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Short communication

Changes in oxylipin synthesis after *Phytophthora infestans* infection of potato leaves do not correlate with resistance

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Abstract

Oxylipins constitute a class of molecules notably involved in host–pathogen interactions. In the potato-*Phytophthora infestans* (Mont.) De Barry (*P. infestans*) relationships, the role of colneleic and colnelenic acids, two oxylipins resulting from the consecutive action of lipoxygenase (EC 1.13.11.12) and divinyl ether synthase (EC 1.-) on respectively linoleic and linolenic acids have been previously reported. In the present paper, five potato cultivars with contrasting resistance to *P. infestans* were submitted to infection. Lipoxygenase pathway response was studied at both transcriptional and metabolic levels. A Northern blot preliminary study revealed that lipoxygenase (*lox1* and *lox3*) and divinyl ether synthase genes were clearly up-regulated 96 h after leaf inoculation with *P. infestans*. Profiling of free and esterified oxylipins performed 24 h, 48 h, 72 h and 96 h after inoculation, showed that esterified oxylipins are mainly produced with 9-derivatives in higher concentrations (esterified forms of colnelenic acid, 9-hydroxy octadecatrienoic acid, 9-hydroperoxy octadecatrienoic acid). Oxylipin accumulation is undetectable 24 h after infection, slightly detectable after 48 h, reaching highest concentrations after 96 h. Cultivars show slightly different oxylipin profiles but the concentration of individual oxylipins differs markedly 96 h after infection. No correlation was found between *P. infestans* resistance levels and oxylipin synthesis rates or concentration. To assess local and systemic effects of colneleic acid application before *P. infestans* infection, Bintje cultivar was sprayed with colneleic acid 72 h before inoculation. Both application modes (local and systemic) resulted in lipoxygenase pathway activation without affecting the resistance level to the pathogen.
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Keywords: Lipoxygenase; Oxylipin; *Phytophthora infestans*; Potato; *Solanum tuberosum*

1. Introduction

Among the various diseases affecting potato crop, late blight is considered as a major threat. Its causing agent is *Phytophthora infestans* (Mont.) De Barry (*P. infestans*), an oomycete fungus [4]. Oxylipins have been shown to be involved in the *P. infestans*-potato relation [26]. Oxylipin is a collective name for oxidized polyunsaturated fatty acids and their metabolites. The first step of their metabolic pathway leads to the synthesis of polyunsaturated fatty acid hydroperoxides (HPO) that can be formed enzymatically by lipoxygenase (LOX, EC 1.13.11.12) or α-dioxygenase (EC 1.13.11) or non-enzymatically. Oxygenation of polyunsaturated fatty acids (PUFA) by LOX can occur either at the carbon 9

Abbreviations: CA, colneleic acid; CnA, colnelenic acid; DES, divinyl ether synthase (enzyme); *des*, divinyl ether synthase (gene); FW, fresh weight; HOD, hydroxyoctadecadienoic acid; HOT, hydroxyoctadecatrienoic acid; HPL, hydroperoxide lyase (enzyme); *hpl*, hydroperoxide lyase (gene); HPLC, high-performance liquid chromatography; HPO, polyunsaturated fatty acid hydroperoxide; HPOD, hydroperoxy octadecadienoic acid; HPOT, hydroperoxy octadecatrienoic acid; LOX, lipoxygenase (enzyme); *lox1*, lipoxygenase 1 (gene); *lox2*, lipoxygenase 2 (gene); *lox3*, lipoxygenase 3 (gene); *Me*, esterified form; *P. infestans*, *Phytophthora infestans*; PUFA, polyunsaturated fatty acid.

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(9-LOX) or 13 (13-LOX) [15]. In potato, three *lox* genes have been described [21]. *Lox1* mainly forms 9-HPO while *lox2* and *3* mainly form 13-HPO. HPO can be further degraded by at least seven different pathways leading to various bioactive compounds: peroxygenase (EC .-) forms epoxy PUFA, allene oxide synthase (EC 4.2.1.92) forms allene oxides that can lead to the jasmonates, the LOX itself forms ketodienes under certain conditions, epoxy alcohol synthase (EC .-) forms epoxy hydroxy PUFA, reductase (EC .-) reduces HPO into the corresponding hydroxy PUFA, hydroperoxide lyase (HPL, EC .-) cleaves HPO into aldehydes and fatty acids while divinyl ether synthase (DES, EC .-) forms divinyl ethers. Among divinyl ethers, colneleic and colnelenic acids are produced from 9-HPO [6].

Weber et al. [26] were the first to report that colneleic (CA) and colnelenic acids (CnA) accumulate more rapidly in a potato variety resistant to late blight (Matilda) than in a susceptible one (Bintje). In the same time, they demonstrated that CA and CnA inhibit in vitro mycelial growth of *P. infestans* [26]. Afterwards, studies focusing on lipoxygenase pathway and considering gene expression, enzyme activity or oxylipins synthesis, revealed the preferential activation of the 9-LOX pathway in potato infected with *P. infestans* [9–11,23]. Stumpe et al. [23] showed that a DES was induced by the pathogen in potato cell suspensions. Furthermore, when 9-LOX is repressed (RNAi repression), the 13-LOX pathway is activated, compensating the 9-LOX down-regulation, and symptoms are unchanged [10]. In tobacco, it has also been shown that the 9-LOX pathway was preferentially activated in plants inoculated with *P. parasitica nicotianae* [7] and that Lox gene overexpression is sufficient to reduce the susceptibility to the pathogen [17,20]. Furthermore, colneleic and colnelenic acids are formed in tobacco roots of plant inoculated with *P. parasitica nicotianae* by the successive action of LOX and DES conferring resistance to the pathogen [5].

