Evaluation of the Antimicrobial Activities of Plant Oxylipins Supports Their Involvement in Defense against Pathogens[1][W]

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Plant oxylipins are a large family of metabolites derived from polyunsaturated fatty acids. The characterization of mutants or transgenic plants affected in the biosynthesis or perception of oxylipins has recently emphasized the role of the so-called oxylipin pathway in plant defense against pests and pathogens. In this context, presumed functions of oxylipins include direct antimicrobial effect, stimulation of plant defense gene expression, and regulation of plant cell death. However, the precise contribution of individual oxylipins to plant defense remains essentially unknown. To get a better insight into the biological activities of oxylipins, in vitro growth inhibition assays were used to investigate the direct antimicrobial activities of 43 natural oxylipins against a set of 13 plant pathogenic microorganisms including bacteria, oomycetes, and fungi. This study showed unequivocally that most oxylipins are able to impair growth of some plant microbial pathogens, with only two out of 43 oxylipins being completely inactive against all the tested organisms, and 26 oxylipins showing inhibitory activity toward at least three different microbes. Six oxylipins strongly inhibited mycelial growth and spore germination of eukaryotic microbes, including compounds that had not previously been ascribed an antimicrobial activity, such as 13-keto-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid and 12-oxo-10,15(*Z*)-phytodienoic acid. Interestingly, this first large-scale comparative assessment of the antimicrobial effects of oxylipins reveals that regulators of plant defense responses are also the most active oxylipins against eukaryotic microorganisms, suggesting that such oxylipins might contribute to plant defense through their effects both on the plant and on pathogens, possibly through related mechanisms.

Plant oxylipins represent a vast and diverse family of secondary metabolites, believed to occur in all higher plants. They originate from oxidation and further conversions of polyunsaturated fatty acids (PUFAs), predominantly linoleic acid (18:2) and linolenic acid (18:3). The enzymatic biosynthesis of plant oxylipins from these PUFAs is mainly initiated by α-dioxygenase (α-DOX) and lipoxygenases (LOXs; Blée, 2002; Feussner and Wasternack, 2002; Howe and Schilmiller, 2002; Supplemental Fig. 1). α-DOX converts 18:2 and 18:3 into highly reactive 2-hydroperoxyoctadecadi(tri)enoic acids, which can be converted into the corresponding 2-hydroxyoctadecadi(tri)enoic acid [2-HO(D/T)] or undergo nonenzymatic decarboxylation into one-carbon-shortened fatty aldehydes and fatty acids (Hamberg et al., 2003). Hydroperoxides formed through the action of 9- and 13-LOXs [9-HPO(D/T) or 13-HPO(D/T)] can be further metabolized by six main enzymatic routes, giving rise to (1) 9- or 13-hydroxyoctadecadi(tri)enoic acid [9- or 13-HO(D/T)] through reduction by a peroxygenase (Blée, 2002) or other peroxidase activity (Weichert et al., 1999; Dietz et al., 2002); (2) trihydroxylated fatty acids through conversion into epoxy alcohols, catalyzed either by an epoxy-alcohol synthase (Hamberg, 1999) or a peroxygenase (Blée, 1998), followed by an epoxide hydrolase (Blée, 1998); (3) fatty acid ketodienes (KOD)

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or ketotrienes (KOT) through dehydration by LOXs (Vollenweider et al., 2000) or by dehydrogenation of fatty acid hydroxides by a recently characterized enzyme (Chechetkin et al., 2004); (4) unstable hemiacetals, which almost immediately dissociate into short-chain aldoacids (C9 or C12) and aldehydes (C9 or C6), via the activity of 9- or 13-hydroperoxides lyases (HPL; Grechkin, 2002; Grechkin and Hamberg, 2004); (5) divinyl ether fatty acids through the action of divinyl ether synthases (Grechkin, 2002); and (6) unstable allene oxides generated by allene oxide synthases (Tijet and Brash, 2002). These latter compounds can undergo nonenzymatic hydrolysis forming α- or γ-ketols, or can be channeled into the generation of cyclic, prostaglandin-like cyclopentenones through enzymatic cyclization by allene oxide cyclases. C18 cyclopentenones, e.g. 12-Oxo-10,15(Z)-phytodienoic acid (12-Oxo-PDA), are further reduced to cyclopentanones and β-oxidized to shorter-chain molecules, such as jasmonic acid (JA; Vick and Zimmerman, 1983). Other oxylipins, such as epoxy and dihydroxy fatty acids, can be formed from C18 PUFAs by the peroxygenase/epoxide hydrolase pathway (Blée, 1998). Overall, more than 100 different oxylipins have been detected so far in a wide range of plant tissues.

A few genes encoding oxylipin biosynthetic enzymes are specifically induced upon inoculation with plant pests or pathogens, and the production of related oxylipins is concomitantly increased as revealed in oxylipin profiling studies (Weber et al., 1999; Göbel et al., 2001, 2002; Ponce de Leon et al., 2002; Hamberg et al., 2003), suggesting a role for these compounds in plant defense. The importance of oxylipins in defense was demonstrated in specific cases. For example, decreasing gene expression of a pathogen-inducible 9-LOX in tobacco (*Nicotiana tabacum*) resulted in increased susceptibility to *Phytophthora parasitica* var. *nicotianae* (hereafter designated as *P. parasitica*), an oomycete pathogen of tobacco (Rancé et al., 1998), while suppression of 13-HPL in potato (*Solanum tuberosum*) resulted in increased aphid fecundity on the transgenic plants (Vancanneyt et al., 2001). Conversely, overexpression of 9-LOX in tobacco or jasmonate methyl transferase in Arabidopsis (*Arabidopsis thaliana*) resulted in increased resistance to *P. parasitica* (Mène-Saffrané et al., 2003) and to *Botrytis cinerea*, a fungal plant pathogen (Seo et al., 2001), respectively.

Indeed, some oxylipins produced in response to pathogen attack were shown to be antimicrobial compounds. Thus, 13-HPOT and 13-HOT (Graner et al., 2003), colneleic acid and colnelenic acid (CL and CLn; Weber et al., 1999), as well as several epoxy- or polyhydroxylated fatty acids (Blée, 1998) were described as antifungal or antioomycete compounds, while cis-3-hexenol and trans-2-hexenal (2-hexenal) were able to strongly reduce growth of *Pseudomonas* in vitro (Croft et al., 1993). Contribution of the oxylipin pathway to plant defense can also proceed from production of signal molecules inducing defense gene expression or regulating localized cell death, a powerful plant defense mechanism against pathogens known as the hypersensitive response (La Camera et al., 2004). Signaling activities were proposed notably for LOX-generated 9- and 13-HPOD/T (Rustérucci et al., 1999; Knight et al., 2001), α-DOX-generated oxylipins (Ponce de Leon et al., 2002; Hamberg et al., 2003), and many 13-LOX-derived compounds, including the hormone-like JA, methyl jasmonate, 12-Oxo-PDA (Farmer et al., 2003), 13-HPL-derived C6 aldehydes (Bate and Rothstein, 1998; Farmer et al., 2003), 13-HPOT, 13-HOT (Weichert et al., 1999; Vollenweider et al., 2000), and the electrophilic fatty acid KODs and KOTs (Almeras et al., 2003). It is not known whether the signaling and antimicrobial activities define two nonoverlapping groups of oxylipins, possibly correlating with different branches of the pathway. Since antimicrobial activity was reported for a few compounds only, and against a limited number of microorganisms, we set up a large-scale approach in which the effect of 43 naturally occurring oxylipins, comprising previously characterized compounds as well as less studied ones, was individually assessed on the in vitro growth of six prokaryotic and seven eukaryotic, agronomically important, microbial plant pathogens. This large-scale screening approach provides a new insight into the antimicrobial activity of oxylipins, and suggests that certain oxylipins might fulfill multiple functions during plant-pathogen interactions.

