# Metabolism of Lipids Containing Arachidonic and Eicosapentaenoic Acids in Race-Specific Interactions Between *Phytophthora infestans* and Potato

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## ABSTRACT

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Lipids containing the radioisotope-labeled elicitors arachidonic (AA) and eicosapentaenoic (EPA) acids were extracted from *Phytophthora infestans* and applied to potato disks (cv. Kennebec), which were subsequently inoculated with spores of an incompatible (race 0) or compatible (race 1,4) isolate of *P. infestans*. Lipids were extracted from the disks at various times after inoculation and analyzed. Inoculation with either race resulted in a significant decline in the proportion of radioactivity recovered in triglycerides within 24 hr after inoculation and concomitant increases in polar lipid and free fatty acid pools. Between 6 and 48 hr after inoculation, the pattern was similar in both interactions. From 24 to 72 hr after inoculation, acyl esterase, phospholipase, and lipase activities were similarly affected in both interactions. However, between 6 and 12 hr after inoculation, phospholipase and lipase activities in the incompatible interaction exceeded the activities in disks inoculated with the compatible race. Lipoxygenase activity was similar in all treatments and declined slightly during this same period. These results indicate that inoculation with *P. infestans* stimulates degradation of lipids containing AA and EPA, and during the initial stages of infection the activities of lipolytic enzymes capable of releasing these elicitors are slightly higher in the incompatible interaction than in the compatible interaction.

*Additional keywords:* hypersensitive response, lipase, lipolytic acyl hydrolase, lipoxygenase.

Studies of the release of elicitors from microbial tissues and their subsequent metabolism by plants may provide clues about the nature of the factors necessary to induce plant defense responses. Although some research has centered on release of oligosaccharides from fungal cell walls (2,15), the release of biologically active lipids has received relatively little attention. Lipid-degrading enzymes such as acyl hydrolases and lipoxygenase may degrade fungal lipids in membranes and lipid bodies, thereby generating products that are cytotoxic or that have specific effects on host and pathogen metabolism. The eicosapolyenoic fatty acids of *Phytophthora infestans* (Mont.) de Bary, principally arachidonic (AA) and eicosapentaenoic (EPA) acids, occur primarily as esters (10) and are specific elicitors of the induced metabolism occurring in potato (*Solanum tuberosum* L.) tissue during hypersensitivity expression (4,5,7). The ability of these compounds to elicit the accumulation of sesquiterpenoid phytoalexins, stimulate key enzymes in terpenoid and phenylpropanoid metabolism (13,28), and elicit a number of other responses characteristic of the hypersensitive response has been documented (7).

Structure-activity studies indicate that free eicosapolyenoic acids with unsaturation at position 5 in the hydrocarbon chain are most active in the induction of the hypersensitive response (4,5,22). Potato lipoxygenase, which forms fatty acid hydroperoxides from free fatty acids (1) and converts AA to 5-hydroperoxy-eicosatetraenoic acid and leukotriene A4 as principal metabolites (27), has been implicated as a receptor for these elicitors (23). The release of eicosapolyenoic acids from fungal lipid during ingress by an incompatible race of *P. infestans* could facilitate induction of the host response by presenting the most active forms of these elicitors to the plant cell (4,22) and also result in the formation of fungitoxic eicosanoids during infection (8).

Previous studies with AA and EPA have primarily investigated the host responses to the pure compounds or to killed tissues of *P. infestans* containing them (5,7,11,22–24). The present study was undertaken to see if direct evidence could be obtained in support of the hypothesis that levels of free elicitor fatty acids increase after inoculation of potato disks with *P. infestans*. Two approaches were taken. The first examined metabolism of exogenously supplied fungal lipids containing esterified $^{14}$C-EPA and $^{14}$C-AA after infection by incompatible or compatible races. The second compared changes after inoculation in lipolytic activities that are capable of releasing esterified AA and EPA. Portions of this work have been reported (3,6).

