Virulence of *Gibberella pulicaris* on Potato Tubers and Its Relationship to a Gene for Rishitin Metabolism

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ABSTRACT

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The ability of field strains of *Gibberella pulicaris* (*Fusarium sambucinum*) to cause dry rot of potato tubers is related to their ability to metabolize the potato phytoalexin rishitin. All highly virulent field strains studied to date have proven tolerant of and able to metabolize rishitin. Preliminary genetic analysis of one potato-pathogenic field strain, R-6380, suggested that multiple loci might confer rishitin metabolism and that not all of these loci are associated with virulence (A. E. Desjardins and H. W. Gardner, Mol. Plant-Microbe Interact. 2:26-34). To investigate these hypotheses, four phenotypically unique meiotic products of a tetratype ascus from a backcross to strain R-6380 were crossed to strains that were low in rishitin tolerance, rishitin metabolism, and virulence. Tetrad progeny from all four crosses were analyzed for these traits. This genetic analysis indicated that rishitin metabolism in strain R-6380 is controlled by genes at two or more loci but that high virulence on potato is associated with only one of these loci, designated as *Rim1*.

Dry rot caused by *Gibberella pulicaris* (Fries) Sacc. (anamorphs: *Fusarium sambucinum* Fuckel & *F. sulphureum* Schlect.) is a serious storage disease of potato (*Solanum tuberosum* L.) worldwide (7,25). Although dry rot seldom reaches epidemic proportions (2), average yearly losses of 6%, with occasional losses of up to 25%, have been reported during long-term storage (9). Crop losses due to dry rot are compounded by the fact that *G. pulicaris* can contaminate tubers with trichothecene toxins that are injurious to human and animal health (14,15,21). Most potato cultivars are susceptible to *G. pulicaris* (22), and susceptibility increases progressively during storage (6,22). Losses are controlled by treatment of tubers with thiabendazole at harvest or before planting (8). Potato breeding programs have given increased attention to development of cultivars with resistance to dry rot, but the biochemical basis of resistance is still poorly understood (22).

Rishitin, lubimin, and related sesquiterpenes accumulate in potato tubers after infection with *G. pulicaris* and many other fungi as well (10,20). Rishitin and lubimin inhibit the growth of many of these fungi in vitro (17,30) and have been proposed to be part of an active defense mechanism in potatoes. It is well established that some plant pathogenic fungi are tolerant of their hosts' phytoalexins and that they often are able to metabolize the phytoalexins to which they are tolerant (28). Analysis of field strains of *G. pulicaris* from a wide range of habitats has demonstrated a strong correlation between the ability to metabolize rishitin and lubimin in vitro and virulence on potato tubers (12,13,16). These kinds of quantitative correlations, however, do not necessarily prove or disprove a role for phytoalexin tolerance in virulence because of the inherent difficulties in comparing in vitro assay conditions with those in infected plant tissues and because of the nonisogenic backgrounds of field strains.

The recent development of a heterothallic genetic system in *G. pulicaris* (11) might allow a more critical genetic test of whether there is a causal relationship between phytoalexin metabolism and virulence on potato tubers. This genetic approach, however, has been encumbered by two major disadvantages of the potato-*G. pulicaris* experimental system. First is the arduous task of purifying from treated potatoes quantities of rishitin and lubimin sufficient for a detailed genetic analysis. Yields of rishitin or lubimin after purification by a series of chromatographic procedures (12,16) are highly variable and sometimes amount to as little as 2 mg/kg of tuber fresh weight. The second major problem is the consistently poor sexual fertility of phytoalexin-sensitive field strains of *G. pulicaris*. None of the more than 120 field strains that we have analyzed to date is both sexually fertile and completely unable to metabolize either rishitin or lubimin (12,13, Desjardins and Gardner, *unpublished*). Nevertheless, one male-fertile (but female-sterile) field strain, R-7843, was found to be low in rishitin metabolism and tolerance and low in virulence and therefore was suitable, although not ideal, for genetic analysis (12).

