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Reprogramming of fatty acid and oxylipin synthesis in rhizobacteria-induced systemic resistance in tomato

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Abstract

The rhizobacterium *Pseudomonas putida* BTP1 stimulates induced systemic resistance (ISR) in tomato. A previous work showed that the resistance is associated in leaves with the induction of the first enzyme of the oxylipin pathway, the lipoxygenase (LOX), leading to a faster accumulation of its product, the free 13-hydroperoxy octadecatrienoic acid (13-HPOT), 2 days after *Botrytis cinerea* inoculation. In the present study, we further investigated the stimulation of the oxylipin pathway: metabolites and enzymes of the pathway were analyzed to understand the fate of the 13-HPOT in ISR. Actually the stimulation began upstream the LOX: free linolenic acid accumulated faster in *P. putida* BTP1-treated plants than in control. Downstream, the LOX products 13-fatty acid hydroperoxides esterified to galactolipids and phospholipids were more abundant in bacterized plants than in control before infection. These metabolites could constitute a pool that will be used after pathogen attack to produce free fungitoxic metabolites through the action of phospholipase A2, which is enhanced in bacterized plants upon infection. Enzymatic branches which can use as substrate the fatty acid hydroperoxides were differentially regulated in bacterized plants in comparison to control plants, so as to lead to the accumulation of the most fungitoxic compounds against *B. cinerea.* Our study, which is the first to demonstrate the accumulation of an esterified defense metabolite during rhizobacteria-mediated induced systemic resistance, showed that the oxylipin pathway is differentially regulated. It suggests that this allows the plant to prepare to a future infection, and to respond faster and in a more effective way to *B. cinerea* invasion.

Keywords : Induced systemic resistance ; Biocontrol ; Plant growth-promoting rhizobacteria ; Oxylipin ; Fatty acid ; Plant defense

Abbreviations

AOS Allene oxide synthase

DES Divinyl ether synthase

ISR Induced systemic resistance

HL Hydroperoxide lyase

HPO Fatty acid hydroperoxide

LOX Lipoxygenase

PGPR Plant growth-promoting rhizobacteria

PLA1 Phospholipase A1

PLA2 Phospholipase A2

PUFA Poly-unsaturated fatty acid

SAR Systemic acquired resistance

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Introduction

Numerous soil microorganisms living in the plant rhizosphere stimulate growth of their host, by different mechanisms. These microorganisms are called plant growth promoting rhizobacteria (PGPRs), a generic name including beneficial bacteria and fungi. PGPRs can promote plant growth by contributing to host nutrition, by secreting phytohormones, and/or by controlling phytopathogen populations (Hayat et al. 2010). Beneficial rhizobacteria can also protect their plant hosts against pathogens by producing antibiotics or lytic enzymes, decreasing nutrient availability to pathogens, or detoxifying disease virulence factors (Mercado-Blanco and Bakker 2007). These defense mechanisms are called direct mechanisms of protection, as the bacteria directly inhibit the pathogen population. PGPRs can also protect their hosts through indirect mechanisms. They can stimulate host defense mechanisms by inducing two kinds of systemic resistance: systemic acquired resistance (SAR) (Bent 2006) and induced systemic resistance (ISR) (Van Peer et al. 1991; Choudhary et al. 2007). Although phenotypically similar, SAR and ISR are different at the molecular level. Accumulation of salicylic acid is associated with the induction of SAR, but no phytohormone accumulation has been reported in ISR (Pieterse et al. 2000). On the contrary of SAR, ISR is dependent of jasmonate and ethylene signaling pathways (Van der Ent et al. 2009; Ahn et al. 2007; Pieterse et al. 2007). Despite the differences between these resistances, their transduction pathways go through the same protein: NPR1, a transcription-factor regulator. Downstream of NPR1, the two pathways diverge again and the defense mechanisms involved in SAR are not the same as those involved in ISR. SAR, unlike ISR, is associated with expression of Patho-genesis-related (*PR*) genes. Two transcription factors, MYC2 and MYB72, are involved in ISR but not in SAR (Van der Ent et al. 2008; Pozo et al. 2008). The expression level of many defence genes participating to ISR is not stimulated upon elicitation by the beneficial microbes in uninfected plants, but expression of these genes is activated more rapidly and/or to higher levels upon the pathogen attack (Van der Ent et al. 2009). Yet some secondary metabolite synthesis pathways, such as the phenylpropa-noid and oxylipin pathways, are involved in both resistances (Pieterse et al. 2009).

The oxylipin pathway can be stimulated by various stress conditions (Mosblech et al. 2009). The key enzyme of the oxylipin pathway, lipoxygenase (LOX), converts polyunsaturated fatty acids (PUFAs) to fatty-acid hydroperoxides (HPOs). In plants, LOX enzymes oxidize linoleic and linolenic acids at carbon C9 (9-LOX activity) or C13 (13-LOX activity) to produce 9- or 13-HPOD from linoleic acid and 9- or 13-HPOT from linolenic acid. The HPO are substrates of the different branches of the pathway. HPOs can be reduced to hydroxy-fatty acids by glutathione peroxidase, peroxygenase or HPO reductase or via a non-enzymatic reaction. The allene oxide synthase (AOS) and the divinyl ether synthase (DES) respectively catalyze the first step of the pathway leading to jasmonic acid and conversion of HPO to divinyl ether fatty acids (Schaller and Stintzi 2009). Volatile oxylipins are synthesized through the action of the hydroperoxide lyase (HL) on HPOs. The LOX enzyme can further convert HPOs to keto-fatty acids (Mosblech et al. 2009); (Fig. 9). Fatty acids and oxylipins can be esterified into phospholipids, galactolipids or non-esterified (free). Divinyl ether fatty acids, volatile oxylipins, keto-fatty acids, hydroxy-fatty acids and HPOs are oxylipins that display antimicrobial activities (Prost et al. 2005) while others such as jasmonic acid and some aldehyde volatiles act as signal molecules (Bate and Rothstein 1998, Chehab et al. 2008). A previous study has shown the ability of the PGPR *Pseudomonas putida* BTP1 to stimulate LOX and the synthesis of one of its products, 13-HPOT, after inoculation of the fungal pathogen *Botrytis cinerea* (Mariutto et al. 2011).

The present study aims to further characterize this induction of LOX observed in tomato after treatment with *P. putida* BTP1. To this end, we have analyzed at the transcriptional, enzymatic, and/or metabolic levels the different branches of the oxylipin pathway that can use the accumulated 13-HPOT. Looking upstream, we have also quantified oxylipin precursors (PUFAs) to test for a possible repro-gramming of fatty acid synthesis upon ISR induction by *P. putida.* Because PUFAs and oxylipins are present in both free and conjugated form in leaves, we have extended our study by quantifying forms esterified to phospholipids or galactolipids.

Materials and methods

Induction of ISR

Induced systemic resistance was induced in tomato (*Lyco-persicon esculentum* cv "merveille des marches") and plants were infected according to the procedure described in Mariutto et al. (2011). Disease severity was determined by the method described by Audenaert et al. (2002). Three independent experiments were carried out, with 48 plants per treatment. In every experiment, infected leaves from 12 plants were randomly harvested just before challenge and also 1, 2, and 4 days after inoculation of the pathogen and immediately frozen in liquid nitrogen. They were then powdered with mortar and pestle and stored at -70 °C until used for analyses.

