Divinyl Ether Fatty Acid Synthesis in Late Blight–Diseased Potato Leaves

Hans Weber, Aurore Chételat, Daniela Caldelari, and Edward E. Farmer
Laboratory of Plant Biology and Physiology, Biology Building, University of Lausanne–Dorigny, 1015 Lausanne, Switzerland

We conducted a study of the patterns and dynamics of oxidized fatty acid derivatives (oxylipins) in potato leaves infected with the late-blight pathogen Phytophthora infestans. Two 18-carbon divinyl ether fatty acids, colnelic acid and colnelenic acid, accumulated during disease development. To date, there are no reports that such compounds have been detected in higher plants. The divinyl ether fatty acids accumulate more rapidly in potato cultivar Matilda (a cultivar with increased resistance to late blight) than in cultivar Bintje, a susceptible cultivar. Colnelenic acid reached levels of up to \(24 \text{ nmol} (7 \text{ mg})/\text{g fresh weight of tissue} in infected leaves. By contrast, levels of members of the jasmonic acid family did not change significantly during pathogenesis. The divinyl ethers also accumulated during the incompatible interaction of tobacco with tobacco mosaic virus. Colnelic and colnelenic acids were found to be inhibitory to \(P.\) infestans, suggesting a function in plant defense for divinyl ethers, which are unstable compounds rarely encountered in biological systems.

INTRODUCTION

Oxylipins (oxidized fatty acid derivatives) play diverse roles in plant biology as signal molecules for defense gene expression and as antimicrobial compounds (Hamberg and Gardner, 1992; Farmer, 1994). Many oxylipins are generated by the action of lipoxygenases. In plants, these enzymes add molecular oxygen to pentadiene fatty acids, such as linoleic acid and linolenic acid. The resultant products, fatty acid hydroperoxides, are subject to a diverse array of modifications leading to the generation of large numbers of other oxylipins whose functions have not been determined definitively.

In plants, two carbon atoms in polyunsaturated 18-carbon fatty acids such as linoleic acid and linolenic acid are targets for the addition of molecular oxygen: C-13 and C-9. The metabolism of 13-hydroperoxylinolenic acid production has been studied in detail because it is metabolized to a family of potent biological regulators, the jasmonate family. Jasmonates regulate the expression of a number of genes necessary for the defense of plants against insect and microbial pathogens (Creelman and Mullet, 1997; Reymond and Farmer, 1998). A 13-lipoxygenase has been shown to be essential for wound-induced jasmonic acid (JA) synthesis in Arabidopsis (Bell et al., 1995). Another lipoxygenase in tobacco has been shown to be essential for defense of this plant against black shank disease (Rancé et al., 1998), although whether this lipoxygenase contributes to the synthesis of jasmonates or to other oxylipins in vivo is not known.

With the aid of a method for the global analysis and quantitation of oxylipins, we recently discovered in potato and Arabidopsis tissues a new 16-carbon member of the JA family, known as dinor-oxo-phytodienoic acid (dnOPDA) (Weber et al., 1997; Farmer et al., 1998). The biological roles of fatty acid 9-hydroperoxides are far less clear. Although oxylipins derived from fatty acid 9-hydroperoxides have been characterized (Gardner, 1991), there is little data on their biological activities. The global analysis of oxylipins, using the oxylipin signature method (Weber et al., 1997), should allow the identification of new oxylipins derived from the action of both 13- and 9-lipoxygenases. Among these compounds might be new regulators and antimicrobial compounds, including phytoalexins. The oxylipin signature method simply involves extraction of tissues and partial purification of the oxylipin fraction, which is enriched in oxidized fatty acids containing between 12 and 18 carbons. These compounds are separated by gas chromatography (GC) and analyzed by mass spectrometry (MS). By using selective ion monitoring, we can follow changes in the profiles of oxylipins ("oxylipin signatures") during development and in disease. We now have chosen to extend the use of the method to study changes in oxylipin signatures during pathogenesis. Our working hypothesis was that oxylipins previously not known to occur in plants might be discovered and characterized.

