Differences in Phytoalexin Elicitation by *Phytophthora infestans* and *Helminthosporium carbonum* in Potato

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ABSTRACT

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Living spores and mycelia of *Helminthosporium carbonum* and an incompatible race of *Phytophthora infestans* elicited the accumulation of rishitin and lubimin in potato tuber disks. The same inoculum from *H. carbonum* lost the ability to elicit rishitin and lubimin after heat, ethanol, or liquid N_2 treatment, but spores and mycelia of *P. infestans* retained eliciting activity after heat or ethanol treatment. Gas chromatography-mass spectrometric analysis of extracts of *H. carbonum* mycelium failed to detect the presence of arachidonic or eicosapentaenoic acid, fungal elicitors from *P. infestans*. Inoculation with living spores of *H. carbonum* or application of arachidonic acid inhibited steroid glycoalkaloid accumulation, but application of dead spores of *H. carbonum* did not. Inoculation with either sporangia or zoospores of a compatible race of *P. infestans* suppressed rishitin and lubimin accumulation in response to a second inoculation with zoospores of an incompatible race of *P. infestans* but not in response to inoculation with spores of *H. carbonum* or application of arachidonic acid. The suppression by *P. infestans* is not likely due to inhibition of the pathway for the synthesis of rishitin and lubimin.

The potato-*Phytophthora infestans* (Mont.) de Bary interaction includes an example of a putative gene-for-gene system in which sesquiterpenoid phytoalexins accumulate faster and to higher levels after infection of tubers by an incompatible race than after infection by a compatible race (1,6,17,25). Compatible races of *P. infestans* develop in potato cells for 3–4 days without eliciting an appreciable accumulation of sesquiterpenoid phytoalexins (30). However, potato tubers react hypersensitively to cellfree sonicates of the mycelium of *P. infestans* regardless of the fungal race or potato cultivar (9,32). Bostock et al (2) reported that arachidonic acid and eicosapentaenoic acid isolated from mycelium of *P. infestans* elicited the accumulation of sesquiterpenoid phytoalexins in potato tuber tissue. Incompatible and compatible races of *P. infestans* do not differ in the quantity of the acids (*unpublished*). Thus, arachidonic and eicosapentaenoic acids per se are not the determinants of race specificity in the potato-*P. infestans* interaction. Inoculation with a compatible race of *P. infestans* has been reported to inhibit the accumulation of sesquiterpenoid phytoalexins in response to a second inoculation with an incompatible race (29,32). This result led to the separation of race-specific glucans from mycelium and germination fluids of compatible races of the fungus. These glucan preparations suppressed hypersensitive cell death and the accumulation of sesquiterpenoid phytoalexins in response to inoculation with incompatible races (8,12). Using protoplasts from tubers of nine different cultivars of potato and seven races of *P. infestans*, Doke and Tomiyama (10) obtained further evidence that supports race-specific suppression of the hypersensitive response. These suppressor glucans may suppress the hypersensitive response by binding to membrane receptors that mediate the "recognition" of incompatible races of *P. infestans* (18).

Potato tuber tissue has been reported to rapidly accumulate sesquiterpenoid phytoalexins when inoculated with a number of nonpathogens (21), including *Helminthosporium carbonum* Ullstrup. The fungus also has been used to elicit phytoalexins in a variety of leguminous plants (16).

The purpose of the work presented in this paper was to compare the elicitation and suppression of sesquiterpenoid phytoalexin accumulation in potato tuber tissue in response to infection by *P. infestans* or *H. carbonum*.

MATERIALS AND METHODS

Inoculation procedure. Potato tuber disks were prepared as previously described (15). The tuber disks were incubated at 19 C in sterile 15-cm petri plates lined with 15-cm Whatman No. 1 filter paper moistened with 2.5 ml of sterile deionized water.

Race 0 and race 1,4 of *P.infestans* (obtained from R. J. Young, University of West Virginia, Morgantown) were maintained on lima bean agar at 19 C and transferred on a weekly basis. Zoospores and sporangia used in experiments were obtained by a method previously described (14). Mycelial mats of *P. infestans*, used in the preparation of mycelial homogenates, were grown on lima bean broth for 2–3 wk. The mycelial mats were washed thoroughly with deionized water and frozen at −20 C. The frozen mycelium was finely chopped with a razor blade and sonicated (Branson, W185) in distilled water (10 g fresh weight per 100 ml of water) until a homogeneous suspension was obtained.

