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Phytochemistry 63 (2003) 89–95
PHYTOCHEMISTRY
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Screening of oxylipins for control of oilseed rape (*Brassica napus*) fungal pathogens

Georg Granér[a], Mats Hamberg[b], Johan Meijer[a,*]

[a]*Department of Plant Biology, Genetics Center, POBox 7080, Swedish University of Agricultural Sciences, SE-750 07 Uppsala, Sweden*
[b]*Department of Medical Biochemistry and Biophysics, Division of Physiological Chemistry II, Karolinska Institutet, SE-171 77 Stockholm, Sweden*

Received 15 August 2002; received in revised form 1 November 2002

Abstract

Oxylipins are products of oxygenase-catalyzed reactions of fatty acids. Oxylipins have been found or implied to participate in a variety of different functions in or between organisms. In this report we investigated the potential of various naturally occurring oxylipins found in plants for their effects as fungicides on a number of fungal pathogens interfering with Brassica cultivation. The fungi investigated were *Alternaria brassicae, Leptosphaeria maculans, Sclerotinia sclerotiorum* and *Verticillium longisporum*. An in vitro growth inhibition assay was used, where the relative growth rate of the fungi were determined in the presence of various concentrations of oxylipins. While no universal fungicidic effect was found for the 10 compounds investigated there were examples of oxylipins having inhibitory effects. In certain cases the inhibitory effects was overcome by time, however. Since several of the oxylipins tested were found to be stable in the absence of the fungus this effect could be explained by induction of the degrading capacity of the fungus or increased tolerance. Several of the oxylipins also inhibited germination of *L. maculans* spores but the relative potency differed compared to the effects on hyphae. The study suggests that selected oxylipins may be used for disease control on *Brassica* plants.

Keywords: *Brassica* crops; *Alternaria brassicae*; *Leptosphaeria maculans*; *Sclerotinia sclerotiorum*; *Verticillium longisporum*; Oxylipin; Fungicide; Pathogen

1. Introduction

The majority of fatty acids stored in plant seeds used as oil crops are unbranched, of 12–22 carbon atom chain length and may contain some double bonds (Thelen and Ohlrogge, 2002). Many plants synthesize unusual fatty acids and hundreds of different fatty acid structures have been identified (van de Loo et al., 1993). Such modifications can create very complicated structures containing branches, cyclic structures, various functional groups, etc. The function of many of these structures is not known and knowledge about the enzymes responsible for their biosynthesis and further metabolism is incomplete. Oxylipins (oxygenated fatty acids) constitute one example of modified fatty acids which are products of monooxygenase- or dioxygenase-catalysed reactions of fatty acids (Hamberg, 1993).

Oxylipins have been found or implied to serve in a variety of different physiological processes in higher organisms. Oxylipins play an important role in the defense system of plants; they constitute the first line of defense by means of the cuticle, as signal molecules and as antimicrobial compounds (Blée, 1998). The cutin polymer is complex and composed of many different fatty acid structures including oxylipins that minimize water loss from the plant but also interfere with pest attack (Post-Beittenmiller, 1996). Jasmonic acid and related compounds formed in a lipoxygenase-dependent pathway have been shown to be essential for systemic

Abbreviations: ω5(*Z*)-etherolenic acid, (9*Z*,11*E*,1′*Z*,3′*Z*)-12-(1′,3′hexadienoloxy)-9,11-dodecadienoic acid; PDA, potato dextrose agar.

* Corresponding author. Tel.: +46-18-673321; fax: +46-18-673389.
E-mail address: johan.meijer@vbiol.slu.se (J. Meijer).

0031-9422/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0031-9422(02)00724-0

signalling during insect attack or by certain pathogens but also during development (Wasternack and Parthier, 1997). It has been shown that plants can make antifungal oxylipins as shown for example, rice, taro and tomato (Kato et al., 1983; Masui et al., 1989; Vernenghi et al., 1986). Obviously multiple oxylipins including volatiles can be formed for several reasons from plants during abiotic or biotic stress and the oxylipin signatures of different plants and stresses seem unique (e.g. Weber et al., 1997; Kramell et al., 2000; Göbel et al., 2001). The number of fatty acid structures present as well as the complexity in gene families such as for the rather well characterized lipoxygenases (Feussner and Wasternack, 2002) suggest that plants have a large potential to form various oxylipins to serve in stress situations and development (Howe and Schilmiller, 2002).

