Terpenoid Accumulation as a Biochemical Response of the Potato Tuber to Phytophthora Infestans

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ABSTRACT

A consistent response of tubers to infection by incompatible races of *Phytophthora infestans* was demonstrated with 11 potato cultivars and three races of the fungus. The response included rapid necrosis and the accumulation of two terpenoids, rishitin and phytuberin. Detection of the terpenoids occurred shortly before or during necrosis, and their accumulation may be responsible for or represent a manifestation of the hypersensitive response. Phytopathology 61:174-177.

Additional key words: mechanism for resistance, gas-liquid chromatography, thin-layer chromatography.

Rishitin, an antifungal terpenoid, was isolated from tubers of the R_1 cultivar Rishiri (*Solanum tuberosum* × *S. demissum*) inoculated with an incompatible race of *Phytophthora infestans* (Mont.) d By. (4). Its structure was established as one of two isomeric bicyclic norsesquiterpene alcohols with a molecular formula of $C_{14}H_{22}O_2$ (1). Rishitin was not detected in freshly sliced tissue or in culture filtrates or extracts of the fungus, but a trace was detected in noninoculated aged slices and slices inoculated with a compatible race. In further studies, Sato et al. (2) demonstrated that rishitin accumulated in incompatible interactions of four cultivars (R_1, R_2, R_3, R_4) inoculated with race 0. In addition, a variety susceptible to all races of the fungus accumulated rishitin when dipped into a cell-free homogenate of the fungus. This paper presents evidence for the accumulation of rishitin and another terpenoid, phytuberin $C_{17}H_{26}O_4$ (5) in incompatible reactions of 11 cultivars, some with multiple *R* gene designations for resistance. In addition, new techniques for the detection of the terpenoids and their quantitation by thin-layer and gas-liquid chromatography are described.

MATERIALS AND METHODS.—*Growth and storage of potato tubers.*—Eleven cultivars were selected: Cherokee and Kennebec, R_1; 3RC-8, R_2; Pentland, R_3; 1563, R_4; WV-13-8, R_1R_4; 3XX-1, $R_1R_2R_4$; Irish Cobbler, Red Pontiac, Katahdin, and Russet Burbank lack *R* genes for resistance and are designated *r* cultivars. With the exception of Kennebec and Cherokee, the *R* gene cultivars (differentials) were obtained from the Potato Introduction Station in Sturgeon Bay, Wisc. The others were certified seed. Due to the limited quantity of differentials, two growing seasons were required to obtain sufficient tissue for research and propagation. Potatoes were grown at the O'Neal Horticulture Farm of Purdue University, and tubers were stored in the dark at 8-10 C.

Culturing of fungus and inoculation.—*Phytophthora infestans* was maintained on lima bean agar medium and transferred every 30 days to slants. The medium was prepared by homogenizing 200 g of frozen lima beans, filtering the homogenate through a double layer of cheesecloth, and diluting the filtrate to 1 liter with water containing 12 g of agar. Mycelial and conidial suspensions were transferred from petri dishes containing lima bean medium for the preparation of inoculum. Twelve- to 15-day-old cultures were used for all inoculations.

Surface-sterilized tubers were sliced in a sterile chamber, and the slices (0.5 cm thick) were placed in petri dishes containing filter paper saturated with water. A conidial suspension, 4-5 × 10^4 spores/ml, was filtered through a double layer of cheesecloth, and the filtrate was uniformly sprayed on the slices with a chromatographic spray apparatus provided with a filtered air supply. Noninoculated slices were sprayed with water.

Plates containing control and inoculated slices were placed into plastic bags containing moistened paper towels and kept dark at 18 C. At intervals after inoculation, the first (upper) and second mm were removed from the slices, frozen in open plastic bags, and lyophilized for 48 hr. The dried tissue was kept in sealed plastic bags at room temp.

Extraction of tissues.—Ten to 20 g of dry, pulverized tissue were extracted in a Soxhlet extractor for 8 hr with 200 ml of anhydrous methanol. The methanol solutions were then concd in rotatory evaporators to 20 ml and transferred to separatory funnels in a final volume of 30 ml. Water and chloroform were added to the methanol solution to a composition of methanol:water:chloroform (3:2:5, v/v). The solvent system was shaken 1 min, and the phases were separated 1 hr. The chloroform phase was removed and the aq phase extracted twice with the same solvent. After complete evaporation of the combined chloroform fractions, the residue was dissolved in a volume of carbon tetrachloride to give a concn equivalent to 7 to 10 g fresh wt of tissue/ml solvent. These solutions were used for thin-layer chromatography (TLC) and gas-liquid chromatography (GLC).

Thin-layer and gas-liquid chromatography.—Compounds were separated on plates coated with nonactivated silica gel 250 μ thick using cyclohexane:ethyl acetate (1:1, v/v) as developing solvent. Compounds were detected by spraying plates with chloroform saturated with antimony trichloride or vanillin-sulfuric acid

reagent (3 g vanillin and 0.5 ml concd sulfuric acid in 100 ml methanol) and heating at 120 C for 3-5 min.