Spores of *H. carbonum* race 1 were obtained from 2- to 3-wk-old cultures grown on V-8 juice agar media. Spore suspensions were filtered through two layers of cheesecloth. Mycelial homogenates of *H. carbonum* were prepared from 2- to 3-wk-old cultures grown on V-8 juice broth. The mycelial mats were washed throughly with deionized water, then homogenized in distilled water (Brinkmann, PT 10-35) (10 g fresh weight per 150 ml of water).

Mycelium and spores of *H. carbonum* and *P. infestans* were killed by either autoclaving at 121 C for 20 min or treatment with 80% ethanol. Mycelium and spores of *H. carbonum* were also killed by freezing in liquid nitrogen. For ethanol-killed treatments, the fungal material was placed in a 30-ml screw-capped test tube containing 80% ethanol, vortexed, and allowed to stand for 10 min. The ethanol was removed under a stream of N_2 at 50 C. Spores of *H. carbonum* used in "frozen" treatments were germinated in water before being frozen with liquid N_2. Preparations treated with 80% ethanol or frozen were plated out on V-8 juice agar to test for viability of the fungus.

Phytoalexin assay. Each treatment was applied to six tuber disks. When the tuber disks were processed for determination of rishitin and lubimin accumulation, slices were removed from each

disk until no browning was apparent in the remaining tissue of the disk. The slices from the six tuber disks were diced with a razor blade and placed in a 50-ml glass-stoppered flask with 30 ml of methanol. The flask was then placed on a shaker (40 strokes/min) at room temperature for 2 days, the methanolic extract was decanted, and the tuber pieces were washed with an additional 20 ml of methanol. Methyl arachidate was added as an internal standard to the combined methanolic extracts. After mixing, a 20-ml aliquot was removed for determination of rishitin and lubimin accumulation as previously described (15).

Steroid glycoalkaloid assay. The top 2 mm of tissue from 18 tuber disks (30 × 7 mm) was removed and weighed to the nearest 0.1 g for the determination of steroid glycoalkaloid content as previously described (3).

Lipid extractions. Lipids from *H. carbonum* were extracted by the methods of Folch et al (11) and Letters (20). For the Folch extraction, mycelial mats from 2- to 3-wk-old cultures were washed extensively with deionized water, homogenized, and centrifuged. The pellet was extracted with 100 ml of $CHCl_3$:MeOH (2:1, v/v) for 24 hr at room temperature. The extraction procedure was then repeated. The same procedure as described for the Folch extraction (11) was followed for the Letters extraction (20), except that the pellet was extracted with 100 ml of an 80% aqueous solution of ethanol at 75 C for 15 min. The extraction with 80% ethanol was repeated twice.

The combined extracts from each extraction were transferred to open screw-capped tubes and dried under a stream of N_2. Five milliliters of 2 N methanolic-HCl and methyl arachidate were then added to each tube. The tubes were sealed with Teflon-lined caps, then placed in an oven at 80 C for 18 hr. After the methanolic-HCl was removed in a stream of N_2, the residue was dissolved in $CHCl_3$ for analysis by gas chromatography. The fatty acid methyl esters were separated on a glass column packed with 10% SP-2330 on 100/120 Chromosorb with a 150–230 C (4 C/min) linear temperature program (24). The retention time and response factor for each fatty acid methyl ester was calculated using a standard mixture of fatty acid methyl esters. The presence or absence of fatty acid methyl esters in the Folch and Letters extracts was confirmed by mass spectrometric analysis (Finnegan, 3300-6110).

