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RESEARCH PAPER

Attacks by a piercing-sucking insect (*Myzus persicae* Sultzer) or a chewing insect (*Leptinotarsa decemlineata* Say) on potato plants (*Solanum tuberosum* L.) induce differential changes in volatile compound release and oxylipin synthesis

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Abstract

Plant defensive strategies bring into play blends of compounds dependent on the type of attacker and coming from different synthesis pathways. Interest in the field is mainly focused on volatile organic compounds (VOCs) and jasmonic acid (JA). By contrast, little is known about the oxidized polyunsaturated fatty acids (PUFAs), such as PUFA-hydroperoxides, PUFA-hydroxides, or PUFA-ketones. PUFA-hydroperoxides and their derivatives might be involved in stress response and show antimicrobial activities. Hydroperoxides are also precursors of JA and some volatile compounds. In this paper, the differential biochemical response of a plant against insects with distinct feeding behaviours is characterized not only in terms of VOC signature and JA profile but also in terms of their precursors synthesized through the lipoxygenase (LOX)-pathway at the early stage of the plant response. For this purpose, two leading pests of potato with distinct feeding behaviours were used: the Colorado Potato Beetle (*Leptinotarsa decemlineata* Say), a chewing herbivore, and the Green Peach Aphid (*Myzus persicae* Sulzer), a piercing-sucking insect. The volatile signatures identified clearly differ in function with the feeding behaviour of the attacker and the aphid, which causes the smaller damages, triggers the emission of a higher number of volatiles. In addition, 9-LOX products, which are usually associated with defence against pathogens, were exclusively activated by aphid attack. Furthermore, a correlation between volatiles and JA accumulation and the evolution of their precursors was determined. Finally, the role of the insect itself on the plant response after insect infestation was highlighted.

Key words: Fatty acid hydroperoxides, insect attack, *Leptinotarsa decemlineata*, mechanical wounding, *Myzus persicae*, volatile organic compounds.

Introduction

To protect themselves against pests, plants have evolved complex strategies in which compounds from different synthesis pathways play key roles. The blend of defensive compounds synthesized depends on the type of attacker and, in the case of insect attack, on the insect feeding behaviour (Walling, 2000; Leitner *et al.*, 2005; Delphia *et al.*, 2007). Interest in the field is mainly focused on volatile organic compounds (VOCs) emission, especially for

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Abbreviations: AOS, allene oxide synthase; CPB, Colorado Potato Beetle; GLV, green leafy volatile; HPL, hydroperoxide lyase; JA, jasmonic acid; LOX, lipoxygenase; OPDA, 12-oxo-phytodienoic acid; PUFA, polyunsaturated fatty acid; SPME, solid phase microextraction; VOC, volatile organic compound.

their impact in tritrophic interactions, and on jasmonic acid (JA) synthesis, mostly for its key role in signal transduction during insect attack, resulting in the activation of an induced defence response (Howe and Jander, 2008). By contrast, little is known about oxidized polyunsaturated fatty acids (PUFAs), such as PUFA-hydroperoxides, PUFA-hydroxides, or PUFA-ketones that are mainly derived from the enzymatic action of lipoxygenases (LOXs; Blée, 1998; Feussner and Wasternack, 2002) and might be involved in the stress response (Weber, 2002). In addition, interest in investigating PUFA-hydroperoxides and their derivative synthesis lies in their demonstrated antimicrobial activities (Prost *et al.*, 2005) as well as in the precursor status of PUFA-hydroperoxides. The starting point of JA synthesis is the dehydration of 13-hydroperoxides of linolenic acid by an allene oxide synthase (AOS) to form 12-oxo-phytodienoic acid (OPDA) and, finally, JA (Blée, 1998; Feussner and Wasternack, 2002) (Fig. 1). Similarly, some VOCs involved in the plant response against insect attack are synthesized through the so-called LOX pathway. The starting point of these volatiles synthesis is the action of a hydroperoxide lyase (HPL) on PUFA-hydroperoxides resulting in the formation of C6- and C9-volatile aldehydes which are involved in both the direct and the indirect plant defence response against insect attack (Matsui, 2006). For example, Vancanneyt *et al.* (2001) showed that suppression of HPL activity in potato results in increased aphid fitness and attack efficiency.

In this paper, the differential biochemical response of a plant against insects with distinct feeding behaviours is characterized not only in terms of the semiochemical compounds involved in stress signalling but also in terms of their precursors synthesized at the early stage of the plant response. For this purpose, two leading pests of potato with distinct feeding behaviours were used. Potato plants were infested either with the Colorado Potato Beetle (CPB, *Leptinotarsa decemlineata* Say), a chewing herbivore, or with the Green Peach Aphid (*Myzus persicae* Sulzer), a piercing-sucking insect. First, VOC signatures and the accumulation in JA content in potato plants after insect attack were determined using GC-MS. In parallel, the content of precursors of the LOX-derived volatiles and JA were studied by HPLC through three days kinetics. Finally, in order to investigate the impact of the insect itself on the early plant response following attack, potato plants were mechanically wounded to mimic either aphid-attack or CPB-attack and precursor profiles were compared with those of potato plants infested with insects.

Materials and methods

Plant and insect rearing

Potato plants (*Solanum tuberosum* L. cv. Désirée) were individually grown from *in vitro* micro-tubers under controlled conditions (18 °C, 16/8 h (light/dark) photoperiod). Aphids and CPB were reared on potato plants in a growth chamber under controlled conditions (20±2 °C, 16/8 h (light/dark) photoperiod).

Plant volatiles

Potato plants (*Solanum tuberosum* L.), with 4–6 developed leaves were used for volatiles collection. Three experiments were conducted: (i) volatiles were collected on intact plants,

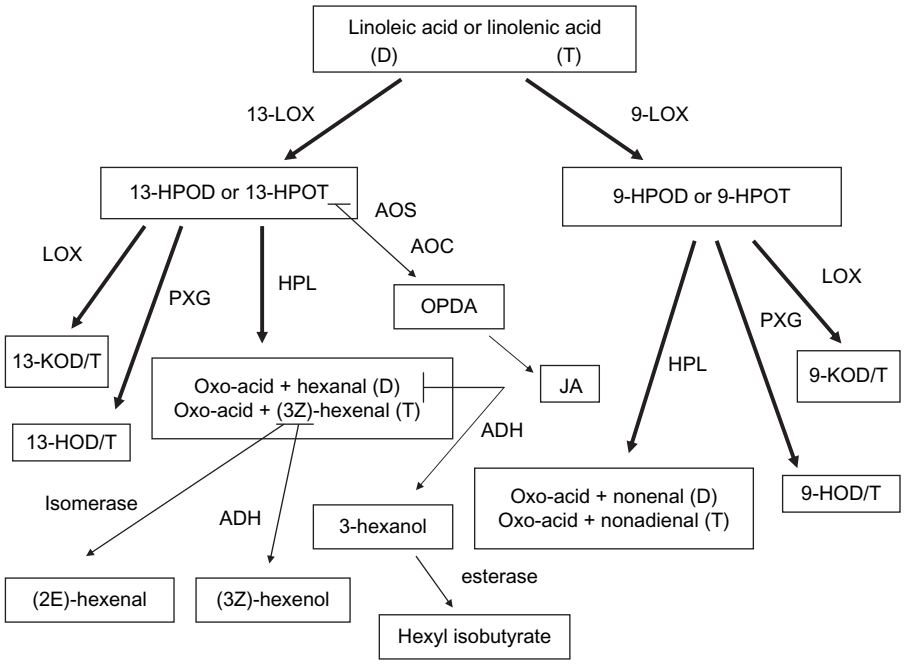

Fig. 1. LOX pathway, simplified scheme. LOX, lipoxygenase; PXG, peroxygenase; HPL, hydroperoxide lyase; AOS, allene oxide synthase; AOC, allene oxide cyclase; ADH, alcohol dehydrogenase; HPOD/T, hydroperoxide of linoleic/linolenic acid; KOD/T, ketone of linoleic/linolenic acid; HOD/T, hydroxide of linoleic/linolenic acid; JA, jasmonic acid, OPDA, 12-oxo-phytodienoic acid.