Table 1 : *Primers used for realtime RT-PCR*

Gene	Accession	Primer name	Primer sequence	Product (bp)
Elongation factor 1-α	X14449	Ef1F	GATTCCCACCAAGCCCATGG	72
		Ef1R	CTGCACAGTTCACTTCCCCT	
Hydroperoxide lyase	AF230372	H1F	GTCTCCCCAGAAATCGCCAA	220
		H1R	ACCCTGTTTGTGCCCTGCTT	
Divinyl ether synthase	AF317515	DesF	CAATATGTTTGCGGGGCTGA	199
		DesR	TGGGCGAAGTCTCAATGTCT	
Allene oxide synthase 1	AF230371	Aos1F	CTACAGTGGGGAATAAACAG	272
		Aos1R	CACCCATTTTGTGAAAACAT	
Allene oxide synthase 2	AJ271093	Aos2F	TTGAACTCAACCTTCGCTTG	310
		Aos2R	CTAATGGTAGTGCATAATGA	

Real-time RT-PCR

Total mRNA was extracted from frozen leaf tissue powder by the Trizol method according to the protocol recommended by the manufacturer (Gibco BRL, Grand Island, NY, USA). Analysis of gene expression was done by realtime PCR. First-strand cDNA was synthesized (Smart PCR cDNA Synthesis Kit, Clontech, Saint-Germain-en-Laye, France) according to the manufacturer's instructions. Realtime PCR step was performed with the qPCR SYBR Green ROX Mix (Thermo Scientific) according to the manufacturer instructions in a Mini Opticon System (Bio-Rad, Richmond, CA) thermocycler. Primers used for qPCR reactions are listed in Table 1. The PCR were performed according the following program: 95 °C for 15 min, 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s. The three last steps were repeated 40 times. Data analysis was carried out using the CFX Manager software (Bio-Rad, Richmond, CA), which created Ct values and extrapolated relative levels of PCR products form standard curves. Melting curves were established to verify the absence of contamination and primer-dimers. *EF1-α* was used as reference housekeeping gene. The analysis was performed in triplicate on three independent biological replicates.

Determination of oxylipin content in leaves

Oxylipins were extracted and analyzed according to Göbel et al. (2002) with a three-step HPLC procedure using internal standards for quantification. Extraction of free oxylipins: 6Z, 9Z, 11E, 13S)-13- hydroxy-6, 9, 11-octa-decatrienoic acid (Cayman Chemical, East Ellsworth, MI, USA) was used as the internal standard and 1 g frozen material was added to 10 mL extraction medium [isohexane/2-propanol, 3/2 (v/v) with 0.0025 % (w/v) BHT]. After homogenization, the extract was centrifuged at 1,300 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 1,300 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.

Oxylipin HPLC analysis: the first step, performed on the reverse-phase column, allowed group separation. Each separated fraction was collected and then injected onto a straight-phase column, allowing separation of individual oxylipins. Reverse-phase HPLC analysis was performed on an EC250/2 Nucleosil 120-5 C18 column (250 mm, diameter 2.1 mm, 5 µm particle size; Macherey and 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 in solvent B up to 40 % over 28 min, a linear increase in solvent B up to 100 % over 30 min, no change for 15 min, a linear decrease down to 20 % solvent B over 5 min, and finally an isocratic post-run at 15 % solvent B for 6 min. The flow rate was 0.18 mL/min up to 30 min. It was then increased linearly to 0.36 mL/min over 35 min and maintained for 10 min. This was followed by a decrease to 0.18 mL/min over 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. The injection volume was 25 µl and the absorbance was recorded at 234 nm. The analysis was performed in duplicate on three independent biological replicates. The enantiomeric composition of the HPOs and hydroxy-fatty acids was analyzed by chiral-phase HPLC on a Chiral OD-H column (150 mm, diameter 2.1 mm, 5 µm particle size; Baker, Phillipsburg, 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. The injection volume was 10 µl. The free jasmonic acid content was determined as described by Göbel et al. (2002). Briefly, jasmonic acid was collected during the straight-phase HPLC step, derivatized with pentafluorobenzylbromide, and analyzed by GC-MS in single-ion monitoring mode with ammonia as the chemical ionization gas. Dihydrojas-monic acid was used as internal standard. The results are

expressed in nmol/g FW. The analysis was performed in duplicate on two independent biological replicates.

Determination of fatty acid content

Free and esterifled fatty acids were extracted by the same method as for cytosolic oxylipins. Quantification was performed by gas chromatography (Hewlett Packard HP6890) on a 30-m CP-Wax 52 CB column (0.25 mm internal diameter, 0.25 µm phase thickness) in the spilt injection mode and with a flame ionization detector. The following temperature program was applied: 40 °C; 30 °C/min to 150 °C; 5 °C/min to 250 °C; and final hold at 250 °C for 10 min. Helium was used as carrier gas at 1 ml/min. Molecular identifications were performed by injection of commercial standards (Sigma). The analysis was performed in duplicate on two independent biological replicates. Results are expressed in nmol/g FW for free fatty acids, in µmol/g FW for esterifled fatty acids.

Determination and quantification of volatile oxylipins

Volatiles were extracted by head-space solid-phase micro extraction (HS-SPME) as described in Gosset et al. (2009). An SPME fiber (Supelco, Bellefonte, PA, USA) coated with a polymethylsilxane/carboxene/divinylbenzene (PDMS/ CAR/DVB) phase was exposed to the headspace for 1 h at room temperature. For determination of the volatiles emitted by whole plants, the fiber was exposed to the phyllo-sphere gases of 8 tomato plants kept in 10-L glass boxes. For determination of the volatiles contained in leaves, the fiber was exposed to gases released by leaf homogenate in a 20-ml SPME glass vials (Filter Service, Belgium), fitted with a sealed cap (white silicone/blue PTFE, Filter Service), according to the method described by Aprea et al. (2011). 0.1 g frozen tomato powder was mixed with 1 ml deionized water containing 6.8 M NaCl, 17 mM citric acid, and 19 mM ascorbic acid. Briefly, extractions were performed using the following method. After equilibration of the vial for 15 min at 30 °C, the fiber was inserted into the headspace for 30 min at the same temperature. After extraction, the volatile compounds were desorbed in pulsed splitless mode for 10 min at 250 °C in the injector of the GC. Analyses were performed on an Agilent Technologies 7890A GC9 System, and compounds were separated on a VF-WAXms column (Agilent technologies, USA; 30 m × 0.250 mm I.D, 0.25 µm film thickness). Helium was used as carrier gas at 1.5 ml/min flow rate. A pulsed splitless injection mode in a 1.5 mm HS-liner was used (injection pulse pressure of 30 psi for 1 min). The following temperature program wase used: 40 °C for 4 min; 15 °C/min to 160 °C; 20 °C/min to 250 °C; and 250 °C for 5 min; 30 °C/min to 300 °C; and final hold at 300 °C for 15 min. Identification of compounds present in the phyllosphere was performed with an Agilent Technologies 5975C Mass Spectrometer. The MS was carried out in EI mode at 70 eV; source temperature, 230 °C; quadrupole temperature, 150 °C; scanned mass range: from 20 to 350 amu, threshold of 150amu; scan speed, 4.27 scans/s. Quantification of molecules was performed with Hewlett Packard HP6890 flam ionization detector (FID). Calibration curves were realized by injecting different dilutions of commercial standards (Sigma). The analysis was performed in triplicate on two independent biological replicates. Results are expressed in nmol/g FW.