In a previous study (12), a cross was made between *G. pulicaris* field strains R-7843 and R-6380, which differ in rishitin metabolism, rishitin tolerance, and virulence, and these traits were followed through one generation of random ascospore progeny (cross 1104) and a second, backcrossed generation of tetrad progeny (cross 1421). All highly virulent progeny of both crosses were highly tolerant of and able to metabolize rishitin, and all rishitin-sensitive progeny were of low virulence. These results supported 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. All 13 tetrads analyzed for virulence in cross 1421 exhibited a 4:4 (high to low) segregation ratio, which is consistent with, but does not prove, a single gene difference between the parents of this cross for this trait. Three of the five tetrads tested from cross 1421 exhibited 4:4 (high to low) segregation ratios for rishitin tolerance and metabolism, whereas two tetrads exhibited 6:2 ratios for these two traits (defining high tolerance and high metabolism as greater than 50–60 and 80%, respectively, of controls). These data suggested that at least two loci for high rishitin tolerance and metabolism were segregating in this cross but that only one of these loci was associated with high virulence. These data also suggest, among other possibilities, that rishitin metabolism alone might not be sufficient for high virulence, that some rishitin metabolism genes might be inactive in vivo, or that the parents of cross 1421 might differ

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at other loci important for virulence on potato. The present study was undertaken to test these hypotheses, to analyze the genetic basis of rishitin metabolism, and to evaluate the relationships among rishitin metabolism, rishitin tolerance, and potato tuber dry rot.

MATERIALS AND METHODS

Cultures. Field strains R-6380 and R-7843 were identified and kindly supplied by P. E. Nelson from the Fusarium Research Center, Pennsylvania State University, University Park, and were single spored before this study. Other strains used in this study are described in Table 1. 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). Cultures were grown routinely on V-8 agar (26) slants or plates on an alternating 12-hr, 25 C light and 12-hr, 20 C dark schedule. For long-term storage, all strains were maintained on V-8 agar slants at 4 C. Field strains also were maintained as lyophilized conidial suspensions in the Agricultural Research Service Collection, Peoria, IL. For all experiments, fresh transfers of the strains were obtained from stock cultures held at 4 C.

Genetic crosses. Crosses were made between strains of opposite mating type, designated Mat1-1 and Mat1-2; crosses between strains of the same mating type are infertile. Techniques for crossing and for tetrad isolations have been described (4,11). Sterile mulberry twigs on water-agar slants were inoculated with the female parent and grown until protoperithecia were well developed (2 mo usually were needed because all strains derived from R-7843 demonstrated low to very low female fertility) and fertilized by the addition of conidia from the male parent. After fertilization, crosses were incubated at 15 C until perithecia were mature. Femaleness was scored as the ability to produce protoperithecia. Mating type was determined by crosses to two tester strains: R-6380 (Mat1-1) and 1810-1-5 (Mat1-2) (3). Cultures were scored for colony color after 10–14 days of growth on potato-dextrose agar. Trichothecene toxin production was scored in 7-day-old liquid cultures as described (4).

Analysis of rishitin tolerance. Rishitin was elicited in potato tuber slices and purified as described (12). The purity of rishitin was 97% or better; weights were determined both gravimetrically and by gas liquid chromatography (GLC) peak areas compared with standards.

Tolerance of fungal strains to rishitin was examined in a V-8 juice agar medium as described (12). Duplicate 35 × 10 mm plastic petri dishes, containing 1 ml of medium and 1% dimethyl sulfoxide with or without rishitin (200 or 220 μg), were inoculated with plugs (3 mm in 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 ± 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 (millimeters per day) on rishitin-amended medium by radial growth rate of controls.

Analysis of rishitin metabolism. After 7 days of incubation, agar cultures from rishitin-tolerance assays (as described above) were extracted with chloroform and methanol (2:1, v/v) and analyzed by GLC (12). Metabolism of rishitin by selected tetrad progeny also was examined by thin-layer chromatography (TLC) with a modification of the tolerance assay method described above. Mycelial plugs (3 mm in diameter, as above) were inoculated onto 0.25 ml of rishitin-amended V-8 juice agar in 7-ml plastic scintillation vials (23). The cultures were incubated for 3 days at 25 C in the dark, then extracted twice with 0.75 ml of chloroform and methanol as above for analysis by TLC (12). Metabolism data are expressed as percentage of rishitin not recovered as compared with an uninoculated, rishitin-amended control plate.

Virulence assay. Virulence was assessed as described (12). Five or 6 days after inoculation of Russet Burbank tuber slices, virulence was measured as the average percentage of tuber tissue dry rotted for three replicate slices and normalized to that of strain R-6380 tested on the same day. Virulence of strain R-6380 varied during this 2-yr study, due in large part to the increase in susceptibility of the tubers to dry rot with time after harvest.

