Effect of Ethylene on Phytuberin and Phytuberol Accumulation in Potato Tuber Slices

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ABSTRACT

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Potato slices treated with Ethrel (2-chloroethylphosphonic acid) and then inoculated with *Helminthosporium victoriae*, *Helminthosporium carbonum*, or an incompatible race of *Phytophthora infestans*, accumulated considerably more phytuberin and phytuberol (a deacetylated derivative of phytuberin) than did slices treated with water followed by inoculation. The accumulation of the two terpenoids also was increased in slices treated with Ethrel followed by cell-free, autoclaved sonicates of *Pythium aphanidermatum* or *Achlya flagellata*. Ethrel did not elicit consistent changes in the accumulation of rishitin and lubimin in tissue accumulating high concentrations of phytuberin and phytuberol. Ethrel did not increase the accumulation of phytuberin, phytuberol, rishitin, or lubimin in noninoculated slices, slices inoculated with compatible races of *P. infestans* or slices treated with sonicates of *H. carbonum*, *H. victoriae*, or *Neurospora crassa*. More phytuberin and phytuberol accumulated in slices treated with Ethrel followed by a sonicate of *P. infestans* at 19 C than at 14 or 25 C, and the terpenoids were not detected in tissues incubated at 30 and 36 C. Ethylene (100 μliters/liter) markedly increased the accumulation of phytuberin and phytuberol in slices treated with cell-free sonicates of *P. infestans*, but alone it did not elicit accumulation of the terpenoids. Ethrel did not alter the resistance or susceptibility of the potato cultivars Kennebec (R_1) or Russet Burbank (r) to race 4 and 1.2.3.4. of *P. infestans* or the resistance of both cultivars to *H. victoriae* and *H. carbonum*.

Additional key words: phytoalexins, plant-parasite interaction.

The terpenoid phytoalexins rishitin, phytuberin, and lubimin have been associated with the resistance of potato to *Phytophthora infestans* (Mont.) de By. (18, 21, 22, 23, 24). They also accumulate in potatoes inoculated with fungi nonpathogenic on potato (23), bacteria (2, 17) and cell-free sonicates of compatible and incompatible races of *P. infestans* (23). Whereas the chemical structures of rishitin, phytuberin, and lubimin have been established, phytuberol appears to be an unsaturated sesquiterpenoid alcohol that is a deacetylated derivative of phytuberin but not deacetyl phytuberin (7, 17).

The alteration of plant metabolism by ethylene has been extensively studied (1). Ethylene and Ethrel (2-chloroethylphosphonic acid), an ethylene-releasing compound (9, 25), elicit the accumulation of the phytoalexins 6-methoxymellein in carrot roots (3, 5, 14), pisatin in pea pods (6), and chlorogenic acid in sweet potato roots (13). Ethylene, however, did not elicit the accumulation of ipomeamarone in sweet potato roots (10) and apparently shunted ipomeamarone or an intermediate of ipomeamarone to 4-impomeanol and 1-ipomeanol (10, 11). Ethylene reduced the accumulation of ipomeamarone in sweet potato slices infected with *Ceratocystis fimbriata* (11), but enhanced its accumulation in slices treated with mercuric chloride (10). Ethylene did not affect resistance in some (4), and increased or decreased resistance in other plant-parasite interactions (1, 12). Preliminary studies by Shih (19) suggested that Ethrel increased the accumulation of phytuberin in potato slices subsequently inoculated with an incompatible race of *P. infestans*. The purpose of this investigation was to study the effect of Ethrel and ethylene on (i) the accumulation of phytuberin and phytuberol, and (ii) the resistance and susceptibility of potato tuber slices to various fungi.

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MATERIALS AND METHODS

Fungus and host.—Unless stated otherwise, all fungi were obtained from stock cultures maintained in our laboratory. Races 4 and 1.2.3.4. of *P. infestans* (obtained from L. Black, Louisiana State University) were maintained on lima bean agar at 19 C and transferred weekly. Zoospores for inoculations were obtained from suspensions of zoosporangia containing 5×10^5 sporangia per ml (8). Mycelial mats for the preparation of sonicates were obtained from 10-day-old cultures of race 4 grown on lima bean broth (200 g homogenized frozen lima beans, 1 g glucose per liter of distilled water). Mats were washed thoroughly with tap water followed by distilled water and stored at −20 C. The frozen material

was homogenized twice in five volumes (w/v) of distilled water in a Waring Blendor. The homogenate then was sonicated three times (4-min intervals of sonication) in a Model W 185 Sonifier Cell disruptor (Heat Systems-Ultrasonics, Inc., Plainview, L.I., NY 11803) at 60 W output. The vial containing the homogenized mycelium was kept in an ice-bath during sonication. Examination of the sonicates under a light microscope revealed that more than 75% of the cells were disrupted. The sonicates were autoclaved at 121 C for 5 min to increase the terpenoid-eliciting activity (16), cooled to room temperature, and applied to the upper surface of potato tuber slices. Autoclaved sonicates did not contain viable cells when plated on lima bean agar. The concentration of sonicates was approximately 1 g (fresh weight) of fungus per 5 ml.

