1999

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Published in *Physiological and Molecular Plant Pathology* **55** (1999), pp. 317–326.

Accepted for publication August 1999.

Pisatin demethylation by fungal pathogens and nonpathogens of pea: Association with pisatin tolerance and virulence

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Abstract

Previous studies have indicated that detoxification of their hosts' phytoalexins is a tolerance mechanism for some true fungi, but not the fungus-like Oomycota, and may be involved in determining the virulence of a pathogen. In the present study, the associations between demethylation of the pea phytoalexin pisatin, tolerance to pisatin, and virulence on pea were examined for 50 fungal isolates which represent 17 species of pathogens and nonpathogens of pea. All isolates of *Pythium coloratum* and *P. irregulare* failed to metabolize and were sensitive to pisatin, consistent with previous observations that members of the Oomycota generally lack the ability to metabolize and are sensitive to their hosts' phytoalexins. Among true fungi tested, the ability to demethylate pisatin was common, regardless of whether the particular isolate was pathogenic on pea or not. However, when the rate of pisatin demethylation was compared to virulence, all but one of the moderate to highly virulent isolates rapidly demethylated pisatin. In addition, the more rapidly demethylating isolates were generally more tolerant of pisatin. These results suggest that a specialized enzyme system for quickly detoxifying pisatin might be present in most pea pathogens. In previous studies a specific cytochrome P450 enzyme for demethylating pisatin was identified in the pea pathogen *Nectria haematococca* mating population VI, and genes (*PDA* genes) encoding that enzyme have been cloned from this fungus. When DNA specific for these genes was used to probe genomic DNA from other fungi that demethylate pisatin, significant hybridization was detected with only one fungus, the pea pathogen *Fusarium oxysporum* f. sp. *pisi*. If the other pea pathogens possess a specific cytochrome P450 system for detoxification of pisatin, the genes encoding these enzymes apparently share limited nucleotide similarity with *N. haematococca PDA* genes.

Keywords: Phytoalexins, cytochrome P-450 monooxygenase, pisatin demethylase, *Pisum sativum* L., detoxification, pterocarpans

INTRODUCTION

In 1962, Cruickshank [*3*] reported on the sensitivity of 45 fungal species to pisatin, the predominant phytoalexin synthesized by garden pea (*Pisum sativum* L.). Only five species were tolerant of pisatin (<50% inhibition by 100 μg ml^{-1}) and all five were pathogens of pea. Only one of the sensitive species was a pea pathogen. Although subsequent surveys of the sensitivity of fungi to phytoalexins, including pisatin, revealed additional exceptions to the correlation between tolerance and host range [*30*, *37*], the remarkable correlation observed by Cruickshank firmly established the concept that tolerance to a phytoalexin might be important in pathogenicity [*4*].

Pisatin tolerance in fungi has been studied most extensively in the pea pathogen *N. haematococca* mating population (MP) VI (anamorph *Fusarium solani* " f. sp. *pisi"*) and two modes of tolerance have been identified [*7*, *38*]. The more thoroughly understood mode is by a substrate-inducible one step demethylation of pisatin to a non-toxic product, which is catalyzed by pisatin demethylase (pda), a cytochrome P450 [*18*]. All isolates of *N. haematococca* MP VI which possess pisatin demethylase activity (Pda^+) are more tolerant of pisatin than those which cannot demethylate pisatin (Pda^-) [*36*]. Additional work has demonstrated that transformation of a *PDA* gene into a Pda^- isolates increases its pisatin tolerance [2], while disruption of a *PDA* gene in Pda^+ isolates reduces their pisatin tolerance [*40*]. These results clearly establish that detoxification of pisatin by this cytochrome P450 is one mode of pisatin tolerance in *N. haematococca* MP VI. Furthermore, these and similar

studies [*29*] demonstrate that pda is a virulence factor : addition of *PDA* to a Pda^- isolate increases its virulence on pea and disruption of the *PDA* gene in a Pda^+ isolate reduces its virulence on pea.

Cytochrome P450s have been called the most versatile biological catalysts known because they catalyze such diverse degradative and biosynthetic reactions [*23*]. While the total number of cytochrome P450s present in filamentous fungi is not known [*34*], most eukaryotic organisms have multiple cytochrome P450s with different substrate specificities and types of regulation. Approximately 1000 cytochrome P450 genes (*CYP*) have been sequenced [*21*] and, despite the biochemical diversity of P450s, all *CYP* genes are considered members of one "superfamily" because of their high degree of conservation at the amino acid (aa) level [*21*]. *CYP* genes whose deduced aa sequences are >40% identical belong to the same family [*21*].

