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Botanical Insecticides for Controlling Agricultural Pests: Piperamides and the Colorado Potato Beetle *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae)

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The efficacy of extracts from two Piperaceae species, *Piper nigrum* L. and *P. tuberculatum* Jacq. were evaluated using larvae and adults of the Colorado Potato Beetle *Leptinotarsa decemlineata* (Say). Young larvae and neonates were the most susceptible; a 24-h LD_{50} of 0.064% extract of *P. tuberculatum* was determined for 4-day-old larvae, while 0.05% extract of *P. nigrum* reduced larval survival up to 70% within one week after treatment of potato *Solanum tuberosum* L. (Solanaceae) plants. When an insecticide resistant strain of *L. decemlineata* larvae was tested with the *P. tuberculatum* extract, there was less than a 2-fold tolerance ratio compared to the 22-fold tolerance ratio to cypermethrin, a pyrethroid. Older larvae, prepupal stage and adults, were less sensitive to the *P. nigrum* extracts; the 24-h LD_{50} was 0.5% (95% C.I. = 0.36, 0.65). However, the same concentration was equally effective under field conditions. In the greenhouse, *P. nigrum* at 0.5% was as effective at reducing adult *L. decemlineata* feeding as combinations with 2 separate botanical mixtures, garlic and lemon grass oil. Under field conditions, the residual activity of the *P. nigrum* extracts was less than 3 h. When adult *L. decemlineata* were placed on treated plants exposed to full sunlight for 0, 1.5, and 3 h, leaf damage progressively increased as the main active compound, piperine, was found to degrade by 80% after 3 h. An in vitro polysubstrate monoxygenase (PSMO) enzyme assay, using the substrate methoxyresorufin O-demethylation (MROD), determined that the principal *P. nigrum* active compound, piperine, is responsible for inhibition of that specific enzyme. The results suggest that *Piper* extracts could be used effectively as contact botanical insect control agents to protect potato plants from developing *L. decemlineata* larvae at concentrations less than 0.1%. There is also potential for *Piper* extracts to control insecticide resistant populations in conjunction with other integrated pest management (IPM) strategies used in conventional and organic agriculture. Arch. Insect Biochem. Physiol. 54:212–225, 2003.

KEYWORDS: Piperaceae; *Piper nigrum*; *Piper tuberculatum*; piperamides; piperine; insecticide resistance; pyrethroids; *Leptinotarsa decemlineata*

INTRODUCTION

The search for new solutions to control insect pests in agriculture and urban areas is currently influenced by 4 concerns: (1) the banning of synthetic insecticide use in municipal areas as recently seen in Canada, (2) public perception that natural compounds are better, (3) products that are Generally Regarded As Safe (GRAS); and (4) the reliance on extracts vs. pure compounds. Research is again focusing on the plant kingdom for solutions since the interaction between plants and herbivores

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has led to the production of a myriad of secondary compounds that can have toxic, growth reducing, and antifeedant properties against insects (Berenbaum and Zangerl, 1996). The diversity of insect defences is exemplified by the family Piperaceae, which includes the common spice black pepper, *Piper nigrum* L., as well as approximately 1,000 other tropical species. Research on the Piperaceae over the past two decades has revealed that *Piper* species contain greater than 200 secondary compounds (Arnason et al., 2002).

The relatively simple amides provide much of the "hot pungent spice" taste as well as the biological activity in many species. The piperamides commonly found in the genus *Piper* are unique since they are bifunctional: an isobutyl amide functionality is combined with a methylenedioxyphenyl (MDP) moeity as seen in piperine (Fig. 1A) found in *P. nigrum* L. fruit and 4,5- dihydropiperlonguminine (Fig. 1B) present in high quantities in foliage of the Central American *P. tuberculatum* Jacq.. The most active piperamide discovered to date is pipercide (Fig. 1C), approximately 100-fold more active than piperine (Miyakado et al., 1979, 1980; Dev and Koul, 1997). The piperamides are also unusual because of their dual biological activities: the amide functionality is neurotoxic and the MDP group is an inhibitor of cytochrome P450 enzymes. The polysubstrate monoxygenase (PSMO) inhibition is demonstrated by similar MDP molecules: the insecticide synergist piperonyl butoxide (PBO) (Farnham, 1998) and dillapiol (Fig. 1D and E, respectively) found in Indian dill *Anethum sowa* and *P. aduncum* (Arnason et al., 2002). The mixture of similar amides found in most *Piper* species, including the West African *P. guineense* Schum and Thonn, suggests these plants have also employed a defence strategy termed analogue syner-