Quantification of HL activity

HL activity was assayed in an NADH-coupled enzyme system as described by Vick (1991). The activity was measured in 100 mM pH 6.8 sodium phosphate buffer, 150 U yeast alcohol dehydrogenase, 0.167 mM NADH, and 0.2 mM 13-HPOT The absorbance increase was monitored at 340 nm with a UVIKON XS spectrophotometer (Beun-De Ronde Serlabo sa, Drogenbos, Belgium). It was recorded for 60 s and the result converted to activity units (amount of enzyme consuming 1 µmol HPO in 1 min) per gram fresh weight by using an extinction coefficient of 6.220 M^{-1}/cm. Each sample was extracted twice, and each extract was analyzed three times.

Quantification of PLA1 and PLA2 activity

Phospholipase activities were extracted from tomato leaves according to Kim et al. (1994) with modifications, and assayed by spectrofluorometry. Powdered frozen leaf tissue (0. 5 g) was extracted with 1 ml extraction buffer (75 mM Tris-HCl pH 7.5, 25 mM $CaCl_2$). Samples were then centrifugea for 5 min at 2,000 g and 4 °C and the supernatants were collected. The PLA1 activity was monitored with the Enzcheck Phospholipase Al Assay Kit (Invitrogen) according to the manufacturer's instructions and the PLA2 activity was determined with the Enzcheck Phospholipase A2 Assay Kit (Invitrogen) according to the manufacturer's instructions, with a 1420 Multilabel Counter Victor3 (PerkinElmer). The enzymatic activity was expressed in milli-enzymatic units per gram fresh weight (mU/g FW).

B. cinerea germination inhibition bioassay

Botrytis cinerea was grown on oat medium (25 g/l oat flour, 12 g/l agar) at room temperature in the dark. After 1 week, the fungus was exposed to UV light for 1 week to stimulate sporulation. Conidia were harvested in spore recuperation solution (1 g/l peptone, 0.085 M NaCl, 5 droplets/L Tween 80). A mix of the four hydroperoxides

at known concentration was added to 200 µl of potato dextrose broth containing 30,000 spores in wells of a 96-well micro-plate. The profile corresponding to bacterized plants contained 2.05 µM 13-HPOD, 1.84 µM 13-HPOT, 0.28 µM 9-HPOD and 0.45 µM 9-HPOT. The profile corresponding to the untreated plants was composed of 1.1 µM 13-HPOD, 1.05 µM 13-HPOT, 0.28 µM 9-HPOD and 0.45 µM 9-HPOT. All the oxylipins were purchased from Larodan (Malmö, Sweden). A negative control HPO-free profile did not contain any oxylipin and its inhibition was considered as equal to 0. The antimicrobial activity of the samples was measured by monitoring growth of *B. cinerea*. The optical density of each well was measured with a microplate reader (Victor^3,1420 Multilabel Counter, Perkin Elmer) at 530 nm after 0 and 16 h of incubation at room temperature in the dark. The assay was carried out in sextuplicate on three independent biological replicates.

Statistical analysis

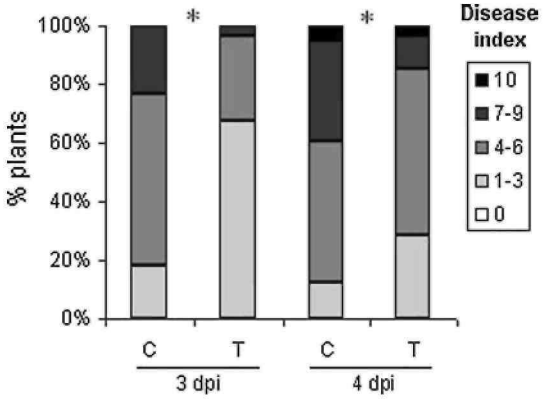
In the figures, each value is the mean and each error bar the standard deviation of one experiment. Results for the different replicates were subjected to ANOVA 2 ($\alpha = 0.05$) so as to pool the data from different experiments but, although similar results were obtained between replicates, the high variability of the data did not allow their grouping. Hence, all graphs shown in the present work were obtained from one experiment. Differences between control and treated plants were tested with the unpaired Student T test ($\alpha = 0.05$).

Results

Disease reduction in P. putida BTP1-treated tomato

Four-week-old tomato plants were infected with the fungal pathogen *B. cinerea*. Ten droplets of spore suspension (10^5 spores per ml) were inoculated onto the third leaves and disease severity was rated according to the number of droplets from which lesions were found to spread clearly out of the inoculation drop zone. The majority of bacterized plants displayed less disease development than controls, and pathogen expansion was delayed in the treated plants. In three independent biological repetitions, *P. putida* BTP1-treated plants showed a disease reduction of 21, 28 and 38 % compared to controls. Figure 1 shows the distribution of disease severity ratings in control and bacterized plant populations in a representative experiment. As the bacteria were unable to migrate to the aerial parts of the plant (Ongena et al. 2002) and were spatially separated from the pathogen, the observed protection could only have been caused by induction of systemic resistance in the leaves.

Fig. 1 *Disease progression in control (C) and P. putida BTP1-treated (T) plants following challenge with B. cinerea. Three-week-old control and bacterized plants were challenged with B. cinerea. Ten 5-µl droplets of a suspension of 10 spores per ml were deposited on the adaxial faces of third leaves. Disease severity was quantified three (3 dpi) and four (4 dpi) days after pathogen inoculation. It is rated as follows: percentage of leaves with no lesions spreading beyond the drop site (0), percentage with 1 to 3 (weak infection), 4 to 6 (medium infection), 7 to 9 (serious infection) and 10 (total infection) spreading lesions. The figure shows data from a representative experiment that was repeated three times with similar results. Stars indicate significantly lesser disease severity in treated versus control plants (ANOVA l, α = 0.05)*

