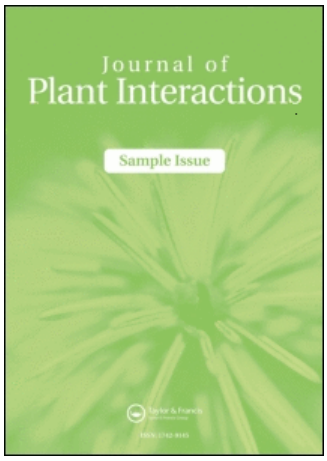

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ORIGINAL ARTICLE

Myzus persicae Sulzer aphid contains oxylipins that originate from phloem sap

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Abstract
Oxylipins are oxygenated fatty acids and their derivatives that play key roles in response to stress. They are widespread in many organisms including mammals or plants but are unknown in arthropods. In this study, we identified oxylipins in *Myzys persicae* Sulzer aphids reared on *Vicia faba* L. plants. Further experiments based on: (i) Radiolabelled substrate incubation with aphid extracts, and (ii) rearing of aphids on an artificial diet revealed that aphids were unable to synthesize oxylipins. As the *V. faba* total leaf oxylipin profile differs from the aphid one, it was assessed whether: (i) Aphids are able to transform leaf oxylipins, or (ii) phloem sap (ingested by aphids when feeding on plants) contains oxylipins different from total leaf ones. Phloem exudates analysis revealed, for the first time, that it contains oxylipins that are different from total leaf ones. These oxylipins are further ingested by aphids.

Keywords: *Oxylipins, aphid,* Myzus persicae*, phloem, fatty acid hydroperoxide, lipoxygenase*

Introduction

Oxylipin is a collective name referring to oxygenated compounds formed from polyunsaturated fatty acids and their derivatives. Oxylipins are known among lower marine organisms (Brash et al. 1996), fungi (Hamberg et al. 1986) and bacteria (Porta & Rocha-Sosa 2001) but are almost exclusively studied among mammals (Kuhn et al. 2002) and plants (Blée 2002).

The first step of the biosynthesis pathway is catalyzed by lipoxygenases and leads to fatty acid hydroperoxides. These compounds are, in turn, transformed by at least seven diverging enzymatic pathways into various biologically active compounds differing in their chemical structures (Blée 2002, Feussner & Wasternack 2002). In mammals, oxylipins are derived mainly from the arachidonic acid cascade and play a major role in inflammatory processes and in stress responses to infection and allergy (Blée 2002). In plants, linoleic (C18:2) and linolenic (C18:3) acids are the main precursors of oxylipins. Oxylipins can exhibit a variety of functions related to plant metabolism, in response to environmental modifications or to biotic or abiotic stress conditions (Howe & Schilmiller 2002).

Oxylipins and fatty acid related compounds play important roles in plant–insect interactions. Among oxylipins, jasmonic acid and more generally jasmonate-related compounds have been extensively studied and their implication in intra- and inter-species signalling has been demonstrated (Schilmiller & Howe 2005). Those compounds induce defence mechanisms conferring protection against insects and pathogens attacks to plants (Browse 2005). It has also been shown that jasmonate and salicylate from host plant can activate cytochrome P450 genes associated with detoxification of allelochemicals in corn earworm (Li et al. 2002). More recently, the signal role upon insect attack of green leafy volatiles resulting from the cleavage by hydroperoxide lyase of fatty acid hydroperoxides into C6-aldehydes and their corresponding alcohols and esters has been demonstrated (Schoonhoven et al. 2005, Matsui 2006). It has been shown that aphid performances increase when feeding on plants lacking hydroperoxide lyase that are unable to synthesize green leafy volatiles (Vancanneyt et al. 2001). A fatty acid derived compound, volicitin, has been identified in caterpillar regurgitate. In maize, volicitin induces the emission of signal molecules that attract parasitoids

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(Alborn et al. 1997). Volicitin chemical structure exhibits a fatty acid (C18:3) and a glutamine moieties (Paré et al. 1998). To our knowledge, oxylipins are not yet described among arthropods.

Among the numerous phytophagous insect species, aphids are considered as major pests because they are widespread and can attack various species of plants (Blackman & Eastop 1994). Aphids can cause direct (phloem sucking) and indirect (phytopathogen virus vector) damage. *Myzus persicae* Sulzer (1776) used here as a model is one of the most generalist aphid species that can be found in temperate weather regions on about 400 host plant species including *Vicia faba* L. (Blackman & Eastop 1994).

In this study, we focus on insect oxylipins and on the elucidation of the origin of the compounds identified in the aphid *M. persicae*.

Materials and methods

Insects and plant materials

Broad beans (*Vicia faba* L.) were grown in a controlled environment at $20 \pm 2^{\circ}C$ under a 16 h photoperiod. Each plant was inoculated with *M. persicae* when emerging. Aphids were collected after 10 days by brushing them carefully from the leaves into liquid nitrogen before storage at $-80^{\circ}C$. Aphids were also reared on artificial diets during 10 days before storage at $-80^{\circ}C$ (Febvay et al. 1995). The diet contains 10 vitamins (amino benzoic acid, ascorbic acid, biotine, calcium chloride, choline chloride, folic acid, myo-inositol, nicotinic acid, pyroxidine and thiamine), 22 amino acids (alanine, β-alanine, arginine, asparagine, aspartic acid, cystein, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine), sucrose, 5 metallic compounds ($CuSO_4.5H_2O$, $FeCl_3.6H_2O$, $MnCl_2.4H_2O$, NaCl and ZnCl), as well as citric acid, cholesterol benzoate and $MgSO_4.7H_2O$. *Vicia faba* leaves were collected in liquid nitrogen from plants grown in the same conditions without aphids.

Collection of phloem exudates

Phloem exudates were collected from plants grown in the same conditions without aphids according to a protocol adapted from King and Zeevaart (1974) that allows the collection of phloem exudates without major contaminations. The petioles of 10 g of *V. faba* freshly cut leaves were placed in 20 ml of a 20 mM Na_2 EDTA solution for 1 h in the dark under 90% of relative humidity. The petioles were then recut and transferred to 20 ml of water in the same conditions for 5 h. The aqueous solution containing phloem exudates was used for oxylipin extraction and analysis.

