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Detoxification of sesquiterpene phytoalexins by *Gibberella pulicaris* (*Fusarium sambucinum*) and its importance for virulence on potato tubers

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SUMMARY

Gibberella pulicaris (*Fusarium sambucinum*) is a major cause of dry-rot of stored potatoes (*Solanum tuberosum*) worldwide. The ability of field strains of *G. pulicaris* to cause dry-rot is correlated with their ability to detoxify sesquiterpene phytoalexins produced by potato. All highly virulent field strains can detoxify the sesquiterpenes rishitin and lubimin. Meiotic recombinational analysis indicates that rishitin detoxification can be controlled at two or more loci. High virulence has been associated with one of these loci, designated *Rim1*. Detoxification of rishitin and lubimin comprises a complex pattern of reactions involving epoxidation, dehydrogenation, and cyclization. To date, seven lubimin metabolites and one rishitin metabolite have been characterized. Genes for rishitin and lubimin detoxification are being cloned from *G. pulicaris* in order to more rigorously analyze the role and regulation of sesquiterpene metabolism in potato dry-rot. Our results indirectly support a role for sesquiterpene phytoalexins in resistance of potato tubers to dry-rot and may enhance research on alternative control strategies for this economically important potato disease.

INTRODUCTION

Dry-rot caused by *Gibberella pulicaris* (anamorph: *Fusarium sambucinum*) is a serious storage disease of potato (*Solanum tuberosum*) worldwide [7,39]. Although dry-rot seldom reaches epidemic proportions [4], average yearly losses of 6%, with occasional losses of 25% or more, have been reported during long-term storage [12]. Yield losses due to dry-rot are compounded by the fact that *G. pulicaris* can contaminate tubers with trichothecene toxins which are injurious to human and animal health [19,29]. All commonly grown potato cultivars, e.g. Russet Burbank, are susceptible to *G. pulicaris* [32], and susceptibility increases progressively during storage [7,32]. During the past 20 years potato growers have relied heavily on thiabendazole, a benzimidazole fungicide, to control dry-rot in seed and in stored potatoes [11]. Recently, general public concern about pesticide residues in food products and in the environment has stimulated research on alternative control strategies for fungal diseases of potatoes and other crop plants. The search for new control methods for potato dry-rot has been accelerated by the emergence of thiabendazole resistant strains of *G. pulicaris* in populations from severely dry-rotted tubers in North America and in Europe ([42]; Desjardins and Secor, unpublished).

Potato breeding programs have given increased attention to the development of cultivars with enhanced disease and insect resistance by incorporating germplasm from wild potato species [14,25]. This empirical approach, however, is not without its pitfalls. Wild plants often owe their disease resistance to higher levels of toxic chemicals than their cultivated relatives [1]. Wild potato species, for example, contain high levels of the bitter glycoalkaloids solanine and chaconine, which are toxic to humans. When a wild potato parent was used as a source of disease resistance in the potato cultivar Lenape in 1970, this new cultivar had to be withdrawn from the market because of its high glycoalkaloid content [1,53]. Potato breeders had not anticipated that selection for increased disease resistance in Lenape would also select for natural toxins, and no biochemical screening had been done before cultivar release.

Cultivated potatoes contain low levels of a number of chemically diverse toxins and potential toxins. These include the glycoalkaloids mentioned above, various phe-

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nols such as chlorogenic acid, caffeic acid and scopoletin, and a large family of sesquiterpenes [30]. Human toxicity of few of these potato chemicals is known, with the exceptions of chlorogenic acid (a mutagen), caffeic acid (a carcinogen), and solanine and chaconine (teratogens and cholinesterase inhibitors) [2]. In light of these findings, it seems important to focus research on toxicology of these natural chemicals and on determining which of them are most important for resistance of potato tubers to major diseases. Although potato phenols and glycoalkaloids have demonstrated antifungal activity in vitro, evidence for their role in disease resistance in potato tubers is largely circumstantial. Much of the research in biochemistry of resistance has focused on the sesquiterpenes, especially rishitin and lubimin (Fig. 1), because they are fungitoxic in vitro and they can accumulate to high levels in potato tubers, but only near infection sites [30]. In addition, no harmful effects have been observed following exposure of mice [10] or chicken embryos [51] to rishitin or related compounds, which suggests that these sesquiterpenes may present a low risk of toxicity to humans.

If rishitin and lubimin are important disease resistance factors (phytoalexins) in potato tubers, then successful pathogens may have developed mechanisms for tolerating them. It is well established that some plant pathogenic fungi are tolerant of phytoalexins, and, furthermore, that they are often able to detoxify the phytoalexins of which they are tolerant [45]. VanEtten et al. [44] have demonstrated that a gene conferring detoxification of the phytoalexin pisatin is essential for virulence of *Nectria haematococca* on pea plants. In this article we will summarize some of the biochemical and genetical evidence that *G. pulicaris* can detoxify the potato phytoalexins rishitin and lubimin, and that detoxification is necessary for a high level of virulence on potato tubers.