Resistance of plants to pathogens depends on physical and chemical defenses that are constitutive and inducible (Karban and Baldwin, 1997). Changing agricultural practice, plant breeding not primarily aiming for pest resistance and rapid evolution of pest strains are examples of factors that has made many crops vulnerable to pests in todays agriculture. *Brassica* cultivation face increasing problems with pathogens and insect pests that limit production and improved resistance to pathogens is therefore important to safeguard rapeseed production. We are interested in the use of oxylipins as potential fungicides against pathogens that constitute major threats to *Brassicas*. Several of the oxylipins chosen in our investigation are expected to be present in oilseed rape tissues under normal and/or stress conditions but the relative levels may vary. As a first step to identify candidate oxylipins with fungicidic effects we used an in vitro screening, where the fungi was grown in the presence of various oxylipins and effects on growth rate or spore germination recorded. The tested pathogens are ascomycetes and nonobligate parasites on *B. napus*; *Leptosphaeria maculans* causes blackleg disease or stem (crown) canker, *Alternaria brassicae* causes blackspot, *Sclerotinia sclerotiorum* causes rot and *Verticillium longisporum* is a wilt pathogen. We here report that certain oxylipins can inhibit growth and germination of *Brassica* pathogens.

2. Results and discussion

By measuring the size of the growing fungal colony on oxylipin-containing potato dextrose agar (PDA) we found a diverse pattern of growth inhibition. We found that (9*Z*,11*E*,1′*Z*,3′*Z*)-12-(1′,3′hexadienoloxy)-9,11-dodecadienoic acid [ω5(*Z*)-etherolenic acid] caused a long-lasting inhibition (e.g. 70% of control at 120 h and 1 mM) of *L. maculans* development (Fig. 1a). The strongest inhibition of *L. maculans* growth after 3 days (ca. 20% of control at 1 mM) was caused by (±)-*threo*-12,13-dihydroxy-9(*Z*)-octadecenoic acid (Fig. 1b). In the latter case the growth rate showed a tendency to increase with time at the 0.5 mM concentration of oxylipin and colonies were of the same size as for the control after 5 days indicating a potential active degradation of this oxylipin or somehow an increased tolerance, while the 1 mM concentration still was toxic. *L. maculans* start by infecting plant tissue biotrophically but behind the front the fungus turn necrotrophic (Howlett et al., 2001). These two states may handle or respond to oxylipins differently showing dose dependent effects. 13(*S*)-Hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid was also a potent inhibitor of *L. maculans* growth (50% of control at 120 h and 1 mM) (Fig. 1c) but again the fungus was not killed but only retarded in growth rate and reached the maximum size of the dish after 8 days (results not shown). The following oxylipins had no effect on *L. maculans* growth when tested at 1 mM concentration or lower; (±)-*cis*-12,13-epoxy-9(*Z*)-octadecenoic acid, (±)-*threo*-9,10-dihydroxy-12(*Z*)-octadecenoic acid, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid, 13(*S*)-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid and 9(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid. None of the oxylipins tested was lethal at 1 mM concentration to any of the pathogens although some of the structures caused strong growth inhibition (summarized in Table 1). The results also showed that the sensitivity towards the oxylipins varied between the phytopathogens.

Both *A. brassicae* and *L. maculans*, that belong to the same order; Pleosporales, was inhibited by about half of the oxylipins but not necessarily by the same ones. *A. brassicae* was strongly affected by ω-5(*Z*)-etherolenic acid (summarized in Table 1), growing at only 14% of the control at 96 h; this was the over all strongest inhibition we recorded. Significant inhibition of fungal growth was also detected for (±)-*cis*-12,13-epoxy-9(*Z*)-octadecenoic acid, (±)-*threo*-12,13-dihydroxy-9(*Z*)-octadecenoic acid, (±)-*threo*-9,10-dihydroxy-12(*Z*)-octadecenoic acid and 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid. The 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid seem to have a growth-stimulating effect on *A. brassicae* at 0.13 mM (data not shown), however.