We chose to study late blight of potato, one of the world’s most problematic plant diseases (Fry and Goodwin, 1997; Judelson, 1997). The causal agent, Phytophthora infestans, a fungal protist, was a principle cause of the devastating
potato famine in Ireland in the mid-1800s, which led to more than one million deaths (Woodham-Smith, 1962). The disease continues to menace world potato production, leading to losses estimated in billions of dollars (Fry and Goodwin, 1997). Little is known about the role that low molecular mass compounds play in the defense of potato plants against infection with *P. infestans*. Thus, we hope to extend our knowledge of the pathosystem by focusing on oxylipins generated specifically during pathogenesis. An additional important consideration is that potato leaves are known to contain 9-lipoxygenase activity (Royo et al., 1996) and are thus a potentially good source of 9-lipoxygenase-derived oxylipins that have not been well studied. Thus, we studied dynamic changes in oxylipin profiles of late blight–diseased potato leaves, concentrating particularly on 9-lipoxygenase–derived oxylipins.

**RESULTS**

**Divinyl Ether Fatty Acids Accumulate in Diseased Potato and Tobacco Leaves**

To study changes in oxylipin levels in the potato–*P. infestans* pathosystem, we extracted healthy, wounded, and diseased leaf tissue and analyzed methylated extracts by GC-MS (Weber et al., 1997). During the analysis of total ion profiles by selective ion monitoring, we noticed the specific accumulation of two compounds having mass-to-charge ratios (m/z) of 308 and 306 in infected leaves but not in uninfected or wounded leaves (Figure 1). The fragmentation patterns and retention times of the two compounds were identical to the divinyl ethers colneleic acid (CA) and colnenolic acid (CnA). These compounds can be made in vitro from linoleic and linolenic acids by using cell-free extracts from potato tubers (Galliard and Phillips, 1972; Galliard et al., 1973) and, as we reported recently, tomato roots (Caldelari and Farmer, 1998). Both CA and CnA (Figure 2) are unstable and, due to this property, are not readily quantitated by GC-MS. Furthermore, for quantitation of the compounds in plant tissues, the development of an internal standard with stability properties similar to those of CA and CnA was essential.

A novel 20-carbon divinyl ether, dorignic acid (DA; Figure 2), was synthesized as an internal standard. Taking advantage of the characteristic UV absorption of the divinyl ethers (Galliard et al., 1973), we devised a reversed-phase HPLC method allowing the facile separation and quantitation of the compounds (Figure 3). For standards, CA and CnA were synthesized in vitro according to an established procedure (Galliard and Phillips, 1972).

We analyzed the accumulation of CA and CnA in two different potato cultivars: Bintje, which is highly susceptible to *P. infestans*, and Matilda, a more resistant cultivar (Strömberg, 1995). Divinyl ether fatty acids were undetectable in healthy leaves of both cultivars throughout the period of study but reproducibly appeared at high levels during late-blight disease. The accumulation of these compounds during disease progression is shown in Figure 4. In infected leaves of both cultivars, no divinyl ether fatty acids were detected 24 hr after infection with *P. infestans* zoospores. On day 2, however, a large difference between the two potato cultivars was apparent. In cultivar Bintje, low levels of CA (0.12 ± 0.03 nmol g⁻¹ fresh weight of tissue) and CnA (0.13 ± 0.05 nmol g⁻¹ fresh weight) accumulated. At the same time point for cultivar Matilda, levels of CA were nearly ninefold higher (1.02 ± 0.26 nmol g⁻¹ fresh weight), and levels of CnA were 14.5-fold higher (1.89 ± 0.67 nmol g⁻¹ fresh weight) than in cultivar Bintje. On day 3, high levels of CA and CnA were detectable in the leaves of both cultivars; however, by day 4, the leaves of cultivar Bintje were too badly damaged to be extracted. On day 4 for cultivar Matilda, CA had reached levels of 15.65 ± 3.74 nmol g⁻¹ fresh weight of tissue, and CnA levels were at 23.63 ± 8.22 nmol g⁻¹ fresh weight.