RESULTS

Screening Oxylipins for Inhibitory Activities against Plant Microbial Pathogens

Fourty-three oxylipins as well as anacardic acid (AA-15:1; Table I; Fig. 1) were prepared and purified in multi-milligram quantities by up scaling previously described procedures (see ''Materials and Methods''). They were chosen to represent as much as possible the diversity of structures of this family of metabolites (Fig. 1; Supplemental Fig. 1). Screening of this collection of oxylipins for their effect on microbial plant pathogens was achieved by evaluating the growth of target organisms in liquid media, 24 h after exposure to each oxylipin at 100 μM, in a microspectrophotometric assay. This concentration was selected on the basis of published data on antimicrobial oxylipins, e.g. CLn or 2-hexenal (Croft et al., 1993; Weber et al., 1999), and of preliminary experiments (data not shown). Target organisms were chosen in the major classes of microbial pathogens of crop plants, i.e. bacteria, fungi, and oomycetes (Table II). In vitro growth effects were measured as compared to controls grown in the presence of the ethanol carrier (1%) alone. Results were organized and visualized using a hierarchical clustering software (Seo and Shneiderman, 2002).

The 44 synthesized compounds as well as 18:2 and 18:3 were assayed at 100 μM on plant bacterial

Table I. *Oxylipins used in this study*

Short names and references to the relevant biosynthetic enzyme or chemical synthesis method are indicated. The references indicated by the numbers in the Reference column are as follows: 1, Hamberg et al., 1999; 2, Gardner, 1996; 3, Lie and Pasha, 1998; 4, Gunstone et al., 1994; 5, Baertschi et al., 1988; 6, Hamberg and Fahlstadius, 1990; 7, Hamberg, 1987; 8, Galliard and Phillips, 1972; 9, Hamberg, 1998; and 10, Hamberg, 1991.

Oxylipin	Short Name	Reference
2(*R*,*S*)-Hydroxy-9(*Z*)-octadecenoic acid	2-HOE	1
2(*R*)-Hydroxy-9(*Z*),12(*Z*),15(*Z*)-octadecatrienoic acid	2-HOT	1
8(*Z*),11(*Z*),14(*Z*)-Heptadecatrienal	17:3-al	1
9(*S*)-Hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid	9-HPOT	2, 3
9(*S*)-Hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid	9-HPOD	2, 3
11(*S*)-Hydroperoxy-7(*Z*),9(*E*),13(*Z*)-hexadecatrienoic acid	11-HPHT	2, 3
13(*S*)-Hydroperoxy-6(*Z*),9(*Z*),11(*E*)-octadecatrienoic acid	13-HPOTω6	2, 3
13(*S*)-Hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid	13-HPOT	2, 3
13(*S*)-Hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid	13-HPOD	2, 3
9(*S*)-Hydroxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid	9-HOT	2, 3
9(*S*)-Hydroxy-10(*E*),12(*Z*)-octadecadienoic acid	9-HOD	2, 3
11(*S*)-Hydroxy-7(*Z*),9(*E*),13(*Z*)-hexadecatrienoic acid	11-HHT	2, 3
13(*S*)-Hydroxy-6(*Z*),9(*Z*),11(*E*)-octadecatrienoic acid	13-HOTω6	2, 3
13(*S*)-Hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid	13-HOT	2, 3
13(*S*)-Hydroxy-9(*Z*),11(*E*)-octadecadienoic acid	13-HOD	2, 3
9-Keto-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid	9-KOT	2, 3
9-Keto-10(*E*),12(*Z*)-octadecadienoic acid	9-KOD	2, 3
13-Keto-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid	13-KOT	2, 3
13-Keto-9(*Z*),11(*E*)-octadecadienoic acid	13-KOD	2, 3
(±)-cis-9,10-Epoxyoctadecanoic acid	9,10-EO	4
9(*R*),10(*S*)-Epoxy-12(*Z*)-octadecenoic acid	9,10-EOE	4
12(*R*),13(*S*)-Epoxy-9(*Z*)-octadecenoic acid	12,13-EOE	4
(±)-threo-9,10-Dihydroxy-12(*Z*)-octadecenoic acid	9,10-DHOE	4
(±)-threo-12,13-Dihydroxy-9(*Z*)-octadecenoic acid	12,13-DHOE	4
9,10,16-Trihydroxyhexadecanoic acid (aleuritic acid)	9,10,16-THH	4
12-Oxo-10,15(*Z*)-phytodienoic acid	12-Oxo-PDA	5, 6
13-Hydroxy-12-keto-9(*Z*),15(*Z*)-octadecadienoic acid	12,13-KHOD	7
2(*E*)-Hexenal	2-Hexenal	–
3(*Z*)-Hexenal	3-Hexenal	4
2(*E*)-Nonenal	2-Nonenal	4
3(*Z*)-Nonenal	3-Nonenal	4
9-Oxononanoic acid	9-Oxo-C_9	4
12-Oxo-9(*Z*)-dodecenoic acid	12-Oxo-12:1(Z)	4
12-Oxo-10(*E*)-dodecenoic acid	12-Oxo-12:1(E)	4
1-Penten-3-ol	5:1-ol	–
Colnelenic acid	CLn	8
Colneleic acid	CL	8
ω5(*Z*)-Etherolenic acid	ω5(Z)-ELn	9
10(*S*),11(*S*)-Epoxy-9(*S*)-hydroxy-12(*Z*),15(*Z*)-octadecadienoate[a]	9,10,11-EHOD	10
11(*S*),12(*S*)-Epoxy-13(*S*)-hydroxy-9(*Z*),15(*Z*)-octadecadienoate[a]	11,12,13-EHOD	10
9(*S*),12(*S*),13(*S*)-Trihydroxy-10(*E*),15(*Z*)-octadecadienoic acid	9,12,13-THOD	10
9(*S*),12(*S*),13(*S*)-Trihydroxy-10(*E*)-octadecenoic acid	9,12,13-THOE	10
9(*S*),10(*S*),11(*R*)-Trihydroxy-12(*Z*)-octadecenoic acid	9,10,11-THOE	10
11(*R*),12(*S*),13(*S*)-Trihydroxy-9(*Z*)-octadecenoic acid	11,12,13-THOE	10
6-[8(*Z*)-Pentadecenyl]salicylic acid (anacardic acid)	AA-15:1	4

[a]Methyl ester.

