Plant Physiol. (1982) 70, 1417–1424
0032-0889/82/70/1417/08/$00.50/0

Factors Affecting the Elicitation of Sesquiterpenoid Phytoalexin Accumulation by Eicosapentaenoic and Arachidonic Acids in Potato[1]

Received for publication April 26, 1982 and in revised form July 26, 1982

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ABSTRACT

Eicosapentaenoic and arachidonic acids in extracts of *Phytophthora infestans* mycelium were identified as the most active elicitors of sesquiterpenoid phytoalexin accumulation in potato tuber slices. These fatty acids were found free or esterified in all fractions with elicitor activity including cell wall preparations. Yeast lipase released a major portion of eicosapentaenoic and arachidonic acids from lyophilized mycelium. Concentration response curves comparing the elicitor activity of the polyunsaturated fatty acids to a cell-free sonicate of *P. infestans* mycelium indicated that the elicitor activity of the sonicated mycelium exceeded that which would be obtained by the amount of eicosapentaenoic and arachidonic acids (free and esterified) present in the mycelium. Upon acid hydrolysis of lyophilized mycelium, elicitor activity was obtained only from the fatty acid fraction. However, the fatty acids accounted for only 21% of the activity of the unhydrolyzed mycelium and the residue did not enhance their activity. Centrifugation of the hydrolysate, obtained from lyophilized mycelium treated with 2 N NaOH, 1 molarity $NaBH_4$ at 100°C, yielded a supernatant fraction with little or no elicitor activity. Addition of this material to the fatty acids restored the activity to that which was present in the unhydrolyzed mycelium. The results indicate that the elicitor activity of the unsaturated fatty acids is enhanced by heat and base-stable factors in the mycelium.

Elicitors are metabolites produced by plant pathogenic fungi and bacteria which elicit phytoalexin accumulation in higher plants (20). Phytoalexins, which may inhibit pathogen development, are a prominent feature of the hypersensitive response in many host-parasite interactions. *Phytophthora infestans* (Mont.) de Bary, the fungus which causes late blight disease in potato, occurs in nature as a number of races which can be distinguished by pathogenicity on potato cultivars containing R genes. Compounds from the mycelium of *P. infestans* elicit a reaction in potato tuber, which is similar to the hypersensitive response elicited by incompatible races of the fungus. The responses are characterized by electrolyte leakage, necrosis, and the accumulation of antimicrobial sesquiterpenes (7, 10, 23, 29).

Recently, we reported the polyunsaturated fatty acids, all-*cis*-5,8,11,14,17-eicosapentaenoic and all-*cis*-5,8,11,14-eicosatetraenoic (arachidonic) acids, as the simplest and most active elicitors of sesquiterpenoid phytoalexins found in extracts of *P. infestans* (4, 5). This was the first report of these fatty acids eliciting responses associated with plant hypersensitivity and of the occurrence of eicosapentaenoic acid in the Pythiaceae. Others had tentatively identified arachidonic acid in *Pythium debaryanum* and *P. infestans* and it has been found in numerous other Phycomycetes (31). Gellerman and Schlenk (14) identified eicosapentaenoic acid as the predominant fatty acid in *Saprolegnia parasitica*. Our findings were a significant departure from those of other studies of fungal elicitors which attributed activity to polysaccharides and glycoproteins obtained from fungal culture filtrates or mycelial cell walls (1).

In this paper, we present further details concerning the elicitor activity of eicosapentaenoic and arachidonic acids and evidence for other factors in mycelial extracts of *P. infestans* which dramatically enhance fatty acid elicitor activity. In addition, we report that cell wall preparations contain small amounts of these fatty acids and only a small proportion of the total elicitor activity present in the mycelium of *P. infestans*.

MATERIALS AND METHODS

General. Unless indicated otherwise, all chemicals used were of reagent grade purity and all solvents were redistilled prior to use. Total carbohydrate was estimated by the phenol-sulfuric acid method (9) and total protein by the method of Lowry *et al.* (24). Glucose and BSA were used as standards, respectively. Concentrations of test solutions for assay on tuber slices were determined by drying 50- to 100-μl aliquots (two or three per sample) on preweighed aluminum cups at 60°C for 1 h and then weighing them on a microanalytical balance. Concentrations of fatty acid were also determined by methylating a small aliquot with diazomethane and analyzing the products by GLC.

In all experiments, certified seed potato tubers or tubers grown from certified seed were used. Potatoes were obtained from Fayette Seed Co., Lexington, KY, Dr. R. J. Young, West Virginia University, Morgantown, and the experimental farm at the University of Kentucky. Kennebec potatoes were used in all of the experiments and tubers were stored at 4°C until 24 h before use.

Isolates of *P. infestans* were obtained from Dr. R. J. Young, West Virginia University, Morgantown. Sporangial zoospore suspensions were prepared from cultures grown on lima bean agar at 18 to 20°C (18) and mycelium was obtained from cultures grown

[1] Supported in part by Grant 78-59-2211-0-1-063-1 of the United States Department of Agriculture, Science and Education Administration, and a grant from the Rockefeller Foundation. Journal Paper No. 81-11-267 of the Kentucky Agricultural Experiment Station, Lexington, KY 40446.

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[3] Recipient of Grants AM25101 and GM23902 from the National Institutes of Health, and Equipment Grants from the Fleischmann Foundation and from the Graduate School of the University of Kentucky.

in lima bean broth for 14 to 17 days (23).

All fatty acid standards were purchased from Sigma Chemical Co. Authentic eicosapentaenoic acid was a generous gift from J. Gellerman, The Hormel Institute, University of Minnesota, Austin.

Assay, Extraction, and Analysis of Rishitin and Lubimin. A semimicro method for the quantitation of potato terpenoids was used throughout this study (17). Discs cut from (3-cm diameter) potato tubers were washed in three changes of sterile deionized H_2O and placed in sterile Petri dishes containing moistened Whatman No. 1 filter paper. Discs were aged at room temperature for 6 to 8 h and then a 100-μl aliquot of the test solution was spread evenly over the upper surface of each disc. The discs were incubated at 18 to 20°C in the dark.