Seven similar cytochrome P450s that encode pda have been identified in *N. haematococca* [*16*, *20*, *27*]. The deduced aa sequences of the three genes that have been sequenced are greater than 88% identical to that of the first *PDA* gene cloned (*PDAT9*) [*13*]. These *PDA* genes are divergent enough from all other known *CYP* genes to define their own cytochrome P450 family (*CYP57*) [*16*]. Three different phenotypes have been identified in *N. haematococca* based on the lag period for induction of pda and the resulting amount of activity induced: Pda^{SH} = short lag, high activity ; Pda^{SM} = short lag, moderate activity ; Pda^{LL} = long lag, low activity. The Pda^{SH} and Pda^{SM} isolates are slightly more tolerant of pisatin than the Pda^{LL} isolates [*14*, *15*]. The most virulent isolates on pea are those with the Pda^{SH} and Pda^{SM} phenotypes [*14*, *15*] and the differential induction observed *in vitro* of PDA^{SH} and PDA^{LL} genes is also observed *in planta* [*13*]. The cytochrome P450 from Pda^{SH} isolates of *N. haematococca* is selectively induced by pisatin, has a high substrate specificity for this compound, and has a low K_m when pisatin is the substrate [*10*, *18*, *38*]. These properties, along with the association of this enzyme with tolerance of pisatin and virulence on pea, suggest that this cytochrome P450 might be a specific enzyme system for detoxification of pisatin. Our hypothesis is that this specialized detoxifying system evolved in *N. haematococca* as it became a pathogen of pea [*38*]: i.e., a readily inducible enzyme detoxification system, PDA^{SH} or PDA^{SM} genes, confers tolerance to pisatin and allows a tolerant isolate to be more virulent on pea than an isolate that more slowly detoxifies pisatin (PDA^{LL}) or lacks pda.

If pda has specifically evolved as a pathogenicity trait for *N. haematococca*, it might be expected that a similar enzyme system would have evolved in other pathogens which encounter this phytoalexin and that pda would be limited to pathogens of pea. However, in a previous screen for pda, it was observed that many fungi had this enzymatic capability regardless of whether or not they were pathogenic on pea [*6*]. The earlier study did not evaluate how rapidly this enzyme activity was expressed or how the activity was related to tolerance of pisatin or the virulence of an isolate. It may be that in most fungi the demethylation of pisatin is carried out by a non-specific cytochrome P450. Studies on mammalian cytochrome P450s indicate that some of these enzymes have very broad substrate specificities [*11*].

The present study was undertaken to characterize the rate of pisatin demethylation in nonpathogens of pea and in pea pathogens other than *N. haematococca* to determine whether the pda activity in pea pathogens is related to tolerance of pisatin and whether these pdas might differ from those in fungi that are not pathogenic on pea. Another objective was to evaluate isolates of the same species for rates of pisatin demethylation, tolerance to pisatin, and virulence on pea to determine if correlations between these traits exist for other pea pathogens as found in the intraspecies comparisons of *N. haematococca* MP VI. The final objective was to determine if other pea pathogens contain a specific cytochrome P450 for the detoxification of pisatin analogous to that from Pda^{SH} isolates of *N. haematococca* by using a portion of a *N. haematococca* PDA^{SH} gene as a heterologous probe in Southern hybridization analysis.

MATERIALS AND METHODS

Cultures

N. haematococca Berk. & Br. MP VI ascospore isolates known to contain single *PDA* genes, 196-10-7 (FGSC 8122) (*PDA4*, a PDA^{SM} gene) [*15*], 77-2-3 (FGSC 8119) (*PDA1*, a PDA^{SH} gene), 62-1 (*PDA3*, a PDA^{LL} gene), or known to lack *PDA* genes, 44-100 [*14*], were used as standards. The other fungi examined, the plant from which they were isolated and their geographic source are listed in Table 1. Fungi were categorized as pea pathogens if reported to be such [*12*] but all isolates, except two isolates of *P. irregulare*, were tested for virulence on pea (Table 1). Isolates deposited in recognized collections are: T393 (ATCC 58662), T394 (ATCC 58660), and T405 (ATCC 44649), American Type Culture Collection, Rockville, MD, U.S.A.; and T415 (JI2), T416 (JI22), T417 (JI33), T418 (JI70), T419 (JI71), T420 (JI88), T421 (JIPD1), T422 (JIPD2), T423 (JIPD3), T424 (JIPD4), T425 (JIPD5), and T427 (JI29), John Innes Institute, Norwich, U.K. All isolates were stored on V-8 agar medium slants (medium 29) [*32*] at 4°C. The inoculum for all experiments, except the virulence assays, was produced by growth on Martin's peptone-glucose agar (PGA) medium [*17*] for 2–3 days at 24 ± 1°C in darkness, unless otherwise indicated.

Preparation of pisatin and 6a-hydroxymaackiain (6a-HM)

Pisatin was extracted from pea [*33*]. Pisatin labeled at the 3-*O*-methyl position with ^{14}C[(^{14}C)pisatin] was prepared by methylation of 6a-HM with [^{14}C]methyl iodide [*36*]. 6a-HM was obtained by demethylation of pisatin by transformant III-202 of *Aspergillus nidulans* strain UCD1 [*25*]. Pisatin was quantified using the molar extinction coefficient in ethanol, log ε = 3.86 at 309 nm [*5*].