Pythium aphanidermatum (Edson) Fitz. was grown on V-8 broth (200 ml V-8 juice per liter of distilled water adjusted to pH 6.0 with 0.5*N* NaOH) for 6 days at 24 C. *Achlya flagellata* Coker was grown on glucose-yeast broth (20 g glucose, 5 g Bacto peptone, and 5 g Bacto yeast extract per liter of distilled water) for 6 days at 19 C. *Helminthosporium carbonum* Ullstrup race 1, *Helminthosporium victoriae* Meehan & Murphy (isolate HW-1), and *Neurospora crassa* Shear & Dodge were grown on glucose-yeast broth for 8 days at 24 C. Mycelial mats were harvested and sonicated as described for *P. infestans*. To obtain spores, *H. victoriae* and *H. carbonum* were grown for 1 wk at 22-24 C on oatmeal and potato-dextrose agar, respectively. Conidia were obtained by adding water to cultures and rubbing their surface with a glass rod. The concentrations were adjusted with water to 10^6 conidia per ml.

The potato cultivars Kennebec (R_1) (compatible with 1.2.3.4. and incompatible with race 4 of *P. infestans*) and Russet Burbank (compatible with both races) were used in the experiments. *Pythium aphanidermatum* is a pathogen of many hosts including potato. *Neurospora crassa* and *A. flagellata* are saprophytes, and *H. carbonum* and *H. victoriae* are pathogens of corn and oats, respectively. Potatoes were stored at 4 C and brought to room temperature 1 day prior to use. Slices were prepared as described by Kuć et al. (15). Slices of each tuber were distributed among all treatments in each experiment. Three or four slices were placed on disks of moist filter paper in petri dishes (14-cm diam). Twenty to 30 slices were used per treatment, and all experiments were repeated at least twice. Approximately 0.3 ml of water, 0.042 M Ethrel or Ethrel hydrolyzed with 0.10 M NaOH for 3 hr and adjusted to pH 3 with HCl (final concentration equivalent to 0.042 M Ethrel) were spread over the upper surface of each slice. After 8 hr, the same surface was covered with approximately 0.3 ml of a sonicate or a suspension of zoospores or conidia. Slices were incubated at 18-19 C except as indicated. An 8-hr interval between Ethrel application and treatment with sonicates or inoculation with fungi and a concentration of 0.042 M Ethrel were found to be optimal for the accumulation of phytuberin and phytuberol. Intervals tested were 1, 6, 8, 12, and 24 hr and concentrations tested were 0.007, 0.042, 0.084, and 0.168 M.

In the experiments with ethylene, potato slices 7 mm thick and 3 cm diameter were placed in an open petri dish and incubated in a 10-liter desiccator in which the atmosphere was saturated with water. Ethylene was injected into desiccators through self-sealing stoppers. Slices were incubated for 72 hr at 18-19 C.

Extraction and quantitation of terpenoids. At the end of the incubation period, the upper 1 mm of tuber slices was removed, weighed, and homogenized in methanol (1 g fresh weight tissue per 5 ml methanol) with a Waring Blendor. The homogenate was filtered on Whatman No. 2 filter paper under reduced pressure, the residue was homogenized in methanol, and the homogenate was refiltered. The filtrates were combined and concentrated almost to dryness under reduced pressure at <40 C. Chloroform, water, and 7.5% acetic acid (200:200:1, v/v) were added to the concentrate and the terpenoids were partitioned into chloroform. The chloroform was evaporated under reduced pressure to dryness and the residue was suspended in methanol (the equivalent of 10 g fresh weight of potato tissue per milliliter) and centrifuged at 600 *g* for 10 min. The clear yellow supernatant liquid was used for quantitative and qualitative analyses of terpenoids. Terpenoids were quantitated by gas-liquid chromatography (GLC) with a Series 1400 Varian Gas Chromatograph equipped with a 180 cm × 0.64 cm OD 2 mm ID Pyrex glass column packed with 3% OV-225 on 177-149 μm particle size (80/100-mesh) Supelcoport (Supelco, Inc., Bellefonte, PA 16823) and a flame ionization detector. Nitrogen at a rate of 40 ml/min was used as the carrier gas. Temperatures of injection port, oven, and detector were 230, 180, and 255 C, respectively. One-μliter samples of the methanol extracts were injected and quantities of the various terpenoids were determined on the basis of peak heights relative to that of the internal standard, methyl arachidate.

