Suppression of Steroid Glycoalkaloid Accumulation as Related to Rishitin Accumulation in Potato Tubers

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ABSTRACT

The accumulation of fungitoxic steroid glycoalkaloids in potato tuber slices is markedly suppressed when the cut surface is inoculated with either *Phytophthora infestans* or *Helminthosporium carbonum* (a pathogen of corn), or treated with a cell-free sonicate of compatible or incompatible races of *P. infestans*. Suppression is greater with an incompatible than with a compatible race of *P. infestans*. Marked suppression of steroid glycoalkaloid accumulation is associated with the accumulation of high levels of rishitin. Exogenous α-solanine has little influence on the accumulation of steroid glycoalkaloids or rishitin. Aging for 72 hr before inoculation or treatment with sonicates, reduced rishitin accumulation and this effect did not appear to be caused by the steroid glycoalkaloids accumulated during aging prior to treatment or inoculation.

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Allen & Kuć (3) found that the antifungal activity of alcoholic extracts of potato tuber peel is mainly due to the steroid glycoalkaloids, α-solanine and α-chaconine. McKee (9) reported that the rapid accumulation of steroid glycoalkaloids near wounds in potato tubers increased resistance to *Fusarium coeruleum* Lib. and he concluded that the free bases of the steroid glycoalkaloids were toxic to the fungus. Cutting followed by aging results in the accumulation of steroid glycoalkaloids in the upper mm of tuber slices. However, only a trace of steroid glycoalkaloids was found in tuber slices inoculated with *Helminthosporium carbonum* race 1 Ullstrup (2). Allen showed that the decrease in accumulation of the compounds was not due to the degradation by the fungus (1).

Many terpenoid compounds accumulate in potato tuber slices inoculated with pathogens and nonpathogens (8). Two of them, rishitin (12, 15,16) and phytuberin (15, 16), accumulate to high levels in the infected area of slices inoculated with incompatible races of *Phytophthora infestans* (Mont.) deBary (resistant reaction) but not compatible races (susceptible reaction). The two compounds are fungitoxic and appear associated with the "R" gene resistance mechanism of potato tubers to *P. infestans*. Rishitin and phytuberin accumulation in potato tuber slices, however, is not a specific response to incompatible races of *P. infestans* (13, 14). Rishitin and phytuberin were detected in tuber slices inoculated with two fungi nonpathogenic to potato, *Ceratocystis fimbriata* Ell. & Halst and *H. carbonum* as early as 24 hr after inoculation. *H. carbonum* stimulated rishitin and phytuberin accumulation to levels comparable to those obtained after inoculation with incompatible races of *P. infestans*. Cell-free sonicates of three races of *P. infestans* applied to the surface of potato slices, including cultivars susceptible to all races of the fungus, caused necrosis and rishitin and phytuberin accumulation (14). Inoculation of tubers with a compatible race of *P. infestans*, 12-36 hr prior to inoculation with an incompatible race or treatment with a cell-free sonicate of the fungus, suppressed rishitin and phytuberin accumulation and necrosis (15). The suppression was accompanied by the accumulation of terpenoids other than rishitin and phytuberin.

The purpose of this investigation was to determine whether marked rishitin accumulation, due to different stimuli, is consistently associated with a suppression of steroid glycoalkaloid accumulation in potato tubers. Since steroid glycoalkaloids and rishitin appear synthesized by the acetate-mevalonate pathway, such data would suggest that a block in the acetate-mevalonate pathway and/or the synthesis or activation of key enzymes at a branch in the pathway are necessary for rishitin accumulation. The utilization of the branch in the pathway appears controlled by the fungus and determines whether rishitin (incompatible reaction) accumulates or other unidentified terpenoids (compatible reaction) accumulate (13). Thus, the metabolic site for the action of "R" genes for resistance and genes for pathogenicity may be at this branch.