## MATERIALS AND METHODS

**General.** The methods for growth and maintenance of cultures of *P. infestans*, and the preparation, inoculation, and incubation of potato tuber disks were those described previously (4,5,7). Tubers of *Solanum tuberosum* L. 'Kennebec' were used throughout this study and were of certified seed quality. This cultivar is incompatible with race 0 and compatible with races 1,4 and 1,2,3,4. All experiments with inoculated plant tissue were conducted at 20 C without supplemental lighting. Protein content in potato extracts was estimated by the Bio-Rad dye-binding method following the manufacturers directions, with $\gamma$-globulin as the standard. All experiments were performed at least twice and in some cases three or more times.

**Labeling of fungal lipids.** Mycelial cultures of *P. infestans* grown in a liquid synthetic medium at 20 C (16) were used as a source of lipids. Cultures were started by seeding the flasks with one or two mycelial plugs from a culture growing on amended lima bean agar. For large-scale preparation of $^{14}$C-labeled lipids, $^{14}$C-acetate, sodium salt (New England Nuclear, 56.2 mCi mmol$^{-1}$) was added to early log-phase cultures (6 days old) at a rate of 5–8 $\mu$Ci per 100 ml of synthetic medium. Cultures were further incubated for 3 days at 20 C before extraction of lipids.

**Lipid extraction and analyses.** Lipids were extracted from mycelium with chloroform:methanol (CHCl:MeOH; 2:1, v/v), and nonlipid contaminants were removed with 0.75% KCl (4,10). Extracts were concentrated by rotary evaporation, and the neutral lipids were separated from the polar lipids by thin-layer chromatography (TLC) on 250 $\mu$m Silica Gel G plates (Analtech, Inc., Newark, DE) developed in hexane:diethyl ether:acetic acid

90:30:5, v/v/v). Bands corresponding to the individual lipid classes were scraped and radioactivity determined by liquid scintillation counting (LSC). Because of their similar mobilities in this solvent system, in certain experiments monoglycerides were pooled with the polar lipid fraction. In some experiments, polar lipids were rechromatographed on silica gel GHL TLC plates (Analtech) developed in chloroform:methanol:acetic acid:water (65:35:9:3, v/v). Bands corresponding to individual phospholipids were located by comparison with authentic standards (Sigma Chemical Co., St. Louis, MO) visualized with a phospholipid spray containing molybdenum blue (Sigma). Ceramide aminoethylphosphonate was purified from mycelium of *P. infestans* as described previously (10). Radioactivity was also determined by LSC.

Fatty acid methyl esters were prepared from the lipid extracts by methanolysis with 1 N methanolic-HCl (4,5). Individual fatty acid methyl esters were separated on Whatman KC-18 TLC plates (Whatman Labsales Inc., Hillsboro, OR) developed in acetonitrile:tetrahydrofuran:acetic acid (80:15:5, v/v/v). Bands corresponding to individual fatty acid methyl esters were located by autoradiography and by comparison with authentic standards (Sigma) visualized with iodine vapor. Radioactivity was determined as above.

**Treatment and inoculation of potato disks.** Fungal lipids containing radioisotope-labeled EPA and AA derived from $^{14}$C-acetate were applied at concentrations lower than that necessary to induce the hypersensitive response (4,5,11). These concentrations were determined by estimating the amount of AA and EPA in the lipids based on previous analyses of similar lipid preparations and were confirmed by the absence of hypersensitive response symptoms in the potato disks after treatment with the preparation and by the lack of any inhibition of the compatible race when disks were pretreated with the lipids before inoculation. The specific activity of the fungal lipid preparation was determined by LSC of a known mass to be approximately $1.37 \times 10^5$ dpm mg$^{-1}$. Approximately 6,700–7,800 dpm of the fungal lipids, suspended in water by sonication, were applied to the upper surface of each 2-cm-diameter tuber disk. One to 2 hr later, 50 $\mu$l of a sporangial suspension (0.5–1 $\times$ 10$^6$/ml) was applied to each disk. Disks treated with lipid but not inoculated served as controls. At various times after inoculation, the top layers (approximately 1 mm) from four disks were removed with a razor blade, combined, and the lipids immediately extracted from the tissues with a glass homogenizer containing 10 ml of chloroform:methanol:butylated hydroxytoluene (0.005%). Nonlipid contaminants were removed with 0.75% KCl. The extracts were concentrated under N$_2$ and analyzed by TLC, autoradiography, and LSC. In a preliminary experiment, 80% of the radioactivity recovered in lipid extracts of successive millimeters of the potato disks was present in the top layer. Regression analysis of the data was performed by using a SAS general linear models procedure (26).

