The Role of Pisatin Tolerance and Degradation in the Virulence of *Nectria haematococca* on Peas: A Genetic Analysis

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ABSTRACT

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A series of crosses were made between isolates of *Nectria haematococca* mating population VI that differed in sensitivity to the pea (*Pisum sativum*) phytoalexin, pisatin. The progeny were tested for sensitivity to pisatin, ability to demethylate pisatin, and virulence on pea. The progeny from some crosses fell into two discrete classes of sensitivity. All progeny in the most tolerant class were able to demethylate pisatin; none in the more pisatin-sensitive class could demethylate this phytoalexin. Tetrad analysis of one of these crosses indicated that at least two loci conferred the tolerant, demethylating phenotype. The progeny from two other crosses did not segregate into distinct classes. In these crosses pisatin sensitivity segregated polygenically, and some of the most sensitive progeny were able to demethylate pisatin. In four crosses, virulence on pea segregated in the progeny. Of the 345 progeny analyzed, all of the moderately or highly virulent progeny were tolerant of pisatin and able to demethylate it. Therefore, either pisatin tolerance and demethylating ability are required for virulence on pea or genes for pisatin tolerance and demethylating ability are closely linked to genes for virulence. The results of this study indirectly support the hypothesis that pisatin accumulation is an active mechanism of resistance in pea.

Additional key words: Fusarium solani.

It has been suggested that phytoalexins function as inducible factors in plant disease resistance (7), but conclusive evidence for their role in resistance has not been obtained. If phytoalexin accumulation is the basis of an active resistance mechanism in plants, then successful pathogens must have a means of evading this mechanism. One hypothesized method for a pathogen to evade a phytoalexin-based mechanism of resistance is by being tolerant of its host's phytoalexins (2,3,11,19). Cruickshank's pioneering study (1) on the differential sensitivity of filamentous fungi to pisatin (a phytoalexin produced by *Pisum sativum* L.) provided evidence consistent with this hypothesis when it was found that pathogens of pea are generally more tolerant of pisatin than are fungi nonpathogenic on pea. A related observation found in several studies (4,5,9,17) is that pathogens are sometimes better able to degrade the phytoalexins of their hosts than are nonpathogens of those hosts.

Results of a study of field isolates of *Nectria haematococca* Berk. and Br. mating population VI (MP VI) were consistent with the hypothesis that tolerance to pisatin and the ability to degrade pisatin are required for a high level of virulence on pea by this pathogen (16). All isolates of MP VI that are highly virulent on pea are tolerant of pisatin and able to metabolize pisatin by an *O*-demethylation to the less toxic compound, 3,6a-dihydroxy-8,9-methylenedioxypterocarpan (DMDP). Isolates of *N. haematococca* MP VI most sensitive to pisatin are all low in virulence and unable to degrade pisatin.

A genetic analysis of the naturally occurring diversity in *N. haematocca* MP VI should provide a critical test of whether or not there is a causal relationship between virulence by this pathogen on pea and phytoalexin tolerance and degradation. *N. haematococca* MP VI is amenable to genetic analysis (14). If these traits are separable, progeny that are sensitive to pisatin or lack the ability to demethylate pisatin but are highly virulent should be recovered. Isolation of progeny that are tolerant of pisatin but unable to demethylate it would also be expected if demethylating ability is not required for tolerance.

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MATERIALS AND METHODS

Maintenance of cultures and the procedures for crossing isolates and isolating and coding ascospores are described in Tegtmeier and VanEtten (14). Both random ascospore and tetrad analyses were performed. Methods for characterizing progeny for mating type, female fertility, and perithecial color are described in Tegtmeier and VanEtten (14).

Pisatin. Pisatin and pisatin specifically labeled with ^{14}C at the 3-*O*-methyl position ([3-*O*-methyl-^{14}C]-pisatin) were obtained by published procedures (16,18). The molar extinction coefficient of $\log \epsilon = 3.86$ at 309 nm for pisatin in ethanol was used to determine pisatin concentration (8).

