

## Class distribution, fatty acid composition and elicitor activity of *Phytophthora infestans* mycelial lipids

M. J. KURANTZ and S. F. OSMAN

Eastern Regional Research Center\*, Philadelphia, Pennsylvania 19118

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Mycelial lipids from *Phytophthora infestans* were separated by class and the fatty acid composition was determined. Lipid fractions were assayed for elicitor activity by determining rishitin accumulation induced in potato tuber tissue. The rishitin-inducing activity of the fungal lipid fractions were compared with the known fatty acid elicitors, arachidonic and eicosapentaenoic acid. Differences in rishitin-inducing activity were observed between the various lipid classes, the monoglyceride fraction exhibiting the highest and the free acid fraction the lowest activity. A soluble glucan from *P. infestans* mycelia was found to enhance the rishitin-inducing activity of the known fatty acid elicitors as well as the mycelial lipid fractions.

### INTRODUCTION

Cell-free extracts of *Phytophthora infestans* mycelia have been shown to be elicitors of the hypersensitive response (HR) and sesquiterpenoid accumulation in potato tubers [19]. The elicitor-active constituent of extracts of *P. infestans* was associated with cell walls [10] and believed to be, in part, a cell wall glucan [16].

Recently, a lipophilic material from *P. infestans* mycelia was found to elicit phytoalexin accumulation in potato [7]. A relationship between lipid content and elicitor activity of *P. infestans* mycelial extracts was demonstrated in other work [13]. The active lipid components were identified by Bostock *et al.* [4] as arachidonic and eicosapentaenoic acids and these fatty acids, isolated from *P. infestans* mycelia, as well as commercially available samples, proved to be effective elicitors of an HR and phytoalexin accumulation in potato tuber.

The finding that certain fatty acids from a phytopathogenic fungus have such a marked biological effect upon host plant tissue emphasizes the need for further characterization of fungal components. We have examined the class distribution and fatty acid composition of *P. infestans* mycelial lipid in the context of elicitor activity both in the presence and absence of other mycelial components. The objective of this investigation was to determine structure-activity relationships which may ultimately aid in elucidating some of the underlying mechanisms involved in disease resistance of plants to pathogenic organisms.

### MATERIALS AND METHODS

#### *Plant material and pathogen*

*Phytophthora infestans* race 0 was grown on a rye steep 2% agar medium [12] supplemented with 1% sucrose. Cultures of 10–14 days in age were flushed with sterile

water and mycelia and sporangia were dislodged by rubbing with a glass rod. Fernback flasks (2800 ml capacity) containing 1500 ml of the liquid rye steep medium were inoculated with the suspension of mycelia and sporangia. The cultures were incubated statically for 2 days, followed with shaking at 85 r min<sup>-1</sup> for 12 days at 20 °C. Mycelia were harvested after 14 days total incubation time.

All bioassays of fungal material were performed on certified Katahdin seed tubers (Agway Corp\*). Tubers were stored at 10 °C for approximately 4 months and conditioned at room temperature for 24 h before use.

#### *Lipid extraction and fractionation*

The mycelial mass from submerged cultures was collected by filtration on coarse, sintered glass funnels and washed three times with 2 l of deionized water. Mycelia were drained by suction on the glass filter to a constant weight.

The drained mycelial mass (31.8 g fresh weight) was ground in a blender with 200 ml of chloroform:methanol (2:1, v/v), filtered, and the residue extracted twice more, as above. The combined filtrates were dried under a stream of nitrogen and the residue was taken up in chloroform:methanol:water (10:5:3, v/v) [ $\beta$ ]. The chloroform layer was removed, dried over anhydrous magnesium sulphate, filtered and dried under nitrogen to a constant weight.

Crude lipid was dissolved in chloroform and separated into classes by thin-layer chromatography (tlc) [21]. Thin-layer plates, 500  $\mu$ m thick of Silica gel GF (Analtech, Inc) were developed in hexane:diethylether:acetic acid (70:30:1, v/v). Standard lipids (Nu Check Prep, Incorporated) were used for  $R_f$  comparisons in order to locate the various lipid classes on the tlc plate. Appropriate zones were removed and the silica gel was eluted with diethylether; polar lipids were eluted with methanol. The extracts were concentrated to dryness under a stream of nitrogen and their weights determined.