The presence of the terpenoids was further confirmed by thin-layer chromatography using 20 × 20-cm plates, coated with a 250-μm layer of silica-gel G, developed in ethyl acetate:cyclohexane 1:1 (v/v). Terpenoids were detected by spraying the plates with concentrated sulfuric acid, vanillin-sulfuric acid, or a saturated solution of antimony trichloride in 60% perchloric acid. Plates were heated for 5 min at 90-100 C after spraying.

In experiments with ethylene, the upper 1-mm of slices were cut into eight segments. The segments were mixed and samples (2 g) were placed overnight in test tubes containing 20 ml methanol. After the methanol was removed by siphoning, the segments were again washed twice with 10-ml volumes of methanol. The methanol extracts were combined and brought to dryness under reduced pressure at 45 C. Five ml of water was added to the residue and the mixture was extracted three times with 5-ml volumes of hexane. The combined hexane fractions were dried and 60-75 μg of methyl-arachidate in *n*-hexane were added to the residue. The mixture was made up to 0.3 ml with *n*-hexane and used for quantitation by GLC as described.

RESULTS

Inoculation with living fungi.— Potato slices treated either with water or Ethrel and subsequently inoculated with zoospores of a compatible race of *P. infestans* (Kennebec with race 1.2.3.4. and Russet Burbank race

1.2.3.4. or 4) did not turn brown, and microscopic investigation revealed that the fungus had ramified through the tissue 60 hr after inoculation. Abundant sporulation and mycelial growth were evident on the surface of the slices 84 hr after inoculation. Little or no phytuberin, phytuberol, rishitin, and lubimin were detected in extracts of these tissues (Tables 1, 3).

The surfaces of potato slices treated either with water or Ethrel and subsequently inoculated with an incompatible race of *P. infestans* (Kennebec with race 4) were intensely browned 72 hr after inoculation. Little or no mycelial development occurred in the tissues and the fungus did not sporulate on these tissues. The accumulation of phytuberin and phytuberol were markedly enhanced and rishitin and lubimin accumulation remained unchanged or decreased in tissues treated with Ethrel (Tables 1, 3).

The surfaces of potato slices treated with either water or Ethrel and subsequently inoculated with *H. carbonum* or *H. victoriae* were intensely browned 72 hr after

TABLE 1. The effect of Ethrel on the accumulation of phytuberin and phytuberol in potato slices inoculated with spores of *Phytophthora infestans*, *Helminthosporium victoriae*, or *Helminthosporium carbonum*

Incubation time (hr)	Inoculum	Potato cultivar[b]	Disease reaction[c]	Quantity of terpenoids and treatment[a]			
				Phytuberin		Phytuberol	
				Water	Ethrel	Water	Ethrel
84	Water	R_1, r	...	0[d]	0	0	0
36	*P. infestans*, race 4	R_1	I	Trace[d]	31	Trace	22
60	*P. infestans*, race 4	R_1	I	Trace	36	Trace	30
84	*P. infestans*, race 4	R_1	I	Trace	44	Trace	37
36, 60, 84	*P. infestans*, race 1.2.3.4.	R_1	C	0	0	0	0
36, 60, 84	*P. infestans*, race 1.2.3.4. or race 4	r	C	0	0	0	0
72	*H. victoriae*	R_1	I	15	82	14	43
72	*H. victoriae*	r	I	9	40	Trace	95
72	*H. carbonum*	R_1	I	5	32	4	41
72	*H. carbonum*	r	I	Trace	48	9	67

[a]Expressed as micrograms per gram fresh weight of top millimeter of potato slices.
[b]R_1 = Kennebec, contains major R gene 1 for resistance to *P. infestans*.
r = Russet Burbank, lacks major R genes for resistance to *P. infestans*.
[c]I = Incompatible interaction: intense browning, no sporulation, little or no growth of fungus.
C = Compatible interaction: little or no browning, sporulation and growth of fungus.
[d]0 = Nondetected; Trace = $\leqslant 3$ μg/g fresh weight of the top 1-mm of potato slices.