Virulence Assay. The virulence of isolates was determined by inoculating the epicotyls of pea seedlings (cultivar Alaska 2B) grown individually in test tubes containing vermiculite (16). Three isolates of *N. haematococca* MP VI ranging from weakly virulent to highly virulent (T76, 6-36, and T8 or T30) (16) were included in each assay. Occasionally the virulence levels of the control isolates were atypical; assay results were discarded when this occurred. Data are presented as the means and standard deviations of the lesion lengths on eight inoculated seedlings. Isolates were classified as highly virulent (lesions $\geq$8 mm long), moderately virulent (lesions 4–7 mm long), or weakly virulent (lesions $<$4 mm long).

Assay for sensitivity to, and demethylation of, pisatin. Mycelial growth bioassays similar to those used in earlier studies (15,16,20) were used to determine the sensitivity of isolates to pisatin. Conidial suspensions were uniformly dispersed on the surface of 8 ml of peptone-dextrose agar medium (6) in 100-mm-diameter petri plates and incubated for 2 days in the dark at 24 ± 2 C. Inoculum plugs (4.0 mm in diameter) from these plates were placed mycelium-side down near the edge of 35-mm-diameter petri plates containing 1 ml of peptone-dextrose agar medium which was

amended to 0.5 mM pisatin and 0.8–0.9% dimethylsulfoxide (DMSO); control plates were amended with DMSO alone. Plates were incubated in the dark at 24 ± 2 C. The radius of the colony formed on pisatin-amended medium was measured when the radius of the colony on unamended medium was 21 ± 1 mm (~6 days), and the percent inhibition of radial growth was calculated.

The ability of an isolate to demethylate pisatin was determined with (3-*O*-methyl-^{14}C)-pisatin. Two different assays were used to determine the loss of ^{14}C during the assay period. The first was the washed-mycelium assay described by VanEtten et al (16) in which the mycelium was grown in succinate medium, rinsed, and resuspended in 0.15 M KH_2PO_4, 0.06 mM (3-*O*-methyl-^{14}C)-pisatin (5.0×10^{-2} μCi/μmole) and 0.5% DMSO. After 2 days the culture was assayed for ^{14}C content (16). In liquid culture the ^{14}C cleaved from the (3-*O*-methyl-^{14}C)-pisatin is given off as $^{14}CO_2$ (16). This assay was used only to test the random ascospore isolates from cross 105. The other assay, which was used to test all other isolates, was a modification of the mycelial growth bioassay given above, and it allowed the concurrent measurement of an isolate's sensitivity to pisatin. In this assay (3-*O*-methyl-^{14}C)-pisatin (1.1×10^{-2} μCi/μmole) was added to the agar medium (final concentration was 0.5 mM pisatin and 0.8–0.9% DMSO). The cultures were incubated until the mycelium completely overgrew the medium (~13 days for pisatin-sensitive isolates). The contents of the petri plates were then placed in scintillation vials. Distilled water (4.5 ml) and 11.5 ml Aquasol (New England Nuclear, 549 Albany St., Boston, MA 02118) were added to the vials. The contents were shaken vigorously to form a gel and to aid in the extraction of pisatin from the agar medium into the scintillation fluid. The ^{14}C content in the vials was determined with a liquid scintillation spectrometer. The efficiency of this procedure in detecting ^{14}C was ~60%.

To test the accuracy of this new assay, isolate T110 (previously shown to be negative for pisatin demethylating ability [16]) was tested four times; in all cases the medium still contained more than 90% of the ^{14}C recoverable from a similarly treated, uninoculated plate. The validity of the assay was monitored by routinely including isolates T110 and T63 (previously shown to be positive for pisatin demethylating ability [16]) in all subsequent assays. In these 14 subsequent experiments 8,200 ± 1,500 cpm were detected in the T110-inoculated medium at the end of the incubation period; T63-inoculated medium contained 2,400±750 cpm. In the Results section, the percent demethylation for a given isolate is expressed as the percent of ^{14}C lost from the medium using the ^{14}C content of medium with isolate T110 as 0% lost. The reliability of this assay was such that most isolates were usually tested only once. However, all progeny of crosses 126 and 127 that were negative for pisatin demethylating ability were tested at least one additional time.

