

Genetic Analysis in *Gibberella pulicaris*: Rishitin Tolerance, Rishitin Metabolism, and Virulence on Potato Tubers

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Twenty-six field strains of *Gibberella pulicaris* were tested for tolerance of the potato phytoalexin rishitin, for ability to metabolize rishitin, and for virulence on potato (*Solanum tuberosum*) tubers. All highly virulent strains were tolerant of rishitin and able to metabolize rishitin. A cross was made between strains that differed in rishitin tolerance, rishitin metabolism, and virulence. These traits were followed through one generation of

random ascospore progeny and a second, backcrossed generation of tetrad progeny. All highly virulent progeny were tolerant of and able to metabolize rishitin, and all rishitin-sensitive progeny were of low virulence. These results support the hypotheses that rishitin metabolism is required for a high level of rishitin tolerance and is either required for or closely linked to genes for a high level of virulence.

Additional keywords: *Fusarium sambucinum*, phytoalexin detoxification, sesquiterpene stress metabolites, *Solanum tuberosum*.

Gibberella pulicaris (Fries) Sacc. (anamorph: *Fusarium sambucinum* Fuckel) is a major cause worldwide of dry rot of potato (*Solanum tuberosum* L.) tubers both in the field and in storage (Boyd 1972). Few potato cultivars have a high degree of resistance to *G. pulicaris*, and losses are controlled by chemical treatment of tubers at harvest or before planting (Leach and Webb 1981). Potato breeding programs have given increased attention to development of cultivars with resistance to *G. pulicaris* (Leach and Webb 1981), but the biochemical factors involved in resistance are poorly understood. Rishitin, lubimin, and other fungitoxic sesquiterpenes have been detected in potato tubers infected with *G. pulicaris* and with many other fungi as well (Corsini and Pavek 1980; Kuć 1982). If these sesquiterpenes are important disease-resistance factors (phytoalexins) in potato, then successful pathogens may have developed mechanisms for tolerating them.

It is well established that some plant pathogenic fungi are tolerant of phytoalexins and that they are often able to metabolize the phytoalexins to which they are tolerant (VanEtten *et al.* 1982). Little is known, however, concerning fungal detoxification of sesquiterpenoid phytoalexins of potato and its role in virulence. *G. pulicaris* is a heterothallic ascomycete and amenable to classical genetic analysis (Desjardins and Beremand 1987), which allows a genetic test of whether there is a causal relationship between tolerance of sesquiterpene phytoalexins, their metabolism, and virulence on potato.

The goals of the present study were to investigate the relationships among rishitin tolerance, rishitin metabolism, and virulence in 26 field strains of *G. pulicaris* and to examine patterns of inheritance among progeny from genetic crosses between parents that differ in these traits.

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MATERIALS AND METHODS

Cultures. Most strains of *G. pulicaris* used in this study were identified and kindly supplied by P. E. Nelson from the *Fusarium sambucinum* collection at the *Fusarium* Research Center, The Pennsylvania State University (strains with the prefix R are from this collection). Other strains were supplied by R. Caldwell, University of Wisconsin-Madison; G. Neish, Agriculture Canada at Ottawa; and H. K. Abbas, University of Minnesota. All strains were reisolated from single conidia before this study. Cultures were routinely grown on V-8 agar medium (Stevens 1974) slants or plates on an alternating 12-hr, 25° C light/ 12-hr, 20° C dark schedule. For long-term storage, all strains were maintained on V-8 agar slants at 4° C. Field strains were also maintained as lyophilized conidial suspensions in the Agricultural Research Service Collection, Peoria, Illinois. For all experiments, fresh transfers of the strains were obtained from stock cultures stored at 4° C.

Genetic crosses. Crosses were made between strains of opposite mating type, designated Mat-1 and Mat-2; crosses between strains of the same mating type are infertile. Techniques for crossing and for random ascospore and tetrad isolations have been previously described (Desjardins and Beremand 1987; Beremand and Desjardins 1988). Sterile mulberry twigs on water-agar slants were inoculated with strain R-6380, the female parent in both crosses 1104 and 1421, and grown until protoperithecia developed (approximately 1 mo) before the addition of conidia from the male parent. After fertilization, crosses were incubated at 15° C until perithecia were mature. Ascospore progeny were cataloged by a series of three numbers: the cross number, the tetrad number (or R for random ascospore progeny), and the ascospore number (1-8 for tetrads and consecutive numbers for random ascospore progeny; Yoder *et al.* 1986). Femaleness was scored as the ability to produce protoperithecia. Mating type was determined by crosses to two tester strains, R-6380 (Mat-1) and R-5455 (Mat-2), of opposite mating type. Cultures were scored for production

of red pigment following 10–14 days growth on potato-dextrose agar (Nelson *et al.* 1983).

Isolation of rishitin and lubimin. Sesquiterpene phytoalexins were elicited in potato slices (for rishitin and lubimin) and in immature seed pods of *Datura stramonium* (for lubimin only) with *Helminthosporium carbonum* Ulstrup (kindly supplied by H. D. VanEtten, Cornell University) with modifications of previously described methods (Lisker and Kuć 1977; Ward *et al.* 1976). Lubimin was isolated by sequential column chromatography and thin-layer chromatography (TLC; Gardner *et al.* 1988). Rishitin was partially purified by the same column chromatographic technique used for lubimin. The residue obtained from ethyl ether extraction of the potato slices (about 0.5 g), containing the sesquiterpenoids and other materials, was slurried in hexane with 2 g of Silic AR CC-7 (Mallinckrodt), and this mixture was applied to the top of a column (2.5 cm i.d.) containing 50 g of Silic AR CC-7 packed in hexane. The column was eluted stepwise with ethyl ether in hexane in the proportions of 0.4 L, 40%; 0.4 L, 50%; and 0.4 L, 55%. Localized by TLC of fraction aliquots, rishitin eluted between 0.68–0.89 L. Eluting rishitin was detected by TLC of 20- μ l aliquots of fractions and visualization of red spots after spraying with 0.5% vanillin in sulfuric acid:ethanol (4:1, v/v). The material from the appropriate column chromatographic fractions was separated by TLC (20 \times 20 \times 0.05 cm) by using ethyl ether:hexane (7:3, v/v) by a threefold development in a paper-lined tank. The rishitin band was localized by the method of Gitler (1972). Occasionally, the rishitin isolated by TLC required an additional separation by TLC by using a threefold development with hexane:acetone (4:1, v/v) without a tank liner. The purity of rishitin and lubimin was 97% or better; weights were determined both gravimetrically and by gas-liquid chromatography (GLC) peak areas compared with standards.