(ii) volatiles were collected on plants infested with 60 aphids, 24 h after infestation, and (iii) volatiles were collected on plants infested with three 3rd- or 4th-instar larvae of CPB, for 3 h. Each experiment was performed in an individual micromesh cage; the three cages were maintained at the same temperature and photoperiod conditions. Each experiment was performed in triplicate.

Collection and analysis of volatiles: For each plant after infestation, insects were carefully removed and the plant was immediately placed in a collection chamber (3.6 l). A SPME fibre (Supelco, Bellefonte, PA, USA) coated with an absorbent phase made up of polydimethylsiloxane/carboxen/divinylbenzene (PDMS/CAR/DVB) was exposed in the headspace for 1 h at 27±2 °C. After sampling, the fibre was directly analysed by GC-MS on an Agilent 6890N gas chromatograph fitted with an apolar column HP5-MS (30 m×0.5 mm, film thickness: 0.2 μm; Palo Alto, CA, USA) coupled with an Agilent 5973 mass selective detector (MS). Helium was used as the carrier gas at a head pressure of 70 kPa. The oven temperature was programmed as follows: 40 °C for 3 min, then to 240 °C at 10 °C min^{-1}, and finally to 280 °C at 20 °C min^{-1} and held for 25 min. The injection was splitless and the injector temperature was 270 °C. The mass spectra were obtained under electron ionization impact at 70 eV and data acquisition was done over a *m/z* range of 35–300. The analytes were identified on the basis of their retention times and by comparison of their mass spectra with those recorded in the Wiley 238.L Spectrometry Library and those related to previous analysis of pure references when commercially available.

Oxylipin analysis

Potato plants (*Solanum tuberosum* L.) were also used for oxylipin analysis including JA and OPDA. Oxylipin profiles were analysed (i) on intact potato plants, (ii) on potato plants infested with aphids or CPB, and (iii) on potato plants mechanically damaged. Each experiment was performed over a 72 h kinetics including five kinetics points (0 h, 8 h, 24 h, 48 h, and 72 h) and conduced in duplicate. Each experiment was performed in an individual micromesh cage; the cages were maintained at the same temperature and photoperiod conditions.

Infestation with aphids: The three first developed leaves of four potato plants were infested with 60 aphids and confined in a micromesh bag in order to prevent aphids from moving on to the other leaves. Infested leaves marked out for oxylipin analysis after 8 h, 24 h, 48 h, and 72 h of infestation were taken 8 h, 24 h, 48 h, and 72 h, respectively, after the beginning of the infestation. Aphids were removed with a soft brush before harvesting. Harvested leaves were directly frozen in liquid nitrogen and stored at –80 °C.

Infestation with CPB: The three first-developed leaves of five potato plants were infested with two CPB larvae for 3 h. CPB larvae were not let continuously on the leaves because of their high voracity. Twenty-four hours after the first infestation, the three first leaves of potato plants dedicated to the oxylipin analysis at the 48 h and 72 h time points were infested once again as described above. Forty-eight hours after the first infestation, the three first leaves of potato plants dedicated to the oxylipin analysis at the 72 h time points were infested once again. Wounded leaves marked out for oxylipin analysis after 8 h, 24 h, 48 h, and 72 h of infestation were taken 8 h, 24 h, 48 h, and 72 h, respectively, after the first wounding. Harvested leaves were directly frozen in liquid nitrogen and stored at –80 °C.

Mechanical pricks: The five leaflets of the three first developed leaves of four potato plants were wounded with two types of needle systems: the first one made up of nine entomological needles and the second one made up of five entomological needles. The biggest leaflet of each leaf was pricked with the nine needles system, the four other leaflets were pricked with the five needles system. Leaves dedicated to the analysis of oxylipin content after 8 h and 24 h were pricked once at t_0. Leaves dedicated to the analysis of oxylipin content after 48 h, were pricked at t_0 and at t_{24h}. Leaves dedicated to the analysis of oxylipin content after 72 h, were pricked at t_0, at t_{24h}, and at t_{48h}. Wounded leaves marked out for oxylipin analysis after 8 h, 24 h, 48 h, and 72 h of infestation were taken 8 h, 24 h, 48 h, and 72 h, respectively, after the first wounding. Harvested leaves were directly frozen in liquid nitrogen and stored at –80 °C.

Mechanical perforations: Mechanical perforations were performed using a single hole (diameter 6 mm) card punch. First, the five leaflets of the three first developed leaves of five potato plants were wounded as follow: the biggest leaflet and one of the middle leaflets was perforated once. Twenty-four hours after the first perforations, the three first leaves of potato plants dedicated to the oxylipin analysis at the 48 h and 72 h time points were perforated once again as follow: a second hole was made in the biggest leaflet and the middle leaflet that had not yet been perforated was perforated. Forty-eight hours after the first perforations, the three first leaves of potato plants dedicated to the oxylipin analysis at the 72 h time point were perforated for the last time as follow: a third hole was made in the biggest leaflet. Wounded leaves marked out for oxylipin analysis after 8 h, 24 h, 48 h, and 72 h of infestation were taken 8 h, 24 h, 48 h, and 72 h, respectively, after the first wounding. Harvested leaves were directly frozen in liquid nitrogen and stored at –80 °C.

