Metabolism of the Phytoalexin Rishitin by *Gibberella pulicaris* Is Highly Reduced in Liquid Culture

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Gibberella pulicaris is a causal agent of potato dry rot. The fungus is able to metabolize the potato phytoalexin rishitin, a trait which is possibly associated with virulence against potato tubers. Metabolism of the plant defence compound on agar medium is completed within 24 h. In contrast, incubations in various liquid media and buffers highly reduced degradation of rishitin with a maximal reduction of substrate down to 30% of the initial concentration within five days. The structurally related sesquiterpene lubimin was degraded completely within 12 hr in all tested liquid media. Our data suggest that rishitin metabolism is under an unusual genetic control requiring growth on a solid surface for efficient metabolism.

Introduction

Gibberella pulicaris (anamorph *Fusarium sambucinum*) is a causal agent of potato dry rot. The fungus enters the tubers via wounds where it is exposed to phytoalexins like rishitin and lubimin as well as phytoanticipins like the saponins α-chaconine and α-solanine. These chemicals are inhibitory to fungi and are known to accumulate in tubers at the site of infection (Kuc, 1982), why they have been proposed to play a role in plant defence against fungi. Several studies have revealed that *G. pulicaris* is able to metabolize all of these compounds leading to less toxic products. The saponins for example are degraded by removing two or all three sugar residues of the side chain of α-solanine and α-chaconine, respectively (Weltring *et al.*, 1997). For lubimin and rishitin several products have been chemically characterized (Gardner *et al.*, 1988, 1994).

A survey of field strains from different geographical regions and habitats demonstrated a strong correlation between the ability of *G. pulicaris* to metabolize lubimin and rishitin *in vitro* and virulence on potato tubers (Desjardins *et al.*, 1989; Desjardins and Gardner, 1989). This correlation was supported for rishitin by a genetic analysis of progeny of crosses between field isolates and several back crosses. The result of these analyses suggested that rishitin metabolism of the virulent strain R-6380 is controlled by genes at two or more loci and that one of these loci, designated *Rim1*, is associated with virulence (Desjardins and Gardner, 1989, 1991).

So far two metabolites of rishitin, 13-hydroxyrishitin and 11,12-epoxyrishitin, have been identified, with the latter showing reduced toxicity towards *G. pulicaris* (Gardner *et al.*, 1994). Since both metabolites only accumulated in small quantities it is not clear which of these products is the first metabolite of rishitin or if both are produced in parallel. Isolation and characterization of the enzymes involved in the breakdown of rishitin would enable us to answer this question. In addition, the sequence of the purified proteins could be used for the design of oligonucleotides which can be employed for isolation of the corresponding gene(s). During establishment of optimal liquid culture conditions for production of sufficient amounts of enzyme we found the interesting and new phenomenon that rishitin metabolism is almost completely reduced in liquid culture suggesting that it is under an unusual genetic control, which has never been observed before for degradation of other phytoalexins or phytoanticipins.

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Materials and Methods

Strains, media and growth conditions

G. pulicaris strain R-6380 was obtained from A. Desjardins (Desjardins and Gardner, 1989). For long-term storage and to produce spores the fungus was grown on V-8 juice agar (Stevens, 1974). Liquid cultures were grown by inoculating spores from a seven to ten day old agar plate culture for three days at 28 °C on a rotary shaker in 100 ml of one of the following media: YEPD: 0.3% yeast extract, 1% casein hydrolysate, 2% glucose; potato-dextrose-medium (PDA): 100 g of cooked potatoes were pressed through cheese cloth and the filtrate was mixed with 10 g glucose, 0.1 g $CaCO_3$, and 0.1 g $MgSO_4 \cdot 7H_2O$ ad 1 liter at pH 5.7; Mantle: Mantle and Nisbet (1976).

Metabolism assays

For metabolism on agar plates spores from a ten day old agar plate were spread on cellophane foil covering the surface of V-8 or Czapek-Dox (CD) agar in a 3.5 cm diameter petri dish. After incubation for three days at 28 °C, the foil together with the mycelial mat was transfered to a fresh plate containing 2 ml of the corresponding agar supplemented with rishitin or lubimin predissolved in DMSO to a final concentration of 150 µg/ml and incubated at 28 °C. At different times the mycelium was removed and the agar was extracted two times with two volumes of ethyl acetate. After evaporation of the organic phase the residue was redissolved in 100 µl ethyl acetate containing 20 µg n-eicosan as internal standard and analysed by gas-liquid-chromatography (GLC) as previously described (Gardner *et al.*, 1988). The amount of rishitin was normalized to the internal standard and these quotients were converted to percent of rishitin remaining in the medium setting the amount of rishitin extractable from uninoculated samples to 100%.

