

Phytoalexins from Potatoes: Evidence for the Conversion of Lubimin to 15-Dihydrolubimin by Fungi

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ABSTRACT

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Lubimin, added to cultures of *Phytophthora capsici*, *Glomerella cingulata*, or *Fusarium sulphureum* was converted to a compound identified as 15-dihydrolubimin. *Monilinia fruticola* and *P. infestans* did not perform the conversion. In potato tubers inoculated with these fungi 15-dihydrolubimin accumulated as a major product with the

first three fungi and not with the last two. The conversion did not appear to be a detoxification mechanism or related to pathogenicity. The significance of the findings is discussed in relation to phytoalexin production in potatoes, particularly with regard to possible fungal or bacterial modification of precursors produced by the plant.

Since the isolation of rishitin (15) as a phytoalexin from potato tubers several other structurally and biogenetically related sesquiterpenes have been demonstrated in incompatible interactions in potatoes both with fungi (4, 8, 16) and bacteria (1, 2, 6). Of these lubimin (Fig. 1) is especially interesting because it has been isolated under similar conditions from two other species of the Solanaceae: *Datura stramonium* (12, 18) and *Solanum melongena* (18). In unpublished experiments with potato tubers (10), we frequently have observed differences in the spectrum of compounds produced in response to different inducing fungi. The studies reported here were carried out following the observation that lubimin was not detectable in all of a series of incompatible interactions examined. The results demonstrate that this was due, at least in part, to the ability of several fungi to metabolize lubimin to 15-dihydrolubimin, and raise the possibility that other compounds isolated as phytoalexins may be derived by fungal agency.

MATERIALS AND METHODS

Fungi used were *Phytophthora capsici* Leonian (ATCC 15399) from pepper, *Glomerella cingulata* (Stonem.) Spauld. and Schrenk from apple, *Fusarium sulphureum* Schlecht. from potato, obtained from W. L. Seaman (9), *Monilinia fruticola* (Wint.) Honey from peach, and *P. infestans* (Mont.) de By race 4, avirulent on potato cultivar Kennebec. Culture conditions and procedures for preparation of spore suspensions for inoculation and assay purposes have been described previously in detail (19) except that *P. infestans* was grown on V-8 juice agar instead of lima-bean agar.

Fungitoxicity of lubimin and 15-dihydrolubimin was determined in standard spore-germination assays as previously described (19).

In studies of lubimin metabolism in vitro, *P. capsici* was grown at 25 C on 15 ml of filtered V-8 juice in 50-ml Erlenmeyer flasks for time-course studies and on 50-ml filtered V-8 juice in 250-ml flasks for preparation of larger quantities of 15-dihydrolubimin. A 5-mm diameter plug cut from the periphery of a culture on V-8 juice agar was added to the medium and incubated for 6 days. Similar procedures were used for *F. sulphureum* and *M. fruticola*, except that the incubation period was 8 days. For *P. infestans*, incubation was at 18 C for 10 days. *Glomerella cingulata* was grown in 50 ml of a medium composed of malt extract 5 g, yeast extract 5 g, and glucose 15 g in 1 liter of distilled water, in 250-ml Erlenmeyer flasks, in shake culture for 5 days at 25 C; each flask received 1 ml of a spore suspension (10^6 spores/ml). All media were sterilized by autoclaving at 121 C at 1.05 Kg-force/cm² for 15 min. Lubimin was added to cultures after the incubation periods indicated at the rate of 0.25 mg in 0.5 ml ethanol per culture flask to the *P. infestans* cultures and to the *P. capsici* cultures on 15 ml of medium, and 0.5 or 1 mg in 1 ml ethanol to each of the other cultures. These rates provided subinhibitory concentrations for each fungus, as indicated by fungitoxicity assays, Table 3 and (17). Treated cultures and controls were extracted after 24 hr or other time intervals as indicated in the Results. Residual lubimin and 15-dihydrolubimin were determined as described below.

Potato tubers (*Solanum tuberosum* L. 'Kennebec') were stored at 4 C. Before use they were scrubbed in running tap-water, immersed in 70% ethanol for 2 min, and allowed to dry in a sterile air stream. They were cut into slices approximately 2 cm thick and a number of depressions about 1 cm deep by 2 cm across were made in one of the cut surfaces using a melon cutter. The slices

were placed in a single layer in sterilized Pyrex trays and 2 ml of a spore suspension (5×10^5 spores/ml) added to each depression. The trays were covered, placed in plastic bags, and incubated at room temperature for 48 hr. The diffusates were collected together with a water rinse of the depressions. The tissue "browned" during the interaction was scooped out of the depressions and weighed.

