Specificity of Rishitin and Phytuberin Accumulation by Potato

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ABSTRACT

Sonicates and boiled aqueous extracts of three races of *Phytophthora infestans* caused necrosis and the accumulation of rishitin and phytuberin in tuber slices. The response was observed with cultivars having *R* genes for resistance as well as those susceptible to all known races of the fungus. *Ceratocystis fimbriata* and *Helminthosporium carbonum*, nonpathogens of potato, also caused accumulation of rishitin and phytuberin. Solutions of chlorogenic acid, L-dihydroxyphenylalanine, catechol, dimethylsulfoxide, mercuric chloride, cupric chloride, or tyrosinase applied to the surface of slices caused necrosis without accumulation of rishitin and phytuberin. The surface of slices briefly exposed to dry ice readily browned without accumulation of the terpenoids. Necrosis accompanied by rishitin and phytuberin accumulation was evident with sprouts of four cultivars inoculated with compatible races, whereas flecking (restricted necrosis) without detection of the terpenoids was observed after inoculation with incompatible races. Rishitin and phytuberin were not detected in extracts of peel or freshly peeled tubers from eleven cultivars. Since accumulation of rishitin and phytuberin is characteristic of the incompatible interaction in tubers, a reassessment of their role as sole or primary agents responsible for restricting development of *P. infestans* in the entire potato plant may be necessary. Phytopathology 61:968-971.

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The hypersensitive response of potato tubers (*Solanum tuberosum* L.) to incompatible races of *Phytophthora infestans* (Mont.) d By. includes rapid necrosis and the accumulation of two terpenoids, rishitin and phytuberin (1, 2, 3, 4). Susceptible reactions in tubers are characterized by a suppression of the above response (5). Investigations reported in this paper were initiated to answer the following questions: Will browning of tissue caused by physical or chemical agents, and fungi other than *P. infestans*, elicit the accumulation of rishitin and phytuberin? Is the accumulation of rishitin and phytuberin associated with the resistance response of the entire plant?

Materials and Methods.—*Chemical and physical injury*.—Aqueous solutions of chlorogenic acid, 1×10^{-2} M; catechol, 1×10^{-2} M; dihydroxyphenylalanine (DOPA), 5×10^{-3} M; cupric chloride, 3×10^{-3} M; mercuric chloride, 3×10^{-4} M; dimethyl sulfoxide (DMSO), 10, 20, and 30%; or tyrosinase, 0.1%, were added (0.3-0.5 ml/slice) to the surface of slices of the cultivars Kennebec (R_1), 3RC-8 (R_2), Cherokee (R_1), 3XX-1 ($R_1R_2R_4$) and Red Pontiac (r). Cultivars with *R* genes for resistance are susceptible only to fungal races with the corresponding *R* genes for pathogenicity. Small *r* cultivars are susceptible to all races. Slices were held at 18 C, and the top 2 mm were removed for analysis 24-30 hr and 60-72 hr after treatment, except for slices treated with chlorogenic acid and tyrosinase. These were held for 72 hr after treatment.

Potato slices of the cultivars Kennebec and 3RC-8 were rubbed on a cake of dry ice and placed in petri dishes. Slices were held at 18 C, and the top 2 mm of tissue were removed 24 hr and 72 hr after treatment.

Treatment with sonicates of P. infestans.—Fifteen-day-old cultures of race 0, 4, and 1.2.4 were grown on lima bean agar in petri dishes and quick-frozen with dry ice. After 1 hr, the fungus was scraped off the frozen surface and stored at −20 C. Ten g of frozen fungal tissue were homogenized in 100 ml of water under asceptic conditions. Homogenization was performed in a Virtis homogenizer (kept cold with an ice bath) at maximum speed for 2 min. The homogenate was then sonicated with a Branson sonifier for 2 min at maximum intensity. The temperature of the solution did not exceed 10 C during the period of sonication. The sonicate was added to lima bean medium in petri dishes, and colonies of P. infestans did not develop. The sonicates were added (0.5 ml/slice) to the surface of tuber slices of the cultivars WV-13-8 (R_1R_4), 3RC-8, Red Pontiac, and Russet Burbank (r). The slices were kept in petri dishes at 18 C for 60 hr. A sonicate of race 0 was placed in a boiling water bath for 10 min and filtered. The filtrate was cooled, then applied to slices of Kennebec, Katahdin (r), and Russet Burbank. Tissue was collected 60 hr after treatment.