An experiment was performed to determine if germinating cystospores in the absence of potato tissue could cause significant degradation of the lipid preparation. Cystospores of race 0 were incubated for 24 hr at 20 C in sterile distilled water with aliquots of the lipid preparation in the same proportions used for the tuber inoculation studies. To each tube, 50 $\mu$l of glacial acetic acid was added, and the lipids were extracted from the mixture with hexane:isopropanol (3:2, v/v) and partitioned against 6.7% Na$_2$SO$_4$ (18). The lipids were then analyzed as above. The lipid preparation without cystospores and the spore-lipid mixtures extracted at the beginning of the incubation period served as controls.

**Evaluation of lipolytic enzyme activities.** Disks were aged at 20 C for 2–3 hr, and then each disk was inoculated with 100 $\mu$l of a suspension containing 0.25–1 $\times$ 10$^6$ zoospores per milliliter (inoculum levels for each race were the same within each experiment). In one set of experiments, where samples were taken at 0, 12, 24, 48, and 72 hr after inoculation, lipolytic activities were evaluated in the top millimeter of potato disks extracted in a buffer containing 0.3 M sucrose, 0.1 M tricine-NaOH, pH

7, and 5 mM 2-mercaptoethanol (18). The tissues (approximately 0.5 g) were homogenized in 10 ml of the extraction buffer with a Ten Broeck glass homogenizer. The extracts were filtered with cheesecloth and centrifuged at 10,000 g for 20 min. All procedures were conducted at 4 C.

To determine more accurately phospholipase and lipase activities, another series of experiments were performed with substrates that better approximate true physiological substrates for these enzymes, and more time points within the first 24 hr after inoculation were examined. Two of these experiments used race 1,4 as the compatible race, and the other two used race 1,2,3,4 as the compatible race. Lipolytic activities were compared at 0, 3, 6, 9, 12, and 24 hr after inoculation. The upper layer of each disk was excised, immediately frozen in liquid N$_2$, and stored at −80 C until extraction. These tissues were homogenized in 3 ml of extraction buffer with the glass homogenizer, and then centrifuged. Acyl hydrolase activity in the supernatant was determined according to the methods of Galliard (14), with $\rho$-nitrophenyl palmitate (Sigma) as substrate at pH 8. Lipase (E.C. 3.1.1.3) and phospholipase B (E.C. 3.1.1.5) activities were determined with trilinolein (1,2,3-tri-[(cis,cis)-9,12-octadecadienoyl]-rac-glycerol; Sigma) and D,L-$\alpha$-phosphatidylcholine (2,3-dihexadecanoyl-sn-glycerol-1-phosphocholine; dipalmitoyl-PC; Sigma), respectively, as substrates. The reaction mixture for lipase contained 0.5 ml of potato extract, and 0.5 ml of a mixture containing 2 mM trilinolein and 0.02% Triton X-100 in 0.2 M tricine-NaOH buffer, pH 7.0. The reaction was allowed to proceed for 1 hr at room temperature on a shaker. The reaction mixture for phospholipase contained 0.1 ml of potato extract and 1 ml of a mixture containing 1 mM substrate and 0.01% Triton X-100 in 0.1 M tricine-NaOH buffer, pH 7.0 (18). The reaction was allowed to proceed for 0.5 hr at room temperature on a shaker.

The liberated fatty acids were extracted and quantified by using a slight modification of the method of Nixon and Chan (21). The reactions were terminated by adding 50 $\mu$l of glacial acetic acid, 5 ml of 6.7% Na$_2$SO$_4$, and 7 ml of hexane:isopropanol (3:2, v/v). The lipids were collected in the upper phase and concentrated under N$_2$. Three milliliters of chloroform:heptane:methanol (2:3:1, v/v/v) were added to each tube and then mixed with 2 ml of freshly prepared copper reagent with shaking for 3 min. The remainder of the procedure was the same (21). The concentration of free fatty acid was derived from standard curves prepared for pure linoleic (lipase assay) and palmitic (phospholipase assay) acids.

