# Induction of Rishitin-Metabolizing Activity in Potato Tuber Tissue Disks by Wounding and Identification of Rishitin Metabolites

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ABSTRACT

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Rishitin, the phytoalexin of potato, was metabolized by fresh and aged potato-tuber disks. In aged disks, radioactivity of rishitin-$^{14}$C began to decrease almost simultaneously after incubation with rishitin-$^{14}$C and continued to decrease at the rate of 3.6 μg rishitin/g fresh wt/hr. The decrease in radioactivity of rishitin-$^{14}$C was accompanied by an increase in radioactivity in the ether-soluble fraction (minus rishitin) and then followed by that in the water-soluble fraction. In fresh disks, on the contrary, there was a lag of a few hours before the initiation of decrease in radioactivity of rishitin-$^{14}$C. These results suggest that intact potato tissue has little activity to metabolize rishitin, but that the activity was induced by wounding. Two $^{14}$C-labeled compounds were isolated from the ether-soluble metabolites of rishitin in tuber tissue treated with $^{14}$C-rishitin. They were identified as rishitin-M-1 and rishitin-M-2 on the basis of $R_f$ values after thin-layer chromatography (TLC), autoradiography, and color reactions after two-dimensional TLC.

*Additional key words*: potato late blight.

The sesquiterpene phytoalexins of potato [rishitin (7, 14, 26), rishitinol (8, 9), lubimin (10, 11, 13, 23), oxylubimin (10, 11), solavetivone (1) etc.] have been reported to play an important role in disease resistance (12, 21, 22). However, it is still not clear how induction of synthesis, accumulation, and disappearance of any of these phytoalexins occur.

In a previous paper (3), it was reported that aged surface tissue of cut potato tuber noninfected or infected by an incompatible race of *Phytophthora infestans* could transform rishitin to other compounds. The metabolism of rishitin in these tissues may play a key role in accumulation and disappearance of rishitin.

In this paper, we report: (i) the identification of metabolites of rishitin in potato tissue, (ii) the time course of rishitin conversion in potato tuber tissue, and (iii) the results of experiments carried out to determine whether fresh potato tuber disks could metabolize rishitin or not.

## MATERIALS AND METHODS

**Plant materials.**—Tubers of the potato cultivar Rishiri carrying the $R_1$ gene were used. They are highly resistant to race 0 of *Phytophthora infestans* which was used in the present experiments. Tubers were stored at 4 C, but were kept at 18 C for 24 hr before they were used for the experiments. Zoospore suspensions of *P. infestans* were prepared as described previously (3).

**Preparation of rishitin-$^{14}$C.**—The $^{14}$C-labeled rishitin was prepared by the method of Horikawa et al. (3). A hole was made in the potato tuber and inoculum consisting of zoospores of race 0 was added. Then the hole was filled with 2 ml of acetate-2-$^{14}$C solution (10 μCi/ml), which was prepared by dissolving acetate-2-$^{14}$C (56 mCi/mmole, Radiochemical Center, Amersham, England) in distilled water. Rishitin-$^{14}$C was isolated from the fluid in the holes and from the tissue surrounding the holes according to the method of Horikawa et al. (3). Fifty mg of nonlabeled rishitin was added to the extract and final purification of the crude rishitin obtained was effected by formation of the crystalline bis(3,5-dinitrobenzoate) followed by hydrolysis and distillation in vacuo (26). When the rishitin-$^{14}$C obtained (336 dpm/μg) was chromatographed on silica gel by thin-layer chromatography (TLC), a single peak of radioactivity was obtained at the proper $R_f$.

**Incubation of tuber tissue with rishitin-$^{14}$C.**—Tissue disks (16 mm in diam, 10-mm thick), were cut out with a cork borer from the central tissue of potato tubers. The disks were washed with running water for 30 min and then used immediately or after incubation at about 18 C for 24 hr. The upper surfaces of the disks were sectioned into slices 0.7 mm thick. Fifteen slices (about 2.2 g) were placed in 1 ml of sterile water containing pure rishitin-$^{14}$C

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($4 \times 10^4$ dpm/ml in the 1st experiment and $3.2 \times 10^4$ dpm/ml in the 2nd experiment), the antibiotic Ceporan (Torii Chem. Co., Chuo-Ku, Nihonbashi 3-3, Tokyo) (3 mg/ml) and 10% acetone, and incubated for 0, 1, 3, 6, 12, 24, 48, and 72 hr at 24 C.