Analysis of tolerance to rishitin and lubimin. Toxicity of rishitin and lubimin to fungal strains was examined in a V-8 juice agar medium as previously described (Desjardins *et al.* 1988). Appropriate controls of fungal cultures without the phytoalexins and of the phytoalexins without fungal inoculation were run. In a preliminary study the growth rate of a phytoalexin-tolerant strain, R-6380, and of a sensitive strain, R-110, was determined at concentrations of 50, 100, 200, and 400 μ g/ml. Growth of both strains with rishitin or lubimin at 50 or 100 μ g/ml was within 20% of the growth rate of controls. The tolerant strain was inhibited less than 20%, and the sensitive strain was inhibited more than 70% at 200 and 400 μ g/ml; thus, about 200 μ g/ml was used for further experiments. Duplicate 35 \times 10 mm plastic petri dishes, containing 1 ml of V-8 juice agar and 2% dimethylsulfoxide (v/v) with or without test compounds, were inoculated with plugs (3-mm diameter) cut from the growing margins of cultures less than 10 days old and placed with the mycelial surface appressed to the surface of the assay medium at the edge of the plate. Plates were incubated at 25 \pm 1° C in the dark. The radius (from the inoculum to the growing margin) was measured daily for 7 days or until growth had reached the edge of the plate. Percent tolerance was calculated by dividing radial growth rate (mm/day) on phytoalexin-amended medium by radial growth rate of controls. Strains with a percent tolerance greater than about 50% at a phytoalexin concentration of 200 μ g/ml were rated

as highly tolerant.

Analysis of metabolism of rishitin and lubimin. After 7 days of incubation, agar cultures from phytoalexin-tolerance assays (as described above) were extracted with chloroform:methanol 2:1 (v/v) and analyzed by GLC as previously described (Desjardins *et al.* 1988).

Metabolism of rishitin by selected tetrad progeny was also examined in a liquid V-8 medium. Inocula were prepared from strains grown on V-8 agar plates for 1–2 wk. Conidia washed from the surfaces of the plates with sterile water were used immediately to inoculate 25 ml of V-8 juice medium (Stevens 1974) at 10⁵ conidia per milliliter in 50-ml Erlenmeyer flasks. Cultures were incubated at 28 \pm 1° C in the dark on a reciprocal shaker at 200 RPM. After 24 hr of incubation, 1-ml aliquots of the mycelial suspensions were transferred to 3.6-ml wells of 24-well polystyrene tissue culture plates (Falcon 3047). Each well contained 150 μ g of rishitin in 10 μ l of dimethylsulfoxide. This concentration of dimethylsulfoxide had no effect on fungal growth. The culture plates were sealed with Parafilm to reduce evaporation and were incubated at 28° C at about 100 RPM on a minishaker (Dynatech Laboratories, Alexandria, VA). Selected cultures were harvested after 0, 16, 24, and 41 hr of incubation and were extracted into chloroform by addition of a threefold volume of chloroform-methanol 2:1 v/v and analyzed by GLC as previously described (Gardner *et al.* 1988).

Analysis of trichothecene toxin production. Trichothecenes were measured in liquid shake cultures incubated for 7 days at 28° C, extracted, and analyzed by GLC as previously described (Beremand and Desjardins 1988). Strains that produce trichothecene toxins oxygenated at carbon number eight (mainly 8-acetylneosolaniol) are called C-8⁺, those that do not (and produce mainly 4,15-diacetoxyscirpenol) are called C-8⁻ (Beremand and Desjardins 1988).

Virulence assay. Tubers of potato cultivars Russet Burbank and Sebago were obtained from the University of Wisconsin, Lelal Starks elite foundation seed potato farm, Rhinelander, WI. Tubers were stored at 4° C, and several hours before use brought to room temperature. Slices were prepared aseptically (0.5–0.8 cm thick and 3 cm in diameter) from the medullary tissue of potato tubers. Three tuber slices were placed in a 10-cm plastic petri dish lined with filter paper and moistened with 2 ml of sterile distilled water. Slices were inoculated immediately by placing an inoculum plug (5-mm diameter), mycelial side down, at the top edge of each slice. Inoculum plugs were cut from the growing margins of cultures less than 10 days old. All cultures used in each experiment were of equal age. The petri dishes were sealed in plastic bags and incubated for 5–6 days at 25° C in the dark. Virulence was assayed gravimetrically. Tuber slices were weighed at the end of each experiment; rotted tissue was then removed with a spatula and the remaining tuber tissue weighed. Virulence was estimated from the average percent of tissue rotted for the three slices.

RESULTS

Rishitin tolerance, rishitin metabolism, and virulence of field strains. Twenty-six strains of *G. pulicaris* from a variety of habitats were each tested twice for tolerance of rishitin by comparing the linear growth rate on rishitin-

amended medium with the linear growth rate of controls. Data on lubimin tolerance of these strains was obtained simultaneously and has been reported previously (Desjardins *et al.* 1988). The 26 strains could be divided into four groups based on their tolerance of both rishitin and lubimin *in vitro*. One group of strains, represented by R-6380 (Table 1, 1–12, and Fig. 1A), was highly tolerant of both phytoalexins. The second group of strains, represented by R-6354 (Table 1, 13–18, and Fig. 1B) showed higher tolerance of rishitin than of lubimin. The two strains, R-7843 and R-7715, in group 3 (Table 1, 19 and 20, and Fig. 1C) showed higher tolerance of lubimin than of rishitin. The remaining six strains in group 4, represented by R-5690 (Table 1, 21–26, and Fig. 1D), were sensitive to both phytoalexins. The ability of the field strains to metabolize rishitin revealed a correlation between rishitin tolerance and rishitin metabolism (Table 1). The eight most rishitin-sensitive strains metabolized rishitin relatively poorly (mean of $136 \pm 38 \mu\text{g}$ recovered of $200 \mu\text{g}$ added), whereas the 18 more rishitin-tolerant strains metabolized rishitin quite well (mean of $19 \pm 26 \mu\text{g}$ recovered). Strain R-5867 appears to be an exception to the correlation between rishitin tolerance and rishitin metabolism in that this strain is quite tolerant but a relatively poor metabolizer of rishitin. This apparent exception is still unexplained. Strain R-5867 is also the only field strain in this study in which disappearance of rishitin

was accompanied by the appearance of a new compound detectable by GLC. An unidentified compound, with a longer retention time than rishitin on gas chromatograms,

