

Accumulation and Transformation of Rishitin and Lubimin in Potato Tuber Tissue Infected By an Incompatible Race of *Phytophthora infestans*

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ABSTRACT

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Holes in potato tubers were filled with zoospore suspensions of an incompatible race of *Phytophthora infestans*. After incubation for various times, the inoculation fluids were collected and examined for inhibitory ethyl ether-soluble compounds. Rishitin, lubimin, rishitinol, and eight unknown compounds were detected by thin-layer chromatography (TLC). Rishitin, lubimin, rishitinol, and five unknown compounds were found in the inoculation fluid but not in the control fluids from healthy tubers. Rishitin levels were determined daily; the amount increased until it reached 245 $\mu\text{g}/\text{ml}$ in the inoculation fluid 6 days after inoculation. Lubimin gradually increased for 96 hours to a maximum level of 28 $\mu\text{g}/\text{ml}$ in the inoculation fluid. Of all the compounds detected by TLC, the rishitin spot was the largest

and had the most intense color reaction, suggesting that rishitin was present in the highest concentration. Rishitin- ^{14}C was administered to healthy tubers and tubers infected by an incompatible race of *P. infestans*. Liquid scintillation spectrometry and autoradiography suggested that rishitin might be transformed to other compounds in both healthy and infected tissues. These results suggested that rishitin synthesized in the healthy tissue around browned tissue in tubers infected by the incompatible race would be transformed to other compounds. Thus, only a slight accumulation of rishitin occurs in the healthy tissue. In contrast, rishitin may accumulate in browned cells or in diffusates from them because of a lack of transformation.

Additional key words: potato late blight.

The production of the sesquiterpenoid phytoalexins, rishitin (6, 13), lubimin (5, 7, 11), oxylubimin (5), rishitinol (4), and phytuberin (1, 2, 14) by potato tissue in response to inoculation with an incompatible parasite has been reported.

In previous papers, the time when rishitin was chemically detectable after infection and the sites where rishitin was synthesized and accumulated were described (3, 8, 9, 10, 12). Rishitin in potato tuber tissue reached maximum concentrations 3-4 days after inoculation with an incompatible race of *Phytophthora infestans*; then concentrations decreased rapidly. It is possible that rishitin is transformed into other compounds in the infected tissue. Rishitin labeled with ^{14}C was used in experiments reported here to study its transformation. Diffusate from the surface of infected and healthy tissues were used to study accumulation and transformation of rishitin.

MATERIALS AND METHODS

Plant materials and inoculation.—Tubers of potato cultivar Rishiri carrying the R_1 gene for resistance to *P. infestans* race 0 were used in this study. Zoospore

suspensions of *P. infestans* race 0 were obtained from mycelial mats growing on the cut surfaces of fresh tubers of a susceptible potato cultivar, Irish Cobbler. The inoculation methods of Metlitsky et al. (7) were used; holes were made in the cut tuber tissues to a depth of approximately 2 cm with a cork borer 12 mm in diameter; the cut surfaces then were washed with water; and the tubers were incubated for 18 hours in a moist plastic box at about 17 C. Two milliliters of zoospore suspension of race 0 (8×10^5 zoospores/ml) were then poured into each hole. The inoculated tubers were incubated in a moist plastic box at about 17 C. At intervals, the inoculation fluid in the holes was taken for analysis of rishitin, lubimin, and other compounds.

To determine if rishitin was transformed to other compounds in the infected tuber tissue, the following method of inoculation was used. Tissue plugs (12 mm \times 10 mm) were cut with a cork borer from the central parenchyma of potato tubers. The plugs were washed with running water for 30 minutes and then incubated for 18 hours on filter paper in a moist plastic box at about 17 C. The upper surface of 30 plugs then was inoculated with 20 μl of the zoospore suspension of race 0 (8×10^5 spores/ml) containing the antibiotic Ceporan (Torii Chem. Co.) (2 mg/ml). As a control, each of the upper surfaces of 30 plugs of noninoculated healthy tissue was treated with 20 μl of the Ceporan solution (2 mg/ml) and as an additional control 30 plugs were autoclaved at