CA and CnA were not found in *P. infestans* mycelia analyzed by using the sensitive HPLC method that we describe herein for the analysis of these compounds from plant leaves of both cultivars.
Divinyl Ether Fatty Acids in Plants

leaves. The finding of divinyl ether fatty acids in infected potato leaves led us to ask whether the production of these compounds during disease was unique to this plant. Tobacco plants (cv Xanthi) were infected with tobacco mosaic virus (TMV) or mock inoculated (Weber et al., 1993). After 4 days, hypersensitive lesions were clearly visible on the infected leaves but not on the control plants. The leaves were extracted and analyzed for the presence of the divinyl ethers CA and CnA. No divinyl ethers were detected in the mock-inoculated leaves. However, both compounds were detected in infected leaves in which CA levels were 1.66 ± 0.15 nmol g⁻¹ fresh weight of tissue and CnA levels were 2.28 ± 0.28 nmol g⁻¹ fresh weight (data not shown).

Divinyl Ether Fatty Acids Inhibit the Growth of P. infestans

Given that CA and CnA accumulate in diseased leaves, we speculated that these compounds might contribute to the defense of potato against P. infestans. The effect of the compounds on the mycelial growth of P. infestans was estimated by adding the oxylipins to P. infestans mycelia grown on agar. Increased mycelial growth was monitored by video image analysis. CnA proved to be inhibitory to the late-blight fungus (Figure 5A), with a concentration of 150 µM reducing mycelial growth by 50%. CA was less effective than was CnA in this assay (data not shown). An alternative toxicity assay (Ricker and Bostock, 1994) based on the ability of compounds to inhibit the germination of P. infestans zoospores was employed. Both CA and CnA proved to be inhibitory to P. infestans in this assay (Figure 5B). CnA was more toxic than was CA, with a concentration of 30 µM CnA reducing zoospore germination from a control level of 83.3 to 3.8%. CA, at a concentration of 30 µM, reduced zoospore germination to 21.3%. The toxicity of the two compounds is additive (data not shown).

Levels of JA, OPDA, and dnOPDA Are Unchanged 3 Days after Infection

The dramatic changes in the levels of divinyl ether fatty acids in late blight-infected tissues were compared with those

![Figure 2. Structures of CA and CnA as Well as the Novel Internal Standard DA.](image)

![Figure 3. HPLC Quantitation of Divinyl Ethers in Late-Blight Diseased Potato Leaves.](image)
of members of the JA family by using a previously developed assay (Weber et al., 1997). Levels of three jasmonate family members, OPDA, dnOPDA, and JA, were simultaneously estimated 3 days after infection of the potato cultivar Matilda with suspension of zoospores ($1 \times 10^5$ zoospores per mL; Table 1). No significant differences in jasmonate levels relative to the noninfected plants were recorded at this time point. JA, the dominant jasmonate family member in unwounded potato leaves, was present at levels of $\sim 50$ pmol g$^{-1}$ fresh weight of tissue. Measurements at earlier disease stages yielded similar results (data not shown), but we cannot rule out the possibility that transient spikes of jasmonate family members appear at earlier time points during the infection process.