After incubation of the discs for 96 h, the upper 1 mm[4] was removed and two to three 1.0-g samples were each extracted for sesquiterpenoid stress metabolites (17). Since rishitin and lubimin were approximately 90% of the total sesquiterpenoid stress metabolites which accumulated, these stress metabolites were quantified by GLC (17). The results are expressed as μg stress metabolites/g fresh weight of the upper 1 mm of potato tissue, which is the sum of rishitin and lubimin accumulations. Specific activity is expressed as μg stress metabolites/μg treatment solutes and was determined using the following formula:

$$\text{Specific activity} = \frac{\mu\text{g stress metabolites}}{\text{g fresh weight}} \times \frac{0.5\text{ g fresh weight/upper mm}}{\mu\text{g solutes applied/upper mm}}$$

Preparation and Analysis of Mycelial Cell Walls and Heat-Released Elicitor Material. Purified cell walls of *P. infestans* (races 4 and 1.2.3.4) were prepared by the method of Ayers *et al.* (2). The resulting purified cell wall fraction was air-dried and either (a) autoclaved for 3 h in distilled H_2O (100 ml/g cell wall) to obtain the "heat-released elicitors" or (b) dried over $CaSO_4$ in a desiccator for 48 h at 4°C, pulverized with a mortar and pestle, and stored at −20°C for further analysis. The heat-released elicitor solutions were clarified by filtration through a 0.22-μm membrane filter and concentrated to about 1% of their volume by rotary evaporation under reduced pressure, and the concentrate was dialyzed against three changes of deionized H_2O.

Further experiments indicated that reducing the volumes of the aqueous wash solutions in the procedure described by Ayers *et al.* (2) did not significantly affect the recovered activity in the heat-released preparations. An experiment was conducted to assess the distribution of elicitor activity in the washings. One hundred grams of mycelium from *P. infestans* race 1.2.3.4 were washed over a 74-μm nylon mesh sieve, supported on a coarse sintered glass tunnel, with 2 L of deionized H_2O, 1 L of 100 mM K-phosphate (pH 7.2), and 1 L of 500 mM K-phosphate (pH 7.2). The residue was homogenized with a Polytron tissue homogenizer in 250 ml of the 500 mM buffer and filtered over the nylon sieve, and the residue was washed with 1 L of the 500 mM buffer, 1 L deionized H_2O, 500 ml chloroform:methanol (1:1, v/v), and 500 ml acetone. The washings were concentrated by rotary evaporation under reduced pressure and the solutions were dialyzed for 48 h at 4°C against several changes of deionized H_2O. Insoluble material was removed by centrifugation at 10,000*g* for 10 min and all the pellets were combined. Centrifugation of the dialyzed organic solvent washings (chloroform:methanol and acetone) gave rise to a pellet, a supernatant, and an oily film overlaying the supernatant. The oily film was collected with a Pasteur pipette. All of the wash fractions, including the combined pellets, were brought to 20 ml with sterile, distilled H_2O and shaken vigorously on a Vortex mixer prior to assay on tuber slices.

The carbohydrate compositions of the cell wall heat-released elicitor preparations were determined by GLC of the products obtained after hydrolysis and conversion of the resulting monomers to their alditol acetates (22). Prior to hydrolysis, xylitol was added as an internal standard to aliquots containing approximately 100 nmol glucose equivalent.

After derivatization, samples were dissolved in 50 μl acetone and analyzed by GLC. Chromatography was performed on a Perkin-Elmer model 3920 gas chromatograph equipped with a flame ionization detector and a 1.6 m × 2 mm i.d. glass column packed with 3% OV-275 on Supelcoport. Oven temperature was 205°C for 8 min and then programmed at 32°C/min to 250°C for 8 min to elute derivatives of the amino sugars.

Extraction of Lipophilic Elicitors. Lipids from mycelial mats of race 4 of *P. infestans* were extracted as described previously (5). The total lipid extract was washed according to the methods of Folch *et al.* (11) or modifications of this method (4). All organic solvents were removed by rotary evaporation under reduced pressure at 30 to 35°C or under a stream of N_2, and lipids were emulsified in sterile, distilled H_2O by sonication prior to assay on tuber discs.

Mycelial sonicates were prepared by homogenizing mycelium in sterile, distilled H_2O with a Polytron homogenizer and then sonicating the mixture at maximum intensity for 10 min. The sonicate was frozen and thawed three times to ensure that there were no viable cells (18). In later experiments, the mycelial sonicate was obtained from lyophilized mycelium which had been pulverized with a mortar and pestle and suspended by sonication in sterile, distilled H_2O. All test solutions were sealed and stored at −20°C until use. Solutions were resonicated prior to assay on tuber discs.

Fractionation and Analysis of Lipid Extracts. The washed, lipid extract from 35 g fresh weight of mycelium was concentrated to a chloroform solution of several ml by solvent replacement and fractionated by chromatography on Sephadex LH-20 as described previously (5). Aliquots from fractions with elicitor activity were methanolyzed in 1 N methanolic HCl for 2 h at 100°C, cooled, and then partitioned at least three times against an equal volume of hexane. FAME[5] in the hexane phase were analyzed with a Varian 1400 gas chromatograph equipped with a flame ionization detector and a 1.8 m × 2 mm i.d. glass column packed with 10% SP-2330 on Chromosorb W AW (Supelco). Column temperature was programmed between 150 and 260°C at 6°C/min and carrier gas flow rate (N_2) was 30 cc/min.

To distinguish between free and esterified forms of fatty acids, portions of the active fractions were treated with diazomethane or with methanolic HCl. Detection of FAME by GLC in extracts after treatment with methanolic HCl but not after treatment with diazomethane indicated that the fatty acids were esterified to other compounds.

Identification of Fatty Acid Elicitors. Methane chemical ionization mass spectra of FAME were obtained as described previously (5). The IR spectrum of methyl eicosapentaenoate was obtained from a thin film sample on KBr windows in a Beckman 283 B spectrophotometer.

Purification of small quantities of eicosapentaenoic acid was accomplished by PTLC (5). Large quantities were purified as FAME by a combination of PTLC and argentation column chromatography (8, 27). FAME (approximately 10 g) were fractionated on a $AgNO_3$-silica gel column (85 × 3.4 cm) and eluted with 200

[4] The average weight of each upper 1-mm disc was approximately 0.5 g.

[5] Abbreviations: FAME, fatty acid methyl esters; PTLC, preparative thin layer chromatography.

ml each of mixtures of diethyl ether in petroleum ether (boiling point, 35 to 60°C) according to the following sequence: petroleum ether, 10, 20, 40, 50, 60, 70, and 80% (v/v) diethyl ether in petroleum ether. The final eluant was 500 ml diethyl ether. Column effluent was monitored by GLC and fractions containing methyl eicosapentaenoate of high purity were pooled.

TLC revealed that some minor contaminants were still present and these were removed by chromatography on a Unisil column (55 × 1.2 cm) eluted with 100 ml hexane, and 100 ml each of 10, 20, and 30% (v/v) diethyl ether in hexane. Fractions containing pure methyl eicosapentaenoate were pooled and the material gave a single spot on Whatman KC-18 thin layer plates developed in a mixture of acetonitrile:tetrahydrofuran:acetic acid (80:15:5, v/v) after charring with chromic acid-H_2SO_4 spray reagent. The material was judged to be about 99% pure by GLC and 265 mg were obtained.

