Regulation of Pisatin Demethylation in \textit{Nectria haematococca} and its Influence on Pisatin Tolerance and Virulence

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\textit{Nectria haematococca}, a fungal pathogen of pea, demethylates the pea isoflavonoid phytoalexin pisatin to yield the less inhibitory product, 3,6a-dihydroxy-8,9-methylenedioxypterocarpan. Among naturally occurring isolates that demethylate pisatin (PDA$^+$ isolates), some can be induced to do so at a high rate by pretreatment with the substrate (PDA$^i$ isolates), while others demethylate pisatin only slowly, regardless of pretreatment (PDA$^a$ isolates). Genetic analysis of these pisatin demethylation phenotypes has indicated that one gene confers the PDA$^i$ phenotype and that two distinct genes at different loci each confer the PDA$^a$ phenotype. We report here the relationship of these PDA phenotypes to pisatin tolerance and to virulence toward pea. The contribution of each PDA gene to these traits was also evaluated. In initial crosses between tolerant, PDA$^+$ parents, recombinant progeny lacking demethylating activity (PDA$^-$ isolates) occurred. These PDA$^-$ progeny were uniformly more sensitive to pisatin than the PDA$^+$ parents and all PDA$^+$ progeny. Progeny having PDA$^i$ phenotypes were more tolerant to pisatin than PDA$^a$ progeny, which in turn were more tolerant than PDA$^-$ phenotype. Further genetic analyses confirmed that a characteristic pattern of pisatin tolerance was associated with each PDA gene. The gene conferring the PDA$^i$ phenotype was associated with the highest level of tolerance, while the two genes conferring PDA$^a$ phenotypes were each associated with an intermediate level of pisatin tolerance. Thus relative tolerance to pisatin appears to depend upon both the presence and amount of pisatin demethylase activity. Virulence toward pea may also be determined by the ability to demethylate pisatin rapidly. In numerous crosses between \textit{N. haematococca} isolates with different pisatin demethylation phenotypes, high virulence in progeny was always correlated to the PDA$^i$ phenotype. All PDA$^a$ and PDA$^-$ progeny were low in virulence. Thus the gene that confers high pisatin demethylase activity in \textit{N. haematococca} is, or is closely linked to, a gene for virulence to pea.

**INTRODUCTION**

\textit{Nectria haematococca} Berk. and Br. (imperfect stage \textit{Fusarium solani}) mating population VI (MP VI), a fungal pathogen of \textit{Pisum sativum} L., can demethylate the major phytoalexin of pea, pisatin, to the less toxic product DMDP (VanEtten et al., 1975). Pisatin demethylase in this organism is accomplished by a microsomal cytochrome P-450 monooxygenase (Desjardins et al., 1984; Matthews & VanEtten, 1983). Recent genetic analysis has indicated that three non-allelic genes are each sufficient to confer pisatin demethylating ability in this organism (Kistler & VanEtten, 1984). Two genes, at the loci \textit{pda-2} and \textit{pda-3}, each confer a phenotype with low enzyme activity (PDA$^+$), while a gene at locus \textit{pda-1} is responsible for a phenotype with

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Abbreviation: DMDP, 3,6a-dihydroxy-8,9-methylenedioxypterocarpan.
inducible, high enzyme activity (PDA'). Isolates with negative alleles at all three loci have a
pisatin non-demethylating (PDA-) phenotype. The purpose of this investigation was to
examine the relationship of different pisatin demethylating phenotypes with pisatin tolerance
and virulence toward pea, as well as to evaluate the influence of individual genes for pisatin
demethylation on these traits. Field isolates and progeny of crosses among field isolates were
examined. If fungal virulence and tolerance to pisatin result from pisatin demethylation,
quantitative differences in demethylating ability between PDA' and PDA' isolates might affect
their relative tolerance to the phytoalexin and their virulence toward pea.

METHODS

Cultures. All cultures of N. haematococca MP VI were maintained on V-8 juice agar (medium 29; Stevens, 1974)
at 20 °C under continuous fluorescent lighting. Isolates T-2 and T-9 were isolated as pea pathogens whereas isolate
T-219 was isolated from soil (VanEtten, 1978; VanEtten et al., 1980). Other cultures were obtained as ascospore
progeny derived from these field isolates. Genetic analysis of progeny has been described previously (Kistler &
VanEtten, 1984), as have techniques for propagation of ascospore progeny in N. haematococca (Tegtmeier &
VanEtten, 1982a; VanEtten, 1978).