#### *Phytophthora infestans mycolaminaran† (ML)*

*Phytophthora infestans* race 0 was cultured and harvested as described previously. Mycelia were homogenized in a blender with deionized water (10 ml g<sup>-1</sup> fresh weight mycelia) and the homogenate was sonicated by use of a Branson sonifier-cell disrupter (Heat Systems Ultrasonics) set at full power for 10 min. The mixture was autoclaved for 3 h at 120 °C, 15 psi, cooled and centrifuged for 1 h at 48 000 g. The resulting supernatant was filtered through a column (25  $\times$  5 cm) of Sepharose 6B (Pharmacia) to remove any remaining insoluble cellular debris. Material retained on the 6B column was eluted with deionized water and passed through a bed of ion exchange resin (Amberlite, MB-3, Mallinckrodt). The neutral effluent was concentrated by lyophilization and rechromatographed on a Sephadex G-75 column (35  $\times$  2.5 cm) equilibrated and eluted with 0.05 M phosphate buffer, pH 7.3. Column fractions were desalted by membrane dialysis and dried by lyophilization.

#### *Chemical methods*

Aliquots of the various lipid fractions from the tlc class separation (1–20 mg, depend-

\* Reference to brand or firm names does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

† Mycolaminaran (ML) is the term used by Wang and Bartnicki-Garcia [20] to refer to water-soluble  $\beta$ -1,3-glucans obtained from the mycelia of various fungi.

ing upon quantities available) were converted to fatty acid methyl esters (FAMES) by the method of Slover & Lanza [17]. FAMES were analysed by gas-liquid chromatography (glc) using a Hewlett-Packard 5880A instrument equipped with an FID detector and a 100-m open tubular capillary column coated with SP 23-40 phase. The carrier gas was helium with a flow rate of 1 ml min<sup>-1</sup>. The glc method of Maxwell & Marmer [15] was used for analysis of the samples.

Protein and carbohydrate in the Sephadex G-75 fractions were monitored by the methods of Lowry [14] and the phenol-sulphuric acid method [11], respectively. Polysaccharide was hydrolysed by the method of Bartnicki-Garcia [2] and component sugars were determined by glc of the aldonitrile [18] and alditol acetate derivatives [1]. Polysaccharide structure was determined by permethylation analysis [3]. Mass spectra were obtained with a Hewlett-Packard 5992 Gas Chromatograph/Mass Spectrometer (GC/MS).

#### *Tissue treatment and rishitin analysis*

Potato tubers were surface sterilized in 5% NaClO solution for 30 min, rinsed, and cut into discs (4 cm diameter × 0.4 cm thick).

*Phytophthora infestans* lipid fractions were freed of solvent by evaporation and then suspended in sterile distilled water by treatment for 1 min in an ultrasonic bath followed by repeated vortex mixing. When soluble ML was to be assayed with lipids, the appropriate amount of dry ML was dissolved in water, autoclaved and added to the various lipids. All test mixtures were applied to tuber discs in 500 µl of water. Discs were incubated for 5 days at 20 °C. Extraction of tuber discs and rishitin quantitation was the same as described previously [9, 13].

## RESULTS

### *Fractionation of lipids*

The total extractable lipid from the *P. infestans* mycelial mass (31.8 g) accounted for 2.24% of the fresh weight of mycelium. Thin-layer class separation revealed a distribution of lipid classes as listed in Table 1. Triglycerides accounted for the majority of total lipid extracted with polar lipids being the next most abundant.

The fatty acid composition of the various lipid classes is listed in Table 2. The identities of fatty acids were assigned by calculation of relative retention times based upon methyl arachidonate and by use of known FAMES. All lipid fractions that were analysed were also bioassayed on tuber discs.

TABLE 1  
*Distribution of P. infestans mycelial lipid*

Lipid class	Weight (%) <sup>a</sup>
Sterol ester	0.8
Triglyceride	76.2
Free fatty acid	4.9
Sterol	1.3
Diglyceride	5.6
Monoglyceride	0.9
Polar lipid	10.4

<sup>a</sup> Recovery of lipids from thin-layer plates was 95.9%.