The diffusates and rinsings in the tuber experiments and the entire medium and mycelium in the experiments with fungus cultures were extracted without further treatment three times with half-volumes of ether. The tuber tissue was similarly extracted three times by steeping overnight in approximately half-volumes of ether and these extracts were combined with those from the diffusates and rinsings. The ether extracts were dried with sodium sulfate, concentrated in vacuo below 35 C and evaporated from ethanol. The major components were separated by thin-layer chromatography (TLC) and quantitated by gas-liquid chromatography (GLC). For TLC, silica gel plates (Camag DF5, 300 μ m thick) were used with *t*-butanol-ethyl acetate-acetic acid (5:95:0.5, v/v) as the solvent system. They were developed by spraying with 5% phosphomolybdic acid in ethanol or 0.5% vanillin in 80% sulfuric acid in ethanol followed by heating for 10 min at 110 C. The 15-dihydrolubimin was isolated from extracts of inoculated tubers and cultures by preparative TLC using essentially the same procedures. Gas-liquid chromatography was done with a Hewlett-Packard 5700A instrument with flame ionization detector and fitted with a glass column (1.83 m long, 1.5 mm i.d.) packed with Gas Chrom Q [177- to 149-

μ m particle size range (80- to 100-mesh)] coated with 3% SE 30. Nitrogen flow rate was 38 ml/min. Column and detector temperatures for lubimin were 160 C and 250 C and for 15-dihydrolubimin 190 C and 250 C, respectively. Quantitative determinations were made following the preparation of standard curves with methyl myristate as an internal standard.

A supply of lubimin was obtained by inoculating potato tubers with *M. fructicola* as above followed by fractionation of the ether extract by column chromatography as described in detail elsewhere (11, 17, 18). Authentic 15-dihydrolubimin, m.p. 127 C, was available from earlier experiments (11).

RESULTS

Ether extracts from tubers inoculated with *P. capsici*, *P. infestans*, *G. cingulata*, *F. sulphureum*, or *M. fructicola*, all contained rishitin as judged by TLC and GLC (13). Lubimin was particularly prominent in chromatograms from *P. infestans*- and *M. fructicola*-inoculated tubers, but was either absent or present in much smaller amounts in extracts from tubers inoculated with *P. capsici*, *F. sulphureum*, and *G. cingulata*.

Extracts of cultures of *P. capsici*, *G. cingulata*, and *F. sulphureum* to which lubimin had been supplied were found, after 24 hr of incubation, to contain a new compound not present in control cultures, and, except for less than 0.04 mg in the case of *P. capsici*, no lubimin (Table 1). There was no significant change in concentration of lubimin supplied to cultures of *P. infestans* or *M. fructicola* during the same period, and lubimin or related compounds were not detected in controls. The new compound migrated more slowly than rishitin or lubimin on TLC and gave a strong purple color with vanillin sulfuric acid spray reagent. The R_{rshitin} for lubimin and the newly formed compound in the system *t*-butanol-ethyl acetate-acetic acid (5:95:0.5, v/v) were 2.3 and 0.5, respectively. The compound had a GLC retention time of 123 sec at 190 C (column temperature) compared to 238 sec for lubimin at 160 C under the conditions described. It was isolated as a crystalline product by preparative TLC from ether extracts of lubimin-fed cultures of each of the three fungi. It was recrystallized from methylene chloride and shown to be

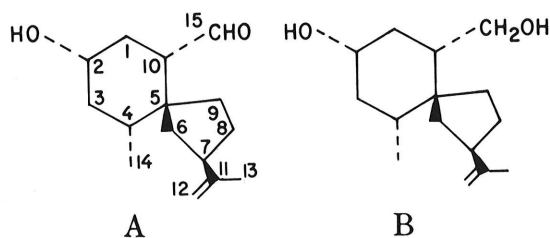


Fig. 1-(A, B). Molecular structures of A) lubimin and B) 15-dihydrolubimin.