Chemicals. Pisatin, DMDP, and pisatin 14C-labelled at the 3-O-methyl carbon (14Cpisatin) were obtained and
quantified as described in the preceding paper (Kistler & VanEtten, 1984).

Pisatin demethylase phenotypes. Procedures for defining and determining pisatin demethylase phenotypes in
liquid and agar cultures were as described in the preceding paper (Kistler & VanEtten, 1984). The substrate-
induced, high activity pisatin demethylating isolates (PDA') had demethylation rates from 31 to 138 pmol pisatin
min⁻¹ (mg wet wt mycelium)⁻¹. When absolute demethylation rates for the low activity, 'non-inducible' PDA-
isolates were determined, they ranged from 0.22 to 1.02 pmol min⁻¹ mg⁻¹. Non-demethylating (PDA-) isolates
showed no detectable rate (0.02 pmol pisatin demethylated min⁻¹ mg⁻¹), or no significant decrease in pisatin
congestion in agar cultures after 10 to 14 d (Kistler & VanEtten, 1984).

Bioassay of pisatin tolerance. The inhibitory effect of pisatin was measured by radial growth of mycelial colonies
on a peptone/glucose agar medium (M-2; VanEtten, 1973) containing pisatin. This procedure has been
described previously (VanEtten et al., 1980). Conidial suspensions of isolates to be tested were spread on Petri plates
containing M-2 and plates were incubated in the dark at 24 °C. After 2 d, 4-0 mm diameter plugs were cut from
these cultures with a cork borer and placed, mycelium-surface down, at the edge of a Petri plate (35 × 10 mm)
containing 1 ml M-2. Media in these plates were supplemented either with 161 μg pisatin in 25 μl DMSO, or with
the solvent alone. Final concentration of pisatin was 0.5 mM. For dose-response experiments on DMDP, the
compound was added in 25 μl DMSO to give final concentrations of 0, 0-03, 0-06, 0-12, 0-24, and 0-50 mM. Plates
were incubated in the dark at 24 °C.

Assessment of pisatin tolerance involved measurement of mycelial growth to the nearest 0.1 mm using a
dissection microscope with an ocular micrometer. The radius of the colony (in mm) at four or more observations
between 72 and 188 h was plotted against time for both treatment and control cultures.

Mycelial growth of fungi on agar medium supplemented with phytoalexins is frequently non-linear, making
quantitative comparison of inhibition sometimes difficult (Bailey et al., 1976; Skipp & Bailey, 1977). Indeed, we
observed non-linear growth kinetics for N. haematococca on agar both with and without pisatin (Fig. 1a). All
cultures, however, were observed to be in the linear phase of growth after 72 h. Therefore the linear regression of
observations beginning at 72 h was used to determine the final, maximum growth rate of a particular isolate in
both treatment and control cultures (Fig. 1b). This final growth rate on medium with pisatin, expressed as a
percentage inhibition of the control growth rate (% control growth rate), was one measure of pisatin effect. The difference
between x intercepts of these regression lines in pisatin-treated and control cultures (∆x) was another measure of
pisatin effect: the additional time required for pisatin-treated cultures to achieve the final, linear phase of growth.
‘End-point’ percentage inhibition (% end-point), the overall inhibition of the colony radius at the time control
cultures had grown to the edge of their Petri plates (6 to 8 d), was a third measure of pisatin effect, encompassing both
the increased length of the lag period and inhibition of the final growth rate.