TABLE 2. The effect of Ethrel on the accumulation of phytuberin and phytuberol in potato slices treated with sonicates of *Phytophthora infestans*, *Pythium aphanidermatum*, *Achlya flagellata*, *Helminthosporium carbonum*, *Helminthosporium victoriae* or *Neutospora crassa*

Incubation time (hr)	Fungal sonicate	Potato cultivar[a]	Browning[b]	Quantity of terpenoids and treatment[c]			
				Phytuberin		Phytuberol	
				Water	Ethrel	Water	Ethrel
84	Water	R_1, r	−	0[d]	0	0	0
36	*P. infestans*, race 4	R_1	+	Trace[d]	14	Trace	6
60	*P. infestans*, race 4	R_1	+	Trace	20	Trace	13
84	*P. infestans*, race 4	R_1	+	Trace	28	Trace	17
36	*P. infestans*, race 4	r	+	Trace	27	Trace	7
60	*P. infestans*, race 4	r	+	11	51	Trace	11
84	*P. infestans*, race 4	r	+	17	69	3	14
72	*P. aphanidermatum*	R_1	+	Trace	12	Trace	25
72	*A. flagellata*	R_1	+	Trace	14	Trace	23
72	*H. carbonum*	R_1	−	0	0	0	0
72	*H. victoriae*	R_1	−	0	0	0	0
72	*N. crassa*	R_1	−	0	0	0	0

[a]R = Kennebec, contains major R gene 1 for resistance to *P. infestans*.
r = Russet Burbank, lacks major R genes for resistance to *P. infestans*.
[b]+ = Intense browning of the surface.
− = Little or no browning of the surface.
[c]Expressed as micrograms per gram fresh weight of the top 1-mm of potato slices.
[d]0 = Nondetected; Trace = $\leqslant 3$ μg/g fresh weight of the top 1-mm of potato slices.

inoculation. Little or no mycelium was apparent and the fungi did not sporulate. Ethrel markedly enhanced the accumulation of phytuberin and phytuberol in the inoculated tissues (Table 1). Earlier reports (16, 20) demonstrated that rishitin and lubimin accumulated in potato slices inoculated with *H. carbonum*, *H. victoriae*, or *N. crassa*.

Treatment with sonicates.—The surface of potato slices treated with water or Ethrel and subsequently treated with cell-free autoclaved sonicates of *P. infestans* (race 4, on Kennebec and Russet Burbank) were intensely browned 72 hr after treatment with the sonicates (Table 2). These tissues accumulated small amounts of phytuberin, phytuberol, and considerably greater amounts of rishitin and lubimin (Table 2, 3). Treatment of slices with Ethrel markedly enhanced the accumulation of phytuberin and phytuberol (Table 2). The accumulation of rishitin and lubimin generally was slightly enhanced by Ethrel (Table 3). Sonicates of *H. carbonum*, *H. victoriae*, and *N. crassa* elicited the accumulation of little or none of the four terpenoids with or without Ethrel treatment (Table 2, 3). In an earlier publication (16), sonicates of *A. flagellata* and *P. aphanidermatum* were reported to elicit rishitin and lubimin accumulation in potato tuber slices. Treatment with Ethrel enhanced the accumulation of phytuberin and phytuberol in slices treated with sonicates of those fungi (Table 2).

Kennebec tuber slices treated with sonicates of *P. infestans*, race 4, accumulated more rishitin and lubimin at 19 C than at 14 or 25 C, and Ethrel enhanced the accumulation of phytuberin and phytuberol at 14, 19, and 25 C (Table 4). Little or no rishitin was detected in slices at 30 or 36 C and low levels of lubimin were detected at these temperatures. Both ethylene and Ethrel enhanced the

TABLE 3. The effect of Ethrel on the accumulation of rishitin and lubimin in potato slices inoculated with *Phytophthora infestans*, or treated with sonicates of *P. infestans*, *Helminthosporium carbonum*, *H. victoriae*, or *Neurospora crassa*