**Extraction and detection of $^{14}$C-labeled compounds.**—At each sampling time, both the disks and $^{14}$C-rishitin solution were placed in a mortar and ground with a mixture of chloroform and methanol (2:1, v/v).. The mixture was filtered through Toyo filter paper No. 6. After evaporation of the solvent, the residue was extracted with ether (ether-soluble fraction) and then extracted with water (water-soluble fraction).

The ethyl-ether soluble compounds were separated by TLC (silica gel G, 0.5 mm thick) with cyclohexane: ethyl acetate (1:1, v/v) as solvent. After development, the chromatogram was scraped off in zones, each 5 mm wide, from the origin to the front. The silica gel from each band was placed in 10 ml of toluene scintillation fluid (4 g of 2.5-diphenyloxazol and 0.2 g of 1.4-di[2-(5-phenyloxazol)]-benzene in 1 liter toluene).

Water-soluble compounds were dissolved in 1 ml of water, placed in a vial, and added to 10 ml of Triton X-100 scintillation fluid (toluene scintillation fluid: Triton X-100, 2:1, v/v). Radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer, Model 3320 (Packard Scientific Instrument Co., Inc., Downer's Grove, IL 60515). The $^{14}$C-labeled compounds in the ether extract also were separated on a Merck TLC aluminum sheet of silica gel G 60 ($5 \times 20$ cm, 0.2 mm thick) and then covered with Fuji X-ray film for autoradiography.

**Extraction and isolation of compounds from nonlabeled rishitin-treated tissue.**—Potato tubers (17 kg) were cut into many thin slices (2 mm thick and 18 mm diameter), which were washed with running water at room temperature for 1 hr and then incubated at 20 C for 20 hr. These aged slices (total 4.4 kg) were placed in petri dishes [22-25 slices (approximately 10 g) in each dish] and then treated with a 1:9 mixture of acetone and water (2 ml for each dish, total 886 ml) containing crystalline rishitin (1 mg for each dish, total 443 mg), and then kept at 23-24 C for 24 hr. The whole slices were extracted in methanol (15 liters) at room temperature for 8-10 days. The methanol extracts were filtered, and the filtrate (6.5-7 liters) was evaporated to a syrup at 30 C or below and then extracted with 2 liters of chloroform. The chloroform solution was washed with water, dried and evaporated to remove resinous materials. The chloroform-insoluble aqueous extracts and water washings were combined and used for isolation of rishitin metabolites. The resinous materials obtained (5.54 g) were fractionated by column chromatography with silica gel [Merck, 212-56 $\mu$m particle size range (70-230 mesh), 160 g]. After thorough elution with ether to remove tarry materials (3.89 g), the mixture was eluted with a 20:1 mixture of ether and methanol (750 ml) and separated into three fractions. The least polar fraction (0.64 g) showed many spots by TLC, including that of rishitin. The middle (86 mg) and the most polar (26 mg) fractions did not contain compounds detected by TLC that were different from those of extracts from slices not treated with rishitin. The middle fraction again was purified by column chromatography with silica gel (7 g) with a 100:1 mixture of ether and methanol (250 ml), and yielded two compounds: (a) obtained (51 mg) in pure state, and (b) present in a mixture (35 mg) with compound (a). The mixture and the most polar fraction were fractionated separately by TLC (silica gel, Merck 60 F 254, 0.2 mm thickness, six and five plates, respectively) with ethyl acetate, developed twice, to give an additional sample of (a) 10 mg, and (b) 8 mg, in pure state. Previously, we had designated these compounds as rishitin-M-1 and rishitin-M-2, respectively, and determined their chemical structures (15) (Fig. 1).