**DISCUSSION**

**Oxylipin Signatures Change during Pathogenesis**

Dramatic changes in the levels of several oxylipins occurred during the infection of potato leaves with *P. infestans*, resulting in a unique and characteristic oxylipin signature in infected leaves (Figure 1). Two compounds, CA and CnA, which were undetectable in healthy leaves of potato and tobacco, were detected when plants were infected. In the case of potato infected with the late-blight fungus (*P. infestans*), the levels of these two divinyl ether fatty acids increased continually during infection, reaching combined levels of $\sim 30$ nmol g$^{-1}$ fresh weight. Levels of isomers of the signal molecule JA 3 days after infecting potato leaves were $\sim 50$ pmol g$^{-1}$ fresh weight, which is far lower than are levels of divinyl ether fatty acids that, in the case of CnA, were nearly 400-fold higher at this time point. In comparison, JA levels rose up to $\sim 714$ pmol g$^{-1}$ fresh weight in wounded potato leaves (Weber et al., 1997), which is still $\sim 33$-fold lower than are the maximum CnA levels that we detected in infected plants. At least in potato leaves infected with the late-blight fungus, CA and CnA are quantitatively important compounds.

It was interesting to note that 3 days after infection of potato leaves with late-blight fungus, no significant changes in the levels of JA, OPDA, and dnOPDA were detected. The 3-day time point, chosen for comparison with divinyl ether levels in potato leaves (Figure 4), corresponded to visual symptom development. Several pathogens are known to cause changes in the levels of JA, one of the best-studied cases being that of the fungus Alternaria brassicicola on Arabidopsis (Penninckx et al., 1996). JA production is known to be essential for the defense of Arabidopsis against the fungus Pythium mastophorum (Vijayan et al., 1998). Our results do not rule out a role of jasmonates in the potato–late-blight pathosystem, but they clearly show that dramatic changes in the pool of one type of oxylipin derived from 9-lipoxygenase(s) can be independent from that of JA derived from

![Figure 4. Accumulation of Divinyl Ether Fatty Acids during the Infection of Potato Leaves with *P. infestans* Zoospores.](image-url)
13-lipoxygenase activity. Our data indicate that pathogens and wounding (Weber et al., 1997) cause different effects on the oxylipin signature in potato leaves. The results draw attention to the fact that 9-lipoxygenases may play roles in the defense of potato against P. infestans.

**Divinyl Ether Fatty Acid Production in Higher Plants**

We report our novel discovery of the occurrence of the divinyl ether fatty acids CA and CnA in two species of angiosperms and describe their relationship to plant disease. Whereas the in vitro synthesis of these compounds in potato, garlic, and tomato extracts has been reported (Galliard and Phillips, 1972; Galliard et al., 1973; Grechkin et al., 1997; Caldelari and Farmer, 1998), divinyl ether fatty acids appear to be rare in nature. However, compounds of this type have been observed in some marine algae (Gerwick, 1996).

We have shown that both potato and tobacco plants synthesize CA and CnA in response to pathogen attack. These results indicate that divinyl ether production is not confined to a single species of plant. Whether divinyl ether fatty acids are more common in nature remains to be seen, but it is likely because enzymes catalyzing the in vitro formation of divinyl ethers structurally related to CA and CnA have been found in extracts from garlic (Grechkin and Hamberg, 1996). Because garlic is a monocotyledonous plant and potato and tobacco are dicotyledonous plants, the ability to synthesize divinyl ether fatty acids may be found in a variety of other plants. Finally, because the genome of TMV does not encode enzymes of fatty acid metabolism, these data confirm that it is the plant that produces these substances. This is highly consistent with the literature in which cell-free extracts of uninfected tubers and roots have been shown to catalyze both CA and CnA production (Galliard and Phillips, 1972; Galliard et al., 1973; Caldelari and Farmer, 1998).

**Potential Roles of Divinyl Ethers**

The new finding of divinyl ether fatty acid accumulation in two plant species during pathogenesis led us to ask what biological role these oxylipins play. The role of these compounds as phytoalexins (which are low molecular mass antimicrobial compounds produced by plants) is possible because, at micromolar concentrations, they are inhibitory to P. infestans (Figure 5). A role of CA and CnA as phytoalexins would be consistent with the data in Figure 4. At the 48-hr time point, levels of CA and CnA in the more resistant potato cultivar Matilda were approximately ninefold higher than in the more susceptible cultivar Bintje. The ability to accumulate divinyl ethers during infection with late blight thus correlates with the higher resistance of cultivar Matilda with respect to cultivar Bintje. The high levels of divinyl ether production in infected potato leaves (Figure 4) would be consistent with a

![Figure 5](image-url).