TABLE 2  
Fatty acid composition<sup>a</sup> of various *P. infestans* mycelial lipids

Fatty acid	Lipid class <sup>b</sup>					
	SE	TG	FA	DG	MG	PL
14:0	7.3	18.7	11.5	10.5	7.4	2.3
16:0	28.5	23.5	40.1	15.0	20.6	19.2
16:1	0.7	1.9	1.0	2.0	1.5	0.8
18:0	6.4	2.3	5.7	2.2	4.4	3.0
18:1	20.7	14.4	11.3	17.4	15.1	8.1
18:2	6.3	17.9	11.5	22.8	14.4	18.1
18:3	3.6	1.6	2.8	1.9	1.4	1.0
20:0	1.4	1.9	0.9	2.1	0.7	1.2
20:3	1.0	1.3	1.2	2.2	1.5	2.3
20:4	11.4	4.4	6.4	6.7	11.7	24.5
20:5	0.8	9.5	3.5	13.6	7.8	12.8
22:0	—	—	0.4	0.3	—	0.3
22:4	1.1	—	—	—	—	0.6
22:5	—	0.1	—	0.2	—	0.2
Unsaturated (%)	51.1	52.4	39.3	68.9	61.7	72.5

<sup>a</sup> Fatty acids are reported as the percentage area of total fatty acids per fraction, determined by peak integration.

<sup>b</sup> SE, sterol ester; TG, triglyceride; FA, free acid; DG, diglyceride; MG, monoglyceride; PL, polar lipid.

<sup>c</sup> Percentage unsaturated fatty acids was calculated as a percentage of total acids listed.

Qualitatively, with the exception of minor amounts of the C<sub>22</sub> fatty acids, all fungal lipid classes were similar in composition. Quantitatively the proportion of certain fatty acids varied considerably with lipid class. This is reflected in the calculated percentages of saturated and unsaturated acids listed in the table. The polar lipids and partial glycerides contained the highest percentage of unsaturated acids, while the free fatty acid fraction contained a high proportion of saturated acids. The elicitor fatty acids, arachidonic (20:4) and eicosapentaenoic (20:5), were present in all lipid classes, with the exception of the sterol fraction, and were a high proportion of the total fatty acid content.

#### Composition of ML

Earlier work in this laboratory demonstrated that insoluble cell wall and a water-soluble fraction, both from *P. infestans* mycelia, enhanced the elicitor activity of arachidonic and eicosapentaenoic acid (unpublished observations). The soluble mycelial preparation was partially purified by gel filtration, ion exchange, and finally Sephadex G-75 gel permeation chromatography. Two fractions were obtained from the Sephadex G-75 column, a small peak in the void volume and a single peak retained by the gel (elution volume = 20 ml). The composition of each peak is listed in Table 3; on the basis of composition and Sephadex G-75 chromatography this material corresponds almost identically with the ML from *P. infestans* mycelia characterized by Wang & Bartnicki-Garcia [20].

TABLE 3  
Chemical composition of G-75 column fractions from *P. infestans* mycelia

Fraction	Protein (%)	Carbohydrate composition (%) <sup>a</sup>		Glucan structure (%) <sup>a</sup>	
1 (void)	8.8	Glucose	97	Unsubstituted glucose	18
		Mannose	2	3-Substituted glucose	63
		Arabinose	Trace	6-Substituted glucose	18
				3,6-Substituted glucose <sup>b</sup>	
2 (retained)	11.1	Glucose	>95	Unsubstituted glucose	20.2
		Mannose	Trace	3-Substituted glucose	74
		Arabinose	Trace	6-Substituted glucose	6
				3,6-Substituted glucose <sup>b</sup>	

<sup>a</sup> Based on relative peak areas.

<sup>b</sup> Identity could not be confirmed by GC/MS due to insufficient quantities.

