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Gene Expression Profiles of *Drosophila melanogaster* Exposed to an Insecticidal Extract of *Piper nigrum*

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Black pepper, *Piper nigrum* L. (Piperaceae), has insecticidal properties and could potentially be utilized as an alternative to synthetic insecticides. Piperine extracted from *P. nigrum* has a biphasic effect upon cytochrome P450 monooxygenase activity with an initial suppression followed by induction. In this study, an ethyl acetate extract of *P. nigrum* seeds was tested for insecticidal activity toward adult *Musca domestica* and *Drosophila melanogaster*. The effect of this same *P. nigrum* extract upon differential gene expression in *D. melanogaster* was investigated using cDNA microarray analysis of 7380 genes. Treatment of *D.* melanogaster with *P. nigrum* extract led to a greater than 2-fold upregulation of transcription of the cytochrome P450 phase I metabolism genes *Cyp 6a8*, *Cyp 9b2*, and *Cyp 12d1* as well as the glutathione-S-transferase phase II metabolism gene *Gst-S1*. These data suggests a complex effect of *P. nigrum* upon toxin metabolism.

KEYWORDS: cDNA microarray; Piper nigrum; D. melanogaster; M. domestica; botanical insecticide; cytochrome P450; Cyp 12d1; Cyp 9b2; Cyp 6a8

1. INTRODUCTION

The black pepper plant, *Piper nigrum* L. (Piperaceae), has traditionally been used as a spice and as a medicine (*1*). The piperamides extracted from *P. nigrum* and other members of the genus *Piper* have shown potent insecticidal activity (*2*, *3*) and could potentially be utilized as alternatives to synthetic insecticides. *P. nigrum* has a high level of acute toxicity toward a number of economically important insects (*4*). Certain piperamides have a bifunctional mode of action linked to the presence of two functional groups. The isobutylamide functional group is a sodium channel agonist (*5*) that binds to site 2 of voltage-gated sodium channels and causes persistent neuronal activation (*6*). The methylenedioxyphenyl functional group is well characterized for its inhibition of polysubstrate monooxygenase (PSMO) activity (*7*) and is responsible for the synergistic properties of piperonyl butoxide (*8*) by slowing the metabolism of insecticides. Recent research has demonstrated that the piperamide piperine does, in fact, inhibit PSMO activity in *Musca domestica* (*9*) and Cyp 3A4 in human Caco-2 cells (*10*). In rats, piperine from *P. nigrum* inhibits cytochrome P450 enzyme activity within 1 h of administration (*8*). In rat hepatoma cells piperine causes a biphasic response in terms of cytochrome P450 mediated arylhydrocarbon hydroxylase activity with an initial inhibition followed by induction (*11*). This may be due to a feedback mechanism whereby an upregulation of transcription of cytochrome P450 genes occurs in response to the inhibition of cytochrome P450 enzymes. This has been shown to be the case for CYP 1A1 in the rat hepatoma 5L cell line where initial inhibition of the enzyme by piperine is followed by an increase in mRNA transcription and protein levels (*12*).

There is currently limited information available concerning the effect of *Piper* extracts and pure compounds upon gene expression. Previous research has found that a compound from *P. nigrum* inhibits the transcription of aflatoxin biosynthetic genes in *Aspergillus parasiticus* (*13*). The lignan piperlactum S isolated from *Piper kadsura* has also been found to suppress the synthesis of RNA and proteins and to stop the cell cycle progression in human T lymphocytes (*14*). In insects, the accumulated evidence concerning the insecticidal activities and physiological effects of phytochemicals obtained from *Piper* species suggests that they are likely to also cause significant perturbations in gene expression.

The goal of this study was to investigate the efficacy of a *P. nigrum* extract as an insecticide against the test insects *M. domestica* L. (Diptera: Muscidae) and *D. melanogaster* (Diptera: Drosophilidae) and to investigate the effect of this extract upon gene expression in *D. melanogaster*. This research was also intended to validate the use of cDNA microarray technology as a novel experimental platform to elucidate the possible mode of action of botanical insecticides and the insect response to these products. At the time of writing, few

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microarray studies of the effect of botanical extracts or compounds on insect gene expression had been published. In this study, cDNA microarrays containing 7380 of the approximately 14 000 *Drosophila melanogaster* genes were used to investigate the expressed transcriptome of this model insect in response to an acute exposure to *P. nigrum*. It was predicted that exposure to *P. nigrum* extracts would lead to the upregulation of a number of cytochrome P450 genes due to the biphasic nature of cytochrome 450 inhibition followed by induction exhibited by compounds with the MDP functional group.