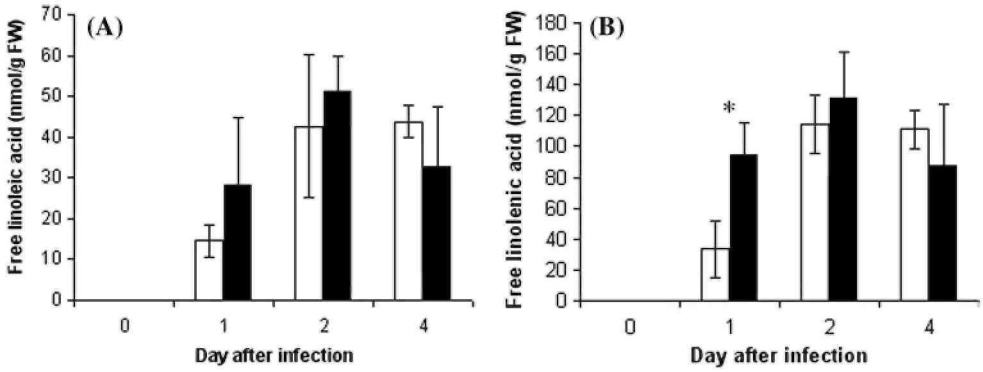


P. putida BTP1-treated plants accumulate free linolenic acid faster than control plants

As first substrates of the LOX pathway, linoleic and linolenic acids were quantified in leaves of control and treated plants before and after inoculation of the fungal pathogen *B. cinerea*. Free and esterified linoleic and linolenic acids as well as their precursors palmitic, stearic, and oleic acids were quantified by gas chromatography in two independent experiments. No significant differences in free or esterified palmitic, stearic, or oleic acid content were observed between control and elicited plants (data not shown). Before infection, linoleic acid and linolenic acid, both direct precursors of HPOs, were detected only as esterified forms in leaves

and no clear changes occurred in that pool after infection (Fig. 2; Supplemental Fig. S1). By contrast, a clear release of free linoleic and linolenic acids was observed in all plants upon infection but only linolenic acid accumulated faster in bacterized plants than in controls (Fig. 2).

Fig. 2 *Time course accumulation of the LOX substrates in control (white) and treated (black) plants. Free linoleic (a), free linolenic (b) acids were quantified before (0), 1, 2 and 4 days after infection by GC-FID. The data presented was from a representative experiment that was independently repeated once with similar results. Stars indicate statistically significant differences between control and treated plants (Student's t test, α = 0.05)*



Free HPOs accumulate to fungitoxic levels in treated plants

In parallel to the quantification of PUFAs, we investigated the time-course evolution of hydroperoxide content in bacterized plants compared to controls. As shown in Fig 3c, d, higher concentrations of esterifled forms of both 13-HPOD and 13-HPOT were present in leaves of BTP1-treated healthy plants. This pool globally decreased after infection while free forms readily accumulate in the first days after this pathogen challenge. This was observed in all plants but free 13-HPOD and 13-HPOT accumulated to a significantly higher extent in bacterized plants and also much faster to reach a maximal concentration only one day after infection (Fig 3a, b). As these 13-HPOs were reported to retain some inhibitory activity against fungi, bacteria, and oomycetes (Prost et al. 2005), we wanted to determine whether their differential accumulation in PGPR-treated compared to control plants may quantitatively correlate with an enhanced direct inhibition of Botrytis growth. Facing the high instability of these hydroperoxide products during extraction and some difficulty to obtain pure compounds from hexane leaf extracts, we used commercial standards to reconstitute the HPO profiles found in both types of leaves. These mixtures were tested at appropriate concentrations similar to those observed on the second day after pathogen inoculation for toxicity against *B. cinerea* in an in vitro spectrophotometric assay. As determined by the change in optical density (560 nm), BTP1-HPO and untreated-HPO profiles inhibited the fungal growth respectively by 86.1 ± 2.6 and 79.4 ± 2.5 %, compared to HPO-free control showing that the levels present in bacterized plants proved to be more inhibitory than those present in untreated plants.

The phospholipase A2 activity is primed in bacterized plants

The faster decrease in esterified 13-HPOD/T upon infection in treated plants than in controls led us to measure the activity of phospholipases A1 (PLA1) and A2 (PLA2), two enzymes involved in releasing free PUFAs/oxylipins from phospho/galactolipids (Blée 2002). PLA1 and PLA2 cleave the lipid substrate respectively at the sn1 and sn2 position. PLA enzymes were extracted from leaves of control and treated plants before and after infection and their enzymatic activity was monitored by spectrofluorometry. As shown in Fig. 4, PLA1 was found not to be upregulated in ISR-expressing plants. PLA2 displayed a different profile: no significant difference in PLA2 activity was observed between control and elicited plants before pathogen challenge, but this activity increased markedly faster in treated plants in response to infection.

Fig. 3 *Metabolic profiling of 13-HPO. Free 13-HPOD (a) and 13-HPOT (b), esterifled 13-HPOD (c) and esterifled 13-HPOT (d) were quantified in control (white) and treated (black) plants before, 1, 2 and 4 days after infection by reverse phase HPLC. The results presented were from a representative experiment that was independently repeated twice with similar results. Stars indicate statistically significant differences between control and treated plants (Student's t test, α = 0.05)*

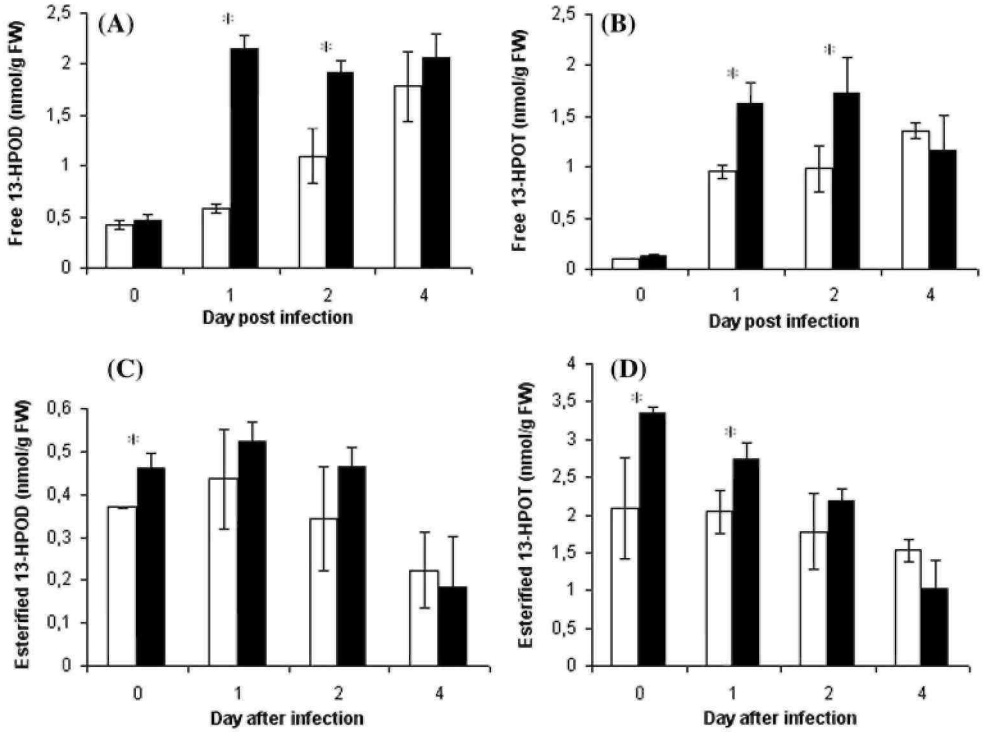
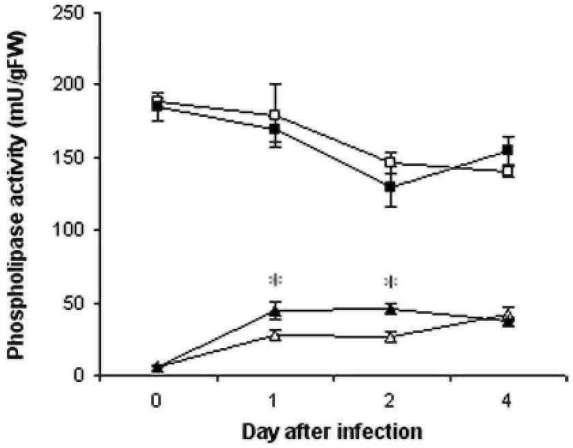