A portion of the pure methyl eicosapentaenoate (approximately 80 mg) was hydrolyzed in 5 ml 20% (w/v) KOH in 50% (v/v) methanol at room temperature under N_2 in the dark for 36 h. Most of the methanol was removed under a stream of N_2. Distilled H_2O (2.5 ml) was added and then the extract was acidified with 5 ml 5 N H_2SO_4 under a layer of 10 ml diethyl ether. The ether phase containing the free acid was collected and the extract was partitioned four times with 10 ml diethyl ether. The combined ether solutions were concentrated to 10 ml and then washed with 5 ml distilled H_2O. The ether solution was evaporated, and the oil was dissolved in 5 ml chloroform and stored at −20°C until use. TLC and the absence of a peak on GLC indicated that hydrolysis of the methyl ester to the free fatty acid was complete.

Fractions from argentation chromatography enriched in methyl arachidonate (*cis*-5,8,11,14-eicosatetraenoic acid-methyl ester) eluted before methyl eicosapentaenoate and were analyzed by GLC/MS for confirmation of structure.

Methanolysis and Base-Borohydride Hydrolysis of Lyophilized Mycelium. To determine the fatty acid composition, 20-mg samples of lyophilized mycelium were methanolyzed for 2 h at 100°C and the resulting FAME were analyzed by GLC. To determine the fatty acid composition of a cell wall preparation, 50-mg samples were methanolyzed.

Lyophilized mycelium was hydrolyzed with acid or base to assess the contribution of the elicitor fatty acids to the total activity. Fifty- or 100-mg samples of lyophilized mycelium were methanolyzed and the FAME in the hexane phase were isolated by PTLC on silica gel plates. The other lipids on the plate were recombined with the hydrolyzed residues obtained after drying the methanol phase (under N_2) and diluted equivalent to their concentration in a 5 mg/ml suspension of lyophilized mycelium.

For base hydrolysis, 50 mg samples were hydrolyzed in 2 ml of 1 N or 2 N NaOH containing 1 M $NaBH_4$ at 100°C. The hydrolysate was acidified with HCl under a layer of diethyl ether and the extracts were partitioned at least three times against an equal volume of diethyl ether. The aqueous phase was centrifuged at 10,000*g* for 20 min and the supernatant was desalted on a Sephadex PD-10 column (Pharmacia). The pellet was washed with distilled H_2O and recentrifuged, and the supernatant was desalted. The pellet and supernatants were recombined and diluted to 10 ml with sterile distilled H_2O (*i.e.* equivalent to material obtained from a 5 mg/ml suspension of mycelium). This suspension was assayed with and without added elicitor fatty acids.

Release of Elicitor Fatty Acids from the Mycelium by Lipase. To determine if elicitor fatty acids could be released from the mycelium enzymically, lyophilized mycelium was incubated with a purified lipase preparation from *Candida cylindracea* (Sigma Chemical Co.). The reaction mixture was a modification of that outlined in the Worthington Enzyme Manual (33) for pancreatic lipase. The procedure was as follows. Mix 0.1 ml Na-taurocholate suspension (70 mg taurocholate/ml 0.1 N NaOH), 0.5 ml 3 M NaCl, 0.25 ml 0.075 M $CaCl_2$, and 3.25 ml distilled H_2O. Adjust pH to 7.5 and add 50 mg lyophilized mycelium. Sonicate to suspend. Add 1 ml lipase solution (5 mg/ml in 0.005 M $CaCl_2$) and mix. The control contained 1 ml 0.005 M $CaCl_2$ and no enzyme. Incubate at 25°C in a water bath. One-milliliter samples were taken at various times and extracted immediately three times each with 2 ml diethyl ether. The combined ether phases were evaporated, the liberated fatty acids were methylated with diazomethane, and the products were analyzed by GLC.

RESULTS

Evaluation of Elicitor Preparations from Mycelial Cell Walls. In light of the evidence that glyceollin elicitors are associated with the cell walls of *Phytophthora megasperma* f. sp. glycinia (syn. *P. megasperma* var. sojae), it seemed logical to examine *P. infestans* mycelial cell walls for similar compounds which elicited potato stress metabolites (1–3). Preparations obtained from autoclaved mycelial cell walls from races 4 and 1.2.3.4 had low activity in eliciting necrosis and sesquiterpenoid phytoalexin accumulation. The specific activities of the preparations from these races were not different and were typically 0.04 to 0.06 μg stress metabolites/μg treatment solutes. These crude preparations consisted predominantly of protein and carbohydrate (4). The sugar composition of a preparation from race 4 was similar to that reported for the elicitors of glyceollin obtained by autoclaving cell walls of *P. megasperma* f. sp. glycinea and contained glucose (91%, mole%) and small amounts of mannose (7%) and glucosamine (2%). Fractionation of these cell wall-released preparations by ion-exchange and gel filtration chromatography yielded preparations with only low elicitor activity (4).

Because of the low specific activity of the cell wall-associated elicitor preparations, the washings obtained during the preparation of the mycelial cell walls were assayed. Activity was found in all fractions, but the material obtained from the organic solvent washes and insoluble residues obtained by centrifugation of the washings had the highest activities (4). This indicated that most of the active material was discarded during cell wall preparation and

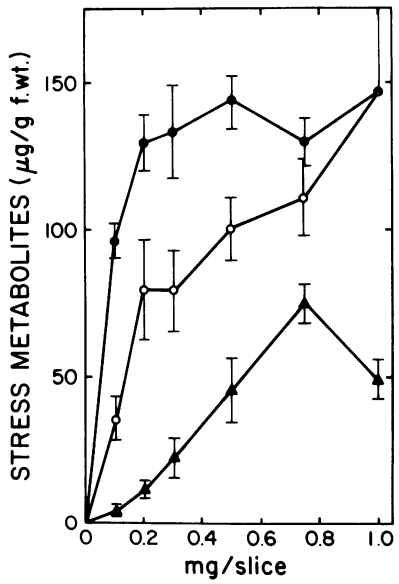

FIG. 1. Concentration response curves of stress metabolite accumulation (rishitin + lubimin) *versus* the concentrations of mycelial sonicate (●), chloroform-methanol extract (○), or mycelial residue after chloroform-methanol extraction (▲). Each value is the mean ± SE of six determinations from three experiments. Eicosapentaenaoic and arachidonic acids are predominantly linked to other compounds and only small amounts are present as free fatty acids in these extracts.