Virulence bioassay. Virulence of individual fungal isolates was determined by the previously described 'test-tube
assay' (VanEtten et al., 1980), except that virulence was judged 6 instead of 4 d after fungal inoculation. Peas
(Alaska 2B, Aggrow Seed Co., Kalamazoo, Mich., USA) were grown in test tubes containing vermiculite and
Hoagland's solution (Hoagland & Arnon, 1938) in a high humidity growth chamber (28 °C) in the dark for 5 d, and
then for 2 d under fluorescent lighting. Epicotyls were then wounded with a small needle and inoculated with a
3 mm diameter plug of 2 d mycelium grown on the surface of Ustilago minimal medium (medium M-100; Stevens,
1974). Inoculated plants were then incubated in the lighted humidity chamber for 6 d. At this time, virulence was
measured by the mean length of the resulting tan to brown epicotyl lesions on six to eight replicate plants.
Wounded but uninoculated control plants displayed little (<1 mm) or no discoloration at the site of the wound.
Pisatin tolerance and virulence in Nectria

Fig. 1. (a) Effects of pisatin on growth of Nectria haematococca on agar medium. For the purpose of this illustration, three isolates with essentially identical growth kinetics in the absence of pisatin were chosen (all denoted by ○). Growth of the PDA' (■), PDA" (▲), and PDA - (●) isolates in the presence of 0.5 mM-pisatin. (b) Linear regression of the final four data points shown in (a). Final growth rate is defined by the slope of the regression lines. Also shown is the lag time (Δt) for growth of the PDA" isolate in the presence of pisatin.

Table 1. Effect of 0.5 mM-pisatin on mycelial growth of parent isolates

<table>
<thead>
<tr>
<th>Parent isolate</th>
<th>Demethylation rate*</th>
<th>Demethylase phenotype</th>
<th>%I growth rate</th>
<th>Δt (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-2</td>
<td>64 ± 40</td>
<td>PDA'</td>
<td>9 ± 6*</td>
<td>1 ± 4*</td>
</tr>
<tr>
<td>T-9</td>
<td>93 ± 30</td>
<td>PDA'</td>
<td>17 ± 5*</td>
<td>5 ± 2*</td>
</tr>
<tr>
<td>T-219</td>
<td>0.33 ± 0.11</td>
<td>PDA&quot;</td>
<td>33 ± 5*</td>
<td>11 ± 4*</td>
</tr>
<tr>
<td>44-100</td>
<td>&lt;0.02</td>
<td>PDA -</td>
<td>55 ± 6*</td>
<td>50 ± 8*</td>
</tr>
</tbody>
</table>

* Pisatin demethylation rates in pmol min⁻¹ (mg wet weight mycelium)⁻¹ as determined by Method 1 (T-2 and T-9) or by Method 2 (T-219 and 44-100), as described in the preceding paper (Kistler & VanEtten, 1984).

RESULTS

Measurement of pisatin tolerance

Pisatin tolerance for four isolates used as parents in genetic studies is given in Table 1. The two PDA' field isolates (T-2 and T-9) were the most tolerant of pisatin. Both showed only slight end-point inhibition, which was reflected in modest values for %I growth rate and Δt. The PDA" isolate (T-219) showed a significantly greater end-point inhibition by 0.5 mM-pisatin. The major component of this difference seemed to be the significantly longer Δt of T-219. End-point inhibition was greatest for the PDA - isolate 44-100, and reflected primarily the contribution of %I growth rate, that is inhibition of the final rate of growth. A similar correlation between growth characteristics affected by pisatin and the pisatin demethylating phenotype was found in the progeny derived from these parents, as will be discussed below.

Dosage response to DMDP

Isolates T-2, T-219, and 44-100 were grown on agar medium containing different concentrations (0.03 to 0.5 mM) of demethylated pisatin (DMDP). All three isolates were highly tolerant (end-point inhibition ≤ 8%) of DMDP at all concentrations tested. No difference in sensitivity to DMDP could be discerned among the isolates.

Pisatin tolerance in progeny of isolates of different pisatin demethylase phenotypes

To examine the three pisatin demethylase phenotypes for their relative contribution to pisatin tolerance, crosses were made between isolates displaying different pisatin demethylase
phenotypes. When all three phenotypes were found among random ascospore isolates of a single cross, the progeny were analysed for pisatin tolerance. These results are summarized in Table 2.