Oxylipin profiling, extraction and analysis

Oxylipins can exist in free and esterified forms (e.g. esterified in phospholipids or galactolipids). Their analysis requires two distinct analytical protocols. In this study, free and esterified oxylipins were extracted in duplicate and HPLC (high performance liquid chromatography) profiling was performed: (1) on aphids reared on plants and on artificial diets, (2) on *V. faba* leaves, and (3) on phloem exudates according to Göbel et al. (2003).

To analyze oxylipins present in aphids, 2 g and 0.5 g of liquid nitrogen-frozen aphids were respectively used for free and esterified oxylipin analysis. For *V. faba* leaves, a sample of 1 g was used for free and esterified oxylipin analysis.

For the extraction of free oxylipins, (6*Z*, 9*Z*, 11*E*, 13S)-13- hydroxy-6, 9, 11-octadecatrienoic acid (Cayman Chemical, East Ellsworth, MI, USA) was used as internal standard and the previously described amount of frozen material was suspended in 20 ml of extraction medium [isohexane/2-propanol, 3/2 (v/v) with 0.0025% (w/v) butyl hydroxytoluene (BHT)]. After homogenization, the extract was centrifuged at 1300 *g* at 4°C for 10 min. The clear upper phase was collected and a 6.7% (w/v) solution of potassium sulfate was added to reach a volume of 32.5 ml. After vigorous shaking, the extract was centrifuged at 1300 *g* at 4°C for 10 min. The upper hexane-rich layer containing the oxylipin fatty acid derivatives was collected and used for further analysis.

For the extraction of esterified oxylipins, triricinoleate (Sigma, St Louis, MO, USA) was used as an internal standard and the amount of frozen material was added to 20 ml of extraction medium [isohexane/2-propanol, 3/2 (v/v) with 0.0025% (w/v) BHT]. After homogenization, the extract was centrifuged at 1300 *g* at 4°C for 10 min. The clear upper phase was collected and a 6.7% (w/v) solution of potassium sulfate was added to bring the volume to 32.5 ml. After vigorous shaking, the extract was centrifuged at 1300 *g* at 4°C for 10 min. The upper hexane-rich layer containing the oxylipin fatty acid derivatives was removed. Subsequently, esterified oxylipins were transmethylated with sodium methoxide according to Göbel et al. (2003). Briefly, the sample was dried under a nitrogen steam. 333 µl of a methanol/toluol (2:1) solution and 167 µl of 0.5 M CH_3NaO were added. The sample was agitated for 20 min at room temperature before addition of 500 µl of a saturated NaCl solution and 20 µl of 32% HCl (v/v). The sample was extracted two times with *n*-hexane (v/v), the organic phases were pooled and dried under a nitrogen stream.

For the extraction of free and esterified oxylipins from phloem exudates, internal standards (6*Z*, 9*Z*,

11*E*, 13S)-13- hydroxy-6, 9, 11-octadecatrienoic acid and triricinoleate respectively for free and esterified oxylpins, were added to the water in which phloem exudates had been collected. The aqueous solution was extracted two times with 15 ml of $CHCl_3$. The organic phases were pooled and evaporated to dryness before HPLC analysis. Esterified oxylipins were transmethylated with sodium methoxide according to Göbel et al. (2003) as described above.

Free and esterified oxylipins were submitted to HPLC analysis for separation, identification and quantification by means of internal standards. The same chromatographic conditions can be applied to free and esterified oxylipin samples. The protocol is divided in three steps: a first step performed on reverse phase column allowed group separation. Each separated fraction was collected and further injected on a straight-phase column that allowed individual separation of oxylipins. When required, individual oxylipins were collected and injected on a chiral phase column that allowed enantiomeric composition determination.

Reverse-phase HPLC analysis was performed on an EC250/2 Nucleosil 120-5 C18 column (250 × 2.1 mm, 5 μm particle size; Macherey & Nagel, Easton, PA, USA) with the following binary gradient system: solvent A (methanol:water:acetic acid (75:15:0.1, v/v) and solvent B (methanol:water:acetic acid (100: 0:0.1, v/v) with the following gradient program: 20% solvent B for 10 min, followed by a linear increase of solvent B up to 40% within 28 min, by a linear increase of solvent B up to 100% within 30 min and held for 15 min, by a linear decrease up to 20% solvent B within 5 min and finally by an isocratic post-run at 15% solvent B for 6 min. The flow rate was 0.18 ml min^{-1} for 30 min then increased linearly to 0.36 ml min^{-1} in the next 5 min, held for 10 min, followed by a linear decrease to 0.18 ml min^{-1} in the next 5 min and a post-run of 6 min. The injection volume was 80 μl. Straight-phase HPLC was performed on a Zorbax Rx-SIL column (150 × 2.1 mm, 5 μm particle size, Agilent, Palo Alto, CA, USA) with *n*-hexane/ 2-propanol/ acetic acid (100:1:0.1, v/v/v) as a solvent system at a flow rate of 0.2 ml min^{-1}. The injection volume was 25 μl. To detect hydroperoxy and hydroxy fatty acids, the absorbance was recorded at 234 nm while the absorbance was recorded at 270 nm for keto fatty acids. The enantiomeric composition of the hydroxy fatty acids was analyzed by chiral-phase HPLC on a Chiral OD-H column (150 × 2.1 mm, 5 μm particle size; Baker, Phillisburg, NJ, USA) with *n*-hexane/ 2-propanol/ acetic acid (100:5:0.1, v/v/v) as a solvent system at a flow rate of 0.1 ml min^{-1}. The injection volume was 10 μl.

The oxylipin content was expressed in terms of nmol g^{-1} fresh weight (FW) except for phloem exudates where it was expressed in nmol of oxylipin coming from the phloem exudates collected from one gram of fresh leaves. The values used in the text and in the figures correspond to the mean of two or three replicates as described in the Materials and methods section and the error bars to the standard deviations.