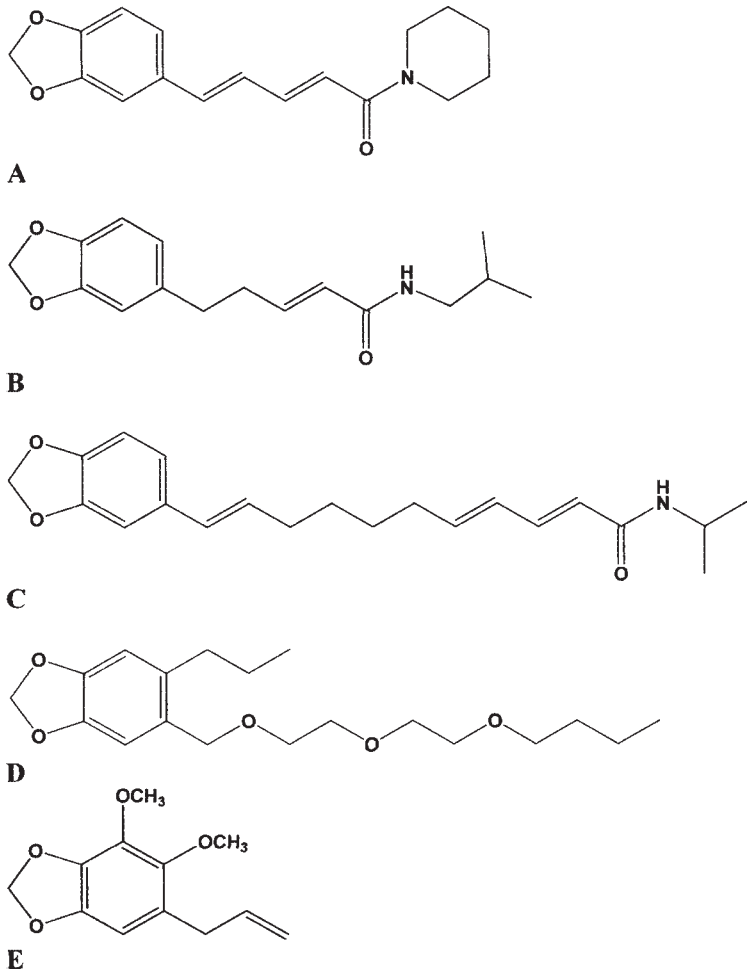

Fig. 1. Structure of Piperine (**A**), 4,5-dihydropiperlonguminine (**B**), Pipercide (**C**), Piperonyl Butoxide (**D**), and Dillapiol (**E**).

gism (McKey, 1979, in Berenbaum and Zangerl, 1996) whereby many similar compounds will increase the toxicity to herbivores and render it difficult for a herbivore to adapt and become resistant (Feng and Isman, 1995). In a previous study (Scott et al., 2002), we demonstrated through combinations of piperamides in binary, tertiary, and quarternary mixtures that they had successively higher toxicity at equimolar concentrations. This combination of useful traits suggests that *Piper* extracts may be good candidates for use in crop protection.

One insect pest that constantly frustrates farmers and agricultural specialists alike is the Colorado potato beetle *Leptinotarsa decemlineata* (Say). A North American native that has been spread to many parts of the world, this species has had a long history of development of resistance to pesticides (Metcalf, 1983). *L. decemlineata*, like several other insect pests of economic importance, shows multiple resistance to several insecticide classes including carbamates, organophophates, organochlorines, and pyrethoids. These factors have led to increased efforts to develop new control strategies. As a target insect, the Colorado potato beetle provides us with the opportunity to test the hypothesis that *Piper* extracts can be used to control an insecticide-resistant pest due to the novel structure and activity of the piperamide molecule and its dual functionality.