TABLE 4  
Elicitor activity of arachidonic and eicosapentaenoic acid standards in his presence and absence of *P. infestans* ML

Treatment	Rishitin <sup>a</sup> ( $\mu\text{g g}^{-1}$ dry weight tuber tissue)	
	AA <sup>b</sup>	AA+2.5 mg ML <sup>c</sup>
AA ( $\mu\text{g}$ per disc)		
0.5	0.9 $\pm$ 0.2	0.8 $\pm$ 0.1
1.0	1.4 $\pm$ 0.5	1.3 $\pm$ 0.1
10.0	9.8 $\pm$ 3.0	12.2 $\pm$ 1.0
25.0	21 $\pm$ 2.4	33 $\pm$ 6.0
50.0	30 $\pm$ 4.0	115 $\pm$ 12
	EPA <sup>d</sup>	EPA+2.5 mg ML <sup>e</sup>
EPA ( $\mu\text{g}$ per disc)		
0.5	Tr <sup>f</sup>	1.8 $\pm$ 0.6
1.0	1.1 $\pm$ 0.1	2.0 $\pm$ 1.0
10.0	6.2 $\pm$ 1.0	36.4 $\pm$ 1.2
25.0	20 $\pm$ 2.0	57.2 $\pm$ 16
50.0	36 $\pm$ 8.0	119 $\pm$ 10

<sup>a</sup> Rishitin is reported as mean  $\pm$  s.d. of three determinations.

<sup>b</sup> AA=arachidonic acid.

<sup>c</sup> AA+2.5 mg ML = arachidonic acid at the concentration shown at left plus 2.5 mg disc<sup>-1</sup> *P. infestans* ML.

<sup>d</sup> EPA=eicosapentaenoic acid.

<sup>e</sup> EPA+2.5 mg ML = eicosapentaenoic acid plus 2.5 mg per disc of *P. infestans* ML.

<sup>f</sup> Tr=Trace, detected by tlc.

#### Elicitor activity of fatty acids

Commercially available arachidonic and eicosapentaenoic acid were applied with and without *P. infestans* ML, to potato tuber discs. Known fatty acids were purified by tlc before use in the tuber disc bioassay (Methods). Concentrations of the acids per disc were representative of the actual amounts of elicitor acids (arachidonic + eicosapentaenoic) found to be present in the lipid fractions.

Elicitor activity was determined by measurement of rishitin accumulation in treated potato tuber discs. We preferred to monitor rishitin exclusively since other sesquiterpenoid stress metabolites, particularly lubimin, were occasionally detected in untreated control discs, while rishitin was not detected in control tissues.

Table 4 shows that both acids tested were active in eliciting rishitin production at all concentrations applied to tuber discs. Rishitin production was, in all cases, accompanied by browning and necrosis of the tuber tissue.

The *P. infestans* ML, in most cases, caused enhanced accumulation of rishitin when applied with the elicitor fatty acids. The presence of ML appeared to cause the greatest enhancement of activity when the concentration of fatty acid was greater than 1.0 µg tuber per disc. The ML applied alone at 2.5, 5.0 and 10 mg tuber per disc failed to elicit rishitin accumulation or tissue necrosis.

#### *Elicitor activity of mycelial lipids*

The various mycelial lipid fractions were assayed for activity on tuber discs at a concentration of 500 µg lipid fraction per disc. Assays were performed on lipids alone and as a mixture with *P. infestans* ML. The mycelial lipids used in the bioassay were from the same material used in the chemical analysis presented earlier.

Table 5 illustrates the results of the elicitor activity assay of the various fungal lipid classes. In these experiments tuber tissue browning and necrosis was again associated with rishitin accumulation.

TABLE 5  
*Elicitor activity of various lipids from P. infestans mycelia in the presence and absence of P. infestans ML*

Lipid classes	Rishitin <sup>a</sup> (µg g <sup>-1</sup> dry weight tuber tissue)				
	Lipid fraction (500 µg)	Specific activity <sup>b</sup>	Lipid fraction <sup>c</sup> (500 µg) ML (+2.5 mg)	Specific activity	Elicitor <sup>d</sup> acids per fraction (µg)
Sterol ester	ND <sup>e</sup>	—	Tr <sup>f</sup>	—	2.0
Triglyceride	3.2 ± 0.8	0.157	11 ± 2.0	0.539	20.4
Free acid	2.0 ± 0.6	0.057	7.5 ± 1.2	0.216	34.8
Sterol	ND	ND		ND	ND
Diglyceride	24 ± 6.0	0.463	53 ± 2.0	1.02	51.8
Monoglyceride	7.8 ± 1.6	0.670	23 ± 7.0	1.96	11.7
Polar lipid	9.0 ± 1.0	0.179	29 ± 2.0	0.578	50.2

<sup>a</sup> Rishitin is reported as the mean ± s.d. of triplicate determinations from two separate experiments.