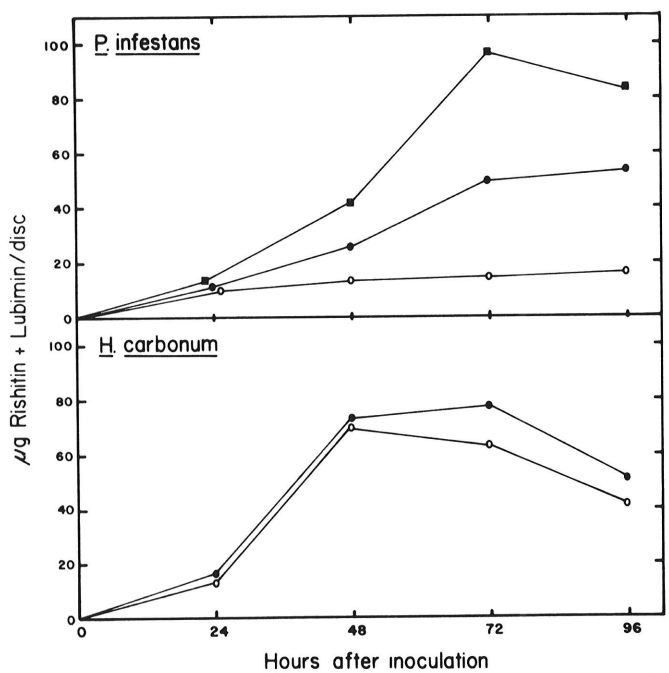

Fig. 1. Time course of rishitin and lubimin accumulation in potato tuber tissue inoculated with *Phytophthora infestans* or *Helminthosporium carbonum*. For *P. infestans*, 10 mg fresh weight of mycelium of race 0 (incompatible) per disk (■), 5×10^4 zoospores of race 0 per disk (●), or 5×10^4 zoospores of race 1,4 (compatible) per disk (o) were applied. For *H. carbonum*, 6.7 mg fresh weight mycelium and spores per disk (●) or 2×10^4 spores per disk (o) were applied.

RESULTS

Both spores and a mycelial homogenate of *H. carbonum* elicited rishitin and lubimin accumulation in potato tuber tissue (Fig. 1). Killing *H. carbonum*, by autoclaving or treatment with 80% ethanol, completely destroyed the ability of both spores and mycelial fragments in a homogenate to elicit rishitin and lubimin accumulation (Table 1). Application of a 100-fold increase in the amount of spores or mycelium of *H. carbonum*, killed by autoclaving or 80% ethanol, did not elicit accumulation of rishitin and lubimin (*data not shown*). Freezing spores and mycelium of *H. carbonum* killed most, but not all, of the fungus; occasionally after extended incubation, growth was evident when the preparation was applied to V-8 juice agar. Preparations of frozen spores and mycelial fragments of *H. carbonum* did not elicit detectable quantities of rishitin and lubimin accumulation up to 96 hr after application to tuber disks. Autoclaving or ethanol treatment did not destroy the ability of sporangia or a mycelial homogenate from *P. infestans* to elicit the accumulation of rishitin and lubimin.

Arachidonic and eicosapentaenoic acids were not detected in extracts of the mycelium of *H. carbonum* (Table 2). The mass spectrometric analysis confirmed the presence of the fatty acid methyl esters, which were quantified by gas chromatography.

Arachidonic acid and living spores of *H. carbonum* suppressed the accumulation of steroid glycoalkaloids, whereas autoclaved and ethanol-killed spores of *H. carbonum* did not (Table 3). These results confirmed the suppression of steroid glycoalkaloid accumulation by arachidonic acid and living *H. carbonum* reported earlier (26,27).

Inoculation of potato tuber tissue with either sporangia or zoospores from a compatible race of *P. infestans* suppressed the accumulation of rishitin and lubimin in response to a subsequent inoculation with zoospores of an incompatible race but not in response to inoculation with spores of *H. carbonum* or application

TABLE 1. Effect of autoclaving, freezing, and treatment with 80% ethanol on rishitin- and lubimin-eliciting activity of inoculum from *Helminthosporium carbonum* and *Phytophthora infestans*

Inoculum	Treatment of fungus and rishitin + lubimin (μg) per disk[a]			
	Untreated	Autoclaved	80% Ethanol	Frozen
H. carbonum spores (2×10^4/disk)	89 ± 12	0[b]	0	0
H. carbonum mycelium and spores (6.7 mg fresh weight/disk)	32 ± 18	0	0	0
P. infestans race 0 sporangia (5×10^4/disk)	24 ± 14	21 ± 18	20 ± 15	...[c]
P. infestans race 0 mycelium (10 mg fresh weight/disk)	50 ± 34	108 ± 54	58 ± 20	...

[a] Tuber disks were assayed for rishitin and lubimin accumulation 96 hr after application of inoculum. Data are means of two to four determinations ± 1 SD. Each determination is average amount of rishitin and lubimin in six tuber disks.
[b] Less than 0.5 μg/disk.
[c] Not determined.