We verified that a substantial proportion of the ^{14}C remaining in the agar medium after the incubation period was still present as (3-*O*-methyl-^{14}C)-pisatin by extracting the agar medium of plates inoculated with isolate T110 and uninoculated medium. For both treatments 91–92% of the recoverable ^{14}C was extracted with $CHCl_3$, and 90–92% of the $CHCl_3$-soluble ^{14}C cochromatographed with a pisatin standard in thin-layer chromatography (13).

RESULTS

Assay for pisatin demethylating ability. In a previous study VanEtten et al (16) found that some field isolates were variable in expressing pisatin-demethylating ability. Several repeated experiments of a laborious assay were required to determine whether an isolate could demethylate pisatin. The second assay described in Materials and Methods is not only a much simpler assay than used previously, but has the advantage of concurrently measuring the sensitivity of an isolate to pisatin.

As a test of the effectiveness of this assay, the ability of 26 field isolates to demethylate pisatin was determined. Previous study using the assay of VanEtten et al (16) had shown that two of these isolates were negative for pisatin demethylating ability, while the other 24 were positive for demethylating ability. Eighteen of these 24 isolates had been variable in the expression of demethylating ability (16). When tested by the new assay procedure, all 24 isolates which had previously been found to be positive for pisatin demethylating ability reduced the ^{14}C content of the medium (36–63% reduction), whereas the two isolates negative for pisatin demethylating ability did not reduce the ^{14}C content of the medium (<0% reduction). In subsequent assays isolates were classified as positive for pisatin demethylating ability (pda$^+$) if more than 35% (mean of 64 ± 14% reduction) and negative for demethylating ability (pda$^-$) if less than 15% (mean of −4 ± 7% reduction) of the ^{14}C was lost from the agar medium.

Association of pisatin tolerance and demethylating ability with virulence on pea in field isolates. For the genetic studies reported here it was desirable to maximize the differences among isolates in their sensitivity to pisatin. Therefore, the concentration of pisatin in the radial growth bioassays was increased from 0.3 mM, used in the earlier work (16), to 0.5 mM for this study. All the previously tested field isolates were reassayed at this concentration. In addition, all isolates that had not been tested by the virulence assay employed in the current study were assayed for virulence. The previously observed association between pisatin tolerance and virulence on pea for the field isolates was maintained at this higher pisatin concentration (Fig. 1), although some rearrangements of ranking of specific isolates by sensitivity levels occurred. The range of pisatin sensitivity increased from 0–41% inhibition of radial growth at 0.3 mM pisatin to 7–76% inhibition at 0.5 mM pisatin. Isolates inhibited by less than 40% in radial growth were all pda$^+$; those inhibited more than 50% were pda$^-$. An area of overlap for demethylating ability occurred at 40–50% inhibition.

Inheritance of virulence, pisatin tolerance, and pisatin-demethylating ability. It was not possible to use the most virulent isolates (Fig. 1) as parents in crosses to further test the association between virulence, tolerance, and demethylating ability. Crosses between those few combinations of highly virulent, pisatin tolerant field isolates and pisatin sensitive, weakly virulent isolates that were sexually compatible were consistently infertile or produced progeny that were all of one parental type for the traits examined. Therefore, the crosses used in the following analyses were between isolates of moderate virulence that are tolerant of pisatin and pda$^+$ and isolates that are sensitive to pisatin, pda$^-$, and low in virulence on pea.

Two different patterns of sensitivity to pisatin were observed in the progeny of these crosses. In one group of crosses the progeny segregated into two (parental) classes of pisatin sensitivity, while in