pathogens *Pseudomonas syringae*, *Xanthomonas campestris*, and *Erwinia carotovora* (Fig. 2; Supplemental Table I). Lower concentrations were also tested for the most active compounds. The highly antibacterial oxylipin, 2-hexenal, that prevented the growth of all the bacteria at low concentration (10 μM) was used as a control in these experiments. About half of the tested oxylipins reduced the growth of at least one bacterial strain above 25%. Sensitivity toward oxylipins was contrasted among and within species. *P. syringae* strains were the most sensitive bacteria, whereas *Erwinia* and *Xanthomonas* strains exhibited low sensitivity toward oxylipins. The most active compounds (CL, CLn, ω5(Z)-etherolenic acid [ω5(Z) ELn], 17:3-al, and AA-15:1) were specific to one species. Interestingly, to our knowledge, inhibition of bacterial growth has not been

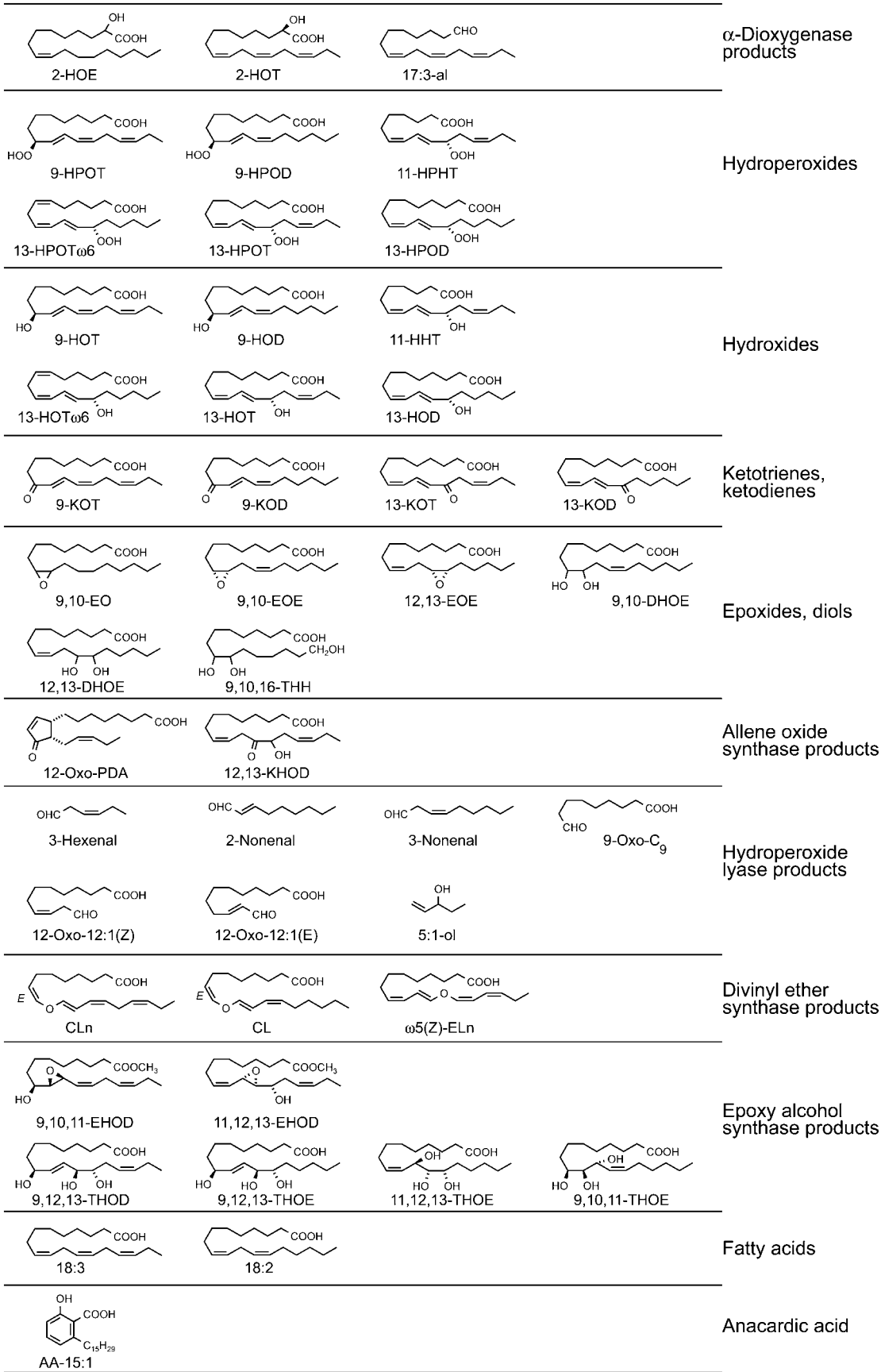

Figure 1. Structures of oxylipins and other compounds used in this work. Abbreviated names for oxylipins are as in Table I.

Table II. *Microbial plant pathogens used in this study*

Species	Disease
Bacteria	
P. syringae pv *syringae*	Bacterial canker of fruit trees, bacterial blight of legumes
P. syringae pv *tomato*	Bacterial speck of tomato and Arabidopsis
P. syringae pv *maculicola*	Bacterial leaf spot of crucifers
P. syringae pv *tabaci*	Bacterial wildfire of tobacco and soybean
X. campestris pv *campestris*	Black rot of crucifers
E. carotovora subsp. *carotovora*	Bacterial soft rot of many vegetables, blackleg of potato
Fungi	
A. brassicicola	Black spot of crucifers
F. oxysporum f. *radicis-lycopersici*	Root and crown rot of tomato
B. cinerea	Gray mold rot on many crops
C. herbarum	Leaf mold, pod, and seed spot on many hosts
Rhizopus spp.	Postharvest decay
Oomycetes	
P. infestans	Late blight of potato and tomato
P. parasitica var. *nicotianae*	Black shank of tobacco

reported before for divinyl ethers. One third of the oxylipins tested did not affect the growth of any bacterial strains.

The same set of compounds was assayed on five fungi, *Alternaria brassicicola*, *B. cinerea*, *Cladosporium herbarum*, *Fusarium oxysporum*, and *Rhizopus* sp., and on two oomycetes, *Phytophthora infestans* and *P. parasitica*. Spores of some of the target organisms, namely, *P. infestans*, *P. parasitica*, *B. cinerea*, and *C. herbarum*, which showed a prolonged lag phase in liquid medium (data not shown), were pregerminated for 16 h prior to oxylipin treatment to actually test the activity of oxylipins on mycelial growth. The results obtained after 24 h of treatment with each oxylipin at 100 μM are gathered in Figure 3 and Supplemental Table II. Eukaryotic microbes appeared to be more sensitive to oxylipins than bacteria. About two-thirds of the molecules tested reduced the growth of at least one eukaryotic plant pathogen above 25% and about half of the compounds were strongly active (around 50% growth inhibition). As observed with bacteria, the eukaryotic microorganisms displayed contrasting levels of overall sensitivity to oxylipins, ranging from high (*P. parasitica* and *C. herbarum*) to intermediate (*B. cinerea*, *P. infestans*, and *F. oxysporum*) or rather low (*A. brassicicola* and *Rhizopus* sp.). One of the most active compounds was 12-Oxo-PDA. This compound is a well established plant signal molecule in wound and stress responses (Stintzi et al., 2001), but its antifungal and antioomycete activity was not previously reported. Four PUFA hydroperoxides (13-HPOT, 9-HPOT, 9-HPOD, and 13-HPOD) as well as their reduced forms (13-HOT, 9-HOT, 13-HOD, and 9-HOD) and dehydration products (notably the ketotrienes, 13-KOT and 9-KOT) were also among the most active oxylipins. In this category, 18:3-derived products seemed slightly more efficient on the strains of the intermediate class of sensitivity to oxylipins than the corresponding 18:2-derived products. Comparison of the effects of 9-LOX-derived compounds to their 13-LOX-derived counterparts, in contrast, did not reveal any obvious relationship between positional specificity and activity.