Oxylipin analysis: Oxylipin analysis was performed as previously described, with some modifications (Göbel *et al.*, 2002). For each experiment, 0.5 g of frozen leaves were extracted by adding 10 ml of extraction medium [*n*-hexane:2-propanol, 3:2 (v/v) with 0.0025% (w/v) butylated hydroxytoluene] and immediately homogenized with an Ultra Turrax homogenizer under streaming argon on ice for 30 s. Prior to extraction, a mixture of internal standards was added, either 100 ng D_6-JA, 100 ng D_5-OPDA and

13γ-HOT. The extract was shaken for 10 min and centrifuged at 3200 *g* at 4 °C for 10 min. The clear upper phase was dried under streaming nitrogen, redissolved in 1 ml isopropanol:chloroform [1:2 (v/v)], and applied to an aminopropyl column (Supelclean LC-NH_2 3 ml SPE tubes; Supelco, distributed by Sigma, Deisenhofen, Germany). Elution was performed with 6 ml of 2-propanol:chloroform [1:2 (v/v)] and 9 ml of diethyl ether:acetic acid [98:2 (v/v)]. The diethylether–acetic acid eluate was dried under streaming nitrogen and redissolved in 80 µl methanol:water:acetic acid (75:25:0.1 by vol.). Analysis of oxylipins was carried out on an Agilent 1100 HPLC system coupled to a diode array detector. At first, oxylipins were purified on reversed phase (RP)-HPLC on an ET250/2 Nucleosil 120-5 C18 column (2.1×250 mm, 5 µm particle size; Macherey-Nagel, Düren, Germany), with a solvent system methanol:water: acetic acid (85:15:0.1 by vol.) and a flow rate of 0.18 ml min^{-1}. For the detection of hydroxy fatty acids, A_{234} nm indicating the conjugated diene system was recorded. Keto fatty acids were detected by monitoring A_{272} nm. For quantification of the hydroxy and keto fatty acids, respectively, straight phase-HPLC was carried out on a Zorbax Rx-SIL column (2.1×150 mm, 5 µm particle size; Agilent) with a solvent system of *n*-hexane:2-propanol:trifluoroacetic acid (100:1:0.02 by vol.), and a flow rate of 0.2 ml min^{-1}. Oxylipins were quantified by using 13γ-HOT as an internal standard to determine the recovery of the hydroxy fatty acids and keto fatty acids as well as divinyl ethers. Calibration curves (five point measurements) for 13-HOD, 13-HOT, 13-KOD, and 13-KOT were established. For detection of JA, OPDA, and dinor-OPDA, these compounds were converted to their pentafluorobenzyl esters after purification by RP-HPLC according to Mueller and Brodschelm (1994). The analysis was performed with a Finigan GCQ GC/MS system equipped with a capillary Rtx-5 column (5% diphenyl–95% polydimethyl siloxane, 30 m×0.25 mm, 0.25 µm coating thickness; Restek, Fuldabrück, Germany). Helium was used as carrier gas (0.7 ml min^{-1}). An electron energy of 70 eV, an ion source temperature of 200 °C, and a temperature of 300 °C for the transfer line was used. The samples were measured in the negative chemical ionization mode using ammonia as ionization gas, and the splitless injection mode (opened after 1 min) with an injector temperature of 220 °C. The temperature gradient was 100 °C for 1 min, 100–300 °C at 8 K min^{-1} and 300 °C for 6 min. For quantification, the ions *m/z* 215 (D_6-JA; R_F=11.26, 11.67 min), *m/z* 209 (JA; R_F=11.32, 11.74 min), *m/z* 296 (D_5-oPDA; R_F=20.55, 21.19, 21.71 min), and *m/z* 291 (OPDA; R_F=20.61, 21.24, 21.76 min) were used, respectively.

Data analysis

The data are the average of two repetitions and are expressed as a ratio between the oxylipin concentration 8 h, 24 h, 48 h, or 72 h after the beginning of the experiment and the oxylipin concentration in control plants (0 h). In the figures, the values correspond to the mean and the error bars to the standard deviations. After one-way variance analysis means were classified using Student's *t* test. Differences between means were considered to be significantly different at $P < 0.05$.

Results

VOC signatures of potato plants attacked by a piercing-sucking insect or a chewing herbivore are compared. In addition, the accumulation of JA and its direct precursor OPDA is presented. The accumulation of oxylipins, which are precursors of volatile compounds and/or JA, is then described (Fig. 1). Finally, the impact of the insect itself on the evolution of oxylipin content in infested potato plants is investigated.

Influence of the insect feeding mode on the VOC signature of infested potato plants

It was clear that potato plants infested with insects emit volatile profiles markedly different from those emitted by intact plants (Fig. 2A, B, C). In addition, volatile profiles of aphid-infested plants and CPB-infested plants (Fig. 2B, C, respectively) revealed a sizeable number of emitted compounds which consist of terpenes as well as C6- and C9-aldehydes, alcohols, and esters. These last compounds form the group of LOX-derived volatiles.

The main identified terpenes were emitted by both aphid-infested and CPB-infested plants. Nevertheless, these terpenes were released in different proportions with β-caryophyllene (22.7%), (*E*)-β-farnesene (13.1%), β-sesquiphellandrene (8.6%), and β-elemene (5.4%) emitted in higher proportions after aphid attack whereas α-pinene (7.0%), δ-3-carene (7.0%), limonene (5.8%), and (*E*)-β-farnesene (5.0%) are released in higher proportions after CPB-infestation (Fig. 2B, C).

Concerning LOX-derived volatiles, it clearly appeared that they are mainly emitted after infestation with CPB. Indeed, LOX-derived volatiles represent 53.0% of the volatiles emitted by CPB-infested plants whereas they represent only 8.9% of the volatiles emitted after infestation with aphids (Fig. 2B, C). As shown in Fig. 3, nonanal, hexanal, and hexyl isobutyrate are released after infestation by both aphids and CPB. On the other hand, (2*E*)-hexenal and (3*Z*)-hexenol are only released after aphid attack whereas 3-hexanol is only released after CPB-attack. In addition, C6-volatiles, derived from 13-hydroperoxides, are mainly emitted by aphid-infested plants whereas CPB-infested plants emit mainly nonanal which is a C9-aldehyde resulting from the non-enzymatic degradation of 9-hydroperoxide of oleic acid (Kipers, 1990). Indeed, as shown in Fig. 1, C-9 aldehydes enzymatically formed are nonenals or nonadienals.

Influence of the insect feeding mode on the synthesis of JA and its direct precursor OPDA in infested plants

JA plays a key role in the signal transduction pathway resulting in the activation of the induced defence response

