FINAL TECHNICAL REPORT

Department of Energy Grant No. DE-FG03-96ER20239 from 7/1/96 to 6/30/98.

TITLE: TOLERANCE TO PHYTOALEXINS AND ITS IMPLICATION FOR THE EVOLUTION OF HOST SPECIFIC VIRULENCE TRAITS

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BACKGROUND: At the time of the submission of our last DOE proposal (summer 1995), we proposed a five year study that had the following three objectives:

- 1) **Determine the importance of non-degradative tolerance (NDT) to pisatin for the virulence of *N. haematococca* MPVI on pea.**
- 2) **Determine the importance of pda for virulence of *F. oxysporum* f. sp. *pisi*, *A. pisi* and *M. pinodes*.**
- 3) **Refine our understanding of those characteristics of the PDA genes that contribute to virulence and the role of non-degradative tolerance in the virulence of other pea pathogens.**

ACCOMPLISHMENTS:

Some of the above objectives were an extension of the objectives of our prior DOE grant (and are objectives of our current proposal). Research results published since 1995 or in final stages of preparation and sponsored in total or in part by past and present DOE grants are given below in the publication section. Progress in the past two year toward the three objectives of Grant No. DE-FG03-96ER20239 is summarized directly below. In addition, the related work of two graduate students that was not part of the above objectives is summarized briefly below. The work of these students relates conceptually to the objective of our prior DOE proposals in that it evaluates the importance of the detoxification of plant antibiotics in pathogenesis. Although the salaries of these students were paid from other sources, they benefited from the materials and supplies used for DOE funded projects. Thus, DOE support has been acknowledged in their publications as partially supporting their work.

1) Determine the importance of non-degradative tolerance (NDT) to pisatin for the virulence of *N. haematococca* MPVI on pea. As outlined in our 1995 renewal we actually had two different objectives that were listed under this one objective that, based on the reviews of our proposal, were not clearly delineated. The first objective was to characterize non-degradative tolerance (NDT) mechanisms to pisatin and the second was to use the NDT genes or other approaches to make *N. haematococca* mutants sensitive to pisatin in order to evaluate the role of pisatin tolerance in pathogenicity.

A) Characterize the mechanism of non-degradative tolerance to pisatin in *N. haematococca* MPVI. The first approach we had proposed to identify the NDT mechanism was to identify the genes for this mechanism without being guided by a preconceived notion of the biochemical basis of tolerance. The method we were going to use involved a standard cDNA-biotin subtraction technique. However, in the interim, a new PCR-based method, representational difference analysis (rda), a technique that has been used successfully to clone differentially expressed transcripts by subtraction and amplification, came to our notice (Hubank & Schatz. 1994. Nucleic Acids Res. 22: 5640). Rda is expected to yield clones of differentially expressed genes, in this case induced by incubation in pisatin. This technique should also include both positive and negative regulatory elements, and all genes involved in tolerance, whether part of a recognized resistance mechanism or not. We have made the induced cDNA pools and the first round of amplification to bulk the cDNA for subtraction but placed this approach on hold until we explore the possibility that a specific ABC transporter(s) is involved in

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NDT to pisatin.

Previously published work from this laboratory has demonstrated that the non-degradative tolerance of *N. haematococca* is energy dependent and relatively specific for pisatin. Since energy dependence and specificity are two hallmarks of the ABC (ATP Binding Cassette) efflux transporters, in a second "educated guess" approach, we used the efflux pump-specific inhibitor verapamil to test whether the NDT mechanism is due to an inducible ABC-like membrane pump. Verapamil (50 μ M) had no effect on the radial growth of *N. haematococca* but pretreatment with 2 μ M verapamil completely abolished inducible tolerance to high concentrations of pisatin, indicating the involvement of ABC efflux transporters in pisatin resistance. We have subsequently used ABC transporter-specific oligonucleotide PCR primers designed from the conserved regions of ABC genes from other species to amplify DNA from *N. haematococca* and obtained at least 12 PCR products. We are currently in the process of refining the PCR protocol to increase stringency and verifying the amplification of ABC transporter genes so that it can be combined with the RT-PCR approach described above.

B) Increase the sensitivity of *N. haematococca* to pisatin by a mutational approach. Prior work by a sabbatical visitor to our laboratory, Dr. Durgadas Kasbekar, has shown that ergosterol mutants (*erg-1* and *erg-3*) of *Neurospora crassa* have a greatly increased sensitivity to pisatin. We proposed to make ergosterol mutants in *N. haematococca* to test the effect of increasing the sensitivity of this fungus to pisatin on its ability to cause disease on pea.

In collaboration with Dr. Kasbekar at the Centre for Cellular and Molecular Biology, Hyderabad, India and Dr. Marc Orbach at the University of Arizona, the *erg-1* (sterol Δ 8,7 isomerase) and *erg-3* (sterol C-14 reductase) genes of *N. haematococca* have been isolated and characterized this past year (10). Complementation of the corresponding mutants in *N. crassa* verified that these are *erg-1* and *erg-3* genes. Surprisingly, *N. haematococca* has two functional *erg-3* genes which have less identity to each other at the amino acid level (56.7%) than to the *N. crassa* *erg-3* gene (59% and 67.8%). Since there is only one *erg-1* gene this will be our primary target for disruption in *N. haematococca*.