The quantitative growth responses of these progeny to pisatin fell into categories correlated with the pisatin demethylase phenotypes. The extent of the variation among progeny within a category is indicated by the standard deviations in Table 2. The nature of the response in each category was similar to that of the parent isolates described above. Overall inhibition (%I end-point) was greater for PDA" progeny than for PDA" progeny, and the latter in turn were significantly more inhibited than PDA' progeny. The effect of pisatin on PDA" progeny was expressed primarily as an increase in the length of the lag phase of growth; these isolates usually had final growth rates not significantly different from those of the most tolerant (PDA') progeny. PDA" progeny, on the other hand, were affected most strongly with regard to their final growth rates, and frequently showed no significant lengthening of the lag phase by pisatin. There was some overlap between the %I end-point values of some individual PDA" progeny and individual progeny of the other two phenotypes. However when effects on %I growth rate and At were considered separately, all of the PDA" isolates were resolved as a distinct, intermediate tolerance class.

It was previously observed in some crosses between pisatin demethylating (PDA') and PDA" isolates of *Nectria haematococca* that progeny displayed a continuous spectrum of sensitivity to pisatin, with PDA' progeny at times being as sensitive to pisatin as PDA" progeny (Tegtmeier & VanEtten, 1982b). It was suggested that tolerance to pisatin can sometimes be a polygenically inherited trait. In the previous report tolerance was measured only with regard to %I end-point. In the present study we found that measurement of response to pisatin in terms of %I growth rate led to discrete classes of pisatin sensitive progeny, which were all PDA", and pisatin tolerant progeny which were all PDA' (either PDA' or PDA") (Table 3). Re-examination of some of the progeny reported by Tegtmeier & VanEtten (1982b) gave similar results (Table 3, cross 126). Therefore we have no clear evidence at this time for polygenic inheritance of pisatin tolerance, or for any genes affecting pisatin tolerance, other than the three identified as controlling pisatin demethylating phenotype (Kistler & VanEtten, 1984).
Table 3. Comparison of ranges of pisatin inhibition expressed as %I end-point and %I growth rate in progeny of selected crosses

<table>
<thead>
<tr>
<th>Cross no.</th>
<th>Demethylase phenotype</th>
<th>n*</th>
<th>Range of inhibition (%)</th>
<th>End-point</th>
<th>Growth rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>74</td>
<td>PDA⁺</td>
<td>20</td>
<td>3-24</td>
<td>1-16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PDA⁻</td>
<td>22</td>
<td>25-50</td>
<td>1-36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PDA⁺</td>
<td>30</td>
<td>53-67</td>
<td>54-69</td>
<td></td>
</tr>
<tr>
<td>77</td>
<td>PDA⁺</td>
<td>23</td>
<td>2-39</td>
<td>-5-22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PDA⁻</td>
<td>10</td>
<td>23-52</td>
<td>8-28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PDA⁺</td>
<td>14</td>
<td>40-71</td>
<td>38-66</td>
<td></td>
</tr>
<tr>
<td>126†</td>
<td>PDA⁺</td>
<td>98 (19)</td>
<td>3-66</td>
<td>(9-35)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PDA⁻</td>
<td>20 (20)</td>
<td>55-70</td>
<td>(45-59)</td>
<td></td>
</tr>
</tbody>
</table>

* Number of progeny tested per phenotypic class.
† Data from Tegtmeier & VanEtten (1982b). PDA⁺, progeny having pisatin demethylating ability. Values in parentheses are from a re-assay of isolates which showed overlapping pisatin tolerance as measured by %I end-point (unpublished data of P. S. Matthews & H. D. VanEtten).

Table 4. Pisatin tolerance conferred at different pisatin demethylase loci

See Table 2 for explanation of headings and values listed.