Fig. 4 *Time course of changes in PLA1 and PLA2 activity in control (respectively open squares and triangles) and elicited (respectively closed squares and triangles) plants. Samples were collected before infection by B. cinerea (0) and two (+2) and four days (+4) thereafter. Stars indicate statistically significant differences between control and treated plants (Student's t test, α = 0.05). Data are means and standard deviations calculated from three measurements on two independently prepared extracts*



Synthesis of free and esterified 13-HOTs is primed in P. putida BTP1-mediated ISR

Fatty-acid hydroperoxides can be reduced to hydroxy-fatty acids by glutathione peroxidase (Dixon et al. 2002), peroxygenase, or HPO reductase (Schaller 2001) or via a non-enzymatic reaction. Control and bacterized plants showed no significant difference in free 9-HOD or 9-HOT before or after pathogen attack (data not shown). They showed similar content of 13-HOD and 13-HOT before inoculation, but afterward, both compounds were found to accumulate to higher levels in the treated plants. Before infection, all four esterified hydroxy-fatty acids accumulated to higher levels in treated plants than in controls, but after pathogen attack, the differences were no longer significant except for esterified 13-HOT (for this compound, the difference remained significant until 2 days after pathogen inoculation) (Fig. 5).

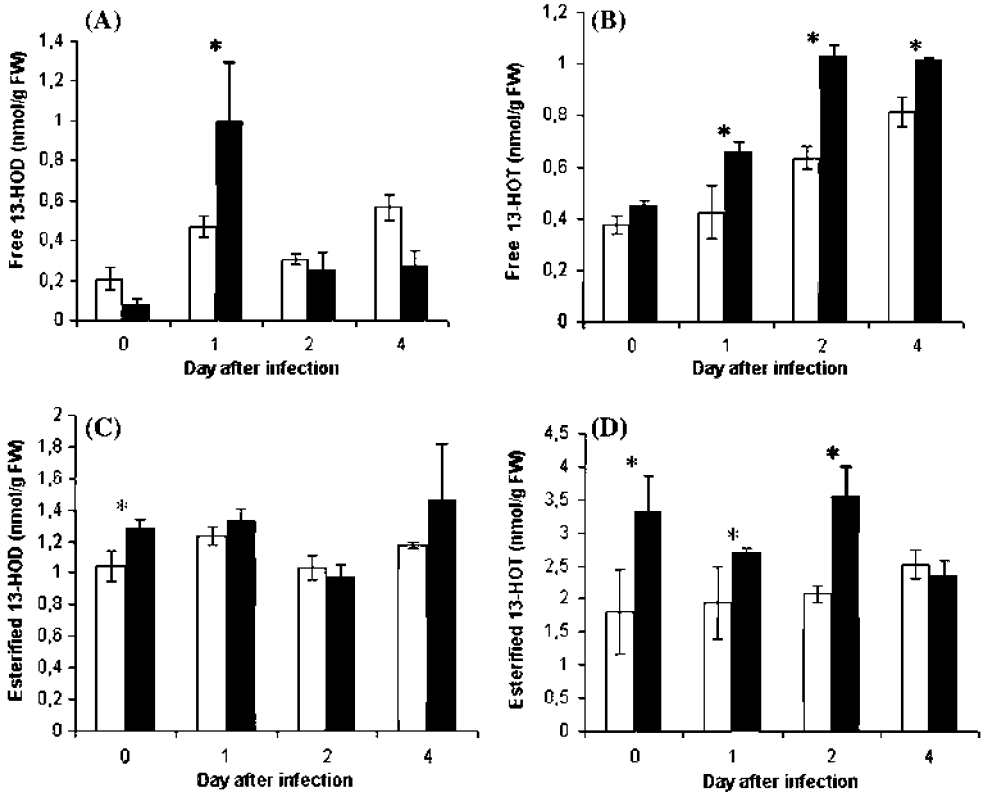
HPOs and hydroxy-fatty acids are produced enzymatically

Besides LOX- and reductase-mediated enzymatic synthesis, which exclusively generates *S*-enantiomers, chemical auto-oxidation and auto-reduction can also yield HPOs and hydroxy-fatty acids, respectively. These latter reactions are not enantioselective; they generate a racemic mix of *S* and *R* forms. The proportion of each form was determined by chiral HPLC in leaf tissues collected before and 1, 2, and 4 days after disease inoculation. A large excess of *S*-enantiomer was always found in extracts from both elicited and control plants showing that most of the accumulating hydroperoxides were generated via an enzymatic process (Supplemental Fig. S2).

The HL branch is not induced in P. putida BP1-mediated ISR

To determine the fate of the transiently accumulating 13-HPOs, we measured the expression and/or activity of enzymes acting downstream in the oxylipin pathway and profiled their possible products in leaves of bacterized and control plants. A first enzyme that can use 13-HPOs is hydroperoxide lyase generating oxylipin volatiles. We measured levels of *Hl* transcripts by RT-qPCR and monitored the corresponding enzymatic activity in a spectrophotometric assay. No significant difference was observed at either transcript (Supplemental Fig. S3a) or enzyme (data not shown) level between control and bacterized plants either before or after pathogen challenging. We confirmed these results by quantifying oxylipin volatiles. The metabolites were collected from leaf homogenates by solid-phase microextraction, identified by GC-MS, and quantified by GC-FID. The volatile compounds hexanal, (Z)-hexen-3-ol, (E)-hex-2-enal, and hexan-1-ol were detected, but as expected no significant difference was observed in the content between control and *P. putida* BTP1-treated plants (Supplemental Fig.5 3b, c, d and e).