Resistance to late blight in potato is a major objective for many breeders [4]. In order to evaluate the potential link between LOX pathway activation intensity and resistance to *P. infestans*, five potato varieties presenting contrasting resistance to the pathogen were inoculated with a virulent strain and disease symptoms were assayed. Northern blotting was used to follow *lox*, *hpl* and *des* gene expression kinetics. Oxylipin profiling of free and esterified forms was performed on the five varieties 24 h, 48 h, 72 h and 96 h after infection. To assess local and systemic effects of CA application before *P. infestans* infection, Bintje cultivar was finally sprayed with CA 72 h before inoculation and oxylipin profiling was performed 48 h and 96 h after infection.

2. Materials and methods

2.1. Biological material

2.1.1. Potato plants

Five different potato (*Solanum tuberosum* L.) cultivars exhibiting different resistance levels to *P. infestans* were used in this study (Esterling, Bintje, Désirée, Cara and Matilda). The potato plants were individually grown from in vitro microtubers in 1-L containers under controlled conditions (18 °C, 16 h photoperiod) during 6 weeks prior to CA treatment or fungus inoculation.

2.1.2. *P. infestans*

P. infestans strain 00091/2 was obtained from the Farming Systems Department of the Walloon Agricultural Research Centre (Belgium). This virulent strain was isolated from an infected potato plant in 2000 and in vitro propagated until its use as inoculums in our experiment.

2.1.3. Treatment with CA

CA (Larodan Fine Chemicals, Malmo, Sweden) was solubilized in acetone/distilled water (1:1, v/v) to reach a 30 μM final concentration. For the local effect studies, this solution was sprayed onto the 1st, 3rd and 5th fully expanded leaves 72 h before inoculation with *P. infestans*. By contrast, the 2nd, 4th and 6th leaves were covered with plastic film to avoid contact with CA solution during spraying. These leaves were used for the systemic effect studies. Finally, control leaves were sprayed with acetone/water instead of CA solution.

2.1.4. Inoculation

Three replicates were used in this study. Each replicate consists of three intermediary fully expanded leaves cut from 6-week-old plants. The petioles were wrapped around with humidified cotton. The leaves were placed in Petri dishes containing sterile water-saturated paper. Each half leaflet was inoculated with 10 μL of sporangial (10^4 sporangia mL^{-1}) suspension on the abaxial leaf face, considering the main nervure as a division line. The leaves pre-treated with the CA solution in a local or systemic way were similarly inoculated. All the Petri dishes were incubated under controlled conditions (18 °C, 16 h photoperiod). After 4 days of incubation, each replicate was frozen in liquid nitrogen and stored at −80 °C. Parallel, control leaves were mock-inoculated with sterile water instead of the sporangial suspension. For Northern blot experiments, samples were taken 0 h, 24 h, 48 h, 72 h and 96 h after inoculation.

2.1.5. Estimation and analysis of lesion growth rate

After 96 h of incubation, *P. infestans* lesions were visually estimated using a graphical reference chart. The data are expressed as percentage of leaf area presenting fungal lesions. Each value is the mean of three replicates ± standard deviation. After one-way variance analysis, the means were classified using the Newman and Keuls test.

2.2. RNA extraction and northern blotting

For each cultivar, potato leaves were sampled after 0 h, 24 h, 48 h, 72 h and 96 h and frozen in liquid nitrogen before RNA extraction according to [16]. Total RNA (20 μg) was separated on 1.2% (w:v) agarose gel containing formaldehyde and transferred to a nylon membrane (Hybond-XL, Amersham-Biosciences) as described by [22]. The membranes were pre-hybridized and

hybridized in 0.5% SDS, 5× Denhardt's solution, 6× SSC, 100 μg mL^{-1} sonicated herring sperm DNA (Sigma–Aldrich). After hybridization membranes were washed 4 times for 5 min in 2× SSC, 0.1% SDS at 25 °C, 2 times for 30 min in 2× SSC, 0.5% SDS at 65 °C and 30 min in 0.1× SSC, 0.1% SDS at 25 °C. The blot was exposed to X-ray films (BioMax MS, Kodak) at −80 °C.

The following cDNA fragments were used as probes: *lox1*, *lox2* and *lox3* [21]; *des* [23]; *hpl*, all kindly supplied by Dr J. Sanchez-Serrano, SSIC, Spain. *Lox1* is a 9-*lox*, *lox2* is a *13-lox*, *lox3* is a *13-lox*, *hpl* is a *13-hpl* and *des* is a 9-*des*. Labelling of the DNA was performed in presence of 30 μCi [^{32}P]dCTP with a random prime labelling system (Rediprime II, Amersham-Biosciences). Equal loading was evaluated with the 18SrRNA signal estimation.

2.3. Oxylipin profiling

Free and esterified oxylipins were extracted and analyzed according to Göbel et al. [9,14] with a two-step HPLC procedure using internal standards for quantification. The first step performed on a reverse phase column allows group separation and CA and CnA determination while the second step performed on a straight phase column allows individual oxylipin quantification. For free oxylipins, 2.0 g of leaf powder was used while 0.5 g was used for esterified compounds. Jasmonates and tri-hydroxy derivatives were not specifically assayed in this study.