2. MATERIALS AND METHODS

2.1. Insect Cultures and *P. nigrum* Extraction. A culture of *Musca domestica*, Benzon Research Inc. (Carlisle, PA), was maintained at room temperature (20−25 °C). Adults aged 7−14 days were used in all trials. An Oregon-R strain of *D. melanogaster* was maintained on instant *Drosophila* medium, formula 4-24, Carolina Biological Supply (Burlington, NC), at a temperature of 23 °C, relative humidity of 60%, and light:dark cycle of 16:8 h. Flies aged between 3 and 14 days were used in the LC_{50} experiments. For the microarray experiments flies aged 10−20 days were used. Adult female flies were used for all experiments, and age was calculated as the number of days after egg hatch.

An extraction was performed on *Piper nigrum* seeds (peppercorns) obtained from Country Bulk Inc. (London, Canada) as previously described (*15*). Voucher seed specimens were retained at the University of Ottawa. For each 200 g of plant material a 300 mL volume of ethyl acetate was used as a solvent and a 300 mL volume of water was used as a wash. The organic fraction was separated and washed a second time with another 300 mL of water. Excess water was removed using anhydrous sodium sulfate, followed by filtering through a Buchner funnel with Whatman No. 1 filter paper, and the solvent was removed using a rotary evaporator at 30 °C under reduced pressure until a resinous material was obtained. Stock *P. nigrum* extract solutions for all insect assays were formulated in 99% ethanol.

2.2. LC_{50} Determination. *Musca domestica.* Flies were anesthetized using CO_2 and maintained on ice prior to use. A total of 20 flies were placed on a filter paper in a 9 cm Pyrex Petri dish and sprayed with 2 mL of *P. nigrum* extract using a Potter's tower at a pressure of 5 psi (34.5 KPa). Nitrogen was used as the carrier gas. The range of *P. nigrum* extract concentrations assayed was 0.8, 1.6, 3.2, and 6.4 mg/mL. All assays included a solvent control. Following treatment, flies were transferred to a mesh-covered cage and provided with a 5% sucrose solution. Mortality was scored after 24 h, and LC_{50} values were calculated using Probit analysis (*16*). The assay was replicated three times.

D. melanogaster. The treatment procedure was similar to that for *M. domestica* except that only female flies were used and the range of *P. nigrum* extract concentrations assayed was 0.4, 0.6, 0.8, 1.0, and 1.2 mg/mL. Flies were placed in 3 dram glass vials plugged with cotton wool soaked in a 5% sucrose solution, and mortality was scored after 24 h. For the calculation of LC_{50} values the assay was replicated six times. All statistical calculations were identical with those used for *M. domestica*.

2.3. Phytochemical Analysis and Potter's Tower Calibration. The concentrations of three marker piperamides [piperine (PIP), 4,5-dihydropiperine (4,5-DHPIP) and piperlonguminine (PLG)] in the stock solutions of *P. nigrum* were quantified using HPLC (Agilent, 1100 series reverse phase LC/MSD) according to an established method (*15*) (**Table 1**). The quantity of the marker compound piperine which reached the Petri plates after each spray application of 0.9 mg/mL *P. nigrum* extract solution using Potter's tower was also determined (**Table 1**). To do this, empty 9 cm Pyrex Petri plates ($n = 3$) were sprayed with 2 mL of the *P. nigrum* solution. The inside walls of each plate were cleaned with a kimwipe dipped in 99% ethanol, and the *P. nigrum* extract present on the bottom of the plate was recovered using 3 mL of 99% EtOH prior to analysis. A 1 mL aliquot of this solution was filtered with a 0.2 μm PTFE syringe filter, Chromatographic Specialties Inc. (Brockville, Canada), and the piperine concentration was analyzed by HPLC as previously described. The total quantity of piperine on the Petri plate and the application rate of piperine/square centimeter were calculated.

Table 1. Phytochemical Characterization of the *P. nigrum* Extract Used in the Microarray Experiment Showing the Mean Application Rate of *P. nigrum* on Petri Plates Using Potter's Tower and the Concentrations of the Piperamides 4,5-Dihydropiperlonguminine, Piperlonguminine, 4,5-Dihydropiperine, and Piperine in the Stock Solution

piperine applicatn[a] (μg/cm^2)	piperamides present in stock soln[b] (μg/mL)			
	4,5-DHPLG	PLG	4,5-DHPIP	PIP
0.67(0.13)	0.59	2.73	20.81	251.40

[a] Mean of 3 replicates with standard error presented in parentheses. [b] Stock solution is 0.9 mg/mL.