MATERIALS AND METHODS.–*Inoculation with P. infestans.*–'Kennebec' or 'Cherokee' tubers (containing the R_1 gene for resistance) were washed with detergent and tap water, surface sterilized by immersing in 70% ethanol for 1 min, air-dried, and cut into 1-cm thick slices with a Vege-O-Matic® cutter (Popeil Brothers, Inc.). The slices were kept in 15-cm diam glass petri dishes lined with moistened Whatman No. 3 paper. Each dish was filled with six to eight slices depending on the tuber size, and slices from a single tuber were distributed among all treatments. Race 4 (incompatible) and race 1.2.4 (compatible) of *P. infestans* were cultured on lima bean agar at 18-20 C with a transfer frequency of

once a month. Spore suspensions (ca. 1×10^5 sporangia/ml) were prepared from 10- to 20-day-old cultures and kept in a refrigerator for an hr to liberate zoospores. The chilled suspension was filtered through a double layer of cheesecloth before use. The top surface of slices was sprayed with the suspension or water under aseptic conditions and incubated at 18 C. The top and second mm layers were removed and either extracted or lyophilized.

Extraction.—Fresh or lyophilized tuber tissue was blended in a mixture of chloroform, acetic acid, and methanol (50:5:45, v/v) (CAM) (ca. 1:10 fresh w/v) using a Virtis homogenizer. The homogenate was allowed to stand overnight, filtered, rinsed with CAM, and the filtrate was evaporated to dryness. Equal volumes of chloroform and 0.2 M acetic acid were introduced into the container, the mixture was shaken, and the two layers were separated. The chloroform layer was dried under reduced pressure and dissolved in methanol to a definite volume. Precoated silica gel plates (20 X 20 cm; Analtech, Inc.) were used to isolate rishitin from the chloroform-soluble fraction. Samples were applied to channels on the plates and the plates were developed in cyclohexane:ethyl acetate (1:1, v/v). Rishitin and phytuberin had R_F values of 0.21 and 0.70, respectively. To locate rishitin, the outer two channels of each TLC plate were spotted with authentic rishitin. After development, the outer channels were sprayed with saturated antimony trichloride in chloroform (Carr-Price reagent) and the compound appeared bright red without heating. Gel within areas extending 0.5 cm on either side of the R_F values for the compound were scraped off the plate and placed in a micro-column made from a disposable pipette by plugging the tip-end with glass wool. Rishitin was quantitatively eluted with 5 ml of a mixture of equal volumes of chloroform and methanol (CM) into a 15-ml test tube, and the eluate was evaporated to dryness using a warm metal block (65 C) under flowing air. The dilute acetic acid fraction which contained steroid glycoalkaloids was concentrated to one-tenth its original volume in a rotary evaporator. Concentrated ammonium hydroxide was added to bring the solution to pH 10. The mixture was warmed in an 80-C water bath for 30 min and cooled in a refrigerator for at least 3 hr. The precipitated alkaloids were collected by centrifugation (10 min at 15,000 *g*), washed with a few ml of 2% NH_4OH, dried in a vacuum desiccator, and finally dissolved in methanolic 5% acetic acid to a definite volume.

Quantitative analyses.—A colorimetric method based on the Alberti reaction (10) was used for analyses of steroid glycoalkaloids. An aliquot of the methanolic 5% acetic acid solution, equivalent to 30-300 μg α-solanine, was put in a 25 ml Erlenmeyer flask. Solvent was removed under reduced pressure, and three ml of a mixture of 50% ethanol and concd sulfuric acid (1:2) were added to the residue. One ml of 1% formaldehyde was then added dropwise to the solution while the flask was swirled in an ice-water bath. The solution was allowed to stand at 23-25 C for 90 min and the purple-red color was measured at 562 nm using a Beckman DB spectrophotometer. Total steroid glycoalkaloid content was determined using a standard curve established with recrystallized commercial α-solanine (mp 228 C, Molar absorptivity $1.4 \times 10^4 M^{-1} cm^{-1}$).