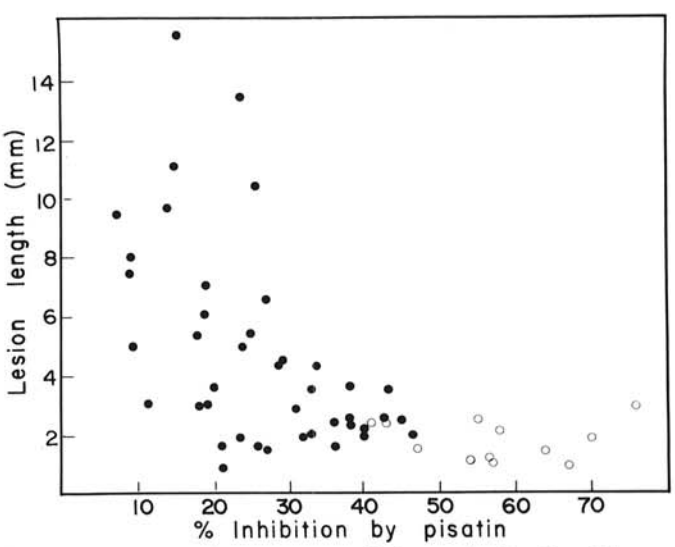

Fig. 1. The relationship between sensitivity to pisatin, the ability to demethylate pisatin, and virulence on pea in field isolates of *Nectria haematococca* MP VI. Field isolates are from a collection reported by VanEtten et al (16). Data on the ability of these isolates to demethylate pisatin and data on the virulence of some isolates are from that study. Data on the sensitivity of the isolates to pisatin is from the present study. Each point represents one isolate; ●, indicates an isolate able to demethylate pisatin, **O**, indicates an isolate unable to demethylate pisatin.

the other crosses the inheritance of this trait was more complex.

The analysis of the progeny of one cross representative of the first pattern of inheritance is presented in Fig. 2. The progeny fell into two classes regarding sensitivity to pisatin: tolerant (5–21% inhibition of radial growth) and sensitive (40–53% inhibition of radial growth). All of the tolerant progeny were pda$^+$, whereas all of the sensitive progeny were pda$^-$.

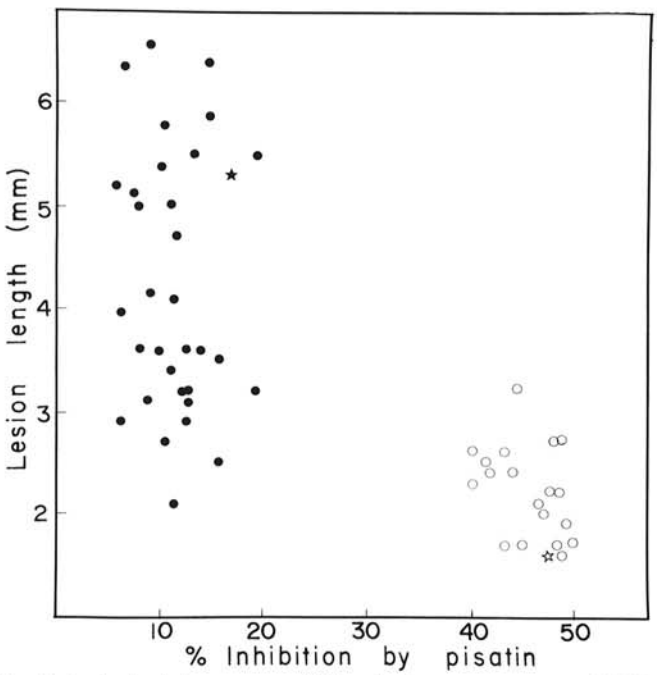

Fig. 2. Analysis of the progeny of *Nectria haematococca* cross 105 for pisatin sensitivity, pisatin demethylating ability, and virulence on pea. Each point represents one isolate. Isolate 6-36 (★) was the maternal parent and is positive for pisatin demethylating ability; isolate T213 (☆)was the paternal parent and is negative for pisatin demethylating ability. Progeny are denoted by circles; ●, isolate able to demethylate pisatin; O, isolate unable to demethylate pisatin.

TABLE 1. Analysis of random ascospore progeny of *Nectria haematococca* from cross 105 and crosses involving its progeny for pisatin sensitivity and pisatin demethylating ability