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Cross no.</th>
<th>Progeny phenotype</th>
<th>Inferred genotype</th>
<th>Control growth rate (mm h⁻¹)</th>
<th>%I end-point</th>
<th>%I growth rate</th>
<th>Δt (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>62</td>
<td>PDA⁺</td>
<td>1 - - + 25</td>
<td>0.175 ± 0.004a</td>
<td>33 ± 4a</td>
<td>15 ± 3a</td>
<td>30 ± 6a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PDA⁻</td>
<td>- - - 21</td>
<td>0.175 ± 0.004a</td>
<td>78 ± 2b</td>
<td>77 ± 3b</td>
<td>2 ± 11b</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>PDA⁺</td>
<td>+ - - 23</td>
<td>0.204 ± 0.005a</td>
<td>31 ± 2a</td>
<td>21 ± 3a</td>
<td>13 ± 4a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PDA⁻</td>
<td>- - - 18</td>
<td>0.201 ± 0.012a</td>
<td>71 ± 4a</td>
<td>73 ± 3b</td>
<td>-5 ± 6a</td>
</tr>
<tr>
<td>3</td>
<td>99</td>
<td>PDA⁺</td>
<td>- + - 18</td>
<td>0.115 ± 0.003a</td>
<td>27 ± 6a</td>
<td>7 ± 3a</td>
<td>34 ± 12a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PDA⁻</td>
<td>- - - 22</td>
<td>0.116 ± 0.003a</td>
<td>56 ± 3a</td>
<td>54 ± 3a</td>
<td>9 ± 7b</td>
</tr>
<tr>
<td>4</td>
<td>77</td>
<td>PDA⁺</td>
<td>+ - - 10</td>
<td>0.187 ± 0.004a</td>
<td>16 ± 2a</td>
<td>9 ± 3a</td>
<td>9 ± 2a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PDA⁻</td>
<td>+ - - 15</td>
<td>0.199 ± 0.004b</td>
<td>15 ± 2a</td>
<td>11 ± 3b</td>
<td>6 ± 4a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PDA⁺</td>
<td>- + - 10</td>
<td>0.189 ± 0.005a</td>
<td>29 ± 5b</td>
<td>11 ± 6a</td>
<td>22 ± 4b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PDA⁻</td>
<td>- - + 10</td>
<td>0.194 ± 0.015a</td>
<td>26 ± 3a</td>
<td>10 ± 4a</td>
<td>18 ± 5a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PDA⁺</td>
<td>- - - 15</td>
<td>0.198 ± 0.005b</td>
<td>51 ± 2a</td>
<td>53 ± 3b</td>
<td>4 ± 4c</td>
</tr>
</tbody>
</table>

* Symbols refer to alleles independently determined to be present at the three pisatin demethylase loci, pda-1 (positive allele confers PDA⁺ phenotype), pda-2 and pda-3 (positive alleles confer PDA⁺ phenotype).

Correlation of pisatin tolerance with genes for pisatin demethylation

Progeny were also analysed in crosses where previously described data (Kistler & VanEtten, 1984) indicated that pisatin demethylating ability was controlled by the difference between alleles at a single locus (Table 4). Alleles at the loci pda-2 and pda-3 each confer a PDA⁺ phenotype and result in progeny with growth characteristics similar to PDA⁺ parental isolates (cross 62 and cross 99 for pda-3 and pda-2, respectively). When a single allele for the PDA⁺ phenotype was segregating (at locus pda-1, cross 94), all PDA⁺ progeny were also tolerant to pisatin when compared to PDA⁻ progeny of the same cross.

We have previously reported (Kistler & VanEtten, 1984) that an active allele at locus pda-1 conferred an epistatic PDA⁺ phenotype, regardless of the alleles present at locus pda-2 or pda-3. PDA⁺ isolates were equally tolerant to pisatin whether active alleles were present at two loci (pda-1 and pda-2, cross 77) or one (pda-1, cross 94). PDA⁺ isolates showed intermediate pisatin tolerance, whether demethylation was conferred by an allele at locus pda-2 (cross 99) or pda-3 (cross 62). Thus all progeny that are capable of demethylating pisatin also have some degree of pisatin tolerance when compared to PDA⁻ progeny, regardless of the gene(s) conferring demethylating ability.
Considerable variation between experiments was observed, with regard to both the growth rates of control cultures and the degree of pisatin inhibition (e.g. experiments 1, 2, and 3 vs 4, Table 4). However when progeny of different crosses were compared directly in the same experiment, there was no significant difference in pisatin sensitivity within a pisatin demethylating phenotype and the differences in control growth rates were much reduced (experiment 4, Table 4).

The small differences in control growth rate observed in experiment 4 of Table 4 were not correlated with pisatin demethylating phenotype. Indeed, whenever progeny of different phenotypes from the same cross were compared directly there was little or no difference in general vigour as measured by this parameter (Tables 2 and 4).