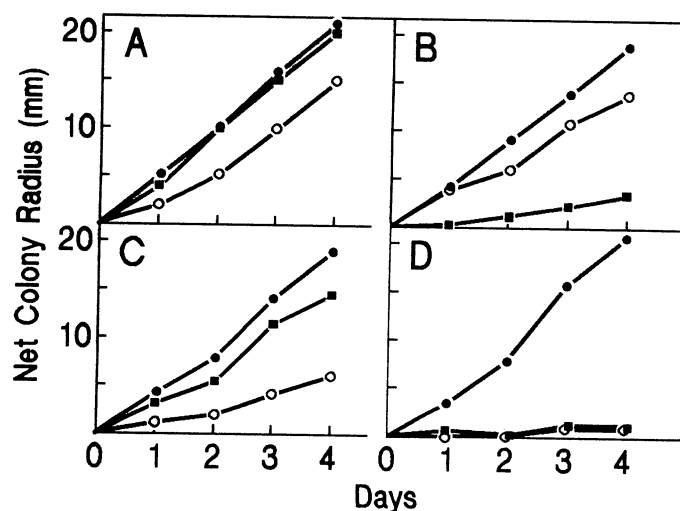


Fig. 1. The linear growth of strains of *Gibberella pulicaris*: A, R-6380; B, R-6354; C, R-7715; and D, R-5690, in agar media amended with rishitin at $200 \mu\text{g/ml}$ (O), lubimin at $200 \mu\text{g/ml}$ (■), or a dimethylsulfoxide control (●). Each point represents the average of two replicate cultures. Coefficients of variation for replicates measured at 4 days were between 0–28%.

Table 1. Rishitin tolerance, rishitin metabolism and virulence of strains of *Gibberella pulicaris* on potato tubers

Strain no. ^y	Habitat ^w	Lubimin tolerance ^x (%)	Rishitin tolerance ^x (%)	Rishitin recovered ^y (μg)	Relative virulence on potato ^z	
1	NRRL-13503	Potato	85 abc	70 abcd	0	98 a
2	R-6380	Potato	90 a	82 a	0	78 b
3	NRRL-13500	Potato	89 a	78 abc	0	74 b
4	R-5389	Potato	91 a	74 abc	9	66 b
5	R-2882	Potato	88 ab	74 abc	0	49 c
6	NRRL-13504	Potato	84 abc	65 abcd	32	36 d
7	R-5390	Potato	92 a	80 a	0	32 de
8	R-7570	Soil debris	52 d	52 d	61	31 de
9	R-5455	Corn	80 abc	61 abcd	3	20 ef
10	R-6112	Unknown	86 abc	57 cd	38	9 fg
11	R-2633	Potato	80 abc	58 bcd	8	7 fg
12	R-583	Knotweed	72 bc	51 d	7	1 g
13	R-5867	Soil	37 e	67 abcd	92	0 g
14	R-5684	Soil	32 ef	68 abcd	10	2 g
15	R-6354	Corn	28 efg	76 abc	11	0 g
16	R-5214	Soil	23 efg	66 abcd	13	6 fg
17	R-5185	Unknown	18 fgh	74 abc	11	6 g
18	R-2155	Unknown	11 h	56 cd	50	0 g
19	R-7843	Carnation	74 c	27 e	126	0 g
20	R-7715	Cactus	70 c	32 e	115	0 g
21	NRRL-13502	Grass	20 fgh	24 e	104	5 g
22	NRRL-13501	Grass	22 efg	20 e	75	2 g
23	R-110	Pine	22 efg	20 e	197	0 g
24	R-5344	Pine	14 gh	20 e	145	1 g
25	R-5753	Soil	18 fgh	18 e	153	1 g
26	R-5690	Soil	12 gh	12 e	170	0 g

^y Strains are listed approximately in order of decreasing virulence.

^w Data on habitat are from the investigator who supplied the strain.

^x Tolerance is expressed as percent of dimethylsulfoxide-treated control culture radial growth rate, mean of two replicate experiments, each with duplicate plates containing rishitin or lubimin at $200 \mu\text{g/ml}$ and incubated for 7 days. All strains were tested simultaneously. Lubimin tolerance data are from a published study (Desjardins and Gardner 1988).

^y Results of a single simultaneous test of all strains. Number of GLC analyses per sample was two to four; initial amount of rishitin was $200 \mu\text{g}$ in each of 1-ml duplicate agar cultures; rishitin recovery was analyzed after 7 days of incubation.

^z For 25 strains, mean of six replicate tests, three of cultivar Russet Burbank and three of cultivar Sebago, three tuber slices per test, scored after 5–6 days of incubation. For strain R-7843, mean of three replicate tests of Russet Burbank. Virulence of each strain was normalized to strain NRRL-13503 in each experiment. Differences among means were evaluated with Duncan's multiple-range test. Data on tolerance of rishitin and on virulence on tubers were analyzed as standardized percentages. In both columns, means followed by the same letter are not significantly different based on 95% confidence difference intervals.

appeared in extracts of R-5867 grown in the presence of rishitin. Rishitin was completely stable in uninoculated control media for up to 7 days. Although *G. pulicaris* is known to produce a variety of sesquiterpenes, rishitin and related compounds have not been found to be produced under any growth conditions in any of the 26 field strains or ascospore progeny in this study. Although there were some differences in growth rates among field strains, these differences did not correlate with phytoalexin tolerance.