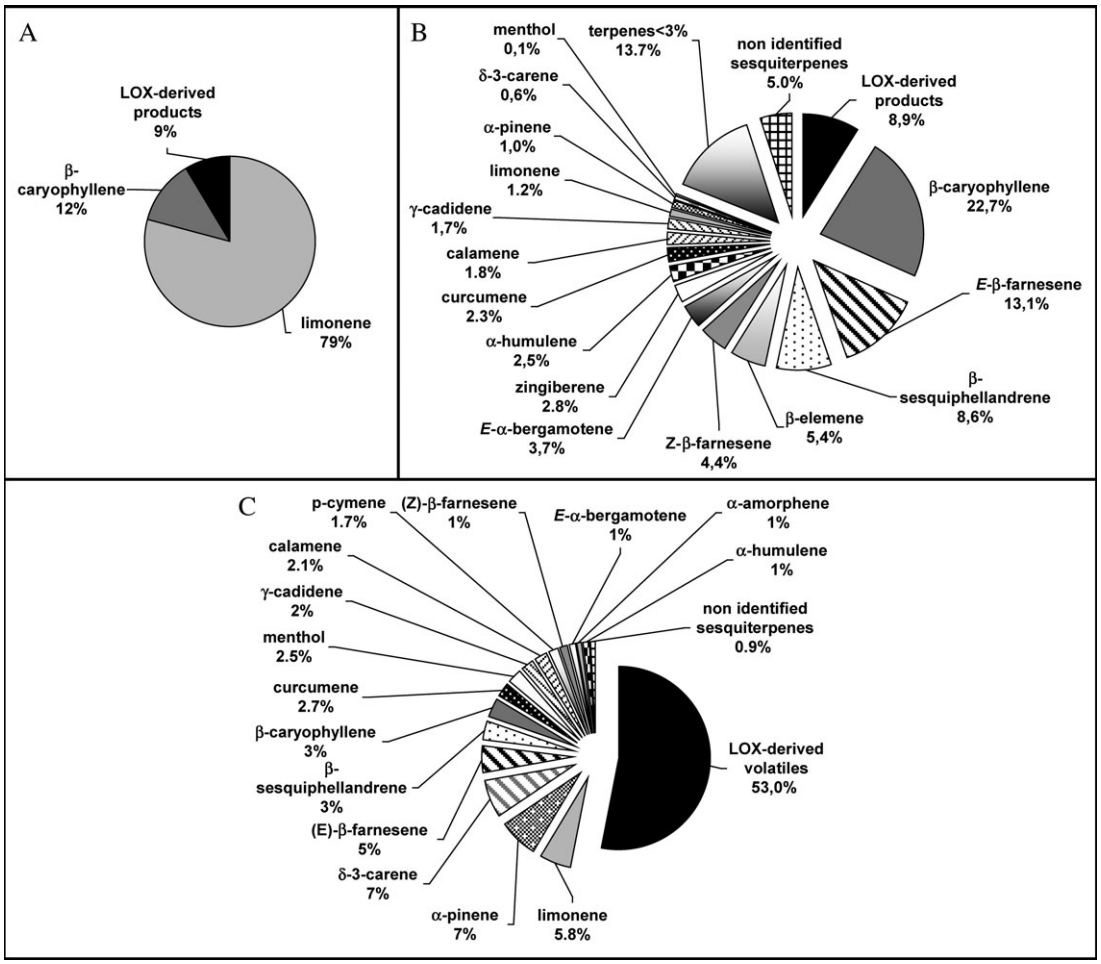

Fig. 2. Relative amount of volatile compounds emitted by (A) control plants, (B) aphid-infested plants after 24 h of infestation, and (C) CPB-infested plants after 3 h of infestation.

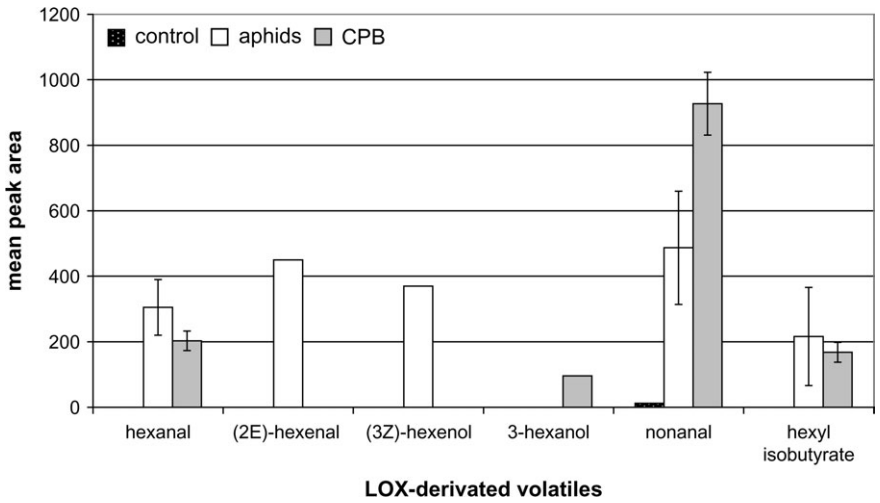

Fig. 3. Peak area of GLVs (green leafy volatiles) emitted by control plants, plants infested with aphids after 24 h of infestation and plants infested with CPB after 3 h of infestation.

against both insects and pathogens. Indeed, JA-deficient plants are usually more susceptible towards herbivore attacks (Kessler *et al.*, 2004; Howe and Jander, 2008) and pathogens (Staswick *et al.*, 1998). The content of JA and its direct precursor OPDA was monitored in control plants and after 8 h, 24 h, 48 h, and 72 h after infestation with aphids or CPB. A significant increase in JA synthesis was only observed for aphid-infested plants 72 h after infestation (Fig. 4A). However, JA content was about twice as high in aphid-infested potato plants after 8 h (0.031 ± 0.006

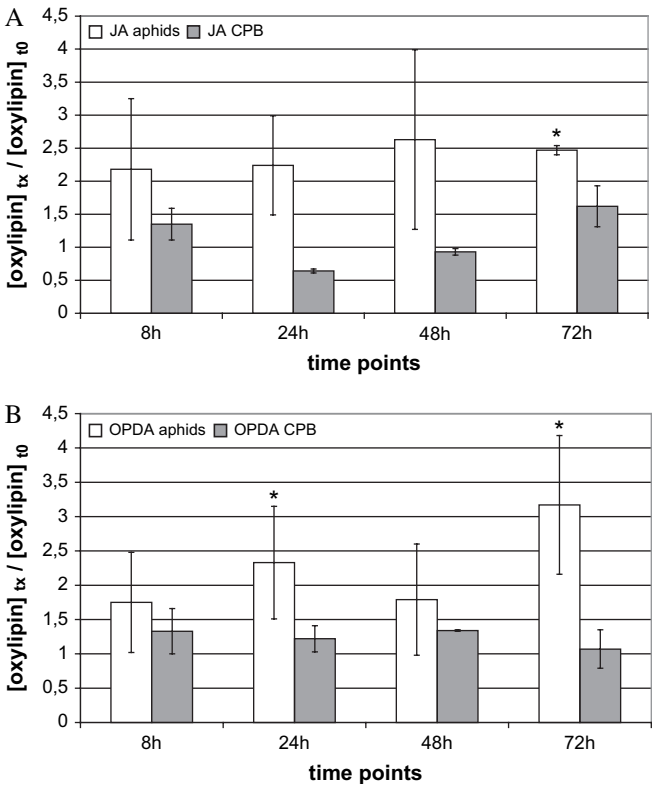

Fig. 4. (A) Ratio between JA content 8 h, 24 h, 48 h, and 72 h after insect attack and in control plants. (B) Ratio between OPDA content 8 h, 24 h, 48 h, and 72 h after insect attack and in control plants. The data are the average of two repetitions and are expressed as a ratio between the oxylipin concentration 8 h, 24 h, 48 h or 72 h after the beginning of the experiment and the oxylipin concentration in control plants (0 h). The values correspond to the mean and the error bars to the standard deviations. After one-way variance analysis means were classified using Student's *t* test. Differences between means were considered to be significantly different at $P < 0.05$.

nmol g^{-1} FW) than in control plants (0.016±0.005 nmol g^{-1} FW) and the JA level in infested potato plants was relatively stable through the kinetics (at 24 h: 0.033±0.001 nmol g^{-1} FW, at 48 h: 0.038±0.008 nmol g^{-1} FW, at 72 h: 0.038±0.011 nmol g^{-1} FW). In addition, the accumulation of JA was higher in aphid-infested potato plants than in CPB-infested potato plants. OPDA content after 24 h (0.135 nmol g^{-1} FW) and 72 h (0.186 nmol g^{-1} FW) of infestation with aphids was significantly higher (P=0.001) than in control plants (0.062 nmol g^{-1} FW). By contrast, the OPDA content in CPB-infested plants was not significantly higher than in control plants (Fig. 4B) at the studied time points and JA and OPDA synthesis was mainly activated after aphid feeding.