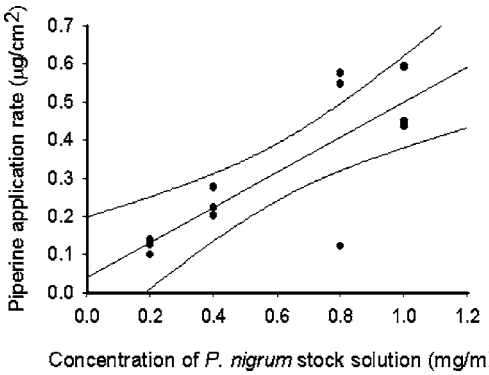

Figure 1. Linear regression of the application rate of piperine applied using Potter's tower to the concentration of the *P. nigrum* solution sprayed.

A simple linear regression was performed to determine the predictive value of the initial concentration of the *P. nigrum* stock solution (using concentrations of 0.2, 0.4, 0.8, and 1.0 mg/mL) in relation to the application rate of piperine using Potter's tower. The application rate of piperine to Petri plates showed a significant linear relationship to the concentration of *P. nigrum* in the solution used to spray the plate ($R^2 = 0.63$, $p = 0.002$, df = 1) (**Figure 1**) demonstrating that the concentration of the marker compound piperine applied using Potter's tower was consistent.

2.4. Insect Treatments for Microarray Analysis. A cDNA microarray analysis was used to investigate the effects of a high concentration of a *P. nigrum* extract (equivalent to the LC_{50} value) upon gene expression in *D. melanogaster* after a 4 h exposure time. Each independent replicate consisted of a sample of RNA from a treated or a control pool of *D. melanogaster*. The day of the experiment, female insects were placed in 5 dram glass vials (60 insects/vial) plugged with cotton wool soaked in a 5% sucrose solution and subsequently returned to the growth chamber. After 4 h the flies were anesthetized on ice, placed on a 90 mm diameter Whatman filter paper in a 90 mm Petri plate, and sprayed with a total of 2 mL of the selected treatment: 99% ethanol (solvent control) or a 0.9 mg/mL *P. nigrum* extract solution. This was done using a Potter's tower as previously described. Flies were returned to the vials and placed in the growth chamber for 4 h. This exposure time was chosen on the basis of a prior study in which insects exposed to immune response inducers had maximum gene expression between 4 and 6 h posttreatment (*17*). At this time all dead flies were removed from each sample and the remaining flies were anesthetized, flash frozen in liquid nitrogen, and stored at −80 °C prior to RNA extraction. Flies from four vials were pooled to give samples of approximately 200 whole insects. Samples to be pooled received the same treatment at the same time to minimize variation.

2.5. RNA Extraction. Total RNA was isolated using the acid guanidium thiocyanate−phenol−choloroform extraction method (*18*) according to the following specifications. Samples were homogenized in 1 mL of solution D using a Kontes pestle VWR (Mississauga, Canada), and insoluble material was removed from samples by centrifuging at 16 000*g* for 3 min. Three washes with cold 80% EtOH were performed to ensure the removal of red colored eye pigments and organic contaminants from the RNA pellet. RNA samples with A_{260}/A_{280} values between 1.8 and 2.0 were considered suitable for

Table 2. Differentially Expressed Genes Discussed in This Publication with a Fold-Change Value Greater or Equal to 1.41 or Less Than or Equal to −1.41 Identified by cDNA Microarray Analysis in Adult Female *D. Melanogaster* Treated with a 0.9 mg/mL Concentration of *P. nigrum* Compared to a Control of 99% EtOH

molecular functn	GO code	biological process	GO code	gene symbol	CG identifier	fold change
monooxygenase activity	GO:0004497	electron transport	GO:0006118	*Cyp9b2*	CG4486	3.12
monooxygenase activity	GO:0004497	electron transport	GO:0006118	*Cyp6a8*	CG10248	3.53
monooxygenase activity	GO:0004497	electron transport	GO:0006118	*Cyp12d1*	CG18240	2.51
monooxygenase activity	GO:0004497	electron transport	GO:0006118	*Cyp6d5*	CG3050	1.74
monooxygenase activity	GO:0004497	electron transport	GO:0006118	*Cyp6w1*	CG8345	1.80
monooxygenase activity	GO:0004497	electron transport	GO:0006118	*Cyp6d4*	CG12800	1.44
glutathione transferase activity	GO:0004364	response to toxin	GO:0009636	*GstS1*	CG8938	2.22
glutathione transferase activity	GO:0004364	response to toxin	GO:0009636	*GstE7*	CG17531	1.41
		response to cold	GO:0009409	*Fst*	CG9434	2.55
RNA binding	GO:0003723	transcriptn from Pol II promoter	GO:0006366	*Aly*	CG1101	2.03
receptor activity	GO:0004872	transmissn of nerve impulse	GO:0019226		CG7896	1.44
phosphoserine phosphatase activity	GO:0004647	L-serine biosynth	GO:0006564	*aay*	CG3705	2.04
					CG5107	−2.08
					CG11892	−2.79

microarray analysis. DNA was removed from the total RNA samples using RNase-free DNase (Promega).