Pisatin metabolism

A slight modification of a previously published procedure was used [*15*]. Plastic scintillation vials (7 ml) contained 0.25 ml PGA amended with 0.1 mM (31 µg ml^{-1}) of [^{14}C]pisatin (1.2×10^5 dpm µmol^{-1}), a pisatin concentration shown to be noninhibitory or only slightly inhibitory to the growth of all fungi used in this study. Each vial was inoculated with one 4 mm diameter agar plug with mycelia, taken from the growing edge of an inoculum culture. In the initial measurements to determine if an isolate was Pda$^+$ or Pda$^-$ vials were incubated in darkness at 24°C for 6 days before the addition of scintillation fluid (4.5 ml of 0.55%2,5-diphenyloxazole in toluene) to stop metabolism. After this length of incubation, the mycelium of all isolates had ramified throughout the media. Unaltered pisatin partitioned into the toluene phase overnight [*15*], and ^{14}C was measured in a Beckman LS355 scintillation spectrometer. To measure the rate of pisatin demethylation in Pda$^+$ isolates the same procedure was used, except five to 19 vials were inoculated per isolate and one vial was removed at each time point to measure pisatin content in the medium. Most isolates were tested at least three times for their ability to metabolize pisatin and three to five time course experiments were carried out on most Pda$^+$ isolates. Isolates of *N. haematococca* of known pisatin demethylation phenotypes (Pda^{SH}, Pda^{LL} and Pda$^-$) were included in each experiment [*15*]. The amount of pisatin remaining at each time period of measurement was plotted and the time (in hours) for 50% of the [^{14}C]pisatin to be metabolized (T50) was interpolated from the graphs (for example Figure 1). If greater than 88% of the pisatin remained at the end of all experiments, the isolate was classified as Pda$^-$.

Detection of 6a-HM

To validate that the decrease in detectable [^{14}C] in the vial assay was due to demethylation of pisatin, selected isolates were assayed for the production of the demethylated product of pisatin, 6a-HM, using modifications of a previously published procedure [*14*]. Spores and/or mycelia were harvested from a culture of the test fungus grown on solid medium and added to 100 ml 2% yeast extract/6% sucrose broth in a 500 ml Erlenmyer flask. Cultures were incubated at 25°C for 3 days on a rotary shaker (150 rpm). Under aseptic conditions, the mycelia were collected by vacuum-filtration on Whatman #4 filter paper, and rinsed several times with water. Rinsed mycelia were resuspended in 50 mM potassium phosphate buffer (pH 6.5), 30 mg (fresh weight) mycelia ml^{-1}, in 25 ml Erlenmyer flasks and [^{14}C]pisatin was added to a final concentration of 0.5 mM. The cultures were incubated at 25°C at 100 rpm.

At intervals over a period of several hours, duplicate 750 µl samples of mycelial suspension were removed. One aliquot was added directly to 4.5 ml scintillation fluid, extracted overnight and counted in the scintillation spectrometer. These data indicated whether a decrease in radiolabeled pisatin was occurring during the assay. From the second aliquot, pisatin and its metabolites were extracted in methylene chloride and separated by thin-layer chromatography (TLC) on silica gel plates containing a fluorescent indicator (Analtech Inc., Type GHLF, 250 mm), in a solvent system of toluene:ethyl acetate (60:40).

Compounds with the same TLC mobilities as the standards of pisatin and 6a-HM as well as other possible metabolites were eluted in ethanol and u.v. absorbance spectra recorded. In addition, samples of the ethanol eluates were added to scintillation fluid and counted as above to verify that the compounds with the same Rf as pisatin were radioactive while those with the same Rf as the 6a-HM standards were not.

Pisatin sensitivity

Inhibition of mycelial growth on pisatin-amended agar medium was determined by a slight modification of a published assay [*14*]. A 4 mm diameter agar plug was removed from the growing edge of an inoculum culture. The plug was placed mycelium-side down on the surface of 1.0 ml PGA amended with 161 µg ml^{-1} of pisatin (0.5 mM) in dimethylsulfoxide (DMSO), in a plastic Petri plate (35 × 10 mm). This concentration was near the maximum solubility of pisatin, given the volume of DMSO used (final concentration of 1%). Control plates contained PGA amended with DMSO (final concentration 1%). Plates containing unamended PGA also were inoculated to determine any growth inhibition due to DMSO, which was generally <10%. The assay plates were incubated in darkness at 24 ± 1°C, a temperature that allowed near-maximum growth rates for most of the fungi tested. The radius of the colony was measured to the nearest 0.5 mm for each plate at 12–24 h intervals, until the mycelia grew to the edge of the DMSO-amended control plates, or for a maximum of 10 days. The endpoint inhibition (EI) value was the colony radius at this point in phytoalexin-amended PGA as a percentage of that in DMSO-amended medium.

Table 1. Rates of pisatin demethylation, sensitivity to pisatin and virulence on pea of fungal species reported as pathogens or nonpathogens of pea [1]