Apart from only two oxylipins, 9(*S*),12(*S*),13(*S*)-Trihydroxy-10(*E*),15(*Z*)-octadecadienoic acid and 11(*R*),12(*S*),13(*S*)-Trihydroxy-9(*Z*)-octadecenoic acid, it is clear that most oxylipins exhibited antimicrobial activities. Inhibitory activity was very often restricted to a small number of microbes, but 26 oxylipins out of 43 significantly reduced the in vitro growth of at least three different microbes in this study, and nine oxylipins, 9-HOT, 13-HOD, 9-KOT, 13-KOT, 12(*R*), 13(*S*)-Epoxy-9(*Z*)-octadecenoic acid, 12-Oxo-PDA, 13-Hydroxy-12-keto-9(*Z*),15(*Z*)-octadecadienoic acid, CLn, and ω5(Z)-ELn, inhibited the growth of six or more different microbes. We next focused on a small number of oxylipins showing inhibitory activities against eukaryotic microbes.

Concentration Required for 50% Growth Inhibition by Selected Oxylipins

The concentration required to inhibit the in vitro mycelial growth of *B. cinerea*, *C. herbarum*, *P. infestans*, and *P. parasitica* by 50% (IC_{50}) was determined for 12-Oxo-PDA, 13-KOT, 13-HPOT, 13-HOT, 9-HPOT, and 9-HOT. A widely used antifungal molecule (benomyl) and an antioomycete compound (metalaxyl) were assayed in the same conditions against the two fungi and the two oomycetes, respectively. For oxylipins, IC_{50} values ranged from 25 to 70 μM with *C. herbarum* and *P. parasitica* as target organisms, from 50 to 130 μM with *B. cinerea*, and were estimated to be higher than 150 μM for *P. infestans*. The IC_{50} for metalaxyl or benomyl were about 1 to 30 μM for the same target organisms, except for metalaxyl and *P. infestans* (100 μM). This suggested that natural oxylipins were slightly less efficient than synthetic molecules at

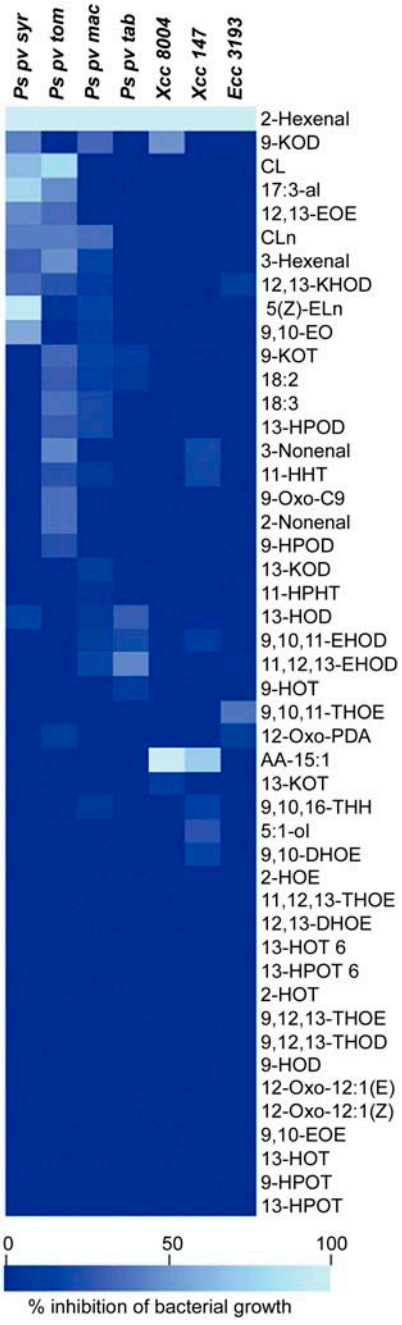

Figure 2. Antibacterial activities of oxylipins. Each test bacterial strain was exposed to each compound (100 μM) in the appropriate liquid medium, for 24 h. For all compounds reproducibly producing a significant reduction in growth of a given organism compared to the ethanol control ($P < 0.05$), inhibition of growth over 24 h was expressed as a percentage relative to the ethanol control: 100 $\times$ (1 $-$ [growth in the presence of oxylipin/growth in control]). Other compounds were scored as causing 0% growth inhibition for this organism. In the diagram, color intensity is inversely proportional to inhibitory activity of the oxylipin toward the organism. Bacteria names are abbreviated as follows: *Ps* pv *syr, P. syringae* pv *syringae*; *Ps* pv *tom, P. syringae* pv *tomato*; *Ps* pv *mac, P. syringae* pv *maculicola*; *Ps* pv *tab, P. syringae* pv *tabaci*; *Xcc, X. campestris* pv *campestris*; and *Ecc, E. carotovora* subsp. *carotovora*. Values represent means of at least three independent experiments, with at least three replicates in each experiment.

inhibiting fungal (or oomycete) growth. We also observed that the growth inhibition effect of these oxylipins was generally transient, with most target organisms overcoming inhibition and resuming growth after 48 to 72 h (data not shown). These observations prompted us to address the stability of the oxylipins in the assay.

Stability of Selected Oxylipins

Many oxylipins are chemically unstable and/or prone to degradation in the presence of biomolecules. Hydroperoxides, for example, are very easily decomposed by metal ions or converted into hydroxides by thiol groups or other reducing agents. Many of the keto-containing oxylipins possess an electrophilic α,β-unsaturated carbonyl partial structure that is prone to Michael addition of glutathione and other thiol-containing compounds (Vollenweider et al., 2000). Additionally, oxylipins may be metabolized by several enzymes (Schaller, 2001; Chechetkin et al., 2004) or enter the mitochondrial or peroxisomal β-oxidation helix, resulting in successive shortening of the carbon chain. To get some insight into their chemical and metabolic stability, the proportion of oxylipins remaining in the assay together with their corresponding metabolites was identified after a 24-h incubation period. Experiments carried out with *B. cinerea*, *C. herbarum*, or *P. parasitica* indicated that the oxylipins introduced were generally hardly detectable in the assay mixture after 24 h (Table III). The structures of the recovered metabolites showed that the hydroperoxides were reduced by the three microorganisms, and 9-HOT, 13-HOT, and 12-Oxo-PDA were degraded through conversion into 15,16-dihydroxylated derivatives or β-oxidation processes, in *B. cinerea* and *C. herbarum*. In contrast, the two hydroxides were recovered nearly unmodified after incubation with *P. parasitica*. CLn and 13-KOT showed low chemical stability in the absence of the fungi, and metabolites could not be detected. CLn may have suffered hydrolysis and chain cleavage into shorter-chain compounds (Galliard et al., 1974), which would have escaped detection by the method used, whereas 13-KOT may have formed Michael adducts with cellular constituents to form water-soluble derivatives (Vollenweider et al., 2000; Almeras et al., 2003). In the case of *P. infestans*, stability of 9,12,13-THOE, ω5(*Z*)-ELn, and CL as well as above-mentioned compounds was assayed. Most oxylipins, including 9-HOT and 13-HOT, showed a very low chemical stability in the noninoculated growth medium of *P. infestans* (data not shown), which might partly explain their low inhibitory activity toward this oomycete as compared to *P. parasitica*. Only 12-Oxo-PDA and 9,12,13-THOE, a chemically quite stable oxylipin (Hamberg, 1991), were less degraded (44% and 83% remaining after 24 h versus 67% and 90% in medium alone, respectively). Altogether, the data suggested that the half-life of oxylipins in the presence of the target organism