Table I. *Effects of Purified Eicosapentaenoic Acid from P. infestans and Authentic Fatty Acid and Fatty Alcohol Standards on Rishitin and Lubimin Accumulation by Potato Discs from Old (10-Month Storage) and New (2-Month Storage) Kennebec Tubers*

Fatty Acid[a]	Old Tubers		New Tubers	
	Rishitin + lubimin[b]	Specific activity[c]	Rishitin + lubimin[b]	Specific activity[c]
	μg/g fresh wt		*μg/g fresh wt*	
Eicosapentaenoic acid from *P. infestans*	136 ± 6	0.680	226 ± 34	1.13
Eicosapentaenoic acid, standard	147 ± 12	0.735	249 ± 22	1.24
Arachidonic acid	162 ± 8	0.810	220 ± 6	1.10
Docosahexaenoic acid	47 ± 2	0.235	124 ± 11	0.574
Eicosatrienoic acid (all-*cis*-8, 11,14)	0	0	22 ± 8	0.110
Eicosatrienoic acid (all-*cis*-11, 14,17)	0	0	0	0
Arachidonyl alcohol	Trace	0	48 ± 13	0.240
Water control	0	0	0	0

[a] One hundred microliters of a 3.3 mM emulsion of the test substance were applied per tuber slice. The pH of the emulsion was approximately 6.6. The following fatty acids were tested and had no activity: myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid (all-*cis*-9,12,15), γ-linolenic acid (all-*cis*-6,9,12), ricinoleic acid, eicosanoic acid, eicosaenoic acid, eicosadienoic acid, docosanoic acid, and docosaenoic acid.

[b] Values listed are the mean ± SE of at least six determinations from two experiments 96 h after treatment of potato discs.

[c] Specific activity measured as μg rishitin and lubimin/μg fatty acid.

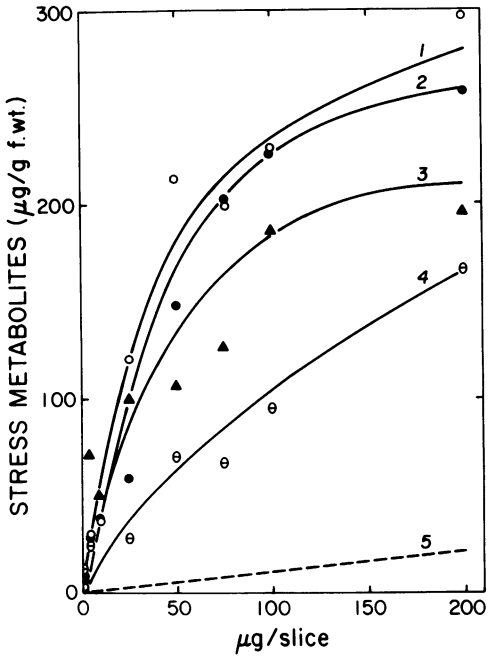

FIG. 2. Concentration response curves of stress metabolite (rishitin + lubimin) accumulation *versus* the concentrations of 1) eicosapentaenoic acid standard (○); 2) eicosapentaenoic acid purified from *P. infestans* (●); 3) arachidonic acid standard (▲); 4) sonicated mycelium from race 4 of *P. infestans* (⊖). Curve 5,- - -, is the expected activity of the mycelial sonicate based on the amount of eicosapentaenoic and arachidonic acids (free and esterified) present as determined by methanolysis. Values for stress metabolite accumulations are the mean of three determinations). Standard errors average approximately 18% of the illustrated values.

that a major portion of the activity was lipophilic. The material released from the cell wall by autoclaving consistently had low activity, less than 10% of that obtained with material from the organic solvent washes.

Analysis of the Lipophilic Elicitor Extracts. The initial experiments with cell wall preparations indicated that lipid extraction procedures were the most efficient to purify and identify the active component(s) in the mycelium. As previously reported, extraction in chloroform:methanol (2:1, v/v) removed a major portion of the activity from the mycelium and this material always had higher specific and total activities than the residue (Fig. 1 and Table 1 in Ref. 5). Routinely, 30% of the dry weight of the mycelium was extracted into chloroform:methanol and the residue accounted for the remaining 70%. The sonicated mycelium, however, usually had higher activity at lower concentrations than the chloroform:methanol extracts (Fig. 1). Details concerning the isolation and identification of eicosapentaenoic acid as one of the active components were reported previously (5). No *trans* double bonds were present, as indicated by the absence of strong absorption in the 950 to 1000 cm^{-1} region of the IR spectrum of methyl eicosapentaenoate.

In subsequent experiments, arachidonic acid (Sigma) was found to be as active as eicosapentaenoic acid (Table I). The presence of arachidonic acid in mycelial extracts was confirmed by GLC/MS of the methyl ester (molecular ion at 318 *m/z*) and comparison with the standard (4). The TLC behavior and GLC retention time of the methyl ester were identical with those of the standard.

Specificity of the Tuber Response to Fatty Acids. We previously reported (5) that of a number of commercially available fatty aids tested for activity, only arachidonic, eicosapentaenoic, and all-*cis*-4,7,10,13,16,19-docosahexaenoic acids were active in eliciting necrosis and stress metabolite accumulation in older tubers (10-month storage, Table I). Docosahexaenoic acid was not as active as the other two acids nor was it observed in mycelial extracts. Subsequently, we found that all-*cis*-11,14,17-eicosatrienoic acid was not active but its isomer, all-cis-8,11,14-eicosatrienoic acid, was 10% as active as arachidonic and eicosapentaenoic acids on new tubers (2-month storage). The results indicated that the tuber response is specific with respect to carbon chain length and degree of unsaturation of the fatty acid. Unsaturation of the δ carbon in the chain appeared to be a requirement for high activity. Eicosapentaenoic and arachidonic acids were the most active elicitors and showed detectable activity at 5 μg/slice (Fig. 2).