2.3.1. Extraction of free oxylipins

(6Z,9Z,11*E*,13S)-13-hydroxy-6,9,11-octadecatrienoic acid (Cayman Chemical, East Ellsworth, MI, USA) was used as the internal standard and 2.0 g 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 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 HPLC analysis.

2.3.2. Extraction of esterified oxylipins

The same protocol was used, with triricinoleate (Sigma, St. Louis, MO, USA) as the internal standard. Subsequently, the esterified oxylipins were transmethylated with sodium methoxide following the method described by [10]. The sample was dried for a short period under a nitrogen stream. After an addition of 333 μL of a methanol/toluol (2:1) solution and 167 μL 0.5 M CH_3NaO, the sample was agitated for 20 min at room temperature. Saturated NaCl solution (500 μL) and 20 μL of 32% HCl (v/v) were then added. The sample was extracted twice with *n*-hexane (v/v), and the organic phases were pooled and dried under a nitrogen stream before HPLC analysis.

2.3.3. Free and esterified oxylipin HPLC analysis

The same chromatographic conditions were used for free and esterified oxylipin samples. The protocol was divided into two steps. The first step, performed on the reverse phase column, allowed group separation. Each separated fraction was collected and then injected on a straight-phase column, allowing for the individual separation of oxylipins. 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) using 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} up to 30 min and increased linearly to 0.36 mL min^{-1} within 35 min, held for 10 min, followed by a linear decrease to 0.18 mL min^{-1} within 50 min and a post-run for 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 and the absorbance was recorded at 234 nm. The results were expressed in terms of nmol g^{-1} FW. The analysis was performed in triplicate and results presented in figures are the mean ± standard deviation. For each oxylipin, one-way variance analysis was performed and means were classified using the Newman and Keuls test.

3. Results and discussion

3.1. Biological observations

The five potato cultivars used in this study were selected based on their reported experimental leaf resistance index [2]. In order to obtain reproducible infection symptoms, we used a well-established detached leaf assay [13,15]. The percentage of leaf area infected by *P. infestans* was observed 4 days after infection for the five varieties (Table 1). The results are consistent with the previously published data. Statistically, 2 groups of cultivars were observed based on their leaf symptoms. For the purpose of this paper, the more susceptible group (Esterling, Bintje, Désirée) is referred as "susceptible" and the less susceptible one (Cara, Matilda) as "resistant".

3.2. Alteration of transcripts levels

Kinetics of *P. infestans* infection of five potato cultivars were followed during 4 days. Transcript levels of genes coding for lipoxygenases (*lox1*, *2*, *3*), divinyl ether synthase (*des*) and hydroperoxide lyase (*hpl*) were analyzed in a preliminary Northern blot experiment (Fig. 1). No clear alteration of gene expression regulation was observed for *lox2* and *hpl*

Table 1
Expected and observed resistance of each cultivar 96 h after *P. infestans* infection and in cultivar Bintje pre-treated, 72 h before infection with *P. infestans*, with acetone/water (1:1 v/v) (SOL) or pre-treated with CA locally (CAD) or systematically (CAI)

Cultivar	Leaf resistance index*	Observed lesions** (% leaf area)
Esterling	2	$38.3^{a} \pm 0.4$
Bintje	3	$36.0^{a} \pm 4.5$
Désirée	4	$33.9^{a} \pm 5.8$
Cara	6	$22.2^{b} \pm 4.3$
Matilda	7	$25.1^{b} \pm 5.2$
Bintje (SOL)	3	$38.2^{a} \pm 3.9$
Bintje (CAD)	3	$34.7^{a} \pm 4.8$
Bintje (CAI)	3	$37.4^{a} \pm 5.1$

Means sharing the same superscript letter are not statistically distinct ($\alpha = 5\%$).
* Data compiled from various European variety databases (ECPD, 2007) were obtained from the Farming Systems Department of the Walloon Agricultural Research Centre (Belgium).
** The observed data are expressed as percentage of leaf area presenting fungal lesions and have been normalized using the corresponding mock-inoculated leaves. Each value is the mean of three replicates ± standard deviation.

during the experiment. On the contrary, *lox1, lox3* and *des* are differentially expressed at the RNA level. *Lox3* and *des* expressions were up-regulated after 96 h but no cultivar-specific differences were observed. After 96 h (4 days), *lox1* was over-expressed in Bintje, Esterling and Désirée leaves, whereas this gene was not up-regulated in Cara and Matilda.

These results indicate that inoculation with *P. infestans* induces differential responses of *lox*-coding genes. Up-regulation of *lox3* 96 h after *P. infestans* infection is consistent with the observations of Göbel et al. [9] showing that the plant presents a detectable response after 3 days and a maximal response during the fourth day. *Lox1* and *lox3* are involved in the 9 and the 13 LOX pathways, respectively [21]. Activation of both pathways could indicate complementary pathogen resistance strategy developed by plants. The present results confirm that *P. infestans* is able to trigger oxylipins metabolism when compared to the control.