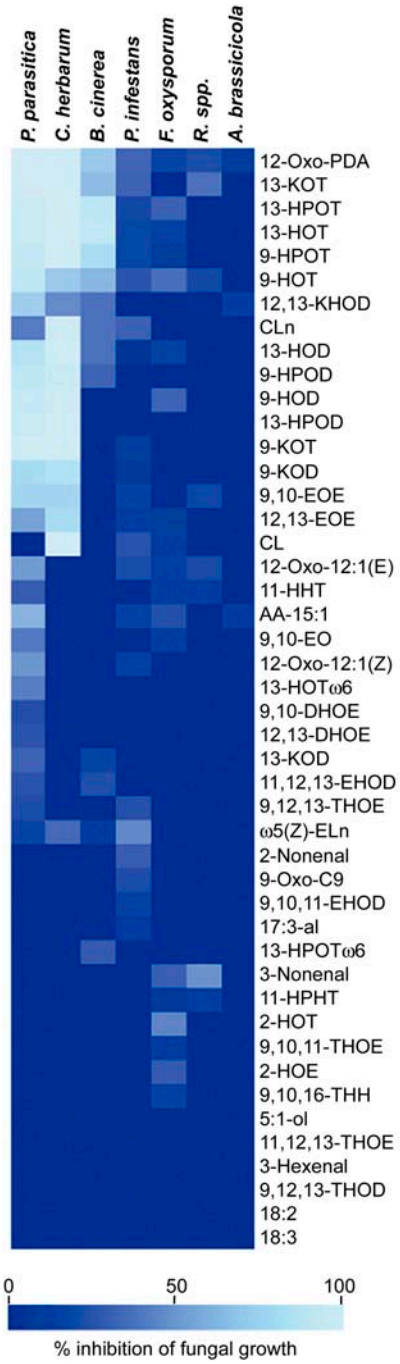

Figure 3. Antifungal and antioomycete activities of oxylipins. Each test fungal or oomycete strain was exposed to each compound (100 μM) in the appropriate liquid medium for 24 h, starting from the time of spore addition to the wells (*F. oxysporum*, *Rhizopus* sp., and *A. brassicicola*) or 16 h later (*P. parasitica*, *C. herbarum*, *B. cinerea*, and *P. infestans*). For all compounds reproducibly producing a significant reduction in growth of a given organism compared to the ethanol control ($P < 0.05$), inhibition of growth over 24 h was expressed as a percentage relative to the ethanol control: 100 × (1 − [growth in the presence of oxylipin/growth in control]). Other compounds were scored as causing 0% growth inhibition for this organism. In the diagram, color intensity is inversely proportional to inhibitory activity of the oxylipin toward the organism. The names of tested organisms are abbreviated as follows: *P. parasitica*, *Phytophthora parasitica nicotianae*; *C. herbarum*, *Cladosporium herbarum*; *B. cinerea*, *Botrytis cinerea*; *P. infestans*, *Phytophthora infestans*; *F. oxysporum*, *Fusarium oxysporum*; *R.* spp., *Rhizopus* species; and *A. brassicicola*, *Alternaria brassicicola*. Values represent means of at least three independent experiments, with at least three replicates in each experiment.

might be quite short. Early degradation might therefore explain why many oxylipins were found to reproducibly but only partially affect the growth of the target microorganisms. In spite of this, the selected oxylipins still displayed significant growth-inhibiting activity toward actively growing mycelium of several target organisms.

Effects on Spore Germination

A microscopic visualization approach was used to investigate the effects of oxylipins on spore germination, an important developmental stage in the life cycle of fungi and oomycetes. Interestingly, this method allowed evaluation of oxylipin activity after shorter time exposure. Oxylipins were added (100 μM final concentration) to freshly prepared spores of *P. parasitica*, *P. infestans*, or *B. cinerea*. The percentage of germinated spores was calculated at optimized time points following treatment, for each organism, and compared to the percentage of germination in controls mock treated with the ethanol carrier (1%) alone or untreated (Fig. 4). Except for CL, the compounds tested highly impaired spore germination of oomycetes such as *P. parasitica* and *P. infestans*, with a maximum of 5% residual spore germination. *B. cinerea* spore germination was also efficiently inhibited by five oxylipins. 12-Oxo-PDA was again the most active oxylipin. It is also noteworthy that some oxylipins that produced the highest levels of inhibition at these early time points, durably inhibited spore germination with no further germination being observed during the next 3 to 4 d (data not shown).

DISCUSSION

Based on the previous, limited knowledge of the biological activities of oxylipins, a large-scale study of direct effects of these compounds on plant pathogens was designed to examine the distribution of antimicrobial activities within the biosynthetic pathway and the spectrum of potential target organisms. Our results reveal that growth-inhibiting activity is unexpectedly widespread among oxylipins. The sensitivity of different pathogens to a given compound may vary greatly, even among closely related strains. The fact that antimicrobial oxylipins were found in all branches of the oxylipin pathway indicated that the production of antimicrobial compounds is not separated from the production of plant signals. Indeed, some of the most active oxylipins against eukaryotic microorganisms in this study, 13-HOT, 13-KOT, 12-Oxo-PDA, as well as

Table III. *Stability of oxylipins in the presence of eukaryotic microbes and structures of main metabolites*

Oxylipins (100 μM) were incubated with suspensions of spores of *B. cinerea, C. herbarum,* or *P. parasitica,* or with growth medium alone, at 25°C. Samples were removed at 0 h and 24 h, were added to ethanol, and 14(*S*)-Hydroxy-10(*Z*),12(*E*),16(*Z*)-nonadecatrienoic acid (3 μg) was added as an internal standard. Levels of oxylipins and structures of metabolites were determined by GC-MS as described in the text. N.D., Not detectable.