Arachidonyl alcohol (Sigma) was inactive on old tubers and

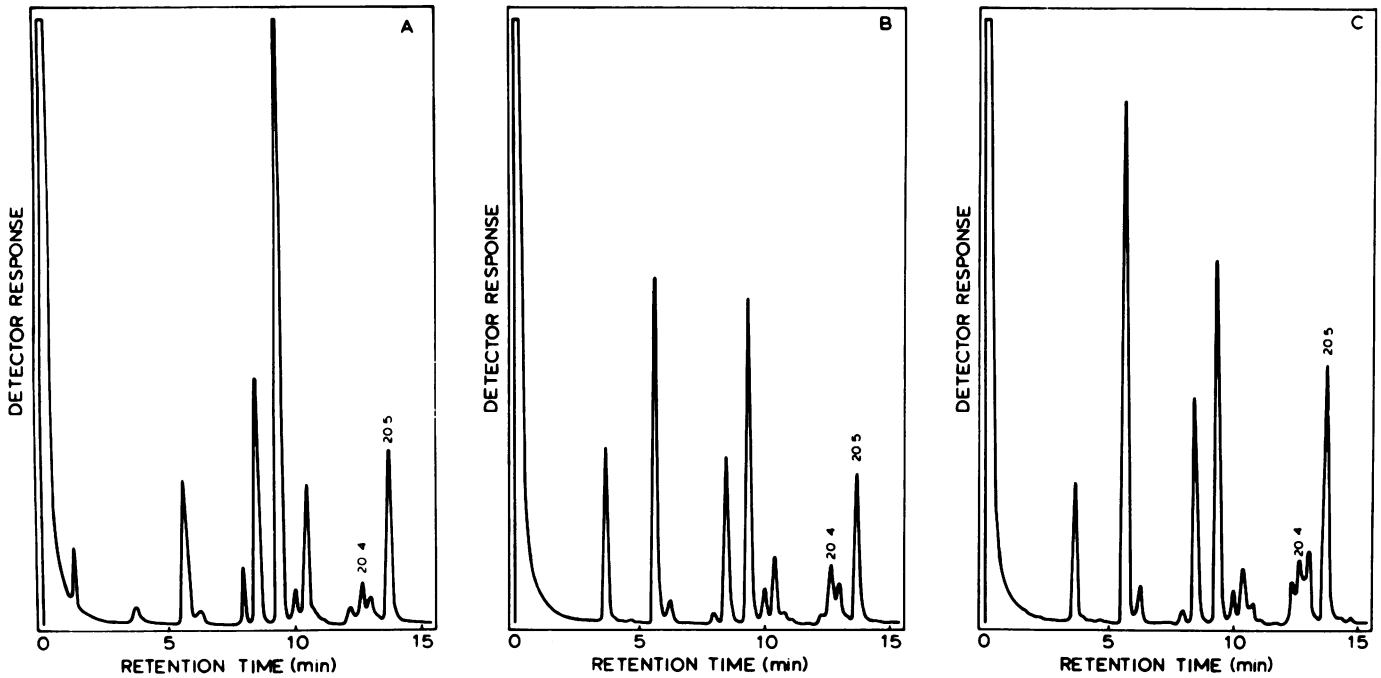

FIG. 3. Gas-liquid chromatograms of FAME fractions from *P. infestans* race 4. A, Sephadex LH-20 chromatography. B, FAME from methanolyzed mycelium. C, FAME from methanolyzed cell wall preparation. The oven temperature was increased from 150 to 260°C at 6°C/min. Peaks corresponding to eicosapentaenoic (20:5) and arachidonic (20:4) acids are indicated.

had low activity on new tubers (Table I), suggesting that the carboxyl function is important for elicitor activity. The methyl esters of the elicitor fatty acids were also active but browning of the tuber slices treated with the methyl ester was consistently slower than in slices treated with the free fatty acid. The browning in the two treatments appeared the same after 48 h. The lag in the response to the methyl ester, however, was not apparent in time course analyses of sesquiterpenoid accumulation.

Methanolysis of Active Fractions, Lyophilized Mycelium, and Cell Walls. Methanolysis revealed that both eicosapentaenoic and arachidonic acids were esterified in all of the active fractions. Methanolysis of lyophilized mycelium from race 4 and analysis of the FAME (Fig. 3) indicated that eicosapentaenoic acid comprised 12.1% of the total fatty acids. Arachidonic acid accounted for 3.5% of the total. The amounts of eicosapentaenoic and arachidonic acids were 17.0 ± 0.1 μg and 5.0 ± 0.1 μg/mg lyophilized mycelium. In addition, FAME with retention times identical with myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, γ-linolenic, and erucic acids were detected in these extracts. Eicosapentaenoic and arachidonic acids were also detected in extracts of *P. infestans* races 1.4 and 1.2.3.4.

Methanolysis of cell wall preparations indicated that the elicitor fatty acids were present (Fig. 3). Eicosapentaenoic acid occurred at a concentration of 5.44 ± 0.13 μg/mg cell wall material and 92% of the recovered eicosapentaenoic acid was present as bound forms. The concentration of arachidonic acid in the cell wall was 0.50 ± 0.01 μg/mg. Total fatty acids accounted for approximately 3.8% (w/w) of the cell wall dry weight. Whether the fatty acids are linked to other lipids associated with the cell wall or to carbohydrate or protein components of the wall remains to be established.

Inhibition of Growth of Compatible Races of *P. infestans* in Tubers Treated with Arachidonic Acid. Because others reported that mycelial growth and sporulation of compatible races were inhibited on tuber slices treated with crude elicitor preparations, it was of interest to know if pure arachidonic acid also had this effect (10, 28). Kennebec tuber slices were treated with H_2O, arachidonic acid, or eicosadienoic acid (a control) and 15 to 30 min later inoculated with a zoospore suspension from race 1 (10^5 zoospores/slice). Arachidonic acid at 100 μg/slice either completely prevented or greatly reduced mycelial growth and sporulation of the compatible race. Infected slices treated with 10 μg arachidonic acid or with eicosadienoic acid gave the same results as the control (H_2O + race 1).

When equal volumes of the fatty acid and zoospore suspensions were mixed, arachidonic acid at both 1.6 mM (500 μg/ml) and 0.16 mM (50 μg/ml) immediately lysed zoospores. Eicosadienoic acid did not lyse zoospores but slightly reduced their motility at 1.6 mM and had no apparent effect at 0.16 mM. Hence, it is unclear whether the inhibition of the compatible reaction is due to toxicity of arachidonic acid to the inoculum or to the elicitation of phytoalexins.

Evidence for Promoting Factors in Mycelial Extracts. Although specific activity of a given fraction depended somewhat on the concentration at which it was assayed, the activity of the pure fatty acid elicitors indicated no more than a 3-fold degree of purification over the starting mycelial material. Time course studies showed that sesquiterpenoid accumulations did not reach maximum levels before 96 h in the fatty acid elicitor-treated slices. This is also generally the case for slices treated with crude elicitor preparations or spore suspensions from incompatible races. Furthermore, the specific activity of arachidonic acid did not exceed more than 5-fold that for the sonicated mycelium at any time after treatment. Concentration response studies demonstrated that the sonicated mycelium was very active at low concentrations (Fig. 2) and that the total amounts of eicosapentaenoic and arachidonic acids in the mycelium could not account for the observed activity. Nevertheless, the purification studies indicated that the response to the fatty acids was highly specific and that they were found in either free or esterified form in all active fractions. These data suggested that there were other factors which contributed to the elicitor activity of the fatty acids.

Methanolysis of the mycelium and removal of the fatty acid fraction revealed that nothing stable to hydrolysis with 1 N methanolic HCl, other than the fatty acids, had elicitor activity (Table II). All of the activity of the fatty acid fraction could be accounted

Table II. *Effects of Fractions from Methanolyzed Mycelium of P. infestans on Rishitin and Lubimin Accumulation in Potato Discs*

Treatment	Rishitin + Lubimin[a]	Elicitor Activity
	μg/g fresh wt	*% mycelial sonicate*
Mycelial sonicate[b]	214 ± 11	100
Fatty acid fraction released from mycelium[c]	46 ± 7	21
Eicosapentaenoic and arachidonic acids[d]	44 ± 5	21
Residues[e]	0	0
Residues + fatty acid fraction released from mycelium	58 ± 9	27
Water control	0	0

[a] Values listed are the mean ± SE of at least six determinations from two experiments 96 h after treatment of potato discs. Mycelium was hydrolyzed in 1 N methanolic HCl for 2 h at 100°C and then extracted with hexane.