2) Determine the importance of Pda for virulence of *F. oxysporum* f. sp. *pisi*, *A. pisi* and *M. pinodes*.

A) The pda in *Phoma medicaginis* var. *pinodella*. Most of the work on biochemical characterization of pda in this fungus has been done under previous DOE funding but the work was completed this past year. As with *A. pisi* and *M. pinodes*, the pda from *Phoma medicaginis* var. *pinodella* is a cytochrome P450, based on cofactor requirements and its inhibition by carbon monoxide, cytochrome P450 inhibitors, and antibodies to NADPH:cytochrome P450 reductase. Also, like pda from the other pea pathogens, it is induced by pisatin, and has a fairly high degree of specificity for pisatin as a substrate. Although the K_m for pisatin is higher than the pdas of other pea pathogens, it is within the range found for specialized cytochrome P450s involved in biosynthetic reactions. The features of this pda, like the pdas from the other pea pathogens, suggest that these fungi have a specific cytochrome P450 for detoxifying pisatin. As with the pda genes from pea pathogens other than *F. oxysporum* f. sp. *pisi*, PDA-specific probes from *N. haematococca* do not detect the PDA gene in *P. medicaginis* var. *pinodella* by Southern analysis of genomic DNA. These results are consistent with the independent evolution of a specific P450 as a virulence trait in at least some of these fungi.

B) Attempts to clone the PDA gene of *M. pinodes*. We completed our attempts to isolate a PDA gene from a cosmid library of *M. pinodes* genomic DNA made in pMocosX by individually testing over 4000 transformants of *N. crassa* for pda. Statistically this is enough transformants to cover the genome of this fungus. Even though the first PDA gene from *N. haematococca* was isolated by transforming *A. nidulans* with a cosmid library of genomic DNA from *N. haematococca* and detecting the

gene by its enzymatic activity, this approach has been unsuccessful with *M. pinodes* whether using *A. nidulans* or *N. crassa* as the recipient fungus. We also transformed pisatin-sensitive *erg-1* mutants of *N. crassa* with the cosmid library and selected for increased tolerance to pisatin. Although we failed to isolate the *PDA* gene from *M. pinodes* by this procedure we did isolate 13 transformants of *N. crassa* that had increased tolerance to pisatin. Eight of these, as judged by PCR amplification using primers from conserved regions of *erg-1* genes, contained the *M. pinodes erg-1* gene. We anticipated cloning *erg-1* during this screen as an indication of the completeness of the cosmid library. Preliminary results indicate the five other cosmid clones that conferred increased tolerance to pisatin contained neither the *erg-1* nor a *PDA* gene. These may confer tolerance by a NDT mechanism.

We do not know the reason we failed to obtain a *PDA* gene from *M. pinodes* by the above approach but to avoid perhaps inadequate recognition of promoter sequences or processing difficulties as well as to more rapidly isolate *PDA* genes from *P. medicaginis* var. *pinodella*, *A. pisi*, and *M. pinodes*, we are developing a screen that is based on expression of *PDA* cDNAs in yeast.

3) Additional projects not mentioned in the 1995 proposal that have benefited from DOE funds.

A) Evaluation of the role of detoxification of the sorghum phytoanticipin cyanide for the pathogenicity of *Gloeocercospora sorghi*. Most of the research on the creation and evaluation of the *cht*⁻ mutant of *G. sorghi* was done prior to 1996. This *cht*⁻ mutant (G-24) was created by transformation-mediated gene disruption and was shown to lack *cht* activity by enzymatic assays. In addition it was demonstrated by Western analysis that G-24 does not produce *cht* protein after induction by HCN. This past year, an analysis of infected sorghum tissue demonstrated that there is no induction of a cryptic *cht* in G-24 *in planta* and a further analysis of the structure of the disrupted *CHT* locus was completed. However, there was no obvious reduction in virulence of this *cht*⁻ mutant which implies that detoxification of cyanogenic compounds is not needed for pathogenicity and that these compounds are not involved in disease resistances.

B) An evaluation of the importance of detoxification of the tomato phytoanticipin α -tomatine in pathogenicity. The saponin α -tomatine occurs as a phytoanticipin in tomato. A survey of fungi for sensitivity to this compound revealed that most pathogens of tomato are tolerant of α -tomatine and are able to degrade this compound to non-toxic products (6). However, some tomato pathogens also have a non-degradative tolerance to this compound. These results parallel those with pathogens of plants that produce either pisatin or HCN: pathogens exposed to toxic compounds from their host appear to develop complementary mechanisms to tolerate these chemicals. Prior work on the tomato pathogen *Septoria lycopersici* had resulted in the cloning of the gene encoding the enzyme, β 2-tomatinase, that detoxifies α -tomatine (1). β 2-tomatinase⁻ mutants of *S. lycopersici* have been made and are being used to evaluate the importance of β 2-tomatinase in pathogenicity on tomato.