Fatty acids determination in aphids

Free and esterified fatty acids were extracted with the same protocols used for free and esterified oxylipins using respectively heptanoic acid and triheptadecenoic acid (Sigma, St Louis, MO, USA) as internal standard. The determination of the corresponding fatty acid methyl esters was performed by gas chromatography using a flam ionization detector with an Agilent 6890 gas chromatograph fitted with a capillary DB-23 column (30 m × 0.25 mm; 0.25 μm coating thickness; J&W Scientific, Agilent, Palo Alto, CA, USA). Helium was used as carrier gas (1 ml min^{-1}). The temperature gradient was 150°C for 1 min, 150–200°C at 8°C min^{-1}, 200–250°C at 25°C min^{-1} and 250°C for 6 min. Identification of individual fatty acids was performed on the basis of retention time using pure fatty acids (Sigma, St Louis, MO, USA) as standards when available and confirmed by gas chromatography mass spectrometry. The mass spectra were obtained using a mass selective detector (Hewlett-Packard model 5973, Palo Alto, CA, USA) under electron impact ionization at a voltage of 70 eV and data acquisition were done over an *m/z* range of 35–350. The confirmation of identified compounds was performed by comparing the observed mass spectra with those recorded in the Wiley 275 L library and by comparison with pure fatty acid spectra (when available). The analyses were performed in triplicate.

Incubation of Myzus persicae *extracts in the presence of* ^{14}C *linoleic acid*

Approximately 0.5 g of aphids, reared either on plants or on an artificial diet for 10 days, were homogenized for 45 s with an Ultra Turrax (IKA, Staufen, Germany) under a stream of nitrogen in 1.6 ml lysis buffer (90 mM Tris/HCl, pH 9.5, 10% glycerol, 500 mM NaCl, 0.1 Tween 20) on ice. The Ultra Turrax was rinsed with 2.4 ml Tris buffer (100 mM Tris/HCl, pH 7.5). Linoleic acid oxygenation assessment was carried out by incubating aphid preparations with 1 μmol of linoleic acid (Sigma, St Louis, MO, USA) containing 1 μCi of ^{14}C labelled C18:2 (PerkinElmer, Boston, MA) for 30 min at room temperature. Five μmols of $SnCl_2$ in methanol were added to the reaction medium to reduce the hydroperoxy moiety into a hydroxy moiety that is more stable. The mixture was incubated at room temperature for 10 min, and 150 μl of acetic acid was added. The mixture was extracted

twice with 4 ml of $CHCl_3$. The organic phases were pooled, evaporated to dryness and the residue was dissolved in acetonitrile before HPLC analysis. Reverse phase HPLC of ^{14}C-labeled oxidized metabolites was performed, at room temperature, using a Licrospher (Merck, Darmstadt, Germany) 100-RP-18 (5μm) column (4.0 × 125 mm) with a solvent mixture of acetonitrile:water:acetic acid (495:500:0.1, v/v/v) at 0.6 ml min^{-1} for 35 min, followed by elution with acetonitrile:water:acetic acid 800:200:0.1 (v/v/v) to complete the run. Radiochromatograms and peak area integrations were obtained using a Flo-one detector (Perkin-Elmer, Courtaboeuf, France).

Two replicates were performed and soybean lipoxygenase (Sigma, St Louis, MO, USA) was used as positive control (2000 units in 1.6 ml lysis buffer).

Incubation of Myzus persicae *extract in presence of 13-hydroxy linoleic acid*

Aphid samples (0.5 g) reared on plants were extracted with an Ultra Turrax during 45 s with 1.6 ml potassium phosphate buffer (100 mM, pH 6) containing 0.1% w/v of Tween 20. The Ultra Turrax was rinsed with 2.4 ml of Tris/HCl buffer (100 mM, pH 7.5). The buffer solutions were pooled and 20 nmol of 13-hydroxy linoleic acid (13-HOT, Cayman Chemical, East Ellsworth, MI, USA) were added. The mixture was incubated 1 h at room temperature and extracted twice with 4 ml of $CHCl_3$. The organic phases were pooled, evaporated to dryness and HPLC analysis was performed in the same conditions as for oxylipin profiling. Two replicates were performed with aphid extracts (without addition of 13-HOT) and two replicates with boiled and crude aphid extracts (with addition of 13 HOT).

Toxicity tests

Before the toxicity tests, aphids were reared for two weeks on the artificial diet and synchronized wingless aphids pre-adults were used for the assays. Toxicity of 13-HOD and 13-HPOD were tested on aphids reared on oxylipin-complemented artificial diet (Febvay et al. 1995). Four concentrations were used for each compound (0.15 nM, 1.5 nM, 15 nM and150 nM). 13-HOD and 13-HPOD were diluted in ethanol and the solvent was further evaporated under a stream of nitrogen. The tested compounds were suspended in artificial diet by a vigorous agitation using a vortex during 2 h. The real concentrations in the diet were checked by extracting 13-HOD or 13-HPOD with two volumes of diethyl ether and by measuring the concentrations at 234 nm with a spectrophotometer (Shimadzu, Kyoto, Japan). A blank was determined in the same conditions. The artificial diet was changed every two days. Five independent repetitions were carried out using 10 aphids for each concentration and the blank. The mortality was evaluated daily for 10 days.

Results

Aphid oxylipin profiles

Free and esterified oxylipins were identified for the first time in aphids reared on *V. faba* (Figure 1). Free oxylipins are predominant. The main free oxylipins identified, in decreasing order, are: 13-hydroxy octadecadienoic acid (13-HOD), 9-HOD, 9-hydroxy octadecatrienoic acid (9-HOT), 13-HOT, 9-hydroperoxy octadecadienoic acid (9-HPOD), 13-HPOD and 12-HOT. 16-HOT, 9-HPOT and 13-hydroperoxy octadecatrienoic acid (13-HPOT) were not detected (Figure 1A). The esterified oxylipin profile is slightly different: methylated 13-and 9-hydroxy octadecadienoic acid (13- and 9-HODs-Me) are also the main aphid oxylipins, but the most abundant after these are 13-HPOD-Me, 9-HPOD-Me, 9-HOT-Me, 9-HPOT-Me and 13-HPOT-Me (Figure 1B); 12- and 16-HOTs-Me were not identified. It can also be noted that jasmonates and 12-oxophytodienoic acid, its precursor was not detectable in the sample.