Oxylipin	Fungus	Stability[a]	Major Metabolite
		%	
13-HPOT	*B. cinerea*	N.D.	13,15,16-Trihydroxy-9,11-octadecadienoic acid
	C. herbarum	N.D.	13-HOT
	P. parasitica	N.D.	13-HOT
	Medium	N.D.	
9-HPOT	*B. cinerea*	N.D.	9,15,16-Trihydroxy-10,12-octadecadienoic acid
	C. herbarum	N.D.	9-HOT
	P. parasitica	N.D.	9-HOT
	Medium	N.D.	
13-HOT	*B. cinerea*	0	13,15,16-Trihydroxy-9,11-octadecadienoic acid
	C. herbarum	5	3,13-Dihydroxy-9,11,15-octadecatrienoic acid
	P. parasitica	80	–
	Medium	100	
9-HOT	*B. cinerea*	0	9,15,16-Trihydroxy-10,12-octadecadienoic acid
	C. herbarum	3	9,15,16-Trihydroxy-10,12-octadecadienoic acid
	P. parasitica	65	–
	Medium	78	
12-Oxo-PDA	*B. cinerea*	1	2,3,4,5-Tetranor-10,11-dihydro-12-oxo-PDA
	C. herbarum	32	3-Hydroxy-12-Oxo-PDA
	P. parasitica	36	–
	Medium	60	
CLn	*B. cinerea*	1	–
	C. herbarum	10	–
	P. parasitica	4	–
	Medium	23	
13-KOT	*B. cinerea*	1	–
	C. herbarum	1	–
	P. parasitica	13	–
	Medium	38	

[a]Level of oxylipin extracted after 24 h relative to oxylipin extracted in 0 h samples.

fatty acid hydroperoxides, were previously identified as signaling molecules and/or cell death inducers in plants. Thus, according to our results, signaling and antimicrobial activities do not define distinct categories of oxylipins.

The mechanisms underlying the antimicrobial activity of oxylipins in vitro, as well as their precise contribution to plant defense in planta, remain to be elucidated. The growth-inhibiting activity of oxylipins, as measured on bacterial or fungal cell populations in liquid medium, was apparently transient, suggesting that these compounds did not affect cell viability and only delayed growth. However, a more drastic, lethal effect is not ruled out since such effect would not necessarily have been detected in the microspectrophotometric assay that was used. After 24 h of treatment, the level of remaining oxylipin is generally low as shown in this work, and a low amount of surviving cells would be sufficient to resume growth after 72 h (data not shown). Moreover, in spore germination assays, long-lasting inhibition of germination was observed, suggesting that oxylipin treatment might result in spore death in some cases. The effects of selected oxylipins on cell viability and growth rates will have to be investigated by dedicated methods.

Regarding the mechanism of action of oxylipins, the rather high IC_{50} measured for selected active oxylipins suggests that growth inhibition might be due to chemical or physical properties of these metabolites rather than to interaction with specific cellular targets. In this respect, the high chemical reactivity of some oxylipins, notably hydroperoxides, is reminiscent of what is observed with hydrogen peroxide, which acts as a plant signal at low concentrations and, at higher doses, is toxic to plants and other cells (Apel and Hirt, 2004) due to its chemical reactivity. However, not all oxylipins bearing a hydroperoxide function caused growth inhibition in our assay. Likewise, it was recently proposed that several bioactive oxylipins, including 12-Oxo-PDA and 13-KOT, might induce plant cell damage and defense gene expression through a mechanism based on their electrophilic nature (Farmer et al., 2003). The effect of these oxylipins on fungi and oomycetes might be based on such reactivity. Consistent with this, 12-Oxo-PDA scored among the most

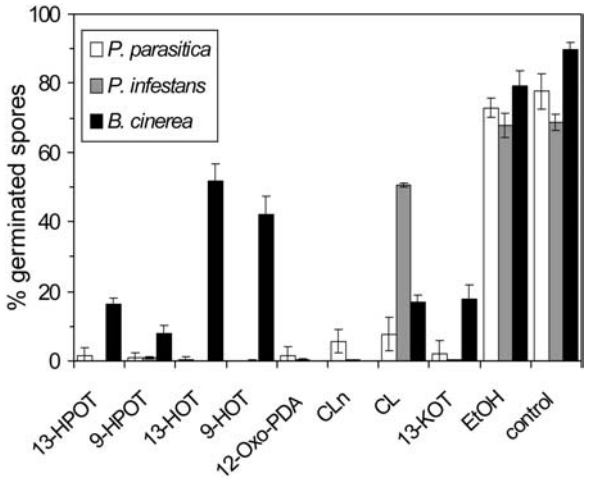

Figure 4. Effect of selected oxylipins on spore germination of oomycetes and fungi. Freshly prepared spores of *P. parasitica*, *P. infestans*, or *B. cinerea* (5.10^4 spores/mL) were exposed to each selected oxylipin (100 μM) and assessed for germination status after 5 h (*P. infestans*), 6 h (*P. parasitica*), or 8 h (*B. cinerea*). Each oxylipin was tested in at least three replicates, in each experiment. Each bar represents mean of three independent experiments ± SD.

active compounds against eukaryotic filamentous microbes, while JA, which derives from 12-Oxo-PDA and shares several structural features except the α,β-unsaturated carbonyl group, did not affect the mycelial growth of *P. parasitica*, *C. herbarum*, and *B. cinerea* in the same conditions (data not shown). Again, not all electrophilic oxylipins were able to cause growth inhibition to a given organism. Overall, our data did not reveal strong relationships between oxylipin chemical structure and pattern of activity.

Besides their chemical reactivity, fatty acid derivatives are likely to interact with biological membranes. It was recently proposed that cis-9-heptadecenoic acid, an antifungal fatty acid, might insert into the hydrophobic layer of fungal membranes, thereby disrupting membrane properties and possibly causing cell collapse (Avis and Bélanger, 2001). Although the hydrophobicity of some oxylipins should be reduced as compared to fatty acids (by the presence of polar oxygenated substituents), hence decreasing their ability to insert into membranes, they might still cause detergent-like disruption of fungal membranes. The effects of selected oxylipins on membrane permeability should be investigated in the future.

The damaging effect of some plant oxylipins on fungi and oomycetes might also result from more specific molecular interactions. Indeed, evidence is accumulating that, as other eukaryotes, fungi and oomycetes produce eicosanoids and/or oxylipins that are involved in the regulation of growth, development, and life cycle (Kock et al., 2003; Noverr et al., 2003; Tsitsigiannis et al., 2004). Whether plant oxylipins interfere with microbial metabolism or signaling by competing with structurally related endogenous lipids should be investigated. Understanding the mechanism of action of oxylipins requires additional studies on specificity and structure-activity relationships, focused on selected compounds such as 12-Oxo-PDA.

Obviously, the mechanism of action might differ depending on the considered microorganism, as suggested by this work that shows rather unexpectedly that plant pathogens display greatly varying levels of sensitivity, ranging from highly sensitive to a large number of oxylipins to mostly unaffected by these compounds. This might result from varying abilities to metabolize oxylipins as pointed out by the stability assays performed on selected oxylipins. Alternatively, these pathogens might also differ greatly in either the uptake of oxylipins from the aqueous culture broth, features of potential targets such as their membrane composition, or their eicosanoid/oxylipin metabolism.

Beyond addressing the direct effect of oxylipins on pathogens in vitro, a crucial point will be to investigate whether growth inhibition is likely to take place in planta during pathogen attack. Open questions concern the local concentration of oxylipins at infection sites and the likelihood that pathogens become in contact with these compounds. The amount of oxylipins in pathogen-challenged plant tissues was investigated in recent years at the organ level through oxylipin profiling/signature methods. In potato, the amount of individual oxylipins reached up to 200 nmol per gram of inoculated leaf tissue during the first 24 h (Göbel et al., 2002) and in tobacco leaves, levels up to 500 nmol per gram were observed (Hamberg et al., 2003). One might expect even higher concentrations at or near infection sites since oxylipins are probably not evenly distributed in inoculated leaves. Additive inhibitory effects might be expected in planta where pathogens have to face the various oxylipins that are produced simultaneously. Regarding cellular location, the production of oxylipins is considered to be compartmentalized, with biosynthetic enzymes occurring in plastids, peroxisomes, cytosol, and possibly other compartments (Feussner and Wasternack, 2002). It will be particularly important to localize the accumulation of oxylipins at the subcellular level, during pathogen attack. For instance, are they released into apoplastic spaces during attack by biotrophic pathogens?