RESULTS

To determine the genetic basis of rishitin metabolism and virulence in field strain R-6380, we selected a putative tetratype ascus from cross 1421, a second-generation backcross to this strain (Fig. 1). In ascus 8 of this cross, twin nonparental-type progeny of high rishitin metabolism but low virulence were recovered, along with twin parental-type progeny of low metabolism and low virulence and three parental-type progeny (one mitotic twin was missing) of high metabolism and high virulence. Based on the phenotypes of these progeny, we have proposed the following hypothetical genotypes for rishitin metabolism for the parents of this cross. Strain R-6380 was designated $Rim1^{+}$ $Rim2^{+}$, and strains R-7843 and 1104-R-6 were designated $Rim1^{-}$ $Rim2^{-}$, where $Rim1^{+}$ and $Rim2^{+}$ (for *ri*shitin *m*etabolism) designate non-allelic genes for a high level of rishitin metabolism but only *Rim1* is associated with high virulence. If these proposed genotypes are correct, then the progeny of tetratype ascus 1421-8 should have the following genotypes: $Rim1^{-}$ $Rim2^{-}$ (strains 1421-8-2 and 4), $Rim1^{-}$ $Rim2^{-}$ (strains 1421-8-1 and 3), $Rim1^{-}$ $Rim2^{-}$ (strain 1421-8-5), and $Rim1^{+}$ $Rim2^{+}$ (strains 1421-8-6 and 7). The proposal of strain 1421-8-6 rather than strain 1421-8-5 as

TABLE 1. *Gibberella pulicaris* strains used as parents in crosses in this study

Strain no.	Virulence[a] (%)	Rishitin tolerance[b] (%)	Rishitin metabolism[c] (%)	Proposed *Rim* genotype	Other traits: Mating type	Other traits: Sex
R-6380	100 (12)	100 (10)	100 (8)	$Rim1^{+}Rim2^{+}$	Mat1-1	$Mln^{+}Fmn^{+}$
R-7843	0 (6)*[d]	33 (2)[d]	37 (2)[d]	$Rim1^{-}Rim2^{-}$	Mat1-2	$Mln^{+}Fmn^{-}$
1104-R-6	9 ± 4 (6)	49 ± 7 (6)	45 ± 3 (4)	$Rim1^{-}Rim2^{-}$	Mat1-2	$Mln^{+}Fmn^{+}$
1421-8-1	12 ± 5 (6)	67 (2)	100 (2)	$Rim1^{-}Rim2^{+}$	Mat1-2	$Mln^{+}Fmn^{+}$
1421-8-2	10 ± 10 (12)	53 ± 4 (8)	54 ± 10 (6)	$Rim1^{-}Rim2^{-}$	Mat1-1	$Mln^{+}Fmn^{+}$
1421-8-5	64 (3)	69 (2)	100 (2)	$Rim1^{+}Rim2^{-}$	Mat1-2	$Mln^{+}Fmn^{+}$
1421-8-6	100 (3)	100 (2)	100 (2)	$Rim1^{+}Rim2^{+}$	Mat1-1	$Mln^{+}Fmn^{+}$

[a] Virulence was assessed on cultivar Russet Burbank (three tuber slices per test) incubated for 5–6 days at 25 C in the dark. Strain R-6380, both parents, and all progeny were tested at the same time, and virulence was normalized to strain R-6380. Data are expressed as mean ± standard deviation (total number of replicates). Virulence of strain R-6380 was 79 ± 24% (12) of tuber tissue rotted.

[b] Tolerance was assessed as percent of control culture radial growth rate, mean of two duplicate plates containing rishitin at 200 μg/ml and incubated for 7 days. Strain R-6380, both parents, and all progeny were tested at the same time, and tolerance was normalized to strain R-6380. Data are expressed as in footnote a. Tolerance of strain R-6380 was 86 ± 5% (10) of control growth rate.

[c] Metabolism was assessed by duplicate gas liquid chromatography assays of the duplicate plates from tolerance assays (footnote b). Data are expressed as in footnote a.

[d] Data are all from a previous study (12).

containing two putative *Rim* genes is based on its higher rate of rishitin metabolism in liquid culture. To test these hypotheses, we crossed each of the four meiotic progeny to a strain of low metabolism and low virulence (proposed genotype $Rim1^-$ $Rim2^-$) and studied inheritance of these traits in several tetrads from each cross. Because strains of the same mating type cannot be crossed, progeny of mating type 2 (strains 1421-8-1 and 1421-8-5) were crossed to strain 1421-8-2, and progeny of mating type 1 (1421-8-2 and 1421-8-6) were crossed to strain 1104-R-6. The parents and sequence of crosses in this study are described in Tables 1 and 2 and Figure 1.