For rishitin analyses, the dried sample was dissolved in 1 ml of cyclohexane and 2 ml of concd sulfuric acid were added to the solution. The mixture was agitated and centrifuged at low speed (ca. 1,000 *g*) for 2-3 min. The red color of the lower sulfuric acid layer was measured at 500 nm 10-20 min after the addition of sulfuric acid. Concentrated sulfuric acid served as a blank.

Inoculation with H. carbonum.—A spore suspension of *H. carbonum* was prepared from two-week cultures grown on potato-dextrose agar. The suspension was filtered through a double layer of cheese-cloth and the filtrate was applied to the top surface of Kennebec tuber slices. Slices were incubated for 72 hr, and the rishitin and steroid glycoalkaloid content in the top mm was determined.

Treatment with sonicates.—*P. infestans*, race 1.2.4 or 1.3.4, was grown on lima bean agar medium in petri dishes. Two-week cultures were frozen with dry ice and the fungus was scraped off the surface of the frozen medium. Ten g of frozen fungus were sonicated in 100 ml of water, cooled in an ice bath for 5 min at maximum intensity using a Branson Sonifier. Two ml of the resulting cell-free milky suspension was added to the top surface of six to eight Kennebec tuber slices per petri dish (ca. equivalent to 2 ml/10 g fresh wt of the top mm). Slices inoculated with *P. infestans* race 1.2.4 or treated with water served as controls. Slices were incubated for 72 hr and the rishitin and steroid glycoalkaloid content in the upper 1-mm of each slice (top mm) was determined.

Aging.—Kennebec tuber slices were aged 24, 48, or 72 hr and their top surfaces treated with a sonicate of *P. infestans* race 1.2.4. Rishitin was determined in the top mm 72 hr after treatment with sonicate. In a second experiment, Kennebec tuber slices were aged 72 hr and then inoculated with *P. infestans* race 4 or

TABLE 1. Total steroid glycoalkaloids[a] in the top and second mm of 'Kennebec' potato tuber slices after cutting or inoculation with *Phytophthora infestans*

Treatment	Expt 1[b]	Expt 2	Expt 3
Fresh (unaged)	15	14	139
Cut	251(215)	265(217)	
Cut and sprayed with water	169(166)	190(177)	615
Inoculated with race 4[c]	30(60)	28(61)	206
Inoculated with race 1.2.4[c]	90(137)	91(149)	252

[a] Expressed as μg α-solanine/g fresh wt. Figures in parentheses, are the contents in the second mm.

[b] Values in Expt 1 and 2 determined 60 hr after cutting or inoculation of potatoes grown in 1970. Values Expt 3 determined 72 hr after cutting or inoculation of potatoes, extremely high in steroid glycoalkaloids, grown in 1971.

[c] Race 4 = incompatible, race 1.2.4 = compatible.