**Figure 5.** Effect of CA and CnA on P. infestans Mycelial Growth and Cytospore Germination.

**(A)** Inhibition of the growth of P. infestans mycelia by CnA. A suspension of CnA in water was added to growing P. infestans hyphae, and the effect on growth was measured by quantitative analysis of hyphal length. As controls, the 18-carbon fatty acid linoleic acid (18:2) or water alone (Con.) was added. Values represent the mean ±SE of 30 hyphal measurements.

**(B)** Inhibition of germination of P. infestans cytospores in the presence of CA and CnA. Suspensions of both compounds in water were inhibitory, whereas suspensions of linoleic acid were not. Values represent the mean ±SE of four experiments.
role as antimicrobial compounds. Indeed, if the production of the compounds is localized to regions near infection sites, their in vivo concentrations could reach high micromolar levels.

CA and CnA, like other divinyl ethers, are unstable compounds, and their effects on mycelial growth of P. infestans in vitro proved to be difficult to assess on solid media. These compounds may react with substances present in many growth media. CnA was inhibitory to mycelial growth in a simple agar medium (Figure 5A). Cytospore germination in vitro was far more sensitive to inhibition by the divinyl ether fatty acids (Figure 5B). In vitro, CA and CnA degrade to carbonyl fragments, one of which is probably 9-oxo-nonanoic acid (Galliard et al., 1974; Caledelari and Farmer, 1998). It is possible that the divinyl ether fatty acids are phytoalexins that could diffuse into and degrade within P. infestans, yielding cytotoxic carbonyl fragments. Phytoalexins have not been found reproducibly in potato leaves, which in the case of P. infestans is the major infection site. Sesquiterpenoid phytoalexins, however, were found in tubers during infection with late blight (Rohwer et al., 1987) or with the soft rot bacterium Erwinia carotovora (Abenthum et al., 1995).

CA and CnA accumulate fairly late in pathogenesis, being first detected 2 days after infection in potato cultivar Matilda (Figure 4). Thus, we term these compounds “late-phase” oxylipins to distinguish them from other oxylipins that accumulate rapidly after a stimulus (e.g., JA, which accumulates rapidly after wounding plant leaves; Farmer, 1994). The late accumulation of CA and CnA correlates with visual symptoms development in potato and tobacco leaves. Thus, the production of divinyl ether fatty acids in these systems may be related to or correlated with cell death. More research on this interesting aspect is in progress, and the possibility that cell death–specific oxylipins exist has been raised (Caledelari and Farmer, 1998). If CA and CnA are such compounds, they would represent a new class of oxylipin in plants. Finally, the possibility that CA and/or CnA are natural regulators of plant defense gene expression remains to be tested. Clearly, more research on the consequences of divinyl ether production in plant pathosystems is necessary.

The first step of CA and CnA synthesis, that is, introduction of oxygen into the C-9 position of linoleic acid and linolenic acid, is catalyzed by 9-lipoxygenase (Galliard et al., 1974). Our results thus suggest a role for 9-lipoxygenases in the defense response of late-blight infected potato leaves. The formation of biologically active molecules from fatty acid 9-hydroperoxides in plants has not received great attention, although our results suggest a biological role for such compounds in plant defense (Caledelari and Farmer, 1998). In tobacco, a lipoxygenase with predominant regiospecificity for the C-9 position of unsaturated 18-carbon fatty acids recently was shown to be essential for the defense against black shank disease caused by P. parasitica var nicotianae (Rancé et al., 1998). In this case, the molecular basis of lipoxygenase action has yet to be established.