Cross 1842. Backcross 1842 was between strain 1421-8-2 ($Rim1^-$ $Rim2^-$) and strain 1104-R-6 ($Rim1^-$ $Rim2^-$), both of which were low in rishitin metabolism, rishitin tolerance, and virulence (Tables 1 and 2). Although many perithecia were produced in this cross, almost half of the asci picked contained fewer than four spores (Table 3). Four randomly selected eight-spored asci were analyzed for rishitin tolerance and virulence and for rishitin metabolism. All 32 progeny tested were low in rishitin tolerance (mean 57 ± 6%) (Fig. 2A) and rishitin metabolism (mean 39 ± 14%) (Fig. 3A) and very low in virulence (mean 8 ± 4%) (Fig. 2A) when compared with the strain R-6380 control. The 0:32 (high to low) segregation ratios for rishitin tolerance, metabolism, and virulence observed in this cross are in agreement with the predicted ratios (Tables 2 and 4). These results are consistent with the proposed genotype of $Rim1^-$ $Rim2^-$ for strains 1421-8-2 and 1104-R-6. Because of the limited quantities of available rishitin, this cross was not analyzed further.

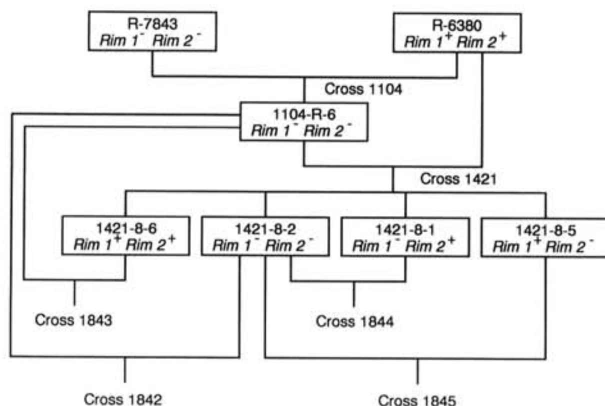

Fig. 1. Strain numbers and proposed genotypes for crosses used in this study.

Cross 1845. Strain 1421-8-5 ($Rim1^+$ $Rim2^-$), which was highly tolerant of rishitin and highly virulent, was crossed as a male to its sibling strain 1421-8-2 ($Rim1^-$ $Rim2^-$). (Forty attempts to cross strain 1421-8-5 as a female were unsuccessful.) Perithecia were abundant in this cross, but one-third of the asci contained four or fewer ascospores (Table 3). Nineteen partial and complete tetrads were analyzed for virulence and for rishitin metabolism by TLC and/or GLC (Table 5). With one exception, segregation ratios were consistent with single-gene segregation of both high metabolism and high virulence. Of the 122 progeny from these asci, 58 were high rishitin metabolizers and highly virulent, and 64 were low rishitin metabolizers and avirulent. High rishitin-metabolizing, avirulent, recombinant progeny were not recovered. Five seven- or eight-spored asci were randomly selected for confirmatory analysis of rishitin metabolism by GLC and for analysis of rishitin tolerance. In this analysis, all five tetrads segregated 4:4 (high to low) for rishitin tolerance (mean 99 ± 8%:47 ± 12%) (Fig. 2C), rishitin metabolism (mean 96 ± 5%:47 ± 9%) (Fig. 3C), and virulence (mean 97 ± 8%:11 ± 7%) (Fig. 2C). One ascus with seven rishitin-sensitive, avirulent progeny was obtained in cross 1845. This tetrad, 1845-11, segregated 4:4 for two other markers: colony color and trichothecene toxin hydroxylation. Only four progeny were fertile, and these were all of mating type 1 (Table 6). The overall 58:64 (high to low) segregation ratios for rishitin tolerance, rishitin metabolism, and virulence observed in cross 1845 are close to the predicted ratios (Tables 2 and 4). These results support the proposed genotypes of $Rim1^+$ $Rim2^-$ for strain 1421-8-5 and $Rim1^-$ $Rim2^-$ for strain 1421-8-2. Except for the single 0:8 ascus, to be discussed later, these data are consistent with the segregation of a single *Rim* gene that is associated with virulence.

