but does not activate a mobile signal to trigger release of terpenes from untreated portions of the plant. MeJA has been shown to trigger the systemic accumulation of PI proteins in tomato (Farmer et al., 1992); however, since earlier studies did not estimate plant exposure levels with MeJA treatment, comparisons are not possible between earlier studies and this work. Data from whole plant volatile analysis showed that MeJA treatment failed to trigger green C_6-volatiles in plants (Fig. 2B); since an exogenous application of (*E*)-2-hexenal triggered systemic VOC emissions (Fig. 4C) these signals may play some role in the systemic activation of volatiles. These data show that C_6-volatiles trigger a systemic indirect defense response which may or may not be related to the previously characterized response of systemin induction (Sivasankar et al., 2000).

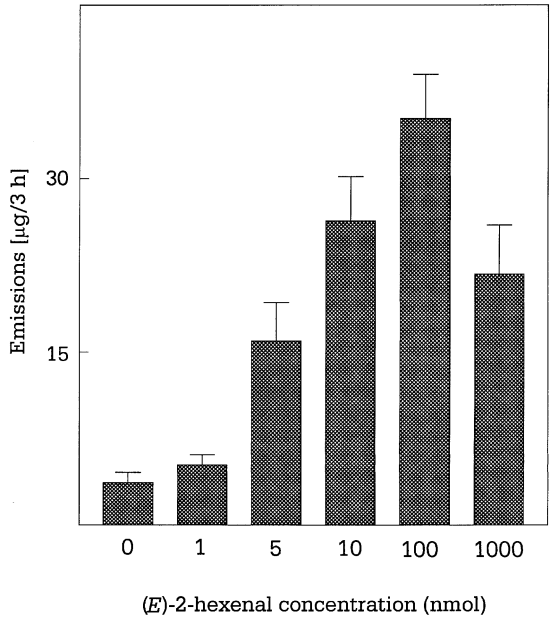

Fig. 3. VOCs collected from whole plants at 3-h intervals with different doses of (*E*)-2-hexenal. Bars represent total monoterpenes and sesquiterpenes. Monoterpenes (C_{10}) include α-pinene, β-pinene, 2-carene, β-phellandrene and sesquiterpenes (C_{15}) include β-caryophyllene, α-humulene, δ-elemene. Error bars represent standard error ($n=3$).