Enzymatically formed oxylipins are characterized by their high degree of enantioselectivity where the *S* enantiomer predominates. On this basis, 13-HOD is the only oxylipin enzymatically formed while 9-HOD, 13-HOD-Me are non-enzymatically or partly enzymatically formed. Other oxylipins are present in too small amounts to allow enantiomeric analysis. HOD derivatives are reduced forms of HPOD oxylipins. It can be observed, except for 13-HPOT-Me, that HOD derivatives are present in higher amounts than HPOD ones (Figure 1A and 1B). Moreover colneleic and colnelenic acids were not identified in aphids (data not shown).

The oxylipin profile in aphids is in accordance with fatty acid composition. Gas chromatography analyses reveal that esterified fatty acids exist in 6 to 16-times higher amounts than free ones (Figure 2).

In both forms, linoleic acid (C18:2) is the predominant fatty acid, followed by oleic (C18:1), stearic (C18:0), palmitic (C16:0) and linolenic acids (C18:3). It must be noted that linolenic acid is present in a particular form typical for aphids: the (*Z, Z*)-9, 12, 17- isomer of octadecatrienoic acid. These last results are in accordance with Febvay et al. 1993.

In order to investigate the origin of aphid oxylipins, two distinct experiments were performed. First, the oxylipin profile was determined in aphids reared on an artificial diet. Secondly, ^{14}C C18:2 was incubated with aphid extracts reared on plants or on an artificial diet.

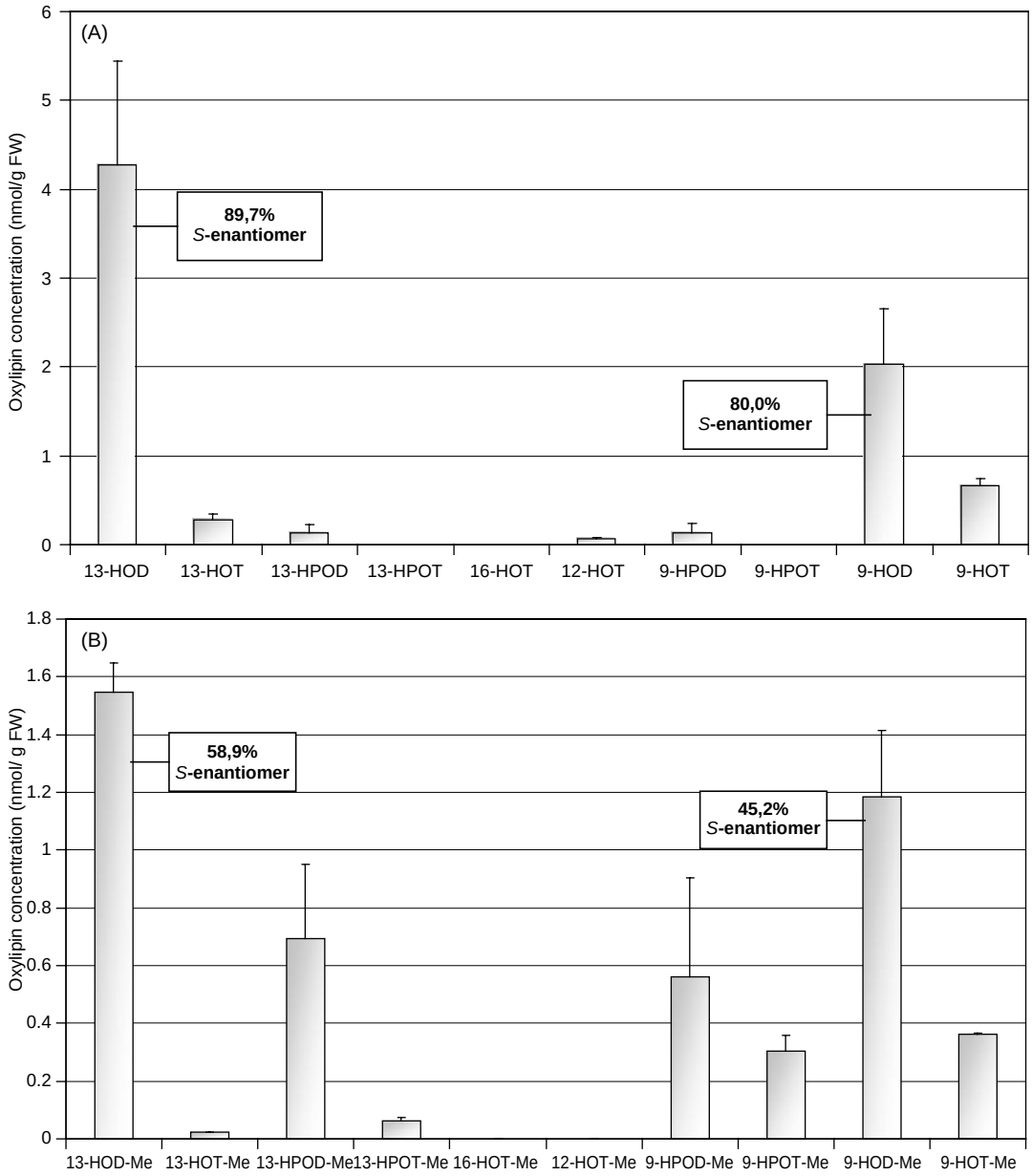

Figure 1. Oxylipin profiles of *Myzus persicae* aphid reared on *Vicia faba* plants. The analysis was performed by HPLC using a three steps procedure: reverse-phase HPLC followed by straight-phase HPLC followed, for the main compounds, by chiral-phase HPLC. (A) Aphid-free oxylipins content. (B) Aphid esterified (Me) oxylipins content. *S*-enantiomer percentage is shown for oxylipins with peak abundance superior to 20 m absorbance units at 234 or 236 nm. Data points show the means $\pm$SE of two independent assays. HPOD (hydroperoxy octadienoic acid), HOD (hydroxy octadienoic acid), HPOT (hydroperoxy octatrienoic acid), HOT (hydroxy octatrienoic acid), Me (methylated).