MATERIALS AND METHODS

Piper Extracts and Piperamide Analysis

Piper nigrum peppercorns were obtained from Country Bulk Inc., a commercial spice supplier in London, Ontario, but originated in Kerala, India. *P. tuberculatum* leaves were collected along the west coast of Costa Rica in May 2001. Leaves and peppercorns were ground and the active constituents extracted following the methods described in Scott et al. (2002). The extracts were formulated as follows: 20% extract, 70% tetrahydrofurfuryl alcohol (THFA, Penn Specialty Chemicals, Memphis, TN), and 10% emulsifier (Alkamuls EL-719 ethoxylated castor oil, Rhodia, Cranbury, NJ). Piperamide concentrations in the extracts and formulations were determined based on the methods of Scott et al. (2002). Degradation of piperamides exposed to full sunlight conditions was determined by placing 50-µL aliquots of 20% extract on glass microscope slides and allowing them to dry overnight on the bench top. The slides were then placed in a full sunlight exposed location for 6 h during peak daylight hours. Readings of solar radiation were taken at 3-h intervals at the location. The glass slides were then rinsed with 5 mL of 99% ethanol to wash off the extract residue. Treated control slides not exposed to full sunlight were rinsed using the same method. The ethanol solutions (1 mL) were further filtered using a 0.2-µm filter and then placed in a 1.5-mL HPLC vial in preparation for analysis. HPLC analysis was conducted using a Varian Prostar model pump, model 330 UV/Vis photodiode array detector and model 410 autosampler, and the column was a Varian reverse-phase C18 (Varian Chromatography Systems, Walnut Creek, CA). The compounds were eluted using a binary gradient of acetonitrile and water where acetonitrile was increased from 30 to 90% in 12 min as described by Scott et al. (2002).

PSMO Study

In an effort to test whether piperamides affect PSMO enzymes directly, an established protocol for measuring microsomal activity in insects was adopted. In this case, the insect used was the housefly *Musca domestica* rather than *L. decemlineata* since an accepted method existed. Microsomes from the housefly were used to test whether microsomal enzymes treated with piperine would show reduced activity or inhibition using the methoxyresorufin O-demethylation (MROD) in vitro technique (Scott et al., 1998). Pyrethroid-resistant houseflies (LPR strain) (Scott and Georghiou, 1985) were used since this strain had increased levels of microsomal activity (Wheelock and Scott, 1992). The adults emerged from pupae for 2 days at 27°C, 70 RH, and 16:8 h LD and fed sugar water ad libitum. Flies were then fed a 15% ethanol/15% sugar water solution for 72 h. Preparation of microsomes

from houseflies followed the techniques of Lee and Scott (1989) allowing for storage of microsomes for at least 2 months at –80°C and repeated use in microsome assays. The MROD assay followed methods described by Lee and Scott (1989). Prepared microsomes were combined with phosphate buffer in microplate wells along with methoxyresorufin substrate and challenged with doses of piperine (0, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, and 5 mM) dissolved in DMSO. A standard curve of 0, 0.5, 1, 2, and 4 µM resorufin was run on each microplate. Piperonyl butoxide and dillapiol at 4.3 $\times 10^{-4}$ mM were used as standards in the assay. The fluorescence of standards and samples was analysed using a SpectraMax Gemini XS microplate fluorometer, Molecular Devices Inc., and SoftMax Pro Version 3.1 software.

M. domestica Study

LPR and insecticide susceptible (Benzon Research Inc., Carlisle, PA) housefly strains were tested using *P. tuberculatum* extract formulated at 10%. Flies were anaesthetized with carbon dioxide and kept on ice until treated. A concentration range of 0.1, 0.2, 0.4, 0.8, and 1% *P. tuberculatum* diluted in distilled water was prepared, along with a formulation blank control. Thirty flies were individually dipped into each 10 mL solution, patted dry on a Whatman No. 1 filter paper, and then placed into a metal cage to recover. Flies were supplied with sugar water ad libitum. Mortality was observed after 1 and 24 h by counting the number of flies unable to stand or fly in each cage. Each trial was repeated at least 3 times for both housefly strains.