TABLE 1. Conversion of lubimin to 15-dihydrolubimin by *Phytophthora capsici*, *Glomerella cingulata*, and *Fusarium sulphureum* in culture^a

Fungus species	Lubimin supplied (mg ^b)	Recovered (mg)	
		Lubimin	15-Dihydrolubimin
<i>Phytophthora capsici</i>	1.00	>0.04	0.48
<i>Glomerella cingulata</i>	0.50	0	0.26
<i>Fusarium sulphureum</i>	0.50	0	0.21
<i>Phytophthora infestans</i>	0.24	0.25	0
<i>Monilinia fructicola</i>	0.97	0.94	0

^a *Phytophthora capsici*, *F. sulphureum*, and *M. fructicola* were grown at 25 C in still culture on 50-ml of filtered V-8 juice in 250-ml flasks, for 8 days prior to addition of lubimin. *Phytophthora infestans* was grown similarly, but at 18 C and for 10 days prior to addition of lubimin. *Glomerella cingulata* was grown at 25 C in shake culture on 50 ml of a medium composed of: malt extract 5 g, yeast extract 5 g, and glucose 15 g in 1 liter of distilled water, for 5 days prior to addition of lubimin. The fungi were incubated for an additional 24 hr after addition of lubimin, when the entire cultures were extracted with ether and 15-dihydrolubimin and residual lubimin was determined by GLC.

^b Supplied in 1 ml of ethanol to culture in 50 ml of medium, except for cultures of *P. infestans* in which 0.5 ml of ethanol was used.

identical with 15-dihydrolubimin (Fig. 1) by TLC, melting point, mixture melting point, ^1H -nuclear magnetic resonance (NMR), infrared (in chloroform), and mass spectra (Varian A60A, Beckman IR 20A, and Varian M66 spectrometers, respectively).

Despite the complete loss of lubimin from cultures, the recovered 15-dihydrolubimin did not represent more than 50% of the lubimin supplied (Table 1). Although this suggests that lubimin and/or 15-dihydrolubimin are metabolized to other compounds, these were not evident in the ether-soluble fraction. The complete recovery of the lubimin supplied to cultures of *P. infestans* and *M. fructicola* suggests that binding of lubimin to other components is improbable. The time-course of lubimin metabolism by cultures of *P. capsici* (Fig. 2) indicates that it occurs rapidly and without any conspicuous lag period.

Examination of ether extracts of tubers inoculated with *P. capsici*, *G. cingulata*, or *F. sulphureum* revealed a compound corresponding in R_f and color reaction on

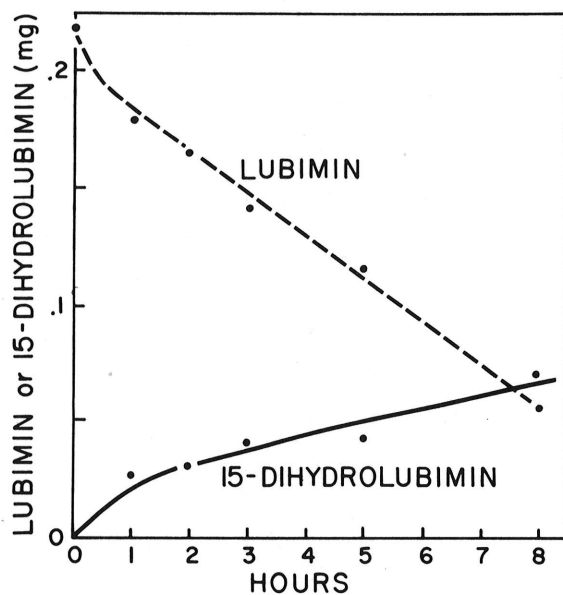


Fig. 2. Time-course of lubimin conversion to 15-dihydrolubimin by cultures of *Phytophthora capsici*.

TLC, and retention time on GLC, to 15-dihydrolubimin. The material was isolated in quantity from *G. cingulata*-inoculated tubers by preparative TLC as a crystalline product and also was shown to be identical with 15-dihydrolubimin by melting point, mixture melting point, and mass spectrum.

Yields of lubimin and 15-dihydrolubimin (Table 2) varied appreciably in different experiments and on two occasions lubimin was not detected in tubers inoculated with *G. cingulata*. The apparently low yields obtained with *P. capsici* probably reflect the fact that this fungus caused extensive maceration of the tuber tissue and a much larger amount of rotted tissue was included in the fresh weight determinations than with the other fungi. A compound that at first appeared to be 15-dihydrolubimin also was found in extracts of tissue inoculated with *P. infestans* or *M. fructicola*. Sufficient material was not available from the *P. infestans* interactions to allow isolation of the compound. However, the compound was isolated from the *M. fructicola*-potato interaction and, although chromatographically similar, it was not identical with 15-dihydrolubimin by melting point, mixture melting point, NMR, and mass spectrum criteria.