VIRULENCE OF GIBBERELLA PULICARIS ON POTATO TUBERS

Gibberella pulicaris is a cosmopolitan soil saprophyte and plant pathogen. Economically, *G. pulicaris* is most important as a storage rot of potato tubers and sugar beets, and as a root and stem rot of cereals, hops and forest trees [5]. Our collection contains more than 160 field strains of *G. pulicaris* isolated from every continent except Antarctica. Approximately one-third of these strains were isolated from soil, one-third from dry-rot potato tubers, and one-third from diseased tissues of more than 20 other plant species, ranging from asparagus in Australia, to cactus in Argentina, cabbage in the F.R.G. and a wingnut tree in Iran. When strains of *G. pulicaris* from a variety of habitats were surveyed for their ability to dry-rot potato tuber slices, a wide range of natural

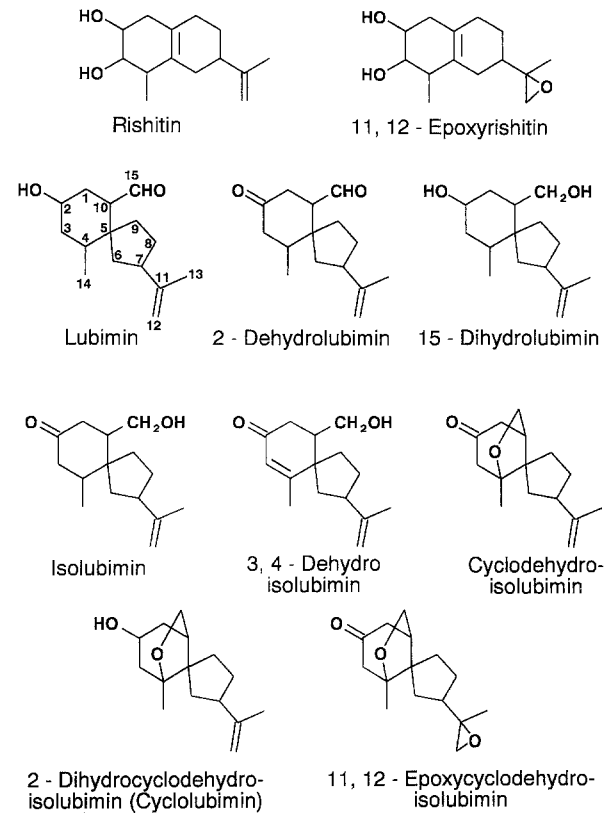

Fig. 1. Structures of potato phytoalexins, rishitin and lubimin, and their fungal metabolites.

variation for this trait was found (Fig. 2). When these strains were surveyed for their tolerance of rishitin and lubimin in vitro, natural variation for these traits was also observed. Furthermore, only those field strains that were highly tolerant of both potato sesquiterpenes in vitro were highly virulent on potato tubers; all strains that were sensitive to either rishitin or lubimin were of low virulence (Fig. 2). In contrast, all field strains that we tested were similar in sensitivity in vitro to the potato glycoalkaloid solanidine, and to the potato phenols chlorogenic acid, caffeic acid, ferulic acid, vanillic acid, quinic acid and scopoletin (Desjardins, unpublished).

Whether the differential tolerance of *G. pulicaris* to rishitin and lubimin was due to their differential metabolism was investigated by gas-chromatographic analysis of the amount of the two phytoalexins recovered from tolerance assays. As shown in Fig. 3, high tolerance was strongly associated with low recovery of both rishitin and lubimin. These data also indicated that different mechanisms were responsible for metabolism of these two phytoalexins because some rishitin-tolerant strains were sensitive to lubimin and vice versa. Furthermore, it was also clear from these results that a high level of metabo-