3.3. Oxylipin profiles

In order to assess if there is a link between resistance to *P. infestans* and oxylipin accumulation, oxylipin profiling was performed on the five varieties 24 h, 48 h, 72 h and 96 h after infection (Fig. 2). Oxylipins exist under two forms: free and esterified (e.g. in phospholipids or galactolipids). Oxylipin profiles were obtained by a two-step high performance liquid chromatography (HPLC) method.

Comparing Fig. 2A and B, corresponding respectively to esterified and free oxylipins, it clearly appears that esterified oxylipins are more abundant than free ones in both control and infected samples for the five varieties at all time points while the concentration of linolenic acid-derived oxylipins is higher than the concentration of linoleic acid-derived oxylipins.

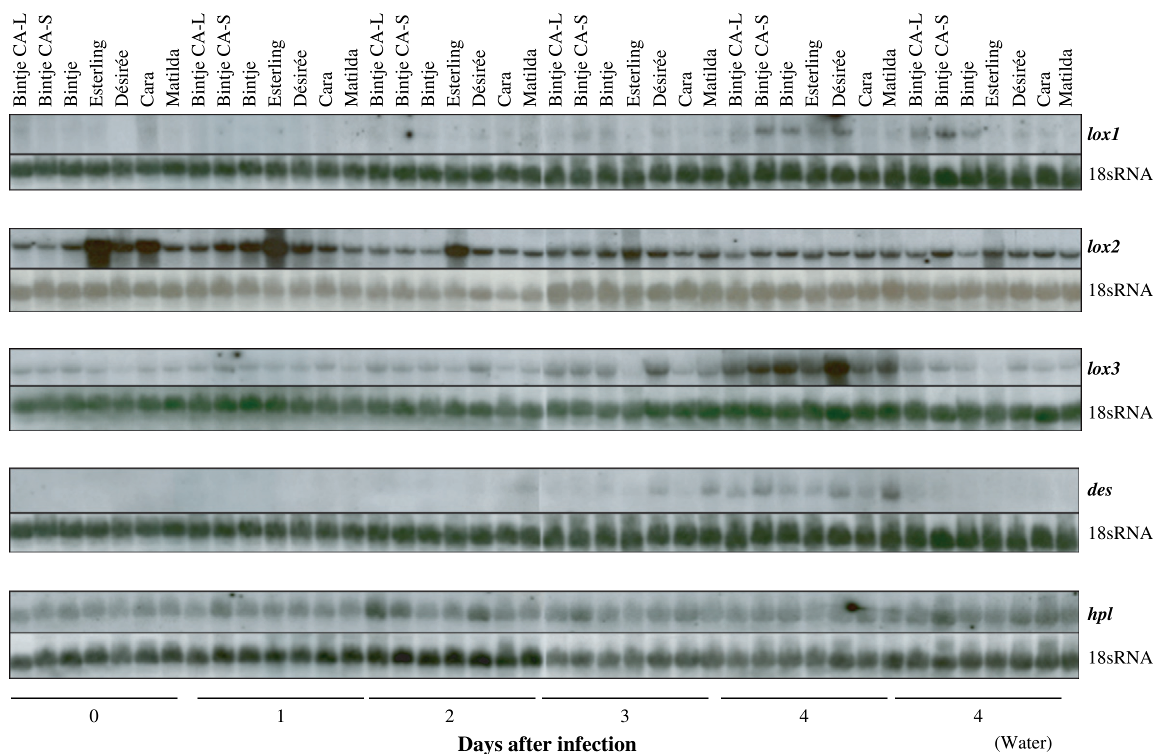

Fig. 1. Expression patterns (Northern blot analysis) of *lox1*, *lox2*, *lox3*, *des* and *hpl* from potato leaves infected with *P. infestans* during 96 h. Control samples were treated with water (water). Application of CA 72 h before infection was realized locally (CA-L) or systemically (CA-S). Lower panels show 18S rRNA hybridization used to verify even loading of samples. After extraction, total RNA was separated on agarose gel containing formaldehyde and transferred to a nylon membrane. Hybridizations were performed with radioactive probes and visualized after X-ray film exposition.