Inoculation with nonpathogens.—Tuber slices of Kennebec were inoculated with two fungi nonpathogenic on potato, *Ceratocystis fimbriata* Ell. & Halst. and *Helminthosporium carbonum* Ullstrup, race 1. Spore suspensions were prepared from 2-week-old cultures grown on potato-dextrose agar medium. Twenty ml of water were added to each petri dish, and spores were released by rubbing the surface with a bent glass rod. The suspensions were then filtered through a double layer of cheesecloth under asceptic conditions and sprayed on Kennebec tuber slices. Tissue inoculated with *C. fimbriata* was kept at 25 C and collected 15, 24, and 74 hr after inoculation. Tissue inoculated with *H. carbonum* was kept at 25 C and collected 12, 24, 36, 48, and 60 hr after inoculation. Extracts were analyzed for rishitin and phytuberin by thin-layer chromatography (TLC). Extracts of potato inoculated with *H. carbonum* were

further analyzed by the gas liquid chromatography (GLC) procedure described (3, 4).

Peel and peeled tuber.—Peel, ca. 1 mm thick, and the freshly peeled tubers of the cultivars Kennebec, Cherokee, 3RC-8, WV-13-8, 3XX-1, 1563 (R_4), Pentland (R_3), Irish Cobbler (r), Russet Burbank, Red Pontiac, and Katahdin were extracted and the extracts analyzed for the presence of rishitin and phytuberin.

Extraction and chromatography.—The 1st and 2nd mm of slices, peel, or peeled tuber were removed and extracted as described (3, 4). Rishitin and phytuberin were detected on silica gel plates developed with cyclohexane:ethyl acetate (1:1, v/v) and sprayed with vanillin-sulfuric acid reagent (4) or chloroform saturated with antimony trichloride (4). The identity of rishitin and phytuberin was confirmed by gas-liquid chromatography and IR, NMR, and mass spectral analyses.

Sprouts.—Green sprouts, 1-3 inches long, cut from stored Kennebec tubers, were washed in detergent and soaked in sterile water for 30 min before a transfer to petri dishes. Seedlings were sprayed with water, race 4 (incompatible) or race 1.2.4 (compatible) of *P. infestans,* and incubated for 5 days at 18 C.

Budding tubers of the cultivars Kennebec, WV-13-8, 3RC-8, and 3XX-1 were placed in moist vermiculite, and the resulting sprouts were grown in the dark at 27 C for 30 days. The sprouts, 6-10 inches long, were cut from the tubers, placed on moistened germination paper, and either sprayed with water or inoculated with suspensions of conidia of race 4 or 1.2.4. The treated sprouts were then covered with another moistened paper, and the papers and sprouts were rolled in wax paper. Incubation was carried out in loosely sealed plastic bags at 18 C for 6 days. Rishitin and phytuberin were extracted from sprouts using techniques described for tuber tissue (3, 4).

RESULTS.—*Effects of chemicals and dry ice treatment.*—Dry ice caused severe browning in the 1st mm and slight browning in the 2nd mm within 3 hr of exposure. Chlorogenic acid, cupric chloride, mercuric chloride, DMSO, and tyrosinase caused browning in the first mm, 24 hr after treatment. DOPA produced an orange coloration on the surface of the tuber within 15 min, which later turned brown and extended into the second mm. Catechol caused a rapid gray-brown discoloration which also extended into the second mm. Though browning was observed with the above treatments, TLC of extracts equivalent to 0.5 g dry wt of tissue did not indicate the presence of rishitin or phytuberin. Aqueous solutions of rishitin (1×10^{-5} M and 1×10^{-4} M, 0.3-5 ml/slice) containing 2% DMSO were added to slices of Cherokee and Kennebec, and the tissues were extracted 60 hr after treatment. An increase in rishitin or phytuberin above that added was not observed.