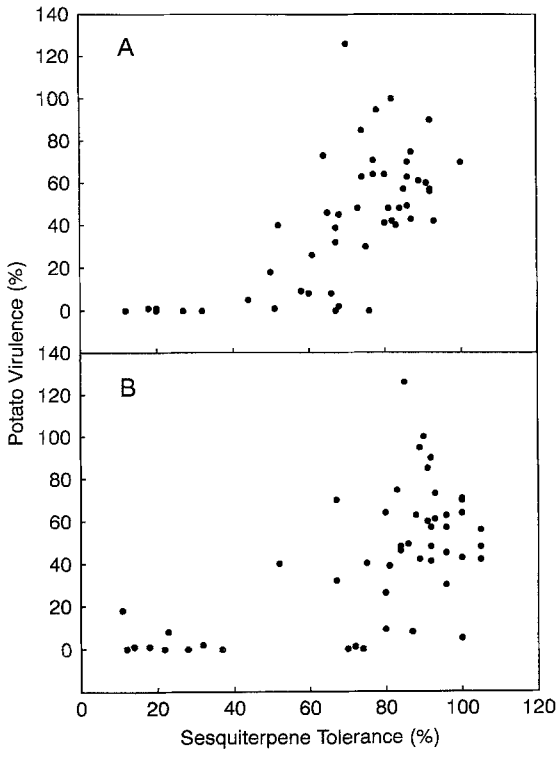

Fig. 2. The relationship between sesquiterpene tolerance and virulence on potato tubers of field strains of *Gibberella pulicaris*. Data for 21 strains are from previous studies [16,18] and for 29 strains are unpublished. Each point represents one strain. Virulence was assayed gravimetrically as the percentage of potato tuber tissue dry-rotted for three or more replicate slices, and was normalized to strain R-6380. Percent tolerance was calculated by dividing radial growth rate on phytoalexin amended agar medium by radial growth rate of controls. A, rishitin tolerance; B, lubimin tolerance: both assayed in agar cultures at 200 μg/ml.

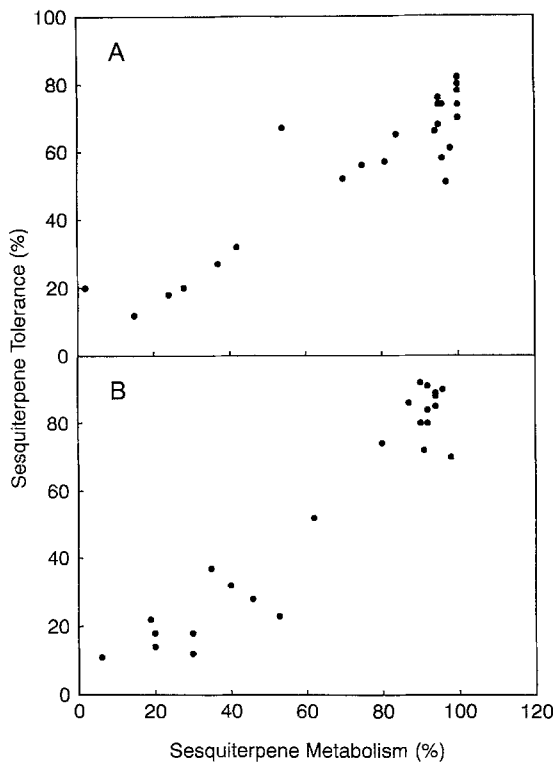

Fig. 3. The relationship between sesquiterpene tolerance and metabolism of field strains of *Gibberella pulicaris*. Each point represents one strain. Data are from [16,18]. Tolerance was assayed as in Fig. 2. Metabolism on agar was assayed by GLC of extracts of tolerance assay plates after 7 days of incubation. A, rishitin; B, lubimin: both assayed in agar cultures at 200 μg/ml.

lism of rishitin and lubimin is not in itself sufficient for virulence, because some strains that are highly tolerant of both phytoalexins are not virulent [16,18]. This is not surprising since pathogenicity is undoubtedly a complex process depending on many genes and resistance of potato tubers to fungal pathogens can apparently involve several defense responses. Our survey of field strains of *G. pulicaris* did demonstrate a strong correlation between the ability to metabolize both rishitin and lubimin in vitro and virulence on potato tubers. These kinds of quantitative correlations, however, do not necessarily prove or disprove a role for rishitin or lubimin metabolism in virulence because of the inherent difficulties in comparing in vitro assay conditions to those in infected plant tissues, and because of the non-isogenic backgrounds of field strains.

GENETIC ANALYSIS OF SESQUITERPENE METABOLISM AND VIRULENCE

Gibberella pulicaris is the teleomorph or sexual stage of *F. sambucinum*. Since *G. pulicaris* is a heterothallic Ascomycete, it is possible to perform crosses between strains that differ in various traits, as long as the strains are sexually fertile and of the appropriate mating type and sex. A special advantage of Ascomycetes is the availability of the haploid products of meiosis in an ascus as four sets of ascospore twins in their original tetrad. Meiosis following a cross between two parental strains results in a 4 : 4 segregation of tetrad progeny with respect to each and every parental difference. If a cross involves two strains that differ with respect to two genes, then there are three possible types of tetrads: (1) a parental ditype in which only parental types are recovered; (2) a non-parental ditype in which all progeny are recombinant; and (3) a tetratype which contains four parental and four non-parental progeny. Although tetrad analysis yields the most infor-

mation, random ascospores can be used for some purposes. However, random selection can be misleading when there are a large number of non-random abortions as has been observed in certain crosses between field strains of *G. pulicaris* [16].