Fungus	Isolate	Source	T50 [1]	EI [2]	Lesion length [3]
Pea Pathogens:					
Ascochyta pisi Lib.	T395	Pea, NY, U.S.A.	>118. (42%)	85.9 (0.9)	2.2 (0.7)
	T411	Pea, Netherlands	21.0 (6.6)	31.0 (2.6)	7.4 (1.8)
	T421	Pea, U.K.	18.3 (2.2)	13.5 (3.7)	7.5 (1.2)
	T422	Pea, U.K.	21.0 (3.9)	30.5 (2.6)	6.3 (2.8)
	T423	Pea, U.K.	79.3 (9.8)	30.2 (1.1)	0.9 (0.4)
	T424	Pea, U.K.	20.0 (5.3)	15.6 (18.5)	3.5 (1.3)
	T425	Pea, U.K.	21.9 (4.1)	24.2 (2.0)	7.1 (1.1)
Colletotrichum pisi Pat.	T403	Pea, WI, U.S.A.	29.7 (1.5)	5.0 (7.0)	0.0 (0)
Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. in Penz. f. sp. *aeschynomene*	T444	*Indigo* sp., TX, U.S.A.	89.5 (0.5)	55.4	1.0 (0)
Cylindrocladium cla.atum C. S. Hodges & L. C. May	T405	Potato, Brazil	75.0 (10)	16.5 (13.6)	11.9 (4.3)
Fusarium oxysporum Schlechtend.:	T247	Pea, unknown	22.3 (6.1)	31.1 (10.2)	4.3 (1.0)
Fr. f. sp. *pisi* (J. C. Hall)	T415	Pea, U.K.	25.3 (4.9)	52.0 (3.1)	5.4 (1.6)
W. C. Snyder & Hansen	T416	Pea, WA, U.S.A.	19.3 (0.9)	39.0 (17.4)	14.1 (2.6)
Mycosphaerella pinodes	T396	Pea, NY, U.S.A.	20.3 (0.5)	17.8 (4.8)	20.1 (1.4)
(Berk. & Bloxam) Vestergr.	T414	Pea, Netherlands	22.3 (1.7)	17.5 (5.7)	12.4 (1.3)
	T417	Pea, U.K.	17.6 (2.0)	25.2 (4.5)	21.0 (1.6)
	T418	Pea, U.K.	19.5 (0.7)	18.7 (1.7)	14.2 (1.6)
	T419	Pea, U.K.	24.0 (2.1)	18.7 (2.1)	14.4 (1.7)
	T426	Pea, U.K.	24.3 (1.7)	25.1 (3.8)	19.2 (1.7)
	T427	Pea, U.K.	21.3 (1.2)	19.5 (4.5)	21.2 (1.8)
Phoma pinodella (L. K. Jones)	T393	Chickpea, WA, U.S.A.	16.5 (1.1)	16.0 (3.9)	17.8 (1.8)
Morgan-Jones & K. B. Burch	T394	Lentil, WA, U.S.A.	18.0 (2.5)	14.5 (7.8)	16.4 (2.0)
	T397	Pea, NY, U.S.A.	17.5 (0.5)	29.3 (8.2)	9.0 (2.4)
	T407	Pea, IL, U.S.A.	17.6 (3.8)	22.6 (2.8)	11.5 (2.2)
	T408	Pea, IL, U.S.A.	17.5 (3.4)	26.6 (7.4)	17.4 (0.8)
	T409	Pea, IL, U.S.A.	18.0 (3.3)	18.3 (2.5)	14.9 (1.9)
	NS413	Pea, Netherlands	17.8 (1.9)	15.0 (5.5)	14.6 (2.2)
	S1413	Pea, Netherlands	18.6 (2.1)	20.2 (4.4)	15.1 (2.5)
	S2413	Pea, Netherlands	16.4 (1.9)	26.8 (0)	15.2 (2.1)
Pythium coloratum Vaartaja	T433	Onion, NY, U.S.A.	Pda^-	93.9	26.5 (4.0)
	T434	Onion, NY, U.S.A.	Pda^-	86.3	22.3 (3.0)
	T445	Onion, NY, U.S.A.	Pda^-	95.8	21.5 (3.0)
	T446	Onion, NY, U.S.A.	Pda^-	95.7	24.8 (3.7)
Pythium irregulare Buisman	T447	Onion, NY, U.S.A.	Pda^-	87.6	ND [4]
	T448	Onion, NY, U.S.A.	Pda^-	77.4	37.9 (9.0)
	T449	Onion, NY, U.S.A.	Pda^-	86.3	ND
Rhizoctonia solani Kühn	T399	Pea, WA, U.S.A.	27.7 (2.5)	70.8 (5.5)	6.3 (0.9)
	T400	Pea, WA, U.S.A.	>260. (20%)	49.7 (9.8)	0.4 (0.7)
	T402	Pea, WA, U.S.A.	>260. (21%)	78.1 (2.5)	1.9 (0.9)
Pea Nonpathogens:					
Fusarium monliforme J. Sheld.	T443	Sorghum	Pda^-	67.5 (2.6)	ND
Gloeocerospora sorghi Bain & Edgerton ex Deighton	T442	Sorghum	>163. (29%)	100.0 (0)	0.5 (0.5)
Colletotrichum graminicola	T435	Sorghum, TX, U.S.A.	30	–12.9 (4.8)	0.5 (0.1)
(Ces.) G. W. Wils.	T438	Sorghum	30	11.7 (1.0)	0.0 (0)
	T441	Corn	>168. (18%)	100.0 (0)	0.0 (0)
Macrophomina phaseolina (Tassi) Goidanich	T437	Sorghum, TX, U.S.A.	65	44.5 (14)	ND
Mycosphaerella rabiei Kovachevski	T398	Chickpea, NY, U.S.A.	115	25.0	ND
Periconia circinata (L. Mangin) Sacc.	T439	Sorghum	>135. (24%)	78.4	1.3 (0.8)
Phoma medicaginis Malbr. & Roum.	T430	Alfalfa, PA, U.S.A.	75.3 (13.7)	87.6 (2.5)	2.4 (0.6)
in Roum.	T431	Alfalfa, NY, U.S.A.	61.7 (8.5)	82.2 (0)	1.6 (0.8)
	T432	Alfalfa, NY, U.S.A.	69. (18.5)	83.3	1.6 (1)

The range of EI mean values for a given isolate, both within and between experiments, generally was less than 20%. In each experiment, a mean EI was calculated based on three or four replicate plates. Unless otherwise indicated, each isolate was assayed three or four times and the experimental means were averaged, resulting in the EI value presented in Table 1.

