

## POTATO PHYTOALEXIN ELICITORS IN *PHYTOPHTHORA INFESTANS* SPORE GERMINATION FLUIDS

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Media from germinating spores of *Phytophthora infestans* contain substances that elicit accumulation of the phytoalexin rishitin in potato tuber slices. Gel permeation chromatography of media extracts indicates the presence of several substances. The active substances can be precipitated with ammonium sulfate, are heat labile and pronase-sensitive, which suggests that they are proteins.

*Key words:* *Phytophthora infestans*; phytoalexin rishitin; potato; ammonium sulfate

### Introduction

Incompatible host-pathogen interactions generally are characterized by hypersensitive cell death and concomitant phytoalexin accumulation. Although the mechanism by which the hypersensitive response is initiated in vivo has not been elucidated, a variety of materials including bacterial and fungal extracts have been shown to induce this response and phytoalexin accumulation [1]. Carbohydrate [2] and glycoprotein [3] elicitors of soybean phytoalexins have been isolated from *P. megasperma* f. sp. *glycinea*; a peptide that elicits phaseollin production in green bean has been isolated from the fungus *Monilinia fructicola* [4], and arachidonic acid, which is a lipid component of some fungi, including the potato pathogen *P. infestans*, has been shown to elicit production of the potato phytoalexins rishitin and lubimin in potato tuber tissue [5]. With the

possible exception of the glycoproteins isolated by Keen [3], these molecules are not determinants of race specificity, i.e. they are found in all races or strains of the microorganism and they elicit phytoalexins on all cultivars of a particular plant species.

In our investigation of race specificity in the interaction of *P. infestans* with potato, the main interest has been in the initial recognition event which determines whether a resistant (hypersensitive) or a susceptible reaction will occur. Although arachidonic acid (or the esterified form) has been shown to act as an elicitor, it has not been demonstrated to be responsible for the hypersensitive response in the interaction in vivo. Little research on elicitors from intact fungal tissue has been conducted since early attempts to isolate elicitors from culture filtrates of *P. infestans* proved unsuccessful [6]. In our study of extracellular enzymes present in *P. infestans* spore germination fluids, we observed a necrotic reaction when these solutions were applied to potato tuber slices which has led us to examine the elicitor activity of this solution.

Abbreviations: FAMES, fatty acid methyl esters; GC/MS, gas chromatography-mass spectrometry; GPC, gel permeation chromatography; TLC, thin-layer chromatography.

## Materials and methods

### Materials

Mature potato tubers (*Solanum tuberosum* cv. Katahdin) were kindly supplied by Dr. R. Webb, Agricultural Research Service, Beltsville, Maryland. An isolate of *P. infestans* (Mont.) de Bary (Race 1,2) was obtained from Dr. R. Goth, Agricultural Research Service, Beltsville, MD and maintained on corn seed media [7] to help retain virulence. Silica gel G plates (Analtech.\*) were used for thin-layer chromatography (TLC) and a Hewlett Packard Model 5990B spectrometer fitted with a 25 M OV-101 capillary column was used for gas chromatography-mass spectrometry (GC/MS). Fatty acid methyl esters and immobilized pronase were obtained from Sigma Chemical Company, St. Louis, MS. Ultrafiltration was accomplished with an Amicon Model 202 ultrafiltration apparatus. Spectrophotometry was carried out with a Beckman Model 35 spectrophotometer.

### Preparation and germination of *P. infestans* spores

*P. infestans*-infected corn seeds were used to inoculate Petri dishes containing 10 ml lima bean agar (Frozen Fordhook lima beans (300 g), 1 l H<sub>2</sub>O and 20 g agar were homogenized for 1 min at high speed with a Waring Blendor and autoclaved for 20 min). After 14 days at 14°C sporangia were harvested by flushing the surface of the agar with 15 ml of distilled water and lightly scraping with a bent glass rod. Mycelia were removed by filtering the sporangia preparation through two layers of cheesecloth. Sporangial suspensions (30-ml aliquots) were transferred to clean Petri dishes and incubated uncovered at 14°C for 2–3 h. Empty sporangia were removed by filtering the motile zoospores through Whatman No. 4 filter paper. The zoospore suspensions (1–3 × 10<sup>6</sup> spores/ml) were vortexed for 60 s to induce

synchronous encystment. Spores were transferred to clean Petri dishes and allowed to germinate at 14°C for up to 20 h. The germination fluid was then decanted and filtered through filter paper (Whatman No. 5, 2.5 μm pore size) to remove spores. Although aseptic techniques were not used during the preparation and germination of spores, there was no evidence of bacterial contamination using light microscopy (600 power). The filtrate was concentrated 40–100-fold by ultrafiltration (PM-10 filter).