Genetic analysis of rishitin and lubimin metabolism in *G. pulicaris* has been constrained by the poor sexual fertility of phytoalexin-sensitive field strains. Of more than 160 field strains tested to date, only one strain was found – only very recently – to be both fertile and sensitive to lubimin (Desjardins and Gardner, unpublished). Genetic analysis of lubimin metabolism using this field strain is now underway. None of the field strains were both fertile and completely unable to metabolize rishitin. One male-fertile field strain from carnation in Chile, 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. Strain R-7843 was crossed to strain R-6380, a highly virulent, rishitin-tolerant strain from potato in the F.R.G., and the traits of rishitin metabolism, rishitin tolerance and virulence were followed for three generations. During the course of this study, more than 300 ascospore progeny were analyzed. High tolerance and high metabolism were defined as greater than 50–60% and 80%, respectively, of controls. All highly virulent progeny were able to metabolize rishitin and were tolerant of rishitin, whereas all rishitin-sensitive progeny were low in virulence [16,17]. This is the strongest evidence to date that a high level of rishitin metabolism is important for the development of dry-rot and, indirectly, that rishitin is important for the resistance of potato tubers to this disease.

Segregation ratios in some tetrads from a cross to field strain R-6380 suggested that two or more loci (designated *Rim1* and *Rim2*) for a high level of rishitin metabolism were present in this strain, and that only one of these loci, *Rim1*, was associated with high virulence. To test this hypothesis, we selected a putative tetratype ascus from this cross (cross 1421, ascus 8, Table 1) and crossed each of the four meiotic progeny to a strain of low metabolism and low virulence. The inheritance of rishitin metabolism, rishitin tolerance and virulence was followed in several tetrads from each of the four test crosses. All results were consistent with the proposed genotype of $Rim1^{+}Rim2^{+}$ for strain R-6380 and, furthermore, suggested 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 (Table 1 and Fig. 4) [16,17].

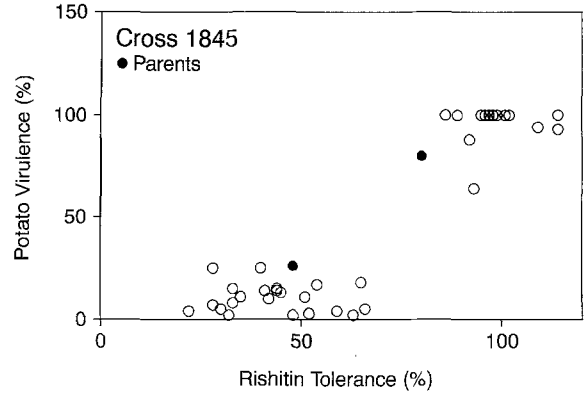

Fig. 4. The relationship between rishitin tolerance and virulence on potato tubers of tetrad progeny from cross 1845 between strain 1421-8-5 ($Rim1^{+}Rim2^{-}$ and strain 1421-8-2 ($Rim1^{-}Rim2^{-}$). Each open circle represents one progeny strain; each solid circle represents a parental strain. Data are from [17]. Normalized virulence and tolerance were assayed as described in Table 1 and Fig. 2.

TABLE 1

Segregation of rishitin metabolism, rishitin tolerance, and virulence in a tetrad of cross 1421 (R-6380 X 1104-R-6) of *Gibberella pulicaris*

Ascus no.	Ascospore no.	Normalized virulence (%)	Rishitin tolerance (%)	Rishitin metabolism		Proposed genotype
				On agar (%)	In liquid (μg/ml/h)	
8	5	104	70	100	2.8	$Rim1^{+}Rim2^{-}$
	6 + 7	101 ± 17	64 ± 1	95 ± 1	3.4 ± 0.3	$Rim1^{+}Rim2^{+}$
	1 + 3	15 ± 2	58 ± 8	80 ± 1	1.2 ± 0.7	$Rim1^{-}Rim2^{+}$
	2 + 4	17 ± 3	22 ± 6	0 ± 2	0.6 ± 0.2	$Rim1^{-}Rim2^{-}$
Parents						
	R-6380	100	96	100	3.4	$Rim1^{+}Rim2^{+}$
	1104-R-6	19 ± 6	24	9	0.2	$Rim1^{-}Rim2^{-}$

Data from [16,18]. Virulence and tolerance were assayed as in Fig. 2. Metabolism on agar was assayed as in Fig. 3. Metabolism in liquid was assayed by GLC of extracts of 1 ml liquid culture medium after the addition of 150 μg rishitin. Metabolism is expressed as the average rate over a 40-h interval. Numbers are mean ± range for ascospore twins, and for duplicate assays of strain 1104-R-6.