Virulence on pea

Fungi were tested for virulence on wounded epicotyls of cv. Alaska 2B, using the "test-tube assay" [*36*]. Inoculum cultures were grown on *Ustilago* minimal medium [*32*] for 2–3 days in darkness at 24 ± 1°C. Eight to 32 plants were inoculated with 4 mm diameter agar plugs with mycelia, and incubated at 25°C. Five days after inoculation, the lesion lengths (mm) of the replicate plants were measured. The values were averaged, resulting in the mean lesion lengths and standard deviations presented in Table 1. Wounded, uninoculated control plants had no discoloration at the wound site. If a lesion larger than the inoculum plug (4 mm) was not produced, the isolate was classified as nonpathogenic.

Southern hybridizations

Representative fungi were grown in GA medium [*36*] and DNA was extracted using a CTAB/phenol extraction protocol [*19*]. DNA was digested with restriction endonucleases following the manufacturer's instructions and fractionated on agarose gels using standard techniques [*28*]. DNA was transferred to nylon membranes (Nytran, Schleicher and Schuell, Keene, NH, U.S.A.) using standard buffers as described by the manufacturer. The central 1.35 kb *Sac*1 fragment (probe SacB) from the *PDA*T9 gene (PDA^{SH}) [*16*] was labeled with ^{32}P for use as a *PDA* gene-specific probe using the random priming technique of Feinberg and Vogelstein [*8*]. Nylon membranes containing genomic DNA from the fungi examined were incubated with probe at 60°C in 7% SDS, 0.25 M $NaPO_4$ (pH 7.0), 0.25 M NaCl and 5 mM EDTA. Membranes were washed twice for 30 min each in 2 × SSC 0.1% SDS at 60°C followed by two 30 min washes in 0.2 × SSC, 0.1% SDS [*28*]. Kodak (Rochester, NY, U.S.A.) XR OMAT film was exposed overnight at –80°C and developed using a Konica automatic film developer.

RESULTS

Ability to metabolize pisatin, Pda phenotypes and determination of T50 values

Most of the fungi tested were able to metabolize pisatin. Among 39 isolates from the 10 fungal species pathogenic on pea only seven isolates representing two *Pythium* species were not able to metabolize pisatin (Table 1). Among the rest of the fungal isolates from species reported as pathogens of pea, all rapidly metabolized pisatin with three exceptions. One isolate of *A. pisi* and two isolates of *R. solani* incompletely metabolized pisatin (<50% remained at the end of the assay) but these particular isolates were not pathogenic on pea (Table 1). Of the 11 isolates representing seven fungal species that are not pathogens of pea, only one was classified as Pda^- (Table 1). Thus, as observed previously, although most pea pathogens were able to demethylate pisatin this is not true of all pea pathogens and pda is not limited to pea pathogens [*6*, *26*].

Previously, the phenotypes of Pda^{SH}, Pda^{SM} and Pda^{LL} were assigned to *N. haematococca* isolates based on the rate of demethylation of pisatin in a liquid suspension of mycelia [*14*, *15*]. A representative isolate of each phenotype was assayed by the more convenient vial assay, which measures metabolism of pisatin in solid medium amended with pisatin, to determine if this assay could distinguish these three phenotypes as well as the Pda^- phenotype [Figure 1(a)]. This assay did not distinguish a Pda^{SH} from a Pda^{SM} isolate but did distinguish between these isolates and a Pda^{LL} isolate or a Pda^- isolate. This assay also allows an estimation of the speed of metabolism in the Pda^+ isolates, namely the time for 50% loss of pisatin (T50 value) [Figure 1(a)]. The vial assay was used subsequently to characterize the pattern of pisatin metabolism and to obtain T50 values of all test isolates in order to compare the relative rates of pisatin metabolism by these fungi (Table 1).

Like the *N. haemaotococca* isolates with PDA^{SH} and PDA^{SM} genes used as standards, all isolates with T50 values less than 40 h had a pattern of metabolism consisting of little or no lag period followed by a fairly linear rate of pisatin metabolism. Representatives of this pattern are shown in Figure 1(b) for *A. pisi* isolate T411,

[Notes to Table 1]

Except for T50, values are means of one to five experiments, followed by the sample standard deviation (SD) in parentheses unless the value is from one experiment and then no SD is value given.

1. T50 = Time (h) for 50% demethylation of 0.1 mm pisatin. A Pda^- indicates less than 12% of the pisatin was metabolized after ≥ 168 h. A value preceded with a > sign indicates that more than 12% was metabolized but less than 50% and the value in parenthesis is the highest % obtained of all the experiments at the time following the > sign.
2. Mean EI = Mean endpoint inhibition in the presence of 0.5 mM pisatin, expressed as a percentage relative to the radial mycelial growth on control plates.
3. Mean lesion length (mm) on pea stems (cv. Alaska 2B) of 8–32 plant 5 days after inoculation.
4. ND = Not determined.