Oxylipin profiling of aphids reared on artificial diet

Oxylipin profiles of aphids reared 10 days on an artificial diet (Figure 3A and 3B) are qualitatively similar to those obtained with aphids reared on plants: oxylipins are present mainly in free form and oxylipins derived from C18:2 (13-HOD, 9-HOD, 13-HOD-Me, 9-HOD-Me) are predominant. The main difference is the absolute amount of compounds: there are around ten times less oxylipins in the aphids reared on artificial diet as compared to their homologues reared on plants. For example, the content of 13-HOD, the main oxylipin, is respectively 0.34 nmol for aphid reared on artificial diet and 4.2 nmol g^{-1} FW for aphid reared on plants.

Incubation of aphid extracts in presence of ^{14}C C18:2

HPLC analysis using a radioactivity detector revealed that aphid extracts obtained both from insects reared on plants or an artificial diet are unable to transform linoleic acid into oxylipins. The radioactivity is found exclusively in a peak which has the same retention time and UV-spectra as linoleic acid (data not shown). Under the same conditions, commercial soybean lipoxygenase used as positive control instead of aphid extract, revealed as expected

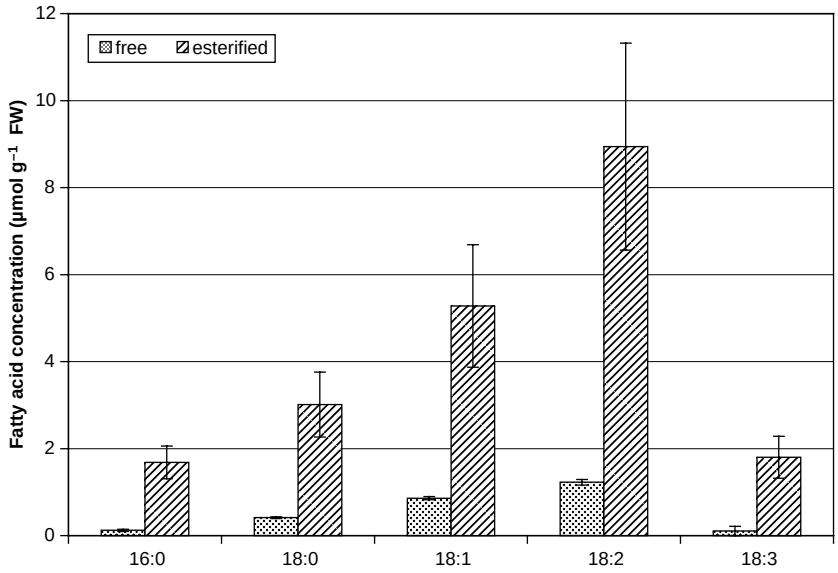

Figure 2. Free and esterified fatty acid profiles of *Myzus persicae* aphids reared on *Vicia faba*. The analysis was performed in duplicate by gas chromatography on crude extracts. Data points show the means ± SD of three independent assays. C16:0: palmitic acid; C18:0: stearic acid; C18:1: oleic acid; C18:2: linoleic acid or octadecadienoic acid; C18:3: linolenic acid or octadecatrienoic acid ((*Z*, *Z*)-9, 12, 17-octadecatrienoic acid).

that the substrate is transformed mainly into 13-HPOD and in a smaller extent into 13-HOD.

Vicia faba *total leaves oxylipin profiling*

Vicia faba leaves exhibit an oxylipin profile typical for green tissues (Figure 4A and 4B). Esterified oxylipins are largely more abundant than free ones. Oxylipin derived from C18:3 (13-HOT-Me, 13-HPOT-ME, 13-HPOT, 13-HOT) are the main compounds and are formed enzymatically.

Incubation of 13-HOT with aphid extracts

Aphids and *V. faba* L. leaf oxylipin profiles are different with, respectively, a predominance of C18:2 and C18:3 derivatives. Whether aphids are capable of transforming 13-HOT into 13-HOD was investigated. HPLC analysis revealed that both the boiled and the crude aphid extracts were unable to transform 13-HOT (data not shown). The results took into account the intrinsic amount of 13-HOD and 13-HOT in the aphids.

Oxylipin profiling of Vicia faba *L. phloem exudates*

Considering that (i) aphids are unable to synthesize and to metabolize oxylipins, (ii) that *V. faba* L. leaves and aphid oxylipin profiles are different, and (iii) that aphids collect phloem sap from plants, it was decided to examine the presence and the composition of oxylipins in phloem exudates. Phloem exudates were collected, extracted and subjected to HPLC analysis for oxylipin identification and quantification. For the first time, several oxylipins were identified in phloem exudates (Figure 5). Oxylipins were only encountered in the free form (no esterified ones). Hydroperoxy fatty acids and hydroxy fatty acids were identified. The oxylipins derived from C18:2 (13-HPOD, 13-HOD, 9-HPOD, 9-HOD) were systematically more abundant than the corresponding compounds originating from C18:3 (13-HPOT, 13-HOT, 9-HPOT, 9-HOT).

Toxicity tests

In order to investigate the toxicity of phloem oxylipins, aphids were reared for 10 days on artificial diets complemented with increasing concentrations of 13-HOD and 13-HPOD.

Mortality was evaluated daily. The results are presented in Table I. It can be concluded that the artificial diet is well-suited to aphids as mortality is low even after 7 days. There is however no significant increase in the mortality of aphids reared with or without 13-HOD or 13-HPOD whatever the concentration tested (Student's *t*-test, $p = 0.05$).