Virulence data for the 26 strains on potato tubers of cultivars Russet Burbank and Sebago are given in Table 1 and Figure 2. All virulent strains produced a dry brown rot with a well-defined edge on tuber slices. Although variability in virulence was observed from experiment to experiment, the relative virulence of the 26 strains was consistent in all experiments with these two potato cultivars. Virulence of selected strains was tested on other potato cultivars and breeding clones, including Kennebec,

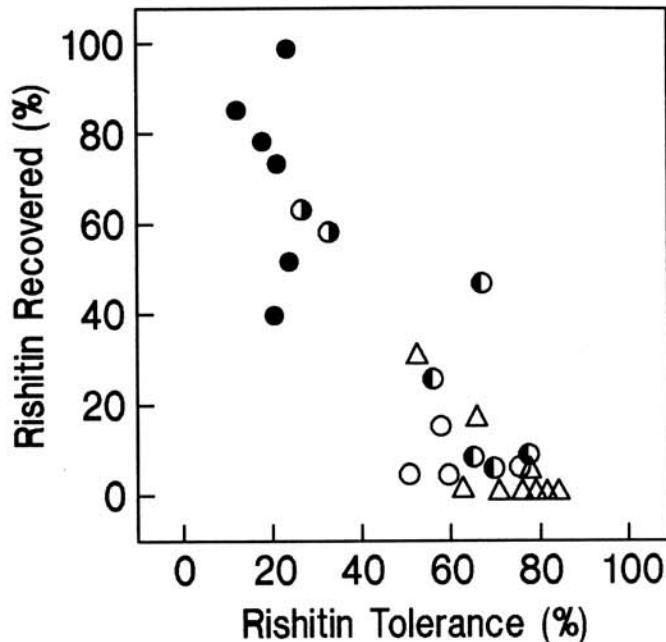


Fig. 2. The relationship between tolerance of rishitin, metabolism of rishitin, and virulence on potato tubers in field strains of *G. pulicaris*. Data on the ability of strains to tolerate lubimin are from a previous study (Desjardins *et al.* 1988). Each point represents one strain. Δ indicates a virulent (relative virulence greater than 20%; see Table 1) strain tolerant (growth rate more than 50% of control; see Table 1) of both rishitin and lubimin; \circ indicates an avirulent strain tolerant of both; \bullet indicates an avirulent strain tolerant of rishitin only; \bullet indicates an avirulent strain tolerant of lubimin only; and \bullet indicates an avirulent strain tolerant of neither. Assays are as described in Table 1.

Centennial Russet, Rosa, NemaRus, B-7200-33, and ND 534-4. Relative virulence on all of these cultivars was consistent with the relative virulence on cultivars Russet Burbank and Sebago (data not shown). Representative virulence assays for four strains on cultivar Centennial Russet are shown in Figure 3. All highly virulent strains were very tolerant of and able to metabolize both rishitin and lubimin, whereas all strains sensitive to either phytoalexin were avirulent (Table 1 and Fig. 2; Desjardins *et al.* 1988). However, tolerance of both phytoalexins was not a sufficient condition for virulence. For example, strains R-6112, R-2633, and R-583 were tolerant of both lubimin and rishitin, but were avirulent on tubers (Table 1).

Inheritance of rishitin metabolism, rishitin tolerance, virulence, and other traits. All rishitin-sensitive strains, with one exception among more than 70 field strains tested, were unable to cross with rishitin-tolerant tester strains. The cross used for the present study was between strain R-6380, which is rishitin-tolerant and highly virulent, and strain R-7843, the only genetically fertile, rishitin-sensitive strain. Both strains were highly tolerant of lubimin and able to

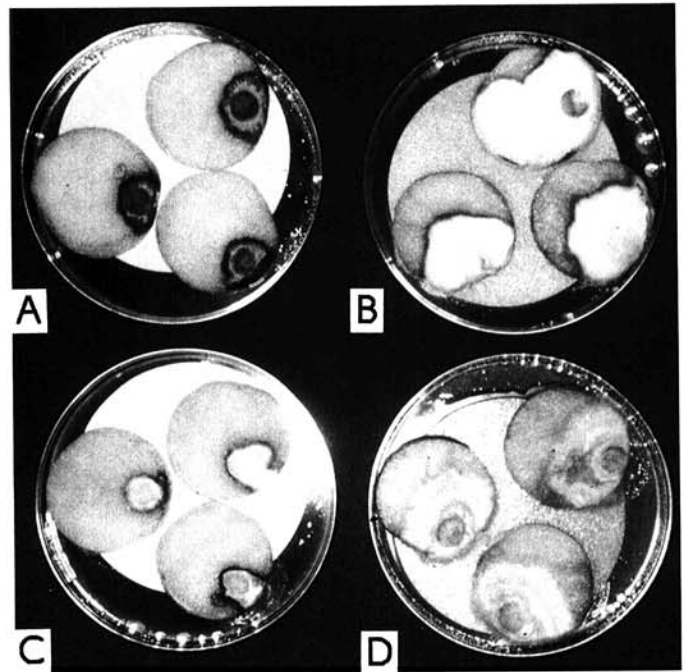


Fig. 3. Potato tuber slices of cultivar Centennial Russet inoculated with agar plugs of mycelium of *G. pulicaris* and incubated for 4 days at 25° C in the dark. Strains R-7570 (B) and A-26511 (D) are tolerant of both lubimin and rishitin, strain R-7715 (A) is tolerant of lubimin but sensitive to rishitin, and strain R-5928 (C) is sensitive to both.

Table 2. *Gibberella pulicaris* strains used as parents in crosses in this study

Strain no.	Virulence on potato	Rishitin tolerance	Lubimin tolerance	Mating type	Sex	Colony color	Major trichothecene	Origin	Host	Source
R-6380	High	High	High	Mat-1	Mln ⁺ Fmn ⁺	Red ⁻	Diacetoxy-scirpenol (C-8 ⁻)	Germany	Potato	P. Nelson
R-7843	Low	Low	High	Mat-2	Mln ⁺ Fmn ⁻	Red ⁺	Acetylneosolanol (C-8 ⁺)	Chile	Carnation	P. Nelson
1104-R-6	Low	Low	High	Mat-2	Mln ⁺ Fmn ⁺	Red ⁺	Acetylneosolanol (C-8 ⁺)	Cross 1104 (R-6380 × R-7843)

metabolize lubimin, but differed in several other traits, including mating type, femaleness, predominant colony color, and trichothecene C-8 hydroxylation (Table 2), which allowed an examination of their inheritance in conjunction with traits related to virulence.

Frequency distribution histograms for the segregation of virulence among random ascospore and tetrad progeny from several perithecia from cross 1104 (R-6380 × R-7843) are presented in Figure 4. Dissimilar patterns of virulence on potato tubers were observed among progeny isolated from different perithecia in this cross. Random ascospores from perithecium 1 were predominately of low virulence (Fig. 4A), whereas those from perithecium 2 segregated into two, parental classes of high and low virulence (Fig. 4B). In contrast, no low-virulence progeny were recovered among random ascospore progeny from perithecium 3 (Fig. 4C), even though perithecia 2 and 3 were selected on the same day from the same mulberry twig. No low-virulence progeny were observed among tetrad progeny from perithecia 5, 6,

and 7 (Fig. 4E). Among tetrad progeny from perithecium 4 a continuous spectrum of virulence was observed (Fig. 4D).