Cross	Parents[a]	Number of progeny pda$^+$	Number of progeny pda$^-$	Germination[c] (%)
105	6-36 pda$^+$ (17%) T213 pda$^-$ (48%)	34(5–21%)[b]	20(40–53%)	ND
134	105-5-8 pda$^+$ (26%) T213 pda$^-$ (48%)	27(12–24%)	3(50–55%)	65
135	105-5-6 pda$^+$ (26%) T213 pda$^-$ (48%)	36(17–31%)	4(55–57%)	82
113	105-43 pda$^-$ (43%) 6-36 pda$^+$ (17%)	26(5–24%)	29(44–67%)	78
142	105-18 pda$^-$ (49%) 101-66 pda$^+$ (9%)	62(5–24%)	51(43–65%)	69
130	105-5-6 pda$^+$ (26%) 105-10-2 pda$^+$ (20%)	24(17–35%)	0	85
131	105-5-6 pda$^+$ (21%) 105-10-5 pda$^+$ (21%)	24(17–30%)	0	51
132	105-5-1 pda$^+$ (24%) 105-10-4 pda$^+$ (26%)	22(15–27%)	0	80
133	105-5-8 pda$^+$ (26%) 105-10-8 pda$^+$ (23%)	24(17–29%)	0	72

[a] The maternal parent in the cross is listed first. The ability of each parental isolate to demethylate pisatin (pda$^+$, pda$^-$) is given and its percent inhibition of mycelial growth by 0.5 mM pisatin is listed in parentheses.
[b] The range of percent inhibition in mycelial growth by 0.5 mM pisatin for each class is given in parentheses.
[c] The percent germination of ascospores in that cross. ND indicates that the value was not determined.

All of the pisatin-sensitive progeny were weakly virulent on pea (Fig. 2), supporting the hypothesis that pisatin tolerance is a trait required for virulence. Some of the pisatin-tolerant progeny were also low in virulence (indicating recombination of loci for tolerance and virulence); this observation, like the finding that some pisatin-tolerant field isolates are low in virulence (Fig. 1), indicates that pisatin tolerance alone is not sufficient for virulence. Evidently the parents used in this cross differ at one or more loci important for virulence on pea in addition to the loci for pisatin tolerance.

The ratio of pisatin-tolerant to pisatin-sensitive progeny (34:20) in cross 105 did not fit the expectation for either one gene (1:1) or unlinked two gene (3:1) segregation for this trait (Table 1). Therefore, tetrad analysis was performed on this cross to determine the number of genes segregating for sensitivity and tolerance (and demethylating ability). Progeny of six tetrads segregated in a 4:4 (tolerant:sensitive) ratio for these attributes, and progeny of one segregated in a 6:2 ratio (representative tetrads in Table 2). The 6:2 tetrad indicates that more than one gene is segregating, and the direction of the ratio indicates that the tolerant allele of either gene is sufficient to confer the tolerant phenotype (and the ability to demethylate pisatin). No tetrads segregated in an 8:0 (tolerant:sensitive) ratio. The frequency of tolerant progeny from the random ascospore analysis of this cross may indicate that the loci segregating for tolerance are linked to each other or to genes affecting the viability of the progeny.

The absolute association between pisatin tolerance and demethylating ability was found in four other crosses (Table 1), all of which involved the progeny of cross 105 as parents. The lack of recombinants from these crosses suggests that pisatin tolerance and demethylating ability are conferred by the same loci or conferred by separate loci not greater than 0.3 map units apart. Pairs of pda$^+$ isolates were crossed in an attempt to obtain pda$^-$ recombinants (Table 1). None was observed in the progeny of these crosses. In cross 142, virulence was segregating in addition to pisatin sensitivity and demethylating ability, giving results similar to those in Fig. 2.

A second segregation pattern for pisatin sensitivity was observed in three other crosses (Table 3, Fig. 3). A continuous spectrum of sensitivity to pisatin occurred in the progeny of these crosses, suggesting that sensitivity was segregating polygenically. All of the most tolerant progeny were pda$^+$, but a few of the highly sensitive progeny were also pda$^+$. The finding that pisatin sensitivity is not always associated with the lack of demethylating ability, suggests

TABLE 2. Inheritance of pisatin sensitivity, demethylating ability, and virulence to pea in tetrads from cross 105 of *Nectria haematococca*