For quantitative analyses, six samples (250 μliters/sample) were applied to channels on TLC plates, and the plates were developed as described. Gel areas extending 1 cm on either side of rishitin and phytuberin were removed and placed in 15-ml centrifuge tubes. Phytuberin was eluted from the gel with ethyl ether, but rishitin required a more polar solvent, acetone. The gel was extracted twice for 1 hr with 1 ml of solvent, using a Cyclo-Mixer, and separated by centrifugation. Combined extracts were dried in 0.5-dr vials using a warm sand bath and a stream of nitrogen. Fifty μliters of solvent containing the internal standard, hexadecane, were added to the vials prior to GLC.

Analyses were performed on a Hewlett-Packard gas chromatograph (F & M, Model 810-DR-12) equipped with dual glass columns, flame ionization detector, and temp programming. The following materials and conditions were used: Carrier gas (He), H_2, & air—45, 22 & 460 cc/min, respectively; Injection port temp—210 C; Detector temp—350 C; Program temp range—140-280 C (10 C/min; postinjection delay of 1 min); Attenuation settings—10 ×16, 32 and 64; Glass columns—4.0 ft × 4 mm ID; Packing—3% OV-1 on Chromosorb Q (60-80 mesh) (Applied Science Labs, Inc., College State, Pa.); Elution temp—hexadecane 180 C, rishitin and phytuberin 200 C. The per cent recovery for rishitin and phytuberin was greater than 95% with the TLC-GLC procedure. Specific amounts of rishitin and phytuberin/g fresh tissue were calculated using their mol wt of 222 and 294, respectively. Linear response ratios between the internal standard and compounds (X) were obtained at the attenuation settings by plotting the ratios of X peak area/internal standard peak area against the quantity of X. This graph was used to equate the sample ratios to the amt of rishitin or phytuberin. The average response ratios, μg X/μg internal standard, were 0.677 and 0.406 for rishitin and phytuberin, respectively.

RESULTS.—*Symptoms.*—Sixty hr after inoculation, slices of the tubers from incompatible interactions were reddish brown on the surface, with numerous dark flecks at the sites of penetration. The underlying second mm of tissue was firm, and flecking was seen on the surface of the remaining tuber. General necrosis did not increase, and the slices were firm 5 days after inoculation. Tissue from compatible interactions with both *R* and *r* cultivars lacked necrosis, with only sparse flecking down to the top of the second mm. The vascular tissue was darker than that of noninoculated tissue. Five days after inoculation, however, aerial mycelia covered most of the tuber surface, and the entire slice was spongy. This severe infection still caused slight discoloration when compared to the incompatible response. Control tissue was firm and succulent without visible contamination. Of the *R* cultivars, only Pentland showed a low-level hypersensitive response with the three races tested. First- and second-mm slices were harvested at intervals after inoculation with incompatible races to determine the relationship between symptom development and the accumulation of rishitin and phytuberin. Marked necrosis of the first and second mm occurred 30-40 hr after inoculation with Cherokee and WV-13-8—race 0 and 20-30 hr with 3RC-8—race 4.

Thin-layer chromatography.—Rishitin and phytuberin had an R_F value of 0.21 and 0.70, respectively. Rishitin appeared turquoise and phytuberin appeared heliotrope after spraying with vanillin-sulfuric acid. After spraying with antimony trichloride in chloroform, rishitin immediately appeared red fading to blue, whereas phytuberin appeared yellow after heating. Estimations of rishitin and phytuberin 60 hr after inoculation are in Table 1. The severity of host necrosis as judged by observation of intact slices and sectioned

TABLE 1. Estimation of rishitin and phytuberin in eleven potato cultivars 60 hr after inoculation with *Phytophthora Infestans*[a]

Variety	Fungal race	Amt spotted (μliters)[b]	Rishitin	Phytuberin
Kennebec	0	150	++	++
(R_1)	4	150	++	+
	1.2.4	150	—	—
WV-13-8	0	150	++	+++
(R_1R_4)	4	150	+++	++
	1.2.4	150	—	—
3XX-1	0	150	+++	++
($R_1R_2R_4$)	4	150	+++	++
	1.2.4	150	—	—
Cherokee	0	150	++	+
(R_1)	4	150	+	+—
	1.2.4	300	—	—
Pentland	0	300	+	—
(R_3)	4	300	+	—
	1.2.4	300	+	—
1563	0	150	+	+
(R_4)	4	150	—	—
	1.2.4	150	—	—
3RC-8	0	150	++	++
(R_2)	4	150	+++	+++
	1.2.4	150	—	—
Russet Burbank	0	300	—	—
(r)	4	300	+—	—
	1.2.4	300	—	—
Irish Cobbler	0	300	+—	—
(r)	4	300	+—	—
	1.2.4	300	+	—
Katahdin &	0	300	—	—
Red Pontiac	4	300	—	—
(r)	1.2.4	300	—	—

[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 and +++ = the largest spot with greatest intensity of color; +— = a trace of compound; and — = the compound not detected.

[b] 100 μliters equivalent to 0.70 g fresh tissue including the combined 1st and 2nd mm.