Discussion

This paper shows for the first time that oxylipins can be identified in insects but demonstrates, at the same time, that they originate from the phloem sap in the case of *M. persicae* reared on broad bean. The main oxylipin encountered in aphids is 13-HOD which is enzymatically formed as attested by chiral phase analysis. When aphids are reared on artificial diet instead of plants, the oxylipin profile is unchanged but the total amount of oxylipin is reduced tenfold. It can be stated that aphids gradually eliminate their oxylipin during the ten days period during which they are fed artificially without any contact with a plant. It is reasonable to conclude that aphids are unable to synthesize oxylipins and take them up from

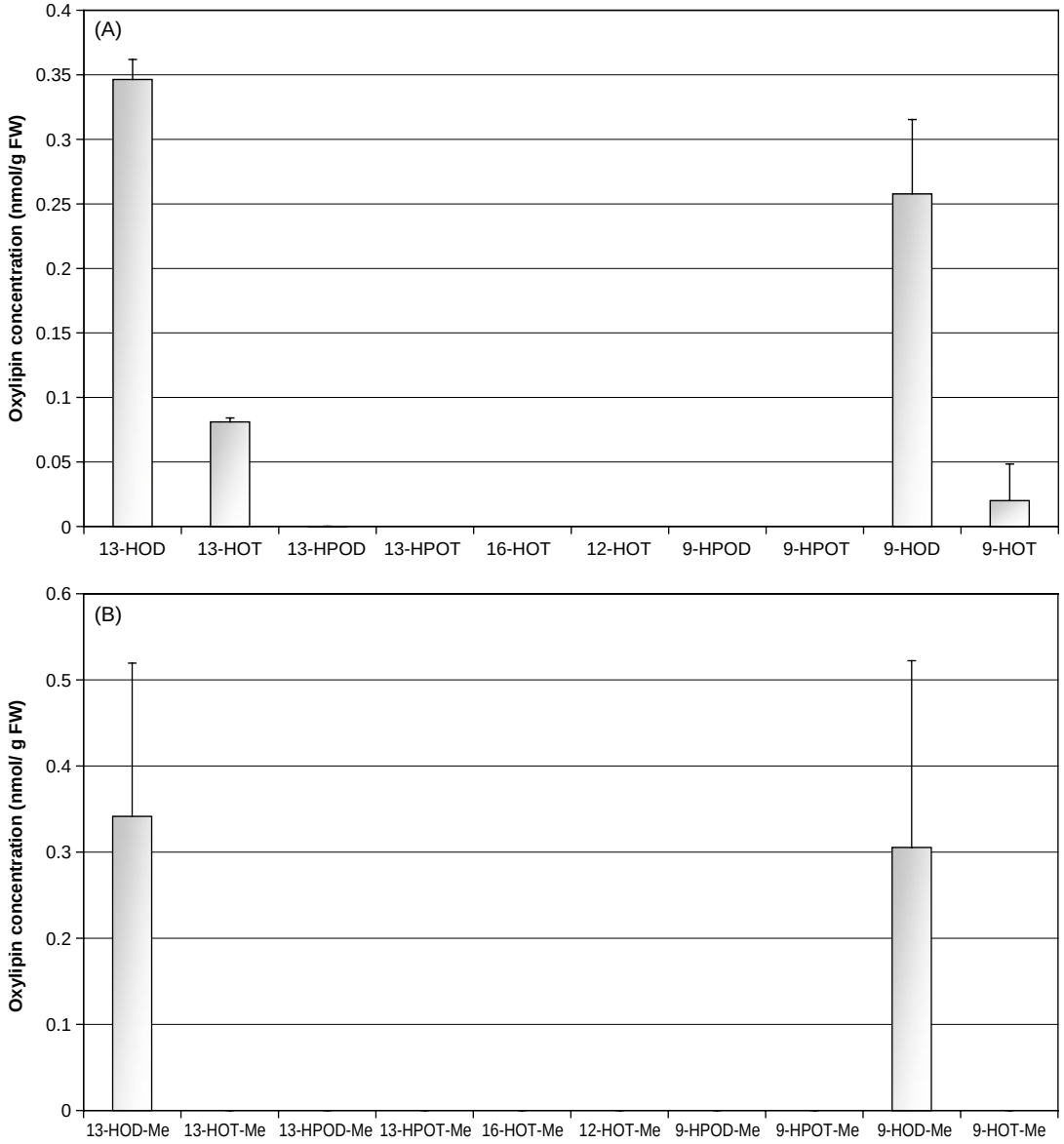

Figure 3. Oxylipin profiles of *Myzus persicae* reared on artificial diet. The analysis was performed by HPLC using a three steps procedure: reverse-phase HPLC followed by straight-phase HPLC. (A) Aphid-free oxylipins content. (B) Aphid esterified (Me) oxylipins content. Data points show the means ± SE of two independent assays. HPOD (hydroperoxy octadienoic acid), HOD (hydroxy octadienoic acid), HPOT (hydroperoxy octatrienoic acid), HOT (hydroxy octatrienoic acid), Me (methylated).

the plant on which they are reared even if they contain the potential substrate to synthesize them (fatty acid profile corresponding to oxylipin profile with mainly C18:2 derivatives).

Nevertheless, it is well known that artificial diets considerably perturb aphid metabolism (Febvay et al. 1995) and could modify the synthesis of oxylipins for example by lack of substrate. The incubation of a crude aphid extract with radiolabeled substrate (^{14}C C18:2) revealed that the substrate is unchanged at the end of the reaction period and that no radioactivity can be detected at the retention times corresponding to the potential reaction products (HPOD and HOD). It seems now clear that aphids are unable to synthesize oxylipins and that they take them up from plants.