In conclusion, as a heterogeneous group, oxylipins have widespread antimicrobial activities, with often a marked and limited range of specificities. Our data show that several oxylipins previously identified as plant signaling molecules or cell death inducers also efficiently affect the growth of eukaryotic microbes, indicating that these biological activities might be somehow related. Although little is known about oxylipin production and their physiological role in filamentous plant pathogens, a complex interplay of bioactive oxylipins produced both by the host and the microbe could take place at the plant-eukaryotic microbe interface.

MATERIALS AND METHODS

Biological Material and Growth Conditions

The plant pathogens used in this work are listed in Table II. The bacterial strains were *Pseudomonas syringae* pv *syringae* (NCPPB2686), *P. syringae* pv *tabaci* (NCPPB1427), *P. syringae* pv *tomato* DC3000 (Whalen et al., 1991), *P. syringae* pv *maculicola* (NCPPB 1820), *Xanthomonas campestris* pv *campestris* 8004, *X. campestris* pv *campestris* 147 (Lummerzheim et al., 1993), and *Erwinia carotovora* subsp. *carotovora* SCC3193 (Vidal et al., 1997). The fungal strains were *Cladosporium herbarum* (Pers.:Fr.) Link, *Botrytis cinerea* (strain MUCL30158), *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Rhizopus* strain unspecified, and *Alternaria brassicicola* (strain MUCL20297; Thomma et al., 1999), kindly provided by B. Mauch-Mani, University of Neuchatel, Switzerland. The oomycete strains were *Phytophthora parasitica* Dastur var. *nicotianae* (Breda de Haan) Tucker race1 (Apple's isolate 1452; Hendrix and Apple, 1967) and *Phytophthora infestans* strain CRA 208 transformed with a green fluorescent protein gene under the control of the Ham promoter (Si-Ammour et al., 2003), kindly provided by Dr Felix Mauch, University of Fribourg, Switzerland. Cultures of *A. brassicicola*, *F. oxysporum*, *Rhizopus* sp., and *B. cinerea* were maintained on potato (*Solanum tuberosum*) dextrose agar (39 g/L, Oxoid) and subcultured on a weekly basis. *P. parasitica* and *C. herbarum* were grown at 24°C on V8 agar medium (50 mL of V8 juice and 20 g agar per liter, pH 5.0) and subcultured every 14 d. *P. infestans* was maintained on oat bean agar and subcultured every 11 d. *A. brassicicola*, *F. oxysporum*, and *Rhizopus* sp. spores were harvested in potato dextrose broth (4.8 g/L, DIFCO) and diluted to a concentration of 1 to 2 $\times$ 10^5 spores/mL and used immediately. For *C. herbarum* conidia production, actively growing mycelium was suspended in sterile distilled water and spread onto a fresh V8 agar plate. After 5 d at 24°C in the dark, a spore suspension was collected by gently scrapping the spores into sterile distilled water. Spore suspension was filtered through a 60 μm scrynell membrane and concentration was adjusted to 4 $\times$ 10^4 spores/mL with water. *B. cinerea* spore suspension was obtained in a similar way, after growing mycelium for 3 to 4 weeks on potato dextrose agar medium (24 g/L, Sigma) with a photoperiod of 12 h to induce sporulation. Concentration was adjusted to 10^5 spores/mL. *P. parasitica* zoospore production was initiated by starvation of 7-d-old mycelium on 1.5% agar plates and zoospores were released in sterile water by cold shock, at 4°C for 30 min, according to Gooding and Lucas (Gooding and Lucas, 1959). The zoospore suspension was collected and density adjusted to 4 $\times$ 10^4 or 10^5 zoospores/mL with sterile distilled water. Similarly, *P. infestans* zoospores were collected in sterile distilled water, after 3 h at 4°C, and spore density was adjusted to 5 $\times$ 10^5 zoospores/mL.

Antimicrobial Tests

In vitro antimicrobial activities of oxylipins were evaluated in a microspectrophotometric assay, according to Broekaert et al. (1990). Microorganisms were grown in sterile, flat-bottom, 96-well microplates, sealed with Parafilm, in a final volume of 100 μL. Bacteria were grown at 28°C starting with approximately 2,000 bacteria per well. *Pseudomonas* strains were grown in King's medium (2% proteose peptone, 2% glycerol, 6.5 mM K_2HPO_4, and 6 mM $MgSO_4$ $7H_2O$), *Erwinia* in LPG medium (0.3% yeast [*Saccharomyces cerevisiae*] extract, 0.5% Bacto-peptone, and 0.5% Glc), and *Xanthomonas* in Kado medium (1% yeast extract, 0.8% casamino acids, 1% saccharose, 6.5 mM K_2HPO_4, and 6 mM $MgSO_4$ $7H_2O$). Fungi and oomycetes were grown in the dark at 25°C, except for *P. infestans*, which was grown at 18°C. *A. brassicicola*, *F. oxysporum*, and *Rhizopus* sp. were grown in potato dextrose broth (4.8 g/L, DIFCO) in an orbital shaker. *B. cinerea*, *C. herbarum*, and *P. parasitica* were grown in clarified V8 juice broth (5% V8 juice in water, pH 5.0, centrifuged three times at 3,000 rpm, for 20 min) without shaking. *P. infestans* was grown in water, onto solid oat bean medium (100 μL each by well), without shaking. Cultures were started with 2,000 spores (*C. herbarum* and *P. parasitica*) or 5,000 spores (*B. cinerea*, *A. brassicicola*, *F. oxysporum*, *Rhizopus* sp., and *P. infestans*). Fatty acids, oxylipins, AA-15:1, metalaxyl (methyl N-methoxyacetyl-N-2,6-xylyl-DL-alaninate, Riedel-deHaën) and benomyl [methyl 1-(butylcarbamoyl)benzimidazol-2-ylcarbamate, Riedel-deHaën] were dissolved in ethanol. Each treatment was performed in three to eight replicates in the same plate, and experiments were repeated three or four times independently. Ethanol concentration was kept to a maximum of 1% final concentration in each well; controls were treated with 1% ethanol.

Except for *P. infestans*, growth was monitored by measuring the absorbance of the microcultures at 595 nm with a microplate reader (Bio-Rad or DYNEX Technologies), at 0 h and after 24 to 72 h of incubation in the presence or absence of compounds to be tested. Growth of *P. infestans* transformed with green fluorescent protein was determined by measuring fluorescence emission at 530 nm after excitation at 485 nm, with a CytoFluor II fluorescence reader (BioSearch, Millipore). For *C. herbarum*, *B. cinerea*, *P. parasitica*, and *P. infestans*, oxylipins were added after 16 h of growth and A_{595} (or fluorescence) measured at 16 h and after a further 24 to 72 h period. At least three wells were used in each experiment and the experiments repeated at least three times independently. Analysis of variance of antimicrobial test results was performed on final absorbance (or fluorescence) values within experiments. The test compounds were tested against different organisms in groups of up to 12 compounds, with all compounds being tested on each of a number of replicate plates alongside control treatments. As a result, individual plates were treated as replicates and the effect of different compounds on the growth of individual organisms was analyzed for small groups of compounds and compared to the values gained for control treatments in that group of plates only. Therefore, the different analyses of variance do not provide comparisons of the effectiveness of one compound against another, but do provide a measure of the impact of individual compounds against the control treatment (1% ethanol) in that experiment.