Lipoxygenase (E.C. 1.13.11.12) activity was determined by a modification of the method of Shimizu et al (27) with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) and AA as substrate. The reaction mixture contained 1.5 ml of 0.1 M potassium phosphate buffer, pH 6.3, 0.1 ml of extract, and 0.1 ml of a mixture containing 350 $\mu$M AA and 0.52 mg/ml of Triton X-100 (1).

Enzyme activity data were log-transformed and analyzed by using the SAS general linear models procedure (26).

## RESULTS

**Characterization of mycelial lipids from *P. infestans* labeled with $^{14}$C-acetate.** $^{14}$C-Acetate was primarily incorporated into neutral lipids, with the majority of radioactivity present in the triglyceride fraction (Table 1). The pattern of incorporation was similar but not identical to the proportion of the various lipid classes determined gravimetrically in an earlier study (10). In that study, the phospholipids comprised only 12.4% by weight of the lipids, whereas in this study the phospholipids comprised approximately 30% or more of the total radioactivity in the lipid preparations from both races.

Methanolysis of the lipids revealed that EPA and AA accounted for 18.0 and 5.4%, respectively, of the total radioactivity of the lipid extract from race 0, and 18.7 and 4.9%, respectively, of the lipid extract from race 1,4. Of the fatty acid methyl esters from both races, the EPA and AA fractions comprised about

35% of the total radioactivity with the other fatty acids accounting for 65% (Table 1). Typically, gas chromatographic analyses of fatty acid methyl esters from mycelial lipids of race 0 indicate that EPA and AA comprise about 20% (mole %) of the total fatty acids (unpublished data).

**Effect of inoculation of potato disks on exogenously supplied $^{14}$C-labeled lipids from *P. infestans*.** Inoculation of potato disks treated with mycelial lipids containing $^{14}$C-EPA and $^{14}$C-AA resulted in a decline in the proportion of radioactivity recovered in the triglyceride fraction and concomitant increases in the free fatty acid and polar lipid fractions (Fig. 1). Generally, the reactions proceeded similarly in both incompatible and compatible interactions. The slopes of the regression lines for the triglyceride fraction in the inoculated treatments were not different from each other but were significantly different from the uninoculated treatment ($F = 16.8$, $P = 0.0002$, and $F = 25.9$, $P = 0.0001$ for race 0 and race 1,4, respectively). The slopes of the regression lines for the polar lipid recoveries in the inoculated disks were significantly different from the control ($F = 8.73$, $P = 0.0051$, and $F = 26.5$, $P = 0.0001$, race 0 and race 1,4, respectively). Inoculation with the incompatible race significantly increased the rate of accumulation of free fatty acid relative to the uninoculated treatment ($F = 4.31$, $P = 0.044$). Although autoradiography revealed that some extracts appeared to have small amounts of radioactivity in the regions of the chromatograms corresponding to oxidized fatty acids (peroxy- and hydroxy-acids), there were no consistent treatment effects in this regard. No treatment differences were detected in the diglyceride fraction.

Analysis of the fatty acid methyl esters obtained by methanolysis of lipids recovered from potato disks indicated that the treatments had no detectable effect on the levels of elicitor fatty acids relative to the other fatty acids. The percentage of the total fatty acids recovered as AA and EPA (combined) ranged from $28.7 \pm 0.7$ to $34.1 \pm 2.3$, but there were no consistent treatment effects. This suggests that the eicosapolyenoic acids were not preferentially metabolized relative to other fatty acids. Recoveries of the applied radioactivity in the lipids were approximately the same for all three treatments over the sampling period and averaged $40.5 \pm 4.0\%$, $33.6 \pm 3.2\%$, and $36.7 \pm 2.4\%$ for the control, race 0, and race 1,4 treatments, respectively.