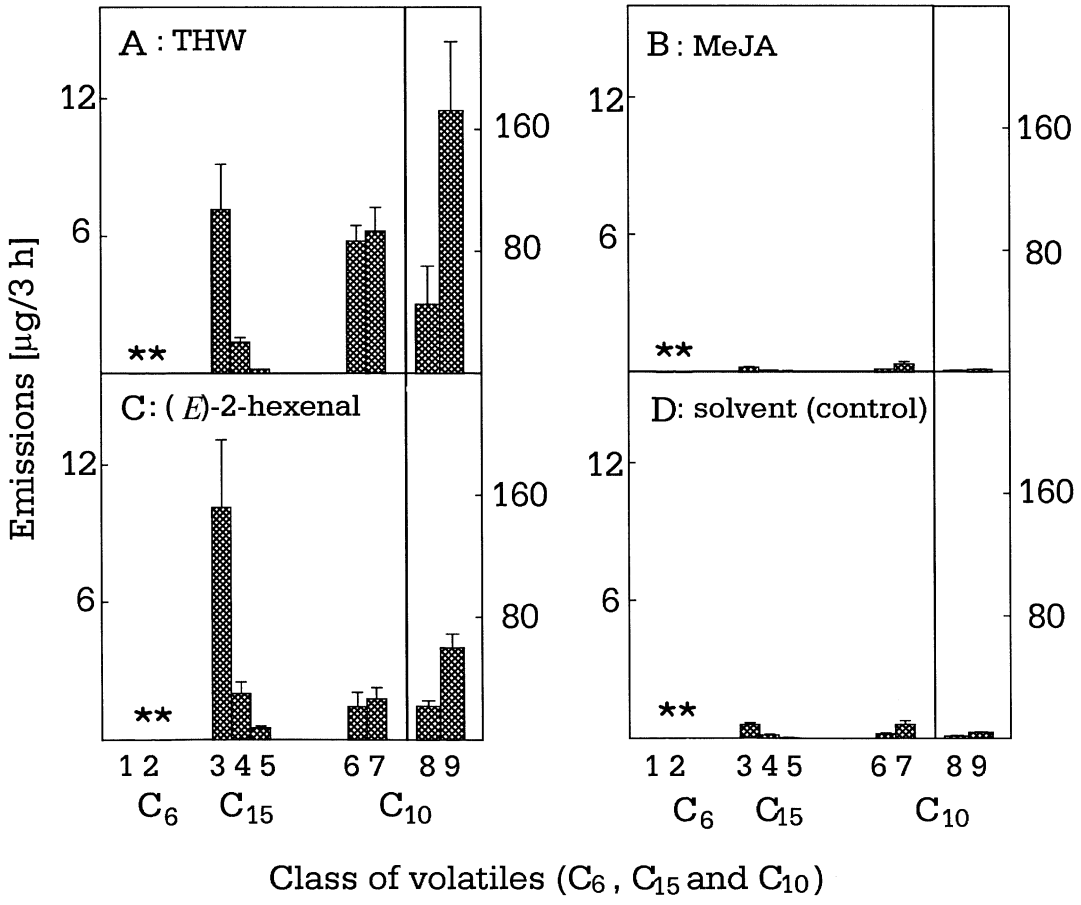

Fig. 4. Systemic VOCs collected at 3-h intervals with THW damage (A), 100 nmol MeJA (B), 100 nmol (*E*)-2-hexenal (C), and control (D). Compounds are grouped as sesquiterpenes (C_{15}) and monoterpenes (C_{10}) and include β-caryophyllene [3], α-humulene [4], δ-elemene [5], α-pinene [6], β-pinene [7]. The scale for 2-carene [8] and β-phellandrene [9] is different that other components as labeled; (*) not detected, (+) not measured. Error bars represent standard error ($n = 3$).

2.3. Light independent volatile emissions

To establish if the head space volatiles from tomato were released from preformed substrates or synthesized with insect damage, plants were exposed to a $^{13}CO_2$ 3 h pulse at the same time that the plant was exposed to THW, MeJA or (*E*)-2-hexenal treatment. Mass spectral analysis of the monoterpenes and sesquiterpenes showed no enhancement in ^{13}C levels during the 3 h labeling period or in subsequent collection periods (data not shown). However (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, a homosesquiterpene was labeled within the first 3 h of $^{13}CO_2$ exposure (54% enrichment of the ^{13}C label). To allow for the activation of synthesis of terpenes in the plant, a $^{13}CO_2$ 3 h pulse was also run after 48 h of insect feeding. The initial induction of (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene occurs within the first 3 h of THW damage, while methyl salicylate (MeSA) does not appear until day 2. When THW damaged plants were exposed to a 3 h $^{13}CO_2$ pulse on the third day of insect damage, MeSA and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene incorporated 34 and 70% of the ^{13}C label respectively (Fig. 5C and D). On the other hand, monoterpenes and sesquiterpenes including 2-carene and α-humulene respectively were not labeled (Fig. 5A and B). With large amounts of mono- and sesquiterpenes stored in trichomes, the possibility exists that dilution of the ^{13}C label may preclude it from being detected. Another possibility is that volatiles are synthesized from pools of stored carbohydrates that are fixed before pulsing. Whether these terpenes are stored as non-volatile precursors, such as terpene glycosides, and volatilized with a biochemical conversion (Boland et al., 1992), or that terpenes are released with changes in the permeability of the storage membrane is unknown. Unlike herbivore damaged cotton plants, in which the release of systemic volatiles is mediated by de novo biosynthesis (Paré and Tumlinson, 1997), in tomato, the endogenous signal that triggers systemic release of volatiles appears to activate the release of terpenes independent of short term synthesis.