S. sclerotiorum, a necrotroph, was only effectively inhibited by (±)-*cis*-12,13-epoxy-9(*Z*)-octadecenoic acid (summarized in Table 1). Some inhibition was also recorded for the (±)-*threo*-12,13-dihydroxy-9(*Z*)-octadecenoic acid and the (±)-*threo*-9,10-dihydroxy-12(*Z*)-octadecenoic acid. *S. sclerotiorum* is a fast growing fungus with a thin hyphal front that made it hard to get accurate measurements. The vascular fungus *V. longisporum* was not significantly inhibited by any of the oxylipins tested (Table 1). Both *S. sclerotiorum* and *V. longisporum* are sclerotia forming fungi. It is unlikely

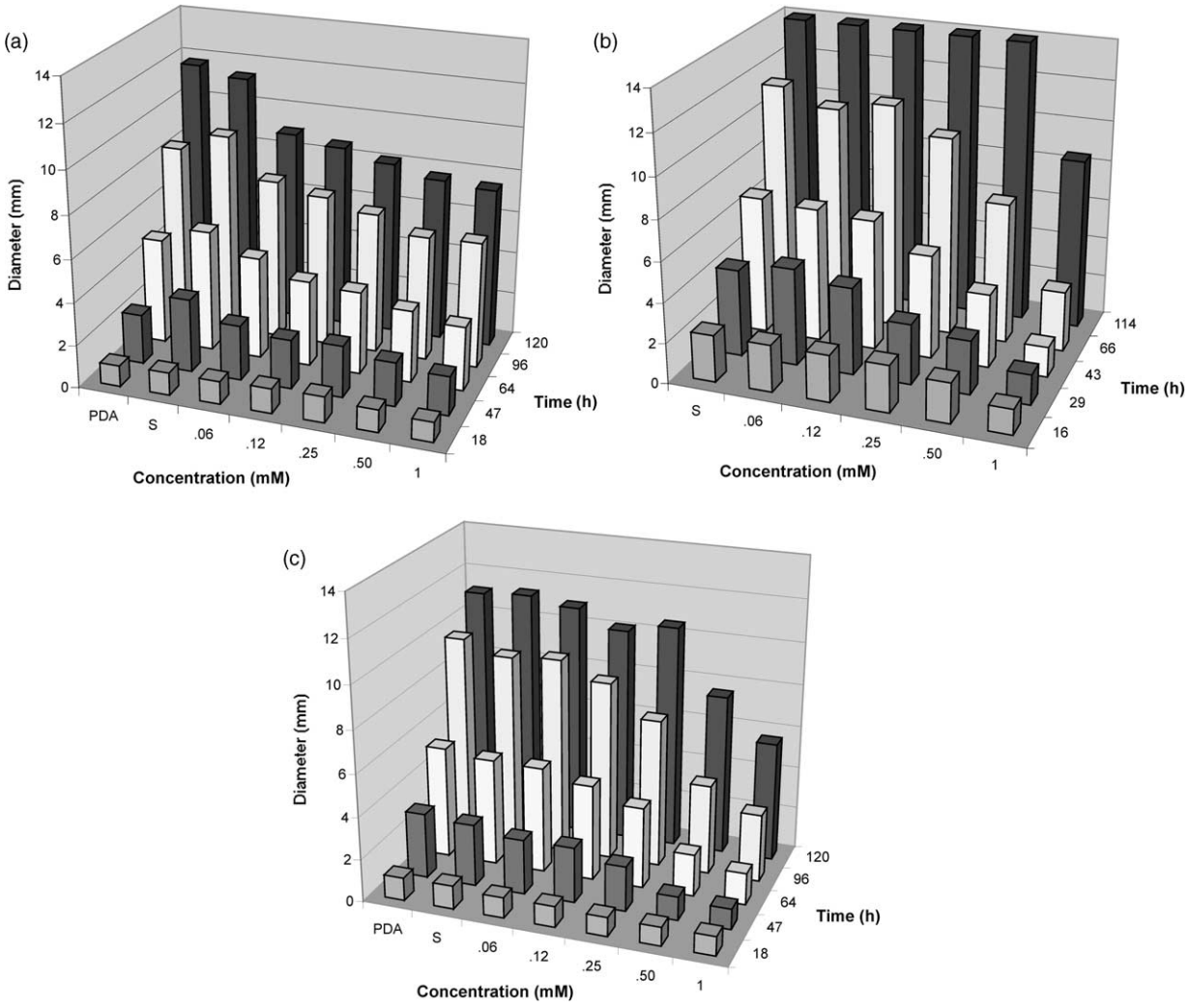

Fig. 1. Growth of *L. maculans* on PDA containing various oxylipins. A disc of *L. maculans* hyphae was grown in the presence of various concentrations (0.06–1 mM) of ω-5(*Z*)-etherolenic acid (a), (±)-*threo*-12,13-dihydroxy-9(*Z*)-octadecenoic acid (b) or 13(*S*)-hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (c) added to PDA and the diameter of the fungus was measured at different time points (16–120 h). The controls consisted of PDA without any additives ("PDA") and PDA containing the solvent used to dissolve the oxylipin ("S") ($n = 3$).

that any of these two fungi will be successfully controlled using oxylipins of the type tested here.