2.6. Microarray Hybridization. Microarray slides were purchased from the Canadian *Drosophila* Microarray Center (CDMC), www.flyarrays.com (Mississauga, Canada). The *Drosophila* array version 7K3 was used in all experiments. This array contains 5756 target cDNAs from the Berkeley *Drosophila* Genome Project, 1078 cDNAs from the National Institutes of Health *Drosophila* testis cDNA library, and 546 gene fragments amplified from genomic DNA (*19*). Direct labeling of RNA and subsequent hybridization to microarray slides and scanning using the ScanArray 4000 XL (GSI Lumonics/Packard Biochips) was performed at the CDMC. Protocol details concerning labeled cDNA synthesis, hybridizations reactions, and scanner settings have been previously described (*19*). For each sample, 80 μg of total RNA was labeled with cyanine 3 or cyanine 5 (Cy3 or Cy5) dye. Two biological replicates and two reverse label hybridizations (using the same RNA samples but reversing the fluorescent labels) were used.

2.7. Analysis of Microarray Data. Quantarray Microarray Analysis Software version 3.0 (Packard BioScience, 2001) was used to quantify the raw images resulting from scans of the microarray slides. Images from the Cy3 and Cy5 channels of the same slide were superimposed, and spots with visually obvious defects were manually flagged for exclusion from analysis. Spot and background intensity were measured using the adaptive circle quantification method. The resulting data was analyzed using Gene Traffic Duo, version 2.8 (Iobion Informatics, 2002).

Data normalization was performed on background subtracted spots on a subgrid basis using the locally weighted scatter plot smoother (LOWESS) algorithm with a smoothing factor of 20. Spots with intensity values less than 100 units and spots with intensity values below the average background intensity value and/or below the local background intensity value were excluded from normalization and analysis. Data were filtered so as to exclude all genes with less than two-thirds of spots being usable as defined by quality filters and by a mean differential expression ratio with a coefficient of variance higher than 100% (*19*).

To determine an initial set of genes of interest for further study $\log_2$ ratio values of −1 and 1 were chosen as cutoff values indicating downregulation and upregulation, respectively. These values correspond to a 2-fold change in gene expression (*20*). An alternative threshold to identify additional differentially expressed genes in the context of this specific microarray platform and experimental design was also established. To do this, samples which had received identical ethanol treatments were hybridized to the same microarray slide. Differential expression ratios were determined for all genes and the number of genes with a given $\log_2$ ratio were plotted as a function of the range of $\log_2$ ratios observed. The selected threshold $\log_2$ ratios for differential expression for this microarray platform were equal to 0.5 and −0.5, equal to a 1.41-fold change in expression. The range between these two values encompassed over 99% of the differential expression ratios for the like−like hybridization (*21*). Differentially expressed genes were tabulated using the *Drosophila* genome annotation available in Flybase (*22*). Genes were presented in tables classified by their molecular function and biological process as listed in the gene ontology (GO) page of Flybase (*22*). Genes were subsequently divided into categories representing the known or inferred role they play in the organism based on the molecular function and/or biological process data.

Since Cyp genes were of special interest in the study, we note that there were 32 Cyp genes on the array. There were 12 Cyp4 genes, 11 Cyp6 genes, 3 Cyp9 genes, 2 Cyp12 genes, and 1 each of Cyp305 and Cyp310 genes.

2.8. Northern Analysis. Northern blot analysis was used to compare the level of differential expression of selected representative genes to the levels determined by microarray analysis. The three genes chosen for study were two upregulated genes, *Cyp 6a8* and *Cyp 9b2*, as well as a control gene, CG2196, which was not differentially expressed. The cDNA clones GH05558 (*Cyp 6a8*), GH08116 (*Cyp 9b2*), and GH19680 (CG2196) from the *Drosophila* Genome Collection release 1 (*23*) were obtained from the CDMC. Clone sequences were verified by sequencing at the Canadian Molecular Research Services Inc. (Ottawa, Canada) and the Core DNA Sequencing and Synthesizing Facility at the University of Ottawa Biotechnology Research Institute (Ottawa, Canada). Sequences were verified using NCBI BLASTn (*24*).

Probe sizes were 1508 bp, 1887 bp, and 2120 bp for *Cyp 6a8*, *Cyp 9b2*, and CG2196, respectively. and were labeled with [α^{32}P]dCTP, Amersham Biosciences (Buckinghamshire, England). Ribosomal RNA was used to normalize RNA loading volumes on each membrane. For each gene a two-sample *t*-test with pooled variances was used to compare expression ratios between the control treatment and the *P. nigrum* treatment using SYSTAT software (version 10, SPSS Inc., 2000).