TABLE 2. Determination of amount of elicitor and nonelicitor fatty acids in spores and mycelium of *Helminthosporium carbonum* by Folch (11) and Letters (20) extraction[a]

Fatty acid	Folch extraction (μg FA/mg dry weight)	Letters extraction (μg FA/mg dry weight)
Palmitate (16:0)	12.2	7.9
Stearate (18:0)	4.4	4.6
Linolenate (18:3)	35.4	32.0
Arachidonate (20:4)	0[b]	0
Eicosapentaenoate (20:5)	0	0

[a] Palmitate, stearate, and linolenate do not elicit sesquiterpenoid phytoalexin accumulation in potato tuber tissue, whereas arachidonate and eicosapentaenoate do.
[b] None detected.

of arachidonic acid (Table 4). The application of arachidonic acid or spores of *H. carbonum* 18 hr after inoculation with the compatible race did not appear to affect the growth of the compatible race. Mycelium and sporangia of *P. infestans* were visible on tuber disks inoculated with the compatible race in all treatments, although it was not possible to differentiate races on disks inoculated with both races. Growth of *P. infestans* was not evident on tuber disks inoculated with only the incompatible race. There was no visible evidence, however, for increased growth of *H. carbonum* on tuber disks previously inoculated with a compatible race of *P. infestans*.

DISCUSSION

H. carbonum and incompatible races of *P. infestans* both elicit sesquiterpenoid phytoalexin accumulation in potato tuber tissue. The inability of killed spores and mycelium of *H. carbonum* to elicit rishitin and lubimin accumulation suggests that, unlike arachidonic acid and eicosapentaenoic acid in *P. infestans*, the elicitor from *H. carbonum* is probably not an endogenous stable component of the fungus. It is known that cell wall components from some fungi elicit phytoalexin accumulation in a variety of plants (5). Living *H. carbonum* may produce an extracellular product that elicits a hypersensitive response. Culture filtrates from 24- to 48-hr-old cultures of *H. carbonum*, when concentrated 20-fold, elicited low levels of rishitin and lubimin accumulation (1.5–10 μg per disk) 60–96 hr after application. Application of a toxin from *H. carbonum* (HC toxin) to potato tuber disks (50 μl of a 2.0 mg/ml solution per disk) did not elicit browning or rishitin and lubimin accumulation (*data not shown*). Since HC toxin is reported to be host-specific, this result was not unexpected.

The demonstration that spores and mycelium of *H. carbonum* do not contain detectable amounts of arachidonic or eicosapentaenoic acids (Table 2) indicates that sesquiterpenoid phytoalexins can accumulate in potato tuber tissue in response to chemically different stimuli. Likewise, the inhibition of steroid glycoalkaloid accumulation by sesquiterpenoid phytoalexin-eliciting agents is nonspecific (Table 3). Since both steroid glycoalkaloids and sesquiterpenoid phytoalexins are synthesized by the acetate-mevalonate pathway, the suppression of steroid glycoalkaloid accumulation may result, in part, from the synthesis or activation of enzymes in the branch of the pathway from farnesyl pyrophosphate that is responsible for sesquiterpenoid phytoalexin synthesis. The data presented in this paper support the observation that accumulation of sesquiterpenoid phytoalexins is consistently associated with inhibition of steroid glycoalkaloid accumulation (26,27), but recent data indicate that inhibition of steroid glycoalkaloid accumulation is not sufficient per se to cause accumulation of sesquiterpenoid phytoalexins (28).

Inoculation of potato tuber tissue with a compatible race of *P. infestans* suppresses sesquiterpenoid phytoalexin accumulation in response to a second inoculation with an incompatible race of the fungus. This is consistent with previously published results (29,32). Inoculation with an incompatible race followed by a compatible race results in rapid sesquiterpenoid phytoalexin accumulation, typical of an incompatible interaction (17,19,33). Inoculation with a compatible race of *P. infestans*, however, did not suppress rishitin and lubimin accumulation in response to inoculation with spores of *H. carbonum* or application of arachidonic acid (Table 4). This result suggests that suppressors from a compatible race of *P. infestans*, produced during infection, interact with potato tuber tissue in some specific manner to suppress sesquiterpenoid phytoalexin accumulation in response to a second infection by an incompatible race of the fungus. The suppression is not likely due to inhibition of the pathway for rishitin and lubimin synthesis.

Growth of the compatible race of *P. infestans* was evident on potato tuber disks that were also inoculated with spores of *H. carbonum* or treated with arachidonic acid despite the high levels of phytoalexins that accumulated in these treatments (Table 4). This observation suggests that once the compatible race is established and ramifying through the tissue, the accumulation of phytoalexins at the surface of the slice does not inhibit its development.