Genetic analyses of rishitin metabolism and virulence in four additional field strains of *G. pulicaris* are in progress. Preliminary results from three tetrads each from crosses with potato pathogenic strains from the United States, Canada and Iran indicated that virulence segregated as a single gene in each cross. One tetrad from each of these four crosses was analyzed in more detail, and virulence was found to segregate with high levels of rishitin metabolism and tolerance in each cross (Table 2). Furthermore, recombinant progeny that metabolized rishitin rapidly, but were not virulent, were recovered, particularly in the cross with strain R-5390, which suggests the presence of multiple genes for rishitin metabolism in these field strains. Allelic relationships between genes for a high level of rishitin metabolism from these four field strains and from strain R-6380 remain to be determined.

Classical genetic analysis has provided convincing evidence that detoxification of sesquiterpene phytoalexins is important for virulence of *G. pulicaris* on potato tubers. For a more rigorous test of the role and regulation of the phytoalexin metabolism genes in potato dry-rot, a molecular genetic approach has been initiated to clone the genes for rishitin and lubimin metabolism. The strategy will be to transform strains that cannot metabolize the phytoalexins with a gene library from a strain that carries the metabolism genes. Transformants resistant to the selectable marker hygromycin B will be screened for the expression of genes for rishitin and lubimin metabolism. From phytoalexin metabolizing transformants, cosmids containing metabolism genes can be re-isolated for subcloning and further analysis.

Isolated genes for phytoalexin metabolism can then be transferred into an avirulent, phytoalexin-sensitive strain of *G. pulicaris*, to conclusively test whether metabolism of rishitin and lubimin is a necessary prerequisite for obtaining potato dry-rot. An important requirement for this molecular approach is an effective transformation system. A preliminary report of a low efficiency DNA-mediated transformation system for *G. pulicaris* has been published [37]. To improve the efficiency of this system, homologous promotors were isolated from a promotor probe library of *G. pulicaris*. One such promotor fused to the hygromycin B gene was used to construct cosmid pGPC1 (Fig. 5). Fragments of genomic DNA (40–45 kb in size) from strain R-6380 were ligated into this vector. The resulting library of approximately 1.5×10^5 recombinant *E. coli* clones is currently being screened for ability to transform *G. pulicaris* to hygromycin resistance and phytoalexin tolerance (Weltring, unpublished).

TABLE 2

Segregation of rishitin metabolism, rishitin tolerance, and virulence in tetrads from crosses of four field strains of *Gibberella pulicaris*

Cross no.	Ascospore nos.	Virulence (%)	Tolerance (%)	Metabolism (%)
2052				
	DAOM 196035, (female parent)	67	78	100
	1,2,3 + 7	9 ± 5	45 ± 7	52 ± 16
	4,5,6 + 8	73 ± 7	77 ± 6	100 ± 0
2050				
	DAOM 192963, (female parent)	81	67	100
	1,2,7	4 ± 1	40 ± 5	56 ± 14
	3,4,5 + 6	72 ± 3	80 ± 6	100
2049				
	NRRL 13707, (female parent)	85	56	100
	1,3,5 + 7	84 ± 3	64 ± 2	100
	2,4,6 + 8	14 ± 4	40 ± 4	77 ± 9
2048				
	R-5390, (female parent)	62	88	100
	3,5,6 + 7	67 ± 8	80 ± 2	100
	1,2,4 + 8	18 ± 6	64 ± 10	97 ± 3
	1104-R-6, (male parent all crosses)	5	27	27

Unpublished data. Assays as in Fig. 2 and 3; rishitin tested at 200 μg/ml in agar cultures.

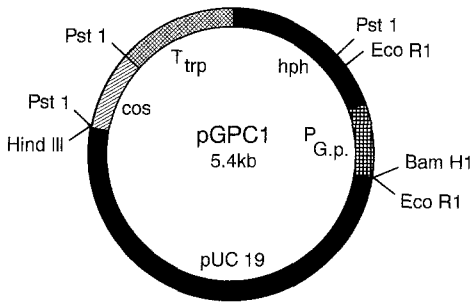

Fig. 5. Structure of plasmid pGPC1.

IDENTIFICATION OF SESQUITERPENE METABOLITES

Fusarium species detoxify phytoalexins by a wide variety of enzymic mechanisms including oxygenation, reduction, hydration and dehydrogenation [45]. *Nectria haematococca*, for example, detoxifies the isoflavonoid pisatin by a demethylation that is catalyzed by a cytochrome P-450 monooxygenase [52]. *Nectria haematococca* also detoxifies the phytoalexins medicarpin and maackiain by one of three alternative oxygenations of the isoflavonoid ring system, two of which are known to be catalyzed by monooxygenases [44]. Preliminary mass spectral data suggest that *G. pulicaris* can metabolize rishitin in agar plates and in liquid culture by epoxidation of the isopropenyl side chain (Fig. 1). After rishitin is fed to fungal cultures, epoxyrishitin is produced only transiently and at very low levels. Consequently, this metabolite has not yet been isolated in amounts sufficient for complete structural characterization, and furthermore, it is not known if this epoxidation pathway represents the principal strategy for detoxification by the fungus or is an unimportant side reaction. Hydroxylation of the isopropenyl side-chain of rishitin can be catalyzed by potato tuber tissue itself [26] and, in that case, the oxygen atom is derived from molecular oxygen, which suggests involvement of a cytochrome P-450 monooxygenase [9]. The origin of the oxygen atom of the putative fungal metabolite of rishitin has not been determined.