Cross 1844. Strain 1421-8-1 ($Rim1^-$ $Rim2^+$), which was highly tolerant of rishitin but avirulent, was crossed to its sibling strain

TABLE 2. Crosses to test the *Rim1Rim2* hypothesis

Cross no.	Proposed parental *Rim* genotypes	Predicted progeny *Rim* genotypes	Traits	Progeny phenotypes (high:low)[a]	
				Predicted	Observed
1842	$Rim1^-Rim2^-$	$Rim1^-Rim2^-$	Rishitin metabolism	0:32	0:32
	$Rim1^-Rim2^-$		Virulence	0:32	0:32
1845	$Rim1^+Rim2^-$	$Rim1^+Rim2^-$	Rishitin metabolism	61:61	58:64
	$Rim1^-Rim2^-$	$Rim1^-Rim2^-$	Virulence	61:61	58:64
1844	$Rim1^-Rim2^+$	$Rim1^-Rim2^+$	Rishitin metabolism	16:16	21:11
	$Rim1^-Rim2^-$	$Rim1^-Rim2^-$	Virulence	0:32	1:31
1843	$Rim1^+Rim2^+$	$Rim1^+Rim2^+$	Rishitin metabolism	24:8 TT[b]	21:11
	$Rim1^-Rim2^-$	$Rim1^+Rim2^-$		16:16 PT[b]	
		$Rim1^-Rim2^+$	Virulence	16:16	16:16
		$Rim1^-Rim2^-$			

[a] For all crosses, high metabolism was scored as >80% metabolized; high virulence was scored as >50% of the virulence of the control strain R-6380.
[b] TT = tetratype; PT = parental ditype.

TABLE 3. Fertility of crosses 1842, 1844, 1845, and 1843

Cross no.	Parents (female × male)	Number of tetrads		Viable:nonviable ascospores in complete tetrads[a]				
		Picked	Complete	8:0	6:2	4:4	2:6	0:8
1842	1421-8-2 × 1104-R-6	15	13	6	1	0	1	5
1845	1421-8-2 × 1421-8-5	36	35	10	11	2	4	8
1844	1421-8-1 × 1421-8-2	11	11	7	1	1	1	1
1843	1104-R-6 × 1421-8-6	17	12	10	1	0	1	0

[a] Asci containing seven or eight spores were counted as complete. Odd numbers of spores were counted as the next highest even number.

Fig. 2. Relationship between rishitin tolerance and virulence on potato tubers of tetrad progeny from: **A,** cross 1842; **B,** cross 1844; **C,** cross 1845; and **D,** cross 1843. Each open circle represents one progeny strain; each solid circle represents a parental strain. Assays are described in Table 1 and in the Materials and Methods section.

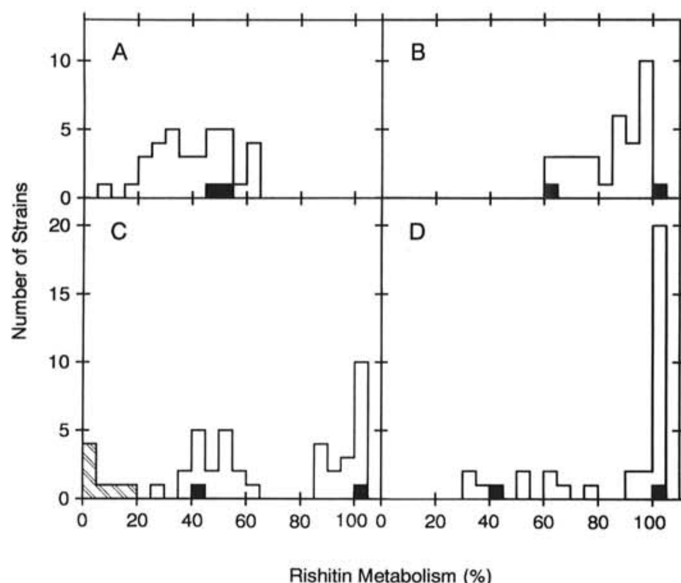

Fig. 3. Frequency distribution histograms of metabolism of rishitin by tetrad progeny from: **A,** cross 1842; **B,** cross 1844; **C,** cross 1845; and **D,** cross 1843. Solid squares represent parental strains; open squares represent progeny strains; hatched squares represent progeny of tetrad 1845-11. Assays are described in Table 1 and in the Materials and Methods section.