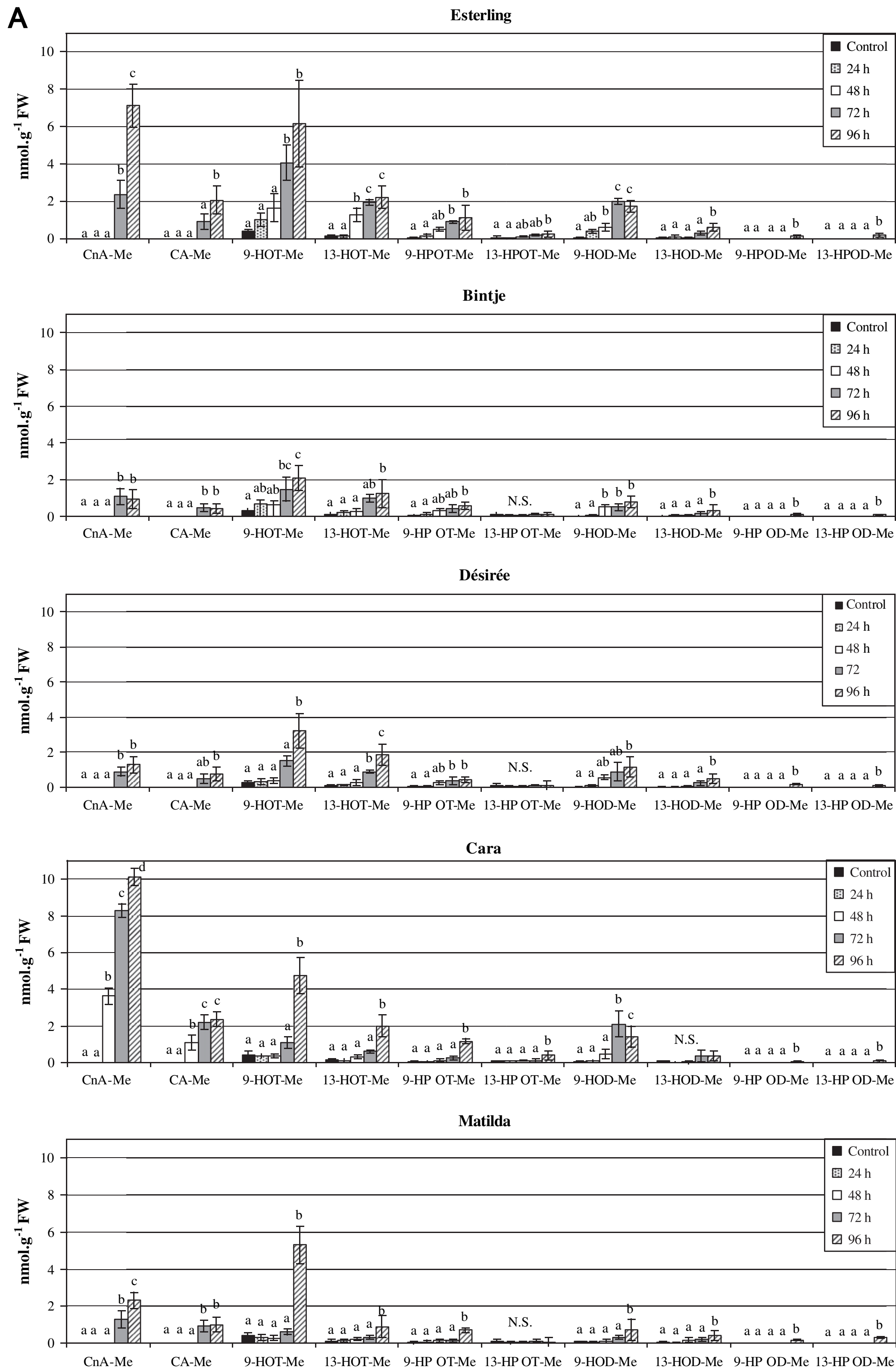

Fig. 2. Oxylipin profile in control leaves (control) and 24 h, 48 h, 72 h and 96 h after infection with *P. infestans* in five potato varieties. (A) Esterified oxylipins. (B) Free oxylipins. Means of three independent measurements ± standard deviation. Oxylipins were extracted, separated and quantified by a two-step HPLC procedure using internal standards. For each oxylipin, one way variance analysis was performed and means were classified using the Newman and Keuls test. Means sharing the same letter are not significantly different.