120 C for 10 minutes. After inoculation, the plugs were incubated at 17 C.

Extraction and determination of rishitin and lubimin.—The inoculation fluid from six holes was collected at each harvest interval (6, 24, 48, 72, 96, 120, and 144 hours after inoculation). The fluids were centrifuged at 7,000 g for 10 minutes, and the supernatant was extracted three times with an equal volume of ethyl ether. The ether extract was evaporated and the residue was dissolved in a small amount of acetone. The acetone extract was subjected to thin-layer chromatography (TLC) (silica gel G, 0.5-mm thick) using chloroform:acetone (85:15, v/v), ethyl ether, or cyclohexane:ethyl acetate (1:1, v/v), as solvents. The compounds were detected by spraying plates with concentrated H₂SO₄ and heating at 110 C for a few minutes.

For quantitative analysis, rishitin and lubimin in the acetone extract were separated by TLC using silica gel plates, with chloroform:acetone (85:15, v/v) as the solvent. The R_f zones containing rishitin and lubimin were packed in a column and eluted with acetone. After evaporation of the acetone, the residue was redissolved in an appropriate amount of acetone. Analyses were performed on a JEOL Model JCC-1100 gas chromatograph equipped with a stainless steel column 1 m × 3 mm ID, and a flame ionization detector. The following materials and conditions were used: carrier gas, N₂; carrier flow pressure, 1.5 kg/cm²; H₂ flow pressure, 1.5 kg/cm²; air flow pressure, 1.5 kg/cm²; injector temperature, 250 C; detector temperature, 250 C; column temperature, 190 C; attenuation setting 16 × 10⁻¹¹; packing, 7.5% STAP on chromosorb W (AW) DMCS [177-149 μm (80- to 100-mesh)] (Nishio Kogyo Co.); chart speed, 5 mm/minute. Amounts of rishitin less than 0.1 μg/ml could not be quantitatively measured under these experimental conditions.

Preparation of rishitin-¹⁴C.—Holes were made in the tuber tissues as described above, and the tubers were incubated in a moist plastic box at about 17 C. After 18-24 hours, each hole was filled with 1 ml of a zoospore suspension of *P. infestans* race 0 (1.1 × 10⁶/ml). Three

hours after inoculation, 1 ml of 10⁻² M acetate buffer (pH 5.6) containing an appropriate amount of sodium acetate-2-¹⁴C (48.0 mCi/mmole) was pipetted into each hole (approximately 5 × 10⁻³ mCi per each hole). The inoculation fluid and browned tissues surrounding the holes were collected for extraction 24-48 hours after inoculation. The fluid was extracted by the method described above. The browned tissues were homogenized with 20 ml of acetone and sea sand in a mortar. The homogenate was filtered through Toyo filter paper No. 6.

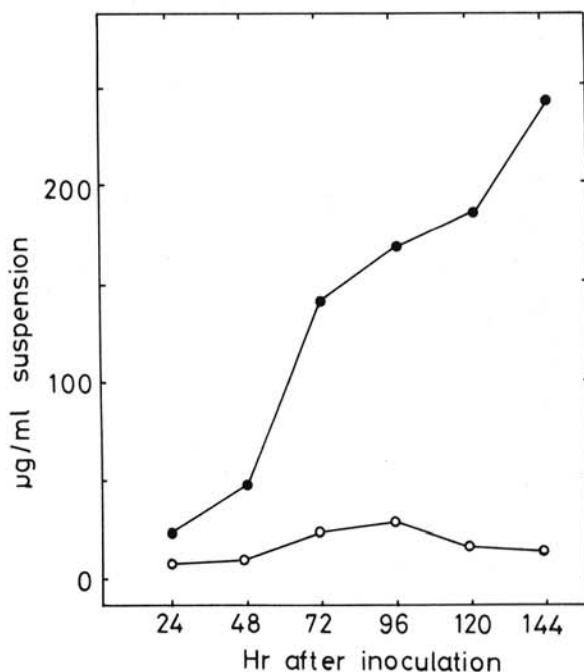


Fig. 1. Time-course of accumulation of rishitin and lubimin in the diffusate (inoculation fluid) from potato tuber tissue infected by an incompatible race of *Phytophthora infestans*. Closed circle = rishitin, open circle = lubimin.