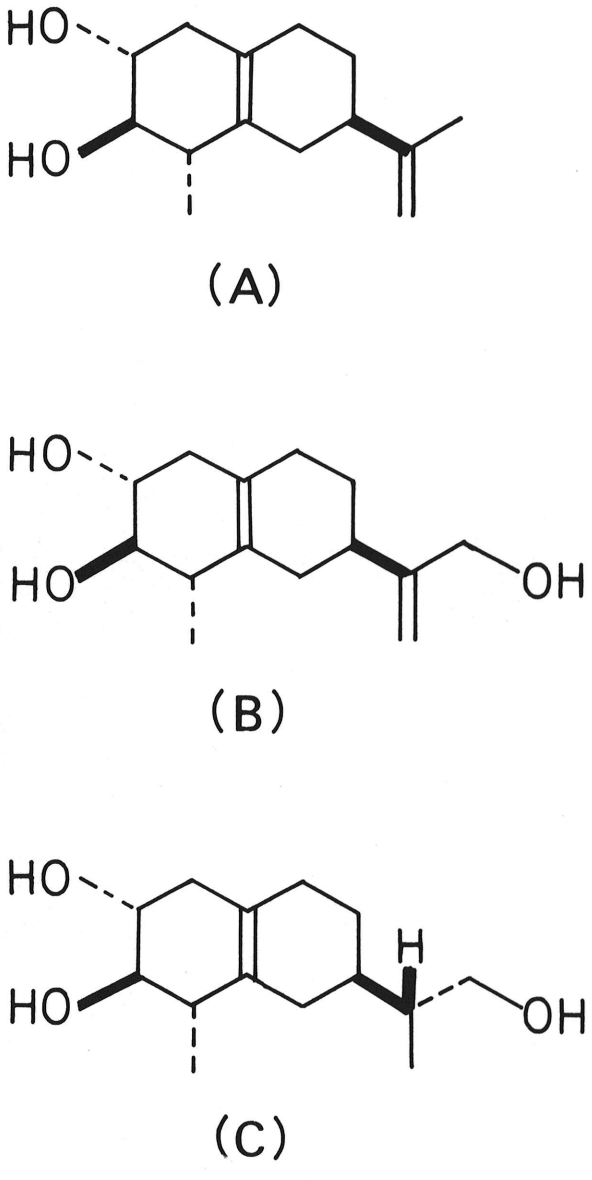


**Fig. 1-(A to C).** Chemical structures of **A)** rishitin, **B)** rishitin-M-1, and **C)** rishitin-M-2.

## RESULTS

**Incorporation of radioactivity from rishitin-$^{14}$C into other compounds in potato disks.**—Rishitin-$^{14}$C (about 120 $\mu$g) was applied to aged tuber disks and incubated in the dark at 24 C. Most of radioactivity of the rishitin fraction disappeared within 24 hr. The radioactivity decreased almost linearly with time at a rate of 100 dpm or 3.6 $\mu$g/g fresh wt/hr. The decrease in radioactivity of rishitin was accompanied by an increase in radioactivity of the ether-soluble fraction. The radioactivity of the ether-soluble fraction (minus rishitin) reached a maximum (about $17.3 \times 10^3$ dpm) about 12 hr after the tissue was fed rishitin-$^{14}$C, and then decreased to about $2.7 \times 10^{-3}$ dpm at 72 hr. The decrease in radioactivity of the ether-soluble fraction was followed with an increase in that of the water-soluble fraction. Radioactivity of the latter continued to increase linearly with time and reached $26.7 \times 10^3$ dpm 72 hr after adding rishitin-$^{14}$C. At each sampling time, the total radioactivity of ether- and water-soluble fractions were almost constant and about the same as the radioactivity of rishitin-$^{14}$C applied. Autoradiography of the ether-soluble, $^{14}$C-labeled compounds derived from rishitin-$^{14}$C showed the rishitin spot and other two spots of $R_f$ 0 and 0.06 (Fig. 2). In the case of fresh disks (Fig. 3-A), the concentration of $^{14}$C-rishitin did not decrease for about 3 hr, but then began to decrease, accompanied by increases in radioactivities of the ether-soluble and water-soluble fractions.