It is known that fatty acid hydroperoxides generated by lipoxygenase action are metabolized to divinyl ethers by the enzyme divinyl ether synthase (DES; Grechkin and Hamberg, 1996). Our results draw attention to the potential importance of the activity of DES in the defense of potato against late blight. DES has yet to be purified and characterized, and no genes known to encode this enzyme are presently available. Our data suggest that DES is likely to be active in infected potato leaves and that the degree of activation of this enzyme at early stages of late-blight disease may depend on the potato cultivar tested. To facilitate further study of DES, it will be worthwhile to isolate the gene(s) encoding this enzyme.

### METHODS

#### Plant Growth

Plants (Solanum tuberosum cvs Matilda and Bintje) were grown from tubers under greenhouse conditions. Three-week-old plants were used for wounding and infection experiments. The leaflets of fully expanded leaves were wounded several times across the lamina and midvein by using forceps such that the apical 30% of each leaflet was wounded. Plants were then kept under light for 4 hr. For infection experiments, plants were transferred 24 hr before inoculation into infection chambers under 16 hr of light at 17°C. Four hours before infection, the relative humidity was set at 100%. Fully expanded leaves were inoculated on the adaxial surfaces of each leaflet with 30 10-μL droplets of zoospore suspension (1 × 10^7 zoospores per mL) per leaf and kept for 1 day at 100% relative humidity and for another 3 days at 50% relative humidity. As controls, leaves were inoculated with 10-μL droplets of water. Phytophthora infestans isolate 94-28 (a gift of P. Malnöe, Federal Research Station, Changins, Switzerland) was propagated for at least two cycles on rye A medium plates, as described by Caten and Jinks (1968), at 17°C before inoculating plants.

For the production of zoospores, mycelium grown for 4 weeks on plates was overlayed with cold water and incubated at 4°C. After 4 hr, the zoospore suspension was recovered, and the titer was adjusted to 1 × 10^8 per mL with cold, distilled water. For infection of tobacco with tobacco mosaic virus (TMV), plants (Nicotiana tabacum

---

### Table 1. Levels of Jasmonate Family Members in Potato Leaves (cv Matilda) 3 Days after Infection with P. infestans

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>dnOPDA</td>
<td>2.54 ± 3.37^a</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>OPDA</td>
<td>21.99 ± 3.56</td>
<td>23.05 ± 1.27</td>
</tr>
<tr>
<td>JA</td>
<td>58.52 ± 11.76</td>
<td>49.57 ± 30.10</td>
</tr>
</tbody>
</table>

^aValues are in picomoles per gram fresh weight ± SE (n = 3). The detection limit for fatty acids was ~7 pmol g⁻¹ fresh weight for OPDA and dnOPDA and 4 pmol g⁻¹ fresh weight for JA. Values for JA are combined for both the 3R,7R and 3R,7S isomers. Similar results were obtained for the cultivar Bintje.

^bValue at the limit of detection.
cv Xanthi) were grown under greenhouse conditions for 7 weeks. Plants were transferred to a culture room (25°C during the day and 17°C during the night, with 16 hr of light), and fully expanded leaves were inoculated with TMV strain U1, as described by Weber et al. (1993).

**Extraction and Gas Chromatography–Mass Spectrometry Analysis of Oxylipins**

Extraction of all oxylipins and the analysis and quantification of jasmonates were as described by Weber et al. (1997).

**Internal Standard Synthesis**

A novel internal standard dorigenic acid (DA; 1’E, 8Z, 11Z, 13E-14(1’-hex-1-en-1-yl)octadeca-8,11,13-trienoic acid) was synthesized from dihomo-γ-linolenic acid (Cayman Chemical Co., Ann Arbor, MI). Dihomo-γ-linolenic acid (3.5 mg) was converted to its corresponding 15-hydroperoxide with the 13-lipoxygenase from soybean (Sigma). For production of the divinyl ether fatty acid, the 15-hydroperoxide of dihomo-γ-linolenic acid was incubated with a microsome preparation from 20 g of peeled garlic bulbs in 60 mL of 0.1 M sodium borate buffer, pH 9.0, for 30 min at room temperature, as described by Grechkin et al. (1997). The solution was acidified with 1 M citric acid (2 mL), and the reaction product was extracted with diethyl ether. Purification of the divinyl ether was by reversed-phase HPLC on a Whatman (Maidstone, Kent, UK) Partisil 10 ODS-3 Magnum 9 column (9.4 × 250 mm) under previously described conditions (De Vries et al., 1997), except that the concentration of trifluoroacetic acid used in the mobile phase was 0.01% (v/v). The column eluate was monitored at 252 nm, and the divinyl ether fatty acid eluted at 14.6 min. The compound had an absorbance maximum at 254 nm.