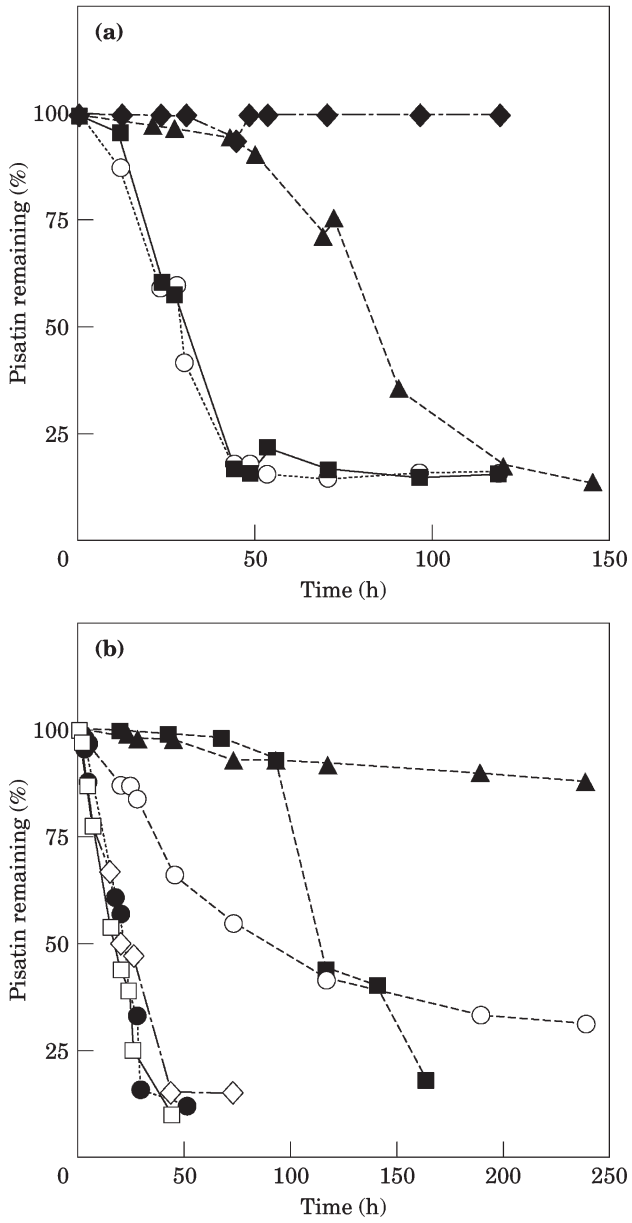

Figure 1. Demethylation of 0–1 mm pisatin by fungi in semi-solid medium. **(a)** Isolates of *Nectria haematococca* MP VI with known Pda phenotypes. Isolate 44-100 (♦), Pda$^-$; 77-2-3 (○), Pda^{SH}; 196-10-7 (■), Pda^{SM}; 62-1 (▲), Pda^{LL}. **(b)** *A. pisi* isolate T411 (◊), *C. gloeosporiodes* f. sp. *aeschynomene* isolate T444 (○), *M. pinodes* isolate T427 (●), *P. pinodella* isolate T408 (□), *P. coloratum* isolate T434 (▲), and *M. rabiei* isolate T398 (■).

M. pinodes isolate T427 and *P. pinodella* isolate T408. Of those isolates which had T50 values greater than 40 h and metabolized more than 50% of the pisatin, all but *C. gloeosporioides* f. sp. *aeschynomene* had a long lag preceding a fairly linear and rapid rate of metabolism. This pattern, illustrated in Figure 1(b) by *M. rabiei*, is similar to that of the *PDA*^{LL} containing isolate of *N. haematococca* used as a standard. The *C. gloeosporioides* f. sp. *aeschynomene* isolate had a pattern of a slow steady rate of metabolism but never removed more than 70% of the pisatin from the medium [Figure 1(b)]. Finally, several of the isolates that we classified as Pda$^-$ appeared to remove some pisatin from the medium in some assays as illustrated for the *P. coloratum* isolate T434 [Figure 1(b)], but because this amount never exceeded the variation seen in the *N. haematococca* Pda$^-$ control, the isolates were classified as Pda$^-$.

Pisatin sensitivity as measured by radial growth

In *N. haematococca*, amendment with pisatin has two different effects on the rate of growth on solid medium [*14*, *15*]. For all isolates there is an initial period of slow growth but following this lag period the Pda$^+$ isolates grow at a linear rate similar to the controls without pisatin. After the lag period, the Pda$^-$ isolates grow at a slower linear rate than the controls without pisatin. Most of the isolates used in this study had short lag periods and most of those with T50 values less than 40 h grew at a rate close to the controls without pisatin after this lag (data not shown). All of the isolates with a T50 greater than 40 h as well as the Pda$^-$ isolates had much slower rates of growth after the lag period than the controls without pisatin. Sensitivity to pisatin (Table 1) is reported as the relative amount of radial growth in the pisatin-amended medium compared to the control (DMSO only) at the end of the assay period (end point inhibition, EI) as this value includes the inhibition caused by pisatin during both growth phases. As originally observed by Cruickshank [*3*], most pea pathogens were tolerant of pisatin while most non-pathogens were not, but there were exceptions (Table 1).