3. RESULTS

3.1. LC_{50} Values. *P. nigrum* seed extract was toxic toward *Musca domestica* with a LC_{50} value (95% confidence interval) of 6.46 mg/mL (4.52, 12.76). For *Drosphila melanogaster* the LC_{50} value (95% confidence interval) was 0.997 mg/mL (0.947, 1.060). Mortality was not present in solvent controls (data not shown).

3.2. Microarrays. The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE3032. The use of a 1.41-fold change in expression as a threshold value resulted in the identification of a total of 63 upregulated and 9 downregulated genes (genes discussed in this publication listed in **Table 2**). The upregulated genes included the cytochrome P450 genes *Cyp6a8*, *Cyp9b2*, *Cyp12d1*, *Cyp 6d4*, *Cyp 6d5*, and *Cyp 6w1* as well as glutathione-S-transferase S1 and glutathione-S-transferase E7.

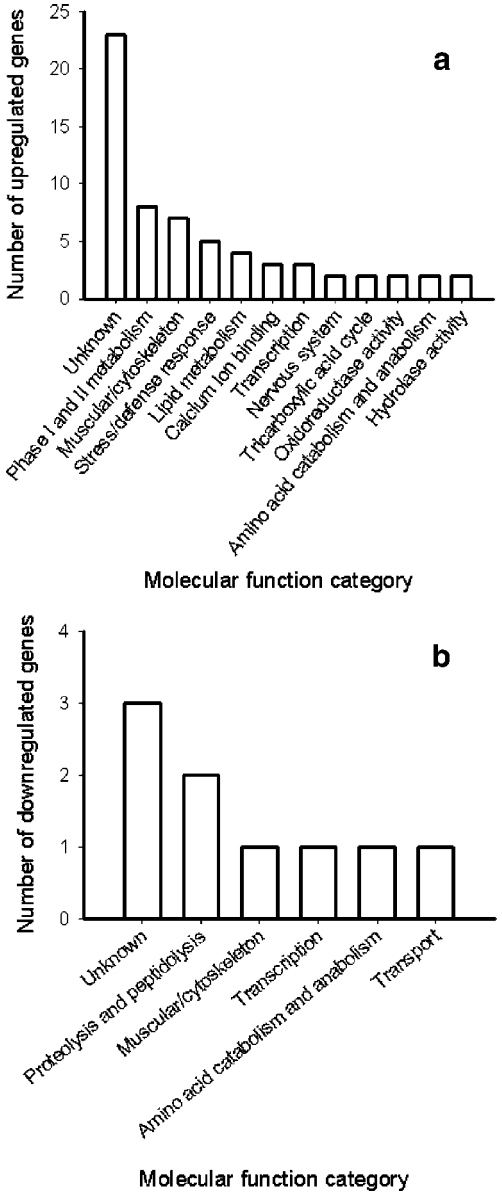

Figure 2. All *D. melanogaster* genes upregulated (a) or downregulated (b) 1.41-fold or more by a treatment of 0.9 mg/mL *P. nigrum* as determined by cDNA microarray analysis. Genes were assigned categories according to their molecular function and associated biological process. A total of 63 genes were found to be upregulated.

All upregulated and downregulated genes with a fold change in expression greater than or equal to 1.41 were classified by their molecular function and/or the biological process to which they are associated (**Figure 2**). The categories of upregulated genes with the greatest number of associated genes were phase I and phase II metabolism (**Figure 3**), muscular/cytoskeleton, and stress/defense response. The largest category of downregulated genes was proteolysis and peptidolysis.

3.3. Northern Analysis. The results of two-sample *t*-tests used to analyze the Northern blot data revealed a significant 2-fold upregulation of mRNA expression for *Cyp 6a8* ($p = 0.003$, df = 4, $n = 6$) and a significant 2.9-fold upregulation of mRNA expression for *Cyp 9b2* ($p = 0.004$, df = 4, $n = 6$) but no significant change in CG2196 (no-change control) mRNA expression level ($p = 0.399$, df = 4, $n = 6$). These values were similar to the values obtained using microarray analysis (**Figure 4**).