[b] Mycelial sonicate refers to the unhydrolyzed mycelium from race 4 assayed at 5 mg/ml (0.5 mg/tuber slice). All other fractions were assayed equivalent to their concentrations in a 5 mg/ml suspension of lyophilized mycelium from race 4.

[c] Free fatty acid fraction obtained from mycelium by methanolysis and subsequent purification as fatty acid methyl esters by PTLC. Free acids were generated from the methyl esters by mild hydrolysis in KOH. Mixture contained 85 μg eicosapentanoic and 25 μg arachidonic acid/ml plus the other fatty acids.

[d] A mixture containing 85 μg pure eicosapentaenoic and 25 μg pure arachidonic acids/ml. This corresponds to the quantity of the acids released from a 5 mg/ml suspension of lyophilized mycelium from race 4.

[e] Residues resulting from hydrolysis of lyophilized mycelium. Includes soluble and insoluble material in the methanol phase after partitioning against hexane and hexane-soluble material after removal of fatty acid methyl esters by PTLC.

Table III. *The Effects of Fractions from Base-Borohydride-Hydrolyzed Mycelium of P. infestans on the Accumulation of Rishitin and Lubimin in Potato Discs*

Treatment	Rishitin + Lubimin[a]	Per Cent of Mycelial Sonicate
	μg/g fresh wt	
Mycelial sonicate[b]	200 ± 9	100
Eicosapentaenoic and arachidonic acids[c]	42 ± 4	21
Base-hydrolyzed suspension[d]	81 ± 19	41
Base-hydrolyzed suspension supernatant[e]	5 ± 4	2
Base-hydrolyzed suspension pellet[e]	76 ± 12	38
Base-hydrolyzed suspension + eicosapentaenoic and arachidonic acids[f]	193 ± 17	96
Base-hydrolyzed suspension supernatant + eicosapentaenoic and arachidonic acids[g]	211 ± 16	106
Base-hydrolyzed suspension pellet + eicosapentaenoic and arachidonic acids[h]	141 ± 6	71
Water control	0	0

[a] Values listed are the mean ± SE of at least nine determinations from three experiments except the values for the base-hydrolyzed suspension/supernatant and base-hydrolyzed suspension/pellet which are the means ± SE of seven and six determinations, respectively, from three experiments. Lyophilized mycelium from race 4 of *P. infestans* was hydrolyzed in 2 N NaOH, 1 M $NaBH_4$ for 3 h at 100°C. Fatty acids released by base hydrolysis were extracted with diethyl ether. See "Materials and Methods" for further details.

[b] Mycelial sonicate refers to the unhydrolyzed mycelium assayed at 5 mg/ml (0.5 mg/slice). All other fractions were assayed equivalent to their concentrations in a 5 mg/ml suspension of lyophilized mycelium.

[c] A mixture containing 85 μg pure eicosapentaenoic and 25 μg pure arachidonic acids/ml. This corresponds to the quantity of these acids released by methanolysis from a 5 mg/ml suspension of lyophilized mycelium of race 4.

[d] Suspension remaining after base hydrolysis of mycelium and extraction with diethyl ether but still containing approximately 6 μg/ml bound eicosapentaenoic and arachidonic acids which were not released by base hydrolysis. Residual elicitor fatty acids were detected by further hydrolysis in methanolic HCl and analysis by GLC.

[e] Two-milliliter aliquots of the base-hydrolyzed suspensions were centrifuged at 10,000*g* for 20 min. The supernatant was collected and diluted to 2 ml with sterile, distilled H_2O and the pellet was suspended in 2 ml sterile, distilled H_2O.

[f] A mixture containing the base-hydrolyzed suspension plus added eicosapentaenoic (85 μg/ml) and arachidonic acids (25 μg/ml).

[g] As in Footnote f except elicitor fatty acids were added to the base-hydrolyzed suspension supernatant fraction.

[h] As in Footnote f except elicitor fatty acids were added to the base-hydrolyzed suspension pellet fraction.

for by eicosapentaenoic and arachidonic acids but the fatty aids only contributed 21% of the activity present in the unhydrolyzed mycelium. The residues and other lipids did not significantly promote the elicitor activity of the fatty acids.

Base-borohydride hydrolysis was destructive to the polyunsaturated fatty acids, as indicated by decreasing recoveries with increasing time of hydrolysis. This is not unexpected because these conditions will cause oxidation of unsaturated fatty acids. The procedure was not as efficient as methanolysis because the methyl esters of eicosapentaenoic and arachidonic acids were released upon methanolysis of base-hydrolyzed residues. Approximately 2.1 μg elicitor fatty acids were recovered per mg hydrolyzed residue. In all cases, addition of the base-hydrolyzed residue to the elicitor fatty acids dramatically increased their activity and this activity was significantly greater than the sum of the activities of the separate components.

Centrifugation of a 3-h hydrolyzed residue for 20 min at 10,000*g* resulted in a supernatant which had little or no elicitor activity but which markedly promoted the activity of the elicitor fatty acids to approach that of the unhydrolyzed mycelial sonicate (Table III). Ninety-four per cent of the elicitor activity in the base-hydrolyzed residue remained in the pellet. The activity of the mixture of pellet and elicitor fatty acids was only slightly greater than the sum of the activities of the two suspensions assayed separately. This indicated that most of the promoting material was in the supernatant and that it was essentially free of elicitor activity.

Release of Eicosapentaenoic and Arachidonic Acids by Yeast Lipase. Over 50% of the eicosapentaenoic and arachidonic acids present in the mycelium, as determined by methanolysis, was released by yeast lipase at the end of the assay (Fig. 4). The results are consistent with Sephadex LH-20 and thin layer chromatography, which indicated that most of the activity of the chloroform-soluble material and, hence, the elicitor fatty acids, was associated with the fractions enriched in acylglycerols.