Spore germination of *L. maculans* was strongly inhibited by ω-5(*Z*)-etherolenic acid (Fig. 2a) with close to 100% inhibition throughout the 4-day test period at the higher concentrations of this oxylipin. Although we could not observe any inhibition of growth of *L. maculans* hyphae in the presence of (±)-*cis*-12,13-epoxy-9(*Z*)-octadecenoic acid, the germination of *L. maculans* spores was significantly inhibited (Fig. 2b). A strong inhibition of spore germination was also observed in the presence of 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (Fig. 2c). The results from the spore germination tests are summarized in Table 2. The results show that spores are more sensitive in general compared with growing hyphae, perhaps because of a lower ability to induce detoxification systems. The rise blast fungus *Pyricularia oryzae* was included as a control for fungicidic effects of oxylipins and our results are in agreement with those reported by Kato et al. (1983).

It is obvious that regioisomers of the same oxylipin core structure can have widely different potency. The change of the relative position of the two hydroxy groups (e.g. 9,10-dihydroxy vs. 12,13-dihydroxy) or the epoxy group (e.g. 9,10-epoxy vs. 12,13-epoxy) change the biological activity of the oxylipin as illustrated for the effects on *L. maculans* growth (Table 1). A similar effect was reported for trihydroxy fatty acids in spraying tests (Hou and Forman, 2000) where the position of the hydroxy groups influenced the bioactivity in other pathogen-host systems as well. The lack of inhibitory effect of the (±)-*cis*-12,13-epoxy-9(*Z*)-octadecenoic acid to *L. maculans* growth (Table 1) suggests that the fungus lack an epoxide hydrolase capable of hydrolyzing this epoxide to the corresponding and inhibitory diol

Table 1
Summary of oxylipin dose response tests and single concentration tests assaying growth rate of hyphae of *Brassica* fungal pathogens during 5 days

Oxylipin[a]	Strain[b]			
	Lm	Vl	Ss	Ab
ω-5(*Z*)-Etherolenic acid	++[c]	–[d]	–[d]	+++
(±)-*cis*-12,13-Epoxy-9(*Z*)-octadecenoic acid	–	–	++	++
(±)-*cis*-9,10-Epoxy-12(*Z*)-octadecenoic acid	++	–	–[d]	–[d]
(±)-*threo*-12,13-Dihydroxy-9(*Z*)-octadecenoic acid	+++	–	+	++
(±)-*threo*-9,10-Dihydroxy-12(*Z*)-octadecenoic acid	–[d]	–[d]	+	++
13(*S*)-Hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid	++	–	–[d]	++
13(*S*)-Hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid	–[d]	–[d]	–[d]	–[d]
13(*S*)-Hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid	+++	–[d]	–	+
13(*S*)-Hydroxy-9(*Z*),11(*E*)-octadecadienoic acid	–[d]	–[d]	–[d]	–[d]
9(*S*)-Hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid	–[d]	–[d]	–[d]	–[d]

[a] The different oxylipins (0.06–1 mM) were dissolved in PDA. As controls served PDA without or containing the solvent ($n = 3$).
[b] The strains tested were *Leptosphaeria maculans* (Lm), *Verticillium longisporum* (Vl), *Sclerotinia sclerotiorum* (Ss), *Alternaria brassicae* (Ab).
[c] Effects are presented as; +++, strong inhibition; ++, inhibition; +, weak inhibition; –, no inhibition.
[d] Results from 1 mM screening.