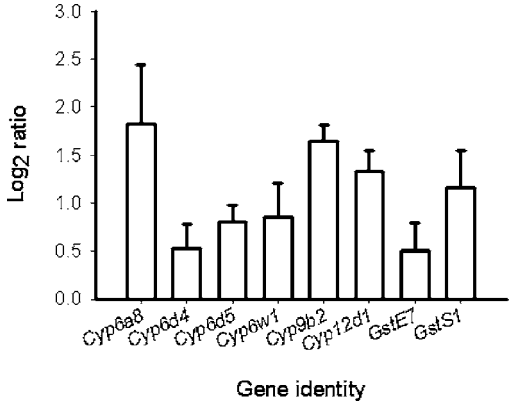

Figure 3. Identities and differential expression ratios of *D. melanogaster* genes implicated in phase I and phase II metabolism of toxins upregulated 4 h after insects were treated with 0.9 mg/mL of *P. nigrum*. Displayed are the mean (+SEM) $\log_2$ expression ratio values (based on two independent samples) for all valid microarray spots associated with a given gene.

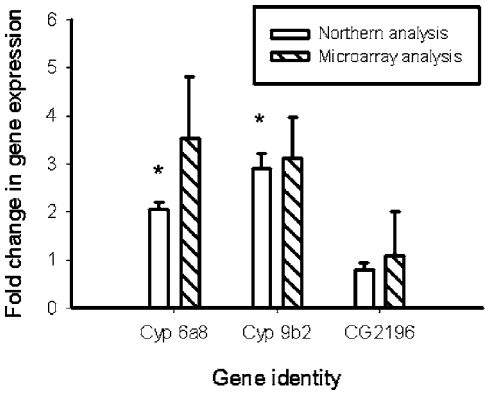

Figure 4. Relative mRNA expression levels represented as the fold change from the control value of *Cyp 9b2*, *Cyp 6a8*, and CG2196 in *D. melanogaster* adult females treated with an ethanol control or a 0.9 mg/mL *P. nigrum* extract and evaluated using Northern analysis and microarray analysis. Values from the Northern analysis marked with an asterisk are significantly different from the control value for the associated gene ($p < 0.05$) in a 2-sample *t*-test ($n = 3$, df = 4). In the microarray analysis, *Cyp9b2* and *Cyp6a8* met the 1.41-fold change criterion for significant for significant change while CG2196 was below the threshold.

4. DISCUSSION

The acute toxicity trials demonstrated that the *P. nigrum* extract is effective against both *M. domestica* and *D. melanogaster*. Prior toxicity trials with *P. tuberculatum* extract against *M. domestica* identified an LC_{50} value of 0.49% (*9*) compared to an LC_{50} value of 0.82% (6.46 mg/mL) for *P. nigrum* against *M. domestica* in the current study. To our knowledge, this study represents the first report of an LC_{50} value of *P. nigrum* extract for *D. melanogaster*.

The gene expression profile exhibited by *D. melanogaster* in response to a *P. nigrum* extract concentration equivalent to the LC_{50} value is indicative both of generalized stress and of a specific response to a toxin (**Table 2**, **Figure 2**). The detoxification response includes six upregulated *Cyp* genes involved in phase I metabolism and 2 upregulated *Gst* genes involved in phase II metabolism (**Figure 3**). The three *Cyp* genes with the highest differential expression ratios are *Cyp 6a8*, *9b2*, and *12d1*. The upregulation of *Cyp* genes is consistent with the biphasic effect of compounds with a MDP group such as piperonyl butoxide which cause an initial inhibition of CYP enzymes

followed by induction (*8*). This second step of enzyme induction by piperine has been linked to an increase in RNA transcription in rat liver cells (*12*). The results of the current study suggest that this is also likely to be the case for the response of *D. melanogaster* to *P. nigrum* extract.

The CYP 6 enzyme family plays an important role in detoxification processes and insecticide resistance and shares sequence similarities with the drug-metabolizing CYP 3 family in humans (*25*). The *Cyp 6a8* gene which was upregulated in *D. melanogaster* exposed to a *P. nigrum* extract in this study is characteristic of detoxification processes. The constitutive overexpression of *Cyp 6a8* is associated with DDT resistance (*26*). DDT-resistant strains of *D. melanogaster* have also been found to overexpress *Cyp 6a2* (*27*, *28*) and *Cyp 6g1* (*29*, *30*). Overexpression of *Cyp 6g1* in the Hikone-R DDT resistant strain of *D. melanogaster* confers cross-resistance to three neonicotinoids (*30*). The selection for constitutive overexpression of *Cyp* 6 enzymes in insecticide-resistant insects has been attributed to this broad range of substrate specificity which can confer an advantage due to an increased level of cross-resistance to other classes of insecticide. Enzymes of the CYP 6 family are also implicated in insect metabolism of plant secondary metabolites. The *Cyp 6b1* and *Cyp 6b3* genes are specifically induced in the gut tissues of the black swallowtail butterfly *Papilio polyxenes* after exposure to the furanocoumarin xanthotoxin (*31*). The implication of genes of the *Cyp 6* family in the detoxification of synthetic products such as DDT and in the induced response to at least two different classes of plant defense chemicals (furanocoumarins and piperamides) provides further evidence of the broad range of substrates available to the Cyp 6 enzymes.