No complete, eight-spored tetrads were recovered from cross 1104. Most asci yielded five or fewer viable ascospores (average of five viable spores per ascus for 17 asci) due mainly to poor germination of the ascospores, which were often unusually small and nonseptate. Three asci with six- or seven-spored tetrads from perithecium 4 were analyzed for segregation of colony color and trichothecene toxin production, as well as for virulence. In these three asci all four products of meiosis were recovered and analyzed. These tetrads appeared to segregate in a 4:4 ratio for both colony color (Red⁺:Red⁻) and trichothecene hydroxylation (C-8⁺:C-8⁻) (Table 3). These findings are consistent with previous reports of single-gene segregation for these traits in this fungus (Desjardins and Beremand 1987; Beremand and Desjardins 1988). In contrast with the simple inheritance of these traits, all progeny of tetrads 4 and 8 were of a high-virulence phenotype, and in tetrad 1, only one progeny was of the low-virulence phenotype of strain R-7843.

Twenty randomly chosen progeny from cross 1104 perithecium 1 were retested for virulence on potato tubers and further analyzed for segregation of colony color, trichothecene toxin production, mating type, femaleness, and rishitin metabolism in agar cultures (Table 4). Results for colony color, trichothecene hydroxylation, mating type, and femaleness were consistent with unlinked, single-gene segregation for these traits as previously reported (Desjardins and Beremand 1987; Beremand and Desjardins 1988). Even though the ability to metabolize rishitin segregated 3:1 (high:low) and virulence segregated 1:3

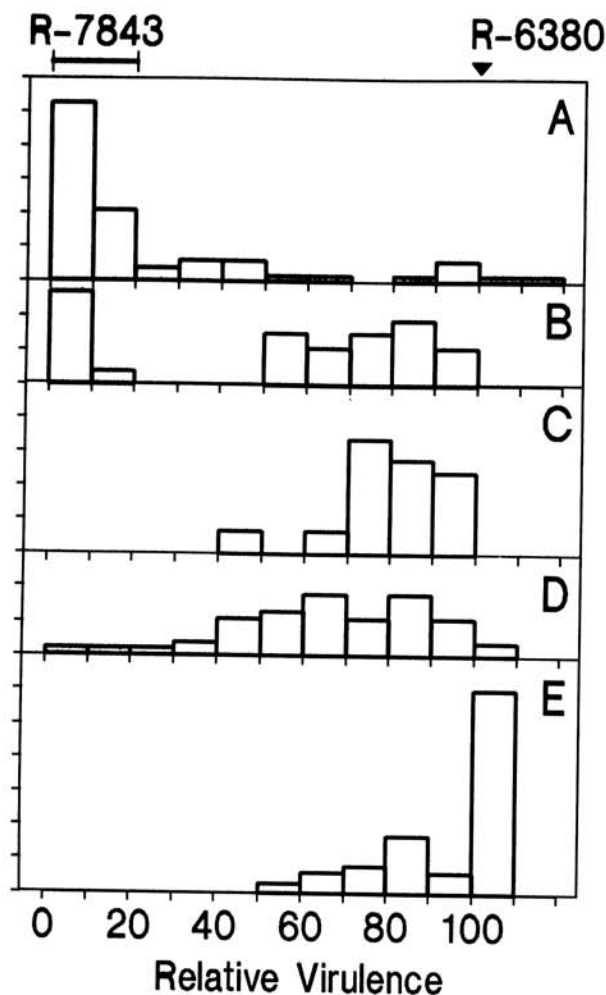


Fig. 4. Frequency distribution histograms of high and low virulence in random ascospore and tetrad progeny from cross 1104 between a high-virulence field strain, R-6380, and a low-virulence field strain, R-7843, of *G. pulicaris*. Each division of the y-axis represents a frequency of 0.10. Virulence was assessed (one to three tests) as described in Table 5 (footnote b) and normalized to R-6380. The range of virulence for R-7843 is shown. **A**, perithecium 1, 53 random ascospore progeny; **B**, perithecium 2, 27 random ascospore progeny; **C**, perithecium 3, 29 random ascospore progeny; **D**, perithecium 4, 44 tetrad progeny; and **E**, perithecia 5, 6, and 7, 35 tetrad progeny.

Table 3. Segregation of virulence and other traits in tetrads of cross 1104 (R-6380 × R-7843) of *G. pulicaris*

Ascus no.	No. ascospores (viable:nonviable)	Ascospore no.	Ascospore Phenotype		
			Color (red)	Major trichothecene toxin (C-8) ^a	Virulence (%) ^b
1	7:1	1 and 4	-	-	58 ± 1
		2 and 3	+	+	88 ± 18
		6 and 8	+	-	77 ± 4
		7	-	+	14
4	6:0	2 and 4	-	+	93 ± 1
		3 and 5	-	-	93 ± 3
		1	+	+	100
8	7:0	6	+	-	110
		2 and 6	+	-	88 ± 12
		4 and 5	-	+	56 ± 8
		1 and 3	+	-	77 ± 6
		7	+	+	110

^aSee Table 2. All asci were from perithecium 4 (Fig. 4D).

^bVirulence assessed as in Table 5 (one test) and normalized to strain R-6380. The average ± range for ascospore twins is reported.

Table 4. Segregation of virulence and other traits among 20 random ascospore progeny of cross 1104 (R-6380 × R-7843) of *G. pulicaris*

Trait	Progeny phenotypes	No. ascospores of each phenotype
Mating type	Mat-1:Mat-2	10:10
Femaleness	Fmn ⁺ :Fmn ⁻	12:8
Colony color	Red ⁺ :Red ⁻	10:10
Major trichothecene toxin	C-8 ⁺ :C-8 ⁻	11:9
Virulence on potato	High:Low	5:15
Rishitin metabolism	High:Low	15:5

(high:low), cosegregation of high virulence with a high level of rishitin metabolism was always observed. Fifteen progeny were able to metabolize most of the rishitin added to agar cultures after 7 days (mean of $5 \pm 5 \mu\text{g}$ recovered of $150 \mu\text{g}$ added); five of these progeny were highly virulent, whereas the remaining 10 progeny were of low virulence. Five progeny were, like the parent strain R-7843, relatively poor metabolizers of rishitin (mean of $60 \pm 8 \mu\text{g}$ recovered); all of these progeny were of low virulence.