PUBLICATIONS: Research publications (since 1995) which were supported in whole or in part by funds from our previous or present DOE grants and acknowledged as such in the publication.

Published:

- 1) Sandrock, R. W., D. DellaPenna and H. D. VanEtten. 1995. Purification and characterization of β 2-tomatinase, an enzyme involved in the degradation of α -tomatine and isolation of the gene encoding β 2-tomatinase from *Septoria lycopersici*. *Molecular Plant-Microbe Interactions* 8: 960-970.
- 2) Covert, S. F., Enkerli, J., Miao, V. P. W. and VanEtten, H. D. 1996. A gene for maackiain detoxification from a dispensable chromosome of *Nectria haematococca*. *Mol. and Gen. Genetics* 251:397-406.

- 3) Hirschi, K. and H. VanEtten. 1996. Expression of the pisatin detoxifying genes (PDA) of *Nectria haematococca* in vitro and in planta. *Molecular Plant-Microbe Interactions* 9: 483-491.
 - 4) Wasmann, C. C. and VanEtten, H. D. 1996. Transformation-mediated chromosome loss and disruption of a gene for pisatin demethylase decreases the virulence of *Nectria haematococca* on pea. *Molecular Plant-Microbe Interactions* 9: 793-803.
 - 5) Ciuffetti, L. M. and VanEtten, H. D. 1996. Virulence of a pisatin-deficient *Nectria haematococca* MPVI isolate is increased by transformation with a pisatin demethylase gene. *Molecular Plant-Microbe Interactions* 9: 787-792.
 - 6) Sandrock, R. W. and H. D. VanEtten. 1998 Fungal sensitivity to and enzymatic degradation of the phytoanticipin α -tomatine. *Phytopathology* 88: 137-143.
 - 7) VanEtten, H., S. Jorgensen, J. Enkerli, and S. F. Covert. 1998 Inducing the loss of conditionally dispensable chromosomes in *Nectria haematococca* during vegetative growth. *Curr. Genet.* 33: 299-303.
 - 8) George, H. L., K Hirschi and , H. D. VanEtten. 1998. Biochemical properties of the products of cytochrome P450 genes (PDA) encoding pisatin demethylase activity in *Nectria haematococca*. *Arch. Microbiol.* 170: 147-154.
- Manuscripts in preparation** for which at least a first draft has been completed:
- 9) Wang, P., R. W. Sandrock and H. D. VanEtten. Disruption of the gene (*CHT*) for detoxifying cyanide in the fungus *Gloeocercospora sorghi* increases its sensitivity to cyanide but does not reduce its virulence on a cyanogenic plant (for *Molecular Plant-Microbe Interactions*)
 - 10) Srinivas, G., T. Harper, K. Aparna1, D. Senfeldova1, R. Sandrock, M. Orbach, H. D. VanEtten, and D. P. Kasbekar. Cloning of the genes encoding sterol Δ 8,7 isomerase and two divergent forms of sterol C-14 reductase from the phytopathogenic fungus *Nectria haematococca* MPVI. (for *Gene*)
 - 11) Hirschi, K., H. George and H. VanEtten. Identification of a new *PDA* gene, an inactive homolog, and the biochemical characterization of *PDA* chimeras of *Nectria haematococca* (for *Gene*).
 - 12) George, H. L. and H. D. VanEtten. Presence of cytochrome P450s in fungal pathogens of pea that detoxify the pea phytoalexin pisatin (for *Microbiology*).
 - 13) Delserone, L. M., K. McCluskey, D. E. Matthews, and H. D. VanEtten. Pisatin demethylation by fungal pathogens and nonpathogens of pea: association with pisatin tolerance and virulence. (for *Phytopathology*)
 - 14) Hirschi, K. D. and H. D. VanEtten. A survey of field isolates of *Nectria haematococca* MPVI and related fungi for pisatin demethylase genes: identification of new active and inactive members of this gene family and association of specific members with pathogenicity on pea.(for *Current Genetics*)

Reviews that have covered research supported in part by DOE and acknowledged in the review:

- 15) VanEtten, H. D., R. W. Sandrock, C. C. Wasmann, S. D. Soby, K. McCluskey, and P. Wang. 1995. Detoxification of phytoanticipins and phytoalexins by phytopathogenic fungi. *Canadian Journal of Botany.* 73 (Suppl.
- 16) VanEtten, H. D. Straney, S. Covert and C. Kistler (in press) Update on selected topics of the genetics of *Nectria haematococca* mating population VI with special emphasis on its conditionally dispensable (CD) chromosomes: a source of habitat specific genes. In B. Summerell (ed.), *Fusarium: Paul E. Nelson Memorial Symposium*, APS Press.