TABLE 1. Thin-layer chromatographic analysis (R_f-values) of phytoalexins and unknown compounds extracted from the diffusate^a of potato tuber tissue infected by an incompatible race of *Phytophthora infestans*

Compounds	R _f values of compounds extracted from diseased potato tuber developed with:			Color reaction to conc. H ₂ SO ₄ and heating
	Ethyl ether	Chloroform : acetone (85:15, v/v)	Ethylacetate : cyclohexane (1:1, v/v)	
Rishitin ^b	0.23	0.13	0.19	Violet
Lubimin ^b	0.37	0.32	0.26	Gray-brown
Rishitinol ^b	0.47	0.36	0.33	Pink
Oxylubimin ^b	0.04	0.05	0.04	Orange
A ^c	0.92	0.88	0.80	Pink
B	0.86	0.80	0.73	Orange-brown
C	0.79	0.72	0.60	Brown
D	0.40	...	0.30	Orange-brown
E	...	0.19	...	Brown
F	0.10	0.08	0.08	Gray-brown

^aInoculation fluid in a hole made in the tuber tissue (see text).

^bAuthentic compounds.

^cA-F, unknown compounds.

The residue was washed four times with 20 ml of acetone. After evaporation of the solvent, the residue was dissolved in a small amount of acetone. Rishitin- ^{14}C in the extract was purified with TLC using chloroform:acetone (85:15, v/v) as a solvent. The silica gel containing the rishitin band (R_f 0.13) was collected and extracted five times with 20 ml of acetone. After evaporation, the residue was dissolved in a small amount of acetone, and again submitted to TLC, using ethyl ether and then cyclohexane:ethyl acetate (1:1, v/v) as solvents. The purified rishitin gave a single spot on TLC in this system. Distribution of radioactivity on the TLC plate gave almost a single peak (94% of the total activity) at the site of the rishitin spot. Autoradiography of the TLC showed that the shape of the radioactive spot clearly coincided with that of the spot having the color reaction

characteristic of rishitin. The shift of the rishitin spot on TLC caused by use of different solvents [chloroform:acetone (85:15, v/v), cyclohexane:ethyl ether (1:1, v/v), and ether] resulted in the same shift of the radioactive spot. These results indicated that most of the radioactivity of the purified ^{14}C -labeled rishitin originated from rishitin itself, although apparently it contained traces of impurities.

Incubation of the tuber tissues with rishitin- ^{14}C .—The upper surface of healthy, inoculated, and autoclaved tissue plugs were sectioned into slices 1-mm thick 24 hours after they were cut and inoculated. The slice taken from the inoculated plug 24 hours after inoculation consisted of brown cells (upper one-fourth) and healthy cells (lower three-fourths of the slices). The slices were placed in 1 ml of 5×10^{-3} M acetate buffer (pH 5.6)