Comparing aphid and broad bean leaf oxylipin profiles, it is surprising to note that they respectively contain mainly C18:2 and C18:3 derivatives. It was postulated that a reductase activity allowing double bond reduction was present in aphid extract. Thus whether aphids are capable of transforming C18:3 oxylipins into C18:2 oxylipins by incubating a crude aphid extract with 13-HOT was evaluated. As 13-HOT was not transformed by aphid extract, it can be concluded that aphid oxylipins must originate from the diet. In the literature, the transformation of an oxylipin by an insect is described (Dabrowska et al. 2006) but the reaction is different: caterpillars (*Spodoptera littoralis*) are able to transform 12-oxophytodienoic acid (a precursor of jasmonic acid) into iso oxophytodienoic acid. The reaction is widespread among Lepidopteran larvae.

The elucidation of the origin of oxylipins in aphids requires taking into account the feeding

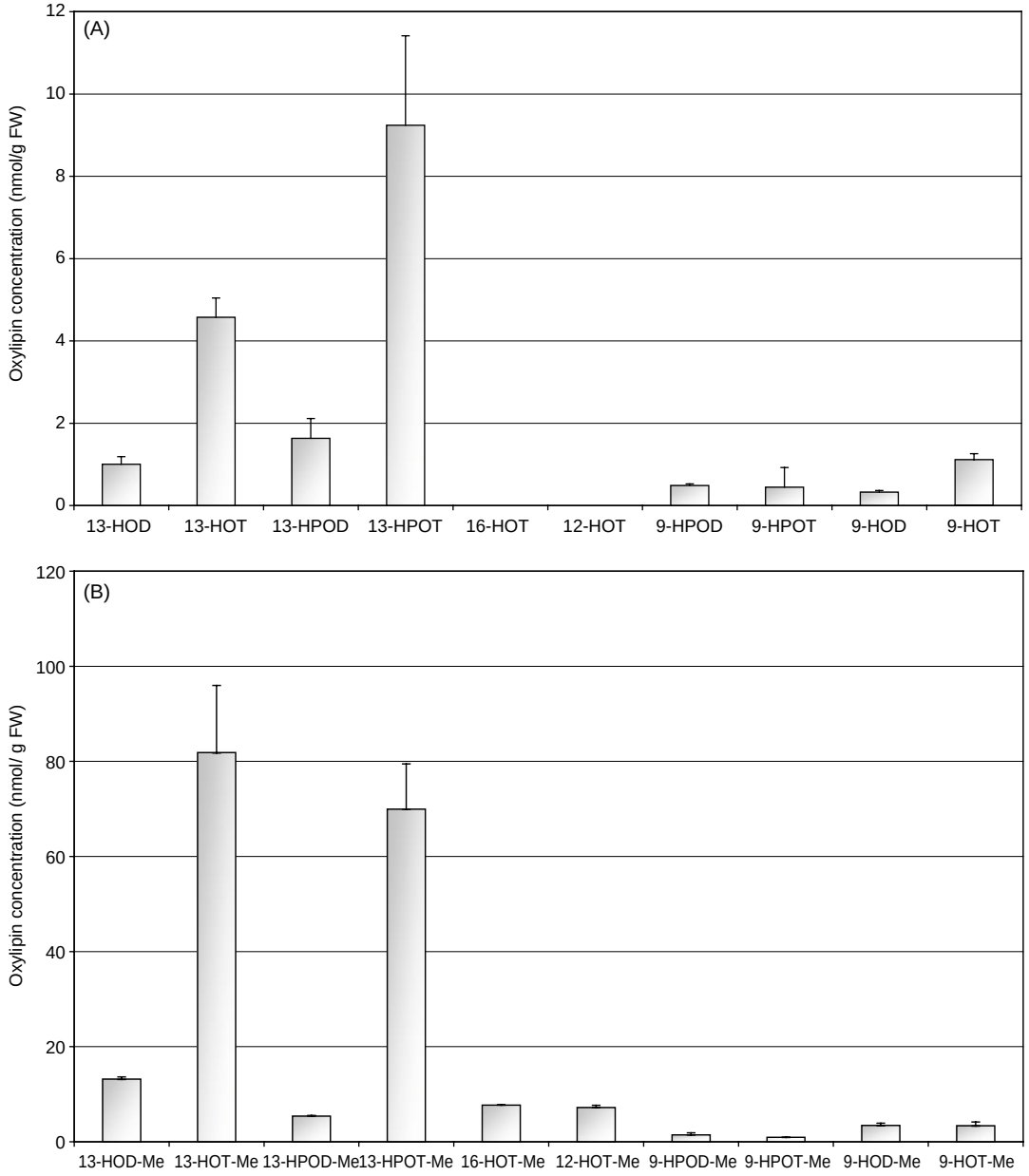

Figure 4. Oxylipin profiles of *Vicia faba* L. leaves. The analysis was performed by HPLC using a three steps procedure: Reverse-phase HPLC followed by straight-phase HPLC. (A) *Vicia faba* L. free oxylipins content. (B) *Vicia faba* L. esterified (Me) oxylipins content. Data points show the means $\pm$SE of two independent assays. HPOD (hydroperoxy octadienoic acid), HOD (hydroxy octadienoic acid), HPOT (hydroperoxy octatrienoic acid), HOT (hydroxy octatrienoic acid), Me (methylated).

comportment of the insect. Aphids are typical phloem sucking insects. They use their flexible stylet to reach the phloem, causing minor damage in the neighboring cells by skirting them (Miles 1999). They ingest relatively large volumes of phloem sap because it contains small amounts of amino acids essential for aphid metabolism (Schoonhoven et al. 2005). Phloem exudates were collected using a method that insures minimal contamination from companion cells (Guelette et al. 2006). In the presence of EDTA, callose (1, 3-ß-glucan) formation is blocked at the incision by chelating Ca^{2+}. It results in enhanced phloem exudation (Madey et al. 2002). Oxylipins were extracted and quantified in phloem exudates, revealing only free oxylipins mainly derived from C18:2.