When rishitin was fed to the disks 24 hr after cutting (Fig. 2-B), it began to decrease immediately and was metabolized to ether- and water-soluble compounds. Generally, the rate of transformation of rishitin was more rapid in the aged disks than in the fresh ones.

**Identification of rishitin metabolites in ether extracts.**—Rishitin-M-1 and rishitin-M-2 were isolated from the tuber tissue treated with non-labeled rishitin (15). These compounds could not be found in non-treated tissue.

To ascertain whether or not rishitin-M-1 and rishitin-M-2 were metabolites of rishitin, the following experiments were carried out. The ether-extracts obtained from the tissue treated with rishitin-$^{14}$C were separated with ethyl acetate, ethyl ether, ethyl acetate:cyclohexane (1:1, v/v), ethyl ether:methanol (9:1, v/v), chloroform: acetone (85:15, v/v), and ethyl acetate:methanol (8:2, v/v) (Table 1). When developed with ethyl acetate, four spots (A, B, C, and D) were

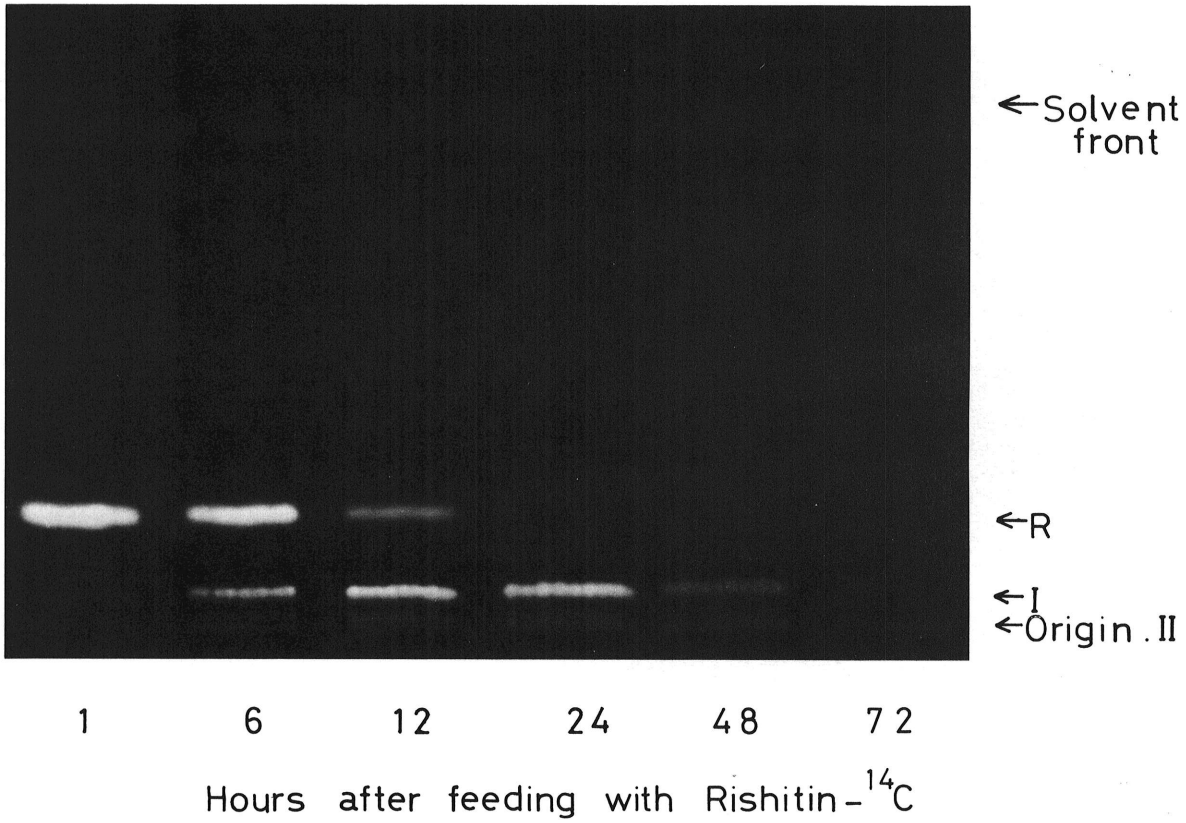


**Fig. 2.** Autoradiogram of thin-layer chromatography (TLC) of ethyl ether extracts from aged potato tuber disks treated with rishitin-$^{14}$C for various lengths of time at 24 C. TLC (Merck, TLC Silica Gel 60, 20 × 20 cm, 0.25 mm thick) was developed with cyclohexane:ethyl acetate (1:1, v/v). Distance from original line to top was 10 cm. The tuber disks were incubated with rishitin-$^{14}$C for 1, 6, 12, 24, 48, and 72 hr. (R): rishitin, I & II: rishitin metabolites.

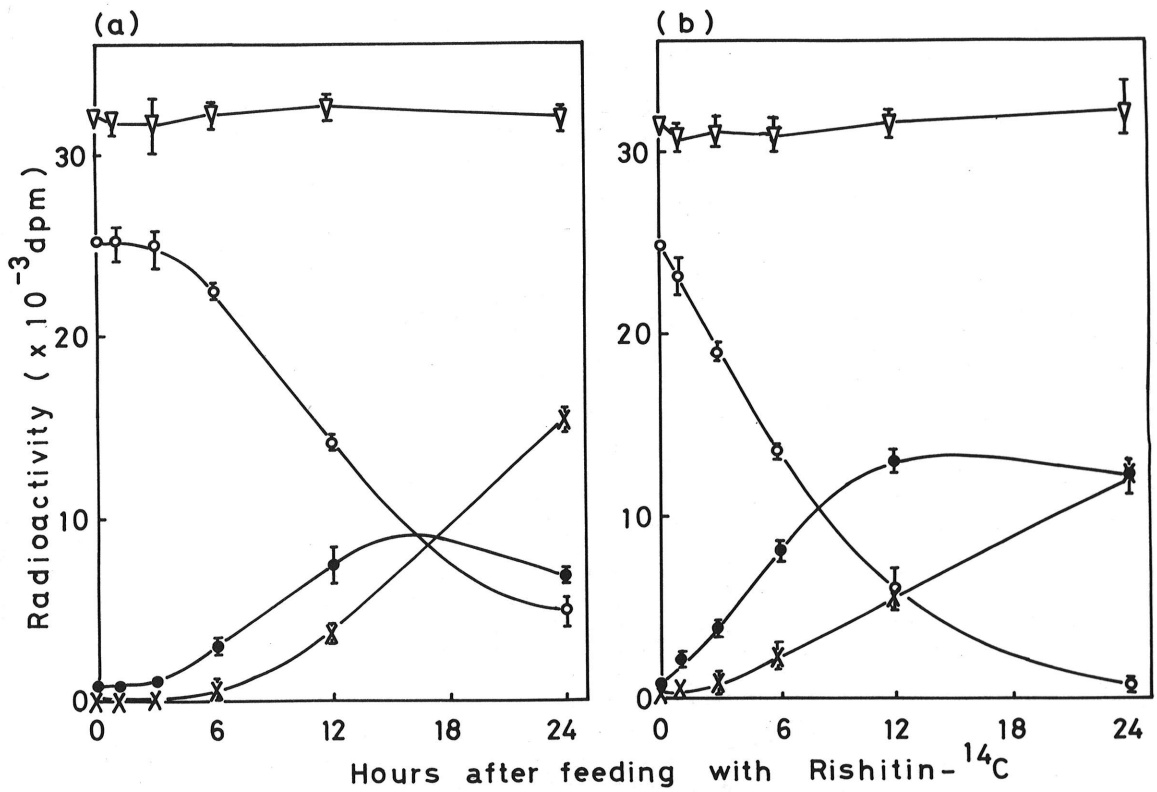


**Fig. 3-(A, B).** Time course of rishitin metabolism in fresh-cut and aged potato tuber disks treated with rishitin-$^{14}$C **A)** 30 min and **B)** 24 hr after cutting. The symbols: ▽= total radioactivity, o = rishitin, ● = ether-soluble rishitin metabolites, and × = water-soluble rishitin metabolites.