Also observed was that monoterpene and sesquiterpene release with insect damage was light independent with the pattern and level of volatiles released being the same whether plants were placed in the dark or exposed to light (Table 1). The C_6-aldehydes also

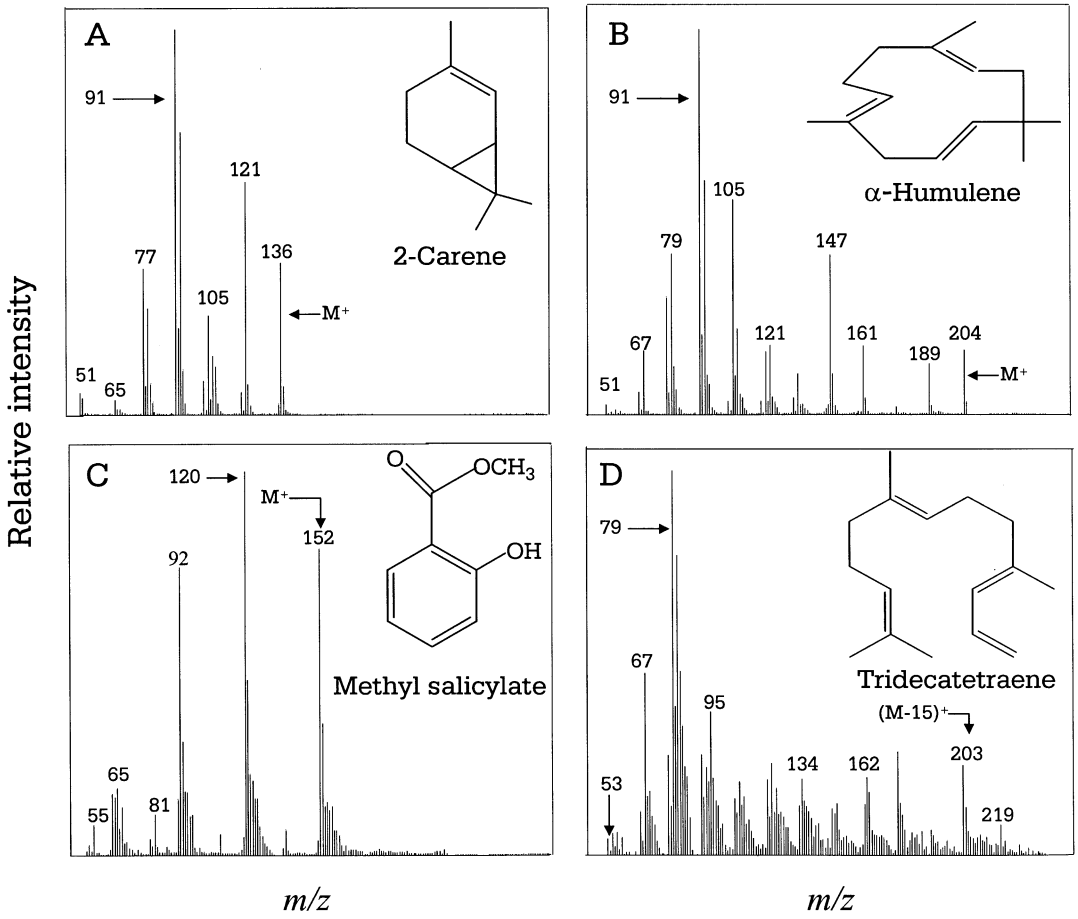

Fig. 5. Electron ionization mass spectra of 2-carene (A), α-humulene (B), methyl salicylate (C), and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (D) collected from tomato after 48 h of THW feeding in an atmosphere consisting of 360 μl l^{-} of >99.9% [^{13}C] CO_2 in synthetic air. M^+ represents the unlabeled compounds; for (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene the M^+-15 ion was used to calculated enrichment levels.