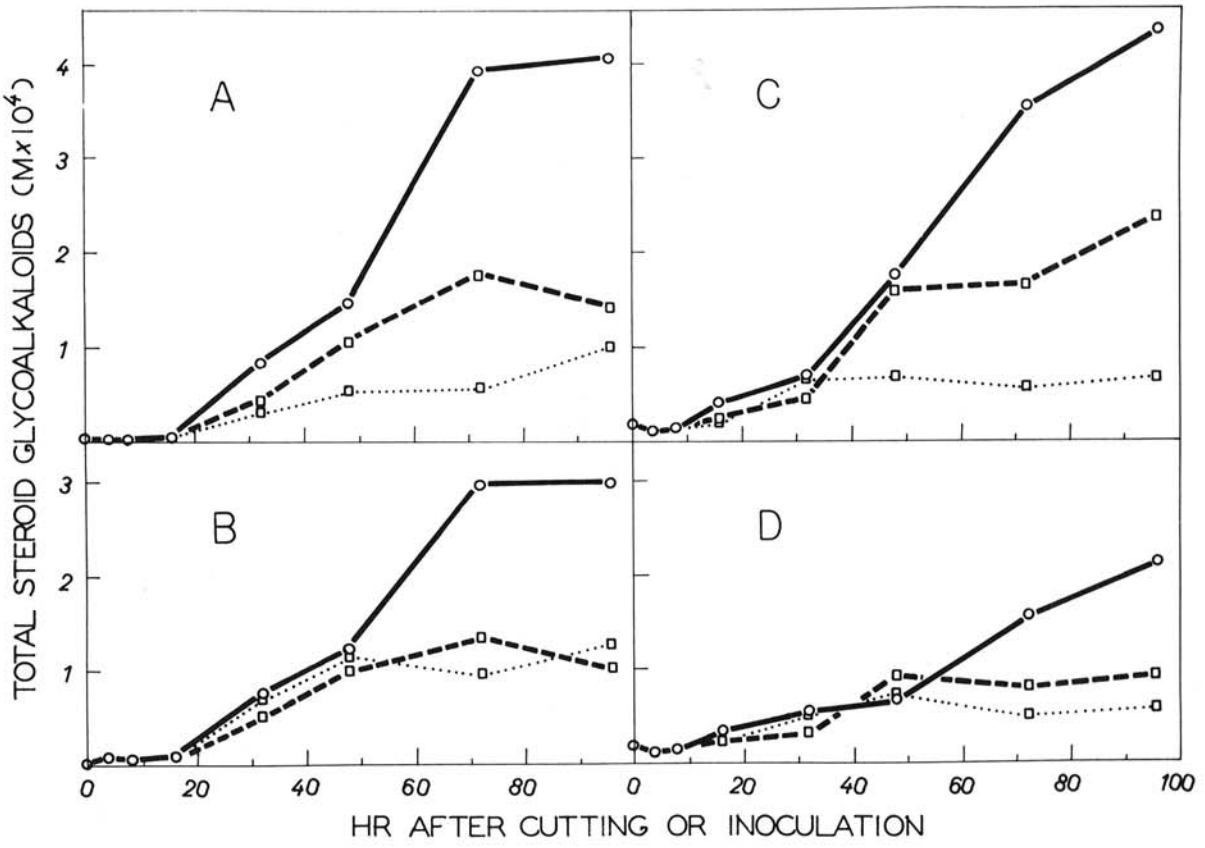

Fig. 1. Effect of *Phytophthora infestans* on the accumulation of steroid glycoalkaloids in the top and second mm of tuber slices. **A)** Top mm; and **B)** second mm, 'Cherokee'; **C)** top mm; and **D)** second mm, 'Kennebec' o——o cut; □---□ race 1.2.4 (compatible); □ · · · □ race 4 (incompatible).

1.2.4. Rishitin and steroid glycoalkaloids were determined in the top mm 72 hr after inoculation. In a third experiment, slices were aged 72 hr, treated with a sonicate of race 1.2.4, the top mm was harvested at intervals after treatment, and rishitin was determined in the top mm.

Exogenous α-solanine.—α-Solanine was added to noninoculated and inoculated potato slices to ascertain whether a feedback control mechanism for steroid glycoalkaloid (α-solanine and α-chaconine) and rishitin could be observed. Levels of α-solanine higher than the ED_{50} values (1) for the inhibition of growth of *P. infestans* were not used since they would have markedly inhibited germination of zoospores and growth of the fungus. Five mg of α-solanine were dissolved in 1 ml of 0.2 M acetic acid, and the solution was diluted with sterile deionized water to 20 ml (250 μg/ml or ED_{50} for *P. infestans*). Two ml of the solution of α-solanine were added to the top surface of 6-8 Kennebec tuber slices/petri dish (ca. 50 μg/g fresh wt of the top mm). The top mm was harvested 18, 32, 48, and 72 hr after treatment and the steroid glycoalkaloid content determined. Slices sprayed with water were the controls. Slices were also treated with α-solanine and inoculated with race 4 or 1.2.4 of *P. infestans* 30 min after treatment. The steroid glycoalkaloids and rishitin in the top mm were determined.