Ascus	Spore[a]	Mating type	Perithecium color	Inhibition (%)	Demethylation (%)	Lesion length (mm)
8	1	−	Red	53	−3	2.6 ± 0.5
	4	−	Red	54	−6	2.9 ± 0.3
	2	−	White	49	5	2.6 ± 0.7
	5	−	White	49	−6	2.6 ± 0.5
	3	+	Red	17	41	5.9 ± 1.5
	6	+	White	25	44	6.7 ± 1.7
	7	+	White	22	45	6.2 ± 1.5
5	1	+	Red	24	45	6.7 ± 0.5
	8	+	Red	26	44	6.0 ± 1.2
	2	+	White	24	39	5.9 ± 0.6
	6	+	White	26	39	4.4 ± 1.3
	4	−	Red	15	48	3.1 ± 0.6
	7	−	Red	15	49	3.6 ± 1.1
	3	−	White	47	−6	2.5 ± 0.5
	5	−	White	47	−1	3.2 ± 0.5

[a] Twins can be identified by mating type and perithecium color.

that in these crosses demethylating ability was conferred by alleles or loci different from those segregating in the previous crosses. A number of the progeny proved to be variable in expressing demethylating ability, a phenomenon that was never observed in the progeny listed in Table 1. In the initial assays for demethylating ability, 40 of 149 progeny from crosses 126 and 127 were pda$^-$. Upon reassaying, however, 10 of the pda$^-$ progeny were characterized as pda$^+$ (these are indicated as pda$^+$ in Table 3 and Fig. 3). Five of the pda-variable progeny were inhibited less than 25% in all of the replicated assays; thus they sometimes grew at a pisatin-tolerant rate in the same assay plate in which they apparently failed to demethylate pisatin. However, it may be that these progeny can occasionally incorporate the ^{14}C from (3-*O*-methyl-^{14}C)-pisatin into a nonvolatile product, giving a false pda$^-$ result in this assay.

Tetrads were isolated from cross 103; four tetrads exhibited a 4:4 segregation ratio (pda$^+$:pda$^-$), one an 8:0 ratio, two a 6:2 ratio, and two a 2:6 ratio. Representatives of the tetrad types are given in Table 4. The recovery of tetrads segregating in 6:2 and 8:0 ratios indicates that at least two loci were segregating for demethylating ability. The recovery of tetrads that segregated in a 2:6 ratio (pda$^+$:pda$^-$) suggests that a third pda$^-$ allele, which is epistatic to pda$^+$, may also have been segregating in this cross. As was seen in the analysis of random ascospore progeny from this cross, generally those progeny from tetrads that were most tolerant of pisatin were pda$^+$ and the most sensitive progeny were pda$^-$, but discrete classes of sensitivity levels were not apparent. Genes that affect the sensitivity of the progeny, in addition to those determining demethylating ability, segregated in this cross but tolerance and demethylating ability were not absolutely linked.

The progeny of crosses 126 (Fig. 3) and 127 also segregated for virulence. All of the most virulent progeny (>7 mm lesions) were tolerant of pisatin (inhibited <30%) and pda$^+$. Loci important for virulence, other than the loci for pisatin tolerance, apparently segregated in these crosses also, since some pisatin tolerant progeny were low in virulence. Thus, despite the more complex inheritance of pisatin tolerance and demethylating ability in these crosses, the results support the hypothesis that tolerance and pisatin demethylating ability are required for virulence.

Recovery of pisatin from pea tissue infected with *N. haematococca* MP VI isolates. If pisatin tolerance and pisatin demethylating ability are required for virulence on pea, seedlings of the pea cultivar Alaska 2B should accumulate pisatin under the conditions of the virulence assay. To verify this, we inoculated seedlings with six different field isolates. The length of lesions formed was determined 4 days after inoculation; the lesion tissue was then excised and pisatin was extracted from the lesions (41–49 lesions for each isolate) using the procedure of Pueppke and VanEtten (10). No pisatin was detected in healthy pea tissue but pisatin did accumulate in the lesions caused by the six isolates (Table 5).