The preliminary data from the 20 random ascospore progeny from cross 1104 suggested an association between high virulence on potato tubers and the ability to metabolize rishitin, but further analysis of this cross was limited because of aberrant segregation ratios for virulence and poor ascospore viability in tetrads. Therefore, one second-generation cross, cross 1421, was undertaken in an effort to obtain higher ascospore viability and more consistent segregation of virulence. Strain 1104-R-6, a female fertile, rishitin-sensitive, low-virulence progeny from cross 1104 ($\text{R-6380} \times \text{R-7843}$) was backcrossed to R-6380, the virulent parent. Most asci yielded six or more large ascospores (average of seven viable spores per ascus for 18 asci). All eight of the seven- or eight-spored asci picked from cross 1421 and five randomly selected six-spored asci from this cross were analyzed for virulence. Frequency distribution histograms for the segregation of virulence among these progeny are shown in Figure 5. Progeny from these 13 asci segregated into two parental classes of high and low virulence. All tetrads exhibited a 4:4 (high:low) segregation ratio for virulence (Fig. 5 and Table 5). Seven tetrads (six-, seven- or eight-spored) were then analyzed for segregation

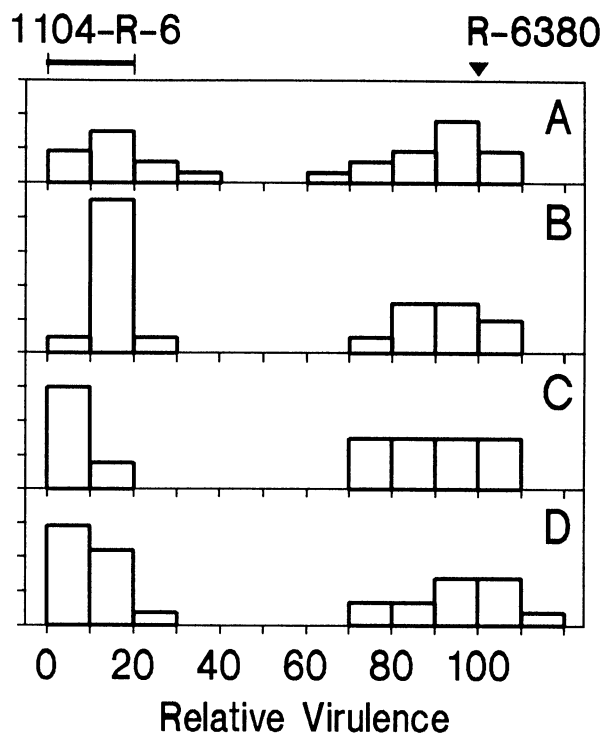


Fig. 5. Frequency distribution histograms of high and low virulence in tetrad progeny from cross 1421 between a high-virulence field strain, R-6380, and a low-virulence ascospore progeny strain, 1104-R-6, of *G. pulicaris*. Virulence was assessed (one to three tests) as described in Figure 4. The range of virulence for 1104-R-4 is shown. **A**, perithecium 1, 26 progeny; **B**, perithecium 2, 20 progeny; **C**, perithecium 3, 13 progeny; and **D**, perithecium 4, 28 progeny.

of colony color ($\text{Red}^+:\text{Red}^-$) and trichothecene hydroxylation ($\text{C-8}^+:\text{C-8}^-$). These tetrads segregated in a 4:4 ratio for these two traits as well (with one exception: $\text{Red}^+:\text{Red}^-$ was 2:6 for tetrad 1421-16). Neither of these traits was tightly linked to each other or to virulence (Table 5; data not shown).

One eight-spored and four randomly selected seven-spored tetrads from cross 1421 were further analyzed for segregation of rishitin tolerance in agar cultures (Table 5). Two tetrads (numbers 4 and 18) exhibited a 4:4 segregation ratio (tolerant:sensitive), two tetrads (numbers 8 and 16) a 6:2 segregation ratio, and one tetrad (number 10) a continuum of phenotypes (possibly 8:0), all of which were considerably more tolerant than the sensitive parent. These data indicate that at least two loci are segregating for rishitin tolerance in this cross and that presence of the tolerant allele of either gene is sufficient to confer the tolerant phenotype.

These five tetrads from cross 1421 were also analyzed for segregation of rishitin metabolism in agar cultures (Table 5 and Fig. 6). In every tetrad, the tolerant progeny showed a high level of rishitin metabolism, and the sensitive progeny showed a low level of rishitin metabolism. These data indicate that the ability to metabolize rishitin causes rishitin tolerance or is closely linked to genes for rishitin tolerance. An additional study of rishitin metabolism by progeny of tetrads 4, 8, and 10 in liquid culture essentially confirmed the results from agar cultures (Table 5). The liquid culture data were more variable and less conclusive than the agar culture data, probably because the liquid culture assays were performed at a lower concentration of rishitin ($150 \mu\text{g}$ versus $220 \mu\text{g/ml}$), and single samples rather than duplicate samples were taken at each time point.

In an additional effort to determine the number of loci conferring a rishitin-tolerant phenotype, nine crosses were made between rishitin-tolerant field strains. Random ascospore progeny from each cross were tested for rishitin tolerance and rishitin metabolism. Among 55 progeny from one cross ($\text{R-5390} \times \text{R-5455}$) and 20 progeny from each of seven crosses ($\text{R-6380} \times \text{R-2882}$, $\text{R-6380} \times \text{R-5389}$, $\text{R-6380} \times \text{R-5455}$, $\text{R-5390} \times \text{R-2882}$, $\text{R-5390} \times \text{R-5389}$, $\text{R-583} \times \text{R-2882}$, $\text{R-583} \times \text{R-5455}$), no rishitin-sensitive progeny were recovered. All progeny were also able to metabolize almost all of the $200 \mu\text{g}$ of rishitin added to agar cultures.

In all tetrads analyzed in cross 1421, all of the rishitin-sensitive progeny were of low virulence on potato tubers (Table 5 and Figure 6), which supports the hypothesis that rishitin tolerance is required for high virulence. Some of the rishitin-tolerant progeny were low in virulence also, which indicates, among other possibilities, that some rishitin tolerance genes may not be active *in vivo* or that rishitin tolerance alone is not sufficient for high virulence and that the parents used in this cross differ at other loci important for virulence on potato. Although both parents are tolerant of lubimin, it is possible that recombination of loci for lubimin tolerance might lead to recovery of lubimin-sensitive, avirulent progeny in this cross. Therefore, lubimin tolerance of the progeny from tetrads 8 and 10 was assessed in agar cultures. All progeny retained the lubimin-tolerant phenotype of the parents (data not shown).