RESULTS.—*Inoculation with P. infestans.*—Inoculation of potato slices with *P. infestans* immediately after cutting suppressed the accumulation of steroid glycoalkaloids (Table 1, Fig. 1). The incompatible race suppressed accumulation more than the compatible race. Accumulation was not detected immediately after slicing and required a lag time of approximately 16 hr (Fig. 1). Potatoes freshly harvested in 1970 had approximately one-tenth the steroid glycoalkaloid content of potatoes freshly harvested in 1971 and the total steroid glycoalkaloid accumulated after cutting in the latter was also much greater (Table 1). Suppression was evident with 1971 potatoes, even though the differences between the magnitude of suppression following inoculation with compatible and incompatible races was small. Slices inoculated with the incompatible race accumulated considerably more rishitin than those inoculated with the compatible race (Table 2, 3, 4).

Treatment with H. carbonum or sonicates.—Slices treated with cell-free sonicates of *P. infestans*, or inoculated with *H. carbonum*, accumulated considerably less steroid glycoalkaloid and more

TABLE 2. Rishitin and steroid glycoalkaloid[a] accumulation in 'Kennebec'[b] tuber slices 72 hr after inoculation or treatment with sonicates

Treatment	Content (μg/g fresh wt) in top mm	
	Rishitin	Steroid glycoalkaloid
Water	4	754
Helminthosporium carbonum	53	264
Phytophthora infestans race 1.3.4[c]	15	349
Sonicate of race 1.3.4	71	118
Sonicate of race 1.2.4	60	150

[a] Expressed as α-solanine.
[b] 1971 crop.
[c] Race 1.3.4 = compatible.

rishitin than did check slices sprayed with water (Table 2). Higher steroid glycoalkaloid accumulation was associated with reduced accumulation of rishitin.

Aging.—Aging slices (1970 and 1971 crop) for 72 hr prior to inoculation with *P. infestans* markedly reduced rishitin accumulation during a subsequent 72-hr period (Table 3). Tubers aged 72 hr before inoculation with the incompatible race did not have the extensive necrosis typical of a hypersensitive reaction. Aging of slices for 48 or 72 hr prior to treatment with sonicates markedly reduced the accumulation of rishitin. Rishitin accumulation was detected 6-12 hr after treating slices (1970 crop), aged for 72 hr, with a sonicate of race 1.2.4 (Fig. 2). Rishitin accumulation reached a peak 20 hr after treatment (28 μg/g fresh wt in the top mm).

Exogenous α-solanine.—The application of α-solanine to the surface of slices did not reduce rishitin or steroid glycoalkaloid accumulation (Table 4). The added α-solanine appeared to be rapidly degraded by the slices.

DISCUSSION.—At harvest and during 7 months of storage, Kennebec tubers from the O'Neal Horticulture Farm of Purdue University in 1971 had a higher content of steroid glycoalkaloids (90-160 as compared to 10-20 μg/g fresh wt) than tubers in 1970. The 1971 crop yield was very high and most tubers had formed very close to the soil surface. Many of them were partially exposed to sunlight and their upper surface appeared green. As early as 1937, Conner (4) demonstrated a tenfold increase in steroid glycoalkaloids of tubers exposed to ultraviolet radiation. Sunlight, therefore, is suggested to be the cause of the high steroid glycoalkaloid content in the 1971 Kennebec potatoes. The authors noticed little or no spore germination and hyphal growth of *H. carbonum* on slices of the 1971 tubers, whereas the fungus made restricted but visible growth on cut surfaces of the 1970 tubers. Tissue of tubers harvested in 1971 showed a definite, but somewhat less severe, necrosis after inoculation with the incompatible race of *P. infestans* and stayed firm longer during compatible interactions than in those harvested in 1970. This suggests that high steroid glycoalkaloid content may have some inhibitory effect on fungal invasion in both compatible and incompatible interactions, but that it is not responsible for "R" gene or hypersensitive resistance.