The upregulated *Cyp 9b2* gene encodes a microsomal enzyme with electron transporter activity. The CYP 9 family of enzymes is exclusive to arthropods (*25*) and has not been studied as extensively as the *Cyp 6* family, but there is still strong evidence that these enzymes play a role in detoxification processes. *Cyp 9a1* shares sequence similarities to the mammalian *Cyp 3* enzymes involved in drug metabolism and also to the insect *Cyp 6* enzymes involved in insecticide metabolism (*32*). The constitutive overexpression of the *Cyp 9a1* gene in the tobacco budworm *Heliothis virescens* is correlated with resistance to the carbamate insecticide thiodicarb (*32*). There is also some evidence that the CYP 9 family is involved in insect defense against plant toxins. Previous work has found that *Cyp 9a2* is induced in *Manduca sexta* after dietary exposure to 2-undecanone, a toxin derived from the wild tomato (*33*). In the current study *Cyp 9b2* is upregulated after *Drosophila* exposure to piperamides, reinforcing the link between the CYP 9 family and the metabolism of plant toxins. To our knowledge, this is the first evidence of *Cyp 9b2* induction in response to a botanical insecticide product.

The upregulation of *Cyp 12d1* in this study suggests that this gene may also play a role in the detoxification of the *P. nigrum* extract. It has previously been demonstrated that *Cyp 12d1* is constitutively overexpressed in two DDT-resistant strains of *Drosophila* and that exposure to DDT results in a further induction of *Cyp 12d1* transcripts (*34*). A genome-wide microarray analysis of DDT-resistant *Drosophila* strains has also identified *Cyp 12d1* as a gene implicated in DDT resistance (*28*). The data presented in this study suggest that CYP 12d1 is probably also involved in the detoxification of *P. nigrum* compounds and possibly other plant toxins. Consequently, CYP 12d1 is likely to possess broad substrate binding capacities similar to the CYP 6 enzymes.

Most of the previously cited studies have examined these *Cyp* genes in relation to their constitutive overexpression and/or inducibility in cases of insect resistance to the synthetic insecticide DDT. The data from the current study provides evidence that *Cyp 6a8*, *9b2*, and *12d1* are induced in susceptible insects by the *P. nigrum* botanical insecticide. These genes are of interest for further study with respect to their functions in the inducible detoxification processes of insects in relation to other plant secondary metabolites. Of particular interest is the lack of differentially expressed genes of the *Cyp 4* family despite the presence of 12 genes from this family on the array. Overexpression of genes of the *Cyp 4* family has previously been implicated in resistance of the Nebraska western corn rootworm to the synthetic insecticides methyl parathion and carbaryl (*35*). The absence of induction in this experiment and the fact that only 6 out of a potential 32 *Cyp* genes present on the array were differentialy expressed reinforces the interest of the upregulated genes that were identified in this study.

Northern blot analysis confirmed the upregulation of *Cyp 6a8* and *Cyp 9b2* in response to *P. nigrum* and demonstrated fold-change values similar to those of the microarray analysis (**Figure 4**). This makes these two genes especially interesting candidates for further study of their role in the interaction between *D. melanogaster* and *P. nigrum*. The 2.9-fold induction of *Cyp 9b2* 4 h after exposure of *D. melanogaster* to *P. nigrum* is similar to the 2−5-fold induction of *Cyp 9a2* in the 2−4 h following dietary exposure of *Manduca sexta* to 2-undecanone (*33*). The change in expression of CG2196 in the microarray analysis was not above the 1.41-fold threshold. In the corresponding Northern analysis there was no significant change in expression indicating that microarray and Northern analysis agree for nonresponding genes.

The phase II metabolism enzymes encoded by the upregulated glutathione S-transferase genes, *Gst-S1* and *Gst-E7*, catalyze the conjugation of glutathione to lipophilic compounds to increase their solubility and facilitate their excretion from the cell (*36*). Resistance to organophosphate and pyrethroid insecticides has been attributed to increased GST activity (*37*, *38*). The upregulation of these two *Gst* genes is probably linked to an increase in phase II metabolism activity to facilitate the elimination of the products of phase I metabolism associated with the upregulation of *Cyp* genes.