**Effect of inoculation with *P. infestans* on lipolytic enzyme activities in potato disks.** Lipolytic acyl hydrolase, the predominant lipid-degrading enzyme in potato tuber, deacylates a variety of lipids, with very high activity towards phospholipids and galactolipids (14). Purified lipolytic acyl hydrolase is essentially inactive against triglycerides and therefore is not a lipase. Total acyl esterase activity in potato tuber, the major

portion of which derives from lipolytic acyl hydrolase, can be estimated with certain *p*-nitrophenyl esters (14). With *p*-nitrophenyl palmitate as substrate, acyl esterase activities were similar in both interactions and generally declined over an extended period of observation (0–72 hr; Fig. 2).

Transient differences between incompatible and compatible interactions in phospholipase and lipase activities were observed
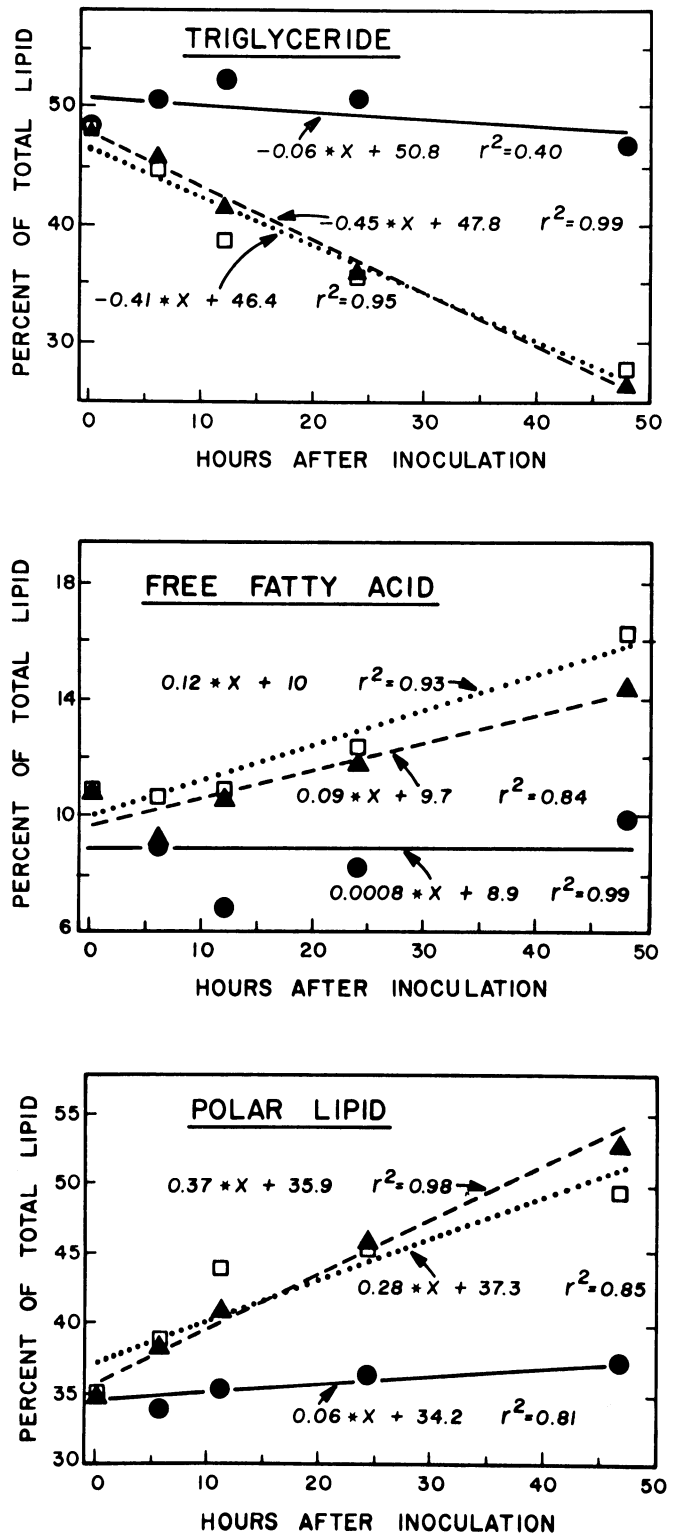






**Fig. 1.** The effect of inoculation with *Phytophthora infestans* on the proportion of radioactivity recovered in the triglyceride, free fatty acid, and polar lipid (including monoglycerides) fractions from potato disks treated with fungal lipids containing $^{14}$C-labeled AA and EPA. ●, control; ☐, race 0; ▲, race 1,4. Values are the means from three separate experiments.