DISCUSSION

The idea that plants produce protective chemicals after exposure to microorganisms was formalized by Müller and

Table 5. Segregation of virulence and other traits in unordered tetrads of cross 1421 (R-6380 × 1104-R-6) of *G. pulicaris*

Ascus no.	Ascospore no.	Colony color (red)	Major trichothecene toxin ^a (C-8)	Virulence (%) ^b	Rishitin tolerance ^c (in agar, %)	Rishitin metabolism	
						In agar (%) ^d	In liquid ^e (μg/ml/hr)
4	7	—	—	71	86	100	1.4
	3 + 4	—	+	101 ± 11 ^f	82 ± 6 ^f	100 ± 0 ^f	3.4 ± 0.2 ^f
	1 + 6	+	—	22 ± 6	33 ± 12	33 ± 4	0.7 ± 0.2
	2 + 5	+	+	31 ± 7	26 ± 2	10 ± 5	0.7 ± 0.1
8	5	+	+	104	70	100	2.8
	6 + 7	+	+	101 ± 17	64 ± 1	95 ± 1	3.4 ± 0.3
	1 + 3	—	—	15 ± 2	58 ± 8	80 ± 1	1.2 ± 0.7
	2 + 4	—	—	17 ± 3	22 ± 6	0 ± 2	0.6 ± 0.2
10	1 + 3	+	—	94 ± 6	74 ± 2	98 ± 1	2.6 ± 0.3
	4	—	+	92 ± 17	60	98	3.7
	6 + 7	+	—	16 ± 2	48 ± 3	72 ± 3	1.9 ± 0.1
	2 + 5	+	+	14 ± 2	42 ± 5	45 ± 2	1.0 ± 0.1
16	4 + 6	+	+	68 ± 5	77 ± 4	99 ± 1	nd ^g
	1 + 2	—	—	90 ± 3	74 ± 5	98 ± 1	nd
	7	—	+	11	51	81	nd
	3 + 5	—	—	7 ± 1	21 ± 1	14 ± 5	nd
18	2 + 6	+	+	74 ± 3	84 ± 3	100 ± 0	nd
	1 + 5	—	—	80 ± 4	80 ± 5	99 ± 5	nd
	3 + 7	—	+	15 ± 6	34 ± 3	46 ± 2	nd
	4 + 8	+	—	6 ± 4	6 ± 4	36 ± 1	nd
R-6380 (female parent)		—	—	100	96	100	3.4
1104-6 (male parent)		+	+	19 ± 6	24	9	0.2

^aSee Table 2.

^bVirulence was assessed on cultivar Russet Burbank, three tuber slices per test, incubated for 5–6 days at 25° C in the dark. Tetrads 4, 8, and 10 were tested three times and the average is reported; tetrads 16 and 18 were tested once. Both parents and all progeny from each tetrad were tested at the same time, and virulence was normalized to that of the virulent parent strain R-6380.

^cTolerance of rishitin and lubimin was assessed as in Table 1 (footnote x), except that 220 μg of rishitin was added to each culture. Both parents and all progeny from each tetrad were tested at the same time.

^dRishitin metabolism was assessed by duplicate GLC assays of extracts of the duplicate plates from tolerance assays (footnote c).

^eRishitin metabolism was assessed by triplicate GLC assays of extracts of 1-ml cultures harvested at 0, 16, 24, and 41 hr after the addition of 150 μg of rishitin. Both parents and all progeny from each tetrad were tested at the same time.

^fAverage ± range for ascospore twins.

^gNot done.

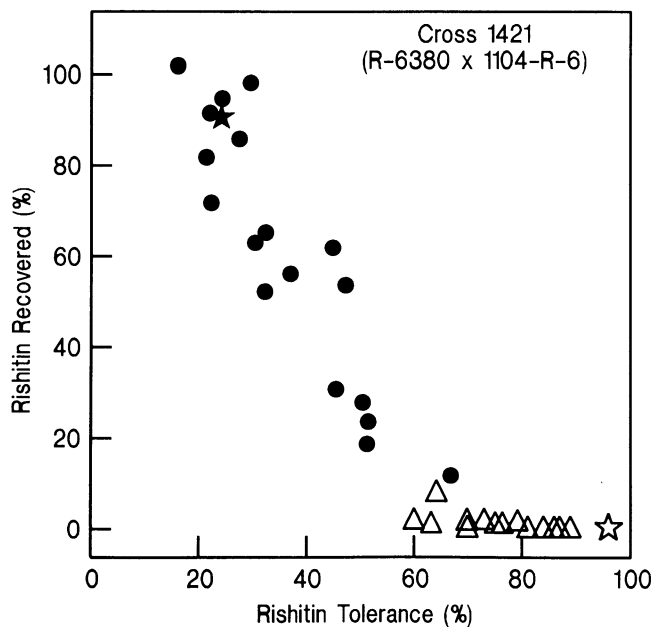


Fig. 6. The relationship between tolerance of rishitin, metabolism of rishitin, and virulence on potato tubers in tetrad progeny from cross 1421. Data are from Table 5. Each point represents one strain. Filled symbols indicate avirulent strains and open symbols indicate virulent strains. Circles and triangles indicate progeny and stars indicate the two parental strains.

Börger in 1940 and was based on their research on potato resistance to *Phytophthora infestans*. Thirty years later, the most active phytoalexins in potato were found to be a series of closely related sesquiterpenes, the most fungitoxic of which are rishitin, lubimin, and phytuberin (Kuć 1982). In potato tubers infected with a variety of fungi, sesquiterpenes can accumulate to concentrations of more than 300 μg/g of fresh weight (Price *et al.* 1976; Henfling and Kuć 1979; Corsini and Pavek 1980), which are sufficient to prevent fungal growth *in vitro*. Fungal tolerance and metabolism of phytoalexins *in vitro* have been correlated with virulence on some phytoalexin-producing plants (VanEtten *et al.* 1982), but studies of rishitin tolerance and its relationship to virulence on rishitin-producing plants have been limited. Among the field strains of *G. pulicaris* in this study, only strains tolerant of rishitin and able to metabolize rishitin *in vitro* were highly virulent on potato tubers; all rishitin-sensitive field strains were avirulent. In contrast, all races of *P. infestans* tested were equally sensitive to rishitin, although their virulence was quite different on certain potato cultivars (Ishizaka *et al.* 1969), whereas *Botrytis fabae* was tolerant of rishitin and able to metabolize rishitin but was avirulent on tomato (Lyon 1976). These kinds of quantitative correlations, however, do not necessarily prove or disprove a role for rishitin tolerance in virulence because

of the inherent difficulties in comparing *in vitro* assay conditions to those in infected plant tissues.