Gibberella pulicaris can metabolize lubimin to a variety of compounds (Fig. 1). Products forming between 1–6 h after addition of lubimin to liquid cultures of a lubimin-tolerant strain R-7715 were two isomers of isolubimin, isomeric 15-dihydrolubimin, two isomers of 2-dehydrolubimin, and the tricyclic compound, cyclodehydro-isolubimin. After 1–2 days, other tricyclic compounds, cyclolubimin (2-dihydrocyclodehydro-isolubimin) and 11,12-epoxycyclodehydro-isolubimin became an increasing proportion of the mixture, apparently at the expense of cyclodehydro-isolubimin. The metabolites 15-dihydrolubimin and isolubimin were common to both lubimin-tolerant and lubimin-sensitive strains, and appeared to still be quite fungitoxic (Table 3). The tricyclic metabolites, in contrast, were not toxic to a lubimin-sensitive strain (Table 3). These results indicate that lubimin detoxification in at least one strain of *G. pulicaris* involves cyclization to cyclodehydro-isolubimin. Relatively high levels of tricyclic lubimin metabolites were found in potato tubers infected with strain R-7715, which provides more direct evidence that *G. pulicaris* detoxifies lubimin during plant pathogenesis [18]. 15-Dihydrolubimin was previously identified in uninfected potato tubers [40] and also as a fungal metabolite of lubimin [48]. Isolubimin and cyclodehydro-isolubimin have also been found as minor components in uninfected or in *Phytophthora*-infected potato tubers [15,40]. Both isolubimin and 15-dihydrolubimin are biosynthetic precursors of lubimin and of rishitin as well [40].

Inspection of the chemical structures suggested several possible schemes for metabolism of lubimin to cyclodehydro-isolubimin by *G. pulicaris*. One pathway involves conversion of lubimin to either 2-dehydrolubimin or to 15-dihydrolubimin followed by conversion to isolubimin. It was not evident from their chemical structures whether 2-dehydrolubimin or 15-dihydrolubimin, or both, were precursors of isolubimin. To select among these alternative possibilities, 2-dehydrolubimin and 15-dihydrolubimin were fed to lubimin-tolerant strains, including R-7715. Within 3 h, added 2-dehydrolubimin was metabolized to a mixture of metabolites that in most respects was qualitatively and quantitatively similar to that derived from lubimin. Since 15-dihydrolubimin appeared so rapidly after feeding 2-dehydrolubimin, the relative importance of the two compounds as isolubimin precursors was not clarified. Moreover, when 15-dihydrolubimin was added to a culture of strain R-7715, 80% was recovered, even after three days of incubation. The lack of metabolism of exogenously supplied 15-dihydrolubimin is puzzling because the test strain apparently metabolized this compound endogenously. 15-Dihydrolubimin was detected within 1 h of the addition of lubimin to strain R-7715, reached a maximum at 2–4 h, and had all disappeared by 8 h. One possibility is that the enzymes responsible for 15-dihydrolubimin metabolism are not constitutively produced and are not induced by 15-dihydrolubimin; for example, there is some evidence for inducibility of fungal metabolism of the related phytoalexin capsidiol [47]. Other possibilities include impermeability of 15-dihydrolubimin to the fungal membrane or that the $NaBH_4$-reduced lubimin used as the 15-dihydrolubimin source was the wrong stereoisomer for metabolism by the fungus.

TABLE 3

Toxicity of lubimin and selected metabolites to a lubimin-sensitive strain of *Gibberella pulicaris*

Compound tested	Compounds recovered								Total recovered (μg)	Radial growth (% control)
	Lubimin	15-Dihydro-lubimin	Isolubimin	2-Dehydro-lubimin	Cyclodehydro-isolubimin	Cyclo-lubimin	11,12-Expoy-cyclodehydro-isolubimin	11,12-Expoxy-cyclolubimin		
Lubimin	69 ± 9	82 ± 10	40 ± 2						191 ± 22	28 ± 0
15-Dihydrolubimin		98 ± 16	80 ± 13						178 ± 30	59 ± 0
2-Dehydrolubimin		38 ± 2	128 ± 5	12 ± 3					179 ± 5	64 ± 4
Cyclodehydro-isolubimin					51 ± 0	68 ± 3			120 ± 3	82 ± 1
Cyclolubimin						128 ± 10			128 ± 10	90 ± 1
11,12-Epoxy-cyclodehydro-isolubimin							146 ± 12	40 ± 3	187 ± 16	96 ± 0

Data from [18]. Assays as in Figs. 2 and 3, lubimin and metabolites were tested at 200 μg/ml in agar culture. Mean ± range for duplicate assays. Blanks indicate none detected.