L. decemlineata Study

Laboratory trials. *L. decemlineata* eggs, insecticide resistant (R strain), and susceptible (S strain) were supplied from the Southern Crop Protection and Food Research Centre (SCPFRC), Agriculture and Agri-food Canada, London, Ontario, Canada. Egg masses containing 25 to 30 eggs (S strain) and 10–15 eggs (R strain) were couriered to the University of Ottawa where they were placed on the leaves of 4-week-old greenhouse grown potato plants (1–2 egg masses per plant), variety Superior Gold and Russett Burbank. Eggs hatched within 2 to 3 days and 4 days after hatching larvae from both the S and R strains were collected for use in acute toxicity bioassays. The R strain *L. decemlineata* tolerance ratios for the conventional insecticides, cypermethrin, azinphosmethyl, and endosulfan were documented as 11 to 22, 18 and 80, respectively (Agriculture and Agri-food Canada, unpublished data). The bioassays to determine the tolerance ratios were conducted by SCPFRC with adult *L. decemlineata* collected from the field in 2000 and tested in the F1 to F3 generation of the strain. The 24-h toxicity trials followed were conducted as described previously by Hilton et al. (1998). S strain adults were sprayed using a Potter spray tower with a series of 5 concentrations for each insecticide, the range selected to kill between 0 and 100%. The >95 % purity technical grade insecticides were diluted in acetone and olive oil (19:1) and 5 mL was sprayed per concentration level. Twenty adults were treated per concentration with 3 replicates per level. The tests were then repeated with the R strain based upon LC_{95} value determined for the S strain. The established LC_{50} for each insecticide with the R strain was then compared to that of the S-strain to determine the tolerance ratio.

Working with a stock solution of 10% *P. tuberculatum* formulated extract, two dose ranges were prepared: 0.02, 0.04, 0.06, 0.08, and 0.1 µg/mL for the S strain and 0.04, 0.08, 0.12, 0.16, and 0.2 µg/mL for the R strain. The control consisted of a formulation blank equal to the highest *P. tuberculatum* concentration. Acute toxicity trials were conducted by dipping individual potato leaves into the solutions, then allowing them to dry on the bench top for 30 min. Larvae were then dipped into the solutions and placed on the leaves with the same treatment inside a glass Petri plate. Ten larvae were used per three leaves and each treatment was replicated at least three times. Petri plates were placed in an incubator at 27°C, 70 RH, and 16:8 h LD. Mortality after 24 h was determined by probing the larvae with tweezers to elicit a response.

After 7 days, S strain larvae were collected from plants and then dipped into treatments of either water (control), formulation blank (emulsifiable concentrate or EC), or 0.1, 0.5, 1, and 2% formulated *P. nigrum*. All treated larvae were placed into Petri plates lined with fresh potato leaves and kept in an incubator following the same environmental conditions as the 4-day-old larvae tests. Mortality after 24 h was determined by probing the larvae with tweezers to elicit a response.

Wild-type adult *L. decemlineata* were collected from organic potato fields or urban allotment gardens having pesticide use restrictions. The adults along with potato leaves were similarly dipped into treatments of *P. nigrum* formulation ranging from 0.25, 0.5, 1, 1.5, and 2%. Ten adults were used per replicate treatment and were placed along with treated leaves in a screen covered 500-mL mason jar.

Greenhouse trials. Single *L. decemlineata* egg masses (S and R) were pinned to leaves on 4–6-week-old potato plants variety Yukon Gold. Plants were sprayed with a 1-litre hand pump sprayer at concentrations of 0.01 and 0.05% *P. nigrum*. The control consisted of a 0.2% EC blank equivalent to the formulation content in the 0.05% *P. nigrum* application. Each plant was sprayed with 100 mL of solution and 3 replicate plants were used for each treatment. Each plant was placed inside a wooden framed cage covered by a fine mesh so that each cage contained one replicate of each treatment. Trays of water in the bottom of each cage kept the plants hydrated during the exposure period. Plants were sampled after 8 days when the number of surviving larvae, developmental stage of larvae, and number of damaged leaves was assessed.

In order to compare the toxic, repellent, and antifeedant effect of *P. nigrum* in combination with other botanical products currently available, a second trial was conducted by spraying potato plants with either 100 mL of 0.5% *P. nigrum* alone, or 0.5% *P. nigrum* mixed with either the recommended dose of Garlic Barrier $^{AG+}$ Insect Repellent (Garlic Research Labs, Glendale, CA) or Ropel® Plant Protect-R™ (Burlington Scientific, Farmingdale, NY), based on garlic *Allium sativum* Linn. and lemon grass oil *Cymbopogan citrates* [DC] Stapf. extracts, respectively. After application, 20 adults were placed on each plant, 3 replicates per treatment and covered with the mesh cage. After 24 h, the number of surviving adults and damaged leaves per plant was assessed.