showed no significant differences in their release between light and dark treatment (Table 1) (Duncans, $P<0.05$). Two light dependent compounds (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene and MeSA were, however, released at significantly lower levels (Duncans, $P<0.05$) in the absence of light, with maximum differences being observed on day 3 with (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene and MeSA emission levels 17 and 5 times higher in the light than that of shaded plants, respectively (Table 1). MeSA has

Table 1
Volatiles collected at 12-h intervals with continuous THW damage (μg 12 h^{-1})

Compounds	Day 1		Day 3	
	Light	Dark	Light	Dark
(*Z*)-3-Hexenal	2.4±0.6	1.9±0.5	2.8±0.4	2.2±0.3
(*E*)-2-Hexenal	1.2±0.2	1.8±0.9	2.2±0.4	3.2±0.9
α-Pinene	3.1±0.7	3.3±0.5	5.5±1.5	7.0±1.5
β-Pinene	4.3±0.7	4.8±0.5	6.1±0.9	8.5±0.8
2-Carene	64±11	68±8.1	98±21	101±19
β-Phellandrene	232±59	225±30	360±40	328±73
β-Caryophyllene	14±2.1	12±2.0	40±8.3	30±6.7
α-Humulene	2.8±0.5	2.9±0.6	8.3±1.7	5.1±0.6
δ-Elemene	0.4±0.1	0.3±0.1	0.9±0.2	0.7±0.1
(*E,E*)-4,8,12-Trimethyl-1, 3,7,11-tridecatetraene	33±4.3	5.4±1.1	35±4.5	1.8±0.3
Methyl salicylate	***	***	4.6±2.0	0.9±0.1

Undamaged control plants showed comparable levels of volatile emissions in dark and light (data not shown); (***) not detected in the headspace collections. Values represent mean±standard error ($n=3$).

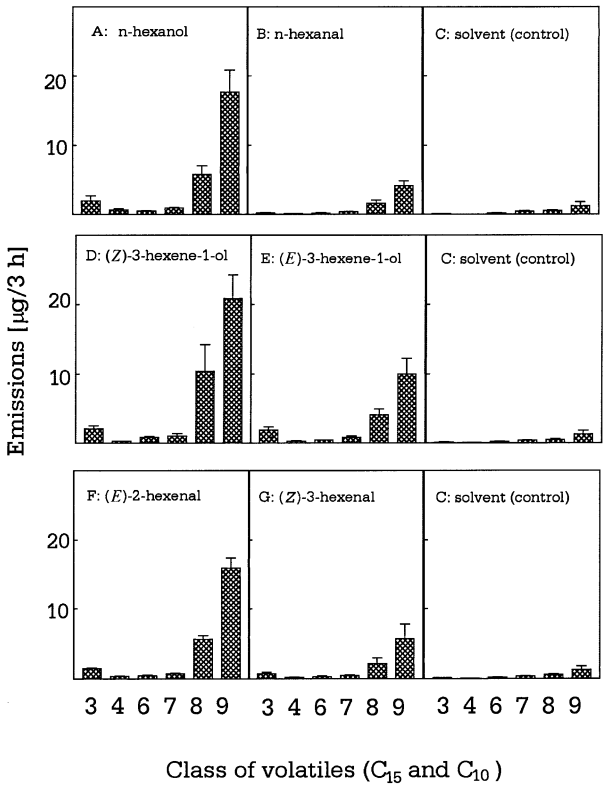

Fig. 6. VOCs collected at 3-h intervals from whole plants treated with different C_6-volatiles. Compounds include β-caryophyllene [3], α-humulene [4], α-pinene [6], β-pinene [7], 2-carene [8], β-phellandrene [9]. Error bars represent standard error ($n=3$).

already been identified as a potential air-borne signal, which when released from tobacco mosaic virus TMV infected tobacco plants was found to activate disease resistance markers in neighboring uninfected plants (Shulaev et al., 1997). This pattern of stored VOCs being light independent, while induced VOCs are light dependent, has also been observed in cotton with studies of diurnal cycle and volatile emissions patterns (Loughrin et al., 1997).