A measure of the fungitoxicity of 15-dihydrolubimin was obtained in spore germination assays (Table 3). Activity was of the same order as that of lubimin.

DISCUSSION

The results demonstrate that *P. capsici*, *G. cingulata*, and *F. sulphureum* readily convert lubimin to 15-

TABLE 3. Inhibition of germination of spores of *Phytophthora infestans*, *Phytophthora capsici*, and *Glomerella cingulata* by lubimin and 15-dihydrolubimin

Fungus species	ED ₅₀ (M × 10 ⁴) ^a	
	Lubimin	15-Dihydrolubimin
<i>Phytophthora infestans</i>	3.4	4.4
<i>P. capsici</i>	3.7	3.4
<i>Glomerella cingulata</i>	>10.0 ^b	>10.0

^aMolar concentrations for 50% inhibition were determined in standard glass slide spore germination assays from dose inhibition curves.

^bApproximately 35% inhibition at this concentration, the highest that was tested.

TABLE 2. Rishitin, lubimin, and 15-dihydrolubimin in ether extracts from potato tuber tissue inoculated with various fungi

Fungus species	Tissue ^a (fresh wt, g)	μg/g tissue (fresh wt)		
		Rishitin	Lubimin	15-Dihydrolubimin
<i>Glomerella cingulata</i> ^b	65.5	11.9	19.6	37.7
<i>Fusarium sulphureum</i>	74.2	11.7	0	12.5
<i>Phytophthora capsici</i>	316.8	3.0	0	1.2
<i>Phytophthora infestans</i>	39.8	13.4	54.5	2.3 ^c
<i>Monilinia fructicola</i>	59.5	14.2	31.3	1.0 ^c

^aBrowned or rotted tissue readily separated from healthy white tissue.

^bResults for *G. cingulata* are the means of five experiments; those for other fungi are the means of two experiments. Tubers were inoculated with spore suspensions of each of the fungi (1 to 5 × 10⁵ spores/ml) and incubated for 48 hr at room temperature.

^cMaterial was chromatographically indistinguishable from 15-dihydrolubimin, but evidently was a different compound because it differed in melting point, mixture melting point, NMR, and mass spectrum.

dihydrolubimin *in vitro*. It seems reasonable to conclude that they bring about the same conversion in tuber tissue, since lubimin accumulated in large amounts after inoculation with *P. infestans* and *M. fructicola*, two fungi that did not metabolize lubimin *in vitro* under the conditions employed. What at first appeared to be a small amount of 15-dihydrolubimin in extracts from the *M. fructicola*-potato interaction turned out to be a different compound whose structure is now under investigation. The same substance probably is produced also in the *P. infestans*-potato interaction. This would be consistent with the chromatographic data and the failure of *P. infestans*, like *M. fructicola*, to metabolize lubimin in culture. On the other hand, the possibility that 15-dihydrolubimin is produced in infected tubers not as a result of fungal metabolism cannot be ruled out at present, especially since it might serve as a biogenetic precursor of lubimin. In this case, the amounts accumulating might be very small or the compound might be bound.

This is the second example of the modification by fungi of a sesquiterpenoid phytoalexin from the Solanaceae. Like the oxidation of capsidiol from peppers (14), the ability of fungi to form 15-dihydrolubimin does not appear to be related to pathogenicity, nor to provide a detoxification mechanism (Table 3). Without the evidence presented here for its formation by a fungal agency it would have seemed reasonable to assume that 15-dihydrolubimin was a phytoalexin of similar status to lubimin. In view of the number of compounds that now have been isolated from potato tuber tissues after incompatible interactions with fungi or bacteria (1, 2, 4, 5, 6, 8, 16), it seems reasonable to question whether other phytoalexins might not also be fungal or bacterial metabolic products of previously identified or unidentified precursors. In our experience, and from the reports of others, only rishitin appears to be produced consistently. Lubimin is notably absent from studies in which bacteria have been used and other related vetispiranes have been found instead (2, 7). It is quite possible that if fungi can metabolize lubimin as demonstrated here they may also metabolize postulated precursors such as germacrene A or farnesol pyrophosphate (13). The fact that potato tuber tissues are seldom free of bacteria (3) adds to the possibility that the sesquiterpenoid compounds produced in incompatible interactions in potato tubers may not all be products of the plant.

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