Electron ionization mass spectrometry (70 eV) of the methyl ester yielded a parent ion (M+) with a mass-to-charge ratio (m/z) of 334. (The intensity of this peak was set to 100%). Other ions had the following characteristics: M–C4H9O, 235 (13%); M–C6H5OH, 234 (4%); 235–CH3OH, 203 (27%); and 203–H2O, 185 (63%). 1H–nuclear magnetic resonance was performed as described previously (Weber et al., 1997) but in the solvent CD3CN. The following chemical shifts (ppm) were observed, where H represents the respective proton: H7, 2.08; H8, 5.39; H9, 5.35; H10, 2.85; H11, 5.22; H12, 5.88; H13, 6.02; H14, 6.65; H14', 6.38; H2', 5.16; H3', 1.95; H4', 1.33; and H5', 0.90. Coupling constants (J, given in Hz) for the pairs of protons indicated in subscript are as follows: J8,9 10.3; J11,12 10.7; J14,13 11.9; and J1,2 12.2.

**HPLC of Divinyl Ethers**

For analysis and quantitation of colnolenic acid (CnA) and colnoleic acid (CA), leaf material was harvested, weighed, and immediately frozen in liquid N2. Frozen leaf material (1 g) was ground in a mortar to a fine powder and added to ice-cold (5 mL) methanol containing 500 ng of the internal standard. The solution was immediately homogenized with a Polytron (Kinematica, Lucerne, Switzerland) for 30 sec on ice. Extraction of the divinyl ether fatty acids was continued by rotating the samples for 30 min at 4°C. After centrifugation, the tissue was reextracted for another 60 min and centrifuged. The supernatants were combined, and ice-cold water (4.3 mL) and 1 M ammonium hydroxide (30 μL) were added. The sample was passed through a 500-mg Bakerbond octadecyl glass column (Baker, Deventer, The Netherlands), which had been prewashed with methanol-water (70:30 [v/v]). The column was washed with 7 mL of methanol-water (75:25, [v/v]), and both the eluates were combined and concentrated to <1 mL under reduced pressure at 40°C in a rotary evaporator. The samples were adjusted to 5 mL with water, acidified with 10% formic acid (120 μL), and extracted twice with 5 mL of chloroform. The organic phase was dried over anhydrous MgSO4, and the solvent was evaporated under a stream of N2.

CnA and CA concentrations in the samples were analyzed by reversed-phase HPLC on a C-18 silica, 15% C, endcapped Chromsphere Inertsil 5 ODS 3 (250 × 3 mm) (Chrompack, Middelburg, The Netherlands). The flow rate was 1 mL per min, and the mobile phase consisted of a gradient formed from solvent A (H2O–trifluoroacetic acid, 100:0.01 [v/v]) and solvent B (acetonitrile–trifluoroacetic acid, 100:0.01 [v/v]), which is as follows: 60% solvent B from 0 to 5 min, 60% solvent B from 5 to 25 min, 72% solvent B from 25 to 30 min, 72 to 100% solvent B from 30 to 32 min, and 100% solvent B from 32 to 37 min. The column eluate was monitored at 252 nm. At this wavelength, the extinction coefficients for the free acids of these compounds were found to be within ±7% of the published values for their methyl esters (Galliard et al., 1973). Elution times were 19.3 min for CnA, 25.7 min for CA, and 28.7 min for the internal standard DA. We used the internal standard DA for quantitation of CA and CnA. Recoveries of divinyl ether fatty acids were as follows: 64% for CnA, 56.4% for CA, and 53% for the internal standard. Reproducibility of recovery was (coefficients of variation; n = 4) 16.3% for CnA, 16.9% for CA, and 15.0% for the internal standard. The detection limit for both CA and CnA was ~44 pmol g−1 fresh weight.