Growth inhibition for each compound and each target organism was calculated using the following expression:

$$100 \times [1 - (\Delta OD_{595nm,\ 0-24\,h} \text{ in oxylipin} / \Delta OD_{595nm,\ 0-24\,h} \text{ in control})].$$

In the experiments involving *P. infestans*, fluorescence was used instead of absorbance. Results were organized and visualized using the Hierarchical Clustering Explorer software (version 2.0 beta; Human-Computer Interaction Lab, University of Maryland [http://www.cs.umd.edu/hcil/hce/]; Seo and Shneiderman, 2002).

Spore Germination Assay

Spore germination in the presence of oxylipins was assessed in 96-well microplates, in clarified V8 juice broth (*P. parasitica* and *B. cinerea*) or water (*P. infestans*). Oxylipins in ethanol were added in each well to a final concentration of 100 μM (1% ethanol final concentration) together with 5,000 spores or zoospores, in triplicate, in a total volume of 100 μL, and the plates incubated for a few hours. For each target organism, time points for spore status assessment were optimized for high number of germinated spores in the controls together with short germ tubes (average length of germ tubes not higher than 8- to 10-fold the spore diameter) for better visualization of each germling. The plates were observed in an inverted microscope (DMIRBB, Leitz or Leitz DM IL, Leica), and for each well two to four nonoverlapping pictures (magnification: 100$\times$) were acquired with a charge-coupled device camera (Color Coolview, Photonic Sciences or Nikon D1x) and 100 to 200 spores or zoospores subsequently assessed for germination status. Spores with germ tubes as long or longer than the spore were counted as being germinated.

Oxylipins

The oxylipins used in this study are listed in Table I and Figure 1. Many of them were prepared from the hydroperoxides 9(*S*)-HPOD, 13(*S*)-HPOD, 9(*S*)-HPOT, or 13(*S*)-HPOT, which were in turn prepared by incubation of 18:2 or 18:3 (Nu-Chek-Prep) with LOX from tomato (*Lycopersicon esculentum*) or soybean (*Glycine max*; Gardner, 1996; Lie and Pasha, 1998). Secondary enzymes used for transformations of hydroperoxides into other oxylipins included allene oxide synthase (Hamberg, 1987; Baertschi et al., 1988), allene oxide cyclase (Hamberg and Fahlstadius, 1990), and divinyl ether synthase (Galliard and Phillips, 1972; Hamberg, 1998; Supplemental Fig. 1). Peroxygenase (Blée and Schuber, 1990; Hamberg and Hamberg, 1990) and epoxide hydrolase (Hamberg and Hamberg, 1996) can be used for the preparation of various epoxy alcohols and trihydroxy oxylipins, however, chemical epoxidation and hydrolysis methods (Hamberg, 1991) are generally more convenient to use in these cases. α-DOX derivatives were produced as previously described (Hamberg et al., 1999). A number of oxylipins, e.g. monoepoxy fatty acids, were isolated as natural products from various seeds (Gunstone et al., 1994), and another group including short-chain aldehydes was prepared from commercially available starting materials using chemical methods. 2-Hexenal and 1-penten-3-ol were purchased from Sigma-Aldrich. 6-[8(*Z*)-Pentadecenyl] salicylic acid (AA-15:1), a nonoxylipin belonging to the anacardic acid family of antibacterial compounds (Kubo et al., 2003), was isolated from cashew nut shell liquid (Paramashivappa et al., 2001). Final purification of each

compound was in most cases carried out by straight-phase HPLC using 2-propanol/hexane-based solvent systems. The structures of the compounds obtained, as well as the stereochemical fidelity and purity in case of optically active compounds, were determined by chemical and physical methods as described either in the references given in Table I or in other studies cited by these references. The purity of each compound was in excess of 97% as determined by straight phase-HPLC and/or gas chromatography-mass spectrometry (GC-MS). Most of the compounds used have recently become commercially available through Larodan Fine Chemicals and/or Cayman Chemical or Biomol.

Estimation of the Stability of Oxylipins

Oxylipins (13-HPOT, 9-HPOT, 13-HOT, 9-HOT, 12-Oxo-PDA, 13-KOT, and CLn, 100 μM each) were added to cultures of *P. parasitica*, *C. herbarum*, or *B. cinerea* in liquid growth medium, or to medium alone, and kept at 25°C. Aliquots were removed at 0 and 24 h and added to ethanol. 14(*S*)-Hydroxy-10(*Z*),12(*E*),16(*Z*)-nonadecatrienoic acid (3 μg) was added as an internal standard and the mixtures extracted with diethyl ether. The material obtained was derivatized by treatment with diazomethane followed by trimethylchlorosilane/hexamethyldisilazane/pyridine and analyzed by GC-MS using a Hewlett-Packard model 5970B mass-selective detector connected to a Hewlett-Packard model 5890 gas chromatograph. A capillary column of 5% phenylmethylsiloxane (12 m, 0.33 μm film thickness) was used with helium as the carrier gas. The column temperature was raised at 10°C/min from 120°C to 260°C. The derivatized samples were analyzed first in the scan mode to characterize metabolites or degradation products formed from the various oxylipins. A second analysis performed in the selected ion monitoring mode was used to determine the levels of oxylipins present in the 24 h samples relative to those in the 0 h samples. The following mass spectral ions were used in these analyses: mass-to-charge ratio (*m/z*) 325 (internal standard; M^+, CH_2-CH=CH-C_2H_5), 311 (13-HOT; M^+, CH_2-CH=CH-C_2H_5), 225 [9-HOT; Me_3SiO^+=CH-(CH=CH)$_2$-C_5H11], 238 (12-Oxo-PDA; M^+, [CH_2-CH=CH-C_2H_5 minus H], 306 (CLn; M^+), and 237 (13-KOT; M^+, CH_2-CH=CH-C_2H5). The percentage level of oxylipin remaining at 24 h relative to the level at 0 h was calculated using the following expression:

$$\%_{24\,h} = 100 \times (\text{intensity of ion/intensity of } m/z\ 325)_{24\,h} / (\text{intensity of ion/intensity of } m/z\ 325)_{0\,h}.$$

In a second series, selected oxylipins were added to cultures of *P. infestans* grown in water on solid medium, or to water on solid medium alone, and samples obtained at 0 and 24 h were treated as described above. The oxylipins used in this case included the above-mentioned ones as well as 9,12,13-THOE (*m/z* 173), ω5(*Z*)-ELn (*m/z* 306), and CL (*m/z* 308).

Estimation of the stability of the hydroperoxides 13-HPOT and 9-HPOT could not be performed using the method described due to their thermal instability, which precluded analysis by GC-MS; however, a number of stable metabolites formed from them could be identified.

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