Incubation time (hr)	Fungus or sonicate (Son.)	Cultivar	Quantity of terpenoids and treatment[a]			
			Rishitin		Lubimin	
			Water	Ethrel	Water	Ethrel
84	Water	R_1, r	0[b]	0	0	0
36	*P. infestans*, race 4	R_1	14	14	31	17
60	*P. infestans*, race 4	R_1	37	28	73	64
84	*P. infestans*, race 4	R_1	42	37	107	87
84	*P. infestans*, race 1.2.3.4.	R_1, r	Trace[b]	Trace	Trace	Trace
84	*P. infestans*, race 4	r	Trace	Trace	Trace	Trace
36	Son. *P. infestans*, race 4	R_1	16	30	6	10
60	Son. *P. infestans*, race 4	R_1	38	42	27	47
84	Son. *P. infestans*, race 4	R_1	50	68	77	89
72	Son. *H. carbonum*	R_1	Trace	Trace	Trace	Trace
72	Son. *H. victoriae*	R_1	Trace	Trace	Trace	Trace
72	Son. *N. crassa*	R_1	Trace	Trace	Trace	Trace

[a]Expressed as micrograms per gram fresh weight of the top 1-mm of potato slices.
[b]0 = Nondetected; Trace = ≤3 μg per g fresh weight of the top 1-mm of potato slices.

TABLE 4. The effect of temperature on the accumulation of phytuberin, phytuberol, rishitin, and lubimin in Kennebec potato slices treated with Ethrel or water, followed by a sonicate of *Phytophthora infestans*, race 4

Temperature (C)	Treatment	Quantity of terpenoids[a]			
		Phytuberin	Phytuberol	Rishitin	Lubimin
14	Water	0[b]	0	10	12
	Ethrel	10	18	25	8
19	Water	0	0	44	26
	Ethrel	43	31	68	21
25	Water	0	0	24	28
	Ethrel	9	14	29	24
30	Water	0	0	Trace[b]	14
	Ethrel	0	0	0	9
36	Water	0	0	0	6
	Ethrel	0	0	0	Trace

[a]Expressed as micrograms per gram fresh weight of the top 1-mm of potato slices.
[b]0 = Nondetected; Trace = ≤3 μg/g fresh weight of the top 1-mm of potato slices.

TABLE 5. The effect of ethylene, Ethrel and hydrolyzed Ethrel on the accumulation of phytuberin and phytuberol in Kennebec potato slices treated with sonicates of race 4 of *Phytophthora infestans*

Treatment[b]	Quantity of terpenoids[a]	
	Phytuberin	Phytuberol
1. Water	7	12
2. Ethrel, 0.042 M	59	24
3. Ethylene, 100 μliters/liter	82	52
4. Water	4	11
5. Ethrel, 0.042 M	35	70
6. Hydrolyzed Ethrel, 0.042 M	3	15

[a] Expressed as micrograms per gram fresh weight in the top 1-mm of potato slices. Values are the average of two experiments.

[b] Potato slices in treatments 1, 2, and 3 were kept in open petri dishes held in sealed 10-liter desiccators.

Potato slices in treatments 4, 5, and 6 were kept in closed petri dishes exposed to the atmosphere.

accumulation of phytuberin and phytuberol in slices treated with a cell-free sonicate of *P. infestans* (Table 5).

DISCUSSION

Ethylene alone did not elicit the accumulation of phytuberin, phytuberol, rishitin, or lubimin. Ethylene enhanced the accumulation of phytuberin and phytuberol in all experiments in which the accumulation of rishitin and lubimin was elicited (Tables 1-5). Ethylene did not enhance the accumulation of phytuberin and phytuberol in the presence of fungi or sonicates of fungi which did not elicit the accumulation of rishitin and lubimin (Tables 1-3). Ethylene, therefore, is not an elicitor of terpenoid accumulation in potato slices, but it can influence the quantities of phytuberin and phytuberol that accumulate. The enhanced accumulation of phytuberin and phytuberol does not appear to be at the expense of rishitin and lubimin accumulation. Ethrel treatment decreased accumulation of rishitin and lubimin in Kennebec potato slices 60 hr after inoculation with race 4 of *P. infestans*, but it increased accumulation of the terpenoids in slices treated with a sonicate of the fungus (Table 3).

The pattern of terpenoid accumulation between 14-36 C (Table 4), agreed with that reported by Currier and Kuć earlier (8). Ethylene enhanced the accumulation of phytuberin and phytuberol only at temperatures that permitted the accumulation of rishitin and lubimin (Table 4). Again, it does not appear that enhanced accumulation of phytuberin and phytuberol is at the expense of rishitin and lubimin.

The enhancement of phytuberin and phytuberol accumulation in slices treated with ethylene, but not hydrolyzed Ethrel, establishes the role of ethylene as the mediator of the response (Table 5).

Ethylene did not appear to affect the susceptibility or resistance of potato slices to the fungi that were tested. The data suggest that the metabolic control of phytuberin accumulation is different from that regulating the accumulation of rishitin and lubimin.

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