For metabolism in liquid medium mycelium from the pre-culture was separated from the culture fluid by filtration through a nylon membrane and washed twice with water. Time course of metabolism of rishitin and lubimin was measured by incubating 1 g mycelium in 10 ml of various media or in 50 mM KH_2PO_4 buffer (pH 5.7), 50 mM KCl buffer or McIlvaine buffer (NaH_2PO_4, Na-citrate, pH 5.0) according to Table I supplemented with 10 µg/ml substrate at 26 °C on a rotary shaker. Samples (0.5 ml) taken at various times were extracted twice with ethyl acetate, evaporated, resuspended in 100 µl ethyl acetate containing 20 µg/ml n-eicosan as internal standard and analysed by GLC as described above.

Metabolism in stationary culture was measured by incubating 10^6 spores in 20 ml PDA medium in a 300 ml Erlenmeyer flask for seven days at 23 °C. After the mycelium had covered the surface the medium was exchanged with fresh Mantle medium supplemented with 20 µg/ml of rishitin and incubation was continued for four days. Each day a 0.5 ml sample was taken and analysed for remaining substrate as described above.

Results and Discussion

Metabolism of rishitin is routinely measured by extracting and quantifying residual substrate from agar medium of tolerance assays or from incubations of 250 µl V-8 juice agar inoculated with a 2 mm^2 mycelial plug for three to four days (Desjardins and Gardner, 1989, 1991). Under these conditions strain R-6380 metabolizes the phytoalexin completely. In a new assay where a mycelial mat grown on cellophane foil is transfered to rishitin amended agar the substrate disappears within 24 h (Fig. 1) regardless if incubated on a complex (V-8) or synthetic (CD) medium. In this test the delay in metabolism present in the other two assays caused by the fact that the fungus first has to grow over the agar surface is avoided. Therefore, the conditions of the new assay most closely resemble the conditions of a liquid culture, which is also inoculated with a pre-grown mycelium.

For purification of enzymes involved in rishitin metabolism it is neccessary to produce large quantities of mycelium containing the protein. Since agar cultures are unsuitable for this purpose we wanted to find liquid culture conditions for the production of metabolically active mycelium. The only liquid assay described so far (Desjardins and Gardner, 1989) is on a small scale and gave inconsistant results. Therefore, we tested various media and buffers listed in Table I to establish conditions for optimal rishitin metabolism in liquid culture.

For growth of mycelium from spores media with complex (YEPD, V-8, PDA) as well as defined

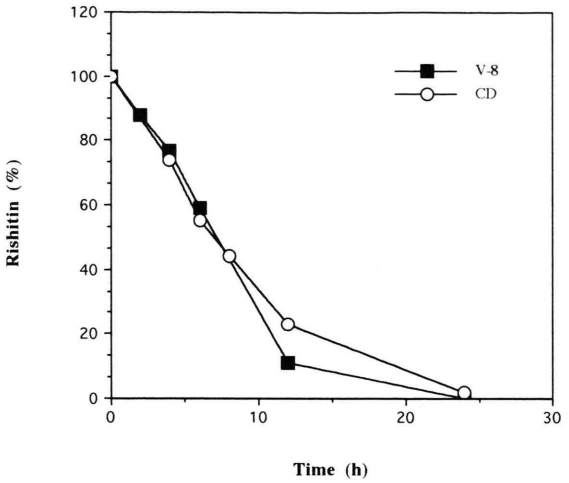

Fig. 1. Time course of decrease of rishitin concentration on V-8 and Czapek-Dox (CD) agar. Petridishes containing 2 ml agar medium supplemented with 200 µg/ml rishitin were inoculated with a mycelial mat grown on cellophane foil. At the indicated time points the mycelium was removed and residual rishitin was extracted from the agar and quantified.

Table I. Recovered rishitin from incubations in various liquid media and buffers. Mycelium (1 g) from various pre-cultures was incubated with 10 µg/ml rishitin (100%) in 10 ml of the listed solutions for five days on a rotary shaker.

Medium of pre-culture	Medium of metabolism assay	Rishitin recovered (%)
YEPD	50 mM KH_2PO_4 buffer	62
YEPD	50 mM KCl buffer	70
YEPD	YEPD	63
YEPD	Mantle	51
Mantle	20 mM McIlvaine buffer	100
V-8	V-8	31
V-8	V-8 + agar powder	48
PDA	PDA	40
PDA*	Mantle*	37
V-8+	V-8+	70+

* Stationary culture
+ Assay conditions as described by Desjardins and Gardner (1989).

carbon and nitrogen sources (Mantle) were used. Metabolism was assayed in these media and in three buffers, which have been used in other studies of metabolism of phytoalexins and corresponding compounds (Denny and VanEtten, 1982; Weltring and Barz, 1980; Mathews and VanEtten, 1983). As shown in Table I no complete metabolism of rishitin was observed within five days under any condition applied. In contrast, the structurally related sesquiterpene phytoalexin lubimin was metabolized within 12 h under all conditions tested (data not shown).