Virulence on pea

Although the common site for disease development by several of the pea pathogens used in this study is not the region of the epicotyl between the cotyledon and the first true leaf, all of these fungi except *C. pisi* will infect this part of the pea plant [*12*]. A "test tube" assay, in which the length of lesion formed on this region of the pea epicotyl is measured, has been developed to determine the relative virulence of *N. haematococca* isolates. The size of lesion produced by different isolates has been shown to correlate with virulence ratings obtained by other assays [*36*]. All of the pea pathogens employed in this study, except *C. pisi*, produced expanding lesions on the epicotyls of pea when this assay was employed (Table 1). In all cases the lesions were tan to dark brown with discrete margins. When isolates of the same species were reassayed, the relative ranking of the isolates according to lesion sizes was reproducible which suggested that the relative virulence of isolates could be estimated by this assay. However, this does not appear to be an appropriate assay to determine the virulence of

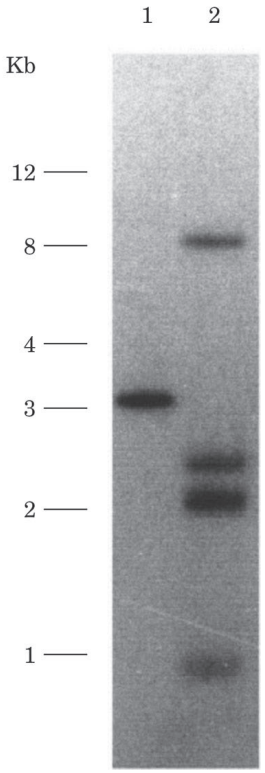

Figure 2. Southern blot hybridization analysis of *Xho*I/*Bam*HI digested DNA of *N. haematococca* and *F. oxysporum* f. sp. *pisi* hybridized with a *N. haematococca PDA* gene probe: Lane 1, *N. haematococca* isolate 77-2-3 which contains a PDA^{SH} gene (*PDA1*); lane 2, *F. oxysporum* f. sp. *pisi* isolate T247. The DNA was hybridized with the SacB fragment of the *PDAT9* gene of *N. haematococca* [*16*].

C. pisi isolates as the isolate employed (T403) was, subsequent to the present study, shown to be virulent on leaves of pea [*22*].

Metabolism of pisatin to 6a-HM and 3-hydroxymaackiainisoflavan

In all instances {*A. pisi* [*41*], *F. oxysporum* f. sp. *pisi* [*9*], and *N. haematococca* [*35*]} where the first step in the metabolism of pisatin by pea pathogens has been identified it has involved the demethylation of pisatin to produce 6a-HM [*37*]. The presence of this metabolite was assayed for in mycelial suspensions of *C. pisi*, *C. gloeosporioides* f. sp. *aeschynomene*, *M. pinodes* (T427) and *P. pinodella* (S2413) and was detected in these cultures when less than 50% of the pisatin had been metabolized (results not shown). In the cultures of *C. pisi*, *M. pinodes* (T427) and *P. pinodella* (S2413) an additional compound, with a lower Rf than 6a-HM and a u.v. absorbance spectrum that was identical to 3-hydroxymaackiainisoflavan [*9*], was detected soon after 6a-HM. This compound had been identified previously as the product of the second step in the metabolism of pisatin by *F. oxysporum* f. sp. *pisi* resulting from the reductive opening of the dihydrofuran ring of 6a-HM.

Presence of N. haematococca *PDA gene homologs*

Southern hybridization analyses at high stringency with the *PDA* specific probe SacB detected putative *PDA* homologs in *Fusarium oxysporum* f. sp. *pisi*, but failed to detect homologous sequences in the other species (Figure 2 and data not shown). Hybridization conducted at reduced stringency failed to reproducibly demonstrate the presence of homologous sequences in any of the other species. The *PDA* genes of *N. haematococca* lack internal *Xho*I and *Bam*HI sites so Southern hybridization analysis with the SacB probe of *Xho*I/*Bam*HI digested genomic DNA detects a single hybridizing band for each *PDA* gene [*16*]. An example is shown in lane 1 (Figure 2) for the *N. haematococca* reference isolate 77-2-3 which contains the PDA^{SH} gene, *PDA1* [*14*]. The detection of multiple bands in the DNA of *F. oxysporum* f. sp. *pisi* (Figure 2, lane 2) suggests that isolates of this species may contain more than one *PDA* homolog and this has been subsequently demonstrated (K. McCluskey, C. Wasmann, and H. VanEtten, unpublished results).

DISCUSSION

As measured by its effect on radial growth, 26 of the 28 fungal isolates that rapidly demethylated pisatin (T50 ≤ 30 h) were relatively insensitive to pisatin (< 50% inhibited) (Table 1 and Figure 3). Thus, whether an isolate is a pea pathogen or not, the ability to readily detoxify pisatin, appears to confer tolerance of this compound, which is not surprising. However, the inability to rapidly demethylate pisatin does not mean that a fungus is highly sensitive to pisatin, as seven of the isolates that were Pda^- or had T50s > 30 h were inhibited less than 50% (Table 1 and Figure 3). This suggests that these fungi have means other than demethylation to avoid the inhibitory effects of pisatin. A number of mechanisms of antibiotic resistance other than detoxification are known [*1*, *39*] and there are indications that fungi have additional means to tolerate the inhibitory effects of pisatin [*24*, *31*]. In particular, *N. haematococca* may possess an inducible mechanism for pisatin tolerance which appears to involve a change in the influx/efflux of pisatin [*7*], and may act in concert with pda to give a high level of tolerance to this plant antibiotic. Similarly some of the other fungi in this study may also have multiple mechanisms of pisatin tolerance.