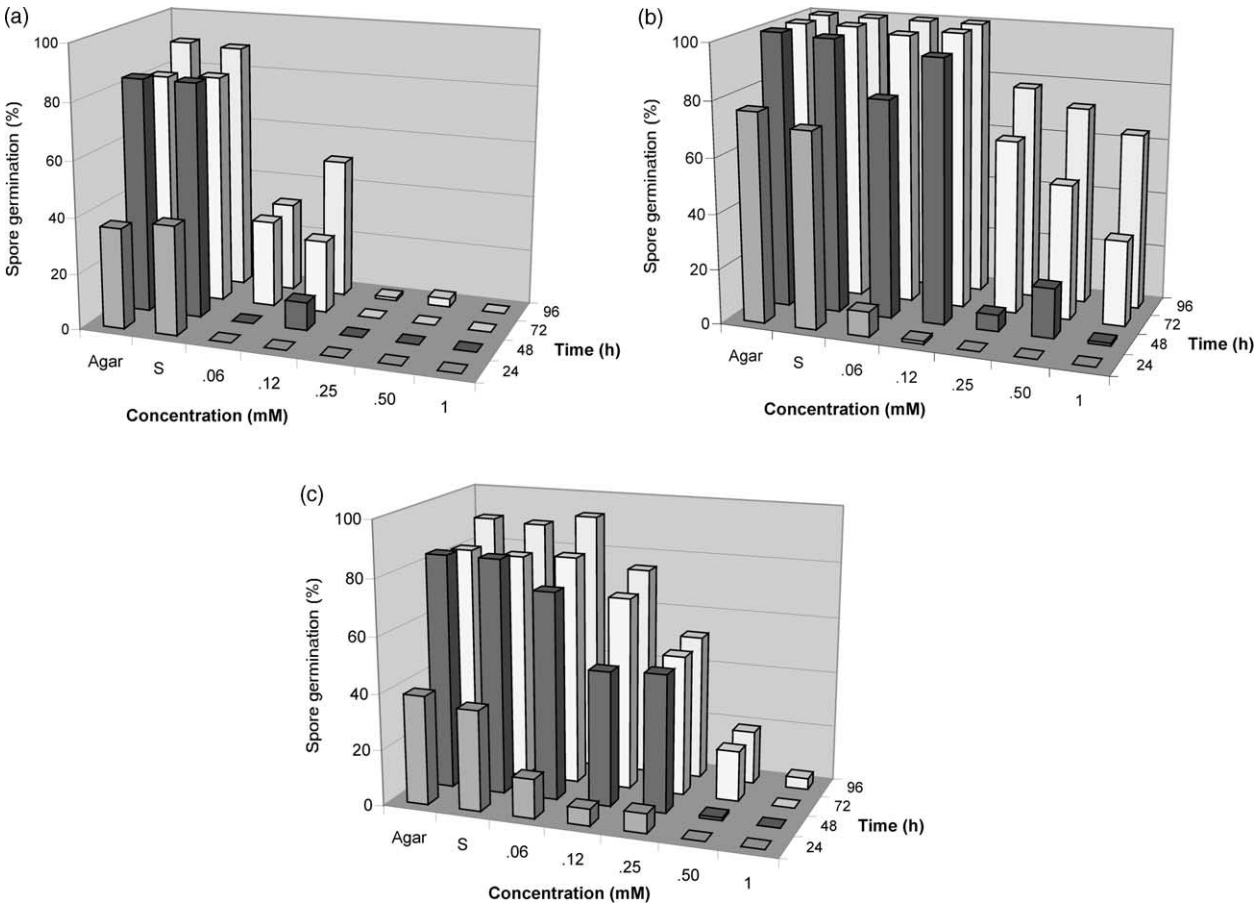

Fig. 2. Percentage germinated *L. maculans* pycnidiospores on water agar containing various oxylipins. *L. maculans* spores were exposed to various concentratios (0.06–1 mM) of ω-5(*Z*)-etherolenic acid (a), (±)-*cis*-12,13-epoxy-9(*Z*)-octadecenoic acid (b) or 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (c) added to water agar and the number of germinated spores measured at different time points (24–96 h). The controls consisted of water agar without any additives (“agar”) and water agar containing the solvent used to dissolve the oxylipin (“S”) ($n = 3$).