Field trials. Field grown potato plants, variety Yukon Gold, each 4–5 weeks old, were sprayed with 100 mL of 0.5% *P. nigrum* to runoff. Control plants were treated with equal amounts of formulation blank. Immediately after application of control solution to 3 replicate plants and 0.5% *P. nigrum* to 9 replicate plants, 20 field collected adult *L. decemlineata* (10 females and 10 males) were placed on each of the control plants and 3 of the 0.5% *P. nigrum* plants. The plants were then covered with a fine mesh net supported by a wire cage to prevent adults from escaping. After 1.5 and 3 h had elapsed, 20 adults were added to the remaining 6 replicate 0.5% *P. nigrum* treated plants and then covered. Solar radiation readings were taken at each time period with a Li-Cor Quantum model LI-192SB sensor. After a 24-h period, plants were sampled and were assessed for leaf damage, survival of adults, and egg mass production.

A second trial to assess contact toxicity consisted of 20 adult *L. decemlineata* (15 females and 5 males) placed on individual potato plants prior to treatment with either formulation blank or 0.5% *P. nigrum*. Plants were then sprayed with 100 mL of each treatment, 3 replicate controls and 4 replicate 0.5% *P. nigrum*, to runoff. Each plant was covered with a mesh cage and left for 24 h. Plants were then sampled and assessed for survival of the adults.

RESULTS

Piperamide Analysis

HPLC analyses determined that piperine concentration in *P. nigrum* stock solutions ranged from 37 to 42.7% by weight. *P. tuberculatum* Jacq. 4,5-dihydropiperlonguminine concentration ranged from 4.1 to 4.7% by weight in the different stock preparations (Table 1).

TABLE 1. Range of Concenterations (μg/mL) for Piperamides Determined by HPLC Analysis of *P. nigrum* and *P. tuberculum* Extracts

Extract	4,5-dihydro-piperlonguminine	Piperlonguminine	4,5-dihydro-piperine	Piperine
P. nigrum	1–2.2	3.6–5.5	35.2–41.7	370–427
P. tuberculatum	41.5–46.6	1.6–2.9	5.8–11.3	4.9–9.1

PSMO Study

The PSMO inhibitory activity of piperine was demonstrated to be almost as effective as PBO against multi-resistant insects. The average IC_{50} for 2 MROD enzyme assays was determined at 1.2 μM piperine (1 mM dose) (Fig. 2) compared to less than 0.43 μM piperonyl butoxide. Dillapiol at 0.43 μM did not affect MROD activity with the LPR housefly microsomes (data not shown).

M. domestica and *L. decemlineata* Laboratory Study

The pyrethroid-resistant housefly strain was more sensitive to the *P. tuberculatum* extract, LC_{50} = 0.49% (95% confidence limits = 0.4, 0.63) than was the susceptible strain, LC_{50} = 0.67% (95% confidence limits = 0.59, 0.78) («Z» test, z = 2.43, z crit. = 1.96). In addition, the insecticide-resistant strain of *L. decemlineata* larvae tested with the *P. tuberculatum* extract showed a less than 2-fold tolerance ratio compared to the susceptible strain. The LC_{50} values calculated for the susceptible and resistant *L. decemlineata* strains were 0.066% (95% confidence limits = 0.058, 0.076) and 0.109% (95% confidence limits = 0.088, 0.149) *P. tuberculatum*, respectively. The acute bioassays indicated that the R strain was significantly less sensitive to the *P. tuberculatum* extract («Z» test, z = 3.43, z crit. = 1.96). This tolerance ratio was 10-fold less than for the pyrethroid cypermethrin when tested with the same R-strain (Fig. 3).