Of particular interest for this study is whether pea pathogens have specific pdas which have evolved for the purpose of pisatin tolerance and virulence on pea. Clearly, as judged by *in vitro* assays, pda and pisatin

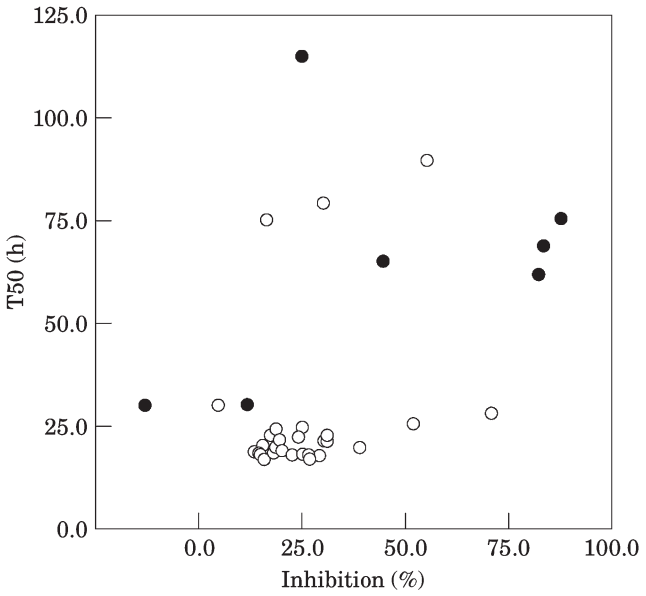

Figure 3. Time for demethylation of 50% of 0–1 mm pisatin vs. the sensitivity of an isolate to 0.5 mM pisatin. The sensitivity to pisatin is expressed as the percentage of inhibition of radial growth in medium containing 0.5 mM pisatin compared to a control that contained 1% DMSO. Data is from Table 1 and only Pda^+ isolates for which it was possible to obtain a T50 value are shown. (○) Pea pathogens and (●) non pea pathogens.

tolerance are not required by all pea pathogens. In this study, isolates of two species of *Pythium* that are highly virulent on pea were shown to be Pda^- and sensitive to pisatin (Table 1). Previous work has demonstrated that the pea pathogen *Aphanomyces euteiches* is also Pda^- and highly sensitive to pisatin [*26*]. The lack of pda in the *Pythium* species extends the observation that members of the Oomycota generally lack the ability to metabolize phytoalexins and are often sensitive to their hosts' phytoalexins [*37*]. It would appear that this group of plant pathogens either avoids physical contact with phytoalexins during infection or the *in vitro* bioassays are not indicative of their sensitivity *in planta* [*33*].

The rest of the pea pathogens studied had pda (Table 1). For those fungal species in which enough isolates were assayed to find large variations in the rates of Pda among isolates (*A. pisi* and *R. solani*), the highly virulent isolates rapidly demethylated pisatin and the isolates which slowly demethylated pisatin (*A. pisi* T395 and T423, *R. solani* T400 and T402) were much less virulent. In addition, all of the highly virulent isolates of *A. pisi*, *F. oxysporum* f. sp. *pisi*, *M. pinodes* and *P. pinodella* had a pattern of demethylation of pisatin similar to the virulent isolates of *N. haematococca*, i.e. none to very short lag periods followed by a rapid demethylation of pisatin [Figure 1(a) vs. Figure 1(b)]. This pattern of expression is consistent with the presence of a readily inducible cytochrome P450 or some other enzyme which demethylates pisatin. Somewhat surprising were the two isolates of a nonpathogen of pea, *C. graminicola* (T435 and T438) which had Pda similar to that observed in many of the pea pathogens (Table 1). Thus, as expected and as observed previously, pathogenicity on pea requires more than the ability to rapidly detoxify pisatin [*14*].

The Southern analyses suggest that only *F. oxysporum* f. sp. *pisi* has a cytochrome P450 that is highly similar to the *PDA* genes of *N. haematococca* (Figure 2). If the demethylations in these other fungi are carried out by cytochrome P450s, their DNA sequences are too divergent to be detected by Southern analysis.

In general, the relationship between pda and pisatin tolerance in the pea pathogens showed that those isolates with the lower T50s were more tolerant but, as with nonpea pathogens, this correlation was not absolute (Table 1 and Figure 3). The vial assay for pisatin demethylation provides a rapid means for measuring pda activity in fungi. However, relating the rate of pda expressed in the vial assay to the expression of sensitivity to pisatin in the radial growth bioassay has one limitation when comparing different species. The vial assay does not accommodate the different growth rates of the fungi, which could affect the apparent rate at which some of these fungi demethylate pisatin. The final determination of the role of pda in the tolerance to pisatin and its importance in the pathogenicity of these fungi, as well how these putative cytochrome P450s relate to each other, will require isolation of the genes encoding these traits.

Acknowledgments — This research was supported in part by National Research Initiative Competitive Grant No. 87-CRCR-1-2510 from the Cooperative State Research Service, grant no. DEFGO2- 89ER14038 from DOE and by USDA Food and Agricultural Sciences National Needs Graduate Fellowship Program Grant 84-GRAD-9-0039 (to LMD). We thank Patty Matthews for technical assistance and advice, and the following individuals who provided isolates for this study: G. C. Bergstrom, M. Gerlagh, W. J. Kaiser, J. Kraft, P. Matthews, T. E. Stasz, G. Templeton, P. C. Vincelli, and P. Wang.

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