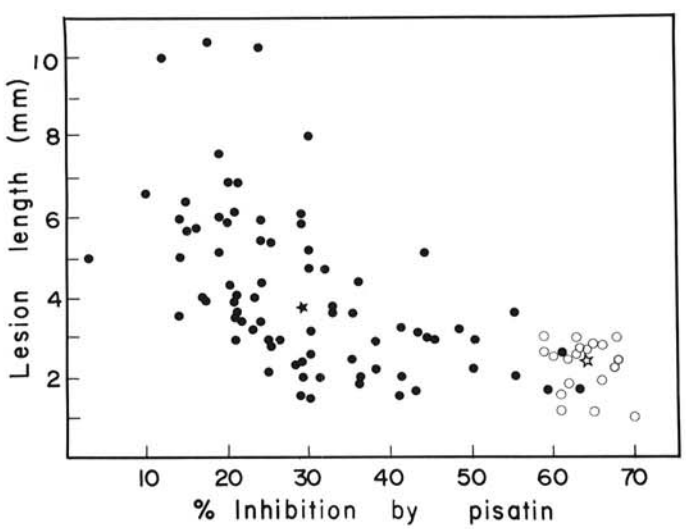

Fig. 3. Analysis of the progeny of *Nectria haematococca* cross 126 for pisatin sensitivity, pisatin demethylating ability, and virulence on pea. Each point represents one isolate. Isolate T33 (★) was the maternal parent and positive for pisatin demethylating ability; isolate T221 (☆) was the paternal parent and negative for pisatin demethylating ability. Progeny are denoted by circles; ●, isolate able to demethylate pisatin; O, isolate unable to demethylate pisatin.

DISCUSSION

A correlative relationship between two traits can be tested to determine whether or not the correlation is causal by selectively preventing the expression of one trait and determining the effect on

TABLE 3. Analysis for pisatin-demethylating ability and sensitivity to pisatin of progeny of *Nectria haematococca* from crosses which segregated polygenically for pisatin sensitivity

Cross	Parents[a]	Number of progeny pda$^+$	Number of progeny pda$^-$	Germination[b] (%)
126	T33 pda$^+$ (15%) T221 pda$^-$ (62%)	98(3–66%)[c]	20(55–70%)	30
127	T33 pda$^+$ (15%) T95 pda$^-$ (60%)	21(20–63%)	10(57–71%)	7
103	T161 pda$^+$ (21%) T110 pda$^-$ (57%)	43(23–59%)	23(45–70%)	ND

[a] The maternal parent in the cross is listed first. The ability of the parental isolate to demethylate pisatin is indicated (pda$^+$, pda$^-$) and its percent inhibition of mycelial growth at 0.5 mM pisatin is given in parentheses.
[b] The percent germination of ascospores in that cross. ND indicates that the value was not determined.
[c] The range of percent inhibition of mycelial growth by 0.5 mM pisatin for each class is given in parentheses.

TABLE 4. Inheritance of pisatin demethylating ability and pisatin sensitivity in tetrads from cross 103 of *Nectria haematococca*

Ascus	Spore[a]	Mating type	Sex	Inhibition (%)	Demethylation (%)
13	4	+	fem$^+$	35	51
	5	+	fem$^+$	35	51
	3	+	fem$^-$	35	52
	1	–	fem$^+$	56	−8
	2	–	fem$^-$	58	−4
	6	–	fem$^-$	62	−4
3	1	+	fem$^+$	29	62
	2	+	fem$^+$	32	60
	4	+	fem$^+$	30	58
	7	+	fem$^+$	29	61
	3	–	fem$^+$	35	56
	5	–	fem$^-$	33	58
	6	–	fem$^-$	33	60
6	3	–	fem$^+$	40	53
	5	–	fem$^+$	42	57
	8	–	fem$^+$	36	58
	6	+	fem$^-$	57	1
	7	+	fem$^-$	48	68
2	1	–	fem$^+$	54	−5
	6	–	fem$^+$	53	−6
	2	+	fem$^+$	70	0
	3	+	fem$^-$	33	49
	4	...	...	33	52
	5	–	fem$^-$	58	−9

[a] Isolate T161 (pda$^+$, 21% inhibition of mycelial growth) was the maternal parent; the paternal parent was isolate T110 (pda$^-$, 57% inhibition of mycelial growth). Twins can be identified by mating type and sex.

the other. Both chemical and genetic techniques of altering expression of activity have been used for this purpose. The lack of selectivity of many chemical inhibitors limits their usefulness, however.