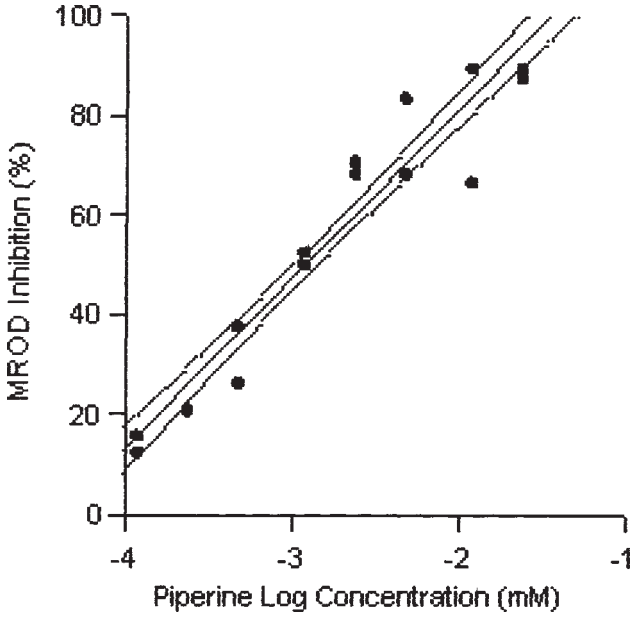

Fig. 2. The inhibitory effect of piperine on methoxyresorufin O-demethylation (MROD) activity as measured in vitro with LPR housefly microsomes.

There was a decrease in sensitivity to *P. nigrum* extracts with increasing age of S-strain *L. decemlineata* larvae. The mid instar stage (7 day) larvae had 75% mortality when exposed to a 0.1% *P. nigrum* treatment while 1% caused 95% mortality (Fig. 4). A 0.5% treatment knocked down approximately 50% of the pre-pupal larvae (data not shown). The adults appear to be equally sensitive as the LD_{50} was determined to be 0.5% (95% confidence limits = 0.36, 0.65) *P. nigrum* (Fig. 5).

Greenhouse Trials

Under more realistic conditions, the *P. nigrum* extract at 0.05% had a similar effect on the R strain *L. decemlineata* larvae compared to the S strain on treated potato plants (Fig. 6). In both cases, there was a 65–70% decrease in the number of larvae that survived from the hatchling stage to the eighth day compared to controls for both larval strains. *P. nigrum* extract at 0.01% had little effect on the developing larvae.

Mixtures of *P. nigrum* and either garlic or lemon grass oil showed no difference in terms of combination effect. No significant difference for either a toxic and repellent effect between *P. nigrum* extract alone or in combination with the two other botanicals was detected (Fig. 7A,B). Although the joint action of *P. nigrum* with lemon grass oil (Ropel®) did not reduce the damage to leaves from *L. decemlineata* feeding, there were on average higher numbers of moribund adults after 24 h. It is im-

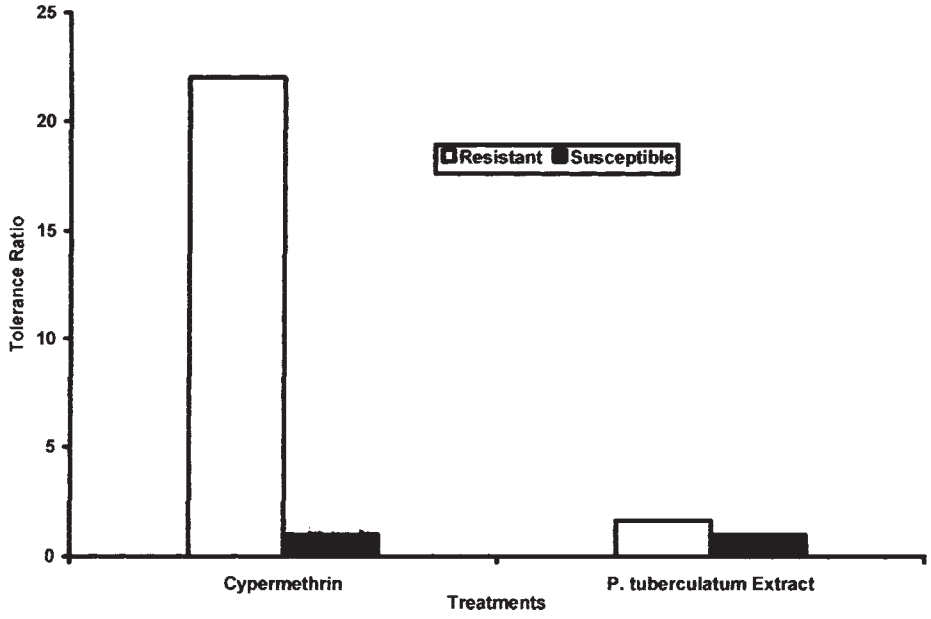

Fig. 3. Comparison of resistance factors between insecticide (pyrethroid) resistant and susceptible *L. decemlineata* larvae treated with a pyrethroid and *P. tuberculatum* extract. The white and black columns show *P. tuberculatum* LD_{50} values for the R and S strain, respectively.

portant to note that the 0.5% *P. nigrum* application did not cause any damage to the treated potato plants while a 1% formulated extract was observed to be phytotoxic.