2.4. Rate of C_6-volatile release with herbivore damage and structural activity relationship

To examine if the applied (*E*)-2-hexenal dose that triggered VOC emissions in the plant was comparable to what C_6-volatiles are released naturally with insect damage, the release rate for synthetic (*E*)-2-hexenal was compared to that of C_6-volatiles [i.e. (*Z*)-3-hexenal, (*E*)-2-hexenal and (*Z*)-3-hexene-1-ol] that were released from the plant with insect damage. Volatiles were collected from small closed chambers (see Experimental). For five continuously damaged THW leaflets, the emission rate for the C_6-volatiles was 2.5 ± 0.5 nmol min^{-1}; by contrast, for the100 nmol synthetic (*E*)-2-hexenal dose that was used for chemically treated plants, the emission rate was 50 ± 10 nmol min^{-1}. Extrapolating C_6-volatile emission levels for damaged leaflets to account for whole plant volatile release (whole plants contain ca. 25 leaflets), it was found that the C_6-green leaf volatiles released from whole plants would be 12.5 nmol min^{-1} or about 4 times less than the 50 nmol min^{-1} of synthetic (*E*)-2-hexenal released in the chambers with C_6 treatment. To estimate the dose of (*E*)-2-hexenal that was observed by the plants, the amount of synthetic (*E*)-2-hexenal that became volatilized over different time intervals was measured. For 2 min collections, $\geqslant 95\%$ of the (*E*)-2-hexenal added to the chamber was recovered from collection filters with air flow through the chamber. Shorter collection intervals significantly reduced recovery (Duncans, $P<0.05$) indicating that an exogenous application of (*E*)-2-hexenal (100 nmol) was volatilized within 2 min of application in the chamber.

To examine structure–activity relationships, a series of commercially available C_6-volatiles (i.e. hexanal, hexanol, (*E*)-3-hexene-1-ol, (*Z*)-3-hexene-1-ol, (*E*)-2-hexenal, (*Z*)-3-hexenal) were tested for their efficacy in activating volatile emissions. Among the blend of monitored terpenes, monoterpene emissions (Fig. 6, compounds 6–9) was more influenced by structural differences among C_6-volatiles than sesquiterpene emissions (Fig. 6, compounds 3 and 4). The most potent C_6-volatile in triggering monoterpene emissions was (*Z*)-3-hexene-1-ol which was twice as effective as its *trans* isomer (Duncans, $P<0.05$) (Fig. 6D and E). Also ca. 3 times higher levels of monoterpene emissions were triggered by the conjugated (*E*)-2-hexenal than a non-conjugated (*Z*)-3-hexenal (Duncans, $P<0.05$) (Fig. 6F and G). Comparing the straight chain saturated alcohol to its aldehyde equivalent, hexanol showed a 4 times higher level of VOCs than hexanal with the later emission levels being indistinguishable from control (Duncans, $P<0.05$) (Fig. 6A and B). Interestingly, the alcohol is more active than the aldehyde in reducing aphid fecundity when insects feed on tomato plants; this is thought to be due to induced changes in the leaves upon which the aphids had fed (Hildebrand et al., 1993).