Virulence of progeny of different pisatin demethylase phenotypes

In previous studies on the relationships between pisatin demethylation and virulence (Tegtmeier & VanEtten, 1982b; VanEtten et al., 1980), some PDA+ isolates were identified that were low in virulence. To determine whether differences in virulence among PDA+ isolates could be explained by differences in pisatin demethylase phenotypes, the virulence of PDA1 and PDA2 isolates was examined in ascospore progeny of crosses where these traits were segregating. When progeny of the initial cross between parents of PDA1 and PDA2 phenotypes (cross 44) were assayed for virulence, all moderately to highly virulent progeny were of the PDA1 phenotype (Fig. 2). PDA2 and PDA- progeny were both lower in virulence.

Three generations of backcrosses were made to a PDA- recurrent parent (44-100) from cross 44, with T-2 (the PDA1 parent of cross 44) or PDA1 progeny of successive crosses serving as the other parent. In all generations, enhanced virulence was always associated with progeny of the PDA1 phenotype. In the final generation, (cross 94, Fig. 3) only parental type PDA1 and PDA- ascospore isolates were found in the progeny. The 1:1 segregation for parental pisatin demethylase phenotypes in random ascospores and tetrads from this cross indicated single gene control of the PDA1 phenotype (Kistler & VanEtten, 1984) and this gene was designated pdu-f.

All PDA- progeny were low in virulence; all PDA1 progeny were higher in virulence. A similar correlation between fungal virulence and the PDA1 phenotype was seen when another, independently isolated PDA1 field isolate (T-9) was crossed with the PDA- isolate 44-100 (Kistler, 1983).

In contrast, when single alleles conferring the PDA2 phenotype were segregating in other crosses (crosses 62 and 99), no difference in virulence was seen between PDA2 and PDA- progeny. Both phenotypic classes were equally low in virulence whether the PDA2 phenotype was conferred at locus pdu-3 (Fig. 4) or pdu-2 (data not shown).

![Fig. 2. Virulence and pisatin tolerance of random ascospore progeny of cross 44. □ PDA1 parent; ▲, PDA2 parent; □, PDA1 progeny; △, PDA2 progeny; ○, PDA- progeny. Pisatin was added at 161 µg ml⁻¹, and tolerance is expressed as %I growth rate, as defined in the text. The standard deviations of the replicate virulence measurements for each isolate averaged 2.3 mm for PDA1 progeny, 0.8 mm for PDA2 progeny and 0.7 mm for PDA- progeny.](image-url)
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Fig. 3. Virulence and pisatin tolerance in random ascospore progeny of cross 94. ■, PDAʻ parent; ●, PDA- parent; □, PDAʻ progeny; ○, PDA- progeny. Pisatin was added at 161 μg ml⁻¹. The standard deviations of replicate virulence measurements for each isolate averaged 3.0 mm for PDAʻ progeny, and 1.0 mm for PDA- progeny.

Fig. 4. Virulence and pisatin tolerance in random ascospore progeny of cross 62. ▲, PDAʻ parent; ●, PDA- parent; Δ, PDAʻ progeny; ○, PDA- progeny. Pisatin was added at 161 μg ml⁻¹. The standard deviations of the replicate virulence measurements for each isolate averaged 1.1 mm.

DISCUSSION

Several lines of evidence suggest that an increased degree of tolerance to pisatin is the direct result of the ability of the fungus to demethylate this phytoalexin. Previous investigation of isolates of N. haematococca MP VI isolated from natural habitats indicated that inability to demethylate pisatin correlated with sensitivity to the phytoalexin (VanEtten et al., 1980). Crossing isolates that differed in ability to demethylate pisatin gave rise to progeny that co-segregated for enhanced pisatin tolerance and pisatin demethylating ability (Tegtmeier & VanEtten, 1982a). In addition, the three isolates used as parents in the present study (T-2, T-219, and 44-100) were equally insensitive to inhibition by the product of the pisatin demethylation reaction, DMDP.

The latter observation suggests that ability to demethylate pisatin alone could explain insensitivity to pisatin inhibition. However, it does not prove that demethylation is the only or necessarily the most critical mode of tolerance, since other mechanisms may also exist in the tested isolates. A 'non-degradative' mode of pisatin tolerance, independent of pisatin metabolism, was recently shown to be expressed during the early growth phase of N. haematococca in liquid culture (Denny & VanEtten, 1983a). The molecular basis for this adaptive, non-degradative tolerance mechanism is as yet unknown (Denny & VanEtten, 1983b).