The upregulation of six different *Cyp* genes as well as two *Gst* genes indicates a classical detoxification response involving both phase I and II metabolism. It also indicates that the effect of the *P. nigrum* extract upon the *Drosophila* detoxification response is complex and may mean that at least eight different phase I and II metabolism enzymes are required in the detoxification process. The presence of analogue synergism between the insecticidal piperamides (*39*) can enhance the efficacy of a botanical extract relative to a pure compound and has been demonstrated to prevent the evolution of resistance (*40*). It is possible that the evolution of resistance of *D. melanogaster* to *P. nigrum* extract may require constitutive overexpression or modification of more than one detoxification gene thereby reducing the probability of the occurrence of resistance. This is in contrast to synthetic molecules such as DDT to which insects have been reported to acquire resistance by overexpressing a single *Cyp 6g1* gene (*41*).

Numerous differentially expressed genes were not associated with detoxification processes. A second biological process category with a large number of upregulated genes was muscle function and cytoskeleton structure (**Figure 2**). This is probably related to the observed knockdown effect of *P. nigrum* which causes uncoordinated movements which is, in turn, related to the neurotoxicity of the piperamides. A third biological process category with a large number of upregulated genes related to the response to physiological stress (**Figure 2**) and comprises

genes with chaperone activity and oxidoreductase activity. This is to be expected considering the overall increase in transcription activity and also the acute toxic effects which may lead to oxidative stress. Both of these could lead to the need for chaperone molecules to mediate the proper folding of newly synthesized proteins and the repair of damaged proteins. The molecular function category associated with the largest number of downregulated genes was proteolysis and peptidolysis (**Figure 2a**), which may indicate a general stress response. This downregulation of proteolysis and peptidolysis associated genes could also potentially represent a component of the toxicity mechanism of the *P. nigrum* extract.

The upregulated *Frost* gene (CG9434) has previously been induced in response to cold shock in *Drosophila melanogaster* (*42*). The expression of this gene in response to *P. nigrum* may indicate that it is not induced solely by cold but by a broader range of stressors. Two other upregulated genes, CG7896 and *Astray* (CG3705), were identified as being linked to the nervous system. The CG7896 gene has receptor activity linked to the transmission of nerve impulses. The *Astray* gene has phosphoserine phosphatase activity which catalyzes the ATP driven final step of biosynthesis of L-serine from glycine (*43*). In mammalian systems these reactions occur primarily in brain synapses (*44*) indicating the probable occurrence of amino acid synthesis in neurons. The upregulation of these two nervous system genes is likely correlated with the neurotoxic activity of the amide moiety of the active *P. nigrum* chemicals (*45*). The upregulated *Aly* gene (CG1101) encodes a product with RNA binding and transcription coactivator activities. This is probably linked to the increased synthesis of proteins that is occurring in response to the treatment.

The downregulated genes were fewer in number than the upregulated genes (**Figure 2**). Limited information is available concerning the two most strongly downregulated genes with a greater than 2-fold change in expression (**Table 2**). The gene identified as CG11892 which was downregulated in the *P. nigrum* treatment has also been found to be downregulated after 4 h in *Drosophila* larvae in response to starvation (*46*). The second downregulated gene, CG5107, is expressed during the embryonic development of *Drosophila melanogaster* (*47*).

The results of this study indicate that the use of cDNA microarrays is promising for identifying novel genes induced by natural products. The observed upregulation of six cytochrome P450 genes is consistent with the theory that suppression of CYP enzymes by the insecticidal compounds of a *P. nigrum* is followed by an increase of mRNA transcription for the associated genes. The upregulated *Cyp 6a8* and *Cyp 12d1* genes have previously been associated with exposure or resistance to synthetic pesticides, but the current data suggest that they are probably also important in the detoxification of *P. nigrum* extract containing piperamides and other phytochemicals and that further study of these genes in the context of plant−insect interactions would be beneficial. The presence of genes previously associated in other studies with starvation, cold shock, and insecticide metabolism such as CG11892, *frost*, and *Cyp 6a8* suggests the presence of a group of genes that are induced in response to a broad range of stressors. This study supports previous work linking enzymes of the CYP 9 family to insect defense against phytochemicals (*33*). In future work, it would be of interest to determine if the gene products do indeed metabolize pepper extracts. Overall, these results suggest that the complexity of the physiological effect of *P. nigrum* extract upon detoxification gene expression could correlate to a reduced potential for the evolution of insecticide resistance compared to insecticides based upon a single active molecule.

ABBREVIATIONS USED

CDMC, Canadian *Drosophila* Microarray Center; 4,5-DHPLG, 4,5-dihydropiperlonguminine; PLG, piperlonguminine; 4,5-DHPIP, 4,5-dihydropiperine; PIP, piperine; MDP, methylenedioxyphenyl; PSMO, polysubstrate monooxygenase.

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Supporting Information Available: Details concerning the fold change and gene ontology of the entire set of differentially expressed genes. This material is available free of charge via the Internet at http://pubs.acs.org.

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