DISCUSSION

Eicosapentaenoic and arachidonic acids were the only compounds with significant elicitor activity detected in mycelial extracts. All fractions with activity, including cell wall preparations,

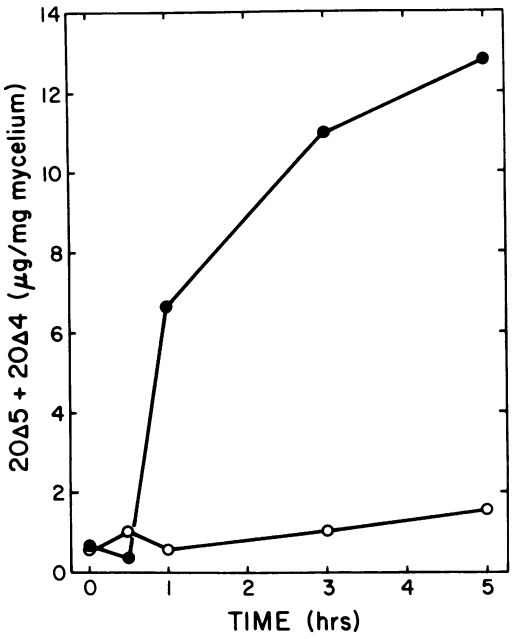

FIG. 4. Release of eicosapentaenoic and arachidonic acids from lyophilized mycelium of *P. infestans* race 4 by yeast lipase. Each value is the mean of two determinations and represents the sum of eicosapentaenoic and arachidonic acids in μg/mg mycelium as determined by GLC of their methyl esters. Mycelium + lipase (●); control (○).

also contained these fatty acids, although the observed activity could not always be accounted for by the amount of elicitor fatty acids present. The elicitor activity of the material released from the cell wall by autoclaving was very low and represented a small portion of the total. The carbohydrate composition of the heat-released material was similar to that found in *P. megasperma* f. sp. glycinea (4). It is conceivable that the glyceollin elicitors released from autoclaved cell wall preparations contain small quantities of elicitor fatty acids because of their reported low rishitin-eliciting activity (6).

Eicosapentaenoic and arachidonic acids at high concentrations did not elicit glyceollin accumulation in soybean cotyledons (J. Kimpel and R. Bostock, unpublished observations, and N. Keen, personal communication), suggesting that the induction of phytoalexin accumulation in potato and soybean tissues is quite different. This is not surprising because pterocarpanoid synthesis requires the involvement of the acetate-malonate, acetate-mevalonate, and shikimate pathways, whereas sesquiterpenoid phytoalexins are derived from the acetate-mevalonate pathway. However, it would be interesting to know if these fatty acids affect prenylation of the isoflavonoid nucleus of glyceollin in soybean tissue.

Base hydrolysis confirmed the presence in the mycelium of factors which promoted the elicitor activity of the fatty acids. Centrifugation of the hydrolysates indicated that the promoting factors were soluble and did not have significant elicitor activity. The promoting factors, when mixed with eicosapentaenoic and arachidonic acids, completely accounted for the activity present in the unhydrolyzed mycelium. The removal of these promoters from lipids containing the elicitor fatty acids during extraction may account for the observed losses in total activity and the rather modest increase in specific activity of the pure fatty acids relative to the mycelial sonicate.

In light of the hydrolysis experiments and the response of tuber slices to specific fatty acids, it seems unlikely that there are other compounds in the mycelium with elicitor activity. During the course of this work, Kurantz and Zacharius (21) reported that significant elicitor activity was observed only when a glucan fraction from the cell wall of *P. infestans* and a lipid extract of pelleted material from an aqueous mycelial homogenate were assayed together. They stated that the lipid extract alone had little or no activity. It is possible that the quantities of eicosapentaenoic and arachidonic acids were very low in their lipid extracts and the inherent elicitor activity of these compounds was overlooked. Our experiments indicate that the elicitor fatty acids alone can induce a response identical with that observed with spores of an incompatible race of *P. infestans*.

Even small amounts of eicosapentaenoic and arachidonic acids, amounts which alone do not elicit detectable quantities of stress metabolites, resulted in significant sesquiterpenoid accumulations if present in combination with the promoting factor(s). The solubility and the behavior of the promoting factor(s) in acid and base and after heat treatment are consistent with those of a polysaccharide. As also observed by Kurantz and Zacharius, the promoting factor(s), if it is a glucan, appears to be more than a simple β-1,3-glucan. Laminaran at 100 and 500 μg/slice (ICN Nutritional Biochemicals) did not promote fatty acid elicitor activity (authors' unpublished data). The nature of the promoting factor(s) is currently under investigation.

Potato lectin quantitatively precipitated activity from a crude, elicitor preparation (13), suggesting that the elicitor fatty acids are associated with compounds containing dimers or trimers of *N*-acetylglucosamine. The reported inhibition by chitobiose of the incompatible reaction (26) could be explained as a hapten inhibition of binding of these complex elicitors to the potato cell.

Henfling *et al.* (18) observed that killed sporangia and cystospores had significantly higher elicitor activity than killed zoospores. Although the authors concluded that an elicitor of sesquiterpenoid phytoalexin accumulation is associated with the cell wall of the fungus, the data also could be interpreted in other ways. One possibility is that, during cell wall synthesis, the promoting factors are produced. Another possibility is that concomitant with cell wall formation is an increase in the amounts of elicitor fatty acids. In other fungi, changes in lipid composition through formation of glycolipid intermediates during cell wall synthesis have been reported (25).

The results of the experiment with yeast lipase support chromatographic observations that the major portion of eicosapentaenoic and arachidonic acids in the mycelium occurs as acyl lipids and not as free fatty acids. The apparent stability of a small portion of the elicitor fatty acids to base hydrolysis for short periods may indicate that this portion is linked to other compounds via amide bonds. The acyl groups of sphingolipids would behave in this manner. Pythiaceous fungi contain high amounts of the unusual phosphonolipid, ceramide aminoethylphosphonate (30), to which the elicitor fatty acids could be *N*-linked. Alkenyl-ether linkages would also be labile to acid but stable to base hydrolysis.

The negative results of experiments with arachidonyl alcohol, the lag in the browning reaction with methyl arachidonate, and the high specific activity of the free fatty acid fraction suggest that the free fatty acid is the active form. The fact that esterified forms are active is not surprising, given that potato tuber is a very rich source for a nonspecific acyl hydrolase and other enzymes capable of deacylating lipids (12). The disruption of lysosomes during the host-parasite interaction could lead to the release of hydrolytic enzymes capable of degrading fungal components to their biologically active forms (32).

The role of these and other fatty acids in plant host-parasite interactions is relatively unexplored. Kato and Misawa (19) observed peroxidation of unsaturated fatty acids as an early event in the hypersensitive response of cowpea leaves to infection by cucumber mosaic virus. Potato tuber contains a very active lipoxygenase (12) and polyunsaturated fatty acids are readily oxidized to hydroperoxides by this enzyme. Perhaps the rate of conversion of the elicitors to hydroperoxides is a key determinant in the

reaction, although in preliminary experiments with α-tocopherol, an antioxidant and inhibitor of lipoxygenase, arachidonic acid elicitor activity was not affected. Unsaturated fatty acids activate polyphenol oxidase from the thylakoid membranes of spinach chloroplasts (15). The formation of toxic quinones by the action of this enzyme on *O*-diphenols during the hypersensitive response might also contribute to disease resistance. The results reported in this paper should lead to definitive studies on the *in vivo* significance of these elicitors and promoter(s) in the host-parasite interaction.