Fig. 5 *Time course accumulation of free and esterified 13-hydroxy-fatty acids. Free 13-HOD (a) and 13-HOT (b), and their esterified form (respectively c and d) were quantified by HPLC from leaves of control (white) and P. putida BTP1-treated plants (black) before, 1, 2 and 4 days after infection. The results presented were from a representative experiment that was independently repeated twice with similar results. Stars indicate statistically significant differences between control and treated plants (Student's t test, α = 0.05)*



Synthesis of esterified keto-fatty acids, but not of free forms, is stimulated in ISR

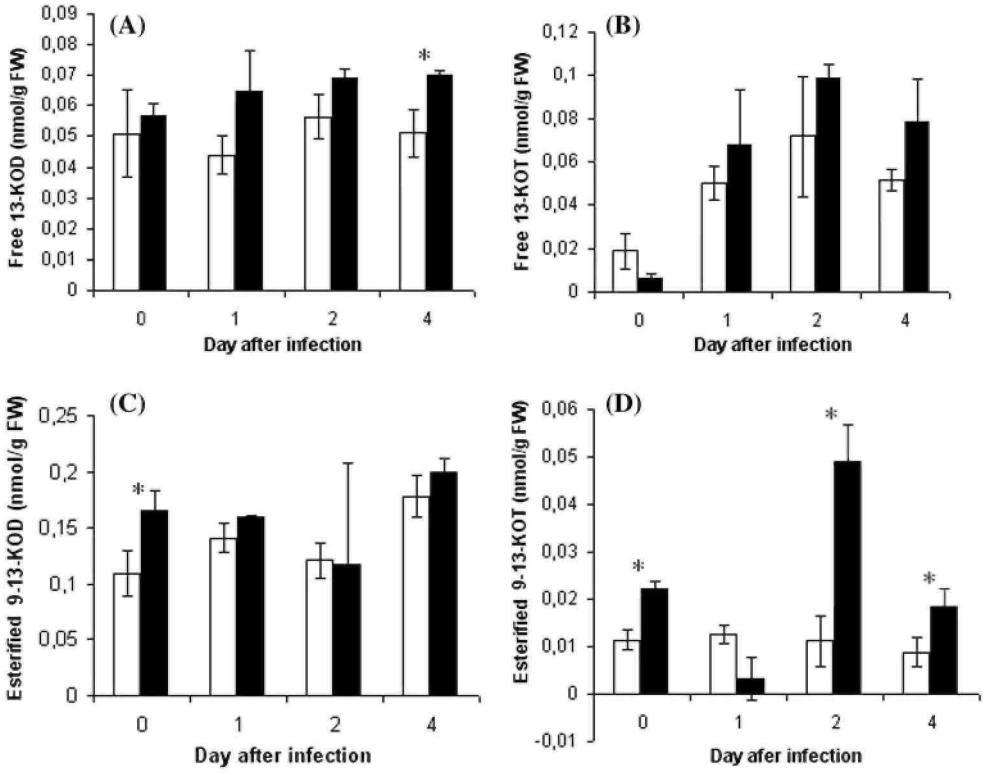
As the LOX enzyme can further convert HPOs to keto-fatty acids, these compounds were extracted in their free forms and as esterified conjugates and quantified by reverse-phase HPLC. Free 9-KOD/T (data not shown) and 13-KOD/T (Fig. 6a, b) were found to accumulate in leaves after infection, but no significant difference was noted between control and treated plants. Esterified forms were detected in only one experiment (out of three),

probably because of their very low content in plant tissues and because poor separation of the 9 and 13 isomers prevented distinguishing 9-KOD from 13-KOD and 9-KOT from 13-KOT. Esterified keto-fatty acids were more abundant in treated plants than in controls before pathogen inoculation. Unlike KODs, 9- and 13-KOT were detected in higher amounts 2 and four days after infection in bacterized plants (Fig. 6c, d).

The AOS and DES branches are partially repressed in bacterized plants upon infection

Allene oxide synthase and DES respectively catalyze the first step of the octadecanoid pathway leading to jasmonic acid and conversion of HPO to divinyl ether fatty acids (Schaller and Stintzi 2009). After inoculation of the pathogen, RT-qPCR analysis showed increased levels of *AOS1, AOS2,* and *DES* transcripts in both bacterized and control plants, but the levels were higher in the latter (Figs. 7a, 8a). Jasmonic acid was quantified by GC-MS but no significant difference was detected before infection among treatments. However, it was found to accumulate in the leaves of control plants after pathogen inoculation but not in those of *Pseudomonas*-treated plants (Fig. 7b). Similarly, quantification of DES products revealed higher colneleic and colnelenic acid contents in untreated plants after pathogen attack (Fig. 8b, c).

Fig. 6 *Time course accumulation of free and esterified keto-fatty acids in control (white) and treated (black) plants before, 1, 2 and 4 days after infection. 13-KOD (a) and 13-KOT (b) were quantified by HPLC. Esterified 9-13-KOD (c) and 9-13-KOT (d) were quantified after transmethylation by HPLC. Peak resolution in the HPLC analysis was not sufficient to fully separate and distinguish the 9 from the 13 isomers. The figure shows data from the only experiment (out of three) where the compounds were detected. Stars indicate statistically significant differences between control and treated plants (Student's t test, α = 0.05)*



Discussion

This study shows that resistance against *B. cinereα* induced in tomato by the nonpathogenic rhizobacterium *P. putidα* BTP1 is associated with significant changes in the oxylipin pathway. Our analyses highlight 13-HPOs and HOD/T, found to accumulate preferentially in esterified form in uninfected bacterized plants and to be released as free fungitoxic compounds upon pathogen inoculation (Fig. 9). Some LOX enzymes can oxidize PUFAs esterified to galactolipids (Nakashima et al. 2011), triacylglycerols, phospholipids, or cholesterol (Andreou and Feussner 2009). Early accumulation of esterified 13-HPOs could be due to the activity of TomLOXF, which is induced by bacterial treatment of plants before infection (Mariutto et al. 2011). As a matter of fact, we observed that recombinant TomLOXF can oxidize dilinoleonyl-phosphatidylcholine to esterified 13-HPOT-phosphatidylcholine (data not shown). Anders-son et al. (2006) also suggested that esterined oxylipins can be produced by acyltransferases which conjugate free oxylipins to phospho/galactolipids. Different LOX isoforms can be located into chloroplasts, cytoplasm or peroxisome (Porta and Rosa Sosa 2002). On the basis of a

bioinformatic analysis of their amino-acid sequences, the two LOX isoforms induced in ISR in tomato are expected to be located in the chloroplasts (Heitz et al. 1997; Mariutto et al. 2011). The localization of the induced LOX is consistent with the relative abundance of esterined lipids in ISR-expressing plants. Indeed, in plants, chloroplast membranes are the site of synthesis of lipids and contain high quantity of conjugated lipids. Several studies have also shown production of esterined oxylipins in plant-pathogen interactions, but not before infection. Indeed, the synthesis of esterined HPOs and hydroxy-fatty acids is induced in potato upon infection with *Phytophtora infestans* (Fauconnier et al. 2008); esterined oxophytodienoic acids (arabidopsides) accumulate during the hypersensitive response in *Arabidopsis* (Andersson et al. 2006); esterined divinyl ether fatty acids (linolipins) are involved in flax-herbivore and flax-pathogen interactions (Chechetkin et al. 2009). The present study is thus the first to demonstrate an accumulation of esterified fungitoxic molecules in uninfected plants during ISR. This accumulation of esterified 13-HPOT and hydroxy-fatty acids before infection may enable the bacterized plant to respond faster and more strongly to pathogen attack.