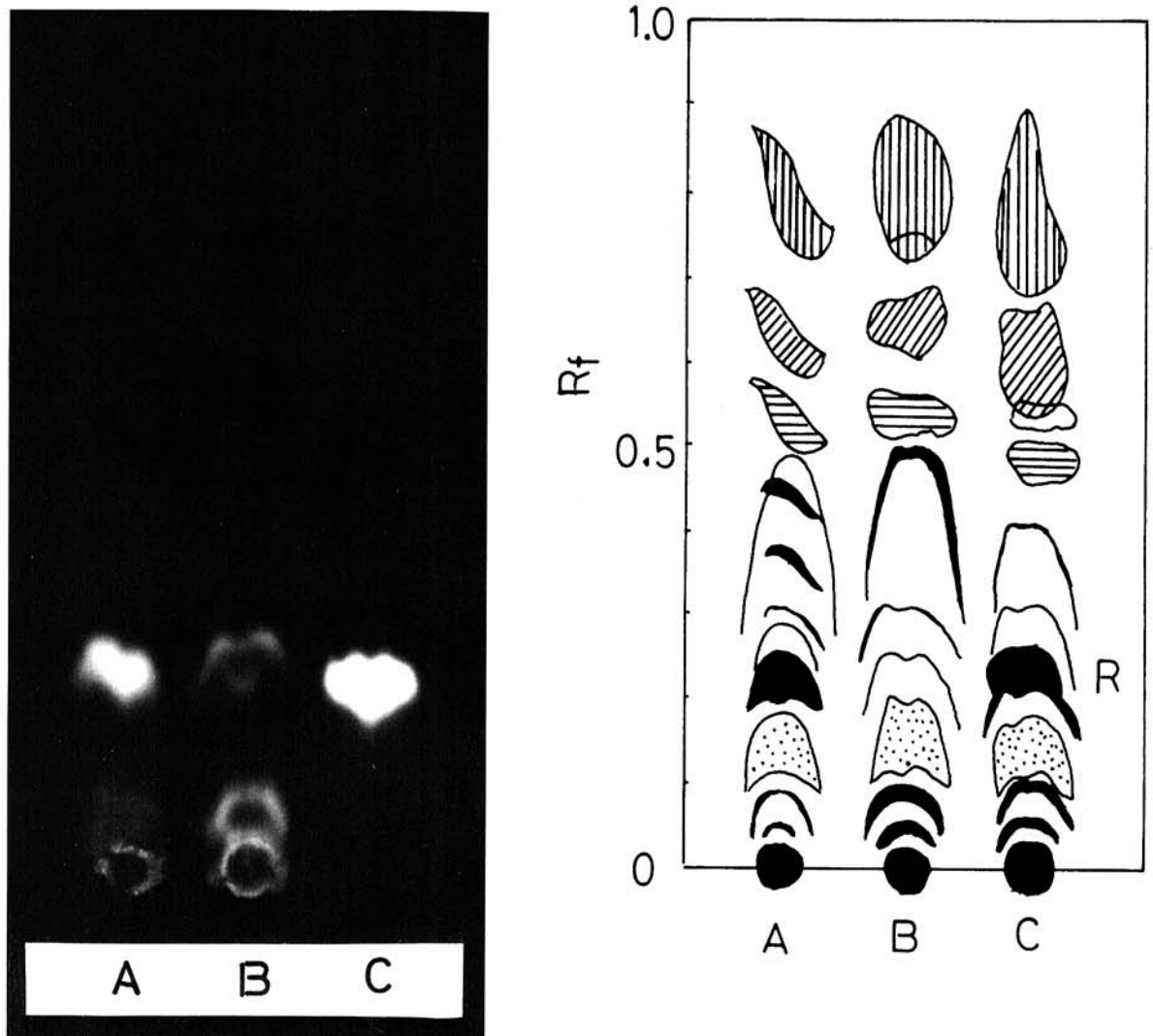


Fig. 2. Autoradiogram (left) of thin-layer chromatography (TLC) of ^{14}C -labeled transformation products of rishitin extracted from the potato tuber tissue incubated with rishitin- ^{14}C for 5 hours. The TLC was developed with cyclohexane:ethyl ether (1:1, v/v). After autoradiography (right) the plate was sprayed with concentrated H_2SO_4 and heated for a few minutes. Legend: A = infected tissue; B = noninfected tissue; C = autoclaved noninfected tissue; and R = rishitin.

containing rishitin- ^{14}C (approximately 10^4 cpm) and Ceporan (2 mg/ml) in a petri dish (5.7 cm in diameter) for 5 or 10 hours.

Extraction and detection of ^{14}C -labeled compounds in tuber tissue.—After incubation, the slices and the acetate buffer were transferred to a mortar and homogenized with sea sand. The homogenate was combined with 20 ml of acetone and then filtered through Toyo filter paper No. 6. The residue was washed four times with 20 ml of acetone. After evaporation, the residue was dissolved in a small amount of acetone. The acetone extract was submitted to TLC, using cyclohexane: ethyl acetate (1:1, v/v) as the solvent. After development, the chromatogram was scraped off in zones 5-mm wide each from the origin to the front. The silica gel of each band was placed in 10 ml of a toluene scintillation fluid [4 g of PPO (Daiichi Chem. Co.), and 0.1 g of POPOP (Wako Chem. Co.) in 1 liter of toluene] and the radioactivity was determined with Packard Tri-Carb liquid scintillation

spectrometers, Model 2003 and Model 3320. Counts were made at 80% efficiency. Autoradiography of the TLC was carried out with Fuji X-ray film, and the compounds were detected as described above.