Marked suppression of steroid glycoalkaloid accumulation was consistently observed when rishitin accumulation was high. This suggests that either a block in the acetate-mevalonate pathway and/or activation of enzymes occurs at the branch to rishitin biosynthesis. The steroid glycoalkaloid content of tubers aged for 72 hr is not suppressed appreciably by inoculation but rishitin accumulation is markedly reduced. The content of steroid glycoalkaloids at the time of inoculation does not appear to be the decisive factor for reduction, since both steroid glycoalkaloid suppression and increased rishitin accumulation were clearly evident with freshly cut 1971 potatoes which were high in steroid glycoalkaloids (Table 1) at the

TABLE 3. Effect of aging on rishitin and steroid glycoalkaloid accumulation[a] in the top mm of 'Kennebec' tuber slices inoculated with *Phytophthora infestans* or treated with sonicates

Treatment	Rishitin		Steroid glycoalkaloid	
	Expt. 1[b]	Expt. 2	Expt. 3	Expt. 4
Water 72 hr[c]	4	1		
Race 4 72 hr[c]	97	72		
Race 1.2.4 72 hr[c]	20	13		
Aged (72 hr) + water 72 hr[d]	trace	2	120	1,000
Aged (72 hr) + race 4 72 hr[d]	35	4	70	980
Aged (72 hr) + race 1.2.4 72 hr[d]	7	2	70	975
Aged 24 hr + sonicate of race 1.2.4[d]		77		
Aged 48 hr + sonicate of race 1.2.4[d]		53		
Aged 72 hr + sonicate of race 1.2.4[d]		18		

[a] Expressed as μg/g fresh wt, steroid glycoalkaloids expressed as α-solanine.
[b] Expt. 1 and 3, 1970 crop; Expt. 2 and 4, 1971 crop which is high in steroid glycoalkaloids.
[c] Slices incubated for 72 hr after spraying with water or inoculation with race 4 (incompatible) or race 1.2.4 (compatible).
[d] Slices cut and held for hr indicated and then sprayed with water, inoculated, or treated with sonicate and held for another 72 hr.

time of inoculation. Aging for 48 or 72 hr prior to treatment with sonicates of *P. infestans* also markedly reduced rishitin accumulation. This eliminates inhibition of fungus by steroid glycoalkaloids as a cause for the reduced accumulation of rishitin. It appears that once the pathway for steroid glycoalkaloid biosynthesis is well established (i.e., after aging, blocking, or activation of enzymes), that a branch for rishitin synthesis is not readily accomplished or is less effective.

α-Solanine added to tuber slices appeared to have little influence on the accumulation of steroid glycoalkaloids or rishitin (Table 4). Instead, the added α-solanine appeared to be lost early after its addition to the cut surfaces. Glycosidases liberated from ruptured cells may hydrolyze the sugars from the steroid glycoalkaloids and initiate their degradation. Such enzymes have been found in sprouts of the potato plant (*Solanum tuberosum* L.) (5) and in leaves of *S. aviculare* (6).

According to Varns (13), sonicates of different races of *P. infestans* increase rishitin accumulation equally in all cultivars regardless of the disease reaction between host and fungus. It appears, therefore, that all races of *P. infestans* may have the potential to induce rishitin accumulation and all potato cultivars the potential to accumulate rishitin. The fungus is contained in incompatible interactions soon after infection (11) and rishitin is detected at the time of containment and increases markedly for an additional 48-72 hr. It is possible, however, that the dead or dying fungus in an incompatible interaction induces rishitin accumulation, in much the same way as a fungal sonicate, and that rishitin is not the principal or only factor responsible for containing or killing the fungus.