In the present study we initiated a genetic analysis to provide a more critical test of whether there is a causal relationship between rishitin tolerance and virulence on potato tubers. Such a genetic approach has been used to demonstrate that pisatin metabolism is required for high virulence of *Nectria haematococca* on pea (Tegtmeier and VanEtten 1982; Kistler and VanEtten 1984a, 1984b). The availability of natural variants and a genetic system in *G. pulicaris* have allowed us to undertake a very similar genetic approach to study virulence of this fungus on potato tubers. Segregation of rishitin tolerance, rishitin metabolism, and virulence was followed among 20 first-generation, random-ascospore progeny and 36 second-generation, backcrossed, tetrad progeny. In both generations, all progeny that were highly sensitive to rishitin were of low virulence on potato tubers, which strongly suggests that rishitin tolerance and rishitin metabolism are either required for virulence or are closely linked to genes for virulence.

Rishitin sensitivity was not recovered in any progeny from crosses between several rishitin-tolerant field strains. Although the number of ascospores tested was relatively small, these results could be interpreted as indicating multiple loci for rishitin tolerance in field strains such that all recombinant progeny inherit at least one gene and, consequently, a rishitin-tolerant phenotype. Alternately, all field strains may have rishitin tolerance genes at the same locus or loci or at closely linked loci, and for that reason, rishitin-sensitive recombinant progeny were not recovered. Crosses between rishitin-sensitive progeny strains and rishitin-tolerant field strains should distinguish among these and other possibilities.

Segregation ratios in some tetrads from cross 1421 also suggest the presence of two or more loci involved in rishitin metabolism in *G. pulicaris*. Multiple genes for pisatin metabolism have been identified by genetic analysis of *N. haematococca* (Kistler and VanEtten 1984a, 1984b). Analyses of the segregation of rishitin tolerance and virulence by further crosses among tetrad progeny from cross 1421 are in progress.

The segregation of virulence was very dissimilar among progeny isolated from different perithecia in cross 1104, which was between two field strains of *G. pulicaris*. Many asci contained fewer than five ascospores, and the results might therefore be explained in part by nonrandom abortion of meiotic products. Among selected random ascospores and tetrads from all perithecia tested in this cross, alleles at the trichothecene C-8 and Red loci segregated as expected for random abortion. In contrast, in some perithecia, very few low-virulence progeny were recovered, suggesting a link between abortion-inducing factors and low virulence. Nonrandom abortion, however, cannot explain the occurrence of only a very small number of low-virulence progeny in tetrads containing a complete or near-complete complement of meiotic products, or reconcile these low segregation ratios for low virulence with the one-to-one segregation of high to low virulence in the second-generation backcross to the virulent parent. Low fertility and aberrant virulence- or avirulence-linked segregation ratios have been observed in crosses between field strains of other filamentous ascomycetes and may be due to a number of factors, including nonrandom abortion

of ascospores (Taga *et al.* 1985) and chromosomal recombination or gene loss (Bronson 1988; Miao and VanEtten 1988). A simple explanation for aberrant segregation ratios would be variation in virulence assays over time. This possibility was tested and refuted by consistent, reproducible results in virulence assays of ascospore progeny over periods of more than 1 yr.

The results of this study indirectly support a role for rishitin in disease resistance of potato tubers. But it is also clear from our results and those of others that rishitin is not the only resistance mechanism in potato tubers. For example, there is also a strong correlation between high virulence of field strains of *G. pulicaris* and their ability to detoxify lubimin and related vetispiranes from potato (Gardner *et al.* 1988; Desjardins *et al.* 1988). All of the more than 20 potato-derived strains of *G. pulicaris* in our collection are very tolerant of both rishitin and lubimin (data not shown). Field strains sensitive to either rishitin or lubimin are of low virulence, and different mechanisms appear to be responsible for metabolism of these two phytoalexins because rishitin-tolerant strains can be sensitive to lubimin and vice versa. Other sesquiterpenes such as phytuberol might accumulate in infected tubers, and the low virulence of some rishitin-tolerant, lubimin-tolerant strains might be due to sensitivity to these other phytoalexins. Indirect evidence against this point is that phytuberin was a minor component of the sesquiterpene response to *G. pulicaris* in an earlier study (Corsini and Pavek 1980) and in our own; in tubers infected with either virulent or avirulent field strains in our study, rishitin and lubimin were the major sesquiterpenes detected (Desjardins, Gardner, and Plattner, unpublished). A role for rishitin in resistance to *G. pulicaris* does not imply that rishitin and related sesquiterpenes are major determinants of potato resistance to all fungi. Bostock *et al.* (1983) and others (Ampomah and Friend 1988) have found that, under certain conditions, potato tubers can show the appropriate compatible and incompatible disease reactions to races of *P. infestans* without any differences in sesquiterpene levels. Biochemical factors, other than sesquiterpenes, which have been previously implicated in potato tuber resistance to fungi, include a variety of phenolics and steroid glycoalkaloids, as well as suberin and lignin (Kuć 1982; Ampomah and Friend 1988).

The metabolites of rishitin produced by tolerant strains of *G. pulicaris* have not yet been identified. Our method for following rishitin metabolism was to determine the disappearance of rishitin by GLC of chloroform-methanol-extracted material. By this method it should be possible to detect, within the time of the GLC profile, metabolites of rishitin containing one additional substituent group. Only one potential and as yet unidentified metabolite was detected in liquid or agar cultures of any of the 26 strains, and none in any ascospore progeny, even when cultures were extracted within minutes or hours of the initiation of rishitin metabolism in some strains. The possibility that rishitin was modified by glycosylation was also investigated by acid hydrolysis of the material remaining after chloroform-methanol extraction of rishitin-amended agar incubated with strain R-6380. The chloroform-extracted material from the hydrolysate did not contain rishitin. The actual fate of rishitin in tolerant strains of *G. pulicaris* continues to be investigated in this laboratory.

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