### Gel permeation chromatography (GPC) of spore germination medium and enzyme assay

A 5.0-ml sample of spore germination fluid concentrate was applied to a column (50 × 2 cm) of LKB ultragel AcA-44 (130 000 mol. wt. exclusion) that had been equilibrated with 20 mM potassium phosphate buffer (pH 7). The column was eluted with the equilibrating buffer (flow rate; 50 ml/h) and 5-ml fractions were collected. Fractions 6–10 which made up the void volume were pooled and concentrated to 5 ml by ultrafiltration. Fractions 11–20, 21–28, and 29–40 were treated in an identical manner and were designated high, medium and low molecular weight cuts, respectively. The total protein and carbohydrate content of the high molecular weight fraction was determined by the Markwell-Lowry method [8] and anthrone method [9], respectively. Esterase activity was measured at pH 8 with *p*-nitrophenyl butyrate as previously described [10]. β-Glucosidase activity was measured with 4-methyl-umbelliferyl-β-glucose (4 Mu-Glu). The reaction mixture contained 1 mM 4 Mu-Glu, 100 mM potassium phosphate buffer (pH 7) and enzyme. Hydrolysis was measured continuously at 25°C with a Sequoia-Turner Model 450 fluorometer equipped with a A<sub>360 nm</sub> excitation filter and a A<sub>>415 nm</sub> emission filter.

### Bioassay of GPC fractions

Tuber slices that had been preconditioned for 6 h at room temperature were inoculated with 150 μl of GPC fractions (per slice). After incubation for 72–96 h in the dark at 19°C the

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

slices were cut into quarters and freeze dried. The dried slices were extracted twice with chloroform/methanol (1:1 v/v) in a Waring blender (100 ml per five slices was used for each extraction). The combined extracts were filtered through Whatman No. 1 filter paper, the filtrate concentrated to dryness under a stream of N<sub>2</sub> at room temperature, and the residue was dissolved in 1 ml of methanol; the insoluble material was separated by centrifugation at 500 × g for 5 min at 23°C. A 10-μl aliquot of the clarified supernatant fluid was analyzed by silica gel thin-layer chromatography using cyclohexane/ethyl acetate (1:1 v/v) as the mobile phase. The rishitin area of the TLC plate was scraped off and extracted with ethyl acetate. The ethyl acetate solution was concentrated to dryness and the residue dissolved in 100 μl of pyridine followed by the addition of 50 μl of acetic anhydride and the solution was heated for 30 min at 70°C. After concentration under a stream of N<sub>2</sub> at room temperature the presumptive rishitin diacetate was dissolved in CHCl<sub>3</sub> (100 μl) and analyzed by GC/MS.

#### Fatty acid analysis

GPC aliquots were dried, taken up in 5 ml of 5% methanolic sodium hydroxide, and heated for 10 min at 100°C. After cooling, 5 ml of a

solution containing 14% boron trifluoride in methanol was added and the solution was reheated for 5 min at 100°C, the solution was then cooled and the fatty acid methyl esters (FAMES) were extracted 3 times with isooctane. The pooled isooctane extracts were dried over anhydrous magnesium sulfate, filtered through Whatman No. 1 filter paper, and concentrated under a stream of N<sub>2</sub> at room temperature. Samples were diluted to the appropriate volume with methylene chloride and analyzed by GC/MS.

#### Results and discussion

The elicitor activity of the germination fluid concentrate is shown with respect to rishitin production in Table I. Rishitin values are low compared to results obtained elsewhere [11], however, they are significantly above control values and compare favorably with the rishitin values obtained with arachidonic acid. The low rishitin values may reflect the condition of the potato tubers used in these experiments. The elicitor-active constituents in the germination fluid were ammonium sulfate precipitable and labile to heat and pronase treatment, which suggests that they are proteins (or glycoproteins). Enhancement of the elicitor activity by ammonium sulfate precipitation (Table I) may

**Table I.** Elicitor activity of germination fluid concentrates and arachidonic acid.

Elicitor	Rishitin concentration (μg/g fresh wt. of tuber tissue) <sup>a</sup>		
	Expt. 1	Expt. 2	Expt. 3
Germination fluid	8.0 ± 1	5.0 ± 1	11 ± 2
Germination fluid (75% ammonium sulfate ppt)	14.0 ± 1	—	—
Germination fluid (boiled)	ND <sup>b</sup>	ND	2.4 ± 0.1
Germination fluid <sup>c</sup> (pronase-treated)	ND	ND	—
Arachidonic acid (200 μg)	5.1 ± 1.5	4.8 ± 1.5	—
Control (H <sub>2</sub> O)	ND	ND	ND