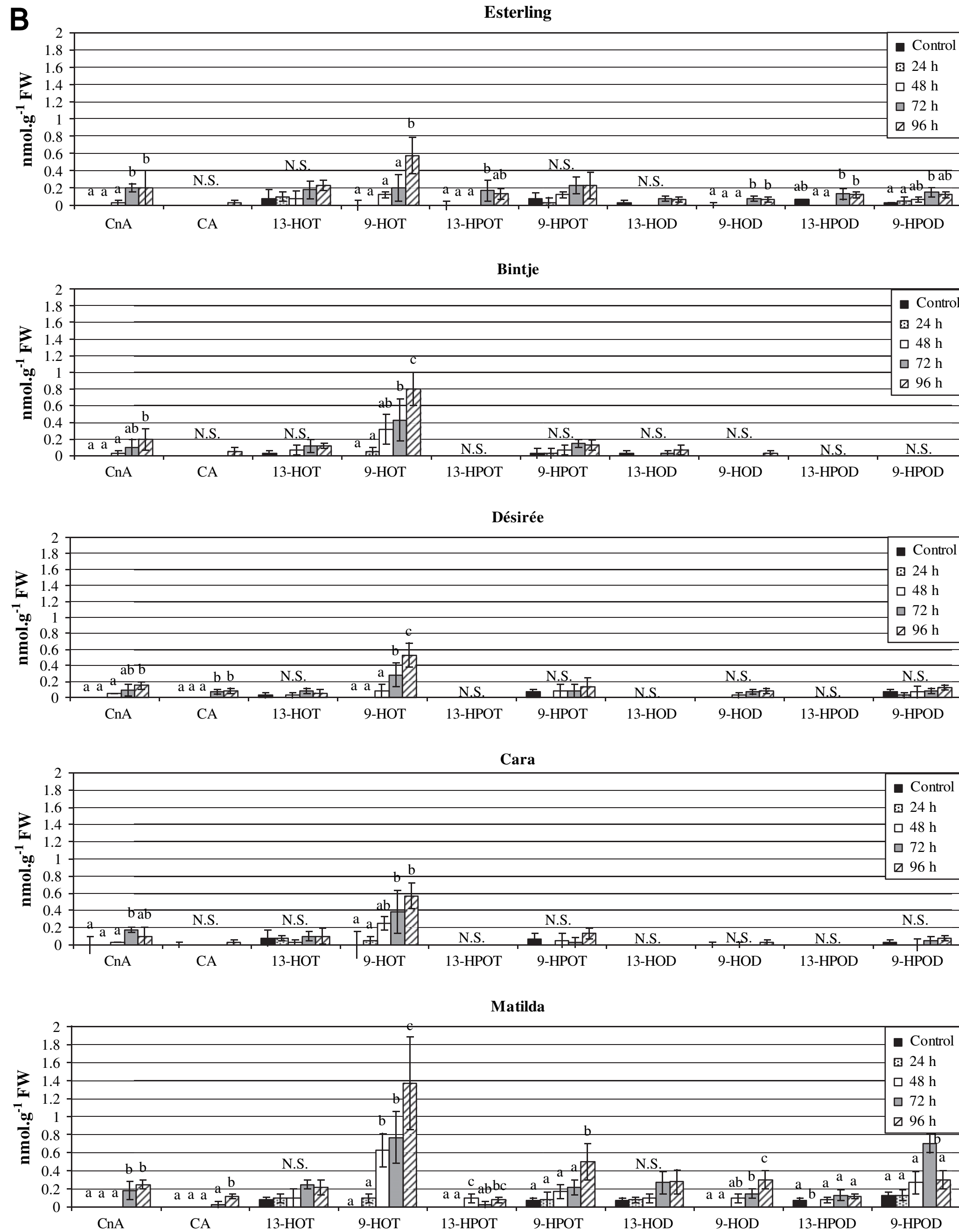

Fig. 2. (*continued*)

In all samples, oxylipins derived from 9-LOX pathway [CA, CnA, 9-hydroxy linolenic acid (9-HOT), 9-hydroperoxy linolenic acid (9-HPOT), 9-hydroxy linoleic acid (9-HOD), 9-hydroperoxy linolenic acid (9-HPOD)] are mainly found both in free and esterified forms compared to compounds derived from 13-LOX pathway.

After inoculation with a virulent strain of *P. infestans*, a drastic change in the oxylipin contents can be observed when compared to the control samples. From a kinetic point of view, no statistically significant difference could be detected in the oxylipin profiles 24 h after infection compared to the control. Afterwards, oxylipin contents increased gradually until 96 h. After 48 h of infection, only a few compounds accumulated significantly compared to the controls (e.g. 13-HOT-Me in Esterling, CnA-Me and Ca-Me in Cara). The majority of the studied oxylipins reache their highest concentration 96 h after infection (except few exceptions, e.g. 9-HPOD in Matilda). Among esterified oxylipins that accumulated during *P. infestans*, CnA-Me, 9-HOT-Me, 13-HOT-Me, 9-HOD-Me and CA-Me are mainly produced in decreasing order. Except 13-HOT-Me, all esterified oxylipins that increase upon pathogen infection resulted from the 9-LOX pathway with a predominance for linolenic acid derivatives. Higher esterified oxylipin concentrations are encountered 96 h after infection in Cara

and Esterling varieties respectively "resistant" and "susceptible" to *P. infestans* (e.g. CnA-Me: 10.1 ± 0.7 nmol g^{-1} fresh weight (FW) in Cara and 7.1 ± 1.0 nmol g^{-1} FW in Esterling). Concerning free oxylipins, a clear difference between control and infected samples can also be observed. In decreasing order, 9-HOT, CnA, 9-HPOT, 13-HOT are formed in all varieties. Higher free oxylipin concentrations are found 96 h after infection in resistant (Matilda) and susceptible (Bintje) varieties (e.g. for 9-HOT: 1.3 ± 0.5 nmol g^{-1} FW in Matilda and 1.0 ± 0.2 nmol g^{-1} FW in Bintje). Considering oxylipin accumulation rates, no clear conclusions can be drawn. CnA-Me and CA-Me accumulated 48 h after infection in the resistant variety Cara but the results are not confirmed with the other resistant variety (Matilda). Indeed, 9-HOT statistically accumulated 48 h after infection both in susceptible (Bintje) and resistant varieties (Matilda and Cara).

The predominance of linolenic acid-derived oxylipins is in accordance with the fatty acid composition of potato leaves [21]. Preferential activation of the 9-LOX pathway in the context of potato infection by *P. infestans* has been reported by several authors [11,13,23,25]. In the past, different oxylipin profiling studies have been performed but they mainly focused on free forms [13,21,26]. The rapid increase in esterified oxylipins observed in our study strengthens the pertinence of a detailed characterisation of oxylipins. Recently, Anderson et al. [1] have reported that more than 90% of the newly formed oxylipins were esterified to glycerolipids in *Arabidopsis thaliana* (L.) Heyn. infected by *Pseudomonas syringae*. Comparison of the results from Northern blot experiments (Fig. 1) and oxylipin profiling (Fig. 2) reveals some divergences. In fact, 4 days after infection, *lox3* gene (13-LOX) is the main up-regulated *lox* gene [21] while accumulated oxylipins mainly results from 9-LOX pathway in our study. This last observation could result from post-transcriptional regulation of gene expression or by an increased consumption of the intermediate compounds 13-HPOT and 13-HPOD that can be transformed into end-products not detected in our analytical conditions (α- and γ-ketols, methyl jasmonate, etc.). However, *des* gene upregulation 4 days after infection is in agreement with CnA and CA accumulation. The relatively slow oxylipin accumulation (maximal concentration 96 h after infection, no detectable response 24 h after infection) is explained by the infection technique using a droplet of sporangial suspension set on the leaf surface. Similar kinetics data was observed by [26] using the same technique while earlier response was observed using infiltration technique [3,10].