1421-8-2 (*Rim1*$^-$ *Rim2*$^-$) (Tables 1 and 2). Perithecia were abundant in this cross, and a majority of the asci contained complete tetrads (Table 3). Four randomly selected eight-spored asci were analyzed for rishitin tolerance (Fig. 2B), rishitin metabolism (Fig. 3B), and virulence (Fig. 2B). Thirty-one of the 32 progeny tested were of low virulence (mean 18 ± 14%), which is in agreement with the predicted ratio (Tables 2 and 4). (The one ascospore progeny scored as highly virulent gave a virulence of only 53% of the strain R-6380 control.) The progeny of ascus 1844-9 segregated 4:4 (high to low, mean 97 ± 3%:66 ± 3%) into the parental phenotypes for rishitin metabolism, but in the three other asci analyzed in this cross, the progeny did not segregate into two discrete classes for rishitin metabolism (Table 4). The overall 21:11 (high to low) segregation ratio for rishitin metabolism observed in cross 1844 is higher than the predicted ratio of 16:16 (Table

TABLE 4. Segregation of rishitin metabolism and virulence phenotypes in progeny derived from R-6380 × 1104-R-6

	Segregation ratios			
Cross	High metabolism, high virulence[a] :	High metabolism, low virulence :	Low metabolism, low virulence	Number of tetrads
1421[b]	4	0	4	3
	4	2	2	2
1843	4	0	4	1
	4	2	2	3
1845	4	0	4	18
	0	0	8	1
1844	0	4	4	1
	0	6	2	3
1842	0	0	8	4

[a] Rishitin metabolism and virulence were assayed and normalized as in Table 1. Normalized metabolism greater than 80% was rated as high, and normalized virulence greater than 50% was rated as high for all crosses.

[b] Cross 1421 data are from a previous study (12).

2). These results support the hypothesis that strain 1421-8-1 contains the *Rim2* gene for rishitin metabolism but lacks the *Rim1* gene associated with high virulence. These data also suggest that the parents of this cross, 1421-8-1 and 1421-8-2, may differ in more than one gene controlling rishitin metabolism and that strain 1421-8-2 may contain a gene or genes for rishitin metabolism in addition to *Rim2*. Because the primary goal of this study was to analyze the association of high virulence with high metabolism, and because all progeny were of low virulence, cross 1844 was not studied further.

Cross 1843. For the final cross of this study, strain 1421-8-6 ($Rim1^+$ $Rim2^+$), which was highly tolerant of rishitin and virulent, was backcrossed to strain 1104-R-6 ($Rim1^-$ $Rim2^-$) (Tables 1 and 2). Over a period of 10 mo, cross 1843 was crossed 60 times with strain 1421-8-6 as the female parent, but was not fertile. Cross 1843 also was crossed 80 times with strain 1104-R-6 as the female parent, and three perithecia were obtained. Most of the asci obtained from these perithecia contained complete tetrads (Table 3). Four randomly selected eight-spored asci were analyzed for rishitin tolerance (Fig. 2D), rishitin metabolism (Fig. 3D), and virulence (Fig. 2D). One of these tetrads segregated as a parental ditype 4:4 (high to low) for rishitin metabolism (mean 100 ± 0%:65 ± 13%) and for virulence (mean 96 ± 19%:18 ± 8%). The remaining three tetrads were tetratypes with three classes of progeny: parental types of high metabolism (mean 100 ± 0%) and high virulence (mean 100 ± 16%), parental types of low metabolism (mean 40 ± 20%) and low virulence (mean 18 ± 8%), and nonparental types of high metabolism (mean 96 ± 4%) but low virulence (mean 13 ± 3%) (Table 4). These results are consistent with the proposed genotypes of $Rim1^+$ $Rim2^+$ for strain 1421-8-5 and $Rim1^-$ $Rim2^-$ for strain 1104-R-6 (Table 2).

TABLE 5. Segregation of rishitin metabolism and virulence in cross 1845

	Tetrad phenotypes			Tetrad phenotypes	
Ascus No.	Virlence[a] (high:low)	Rishitin metabolishm[b] (high:low)	Ascus No.	Virulence (high:low)	Rishitin metabolism (high:low)
2	2:3	2:3	16	4:4	4:4
5	3:3	3:3	17	4:4	4:4
6	2:2	2:2	18	4:4	4:4
7	3:3	3:3	20	4:4	4:4
8	3:2	3:2	26	4:3	4:3
11	0:7	0:7			
12	2:3	2:3	28	3:3	3:3
13	3:4	3:4	29	4:3	4:3
14	4:3	4:3	30	3:3	3:3
15	3:3	3:3	35	3:3	3:3
Total				58:64	58:64

[a] Virulence was assayed and normalized as described in Table 1. Normalized virulence greater than 70% was rated as high, and normalized virulence less than 30% was rated as low.