Table 2
Spore germination in the presence of oxylipins; spores were plated on water agar and germination ratios were determined using light microscopy after 96 h

Oxylipin[a]	Strain[b]	
	L. maculans	*P. oryzae*
ω-5(*Z*)-Etherolenic acid	+++[b]	–[c]
(±)-*cis*-12,13-Epoxy-9(*Z*)-octadecenoic acid	++	+[c]
(±)-*cis*-9,10-Epoxy-12(*Z*)-octadecenoic acid	++	–[c]
(±)-*threo*-12,13-Dihydroxy-9(*Z*)-octadecenoic acid	+	–[c]
13(*S*)-Hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid	++	+[c]
13(*S*)-Hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid	++	+[c]
13(*S*)-Hydroxy-9(*Z*),11(*E*)-octadecadienoic acid	++[c]	nd

[a] The concentrations of oxylipins tested were 0.06–1 mM if not indicated else. As controls served agar without or containing the solvent ($n=3$).
[b] Effects are presented as; +++, strong inhibition; ++, inhibition; +, weak inhibition; –, no inhibition; nd, not determined.
[c] Results from 1 mM screening.

(Fig. 1b). A lack of epoxide hydrolase activity is also supported by the other epoxide-diol pair where the (±)-*cis*-9,10-epoxy-12(*Z*)-octadecenoic acid was inhibitory to *L. maculans* while the (±)-*threo*-9,10-dihydroxy-12(*Z*)-octadecenoic acid not was inhibitory at all (Table 1).

The stability of oxylipins during the time course of the experiment was also tested. The stability of three oxylipins, i.e. 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid, 13(*S*)-hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid and (±)-*threo*-12,13-dihydroxy-9(*Z*)-octadecenoic acid, in the presence of *L. maculans* or *S. sclerotiorum*, or growth medium alone, was examined by GC–MS. Analysis of samples removed 24 h following addition of 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid to fungi or growth medium revealed the presence of 13(*S*)-hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid as the main compound. 13(*S*)-Hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid is the reduction product formed from 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid in the stannous chloride reduction step (see Experimental section) and accordingly, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid was largely stable in the time interval 0–24 h. Samples removed 48 or 72 h following addition of 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid showed lower levels of 13(*S*)-hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid and instead increasing levels of the epoxy alcohol 12,13-epoxy-11-hydroxy-9,15-octadecadienoic acid and the trihydroxy derivatives 9,12,13-trihydroxy-10,15-octadecadienoic acid and 9,10,13-trihydroxy-11,15-octadecadienoic acid. These compounds are formed nonenzymatically from the hydroperoxide by the homolytic degradation route (Hamberg, 1996). With the samples tested, the rates of hydroperoxide degradation were greater in the presence of fungi than in their absence. Samples removed 24, 48, or 72 h after addition of 13(*S*)-hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid or (±)-*threo*-12,13-dihydroxy-9(*Z*)-octadecenoic acid to fungi or growth media alone contained the oxylipin added with less than 10% degradation being observed.

Recently oilseed rape has become the major oil crop within the EU and one of the major three oilcrops of the world. In spite of this there is an apparent lack of pest resistant varieties and chemical control is necessary in many areas to maintain production at high levels. Plant breeding is time-consuming and expensive so development of resistant varieties should ideally match both present and predicted future problems in different regions. Alternative pest management strategies for *Brassicas* that allow a sustainable agriculture are therefore necessary. Oxylipins have been shown to constitute an important part of the plant's defense system and it has been shown that certain oxygenated fatty acids have antifungal effects in other systems (reviewed in Blée, 1998). Use of oxylipins in agriculture have several advantages. They are naturally occurring, reasonally stable but biodegradable, can be produced in large volumes in natural hosts or through bioengineering in crop plants, and are fairly non-toxic during handling. Although the repertoire of oxylipins present in *Brassicas* not really have been analyzed these plants seem to contain most of the enzymes involved in oxylipin metabolism. Several lipoxygenases, have been purified from *B. napus* (e.g. Terp and Brandt, 2000). Treatment of *Brassica oleracea* plants with jasmonic acid or the microbial phytotoxin coronatine stimulated endogenous synthesis of 12-oxophytodienoic acid indicating the presence of 13-lipoxygenase, allene oxide synthase and allene oxide cyclase (Stelmach et al., 1999). We have recently isolated and characterized an epoxide hydrolase from *B. napus* (Bellevik et al., 2002). Furthermore, leaf homogenates of *B. oleracea* have been shown to promote formation of fatty acid epoxides during cell damage (Thums and Spiteller, 1997). Although there is always an element of arbitrariness in choosing oxylipins for testing in this kind of study, the earlier-cited results show that *Brassicas* have the capacity of forming the hydroperoxy and hydroxy derivatives used as well as the