RESULTS

Accumulation of rishitin, lubimin, and other compounds in the inoculation fluid.—Inoculation fluids from incompatible host-pathogen combinations were analyzed by TLC, using ethyl ether, chloroform: acetone (85:15, v/v), and ethyl acetate: cyclohexane (1:1, v/v) as solvents. Rishitin (6), lubimin (5, 7, 11), rishitinol (4), and eight unknown compounds were detected (Table 1). Rishitinol, lubimin, and rishitin were identified from the color reaction and R_f values of these spots by comparing them with the spots of the authentic compounds on the same plate. We could not determine which of the spots was identical with phytuberin (2, 11) and we found no

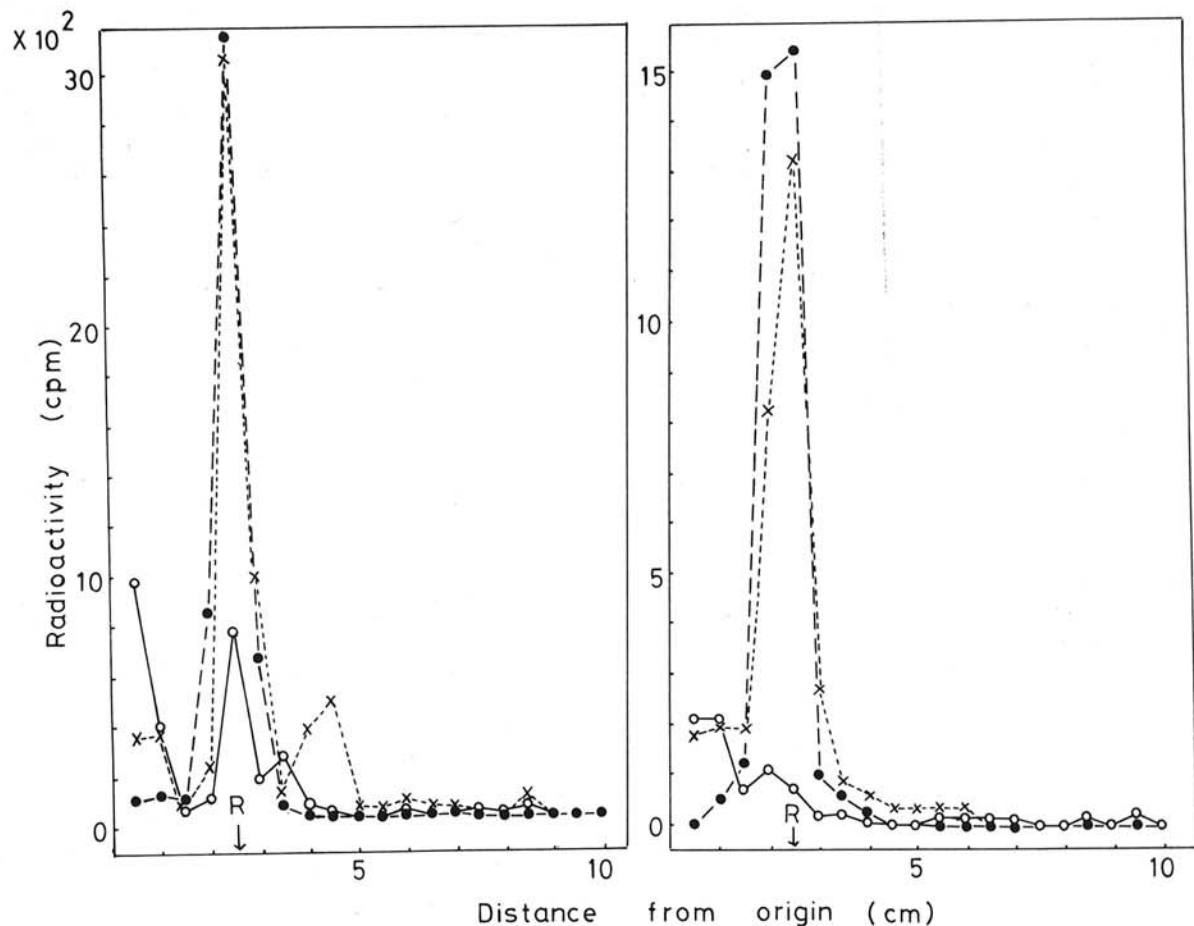


Fig. 3. Distribution of radioactivity in the thin-layer chromatogram of transformation products of rishitin extracted from potato tuber tissue incubated with rishitin- ^{14}C for 5 hours (left) and 10 hours (right). The thin-layer chromatogram was done in the same way as described in Fig. 1, and the radioactivity was measured in a liquid scintillation counter. Legend:

- = autoclaved tissue.
- = healthy tissue.
- x-----x = tissue infected with *Phytophthora infestans*.
- R = rishitin.

oxylubimin. Among these compounds, rishitin, lubimin, rishitinol, and five unknown compounds (C, D, E, F, G, and H) were not detected in the control. Rishitin and lubimin were detected 24 hours after inoculation, and the rishitin continued to increase up to 144 hours after inoculation (Fig. 1). The amount of lubimin always was lower than that of rishitin. Rishitinol was first detected as a spot 48 hours after inoculation, and then the color density of the spot increased and reached a maximum 120 hours after inoculation. No appreciable amount of oxylubimin was found on the TLC through 144 hours after inoculation. Metlitsky et al. (7) reported that eight spots, including rishitin and lubimin, were detected in the exudate from infected potato tubers under similar conditions.

Distribution of radioactivity in extracts from ^{14}C -rishitin-treated tissue.—The distribution of radioactivity in compounds in the extract from the potato tuber tissue was examined by TLC and autoradiography techniques (Fig. 2). After exposure of the X-ray film, the radioactivity distribution on the TLC plate was measured with a liquid scintillation counter (Fig. 3). In extracts obtained from healthy and infected tissue treated with rishitin- ^{14}C , the radioactive spots were found on the TLC plates developed with cyclohexane: ethyl ether (1:1) at R_f values of 0, 0.03, 0.07, 0.13, 0.22, 0.26, and 0.79. The R_f of authentic rishitin on the same plate was 0.22. In the extract from the autoclaved tissue treated with rishitin- ^{14}C , little radioactivity was found on the TLC plates except in the rishitin spot. The radioactivity of the rishitin spot was less in the extract from healthy tissue than in that from the infected tuber (Table 2). More radioactivity was found in the other spots on TLC plates from healthy tissue extracts than from plates of corresponding infected tissues. The transfer of ^{14}C from rishitin to other compounds seemed to be more pronounced 15 hours than 5 hours after treating with rishitin- ^{14}C . Values of R_f for the new radioactive spots on the TLC plates did not coincide with those of authentic lubimin or rishitinol. The results suggested that lubimin and rishitinol might not be derived from rishitin.

DISCUSSION

The inoculation fluid from a hole in a tuber can be considered a diffusate from infected tissues. The experiments reported here clearly indicated that several

compounds, especially rishitin, can accumulate in the diffusate. Previous papers (3, 8, 10, 12) indicated that rishitin accumulated in the brown infected zone, and was found only slightly in the adjacent healthy tissue. Therefore, it is concluded that rishitin can accumulate in the diffusate and brown zone of the infected tissue, but only slightly in the adjacent healthy tissue.

In a previous paper (8) it was demonstrated that rishitin was synthesized in the healthy tissue adjacent to the infected brown zone, but possibly not in the brown dead tissue. Nevertheless, rishitin continued to accumulate in the brown tissue after the browning. It was, therefore, suggested that rishitin synthesized in the healthy tissue might be transported to the browned tissue.

The present experimental results suggested that rishitin possibly may be transformed into other compounds in the healthy tissue around browned areas, resulting in the accumulation of only a trace of rishitin in the healthy tissues. Rishitin transported to the nonliving brown tissue may not be transformed there and thus would accumulate. However, it is still difficult to interpret how the amount of rishitin decreased in the browned lesion after it once reached a maximum.