Representative examples of the various culture conditions shown in Fig. 2 reveal that, overall, rishitin with the exception of the buffer incubations is slowly disappearing from the medium with the initial drop in substrate concentration being most likely due to adsorption of rishitin to the mycelium. Since metabolites of rishitin do not accumulate, it cannot be excluded that adsorption is also responsible for the slow reduction in substrate concentration in the different media, because the mycelium continues to grow in these cultures. This is supported by the fact that rishitin concentration after the initial drop during the first day remained almost unchanged in all buffer cultures where the mycelium does not grow any further.

The possible adsorption of rishitin to the mycelium might also explain the discrepancy between our results and the data described by Desjardins and Gardner (1989), who reported metabolism rates of rishitin between 0.6 and 3.4 µg/ml/hr. Applying this kind of calculation to our data shown in Fig. 2 would convert the 32% reduction of the

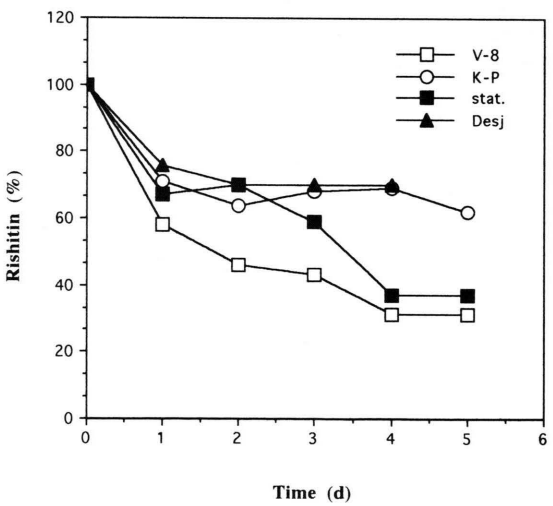

Fig. 2. Time course of decrease of rishitin concentration in different liquid media. Residual rishitin was measured in extracts of 0.5 ml samples from 10 ml incubations taken at the indicated time points. The initial concentration (100%) was 10 µg/ml rishitin in V-8 medium, KH_2PO_4 buffer (K-P) and stationary (stat.) culture, and 150 µg/ml in the assay described by Desjardins and Gardner (1989) (Desj.). The latter assay was only analysed for four days.

substrate (150 µg/ml) within the first 41 hr to a rate of about 1.1 µg/ml/hr, a range corresponding to the data described by Desjardins and Gardner (1989). However, the fact that rishitin did not continue to disappear from the medium in the following two days in our assay and the lack of data about the production of metabolites in both investigations make it difficult to asses the value of these data. Regardless of this problem, even if the observed disappearence of rishitin in both investigations is due to degradation, the metabolism of 200 µg rishitin in 1 ml V-8 agar within 24 hr (Fig. 1) corresponding to a rate of 8.3 µg/ml/hr is still much higher than any rate measured in liquid culture supporting our hypothesis that rishitin metabolism is very much reduced under these conditions.

The lack of metabolism in buffer excludes the possibility that rishitin metabolism is subject to glucose repression, which has been reported for example for pisatin demethylase (VanEtten and Barz, 1981). This demethylase is a cytochrome P-450 monooxygenase, a type of enzyme, which needs molecular oxygen for the removal of the methyl group of pisatin (Desjardins *et al.*, 1984). The same kind of enzyme may be involved in the production of 11,12-epoxyrishitin (Gardner *et al.*, 1994), which could make a reduced oxygen supply in liquid culture compared to growth on agar a limiting factor for break down of rishitin. The fact that pisatin demethylase can be measured in liquid culture proves that this kind of enzyme in general is active under submerged conditions. In addition, the lack of metabolism of rishitin in stationary culture where the mycelium is exposed to air like on agar medium make it highly unlikely that limited oxygen supply is preventing rishitin metabolism in liquid culture.

We routinely used 10 µg rishitin/ml medium to prevent inhibition of metabolism by damage of the mycelium by the phytoalexin. To exclude the possibility that this concentration might be too low for induction of metabolism, we increased the concentration of rishitin up to 150 µg/ml of medium or buffer without any effect on metabolism rate (data not shown). The facts that α-chaconine metabolism by *G. pulicaris* is inducible with 5 µg/ml (Becker and Weltring, unpublished) and that lubimin is also degraded at a concentration of 10 µg/ml supports the conclusion that lack of induction is not responsible for the reduced rishitin metabolism in liquid culture.

The greatly reduced metabolism of rishitin in liquid culture suggests that *G. pulicaris* needs a solid surface for efficient degradation of this phytoalexin. This would be a unique regulation for catabolism of a plant defence compound by a fungus, which has never been described before and which is obviously also not neccessary for metabolism of lubimin and the saponins α-chaconine, α-solanine and α-tomatine by *G. pulicaris* (Desjardins *et al.* 1989; Weltring *et al.* 1997; Weltring *et al.* 1998). To study this unusual regulation of rishitin metabolism in more detail we are currently attempting to isolate one of the *Rim* genes by different approaches.

Acknowledgements

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