[b] Metabolism was assayed by gas liquid chromatography (asci 13, 14, 16, 17, and 18) or thin-layer chromatography (all other asci) as described in the Materials and Methods section. Rishitin recovery for most high metabolizers was zero and for low metabolizers was approximately 50% (by gas liquid chromatography).

DISCUSSION

The primary purpose of this study was to investigate the relationship between rishitin metabolism and virulence of *G. pulicaris* on potato tubers. Among the 26 field strains and the nearly 300 ascospore progeny of *G. pulicaris* analyzed in this and the previous study (12), the presence of a high level of rishitin metabolism was always necessary for a high level of dry rot on potato tubers. This is the strongest evidence to date that rishitin metabolism is important for the development of dry rot and, indirectly, that rishitin is important for resistance of potato tubers to this disease. But it also is clear from our studies, and those of others, that rishitin accumulation is not the only resistance mechanism in potato tubers. For example, there also is a strong correlation between high virulence of field strains of *G. pulicaris* and their ability to detoxify lubimin, isolubimin, and dihydrolubimin from potato (13,16). It should be noted that all parent strains in this study are high metabolizers of lubimin (12). 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 field strains can be sensitive to lubimin and vice versa. This conclusion is supported further by results from the present study. Three ascospore progeny of tetrad 1845-11 that had lost all detectable rishitin-metabolizing ability were all highly tolerant of and able to completely metabolize lubimin in agar cultures (data not shown) as would be expected from their parental phenotypes.

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. Ampomah and Friend (1) and Bostock et al (5) have found that, under certain conditions, potato tubers can show the appropriate compatible and incompatible disease reactions to races of *Phytophthora infestans* without any differences in sesquiterpene levels. Biochemical factors, other than sesquiterpenes, that have been implicated in potato tuber resistance to fungi include a variety of phenolics and steroid glycoalkaloids, as well as suberin and lignin (1,20).

The second goal of this study was to determine the number of genes controlling a high level of rishitin metabolism in field strain R-6380. Segregation ratios for rishitin tolerance, rishitin metabolism, and virulence among progeny from crosses 1842, 1843, and 1845 were consistent with our original hypotheses that there are at least two nonallelic genes for high rishitin metabolism in strain R-6380 and that only one of these genes, *Rim1*, is associated with high virulence. Crosses between putative $Rim1^+$ $Rim2^-$ and $Rim1^-$ $Rim2^+$ progeny are necessary to confirm that these genes are indeed at different loci. Several such crosses between the appropriate progeny of crosses 1844 and 1845 have been attempted but have to date been infertile. Preliminary results from crosses between strain 1104-R-6 and four additional potato-pathogenic field strains of *G. pulicaris* support the association of virulence with a high level of rishitin metabolism, as well as the presence of multiple loci for rishitin metabolism (Desjardins and Gardner, *unpublished*).

A final goal of this study was to investigate the low virulence of some *G. pulicaris* strains that are highly tolerant of rishitin. It is obvious that pathogenicity is a complex process and that phytoalexin-tolerant strains may lack other essential pathogenicity

TABLE 6. Segregation of various traits in tetrad 1845-11

Ascospore no.	Mating type	Colony color	Major trichothecene toxin[a]	Virulence[b] (%)	Rishitin tolerance[b] (%)	Rishitin metabolism[b] (%)
1 + 5	1	Orange	AcNeo	11 ± 4	30 ± 3	0
2 + 3	1	Orange	DAS	3 ± 1	27 ± 5	0
4	Not fertile	White	AcNeo	5	30	6
7 + 8	Not fertile	White	DAS	12 ± 1	43 ± 1	13 ± 3

[a] 4,15-Diacetoxyscirpenol and 8-acetylneosolaniol differ in hydroxylation of C-8, which previously has been shown to be controlled by a single gene in this cross (4,12).

[b] Assays were conducted as described in Table 1.

or virulence factors. In the present study, it could be hypothesized that the parents R-6380 and 1104-R-6 differ in some additional virulence gene, *Vir*, that is unlinked to any genes for rishitin metabolism. If so, then some tetrads with segregation ratios of 2:6 and 0:8 (high to low) for virulence should have been observed in crosses 1421, 1843, and 1845. With the unique exception of tetrad 1845-11, which lost both virulence and rishitin metabolism, only 4:4 segregation ratios for virulence were observed. The total number (36) of tetrads analyzed for virulence in these three crosses is relatively small, but these preliminary results are not consistent with the hypothesis of a third *Vir* gene independent of the *Rim1* and *Rim2* genes already proposed.