These data demonstrate that in tomato, insect feeding triggers the release of a blend of VOCs locally (terpenes and C_6-volatiles) and terpenes systemically. The model that was set forth with cotton in which systemic volatiles are synthesized and then released from undamaged portions of the plant (Paré and Tumlinson, 1997) does not seem to be the case with several monoterpenes and sesquiterpenes in tomato, in which systemically released volatiles are not synthesized de novo. Exogenous C_6-aldehyde treatments trigger a profile of terpenes similar to MeJA treatment. Volatile analysis of systemic emissions with aldehyde treatment indicates that C_6-components do not activate C_6-volatile emissions; however, aldehyde treatment does trigger systemic terpene emissions. MeJA

treatment triggers local terpene release however neither C_6-volatiles nor systemic terpenes were emitted. The absence of local C_6-emissions with MeJA treatment raises the question if C_6 activation is required for systemic VOC emissions.

3. Experimental

3.1. Plants, insects and reagents

Tomato plants (*Lycopersicon esculentum* var. Solar Set) were maintained in an insect free-facility in which temp. was maintained at 29±40 °C with a relative humidity 40±10%. Plants were grown under metal halide and high pressure sodium lamps for a 16-h/8-h light/dark photoperiod with a total light intensity of 700 μmol/m^2/s. Plants were grown in 16 -cm diameter pots using Pro-gro potting soil having a controlled release fertilizer Osmocot (Scotts-Sierra Horticulture, Marysville, OH). Six week-old plants that were 40–50 cm tall and had not set flower buds were used.

Tobacco hornworm (*Manduca sexta*) eggs and diet were obtained from North Carolina State University Insectary (Raleigh, NC). The eggs were incubated with hornworm diet at 26 °C, relative humidity ca. 90% and a 16-h/8-h light /dark cycle. Three-week old larvae were starved for 7 h and then placed on plants to generate insect damage.

(*Z*)-3-Hexenal, (*E*)-3-hexene-1-ol and MeJA (20% (*Z*)-*epi*- form content) were obtained from Bedoukian Research, Inc. (Danbury, CT) and were of ⩾99% purity as determined by capillary GC–FID analysis. Other C_6 compounds were purchased from Sigma-Aldrich (St. Louis, MO) and were also of ⩾99% purity. Solvents used were of GC grade purity.

3.2. Volatile collections and plant treatments

To analyze for volatiles emitted from intact plants, a glass cylinder 60×14-cm-diameter (Analytical Research Systems, Gainsville, FL) was placed over the aerial portion of the plant. At the base of the chamber a split plate with a hole in the center fit loosely around the stem of the plant like a guillotine. Charcoal-purified air was passed over the plant at a rate of 5 l min^{-1} and plant volatiles were collected by putting 1 l min^{-1} by vacuum through Super-Q adsorbent traps located at the base of the collection chamber. Plants were allowed to equilibrate within the chamber for 8 h before volatile sampling; the beginning point for volatile collections was also the start time for chemical treatment of the plant or herbivore damage to the leaves. For insect damage, 4 larvae were placed randomly on the leaves and allowed to feed continuously. For chemical treatment, 100 nmol of either MeJA [or a given C_6-volatile including hexanal, hexanol, (*E*)-3-hexene-1-ol, (*Z*)-3-hexene-1-ol, (*E*)-2-hexenal, (*Z*)-3-hexenal] was dissolved in 100 μl of dichloromethane and applied on filter-paper discs placed at the top inside the collecting chamber. Flow in and out of the chamber was stopped for 5 min at the time of chemical treatment. Plants were exposed to solvent without aldehyde or MeJA as a solvent control.

For analysis of volatiles from insect damaged light unexposed plants, plants were placed in collection chamber wrapped with aluminum foil. To extend the period of volatile collection from insect damaged plants, one larvae instead of four was placed in each chamber and insect damage was monitored daily to insure equal feeding on light exposed and shaded plants.

For analysis of volatiles from untreated portions of treated plants, potted plants were shifted ca. 15 cm from the collection chamber such that the 2 lowest true leaves were positioned outside the collection chamber. For insect damage, THW larvae were allowed to feed continuously and for chemical treatment, the lower leaves were wrapped in Teflon bags (8×18 cm; VWR Scientific) for 3 h with the chemically treated filter paper placed inside.