Effect of sonicates.—Twenty to thirty hr after application of the sonicates, the tuber slices of all four cultivars developed necrosis typical of the hypersensitive response. The degree of necrosis did not differ with the cultivars, and no difference in necrosis was seen for the reaction of a cultivar to the sonicates of any of the three races. The discoloration of the tissue extended to the top of the 2nd mm in all cases.

Thin-layer chromatography of the extracts indicated that all the cultivars responded to sonicates of all three races with the accumulation of rishitin and phytuberin (Table 1). The hypersensitive response and levels of rishitin and phytuberin accumulated in the small r cultivars Red Pontiac and Russet Burbank were approximately equal to those in tuber slices inoculated with incompatible races. Heated sonicates applied to the cultivars Kennebec, Katahdin, and Russet Burbank caused less necrosis than unheated sonicates, but the accumulation of rishitin and phytuberin was not changed.

Inoculation with H. carbonum and C. fimbriata.—*Ceratocystis fimbriata,* the pathogen responsible for black rot in sweetpotatoes, grows and sporulates well on white potato tubers at 25 C. At 50 to 60 hr after

TABLE 1. Estimation[a] of rishitin and phytuberin in potato slices 60 hr after inoculation or treatment with sonicates of *Phytophthora infestans*

		Cultivars[b], rishitin (R) and phytuberin (P)							
		WV-13-8 (R_1R_4)		3RC-8 (R_2)		Red Pontiac (r)		Russet Burbank (r)	
Treatment	Race	R	P	R	P	R	P	R	P
Fungus	0	++	+++	++	+	—	—	—	—
	4	+++	+	+++	++	—	—	—	—
	1.2.4	—	—	—	—	—	—	—	—
Sonicate	0	+++	+++	+++	++	+++	++	+++	++
	4	+++	++	+++	+++	+++	+++	++	++
	1.2.4	+++	++	+++	++	+++	++	+++	++
Boiled sonicate	0							+++	++

[a] Compounds were separated on plates coated with Silica Gel-G using cyclohexane:ethyl acetate (1:1,v/v). Estimations were made by visual observation after plates were sprayed with vanillin-sulfuric acid reagent. Estimations are relative; +++ = the largest spot with greatest intensity; — = the compound not detected. One hundred and fifty-μliters equivalent to 1.5 g fresh tissue applied to plates/treatment.

[b] Cultivars WV-13-8 and 3RC-8 are resistant to race 0 and 4 and susceptible to race 1.2.4. Red Pontiac and Russet Burbank are susceptible to the three races.

TABLE 2. Accumulation of rishitin and phytuberin in tuber slices of Kennebec inoculated with *Helminthosporium carbonum*[a]

Hr after inoculation	μg Compound/g dry wt	
	Rishitin	Phytuberin
12	—[b]	—
24	3	1
36	36	2
48	139	9
60	194	57

[a] Data for combined 1st and 2nd mm slices.
[b] — = the compound not detected.

inoculation, mycelium covered most of the surface, and the tissue underneath was soft and yellowed into the 3rd mm. Phytuberin and rishitin accumulation was detected 24 hr after inoculation and both compounds increased after further incubation. *Helminthosporium carbonum*, the pathogen causing leaf spot on corn, grows to a limited extent on the tuber surface and causes limited necrosis without loss of tissue firmness. Accumulation of rishitin and phytuberin was detected 24 hr after inoculation, and the concentration of both compounds increased up to and including the 60-hr incubation (Table 2).

Apparently *H. carbonum* can stimulate levels of rishitin and phytuberin comparable to those obtained by inoculation with *P. infestans* without marked necrosis. The compounds were detected about 10 hr before their detection in tissue inoculated with *P. infestans*.

Rishitin and phytuberin in peel or peeled tuber.—Rishitin and phytuberin were not detected in extracts by TLC even when the concentration of tissue applied was 4-5 times that used to detect the compounds in inoculated tuber slices.