Another possible explanation of the low virulence of some *G. pulicaris* strains that can metabolize rishitin is that high virulence requires a relatively high level of metabolic activity. VanEtten and co-workers (18,19,23,27,28) have conducted an extensive analysis of the demethylation and consequent detoxification of the pea phytoalexin pisatin by *Nectria haematococca* (*F. solani*). They have crossed many different pisatin-demethylating field strains and analyzed ascospore progeny for their ability to demethylate pisatin in liquid culture. These studies have resulted in the identification of a family of genes that differ in demethylase phenotype, and in the finding that only those genes that confer phenotypes with shorter lag times and higher rates of demethylation are associated with high levels of virulence.

Our attempts at a similar biochemical approach to characterization of rishitin metabolism genes in *G. pulicaris* have met with limited success. In a previous study of progeny from tetrad 1421-8 (12), the highest rates of rishitin metabolism in liquid culture were 3.4 and 2.8 μg/ml/hr for strain 1421-8-6 and 1421-8-5, respectively. The rates for strains 1421-8-1 and 1421-8-2 were 1.2 and 0.6 μg/ml/hr, respectively. These results are consistent with the hypothesis that the higher rate of rishitin metabolism associated with the *Rim1* gene is sufficient for virulence but that the lower rate associated with the *Rim2* gene is not. Our continuing efforts to more thoroughly compare these two phenotypes have been unsuccessful because of our inability to consistently obtain rishitin metabolism in liquid culture. This lack of metabolism is puzzling because these strains previously were able to metabolize rishitin under the same liquid culture conditions and still can consistently metabolize rishitin in agar cultures.

In the present study, rishitin tolerance and metabolism were measured as the inhibition of radial growth and the percentage of rishitin remaining in 1-ml agar cultures at a single end point (7 days). Using this assay, the tolerance phenotypes of the *Rim1* gene from strain 1421-8-5 and of the *Rim2* gene from strain 1421-8-1 could be distinguished, but their metabolism phenotypes could not. That is, the normalized rishitin tolerance averaged 99 ± 8% for the 19 virulent progeny of cross 1845 and 61 ± 16% for the 16 most tolerant progeny of cross 1844, whereas normalized rishitin metabolism was 95 ± 4% and 96 ± 5%, respectively, for these progeny. VanEtten and co-workers (18, 19,23,27) have shown that measurements of the rate of phytoalexin metabolism are superior to single end-point assays in distinguishing different metabolism phenotypes. To further compare rishitin metabolism phenotypes, duplicate rishitin-amended 0.25-ml cultures of representative, high rishitin-metabolizing progeny from crosses 1845 and 1844, and a representative progeny from cross 1842, were sampled at 24-hr intervals over 5 days and analyzed for remaining rishitin by GLC. The times required for 50% metabolism of rishitin were 1–2 days for strain 1845-17-3, 2–3 days for strain 1844-5-8, and more than 4 days for strain 1842-10-2. These preliminary data are consistent with the hypothesis that the virulence associated with *Rim1* is due to a higher rate of rishitin metabolism, but a reliable liquid culture assay system is needed to confirm these results.

The reason for the loss of rishitin metabolism and virulence among the progeny of tetrad 1845-11 is still unknown. This unusual segregation ratio may have arisen by an error in ascospore isolation, although the 4:4 segregation of other markers (color and trichothecene toxins) in this tetrad argues against this simple explanation. It also is possible that tetrad 1845-11 is a nonparental ditype and that rishitin metabolism requires two genes. If so, these two genes must be linked because only one such tetrad was observed among 36 relevant asci analyzed (crosses 1421, 1843, and 1845). This hypothesis cannot be tested by crosses among progeny of tetrad 1845-11 because the four sexually fertile progeny are all of the same mating type.

The unique loss of rishitin metabolism and virulence in tetrad 1845-11 could result from loss or inactivation of the *Rim1* gene during meiosis. Loss of the pisatin demethylase phenotype and virulence during meiosis in *N. haematococca* has been shown to correlate with physical loss of a pisatin demethylase structural gene (24,29) as detected by hybridization of a cloned gene to electrophoretically separated chromosomes.

The rishitin metabolites produced by tolerant strains of *G. pulicaris* have not been identified yet. Preliminary analyses by TLC and gas chromatography-mass spectroscopy indicate that rishitin metabolism comprises a complex pattern of sequential and possibly competitive reactions. Rishitin metabolites currently are being purified and characterized in this laboratory.

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