epoxides and diols. Whether *Brassicas* also can produce the divinyl ether fatty acid, ω-5(*Z*)-etherolenic acid, is not known. Adding high amounts of ω-5(*Z*)-etherolenic acid directly on plants did not give rise to any significant phytotoxic effects (unpublished results), making this a promising candidate to control *L. maculans*, which is an increasing problem in Europe and Australia (Howlett et al., 2001). By screening additional oxylipins for fungicidal properties and generating novel structures it would probably be possible to identify more potent fungicides. Elucidation of the mechanism(s) behind the fungicidic effect by oxylipins will also assist in identification of potent fungicidic structures. Since many oxylipins may not only have fungicidal effecte per se but also act on the host plant and affect e.g. fatty acid metabolism, gene regulation, and pollination (Howe and Schilmiller, 2002; Pichersky and Gershenzon, 2002) they must be carefully evaluated for longterm effects.

3. Experimental

3.1. Biological material

The fungal strains *A. brassicae* 950:31, *L. maculans* (Desm.) Ces. & de Not. (anamorph: *Phoma lingam* (Tode ex Fr.) Desm.) strain 950:14, *S. sclerotiorum* Sc13 and *V. longisporum* Vd11 were supplied by Svalöf Weibull AB (Svalöv, Sweden). Many isolates that cause wilt disease were originally referred to as *V. dahliae* but have recently been renamed to *V. longisporum* (e.g. Steventon et al., 2002) and this is also the case with the Vd11 strain. Fungal strains were grown on PDA in the dark at room temperature and was frequently passed on the host to maintain virulence. The plant used was oilseed rape (*B. napus*) var. Hanna originally from Svalöf-Weibull AB. The *P. oryzae* (*Magnaporthe grisea*) fungus was provided by Dr. Robert Dudler, Institute of Plant Biology, University of Zürich, Switzerland. This fungus was kept on oat-agarose plates in darkness at 28 °C.

3.2. Oxylipins

The oxylipins used in the present study was purchased from Larodan Fine Chemicals AB, Malmö, Sweden. The purity of all compounds was in excess of 97%.

3.3. Fungal toxicity tests

A disc of hyphae (mycelium), 1 mm in diameter, was transferred to PDA containing oxylipins at concentration ranging from 0 to 1 mM. Each well contained 500 μl PDA and the oxylipin dissolved in 10 μl acetonitrile. Plates were incubated in the dark at room temperature for up to 4 days. Growth of the fungus was determined by measurement of the diameter of the colony with a ruler or using a digital camera (Hamamatsu, Täby, Sweden) and the Image Pro v4 software (Media Cybernetics, USA). Since the fungal colony not always is a perfect circle multiple measurements across the colony at different angles was made (or the area estimated) and a value averaged by the image analysis software. Triplicate tests were performed and the overall standard deviation varied between 10 and 15%. PDA only or PDA with 10 μl acetonitrile was used as negative control. For dose effect comparisons the solvent control was set at 100%. Sporulation tests were performed using thin slices of oxylipin-containing water agar plated with spore solution. The number of germinated spores was counted under a light microscope where the minimum number of spores used were >300 in each experiment.

3.4. Test of toxicity to the plant

This was tested by applying 10 μl of the oxylipin dissolved in ethanol to the cotyledon of young *B. napus* plants. Ethanol only was used as negative control and the plants were screened phenotypically.

3.5. Analysis of oxylipins

In order to determine the stability of the oxylipins in the presence of fungi, aliquots were removed from the cultures at different times and extracted with diethyl ether. Extracts were treated with stannous chloride in ethanol in order to reduce hydroperoxides into alcohols (Hamberg, 1971) and thereafter derivatized by treatment with diazomethane followed by trimethylchlorosilane/hexamethyldisilazane in pyridine. The resulting methyl ester/trimethylsilyl ether derivatives were analyzed by GC–MS using a Hewlett-Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph fitted with a 5% phenyl methylsilicone capillary column (length, 12 m; film thickness, 0.33 μm). In most runs the initial column temperature was 120 °C and raised at 10 °C min^{-1} until 240 °C.

Acknowledgements

The pathogens were kindly provided by Svalöf Weibull AB, Sweden or by Dr. Robert Dudler, University of Zürich. This study was supported by Lamms Stiftelse, the Foundation for Strategic Research, and the Swedish Research Council for Agriculture and Forestry.

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