TABLE 4. Effect of exogenous α-solanine on steroid glycoalkaloid and rishitin accumulation[a] in the top mm of 'Kennebec' potato tuber[b] slices

Treatment	Time after cutting or inoc. (hr)	Content (μg/g fresh wt) Without α-solanine	With α-solanine[d]
Noninoculated[c]	0	14	64
	18	20	41
	32	55	57
	48	60	70
	72	116	119
P. infestans 1.2.4[e]	72	62(20)	75(20)
P. infestans 4	72	12(97)	28(98)

[a] Rishitin values in parentheses, steroid glycoalkaloid expressed as α-solanine.

[b] 1970 crop.

[c] Sprayed with deionized water or treated with α-solanine after cutting.

[d] Approximately 50 μg α-solanine added per g fresh wt (top mm) to the surface of slices at 0 time. Values reported are total steroid glycoalkaloid or rishitin in tissue at different time intervals after treatment.

[e] Slices inoculated with race 1.2.4 (compatible) or race 4 (incompatible) 30 min after cutting, or cutting and treatment with α-solanine.

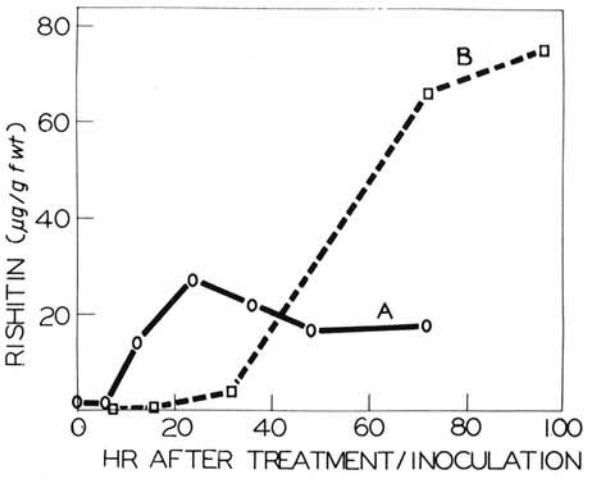

Fig. 2. Rishitin accumulation in the top mm tissue of 'Kennebec' tuber slices (1970 crop) **A)** after aging for 72 hr and treatment with sonicate of *Phytophthora infestans* race 1.2.4; **B)** 72 hr after inoculation with *P. infestans* race 4.

The pattern of isoprenoid accumulation matches the respiratory rate (7) of potato quite well; i.e., respiration is low in intact potato tubers and so is terpenoid accumulation. Respiration displays a sudden jump in freshly cut slices and they accumulate terpenoids.

Currier & Kuć (*unpublished*) detected rishitin in freshly sliced potatoes treated with fungal sonicates or inoculated with an incompatible race of *P. infestans* after a lag period of 24-30 hr. The peak of rishitin accumulation occurred 70-100 hr after treatment with sonicates or inoculation. The time study of rishitin accumulation in slices aged 72 hr before sonicate application (Fig. 2) indicates a lag time of 6-12 hr and a peak accumulation 20 hr after treatment. Rishitin accumulation maxima in tissue aged 72 hr before sonicate application (1970 crop) were approximately one-fourth that in tuber slices inoculated or treated with sonicate immediately after cutting. It is possible that early precursors of the acetate-mevalonate pathway, common to both risthitin and steroid alkaloid biosynthesis, rapidly increase during a lag period which is provided by cutting the tuber and aging for 24 hr or less. Later steps in the pathway for steroid glycoalkaloid accumulation may require 24 hr or more for activation of enzymes or their synthesis. Once these have been established; i.e., after aging for 48 or 72 hr, inoculation with *P. infestans* or treatment with sonicates cannot suppress steroid glycoalkaloid accumulation and rishitin accumulation is reduced.

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