Oxylipin antimicrobial effect on various plant pathogens has been studied in vitro revealing that most oxylipins tested were able to impair growth of some pathogens [19]. CnA was for example able to inhibit *P. infestans* growth [19]. The difference between 9- and 13-derivatives was unclear [10] while linolenic acid derivatives seem more efficient in inhibiting pathogen growth than linoleic acid derivatives [19].

Finally, our results are not fully in agreement with Weber et al. [21] as no more rapid accumulation of CnA or CA was observed in resistant varieties compared to susceptible ones after infection with *P. infestans*. Nevertheless, they are in accordance with Eschen et al. [3] who have shown that infection symptoms were unchanged in potato lines producing a reduced amount of CnA after RNAi inhibition of *des*.

3.4. Local and systemic effect of CA application

To assess the role of exogenous application of CA before infection with *P. infestans*, Bintje cultivar was sprayed with CA 72 h before infection. Local and systemic effects were tested while a blank was performed in the same conditions with the solvent in which CA was dissolved (acetone/water, 1:1, v/v). The percentage of leaf area infected by *P. infestans* observed 4 days after infection is presented in Table 1. Symptoms are similar in the leaves, sprayed 72 h before infection, with the solvent (SOL), locally with CA (CAD) or systematically with CA (CAI).

At the RNA level (Fig. 1), *lox2* and *hpl* gene expression are not affected by the treatments while for *lox1*, the effect is unclear. *Lox3* and *des* are clearly up-regulated 4 days after *P. infestans* infection compared to water treatment in both contexts (local and systemic CA applications) but the up-regulation is comparable to the one observed without pre-treatment with CA. It can be noted that spraying the solvent in which CA has been dissolved does not cause any modifications in the steady state RNA levels (data not shown).

Esterified and free oxylipin profiles of leaves pre-treated with CA are presented respectively in Fig. 3A and B. "SOL 48 h/96 h Phyto" corresponds to the leaves sprayed with the solvent (without CA) and infected by *P. infestans* after respectively 48 h and 96 h. "CAD 48 h/96 h Phyto" and "CAD 48 h/96 h" correspond respectively to plant pre-treated locally with CA and infected or not by *P. infestans*. "CAI 48 h/96 h Phyto" and "CAI 48 h/96 h" correspond respectively to plant pre-treated systematically with CA and infected or not by *P. infestans*. It can be noted that solvent spraying has no effect on oxylipin profiles (SOL 48 h/96 h Phyto) compared to the untreated leaves (Fig. 2A and B, Bintje control). Local application of CA in control conditions (CAD 48 h/96 h) causes a slight increase in esterified and free oxylipins but to a smaller extent than when plants, not pre-treated by CA, are infected by *P. infestans* (Fig. 2A and B, Bintje 48 h/96 h). Indirect application of CA has no effect (CAI 48 h/96 h) on oxylipin profiles. When leaves pre-treated locally with CA are infected by *P. infestans* (CAD 48 h/96 h Phyto), a drastic change can be observed in oxylipin contents. After 48 h of infection, statistically significant differences can be noticed for several free and esterified oxylipins (13-HOT, 9-HPOT, 13-HOD, 9-HPOD, CnA-Me, CA-Me, 13-HPOT-Me). The highest esterified oxylipin concentrations are reached 96 h after infection (e.g. CnA-Me, CA-Me, 13-HOT-Me, 9-HOT-Me, 9-HPOT-Me in decreasing order). Accumulated esterified oxylipins are very similar to the ones in leaves infected by *P. infestans* without pre-treatment with CA (Fig. 2A, Bintje 96 h) but the amounts reach higher levels in CA pre-treated samples (e.g. for CnA-Me 1.0 ± 0.5 nmol g^{-1} FW and 7.1 ± 1.5 nmol g^{-1} FW respectively). The free oxylipin profile reveals that 9-HPOT is the main oxylipin formed in CA-pre-treated samples