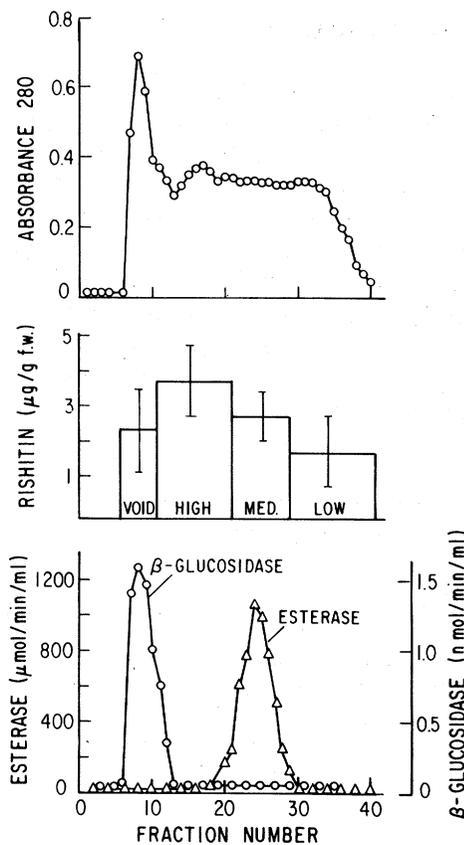
<sup>a</sup>Data for each experiment represents the mean of three repetitions (7 slices per repetition per experiment) ± S.D. Slices were from Katahdin tubers.

<sup>b</sup>ND None detected.

<sup>c</sup>Germination fluid (2 ml) was shaken with 15 mg insoluble (immobilized) pronase for 6 h at 25°C and then the pronase was removed by filtration through glass wool.

result from the removal of elicitor inhibitors (repressors) such as those described by Duke [12].

The germination fluid was fractionated into four molecular weight fractions designated void, high, medium and low (Fig. 1). Although each of these fractions elicited rishitin in tuber slices, use of the high molecular weight fraction resulted in consistently higher rishitin values. This suggests that more than one species has elicitor activity. When  $\beta$ -glucosidase and esterase activities were assayed in the



**Fig. 1.** Fractionation of concentrated germination medium by gel filtration on an LKB-ultragel AcA-44 column. Protein was estimated by measuring the absorbance at 280 nm. Fractions were pooled to yield four molecular weight fractions; each was concentrated to 5 ml, applied to tuber slices, and rishitin was quantitated after 72–96 h. Each rishitin value is the mean  $\pm$ S.D. Esterase and  $\beta$ -glucosidase activities were measured as described in Materials and methods.

column fractions (Fig. 1), the former was eluted in the void ( $>130\,000$  mol. wt.) fraction and the latter in the medium fraction. None of the enzymes that we have tested are eluted in the high molecular weight fraction which elicited the most rishitin. The high molecular weight fraction contained 0.94 mg/ml protein and 0.80 mg/ml carbohydrate (hexose).

Since it has been demonstrated that arachidonic and eicosapentaenoic acids, both components of *P. infestans*, elicit rishitin and lubimin accumulation [5] the fatty acid composition of the germination fluid and GPC fractions was determined (Table II). Although some arachidonic acid was present in the unfractionated samples, it could not be detected in any of the GPC fractions. The sensitivity of the arachidonic acid assay was such that less than 1  $\mu$ g arachidonic acid per 100  $\mu$ l of fraction could be detected.

The formation of endogenous elicitors in plant tissue by the action of fungal pectinases appears to be a common mode of phytoalexin induction [13,14]. In an analysis of specific enzymes in the spore germination fluid there was no detectable polygalacturonase or polygalacturonic acid lyase activity [15] which argues against such a mechanism in this case.

**Table II.** Fatty acid composition of spore germination fluid fractions.

	GM <sup>a</sup>	Void <sup>b</sup>	High	Med	Low
C <sub>14:0</sub>	13 <sup>c</sup>	ND <sup>d</sup>	ND	ND	ND
C <sub>14:1</sub>	15	ND			
C <sub>16:0</sub>	100	100	100	100	100
C <sub>18:0</sub>	43	30	50	20	ND
C <sub>18:1</sub> , C <sub>18:2</sub>	54	30	30	ND	ND
C <sub>20:4</sub>	20	ND	ND	ND	ND
C <sub>20:5</sub>	ND	ND	ND	ND	ND

<sup>a</sup>GM, germination medium.

<sup>b</sup>Column fractions described in Materials and methods. High, medium and low refer to molecular weight ranges of material eluted.

<sup>c</sup>Values are percent referred to C<sub>16:0</sub> taken as 100 for each fraction. Analysis sensitivity was such that 10 ng of fatty acid could be detected. An aliquot corresponding to 10% of the total sample was analyzed.

<sup>d</sup>ND, none detected.

In conclusion we have found that the media in which spores of *P. infestans* are germinated contain compounds that elicit the accumulation of the phytoalexin rishitin in potato slices. The evidence suggests that more than one molecular weight species (different proteins or protein aggregates) have activity. The elicitor activity of these solutions is not due to arachidonic acid or pectinases.

The purification of the elicitors, their identification and their specificity are subjects for future work.

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