TABLE 1. Incorporation of $^{14}$C-acetate in mycelial lipids of *Phytophthora infestans*

| | % Total radioactivity | |
|---|---|---|
| | *P. infestans* | |
| | Race 0 | Race 1.4 |
| Lipid class | | |
| Triglyceride | $56.1 \pm 0.4$ | $53.4 \pm 0.8$ |
| Free fatty acids | $2.7 \pm 0.2$ | $5.8 \pm 0.2$ |
| Diglyceride | $3.7 \pm 0.1$ | $5.5 \pm 0.1$ |
| Monoglyceride | $3.4 \pm 0.3$ | $5.5 \pm 0.2$ |
| Diphosphatidyl/ phosphatidyl glycerols | $11.5 \pm 0.3$ | $11.5 \pm 0.3$ |
| Phosphatidyl ethanolamine | $7.0 \pm 0.2$ | $5.5 \pm 0.1$ |
| Ceramide aminoethyl- phosphonate | $5.8 \pm 0.1$ | $4.6 \pm 0.2$ |
| Phosphatidyl inositol | $2.7 \pm 0.1$ | $2.0 \pm 0.1$ |
| Phosphatidyl choline | $4.2 \pm 0.1$ | $2.5 \pm 0.3$ |
| Unknowns | 2.9 | 3.6 |
| Fatty acid composition[a] | | |
| Arachidonic acid | 8.2 | 7.3 |
| Eicosapentaenoic acid | 27.5 | 27.4 |
| Other fatty acids | 64.3 | 65.3 |

[a] Determined by methanolysis of the total lipid extract.

during the first 12 hr after inoculation (Fig. 3). Phospholipase activity was significantly less ($P \leq 0.05$; by LSD) in the disks inoculated with the compatible race relative to the incompatible race at 6 hr after inoculation. Before and after this time, significant differences in phospholipase activity were not observed among any of the treatments. In the uninoculated disks, phospholipase activity declined during the period of observation, and the corresponding values at 0, 3, 6, 9, 12, and 24 hr were 121, 118, 87, 91, 69, and 91 nmoles palmitate $\mathrm{min}^{-1}$ $\mathrm{mg}^{-1}$ of protein, respectively.

Lipase activity in the incompatible interaction and uninoculated control exceeded that in the compatible interaction at 9 hr after inoculation (Fig. 3). This activity was also suppressed at 3 and 12 hr after inoculation in the compatible interaction relative to the uninoculated control ($P \leq 0.05$; by LSD). Lipase activity in the uninoculated disks was relatively constant during the first 24 hr after preparation of the disks, and the corresponding values at 3, 6, 9, 12, and 24 hr were 2.53, 2.35, 2.13, 2.18, 2.54, and 2.40 nmoles linoleate $\mathrm{min}^{-1}$ $\mathrm{mg}^{-1}$ of protein, respectively. When the lipase data were analyzed by combining the data for all timepoints within a treatment, the overall lipase activity in the compatible interaction was significantly less relative to the incompatible interaction and uninoculated controls ($P \leq 0.05$; by LSD). When the phospholipase data were similarly analyzed, the overall phospholipase activity in the compatible interaction was significantly less than the uninoculated control.

There were no apparent treatment effects on lipoxygenase activities (Fig. 3). The values for the uninoculated control were relatively constant during the period of observation, and ranged from 849 to 929 nmoles $O_2$ $\mathrm{min}^{-1}$ $\mathrm{mg}^{-1}$ of protein.

## DISCUSSION

The results from the experiments illustrated in Figure 1 indicate that infection by *P. infestans* stimulates degradation of lipids containing esterified eicosapolyenoic acids, with a small, corresponding increase in the free fatty acid pool. However, significant differences in the metabolism of exogenously supplied fungal lipids were not apparent between incompatible and compatible interactions. Only during the first 12 hr after inoculation could small, albeit significant, changes in lipolytic activities be discerned between the two treatments. After this period, lipolytic activities were similar in both interactions, in spite of the fact that host symptoms after inoculation with these races were typical and clearly distinguishable.