To analyze volatiles from THW damaged excised leaves, detached leaves containing five leaflets were placed in 37×4-cm-diameter closed glass cylinders with moistened cotton wrapped around the base of the petiole to reduce dessication. Charcoal-purified air was passed over the cut portion of the plant at a rate of 0.5 l min^{-1} and exited the chamber through Super-Q adsorbent traps located at the tip end of the chamber. For insect damage, two larvae were placed on a leaf and allowed to feed continuously.

To compare the head space release of synthetic aldehydes to that of insect damaged excised leaves, a range of doses (1 nmol to 10 μmol) of (*E*)-2-hexenal dissolved in dichloromethane and applied to filter paper were placed in 37×4-cm-diameter closed glass cylinders. Two volatile collection filters were serially connected to the outlet to ensure that all the aldehyde was collected. Volatiles were collected for 5 min as all aldehydes were flushed from the chamber within that time period.

3.3. Chemical analysis of volatiles

Volatiles collected on the Super-Q adsorbent traps for a 3-h interval were eluted with 150 μl of dichloromethane, nonyl acetate was added as an internal standard and extracts were analyzed by capillary GC on a 15-m×0.25-mm (i.d.) fused silica column with a 0.25-μm-thick bonded methyl siloxane (Quadrex, New Haven, CT). Injections were made in the splitless mode for 30 s, and the gas chromotograph was operated under the following conditions: injector 230 °C, detector 250 °C, column oven 40 °C for 0.5 min, then programmed at a rate of 12–180 °C and finally ramped at a rate of 40–220 °C for 2 min, He carrier gas linear flow

velocity 50 cm/s. The C_6-volatiles, samples were prepared in the same manner except that they were analyzed on a 30-m×0.25-mm (i.d.) DB5 column (J&W Scientefic, Folsom, CA) using the same GC conditions as listed above. Quantification was based on comparison of area under the GC–FID peak with the internal standard added at an amount of 800 ng. For comparisons of the same compound under different treatments, response factors for individual compounds was assumed to be equal. Selected samples were also analyzed by GC–MS on a (ion trap) mass spectrometer (GCQ plus, Thermoquest, Austin, TX) interfaced to a gas chromatograph (Trace GC2000) and operated in the electron impact mode. Injections were made in the splitless mode for 30 sec and samples were analyzed on a 30-m×0.25-mm (i.d.) DB5 column (J&W Scientific, Folsom, CA) under the same conditions previously mentioned in GC/FID analysis. The transfer line and ion-source temp. were adjusted at 230 and 180 °C respectively. The components of the plant volatile emission were identified by comparison of GC retention times with those of authentic standards and by comparison of mass spectra with spectra of an EPA/NIH database.

3.4. In vivo labeling

Synthetic premixed air (Cambridge Isotope Laboratories, Andover, MA; Airco, Riverton, NJ), which contained 360 μl l^{-1} CO_2 (^{13}C 99%), 20.7% oxygen, and the remainder as nitrogen, was introduced into the volatile collection chamber by flushing the chamber at 5 l min^{-1} for 3 min and then reducing the flow to 2.5 l min^{-1}. The same purging procedure was followed to switch back to atmospheric air. THW feeding of the leaves began on day 1 at 800 h; the $^{13}CO_2$ label was added to the chamber on day 3 at 800 h for a 3 h interval and $^{13}CO_2$ gassing was repeated on day 4. Plant volatiles were collected for 3 h intervals between 800 h and 2000 h on days 3 and 4. To determine the amount of ^{13}C incorporated in each compound, samples were analyzed by GC–MS and selected mass ions were quantified via computer software analysis. The fraction of each compound that incorporated ^{13}C was computed on a molecule basis (Paré and Tumlinson, 1997).

3.5. Statistics

Analysis of variance was run using a SAS statistical software. Means were separated using Duncans multiple range test at P value less than 0.05.

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