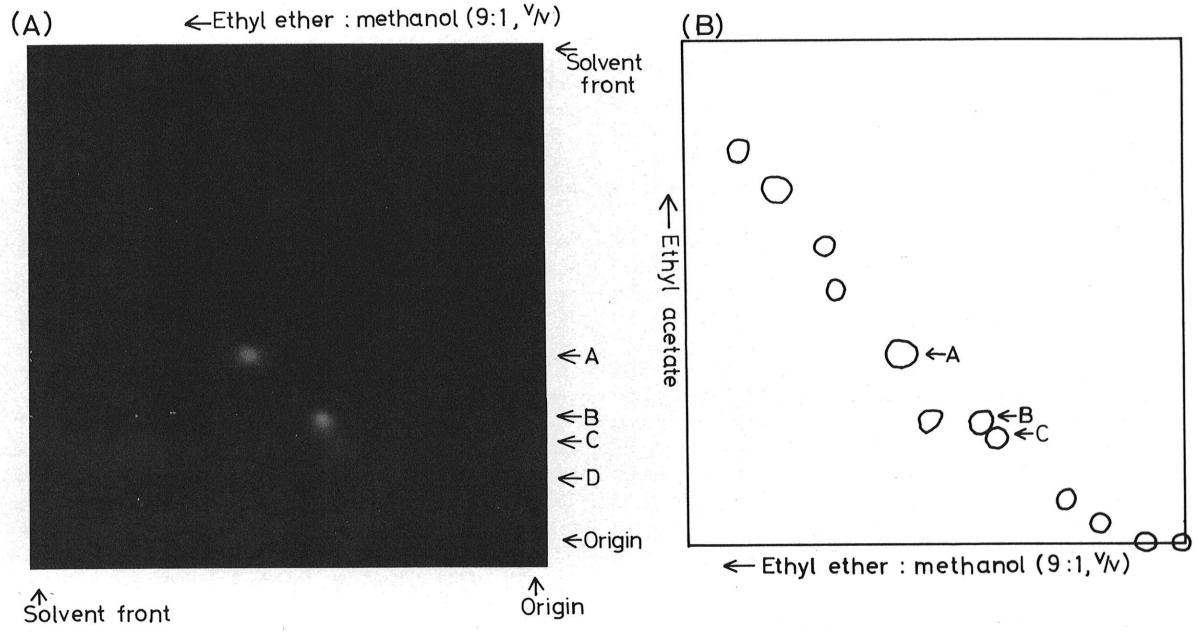


**Fig. 4-(A, B).** **A)** Autoradiographic and **B)** diagrammatic representation of two-dimensional thin-layer chromatography (TLC) of ether extracts from aged potato tuber disks treated with rishitin-$^{14}$C. The plates first were developed with ethyl acetate and then with ethyl ether:methanol (9;1, v/v). After autoradiography the plate was sprayed with concentrated $H_2SO_4$, and then with cerium (IV) sulphate (5% in 1 M $H_2SO_4$) and heated. The spots were: A = rishitin, B = rishitin-M-1, C = rishitin-M-2, and D = unknown compounds.

TABLE 1. The $R_f$ values of labeled compounds separated by thin-layer chromatography (TLC) of an ether-soluble extract from aged potato tuber disks treated with rishitin-$^{14}C$

| Solvent system | $R^f$ values of compounds[a]: | | | |
|---|---|---|---|---|
| | A | B | C | D |
| Ethyl acetate | 0.42 | 0.27 | 0.21 | 0.12 |
| Ethyl ether | 0.28 | 0.06 | 0.05 | 0.00 |
| Ethyl acetate: cyclohexane (1:1, v/v) | 0.25 | 0.07 | 0.06 | 0.00 |
| Ethyl ether: methanol (90:10, v/v) | 0.64 | 0.45 | 0.44 | 0.31 |
| Chloroform: acetone (85:15, v/v) | 0.21 | 0.06[b] | | 0.00 |
| Ethyl acetate: methanol (80:20, v/v) | 0.66 | 0.57[b] | | 0.50 |

[a]Compounds A, B, C, and D represent four autoradiographic spots obtained from TLC of ether extracts of potato tuber disks treated with rishitin-$^{14}C$ with the indicated solvent systems. Separation was done on a Merck TLC aluminum plate of silica gel G 60 (5 × 20 cm, 0.2 mm thick).