Field Trials

Knockdown of adult *L. decemlineata* was observed 24 h after a spray application of *P. nigrum* to potato plants under field conditions. The effect of a 0.5% *P. nigrum* extract treatment on adult *L. decemlineata* feeding on field grown potato plants was comparable to that observed with the LD_{50} dose (Fig. 8). Less than 40% of adults survived compared to 70% in controls while the number of dead and moribund adults found were significantly greater than for the controls after 24 h. On both control and

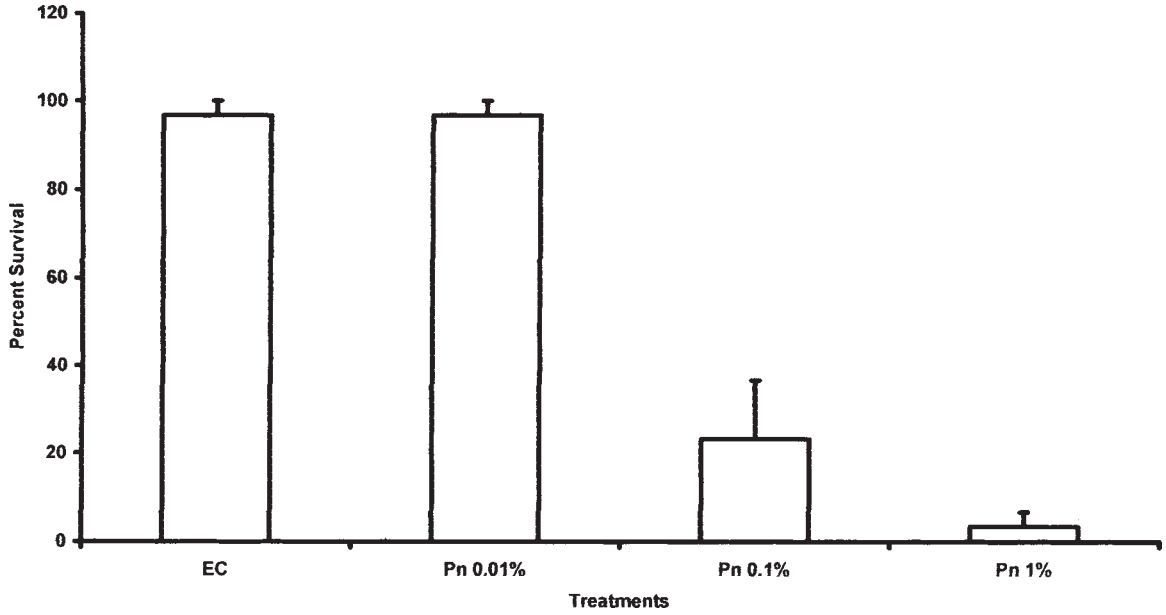

Fig. 4. Percent survival (± standard error) of mid stage *L. decemlineata* larvae treated with 3 concentrations of *P. nigrum* extract compared to the formulation control (EC).

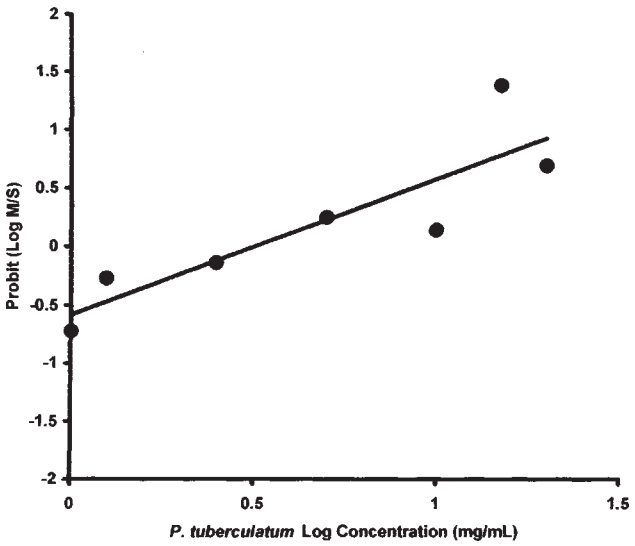

Fig. 5. Dose-response curve for adult *L. decemlineata*. The *P. nigrum* LD_{50} was calculated as 0.5% with 0.36, 0.65 95% confidence limits.

pepper-treated plants, 25% of the adults were not found. Since they could not escape from the cages, it is assumed that they were hiding either in the foliage or the soil. A 1% extract application was not tested due to the phytotoxicity of the formulation.