<sup>b</sup> Specific activity is calculated as the ratio of rishitin to eicosapentaenoic + arachidonic acid present in each lipid fraction.

<sup>c</sup> 2.5 mg of *P. infestans* ML.

<sup>d</sup> Elicitor acids (µg) is the sum of arachidonic and eicosapentaenoic acid in 500 µg of the appropriate lipid fraction. These values were calculated from standard curves obtained by using standard methyl esters of arachidonic and eicosapentaenoic acid.

<sup>e</sup> ND = None detected.

<sup>f</sup> Tr = trace, detected by tlc.

Lipid fractions applied alone to tuber discs showed elicitor activity as reflected by rishitin accumulation. The sterol and sterol ester fractions were the only exceptions. With ML present, however, the sterol ester, demonstrated elicitor activity but the sterol fraction did not. All other lipid fractions showed an enhancement of elicitor activity when ML was present on discs.

Comparison of these rishitin values with those produced by treatment with the standard fatty acids, revealed diminished rishitin accumulation by all active fractions. The actual amounts of arachidonic and eicosapentaenoic acid found in 500  $\mu\text{g}$  of each lipid fraction is also shown in Table 5. In addition, there appear to be some differences in activity associated with lipid class. On the basis of specific activity ( $\mu\text{g}$  rishitin  $\text{g}^{-1}$  dry weight tuber tissue  $\mu\text{g}^{-1}$  elicitor acids), the monoglyceride fraction is the most active followed closely by the diglyceride fraction. The least active fraction by comparison of specific activities was the free acid fraction. These differences in specific activity were retained even when lipid fractions were applied to tuber discs with the mycelial ML. In all cases, overall activity, as determined by rishitin levels, increased but the relative order of activity of the various lipid classes remained unchanged.

## DISCUSSION

Arachidonic and eicosapentaenoic acid, both present in the lipid fraction of *P. infestans* mycelia but not found in potato tuber, elicit phytoalexin accumulation and hypersensitive cell death of potato tuber tissue [4].

Our analysis of the *P. infestans* mycelial lipid indicate that all lipid classes contained arachidonic and eicosapentaenoic acid; however, no direct correlation between the concentrations of these acids in the various fractions and the amount of rishitin produced was found. The fungal lipid fraction which was determined to have the highest specific activity (monoglycerides), caused accumulation of significantly less rishitin than the pure acids at similar concentrations. The arachidonic and eicosapentaenoic acid in the *P. infestans* free acid fraction had much lower specific activities than the pure acids. These facts suggest that the other fatty acids or some undetected fungal component of the lipid fractions attenuate the activity of arachidonic and eicosapentaenoic acid with respect to hypersensitivity and rishitin accumulation. Differences in specific activity between the various forms of esterified arachidonic and eicosapentaenoic acids were also found. It was not clear from this work, however, whether glyceride structure *per se*, or attenuation by other components present in the fractions were responsible for this effect.

In agreement with the results of Bostock, Laine & Kuć [5], we have also found that a non-lipid fraction from *P. infestans* mycelia enhanced the elicitor activity of the active lipid fractions. Partial purification and characterization of this material demonstrated that enhancement activity was associated with an ML fraction analogous to the *P. infestans* mycolaminaran previously described by Wang & Bartnicki-Garcia [20]. Activity of all *P. infestans* active lipid fractions were enhanced when the ML was present and the order of specific activities of the various lipids was maintained. The ML itself, did not elicit rishitin accumulation or hypersensitivity in contrast to other systems in which such materials are active [6].

We are presently investigating the influence of composition and structure on

elicitor activity of fungal materials and whether or not these factors are relevant in the actual host-pathogen interaction.

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