**Synthesis and Bioassay of CA and CnA**

Synthesis of CnA and CA was performed by incubation of a potato tuber extract (cv Désirée with linolenic or linoleic acid, respectively, according to Galliard and Phillips (1972). Peeled potato tubers (46 g) were homogenized in 88 mL of ice-cold 0.1 M sodium phosphate buffer, pH 7.5. The extract was centrifuged at 500g for 2 min at 4°C, and 8 mg of the ammonium salt of linolenic or linoleic acid was added to the supernatant. After a 15-min incubation at room temperature, the solution was acidified with 1 M citric acid (6.6 mL) and extracted twice with 1 volume of diethyl ether. The organic phase was dried over anhydrous MgSO4 and concentrated under a stream of N2.

The extract was then applied to a 20 × 20-cm silica gel 60 thin-layer chromatography plate (Merck). The plate was developed three times to the top in petroleum ether-diethyl ether-formic acid (70:30:1 [v/v/v]). The divinyl ether fatty acid was scraped from the plate and eluted with diethyl ether. After evaporation of the solvent under a stream of N2, the divinyl ether fatty acid was purified further by preparative HPLC, as described for the purification of the internal standard. Elution times were 11.8 min for CnA and 14.0 min for CA. To test the effect of divinyl ether fatty acids on the mycelial growth, we transferred 100 zoospores of isolate RDA-49 (Sandoz AG, Straw Collection, Witterswil, Switzerland) in 25 μL water to one well of a 96-well microtiter plate containing 100 μL of 1.5% (w/v) agar and 0.5% (w/v) sucrose.
Spores were allowed to germinate for 4 hr, and the fatty acids were added as aqueous suspensions in 25 µL water. The length of the hyphae was measured after 18 hr of incubation at 17°C in the dark by using a video camera and the image analysis software Object-Image (Norbert Vischer, University of Amsterdam, The Netherlands). The toxicity of CA and CnA to P. infestans cytophores was investigated using a standard assay (Ricker and Bostock, 1994). The spore titer was adjusted to 50,000 per mL with distilled water. Fifty microliters of the spore suspension was transferred to one well of a 96-well microtiter plate and mixed with 50 µL of 2% (w/v) glucose in water containing the fatty acids or oxylipins. (Fatty acid and oxylipin suspensions were obtained by brief sonication.) The plate was incubated for 3 hr in the dark at room temperature to allow cytophere germination. One-hundred cytophores per well were observed. Cytophores with germ tubes as long or longer than the spore were counted as being germinated.

ACKNOWLEDGMENTS

NMR analysis of dorignic acid was generously and expertly conducted by J.rice Huwe (U.S. Department of Agriculture, Agricultural Research Service, Fargo, ND). We thank Pascal Dumy for help in assigning chemical shifts. Bertrand Ménard helped with image analysis, and Boris Künstner provided expert care of plants. We thank the following members of the Federal Agricultural Research Station, Changins, Switzerland: Werner Rüst for virus-free potato tubers and Pia Malmöe and Laurent Zimmerli for Phytophthora isolates. Pierre Vogel helped with the International Union of Pure and Applied Chemistry name for dorignic acid. This work was supported by the Etat de Vaud and by the Swiss National Science Foundation (Grant No. 31-36472-92).

Received October 5, 1998; accepted December 31, 1998.

REFERENCES


(Solanum tuberosum) to zoospores or elicitors from Phytophthora infestans. Planta **170**, 556–561.