The sequence of changes in phospholipase and lipase activities suggests that during the determinative stages of the interaction, specifically during the first 12 hr after inoculation, these activities are suppressed in the compatible interaction but not the incompatible interaction. Triglycerides are the most abundant source of AA and EPA in *P. infestans* (10), and suppression of lipase activity would seem to favor compatibility by reducing the availability of these elicitors. Cystospores of *P. infestans*
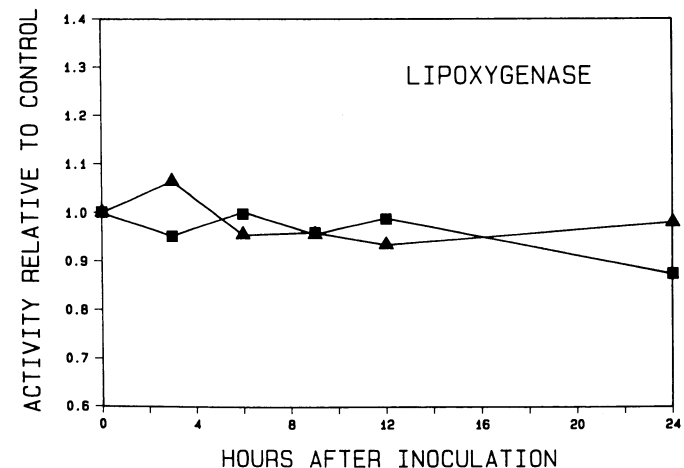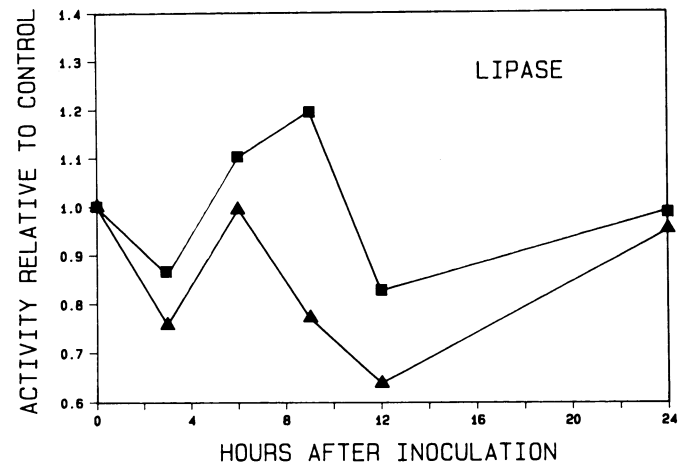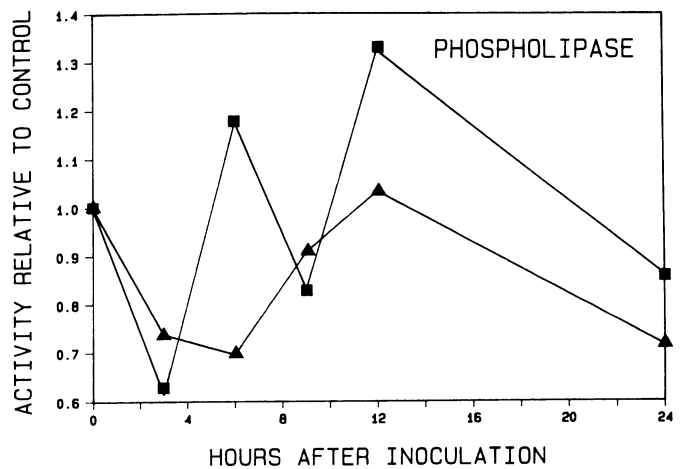