After *B. cinerea* inoculation, different steps in the oxylipin pathway appear modulated. Free linolenic acid and its products 13-HPOD/T accumulate faster in treated plants than in controls. This result is consistent with those of a preliminary study showing that two linolenic-acid-consuming 13-LOX enzymes are stimulated in tomato leaves during ISR (Mariutto et al. 2011). Two other studies have shown an accumulation of HPOs in plant-PGPR interactions: 13-HPOD and 13-HPOT accumulate in groundnut seedlings inoculated with the nonpathogenic bacterium *Bacillus subtilis* AF1 (Sailaja et al. 1998), and *P. putida* BTP1 induces accumulation of free 13-HPOT in uninfected bean leaves (Ongena et al. 2004). This stimulation of the oxylipin pathway could be a common feature of ISR induced by PGPR in plants.

The accumulation of high level free HPOs in ISR-expressing infected leaves could originate from two sources. The increase of PLA2 activity could contribute to the release of free HPOs and hydroxy fatty acid from esterified pool of oxylipins that accumulate at elevated levels prior to fungal infection. In addition, the increase of LOX activity during pathogen inoculation also suggests that free HPO could also be synthesized de novo from linolenic acid released by phospholipase A2. HPOs appear to be converted mostly to hydroxy-fatty acids. The reduction branch of the pathway is depending on peroxygenase, hydroperoxide reductase (Schaller 2001), and gluthatione peroxidase (Dixon et al. 2002) and/or on non-enzymatic auto-reduction (Howe and Schilmiller 2002). No peroxygenase or hydroperoxide reductase genes have been found to date in the tomato genome, and our chiral analyses show that the detected hydroxy-fatty acid products were generated enzymatically. Thus, accumulation of these compounds in tomato leaves could result from an enhanced activity of gluthatione peroxidase, an enzyme involved in reducing a wide variety of inorganic and organic hydroperoxides to their corresponding hydroxy 1 forms (Kühn and Borchert 2002). Some hydroxy-fatty acids show the same trends as the corresponding HPOs: free 13-HOT accumulates faster in treated plants after infection, and esterifled 13-HOT, like its 13-HPOT precursor, is more abundant in uninfected bacterized plants, suggesting that the downstream reductase activity is not limiting compared to the LOX activity. Free keto-fatty acids generated by the LOX branch do not accumulate preferentially in either control or treated plants, but their esterifled forms are more abundant in treated plants both before and after infection and might participate in defense against the fungus.

Unlike reductase and LOX activities, the AOS and DES branches of the oxylipin pathway are clearly stimulated following infection. However, it is interesting to note that such increases in AOS and DES activities were significantly attenuated in rhizobacteria-treated plants. This is the first reported case of ISR in which defense enzymes induced by infection are less stimulated in treated plants than in controls. Prost et al. (2005) have demonstrated that compounds from the branches of the oxylipin pathway down-regulated in our study (DES, AOS products) are less toxic towards *B. cinerea* than the products of the branches shown here to be stimulated by treatment: 13-HPOs and 13-hydroxy-fatty acids, the compounds most overproduced in *P. putida* BTP1-mediated ISR, are the oxylipins most toxic towards *B. cinerea* (Prost et al. 2005). This suggests that the plant can respond to pathogen attack in the most effective way by stimulating production of the most toxic compounds while reducing synthesis of less toxic ones. However, previous studies have shown that JA is involved in ISR (Ann et al. 2007). In *Arabidopsis*, JA does not accumulate in ISR expressing plants, but the ISR requires the JA-signaling pathway (Pieterse et al. 2000). Authors hypothesize that ISR is accompany with increase of sensitivity to JA. In our model, JA does not accumulate in induced plants before infection. After pathogen inoculation, its level is higher in control plants compared to ISR-expressing plants. Molecular events involved in ISR in tomato seem different to the events occurring in the model plant *Arabidopsis thaliana.* On the other hand, some PGPR can induce a JA-independent and/or a NPR1-independent resistance in plants (De Vleesschauwer and Höfte 2009). The molecular bases of the ISR seem to depend on the complex interaction between a plant species and a PGPR strain.

The most notable differences in leaf concentrations of 13-HPOs and 13-hydroxy-fatty acid oxylipins between control and treated plants were observed during the two first days after *B. cinerea* inoculation. This is a crucial

moment for the development of conidia on leaves, which can be affected in the early stages of germination. The high oxylipin levels in elicited plants might restrict the early stages of the pathogen and delay its development, as suggested by the disease evolution in control and *P. putida* BTP1-treated plants (Fig. 1).

The HL branch leading to fungitoxic volatile oxylipins (Croft et al. 1993) is not modulated by the bacterial treatment tested here. A previous study has demonstrated that trans-2-hexenal should be viewed as a component of the resistance against *B. cinerea* infection induced in bean plants by *P. putida* BTP1 (Ongena et al. 2004). As the same PGPR and pathogen strains were used in both studies, this suggests that the defense response induced by the rhizobacteria depends on the plant species.

In conclusion, our study illustrates some oxylipin-related molecular priming during ISR. Indeed, systemic resistance mediated by *P. putida* BTP1 is associated with storage of some fungitoxic oxylipins by the plant in esteri-fied form and with a faster defense response after infection. Actually, many defense mechanisms can be potentially involved in ISR. Our results provide supplemental explanation for ISR in addition to a growing list of possible mechanisms reported by others (Bent 2006). The production of the most fungitoxic oxylipins is up-regulated, while synthesis of less effective ones is down-regulated. The results of the present study, combined with those published in Mariutto et al. (2011), represent the first global analysis of a metabolic pathway that could be involved in ISR. It should be interesting to analyze the oxylipin pathway upon infection with a pathogen such as *Phytophtora infestans,* which is sensitive to 9-LOX derivatives but not to 13-LOX derivatives (Göbel et al. 2001, Prost et al. 2005), to see if rhizobacterial treatment leads to an efficient response of the plant against the pathogen. Preliminary data with an elicitor from *P. putida* BTP1 (the N-alkylated benzylamine derivative) (Ongena et al. 2005) has shown that it also stimulates the oxylipin pathway. Other ISR-inductive rhizobacteria and elicitors are being tested to determine if priming of this pathway is a phenomenon occurring generally in ISR.