Evolution of LOX-derived volatile precursors and JA precursors and their derivatives through insect attack kinetics

Fatty acid hydroperoxides are the starting point of LOX-derived volatiles and jasmonic acid through two different enzymatic pathways (Fig. 1). The hydroperoxide lyase (HPL) cleaves 13- and 9-hydroperoxides into C6- and C9-aldehydes, respectively, and the allene oxide synthase (AOS) leads to the formation of JA from 13-hydroperoxide of linolenic acid (13-HPOT). C6-aldehyde volatiles previously identified are hexanal, the precursor of which is 13-hydroperoxide of linoleic acid (13-HPOD), and (2*E*)-hexenal, the precursor of which is 13-HPOT. Nonanal is the only C9-aldehyde previously identified and it is formed through the non-enzymatic degradation of 9-hydroperoxides of oleic acid. In addition, 3-hexanol and hexyl isobutyrate are the corresponding alcohol and ester, respectively, of hexanal, and (3*Z*)-hexenol is the corresponding alcohol of hexanal. Besides their precursor status, fatty acid hydroperoxides, as well as their derivatives (fatty acid hydroxides, fatty acid ketones, fatty acid divinyl ethers from 9-hydroperoxides), are likely to be involved in plants stress response (Weber, 2002) and exhibit antimicrobial activities (Prost *et al.*, 2005).

Impact of insect attack

A decrease in hydroperoxide content was usually observed after infestation with aphids and CPB except for 9-HPOD after aphid-infestation and for 13-HPOT content after CPB-infestation (Fig. 5A, B, respectively).

The overall decrease observed after infestation is correlated with an increase in the synthesis and emission of hydroperoxide-derived metabolites including LOX-derived volatiles and JA. Indeed, a significant decrease ($P = 0.001$) in 13-HPOT content was observed in aphid-infested potato plants at 48 h and 72 h (Fig. 5B). This decrease is correlated with a significant increase in OPDA content at 24 h and 72 h (Fig. 4B) and with the activation of (2*E*)-hexenal and (3*Z*)-hexenol synthesis (Fig. 3). In addition, 13-HPOD content significantly decreased ($P = 0.013$) after infestation with aphids (0.276±0.028 nmol g^{-1} FW in control plants versus 0.106±0.024 nmol g^{-1} FW at 8 h and 0.082±0.028 nmol g^{-1} FW at 24 h) (Fig. 5C). This decrease is correlated with an activation of hexanal synthesis after 24 h of aphid infestation as shown in Fig. 3. Finally, 9-HPOD content decreased significantly ($P = 0.021$) after CPB-attack (Fig. 5A) and a significant decrease in 9-hydroperoxides of linolenic acid (9-HPOT) was observed after both aphid ($P = 0.003$) and CPB ($P = 0.009$) attack (Fig. 5D). However, no significant increase in either 9-HPOD or 9-HPOT derivatives (hydroxides, ketones, aldehydes) was detected (data not shown).

Although 9-HPOD content increased after aphid attack, no divinyl ethers were detected after infestation. Moreover, no significant increase in 9-hydroxides of linoleic acid (9-HOD) or 9-ketones of linoleic acid (9-KOD) content has been observed (data not shown). Therefore after aphid attack, the 9-HPOD produced seems to be used mainly for its own biological activity.

Concerning 13-HPOT, a significant increase in 13-HPOT content 48 h and 72 h after CPB feeding can be observed in Fig. 5B. However, no significant JA or OPDA increase was

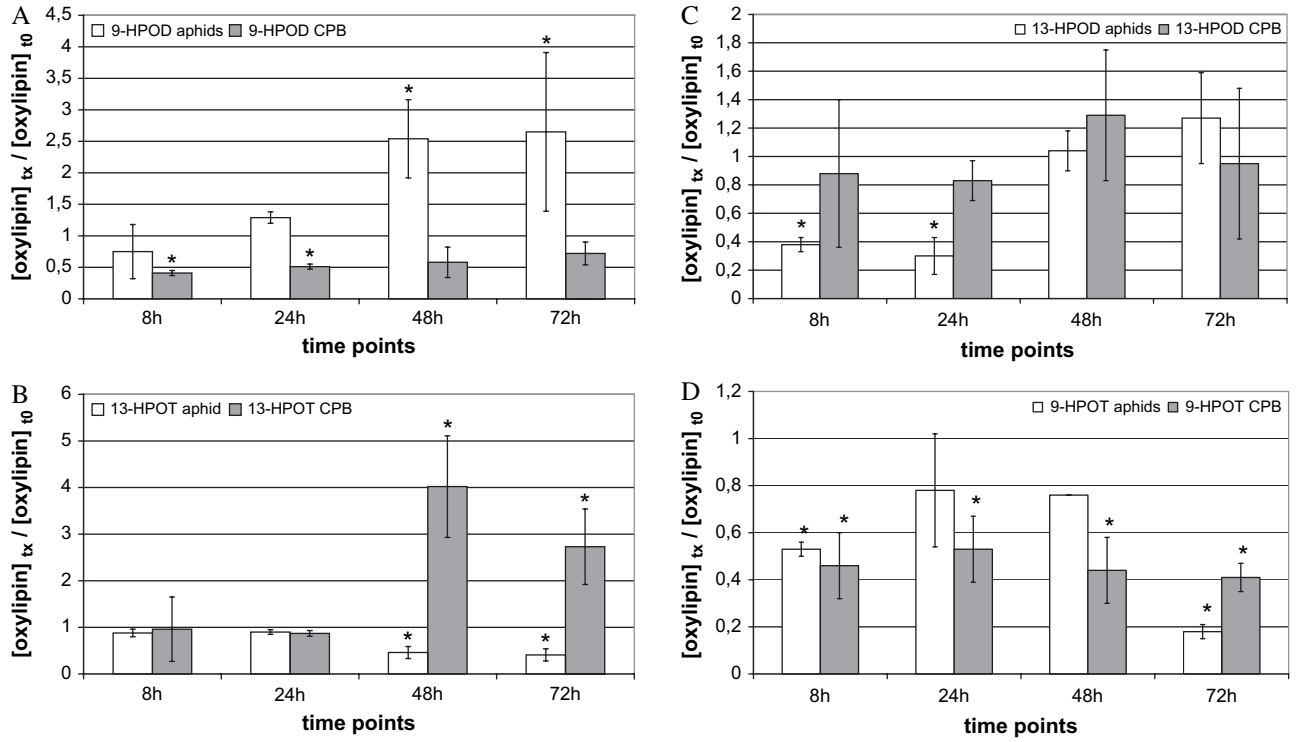

Fig. 5. (A) Ratio between 9-HPOD content 8 h, 24 h, 48 h, and 72 h after insect attack and in control plants. (B) Ratio between 13-HPOT content 8 h, 24 h, 48 h, and 72 h after insect attack and in control plants. (C) Ratio between 13-HPOD content 8 h, 24 h, 48 h, and 72 h after insect attack and in control plants. (D) Ratio between 9-HPOT content 8 h, 24 h, 48 h, and 72 h after insect attack and in control plants. The data are the average of two repetitions and are expressed as a ratio between the oxylipin concentration 8 h, 24 h, 48 h or 72 h after the beginning of the experiment and the oxylipin concentration in control plants (0 h). In all figures, the values correspond to the mean and the error bars to the standard deviations. After one-way variance analysis means were classified using Student's *t* test. Differences between means were considered to be significantly different at $P < 0.05$.