[b]These spots consisted of a mixture of compounds B and C.

detected on the autoradiogram. The $R_f$ of spot A with each solvent system coincided with that of authentic rishitin. Rishitin and the B and C spots developed a color reaction with cerium (IV) sulfate (5% in 1M $H_2SO_4$), but D did not. The substance in spot D was present only in trace amounts. The $R_f$ values of spots B and C coicided with those of authentic samples of rishitin-M-1 and rishitin-M-2, respectively.

The $^{14}C$-labeled spots B and C and authentic rishitin-M-1 and rishitin-M-2 were co-chromatographed on two-dimensional TLC with ethyl acetate in the first direction and with ethyl ether:methanol (9:1, v/v) in the second. After two-dimensional chromatography, the plate was first autoradiographed, they sprayed with concentrated $H_2SO_4$, followed by cerium (IV) sulphate (5% in 2N $H_2SO_4$) and then heated for a few minutes (Fig. 4). Compounds in spots B and C exactly coincided with authentic rishitin-M-1 and rishitin-M-2, respectively.

## DISCUSSION

In a previous paper (3), it was reported that rishitin was transformed to other compounds in aged, cut tissue of potato-tubers which were noninfected or infected with an incompatible race of *P. infestans*. The present results (Fig. 3-B) showed that rishitin was converted primarily to rishitin-M-1 and rishitin-M-2 and then metabolized to water-soluble compounds. These results provide evidence that rishitin may not be a stable end product but an intermediate in plant metabolism.

Fresh disks cannot metabolize rishitin, but the metabolizing system comes into operation a few hours after wounding. This indicates that wounding induces the rishitin-metabolizing system. Considering that rishitin is an intermediate in a biosynthetic pathway, it may be presumed that the synthesis of rishitin also can be induced by wounding. Experimental evidence supporting the concept that rishitin synthesis occurs in the healthy cut surface cells a few hours after cutting was presented previously (27).

It is known that the tricarboxylic acid cycle does not operate in fresh tuber disks, but begins to operate a few hours after wounding (6). It seems reasonable to suppose that there may be a relation between the onset of the rishitin synthesizing or metabolizing systems and the transition from resting state of the various physiological process to activated state caused by cutting (6, 19). However, the possibility that uptake of rishitin is not yet active in the fresh disk may not necessarily be excluded. Further investigation is necessary to confirm this point.

It is well known that phytoalexin production is not a specific response to infection by pathogens (2, 12, 22). Phytoalexins accumulate in plant tissues treated with many toxic chemicals or placed under temperature stress, etc. As described above, preparation of the tuber disks induced activity of the rishitin-metabolizing system. The rishitin-synthesizing system also is induced by cutting (27). However only traces of rishitin could be detected in the freshly cut tissue (5). Synthesis of sesquiterpene phytoalexins occurs in the healthy tissue neighboring the infected brown zone (4, 16), but little rishitin can be detected by chemical analysis in the rishitin-synthesizing healthy tissue. It has been reported that rishitin accumulated almost exclusively in brown lesion of the infected tuber (21, 25). The present experimental results support the hypothesis (3) that rishitin synthesized in healthy tissue next to the infected brown tissue may be metabolized to other compounds and thus is not accumulated. If rishitin is transported to the infected brown zone, it may be accumulated there, but it is not metabolized in dead cells or on the surface of dead cells. These results also suggest a possibility that suppression of rishitin metabolism by some factor also relates to accumulation of rishitin in brown lesion.

The disappearance of pisatin in pea tissue and of capsidiol in pepper tissue have been reported (17, 18, 20, 24). Transformation of phytoalexins in plant tissue seems to be a general phenomenon.

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