Fig. 7 *Analysis of the AOS branch of the oxylipin pathway, a Expression of the AOS1 (in control (open squares) and bacterized plants (closed squares)) and AOS2 (in control (open triangles) and bacterized plants (closed triangles)) genes was analyzed at transcript level by real-time RT-PCR. b Time course accumulation of jasmonic acid in leaves of control (white) and P. putida BTP1-treated (black) tomatoes. Analyses were realized before (0), 1, 2 and 4 days post infection (dpi). Assay was repeated in another independent biological experiment and showed similar results. Stars indicate statistically significant differences between control and treated plants (Student's t test, α = 0.05)*

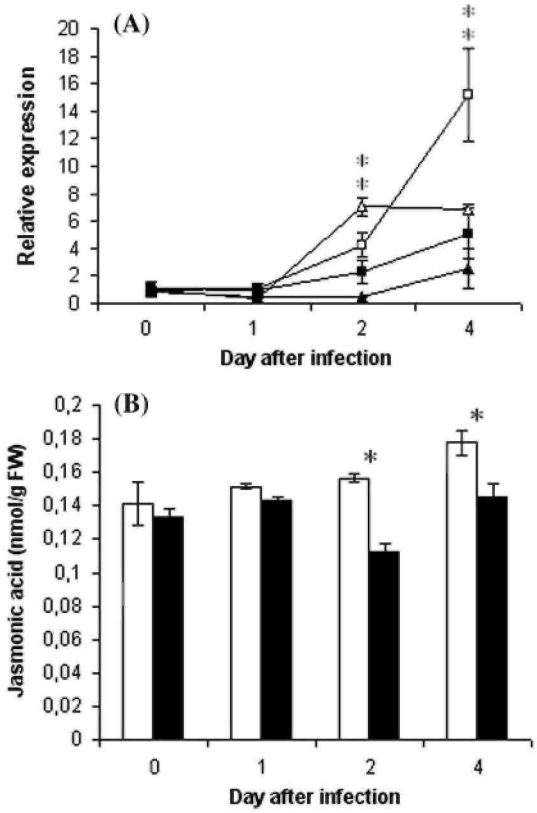


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Fig. 8 *Analysis of the DES branch at the transcriptional and metabolic levels, a Comparison of DES gene transcript levels in control (open squares) and P. putida BTP1-treated (closed squares) plants. Total RNA was extracted from leaves and subjected to real-time RT-PCR. b Time course accumulation of DES products. Colneleic (a) and colnelenic (b) acids,were quantified in leaves of control (white) and P. putida BTP1-treated (black) plants. Analyses were realized before (0), 1, 2 and 4 days post infection (dpi). Assay was repeated in another independent biological experiment and showed similar results. Stars indicate statistically significant differences between control and treated plants (Student's t test, α = 0.05)*

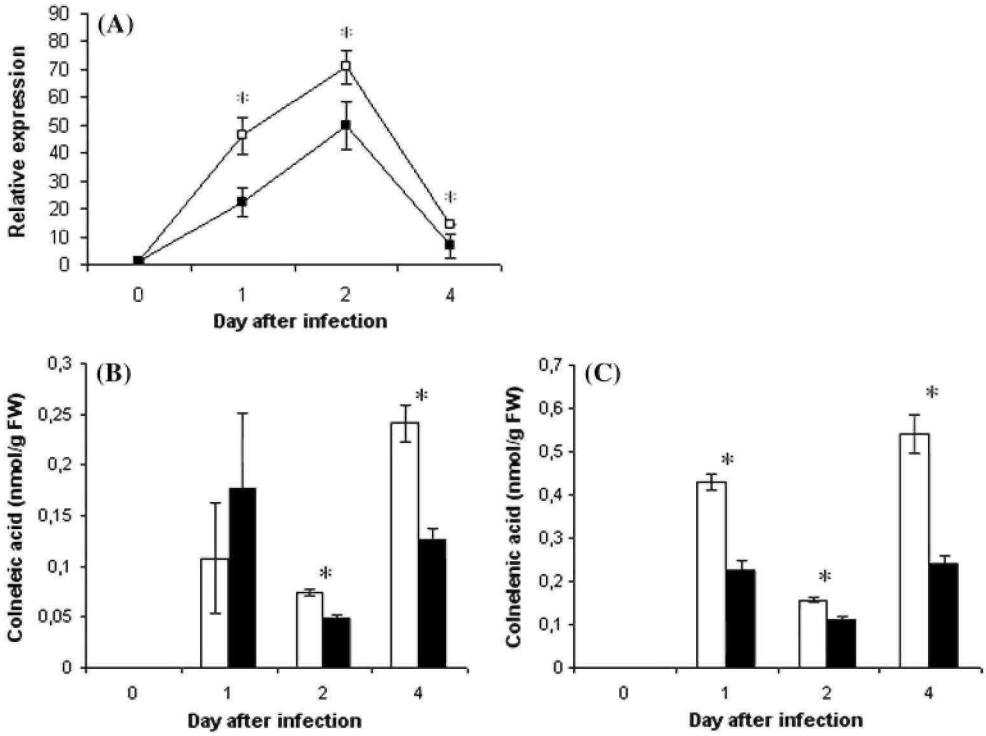
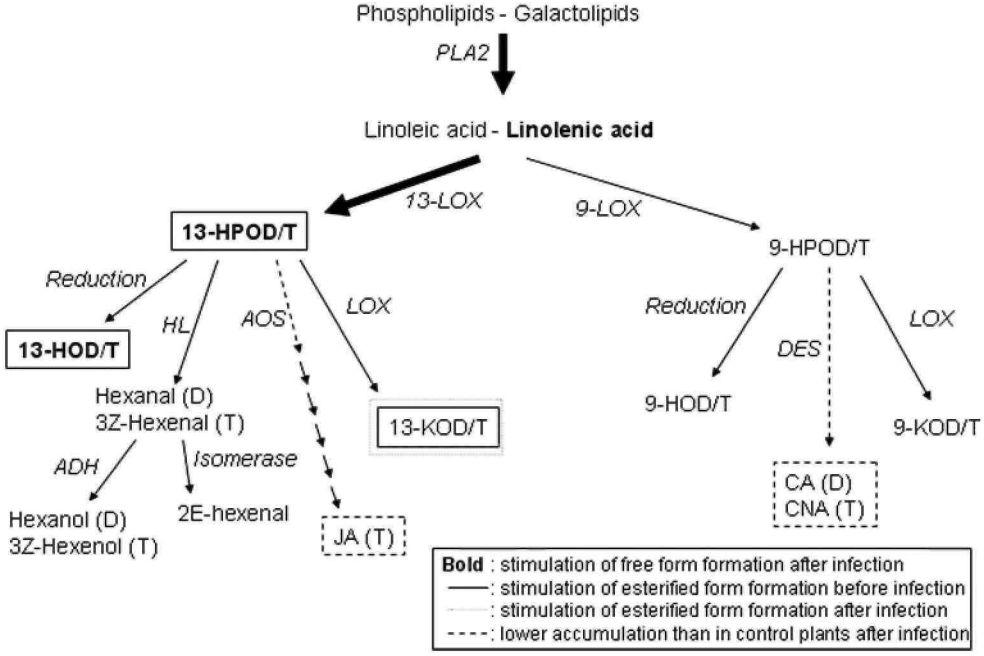


Fig. 9 *Schematic representation of the stimulation of the oxylipin pathway in tomato plants following elicitation by P. putida BTP1 (based on the results from this study). PLA2 phospholipase A2, LOX lipoxygenase, HL hydroperoxide lyase, AOS allene oxide synthase, ADH alcohol dehydrogenase, DES divinyl ether synthase, HPOD/T hydroperoxy-octadecadi/trienoic acid, HOD/T hydroxy-octadecadi/trienoic acid, JA jasmonic acid, KOD/Γ keto-octadecadi/trienoic acid, CA/CNA colnele(n)ic acid. (D) derived from linoleic acid, (J) derived from linolenic acid. Bold arrow, stimulation after infection, disrupted arrow. repression of the increase after infection*



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