observed in Fig. 4A and B, respectively. Moreover, no significant accumulation in 13-hydroxides of linolenic acid (13-HOT) or in 13-ketones of linolenic acid (13-KOT) was observed. Nevertheless, 13-HOT content after 48 h of infestation is about twice as much as that in control plants (0.803 ± 0.248 nmol g^{-1} FW versus 0.429 ± 0.235 nmol g^{-1} FW; data not shown). In parallel, C6-aldehydes derived from 13-HPOT were not detected during volatile collection (Fig. 3). Therefore, 13-HPOT and its corresponding hydroxide (13-HOT) synthesized in response to CPB-attack seem to be accumulated as an end-product.

Impact of mechanical wounding

In order to investigate the impact of the insect itself on oxylipin synthesis, potato plants were mechanically wounded to mimic either aphid attack or CPB attack and oxylipin profiles were compared with those of potato plants infested with insects. Compounds whose synthesis was significantly modified after insect attack are highlighted and the evolution of their synthesis after wounding is discussed. For aphid infestation, OPDA, 9-HPOD, and 13-HPOT are used while 13-HPOT is used for CPB-infestation.

A significant activation of OPDA synthesis after 24 h and 72 h of aphid infestation was observed (Fig. 4). On the other hand, no significant increase in OPDA content or in JA content after wounding with needles was observed (data not shown). Therefore, as mechanical wounding resulting from aphid feeding does not trigger the synthesis of OPDA, it seems that the aphid itself is responsible for the activation of OPDA and JA synthesis after infestation.

9-HPOD and 13-HPOT synthesis were activated after aphid attack and CPB attack, respectively (Fig. 5). The same compounds were activated after the corresponding mechanical wounding but with different kinetics (Fig. 6). Indeed, whereas the activation of 13-HPOT synthesis was observed 48 h and 72 h after CPB infestation and the activation of 9-HPOD synthesis was observed 48 h and 72 h after aphid infestation (Fig. 5), a significant increase in 9-HPOD was observed 72 h after needle pricks (Fig. 6A) and a significant increase in 13-HPOT content was observed 8 h, 24 h, 48 h, and 72 h after perforations (Fig. 6B). Therefore, the activation of 9-HPOD synthesis is faster against aphids than against needle pricks but 9-HPOD content 48 h (0.362 ± 0.012 nmol g^{-1} FW) and 72 h (0.366 ± 0.079 nmol g^{-1} FW) after aphid attack as well as 9-HPOD content 72 h (0.439 ± 0.065 nmol g^{-1} FW) after needle pricks are statistically similar. On the other hand, 13-HPOT activation is faster after perforations than after CPB-attacks and the maximum content in 13-HPOT after perforations (0.781 ± 0.033 nmol g^{-1} FW) is significantly higher than the maximum content in 13-HPOT after CPB infestation

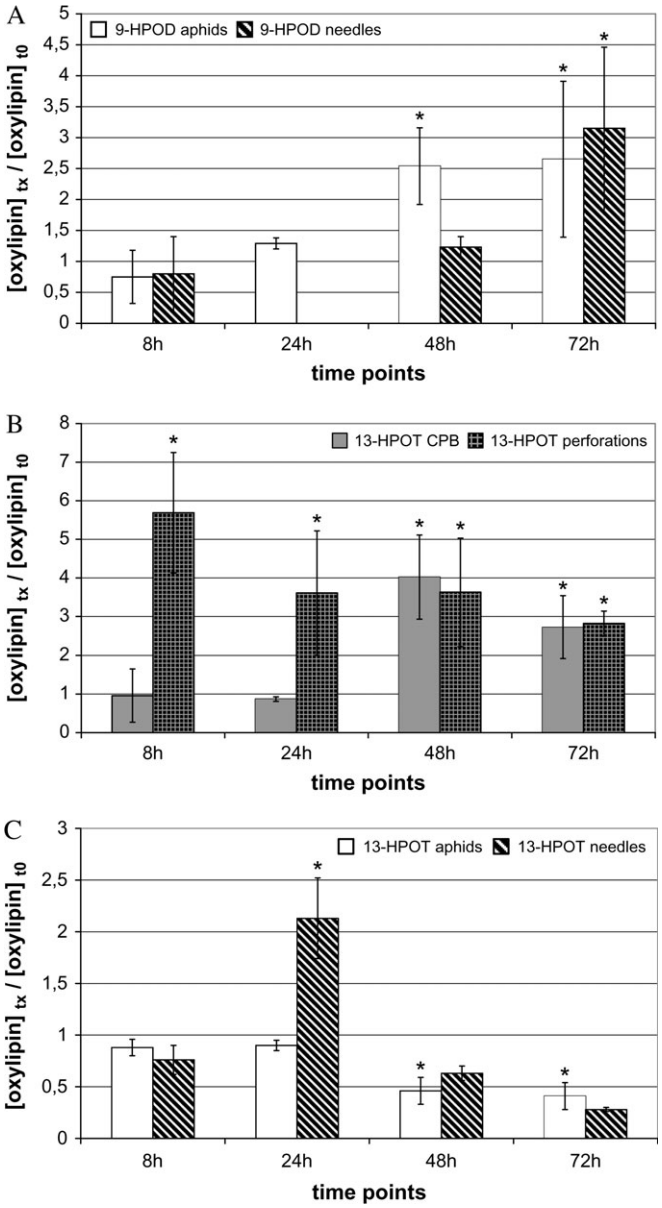

Fig. 6. (A) Ratio between 9-HPOD content 8 h, 24 h, 48 h, and 72 h in aphid-infested or mechanically pricked plants and in control plants. (B) Ratio between 13-HPOT content 8 h, 24 h, 48 h, and 72 h in CPB-infested or mechanically perforated plants and in control plants. (C) Ratio between 13-HPOT content 8 h, 24 h, 48 h, and 72 h in aphid-infested or mechanically pricked plants and in control plants. The data are the average of two repetitions and are expressed as a ratio between the oxylipin concentration 8 h, 24 h, 48 h or 72 h after the beginning of the experiment and the oxylipin concentration in control plants (0 h). In all figures, the values correspond to the mean and the error bars to the standard deviations. After one-way variance analysis means were classified using Student's *t* test. Differences between means were considered to be significantly different at $P < 0.05$.

(0.552 ± 0.022 nmol g^{-1} FW) ($P = 0.015$). Therefore, activation of 13-HPOT synthesis is faster and stronger after perforations than after CPB-attacks.

On the other hand, whereas 13-HPOT content decreased significantly 48 h and 72 h after aphid-attack, a significant increase ($P = 0.003$) in 13-HPOT content 24 h after needle pricks was observed (0.850 ± 0.194 nmol g^{-1} FW at 24 h versus 0.398 ± 0.018 nmol g^{-1} FW in control plants (Fig. 6C). Therefore, compared with needle pricks, aphid impact is more pronounced on 13-HPOT than on 9-HPOD.