The role of phytoalexin tolerance and phytoalexin degradation in the virulence of a pathogen has not been tested previously by genetic analysis. *N. haematococca* MP VI was selected in part because results of assays on field isolates support the hypothesis that phytoalexin tolerance and degradation are required for significant virulence on this phytoalexin-producing host. The naturally occurring variability in phytoalexin sensitivity among isolates of this species eliminated the need to employ mutagenesis and selection procedures to obtain pisatin-sensitive isolates. This natural variability in pisatin sensitivity may be due to the ability of *N. haematococca* MP VI to grow in nature on hosts and in habitats that do not contain or synthesize pisatin (15).

In tests of over 300 progeny derived from the four crosses that were segregating for virulence, pisatin sensitivity, and pisatin demethylating ability, no progeny that were highly sensitive to pisatin or lacked pisatin demethylating ability were even moderately virulent on pea. This finding represents strong evidence that in this organism pisatin tolerance and pisatin-demethylating ability are required for significant virulence on pea. Indirectly, it also provides a new kind of evidence for the hypothesis that the accumulation of pisatin in pea is indeed an active mechanism of resistance. Among the weakly virulent, pisatin-sensitive progeny already obtained there may exist some for which the *only* virulence trait that is lacking is pisatin tolerance or pisatin-demethylating ability. The resistance of a pea plant to any *N. haematococca* MP VI isolate of this type would be mediated solely by its accumulation of pisatin.

A few isolates that were sensitive to pisatin did have the ability to demethylate pisatin. Apparently demethylating ability alone is not always sufficient to produce tolerance. Also, growth of the isolates of MP VI that are most sensitive to pisatin is not completely inhibited by 0.5 mM pisatin, a concentration that completely inhibits the growth of some fungi (12,16,20). The most sensitive isolates of MP VI do not have detectable demethylating ability, and therefore, the limited tolerance they do express probably originates from a mechanism other than the breakdown of pisatin. The biochemical or genetic basis of this tolerance mechanism is unknown but presumably all the isolates used in this study possess the same tolerance mechanism upon which demethylating ability may be superimposed.

One unexpected finding was that the ability to demethylate pisatin can be conferred by more than one locus. In both crosses in which tetrads were analyzed, segregation at more than one locus was evident. In the progeny from some crosses the association between tolerance and demethylating ability was absolute, in others it was not. Whether the alleles segregating in the two kinds of crosses are at the same loci or at different loci was not determined. The loci segregating in these crosses may be different structural genes, duplications of the same structural genes, or may be regulatory genes. Since several loci can confer the pda$^+$ phenotype, it was anticipated that pda$^-$ progeny might be recovered from crosses between different pda$^+$ isolates. No pda$^-$ progeny were isolated from four such crosses. However, epistatic effects between genes for pisatin demethylating ability may occur in the progeny of cross 103.

TABLE 5. Recovery of pisatin from pean tissue infected by *Nectria haematococca*

Isolate	Inhibition by pisatin at 0.5 mM (161 μg/ml) (%)	Demethylating ability	Lesion length[a] (mm)	Pisatin per cm^3 of lesion tissue[b] (μg)
T30	16	+	11.5 ± 1.2	96
T8	15	+	9.5 ± 2.1	115
T221	52	−	2.9 ± 0.6	295
T110	57	−	1.7 ± 0.9	482
T95	55	−	2.4 ± 0.7	505
T213	47	−	2.9 ± 1.0	551
control				N

[a]The length of lesions produced by the isolates was determined and then 41–49 lesions produced by each isolate were extracted and assayed for pisatin.

[b]The values given were not corrected for extraction efficiency. *N* = none detected.

The results of this study suggest that pisatin tolerance and the ability to degrade this phytoalexin are required for the virulence of *N. haematococca* MP VI on pea or that genes for these traits are closely linked. While the lack of pisatin sensitive, pda$^-$, virulent recombinants of *N. haematococca* MP VI does not prove that the relationship is causal, it does provide strong circumstantial evidence in support of it. The development of near-isogenic isolates differing in sensitivity to pisatin and/or demethylating ability, but having a highly virulent genetic background, would be an even more stringent test of this association. However, the results obtained from the current study provide the strongest evidence to date that the ability to degrade and tolerate phytoalexins can determine the virulence of a plant pathogen.

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