Response of sprouts to inoculation with P. infestans.—The green sprouts were covered with tiny flecks after inoculation with the incompatible race, and tissue remained firm. Inoculation with the compatible race caused the appearance of large lesions and brown, water-soaked areas over most of the sprout surface. Etiolated tissue inoculated with the incompatible race was covered with minute flecks in the apical and central portions of the sprout, and the tissue was firm. Lesions on etiolated sprouts inoculated with the compatible race coalesced, giving rise to brown, soft regions extending more than 1 inch in length. Small branch shoots from the stem were destroyed in all cultivars after inoculation with the compatible race. The severity of damage by the compatible race was rated 3XX-1 > 3RC-8 > WV-13-8 or Kennebec in the order of decreasing necrosis of the sprouts. Rishitin and phytuberin were detected in the green and etiolated sprouts inoculated with the compatible race, but not in sprouts inoculated with the incompatible race (Table 3). The accumulation of rishitin in etiolated sprouts of the cultivars paralleled the degree of necrosis. Necrosis and flecking were evident 60-72 hr after inoculation with compatible or incompatible races. Necrosis is not evident with

TABLE 3. Estimation of rishitin and phytuberin in sprouts inoculated with *Phytophthora infestans*[a]

Cultivar	Compatible race[b]		Incompatible race[b]	
	Rishitin	Phytuberin	Rishitin	Phytuberin
Green Kennebec	+++	+	—	—
Etiolated Kennebec	+	+—	—	—
WV-13-8	+	+—	—	—
3RC-8	++	+—	—	—
3XX-1	+++	+—	—	—

[a] Fifty μliters of extract (equivalent to 5 g dry wt of tissue/ml) were applied per spot on plates coated with Silica Gel-G. Plates were developed in cyclohexane:ethyl acetate (1:1, v/v), and estimations were made after plates were sprayed with vanillin-sulfuric acid reagent and heated for 3 min at 120 C. Estimations are relative and based on visual observations; +++ = highest concentration; +— = barely detectible; — = not detected.
[b] Compatible race = race 1.2.4; incompatible race = race 4.

compatible reactions on tubers 60-72 hr after inoculation.

DISCUSSION.—Necrosis of tuber tissue without the presence of fungus or fungal products does not cause the accumulation of rishitin and phytuberin. Physical and chemical induction of necrosis in the 1st and 2nd mm of slices did not result in the accumulation of terpenoids. Dilute solutions of rishitin also did not cause an increase of rishitin in slices. The ability of two nonpathogens of potato, *H. carbonum* and *C. fimbriata*, to cause the accumulation of rishitin and phytuberin indicates that the response is not specific for incompatible races of *P. infestans*. The response of small *r* and large *R* cultivars to sonicates of three races of the fungus suggests that the genetic information for the hypersensitive response is present in all tubers, and resistance is determined by the expression of this information. Crosses of *S. tuberosum* × *S. demissum* apparently do not give the cultivar new genetic information for the synthesis of rishitin and phytuberin which was lacking in the small *r* cultivars, but rather an enhanced ability to utilize the genetic information already present. The key to an incompatible interaction appears to be the ability of the host to respond to the fungus. The ability of *P. infestans* and nonpathogens of potato to elicit the accumulation of rishitin and phytuberin adds evidence to the report that susceptibility depends upon an active mechanism that either blocks the resistance response or interferes with the recognition by the tuber of compatible races of *P. infestans* as foreign entities, and hence suppresses the response (5). Rishitin and phytuberin were not detected in peel, though peel is high in chlorogenic and caffeic acids, α-solanine and α-chaconine. The reason for the accumulation of rishitin and phytuberin in sprouts inoculated with compatible races is unknown. The rapid necrosis in compatible reactions suggests that sprouts are resistant to all races of the fungus (rapid necrosis is typical of hypersensitive resistance). The limiting of necrosis to minute flecking in the incompatible reaction may further reflect

an extremely high level of resistance in the sprouts and localization of rishitin. The inability to detect rishitin in sprouts inoculated with incompatible races may, therefore, be due to its dilution by unaffected tissue. Caution, nevertheless, is advised in drawing conclusions for a general resistance mechanism for an entire plant based on evidence using only a portion of that plant.

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