Discussion

The composition of the released volatile blend is clearly dependent on the insect feeding habit as reported by Leitner *et al.* (2005). Indeed, 32 terpenes and 5 LOX-derived volatiles were detected in aphid-infested plants whereas 16 terpenes and four LOX-derived volatiles were identified on CPB-infested plants. So the aphid, which is the insect that causes the least damage, leads to the emission of a larger range of volatiles by infested potato plants. However, comparisons between insects showing distinct feeding behaviours resulting in completely different kinds of damage, in particular, in terms of area and timing, are very difficult. Indeed, volatiles were collected after 3 h and 24 h of infestation with CPB and aphids, respectively. These collection times were determined in order to collect volatiles emitted at the beginning of the emission kinetics according to literature (Loughrin *et al.*, 1994; Turlings *et al.*, 1995; Engelberth *et al.*, 2004, Harmel *et al.*, 2007*a*; Girling *et al.*, 2008).

Some of the volatiles identified are involved in tritrophic interactions. Indeed, (*E*)-β-farnesene (EBF) is emitted by insect-infested plants and in higher proportion by aphid-infested plants. EBF is not only an aphid alarm pheromone leading to their dispersion in case of danger, it also plays a key role in tritrophic interaction by attracting aphid predators and parasitoids such as *Episyrphus balteatus* (Francis *et al.*, 2005; Harmel *et al.*, 2007*a*) and *Adalia bipunctata* (Francis *et al.*, 2004). Volatiles released by CPB-infested plants also play a role in tritrophic interactions. Indeed, nonanal, which is the major GLV (green leafy volatile) released after CPB infestation, is an attractant for *Podisum maculiventris* and *Perillus bioculatus* which are two predators of CPB (Dickens, 1999). Furthermore, Dickens (2006) showed that, as alternative methods for management of CPB populations in the field, nonanal can be used in combination with other volatiles, including a CPB pheromone, to attract these pests in traps. Increased amounts of emitted nonanal have already been observed in potato plants infested with CPB (Schütz *et al.*, 1997; Dickens, 2006)

On the LOX-derived volatiles basis, the LOX pathway was mainly activated after CPB infestation. However, 13-LOX derived volatiles were mainly encountered after aphid attack while a non-enzymatic degradation product of the 9-LOX pathway was found after CPB attack. A study of the key intermediates, PUFA-hydroperoxides, precursors of LOX-derived volatiles, and of their metabolites (JA, OPDA, PUFA-hydroxides, PUFA-ketones) is essential to

understand the underlying mechanism of the plant response to biotic stress.

In aphid-infested plants, a clear correlation was found between OPDA, JA, and the increase in LOX-derived volatiles (C6-aldehydes and derivatives) and the decrease in the corresponding PUFA-hydroperoxides precursors (13-HPOT and 13-HPOD). The key intermediates, PUFA-hydroperoxides, can also be considered as end-products. Indeed, in CPB-infested plants 13-HPOT and to a lesser extend 13-HOT accumulated without the formation of JA, OPDA, 13-KOT/D or 13-LOX-derived volatiles. The same phenomenon was observed with aphid-infested plants where 9-HPOD accumulated while no derivatives accumulation was found.

Concerning 9-HPOD synthesis via the activation of the 9-LOX pathway in aphid-infested plants, it can be added that it is known that pathogens mainly induce the synthesis of 9-LOX products (Weber *et al.*, 1999; Göbel *et al.*, 2001, 2002; Howe and Schilmiller, 2002; Fammartino *et al.*, 2007; Fauconnier *et al.*, 2008). Previously, it has been suggested that aphid feeding tends to induce gene sets similar to those activated by fungal or bacterial pathogens (Walling, 2000; Kempena *et al.*, 2007). Indeed, aphid attack on *Arabidopsis thaliana* induces the transcription of genes associated with salicylic acid-dependent responses to pathogens such as *PR-1* (Moran and Thompson, 2001; De Vos *et al.*, 2005; Thompson and Goggin, 2006). It appears here that aphid attacks mostly trigger the synthesis of defence compounds that are also involved against pathogens. The parallel that can be drawn between the intimate and long-lasting interaction of the aphid stylet with plant cells during feeding and the pathogen–plant cells interaction during infection could explain this similarity (Walling, 2000, 2008; Kempena *et al.*, 2007).

Prost *et al.* (2005) showed that 9-HPOD exhibited antimicrobial activities. However, though plant hydroperoxides are ingested by *M. persicae* during feeding on *Vicia faba* (Harmel *et al.*, 2007*b*), no toxic effect of these hydroperoxides on insects has yet been reported.

Comparison of insect-infested and mechanically wounded plants reveals contrasting situations. 13-HPOT synthesis increased in perforated plants (wounding mimicking CPB attack) compared with the CPB-infested plants while 9-HPOD was synthesized more rapidly in aphid-infested plants compared with plants wounded with needles. OPDA and JA synthesis increased markedly in aphid-infested plants while this was not the case in needle-wounded plants. The role of the aphid itself in the plant response can be thus highlighted in the case of 9-HPOD and OPDA/JA. Accumulation of JA and its precursor OPDA is frequently associated with wounding (Weber, 2002), but the subtle damage caused by needles in our experimental conditions do not seem to trigger the JA pathway. By contrast with aphids, CPB, which causes visible damage, seems to be unable to activate the JA pathway.

Furthermore, some oxylipins are likely to be involved in signal transduction pathways. Indeed, treatment of barley leaves with the 13-hydroxide of linolenic acid (13-HOT) induced the expression of a pathogenesis-related protein in barley which is usually activated after salicylic acid treatment; suggesting that salicylic acid signalling in barley might be mediated, at least in part by 13-HOT (Weber, 2002). Therefore, 13-HPOT, synthesized in response to CPB-attack and perforation wounding, seems to be accumulated, perhaps as a protective compound (Prost *et al.*, 2005) or as a signal molecule.

Furthermore, a decrease in 13-HPOT content was observed after aphid attack while it increased after needle pricks, suggesting that 13-HPOT might be involved in the strategy to avoid plant defences attributed to aphids.

Conclusion

This study demonstrates that the VOC signatures of potato plants clearly differ according to the feeding behaviour of the attacker. Although aphids cause less damage than CPB, it triggers the emission of a higher number of VOCs by the infested plant. Focusing on LOX-derived volatile precursors allowed us to conclude that 9-HPOD synthesis is exclusively activated after aphid attack, suggesting a parallel between aphid infestation and pathogen infection. With regard to 9-HPOD and 13-HPOT, which are accumulated after aphid attack and CPB attack, respectively, further investigations have to be performed in order to determine their possible toxic effect on the insect and/or signalling role in the plant. Their signalling role could be investigated by treating potato leaves with 9-HPOD in the case of aphid attack or 13-HPOT in the case of CPB attack and studying the physiological responses. Furthermore, our study revealed a correlation between volatiles or JA activation and their precursors decrease. Finally, with regard to the distinct oxylipin profiles between insect attack and the corresponding mechanical wounding, it appears that the insect itself plays a significant role in the defence plant response, especially in terms of kinetics.