The inability of a compatible race of *P. infestans* to suppress sesquiterpenoid phytoalexin accumulation in response to the application of arachidonic acid (Table 4) does not prove that arachidonic acid is not involved in the interaction of potato tuber tissue with an incompatible race of *P. infestans*. The arachidonic acid applied to tuber tissue in a suspension and the arachidonic and eicosapentaenoic acids present in an infecting hypha from an incompatible race (less than 1% present as free acids) may interact differently with a potato cell. Also, partially purified glucans from compatible races of *P. infestans*, which suppress sesquiterpenoid phytoalexin accumulation in response to infection by incompatible races of the fungus (8,12), promote sesquiterpenoid phytoalexin accumulation in response to the application of arachidonic acid (22,24). It is possible that a "bound form" of arachidonic acid (i.e., arachidonic acid bound to lipid or a carbohydrate moiety in the fungal membrane or wall) is responsible for triggering the response in potato tuber tissue that results in the hypersensitive response to infection by incompatible races of *P. infestans*. Release of arachidonic and eicosapentaenoic acids may influence the speed and magnitude of the hypersensitive response.

Varns (31), Heath (13), and Bushnell and Rowell (4) proposed that a suppressor from the compatible race of a pathogen prevents

TABLE 3. Effect of arachidonic acid or untreated, autoclaved, or ethanol-killed spores of *Helminthosporium carbonum* on steroid glycoalkaloid accumulation in potato tuber tissue

Treatment[a]	Steroid glycoalkaloids[b] (μg/g fresh weight)
Water	585 ± 31
Arachidonic acid	58 ± 24
Untreated spores of *H. carbonum*	52 ± 3
Autoclaved spores of *H. carbonum*	545 ± 42
Ethanol-killed spores of *H. carbonum*	511 ± 61

[a] Arachidonic acid (50 μg/disk) or 2×10^4 spores of *H. carbonum* were applied to tuber disks 24 hr after slicing.
[b] All tuber disks were processed for determination of steroid glycoalkaloid accumulation 96 hr after treatment. Data are average of three determinations ± 1 SD. Each determination is result of processing a single group of slices taken from top 2 mm of tissue of 18 tuber disks.

TABLE 4. Effect of inoculating potato tuber disks with compatible race of *Phytophthora infestans* on accumulation of rishitin and lubimin in response to subsequent inoculation with incompatible race of *P. infestans*, inoculation with *Helminthosporium carbonum*, or application of arachidonic acid

	Rishitin + lubimin (μg)/g fresh weight[a]			
	First treatment[b]			
		Inoculum of race 1,4 per disk		
Second treatment[c]	Water	Sporangia (1×10^5)	Sporangia (1×10^4)	Zoospores (5×10^4)
Arachidonic acid (100 μg/disk)	112 ± 48	185	107 ± 42	122 ± 24
P. infestans, race 0 (5×10^4 zoospores/disk)	121 ± 12	42 ± 4[d]	59 ± 6[d]	45 ± 25[d]
H. carbonum (2×10^4 spores/disk)	104 ± 56	122 ± 10	96 ± 37	125 ± 47
Water	0[e]	44 ± 18	16 ± 1	15 ± 6

[a] Tuber disks were assayed for rishitin and lubimin accumulation 72 hr after second treatment. Data for water as first treatment are average of three determinations ± 1 SD. Data for inoculum of race 1,4 as first treatment are average of two determinations ± 1 SD except for first treatment with 1×10^5 sporangia of race 1,4 and second treatment with arachidonic acid; this value was based on one determination. Each determination is average amount of rishitin and lubimin in six disks.
[b] Applied 2 hr after slicing (i.e., preparation of tuber disks from intact tubers).
[c] Applied 18 hr after slicing.
[d] Rishitin and lubimin accumulations are significantly different from appropriate water control ($P = 0.01$, Student's t test).
[e] Less than 0.5 μg/g fresh weight.

binding of the elicitor. In the incompatible interaction, the inability of the suppressor to bind to the host plant's receptor allows binding of the elicitor and subsequent activation of the plant's defensive mechanisms. There is indirect evidence for the existence of membrane-bound receptors in potato tuber tissue that are responsible for initiating hypersensitive cell death in response to infection by incompatible races of *P. infestans* (7,10,19,23). A fungal elicitor-binding receptor from soybean membranes has been partially characterized (34).

The data presented in this paper provide evidence of the degree of specificity present in the potato-*P. infestans* interaction. Arachidonic and eicosapentaenoic acids are not present in all fungi that are able to elicit sesquiterpenoid phytoalexin accumulation, and compatible races of *P. infestans* apparently do not suppress sesquiterpenoid phytoalexin accumulation in response to all sesquiterpenoid phytoalexin-eliciting agents. Suppressors from a compatible race may affect some event necessary for sesquiterpenoid phytoalexin accumulation that occurs only in the potato-*P. infestans* interaction.

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