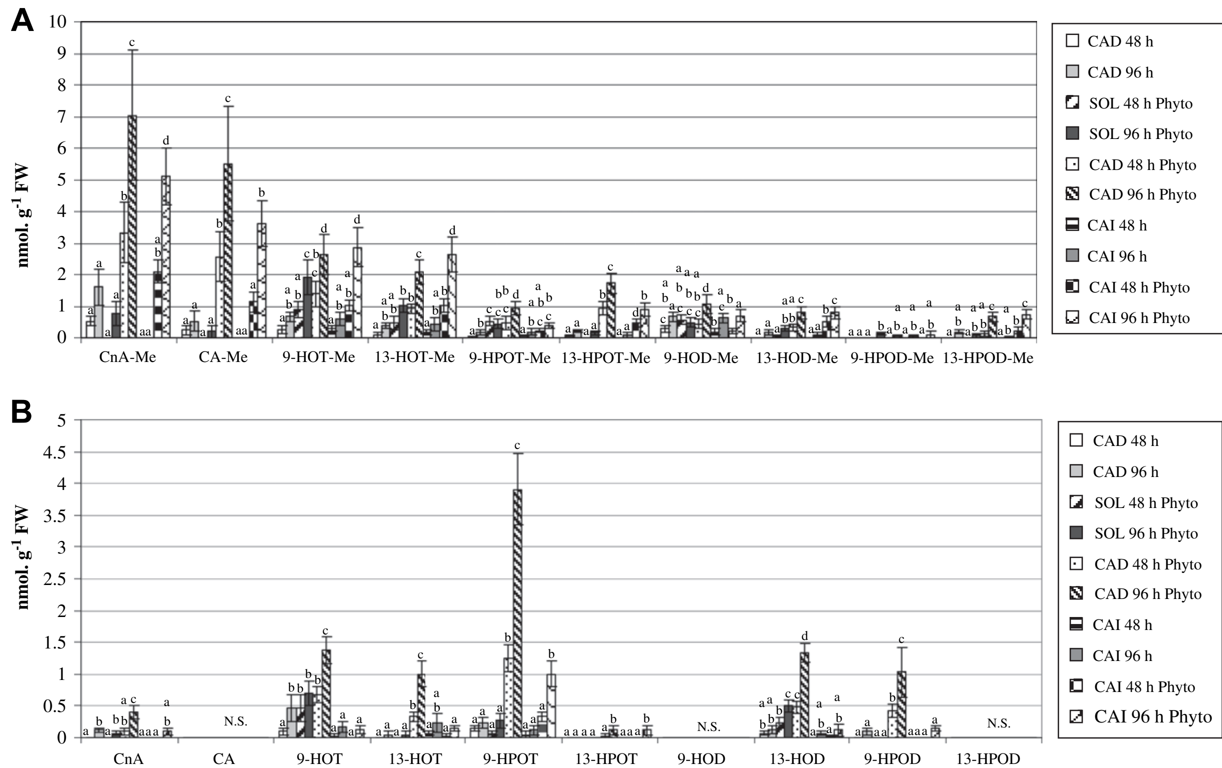

Fig. 3. Oxylipin profile in Bintje leaves treated with acetone/water solution 72 h before infection by *P. infestans* (SOL 48 h/96 h Phyto), in Bintje leaves sprayed directly with CA but not infected by *P. infestans* (CAD 48 h/96 h), in Bintje leaves sprayed directly with CA 72 h before infection by *P. infestans* (CAD 48 h/96 h Phyto), in Bintje leaves treated systematically with CA (systemic effect) but not infected by *P. infestans* (CAI 48 h/96 h), in Bintje leaves treated systematically with CA 72 h before infection by *P. infestans* (CAI 48 h/96 h Phyto). (A) Esterified oxylipins. (B) Free oxylipins. Mean of three independent measurements ± standard deviation. Oxylipins were extracted, separated and quantified by a two-step HPLC procedure using internal standards. For each oxylipin, one-way variance analysis was performed and means were classified using the Newman and Keuls test. Means sharing the same letter are not significantly different.

96 h after infection by *P. infestans*. In the samples not pre-treated with CA, 9-HOT was the major free oxylipin (Fig. 2B). As 9-HOT is the reduced (enzymatically or not) form of 9-HPOT, this last result is not contradictory. Again, concentrations reached after 96 h of infection depend on whether the leaves are pre-treated with CA or not (e.g.: 2.0 ± 0.3 nmol g^{-1} FW of 9-HOT without CA pre-treatment and 3.9 ± 0.6 nmol g^{-1} FW with CA application). Systemic effect of CA, applied prior to infection, can be noticed (CAI 48 h/96 h Phyto), revealing almost the same effect as direct application (CAD 48 h/96 h Phyto) on esterified oxylipins. The effect is noticeable but less marked on the free oxylipins from the same samples.

3.5. Concluding remarks

This paper strengthens the previous observation of the activation of the 9-LOX pathway by *P. infestans* in potato leaves [9,11,13,23] The clear impact of the pathogen on esterified oxylipins accumulation is noticeable as this class of compounds is frequently not taken into account in studies focusing on free or total oxylipins. In spite of the obvious activation of the 9-LOX pathway leading to the accumulation of different oxylipins, no correlation can be established either between rates of oxylipin synthesis after infection by the pathogen or between oxylipin concentrations reached after 96 h of infection and resistance to *P. infestans*. Oxylipin contents or synthesis rates after infection with *P. infestans* cannot thus be proposed as a potential marker in potato varieties selection. In fact, the role of oxylipins in *P. infestans* resistance seems to be more complex, contrarily to tobacco in which Lox pathway activation is sufficient to confer resistance to *P. nicotianae* [17]. Oxylipins that have been shown to have an antimicrobial effect in vitro can play several roles in vivo among which signalling should not be neglected [24,27]. Surprisingly, Gao et al. [8] have shown that 9-LOX products were required to cause conidiation of *Fusarium verticillioides* in infected maize. Moreover, other metabolic pathways are also related to *P. infestans* resistance, involving for example salicylic acid or antioxidants [12,18].

In potato leaves, pre-treatment with CA before *P. infestans* infection induces oxylipin accumulation with a systemic effect. But, as the plant response is not correlated with pathogen resistance, it should be necessary to evaluate the effect on resistance of different pre-treatment times or CA concentrations. As numerous oxylipins (e.g. 12-oxo-phytodienoic acid, 2-nonenal, etherolenic acid) result in in vitro antimicrobial growth inhibition [19], it should be interesting to test their effectiveness as pre-treatment before *P. infestans* infection.

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