Acknowledgment—The authors wish to express their appreciation to Ms. Etta Nuckles for her capable assistance throughout this investigation.

LITERATURE CITED

1. ALBERSHEIM P, BS VALENT 1978 Host-pathogen interactions in plants. Plants, when exposed to oligosaccharides of fungal origin, defend themselves by accumulating antibiotics. J Cell Biol 78: 627–643
2. AYERS AR, J EBEL, B VALENT, P ALBERSHEIM 1976 Host-pathogen interactions. X. Fractionation and biological activity of an elicitor isolated from the mycelial walls of *Phytophthora megasperma* var. sojae. Plant Physiol 57: 760–765
3. AYERS AR, B VALENT, J EBEL, P ALBERSHEIM 1976 Host-pathogen interactions. XI. Composition and structure of wall-released elicitor fractions. Plant Physiol 57: 766–774
4. BOSTOCK RM 1981 The identification of polyunsaturated fatty acids from *Phytophthora infestans* as elicitors of hypersensitivity in potato tuber and studies of related terpenoid metabolism. PhD thesis. University of Kentucky, Lexington
5. BOSTOCK RM, JA KUĆ, RA LAINE 1981 Eicosapentaenoic and arachidonic acids from *Phytophthora infestans* elicit fungitoxic sesquiterpenes in the potato. Science (Wash DC) 212: 67–69
6. CLINE K, M WADE, P ALBERSHEIM 1978 Host-pathogen interactions. XV. Fungal glucans which elicit phytoalexin accumulation in soybean also elicit the accumulation of phytoalexins in other plants. Plant Physiol 62: 918–921
7. CURRIER WW 1974 Characterization of the induction and suppression of terpenoid accumulation in the potato-*Phytophthora infestans* interaction. PhD thesis. Purdue University, Lafayette, IN
8. DE VRIES B 1963 Quantitative separations of higher fatty acid methyl esters by adsorption chromatography on silica impregnated with silver nitrate. J Am Oil Chem Soc 70: 184–186
9. DUBOIS M, KA GILLIS, JK HAMILTON, PA REBES, F SMITH 1956 Colorimetric method for the determination of sugars and related substances. Anal Chem 28: 350–356
10. ERSEK T 1977 A lipoid component from *Phytophthora infestans* inducing resistance and phytoalexin accumulation in potato tuber. *In* Z Kiraly, ed, Current Topics in Plant Pathology. Akademiai Kiado, Budapest, pp 73–75
11. FOLCH J, M LEES, GH SLOANE STANLEY 1957 A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226: 497–509
12. GALLIARD T 1978 Lipolytic and lipoxygenase enzymes in plants and their action in wounded tissues. *In* G Kahl, ed, Biochemistry of Wounded Plant Tissues. De Gruyter & Co, Berlin, pp 155–201
13. GARAS NA, J KUĆ 1981 Potato lectin lyses zoospores of *Phytophthora infestans* and precipitates elicitors of terpenoid accumulation produced by the fungus. Physiol Plant Pathol 18: 227–237
14. GELLERMAN JL, H SCHLENK 1979 Methyl-directed desaturation of arachidonic to eicosapentaenoic acid in the fungus, *Saprolegnia parasitica*. Biochim Biophys Acta 573: 23–30
15. GOLBECK JH, KV CAMMARATA 1981 Spinach thylakoid polyphenol oxidase. Isolation, activation, and properties of the native chloroplast enzyme. Plant Physiol 67: 977–984
16. HENDERSON SJ, J FRIEND 1979 Increase in PAL and lignin-like compounds as race-specific resistance responses of potato tubers to *Phytophthora infestans*. Phytopathol Z 94: 323–334
17. HENFLING JWDM, J KUĆ 1979 A semi-micro method for the quantitation of sesquiterpenoid stress metabolites in potato tuber tissue. Phytopathology 69: 609–612
18. HENFLING JWDM, RM BOSTOCK, J KUĆ 1980 Cell walls of *Phytophthora infestans* contain an elicitor of terpene accumulation in potato tubers. Phytopathology 70: 772–776
19. KATO S, T MISAWA 1976 Lipid peroxidation during the appearance of hypersensitive reaction in cowpea leaves infected with cucumber mosaic virus. Ann Phytopathol Soc Jpn 42: 472–480
20. KEEN NT 1975 Specific elicitors of plant phytoalexin production: Determinants of race specificity in pathogens? Science (Wash DC) 10: 74–75
21. KURANTZ MJ, RM ZACHARIUS 1981 Hypersensitive response in potato tuber: elicitation by combination of non-eliciting components from *Phytophthora infestans*. Physiol Plant Pathol 18: 67–77
22. LAINE RA, WJ ESSELMAN, C SWEELEY 1972 Gas-liquid chromatography of carbohydrates. Methods Enzymol 28: 159–167
23. LISKER N, J KUĆ 1977 Elicitors of terpenoid accumulation in potato tuber slices. Phytopathology 67: 1356–1359
24. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin-phenol reagent. J Biol Chem 193: 265–275
25. MILLS GL, EC CANTINO 1980 The glycolipid involved in chitin synthesis by zoospores of *Blastocladiella emersonii* is a monoglycosyldiacylglycerol. Exp Mycol 4: 175–180
26. NOZUE M, K TOMIYAMA, N DOKE 1980 Effect of *N,N'*-diacetyl-D-chitobiose, the potato-lectin hapten, and other sugars on hypersensitive reaction of potato tuber cells infected by incompatible and compatible races of *Phytophthora infestans*. Physiol Plant Pathol 17: 221–227
27. PRIVETT OS, E CHRISTENSE NICKELL 1963 Preparation of highly purified fatty acids via liquid-liquid partition chromatography. J Am Oil Chem Soc 40: 189–193
28. VARNS JL, J KUĆ 1971 Suppression of rishitin and phytuberin accumulation and hypersensitive response in potato by compatible races of *Phytophthora infestans*. Phytopathology 61: 178–181
29. VARNS JL, WW CURRIER, J KUĆ 1981 Specificity of rishitin and phytuberin accumulation by potato. Phytopathology 61: 968–971
30. WASSEF MZ, JW HENDRIX 1977 Ceramide amino-ethylphosphonate in the fungus *Pythium prolatum*. Biochim Biophys Acta 486: 172–178
31. WEETE JD 1974 Fungal Lipid Biochemistry. Plenum Press, New York, 393 pp
32. WILSON CL 1973 A lysosomal concept for plant pathology. Annu Rev Phytopathol 11: 247–272
33. Worthington Biochemical Corp (Freehold, NJ) 1972 Worthington Enzyme Manual: Enzymes, Enzyme Reagents, Related Biochemicals