ISOTOPE LABELLING EXPERIMENTS ON THE METABOLISM OF LUBIMIN

Previously, it was proposed that a possible route to the nontoxic tricyclic metabolites proceeded through isolubimin and subsequent desaturation of the latter between carbons-3 and -4 to give 3,4-dehydro-isolubimin [20]. This conclusion was based on the observed incorporation of one deuterium (65% incorporation) into carbon-3 of the tricyclic metabolite, cyclodehydro-isolubimin, after lubimin was incubated with strain R-7715 in the presence of ^2H_2O. The incorporation of deuterium at carbon-3 was detected both by ^{1}H-NMR and ^{13}C-NMR [20].

Subsequent to the deuterium labelling experiment, lubimin was incubated with strain R-7715 in an $H_2{}^{18}O$-amended medium using $H_2{}^{18}O$ with 97–98% isotopic purity (Desjardins, Gardner and Plattner, unpublished). For comparison, the lubimin-sensitive strain, R-110 was also incubated with lubimin in $H_2{}^{18}O$ media. It was found that lubimin isolated after incubation with strain R-110 had incorporated one atom of oxygen ($^{16}O_2 : {}^{18}O{}^{16}O$; 1/0.5). This was in accord with previous work which showed spontaneous exchange of the aldehydic oxygen of lubimin with H_2O [9]. On the other hand, the corresponding experiment using the tolerant strain R-7715 resulted in the formation of cyclodehydro-isolubimin with incorporation of two atoms of labelled oxygen ($^{16}O_2 : {}^{16}O{}^{18}O : {}^{18}O_2$; 1/3.1/1.8). It follows then that the ^{18}O-label of the newly formed furan-ring could have originated from exchange of $H_2{}^{18}O$ with the aldehyde group of the precursor, lubimin, or by another unknown mechanism. In regard to incorporation of the second oxygen-label at the ketone of carbon-2, this result was unfortunately ambiguous. Although many investigators have successfully used ^{18}O-label to trace biosynthesis of similar ketones, such as the cyclopentanone, jasmonic acid [46], these experiments were generally enzymic incubations accomplished in less than a few hours. In the present labelling study, 24 h were needed to generate the metabolite cyclodehydro-isolubimin. This period of time possibly would permit significant spontaneous exchange with $H_2{}^{18}O$, especially under acidic or basic conditions [38]. However, the result of this labelling study does point to the possibility of alternative pathways to cyclodehydro-isolubimin other than via isolubimin as previously suggested [20]. Further research in this area is required.

THE ROLE OF SESQUITERPENES IN DISEASE RESISTANCE OF POTATO TUBERS

The idea that plants produce protective chemicals after exposure to microorganisms was formalized by Muller and Borger in 1940 [35] and was based on their experiments with potato tubers infected with *P. infestans*. Thirty years later, the most active phytoalexins in potato were found to be a series of closely-related sesquiterpenes, the most prevalent of which are rishitin and lubimin [30]. Rishitin was originally isolated and characterized in 1968 from diseased tubers of the Rishiri variety of potatoes in Japan [29,43]. Lubimin was similarly isolated from Lyubimets potatoes in the Soviet Union in 1971 [34], and a correct, revised structure was later published [41]. Although the chemical structures of rishitin, lubimin and many other sesquiterpenes have been known for more than 20 years, their role in causing disease resistance in potato tubers is still controversial.