LITERATURE CITED

1. COXON, D. T., R. F. CURTIS, K. R. PRICE, and B. HOWARD. 1974. Phytuberin: a novel antifungal terpenoid from potato. *Tetrahedron Lett.* 27:2362-2366.
2. HUGHES, D. L. 1974. Phytuberin: revised structure from the X-ray crystal analysis of dihydrophytuberin. *Chem. Commun.* 1974:822-823.
3. ISHIZAKA, N., and K. TOMIYAMA. 1972. Effect of wounding or infection by *Phytophthora infestans* on the contents of terpenoids in potato tubers. *Plant Cell Physiol.* 13:1053-1063.
4. KATSUI, N., A. MATSUNAGA, K. IMAIZUMI, T. MASAMUNE, and K. TOMIYAMA. 1971. The structure and synthesis of rishitinol, a new sesquiterpene alcohol from diseased potato tubers. *Tetrahedron Lett.* 2:83-86.
5. KATSUI, N., A. MATSUNAGA, and T. MASAMUNE. 1974. The structure of lubimin and oxylubimin, antifungal metabolites from diseased potato tubers. *Tetrahedron Lett.* 51/52:4483-4486.
6. KATSUI, N., A. MURAI, M. TAKASUGI, K. IMAIZUMI, T. MASAMUNE, and K. TOMIYAMA. 1968. The structure of rishitin, a new antifungal compound from diseased potato tubers. *Chem. Commun.* 1968:43.

TABLE 2. Ratio of radioactivity of the rishitin fraction to total activity of the ether extract obtained from potato tuber tissue incubated with rishitin- ^{14}C for 5 hours

Radioactivity	Healthy tuber tissue in experiments:			Infected tuber tissue in experiments:			Autoclaved tuber tissue in experiments:		
	1	2	3	1	2	3	1	2	3
Incorporation rate of radioactivity into the ether fraction (%)	44.6	28.4	...	74.2	65.0	...	69.4	60.1	...
Percent of cpm of rishitin fraction to total cpm of ether fraction (%)	34.0	36.2	30.6	75.2	73.4	56.2	96.8	94.9	91.1

^aIn the first and second experiments the total counts of rishitin- ^{14}C applied to healthy and infected tubers was 10,866 cpm, but in the third experiment the total counts were not measured. When the rishitin- ^{14}C solution used for incubation was developed on silica gel thin-layer chromatography plates with the ether solvent, 94.1% of radioactivity was found in the rishitin spot.

7. METLITSKY, C. V., Y. T. YURGANOVA, L. I. CHALOVA, and N. I. VASXUKOVA. 1971. Induction of potato phytoalexin. Ser. Biologicheskaya Akad. Nauk. SSSR 3:399-407.
8. NAKAJIMA, T., K. TOMIYAMA, and M. KINUGAWA. 1975. Distribution of rishitin and lubimin in potato tuber tissue infected by an incompatible race of *Phytophthora infestans* and the site where rishitin is synthesized. Ann. Phytopathol. Soc. Jap. 41:49-55.
9. SATO, N., K. KITAZAWA, and K. TOMIYAMA. 1971. The role of rishitin in localizing the invading hyphae of *Phytophthora infestans* in infection sites at the cut surfaces of potato tubers. Physiol. Plant Pathol. 1:289-295.
10. SATO, N., and K. TOMIYAMA. 1969. Localized accumulation of rishitin in the potato-tuber tissue infected by an incompatible race of *Phytophthora infestans*. Ann. Phytopathol. Soc. Jap. 35:202-217.
11. STOESSL, A., J. B. STOTHERS, and E. W. B. WARD. 1974. Lubimin a phytoalexin of several Solanaceae. Structure revision and biogenetic relationships. Chem. Commun. 1974:707-709.
12. TOMIYAMA, K., and M. FUKAYA. 1976. Accumulation of rishitin in dead potato-tuber tissue following treatment with HgCl₂. Ann. Phytopathol. Soc. Jap. 41:418-420.
13. TOMIYAMA, K., T. SAKUMA, N. ISHIZAKA, N. SATO, N. KATSUI, M. TAKASUGI, and T. MASAMUNE. 1968. A new antifungal substance isolated from resistant potato tuber tissue infected by pathogens. Phytopathology 58:115-116.
14. VARNIS, J., J. KUĆ, and E. WILLIAMS. 1971. Terpenoid accumulation as a biochemical response of the potato tuber to *Phytophthora infestans*. Phytopathology 61:174-177.