However, results from the present study further support the necessity of demethylation for
maximum pisatin tolerance in the type of bioassay employed here. Relative tolerance is correlated with the rate at which different isolates demethylate pisatin. Isolates with the ability to demethylate pisatin rapidly (PDA\(^+\) isolates) are the most pisatin tolerant, both as parents (Table 1) and as progeny (Tables 2 and 4). Isolates that can demethylate pisatin only slowly (PDA\(^-\)) show intermediate pisatin sensitivity, while isolates that lack detectable pisatin demethylase activity (PDA\(^-\)) are the most pisatin sensitive. Thus quantitative as well as qualitative differences in pisatin demethylating ability are reflected in differences in pisatin tolerance.

Relative tolerance to the phytoalexin co-segregates with corresponding pisatin demethylase phenotypes in all crosses studied (Tables 2 and 4). Recombinant PDA\(^-\) progeny, derived from parents both of which could demethylate pisatin, were more sensitive to pisatin than was either parent (Table 1 and Table 2, cross 44). These results would be difficult to explain if pisatin demethylation were not acting as a basis for pisatin tolerance.

Previous work (Kistler & VanEtten, 1984) suggested that genes at three loci each are capable of conferring pisatin demethylase activity in progeny. In crosses where single genes for pisatin demethylating ability are segregating (crosses 62, 94, and 99), pisatin tolerance again co-segregates with demethylase activity (Table 4), regardless of the locus involved. This implies that pisatin tolerance results from the genes for pisatin demethylation, and not from closely linked loci.

Several generations of backcrosses of virulent, PDA\(^+\) isolates to a low virulence PDA\(^-\) isolate revealed no independent segregation of high virulence and the PDA\(^+\) phenotype. A cross between a virulent PDA\(^+\) field isolate of independent origin and the same PDA\(^-\) parent gave similar results (Kistler, 1983). A potential for virulence therefore either results from, or is closely linked to, the gene (or genes) that confer the high activity pisatin demethylase.

Virulence to pea is undoubtedly the result of many factors. It should be noted that even among PDA\(^+\) progeny of a third generation backcross (cross 94, Fig. 3), a good deal of variation still occurs with respect to virulence. Also, since virulence of fungal isolates in this study was measured by the reaction of wounded pea plants, attributes of the fungus that allow for successful penetration of intact pea surfaces were not considered. Enzymes involved in successful penetration of intact plants, such as cutinase, may also influence the virulence of *N. haematococca* toward peas (Koller et al., 1982).

An additional complicating factor in this study is the fact that many cytochrome P-450 monooxygenases have broad substrate ranges, and apparently homogeneous preparations of such enzymes are capable of mediating a range of oxidative reactions (Guengerich et al., 1982; Sato et al., 1982). Thus it is conceivable that the pisatin demethylase enzyme may confer virulence by way of an enzymic reaction unrelated to the demethylation of pisatin. However, a more straightforward interpretation is that pisatin demethylation results in rapid pisatin detoxification during infection, which in turn enhances the ability of the pathogen to colonize pea tissue.

Since PDA\(^+\) and not PDA\(^-\) isolates of the fungus are virulent pea pathogens, the difference between high and low virulence isolates may be determined by the ability to regulate a pisatin demethylase. However, this difference could also be due to structurally distinct enzymes with different maximum rates for pisatin demethylation. Since it is known that the different pisatin demethylase phenotypes result from separate genetic loci (Kistler & VanEtten, 1984), and since in some organisms multiple forms of cytochrome P-450 with overlapping substrate specificities are known to exist (Guengerich et al., 1982; Sato et al., 1982), factors other than regulation of a single enzyme may result in the different biological properties of PDA\(^+\) and PDA\(^-\) isolates.

Regardless of the reasons for different pisatin demethylase phenotypes, the results of this study suggest that isolates of *N. haematococca* incapable of pisatin demethylation, or capable of demethylation only at low rates, are restricted in their colonization of the plant by the rapid and extensive accumulation of this antifungal compound. A corollary is that pisatin is indeed acting in some instances as a disease resistance mechanism in pea.

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