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References

Blée E. 1998. Phytooxylipins and plant defence reactions. *Progress in Lipid Research* **1,** 33–72.

De Vos M, Van Oosten V, Van Poecke R, *et al.* 2005. Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Molecular Plant–Microbe Interactions* **18,** 923–937.

Delphia C, Mescher M, De Moraes C. 2007. Induction of plant volatiles by herbivores with different feeding habits and the effects of induced defences on host-plant selection by thrips. *Journal of Chemical Ecology* **33,** 997–1012.

Dickens J. 1999. Predator–prey interactions: olfactory adaptations of generalist and specialist predators. *Agricultural and Forest Entomology* **1,** 47–54.

Dickens J. 2006. Plant volatiles moderate response to aggregation pheromone in Colorado potato beetle. *Journal of Applied Entomology* **130,** 26–31.

Engelberth J, Alborn H, Schmelz E, Tumlinson J. 2004. Airborne signals prime plants against insect herbivore attack. *Proceedings of the National Academy of Sciences, USA* **101,** 1781–1785.

Fammartino A, Cardinale F, Göbel C, Mene-Saffrane L, Fournier J, Feussner I, Esquerre-Tugaye M-T. 2007. Characterization of a divinyl ether biosynthetic pathway specifically associated with pathogenesis in tobacco. *Plant Physiology* **143,** 378–388.

Fauconnier ML, Rojas-Beltran J, Dupuis B, Delaplace P, Frettinger P, Gosset V, du Jardin P. 2008. Changes in oxylipin synthesis after *Phytophthora infestans* infection of potato leaves do not correlate with resistance. *Plant Physiology and Biochemistry* **46,** 823–831.

Feussner I, Wasternack C. 2002. The lipoxygenase pathway. *Annual Review of Plant Biology* **53,** 275–297.

Francis F, Lognay G, Haubruge E. 2004. Olfactory responses to aphid and host plant volatile releases: (*E*)-β-farnesene and effective kairomone for the predator *Adalia bipunctata*. *Journal of Chemical Ecology* **30,** 741–755.

Francis F, Martin T, Lognay G, Haubruge E. 2005. Role of (*E*)-β-farnesene in systematic aphid prey location by *Episyrphus balteatus* larvae (Diptera: Syrphidae). *European Journal of Entomology* **102,** 431–436.

Girling R, Madison R, Hassall M, Poppy G, Turner J. 2008. Investigations into plant biochemical wound-response pathways involved in the production of aphid-induced plant volatiles. *Journal of Experimental Botany* **59,** 3077–3085.

Göbel C, Feussner I, Hamberg M, Rosahl S. 2002. Oxylipin profiling in pathogen-infected potato leaves. *Biochimica et Biophysica Acta* **1584,** 55–64.

Göbel C, Feussner I, Schmidt A, Scheel D, Sanchez-Serrano J, Hamberg M, Rosahl S. 2001. Oxylipin profiling reveals the preferential stimulation of the 9-lipoxygenase pathway in elicitor-treated potato cells. *Journal of Biological Chemistry* **9,** 6267–6273.

Harmel N, Almohamad R, Fauconnier ML, Verheggen F, Marlier M, Haubruge E, Francis F, du Jardin P. 2007*a*. Role of terpenes form aphid-infested potato on searching and oviposition behaviour of *Episyrphus balteatus*. *Insect Sciences* **14,** 57–63.

Harmel N, Delaplace P, Blée E, du Jardin P, Fauconnier ML. 2007*b*. *Myzus persicae* Sulzer aphid contains oxylipins that originate from phloem sap. *Journal of Plant Interactions* **2,** 31–40.

Howe G, Jander G. 2008. Plant immunity to insect herbivores. *Annual Review of Plant Biology* **59,** 41–66.

Howe G, Schilmiller A. 2002. Oxylipin metabolism in response to stress. *Current Opinion in Plant Biology* **5,** 230–236.

Kempena L, Cui X, Holzer F, Walling L. 2007. Arabidopsis transcriptome changes in response to phloem-feeding silverleaf whitefly nymphs. Similarities and distinctions in responses to aphids. *Plant Physiology* **143,** 849–865.

Kessler A, Halitschke R, Baldwin IT. 2004. Silencing the jasmotate cascade: induced plant defences and insect populations. *Science* **305,** 665–668.

Kipers K. 1990. Autoxidation of fatty material: a development of taste and odors in can making. *Lubrication Engineering* **46,** 418–427.

Leitner M, Boland W, Mithöfer A. 2005. Direct and indirect defences induced by piercing-sucking and chewing herbivores in *Medigaco truncatula*. *New Phytologist* **167,** 597–606.

Loughrin J, Manukian A, Heath R, Turlings T, Tumlinson J. 1994. Diurnal cycle of emission of induced volatile terpenoids by herbivore-injured cotton plants. *Proceedings of the National Academy of Sciences, USA* **91,** 11839–11840.

Matsui K. 2006. Green leaf volatiles: hydroperoxides lyase pathway of oxylipin metabolism. *Current Opinion in Plant Biology* **9,** 274–280.

Moran P, Thompson G. 2001. Molecular responses to aphid feeding in Arabidopsis in relation to plant defence pathways. *Plant Physiology* **125,** 1074–1085.

Mueller M, Brodschelm W. 1994. Quantification of jasmonic acid by capillary gas chromatography-negative chemical-ionization mass-spectrometry. *Analytical Biochemistry* **218,** 425–435.

Prost I, Dhondt S, Rothe G, *et al*. 2005. Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defence against pathogens. *Plant Physiology* **139,** 1902–1913.

Schütz S, Weiβbecker B, Klein A, Hummel HE. 1997. Host plant selection of the Colorado Potato Beetle as influenced by damage induced volatiles of the potato plant. *Naturwissenschaften* **84,** 212–217.

Staswick P, Yuen G, Lehman C. 1998. Jasmonate signaling mutants of Arabidopsis are susceptible to the soil fungus *Pythium irregulare*. *The Plant Journal* **15,** 747–754.

Thompson G, Goggin F. 2006. Transcriptomics and functional genomics of plant defence induction by phloem-feeding insects. *Journal of Experimental Botany* **57,** 755–766.

Turlings T, Loughrin J, McCall P, Röse U, Lewis J, Tumlinson J. 1995. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proceedings of the National Academy of Sciences, USA* **92,** 4169–4174.

Vancanneyt G, Sanz C, Farmaki T, Paneque M, Ortego F, Castaneara P, Sanchez-Serrano JJ. 2001. Hydroperoxide lyse depletion in transgenic potato plants leads to an increase in aphid performance. *Proceedings of the National Academy of Sciences, USA* **98,** 8139–8144.

Walling LL. 2000. The myriad plant responses to herbivores. *Journal of Plant Growth Regulation* **19,** 195–216.

Walling LL. 2008. Avoiding effective defences: strategies employed by phloem-feeding insects. *Plant Physiology* **146,** 859–866.

Weber H. 2002. Fatty acid-derived signals in plants. *Trends in Plant Science* **7,** 217–225.

Weber H, Chételat A, Caldelari D, Farmer E. 1999. Divinyl ether fatty acid synthesis in late blight-diseased potato leaves. *The Plant Cell* **11,** 485–493.