TABLE 2. Accumulation of rishitin in five potato cultivars 60 hr after inoculation with *Phytophthora infestans*[a]

Variety	Fungal race	μg rishitin/g fresh wt[b]
Kennebec	0	20.6
(R_1)	4	27.1
	1.2.4	—[c]
Cherokee	0	20.6
(R_1)	4	10.5
	1.2.4	—
3RC-8	0	27.0
(R_2)	4	20.3
	1.2.4	—
3XX-1	0	19.5
($R_1R_2R_4$)	4	17.4
	1.2.4	—
WV-13-8	0	12.9
(R_1R_4)	4	26.0
	1.2.4	—

[a] Analyses by combined thin-layer and gas-liquid chromatography.
[b] Combined 1st and 2nd mm of affected tissue.
[c] Compound not detected.

tissue was directly correlated with rishitin and phytuberin accumulation. Kennebec, WV-13-8, 3RC-8, and 3XX-1 were more highly necrotized in their incompatible interactions than Cherokee, 1563, or Pentland, and accumulated more rishitin and phytuberin. Although Kennebec and WV-13-8 accumulated less rishitin after inoculation with race 0 than race 4, Cherokee and 3RC-8 gave the opposite result, and 3XX-1 showed little difference in accumulation with the two races (Table 2). The small *r* cultivars Russet Burbank and Irish Cobbler accumulated a trace of rishitin after inoculation, but Katahdin and Red Pontiac did not.

Quantitation of rishitin by gas-liquid chromatography.—Using TLC estimations as a guide, five cultivars were selected to quantitate rishitin accumulation 60 hr after inoculation. Duplicate samples were run for each cultivar-race interaction, and the results were averaged (Table 2). Duplicate analyses were also run for three cultivar-race interactions at intervals after inoculation (Table 3). In assays of the first mm, rishitin was detected 30 to 36 hr after inoculation and reached max levels of 1×10^{-4} molal to 2×10^{-4} molal at subsequent time intervals. Phytuberin was first detected in Cherokee—race 0, 3RC-8—race 4, and WV-13-8—race 0 37, 45, and 53 hr after inoculation, respectively. Maximum levels of phytuberin ranged from approximately 1×10^{-6} molal to 6×10^{-5} molal in the first mm. Rishitin was detected in the second mm about 10 hr after it was detected in the first mm with 3RC-8 and WV-13-8, but Cherokee exhibited a nearly concomitant increase in both layers. A parallel rate of phytuberin accumulation was demonstrated initially in Cherokee and 3RC-8 between the first and second mm, but the rate in the second mm slowed during the later stages of infection.

DISCUSSION.—A consistent tuber response to infection by *P. infestans* was demonstrated with 11 potato cultivars and three fungal races. The response, in part, included the accumulation of rishitin and phytuberin. These compounds accumulated in incompatible host-pathogen interactions, and were detected 30-40 hr after inoculation. Little or none of the compounds accumulated in compatible interactions. The time of most striking symptom expression correlated with the accumulation of rishitin and phytuberin with Cherokee—race 0 and WV-13-8—race 0; however, accumulation in 3RC-8—race 4 occurred approximately at the same time as with the above interactions, even though visible symptoms appeared earlier. It appears that marked symptom expression does not always coincide with the detection of rishitin and phytuberin.

Tomiyama et al. (4) reported 80-90 μg rishitin/g fresh tissue in an incompatible reaction 60 hr after inoculation. The quantities reported by us are lower using freshly harvested tubers. Results are comparable with those reported by Tomiyama et al. (4) when stored tubers which had started sprouting were used.

The difference between compatible and incompatible interactions, as reflected in rishitin and phytuberin accumulation, appears to be due to the extent of accumulation and not to the presence of genetic information for the biosynthesis of these compounds.

TABLE 3. Accumulation of rishitin and phytuberin in three incompatible potato-*Phytophthora infestans* interactions[a]

Var., fungal race	Hr incubation	First mm		Second mm	
		Rishitin[b]	Phytuberin[b]	Rishitin[b]	Phytuberin[b]
Cherokee—	20	—[c]	—	—	—
Race 0	29	0.8	—	0.8	—
	37	9.2	0.2	2.4	0.2
	45	11.0	1.1	4.0	0.8
	53	30.0	2.6	5.2	1.9
	65	37.3	19.6	9.3	5.8
	94	29.8	12.0	6.4	3.5
WV-13-8—	18	—	—	—	—
Race 0	26	—	—	—	—
	34	2.2	—	—	—
	43	6.1	—	0.1	—
	53	42.2	0.1	15.4	—
	65	44.2	0.4	14.8	0.2
	89	Lost	0.1	38.0	0.5
3RC-8—	20	—	—	—	—
Race 4	28	—	—	—	—
	36	1.0	—	—	—
	45	7.5	0.2	—	0.2
	53	10.0	1.4	0.8	1.4
	66	19.4	3.8	0.4	3.0
	80	28.7	11.6	trace	4.4

[a] Analyses by combined thin-layer and gas-liquid chromatography.
[b] μg Compound/g fresh wt potato tissue.
[c] Compound not detected.

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