Rishitin and lubimin are certainly toxic in vitro to potato pathogens, including soft-rot *Erwinia* species [33], dry-rot *Fusarium* species [16,18,48], and several races of *Phytophthora infestans* [27]. They are, however, also toxic to a wide range of nonpathogenic fungi and, amongst 15 fungal species tested in one study, there was no correlation between sesquiterpene sensitivity and potato pathogenicity [49]. The sesquiterpenes appear to be fungitoxic only at fairly high concentrations (10^{-3}–10^{-5} M) [48] and to be fungistatic rather than fungicidal [28]. Moreover, rishitin has been shown to be phytotoxic [21] and to retard plant growth [27], although at concentrations considerably higher than those at which it is fungitoxic. These observations suggest that the sesquiterpenes are, as many other phytoalexins, not very specific or very effective as fungal growth inhibitors in vitro. Whether sesquiterpenes inhibit fungal growth in plant tissues has been investigated in several laboratories in Europe, Japan and North America. In potato tubers infected with a wide variety of fungi, sesquiterpenes were found to accumulate to concentrations of more than 300 μg per g fresh weight [13,22,36], which levels are sufficient to prevent fungal growth in vitro. Much of the work in this area (reviewed by Kuc [30]) has focussed on the time course of accumulation of sesquiterpenes in potato tubers inoculated with virulent and avirulent races of *P. infestans*, which causes the late-blight disease. Rishitin and related compounds, in general, accumulated faster and to higher levels in potato tubers inoculated with avirulent races of *P. infestans*, i.e., inhibition of fungal growth correlated with accumulation of sesquiterpenes in vivo. Since *P. infestans* has not been shown to detoxify sesquiterpenes ([30]; Desjardins and Gardner, unpublished), Kuc and others [30] have proposed that virulent races of late-blight are able to suppress sesquiterpene accumulation. More recently, however, Bostock et al. [6] have found that, under certain conditions, potato tubers can show the appropriate reactions to races of *P. infestans* without any differences in sesquiterpene levels. These results indicate that, as might be expected in a complex biological system, sesquiterpenes

are not the only disease-resistance mechanism in potato tubers. Other biochemical factors that have been implicated in resistance include the phenols and glycoalkaloids mentioned above, and the polymers suberin and lignin that are involved in wound healing [3].

Quantitative correlations of disease resistance with sesquiterpene accumulation do not in themselves prove or disprove that sesquiterpenes cause resistance in potato tubers. In theory, such quantitative correlations between traits can be tested by selective manipulation of one trait and observation of its effects on the other. In practice, genetic manipulation and screening of polyploid potatoes presents many technical difficulties. In contrast, certain haploid pathogens such as *G. pulicaris* are highly amenable to both classical and molecular genetic analysis. As outlined in the previous sections, the results of classical genetic studies strongly support the hypothesis that rishitin detoxification is necessary for high virulence of *G. pulicaris* on potato tubers. If this is so, then we can infer that rishitin is a resistance mechanism of potato tubers, at least in dry-rot.

If sesquiterpene phytoalexins are important for potato resistance to *G. pulicaris*, then serious thought should be given to exploitation of sesquiterpenes for dry-rot control. Various strategies for enhancing disease-resistance to phytoalexin-detoxifying fungi were reviewed recently by VanEtten [44] and this discussion follows his outline. One straightforward approach is to enhance the accumulation of naturally-occurring phytoalexins of the host plant by chemical treatment, by conventional plant breeding, or by genetic engineering. Ward and coworkers [50] reported some control of *P. infestans* on tomatoes following application of capsidiol, but this approach is unlikely to be practical due to the high cost of chemical synthesis of the complex sesquiterpene ring structures. Although dry-rot resistant germplasm has been identified in wild *Solanum* species as well as in some cultivated potatoes [14,25], there is very little information available on sesquiterpene levels in resistant plants. In four non-isogenic cultivars of potato that ranged in response from resistance to susceptibility to one strain of *F. sambucinum*, Corsini and Pavek [13] found no correlation between resistance and rishitin levels in infected tuber tissues. Although this approach warrants further study, it may not be productive because nearly all virulent strains of *G. pulicaris* isolated from potato tubers appear able to tolerate high levels ($> 10^{-3}$ M) of sesquiterpenes [16,18]; Desjardins and Gardner, unpublished data). Accordingly, enhancement of the level of naturally-occurring sesquiterpenes may not be sufficient to increase resistance to dry-rot in the field.

An alternative strategy for improving disease resistance is based on the general observation that pathogens are often more sensitive to phytoalexins that are different from those normally produced by their hosts [44]. If sesquiterpene biosynthesis in potato could be altered to produce novel compounds less susceptible to detoxification by *G. pulicaris*, then resistance to dry-rot might be enhanced. Although this approach is highly speculative, there are some promising preliminary results in potato and related systems. The pathway and regulation of sesquiterpene biosynthesis in the *Solanaceae* is under active investigation in several laboratories. An early step in plant sesquiterpene biosynthesis involves the conversion of farnesyl pyrophosphate to aristolichene by a sesquiterpene cyclase. Farnesyl pyrophosphate also serves as substrate for trichodiene synthase, a sesquiterpene cyclase which produces an early intermediate in trichothecene biosynthesis. The trichodiene synthase gene has been cloned from *Fusarium* [23] and the cloned gene has been stably integrated into tobacco plants [24]. Preliminary results indicate that the transgenic plants produce small amounts of the fungal metabolite trichodiene [24]. Thus, it would appear possible to transfer novel genes, which might affect phytoalexin biosynthesis, from other organisms to confer new mechanisms of resistance to potato.

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