Phloem sap is responsible for long distance transport of assimilates to non-photosynthesizing organs of the plant (Madey et al. 2002). Phloem sap has been considered for a long time as a mere concentrate of carbohydrates. It increasingly appears that it has a complex composition containing small molecules (Corbesier et al. 2003), peptides and proteins (Giavalisco et al. 2006), nucleic acids (Haywood et al. 2005) and lipids (Madey et al. 2002). Guelette et al. (2006) have shown that phloem exudates lipids and total leaf lipids are different. Linolenic acid represents less than three percent of the total fatty

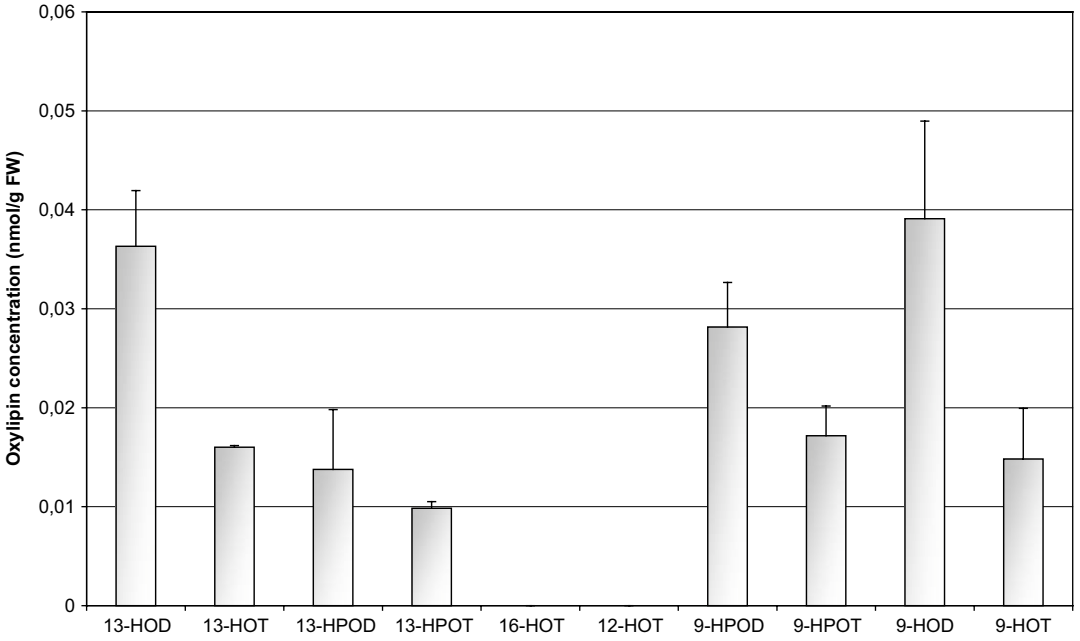

Figure 5. Free oxylipin profile of *Vicia faba* L. ploem exudates. The analysis was performed by HPLC using a three steps procedure: reverse-phase HPLC followed by straight-phase HPLC followed, for the main compounds, by chiral-phase HPLC. Data points show the means ± SE of three independent assays. HPOD (hydroperoxy octadienoic acid), HOD (hydroxy octadienoic acid), HPOT (hydroperoxy octatrienoic acid), HOT (hydroxy octatrienoic acid).

acids in phloem but constitutes 20–40% of the total fatty acids in leaf microsomes and cytosol (Madey et al. 2002). Jasmonates formed through the octadecanoic acid pathway are thought to be transported systematically within wounded plants (Sembdner & Parthier 1993). Jasmonates are the only lipoxygenase-derived metabolites that have been shown to exhibit systemic action. To our knowledge this paper is the first report on a complex array of oxylipins identified in phloem exudates. It can be noted that aphids and host plant phloem exudates have similar oxylipin profiles containing mainly 18:2 derivatives. The relative proportion of HOD/HPOD differs in the two matrices but, as HOD is the direct reduction product of HPOD, this difference may be due to extraction conditions or to the matrix itself.

The role of oxylipins except jasmonic acid in plant–insect interactions is far from clear. In order to investigate whether plant oxylipins that accumulate in aphids can exhibit toxic effects, different concentrations of two oxylipins (13-HPOD and 13-HOD) were added to the diet. 13-HOD is the main oxylipin encountered in aphids while 13-HPOD is notably cytotoxic due to the hydroperoxy moiety (Kaneko et al. 1994, 2001). A large range of concentrations were tested from low physiological concentrations to high, potentially lethal, concentrations. It can be concluded that both compounds even at high concentration (150 nM) do not significantly increase mortality compared to the reference. Aphids are known as formidable pests capable of infesting various host plants (Blackman & Eastop 1994) and of resisting to herbicides (Lan et al. 2005), parasites (Oliver et al. 2003) and secondary metabolites (Francis et al. 2005).

The role of phloem sap oxylipins in aphid–plant interactions must be other than a direct toxic effect. Fatty acid hydroperoxides are substrates of hydroperoxide lyase that leads to the synthesis of C-6 aldehydes. As it has been shown that C-6 aldehydes negatively influence aphid performances (Vancanneyt et al. 2001), an indirect role of phloem sap oxylipins can be envisaged.

Several questions remain to be elucidated. What is the precise role of oxylipins in plant–insect interactions via the phloem? Are oxylipins synthesized by companion cells and transferred into sieve tubes? Do those compounds play any systemic role in the plant?

It could also be interesting to study the localization of oxylipins in the insect body by dissecting them.

Table I. Effect of 13-HOD and 13-HPOD on aphid mortality. Five repetitions of 10 aphids were used to calculate each mean (± standard deviation).

Number of aphids alive after	Blank	13-HOD 0.15 nM	13-HOD 1.5 nM	13-HOD 15 nM	13-HOD 150 nM	13-HPOD 0.15 nM	13-HPOD 1.5 nM	13-HPOD 15 nM	13-HPOD 150 nM
2 days	10 ± 0	9.8 ± 0.4	10 ± 0	10 ± 0	9.8 ± 0.4	10 ± 0	10 ± 0	9.6 ± 0.5	9.6 ± 0.5
7 days	8.8 ± 0.8	7.6 ± 0.5	8.4 ± 1.1	8.2 ± 0.4	8.4 ± 1.5	7.6 ± 0.9	7.6 ± 0.9	8.8 ± 0.8	8.2 ± 0.5

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