Fig. 3. Top, Phospholipase activity in potato disks during the first 24 hr after inoculation with *Phytophthora infestans*. Activities are expressed as a proportion of the uninoculated control. Each value is the mean of 14 determinations from four experiments. ●, race 0; ▲, races 1,4 or 1,2,3,4. Middle, Lipase activity in potato disks during the first 24 hr after inoculation with *P. infestans*. Each value is the mean of 14 determinations from four experiments. Bottom, Lipoxygenase activity in potato disks during the first 24 hr after inoculation with *P. infestans*. Each value is the mean of 16 determinations from four experiments.
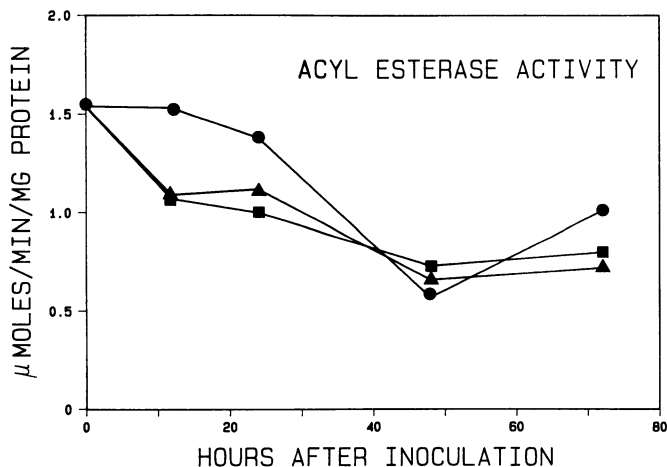


Fig. 2. Acyl esterase activity determined with *p*-nitrophenyl palmitate in the upper 1 mm of potato disks uninoculated (●) or inoculated with *Phytophthora infestans* races 0 (■) or 1,4 (▲). Values are the means from three separate experiments.

secrete a battery of hydrolytic enyzmes during germination, including phospholipase and lipase (19). Although Moreau and Rawa (18) clearly demonstrated an increase in phospholipase activity that appears to be of fungal origin in potato leaves infected with *P. infestans*, this activity did not appear until several days after inoculation. It is likely that the major effects observed in the present study during the first 12 hr after inoculation are on host enzymes since 1) the main effect on lipolytic activities is a general suppression after inoculation with the compatible races, 2) the analyses were performed on host tissues at a stage of infection when only minuscule amounts of fungal tissue are present, and 3) there was no detectable degradation of the lipid preparation by germinating cystospores over a 24-hr period. A more complete characterization of lipase and phospholipase activities with respect to their origin, regulation, and localization at the host-parasite interface would help determine their importance in hypersensitivity expression.

Localization of the 5-lipoxygenase during early stages of infection would also be helpful for evaluating its involvement, if any, in hypersensitivity expression. The absence of a significant effect on lipoxygenase activity after inoculation was surprising, particularly in the light of studies that have reported increased lipoxygenase activity during the hypersensitive response in other host-pathogen interactions (30). Potato tuber is a rich source of this enzyme, the cellular location of which is uncertain but is likely associated with a labile organelle (29). Perhaps infection induces changes in the compartmentation of lipoxygenase or in the activities of specific isoforms (20) that are otherwise not detected in assays of unfractionated preparations.

Several observations are consistent with a scenario in which the availability of fatty acid elicitors is greater in the incompatible interaction. These include 1) the slight suppression of lipid-degrading activity during the first 12 hr after inoculation with compatible races reported in the present study; 2) the presence of superoxide anion, which can deacylate lipids (25) and which increases in potato tissues soon after inoculation with an incompatible race (12); and 3) the high elicitor activity of free AA and EPA, and inactivity of certain nonhydrolyzable esters (4,5,22). Nevertheless, the results of experiments concerning the metabolism of the complex fungal lipid preparation indicate that the increase in the free fatty acid pool after inoculation is rather modest, at best, and does not reflect the dramatic differences in the host responses induced by incompatible and compatible races. The release of specific $\beta$-glucans, which enhance the elicitor activity of eicosapolyenoic acids, could be important in affecting a large response in the presence of low concentrations of AA and EPA, and this aspect should be examined (5,11,17). The use of spores containing radioisotope-labeled elicitor fatty acids coupled with microautoradiography of the inoculated tissues may also provide more precise information regarding the disposition of these elicitors during early stages of infection (9).

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