The protective effect of a 0.5% *P. nigrum* treatment was observed to decrease within 3 h as the number of leaves damaged by adult *L. decemlineata* increased with the length of time that the extract was exposed to full sunlight (Fig. 9A). This decrease in bioactivity is likely related to the almost 80% degradation of piperine, which was observed to occur over a similar length of sunlight exposure (Fig. 9B). Light levels were measured between 1,830 and 2,250 µEinsteins m^{-2} s^{-1}. No difference in the survival of adults or the number of egg masses was found between treated potato plants exposed to sunlight for the three time periods. The survival of adults placed on the plants after the 0.5% *P. nigrum* treatment was noted to be much higher than when they are exposed directly to the spray as in Figure 8.

DISCUSSION

The present experiments clearly show that *Piper* extracts can knock down larvae and adults of *L. decemlineata* feeding on potato plants. Our initial hypothesis regarding the importance of the bifunctional nature of piperamides and the dual activity

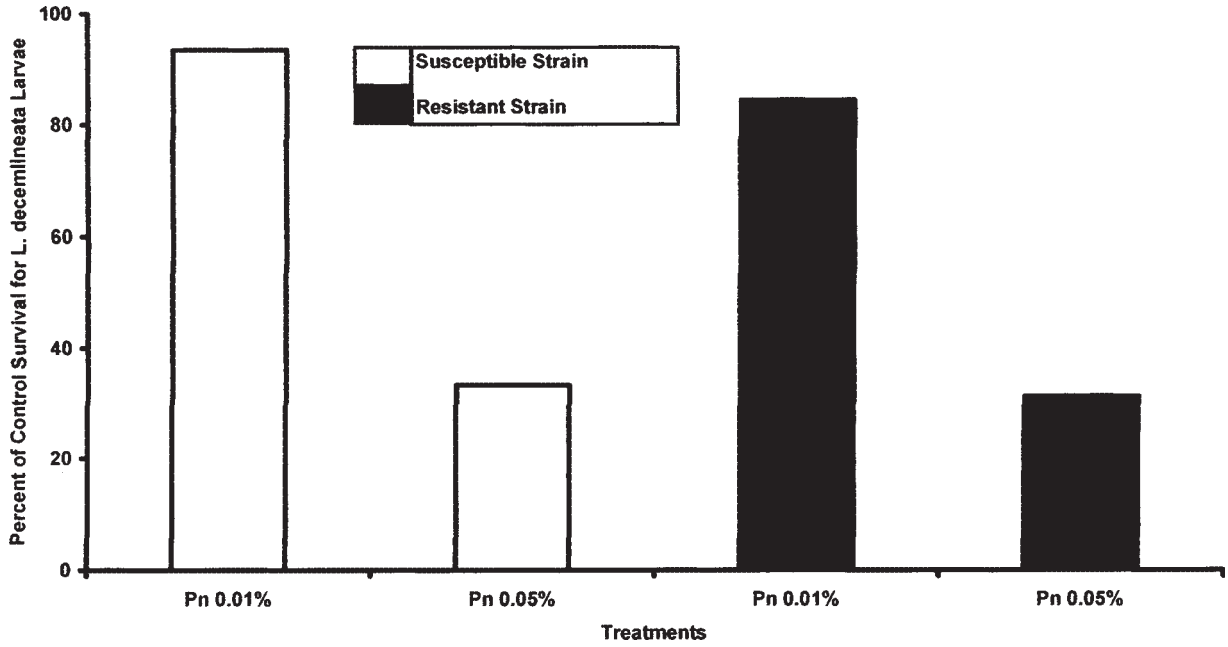

Fig. 6. Mean percent of control survival for susceptible (S) and pesticide resistant (R) strain *L. decemlineata* larvae 8 days after treatment of hatchlings on potato plants with 2 concentrations of *P. nigrum* extract.

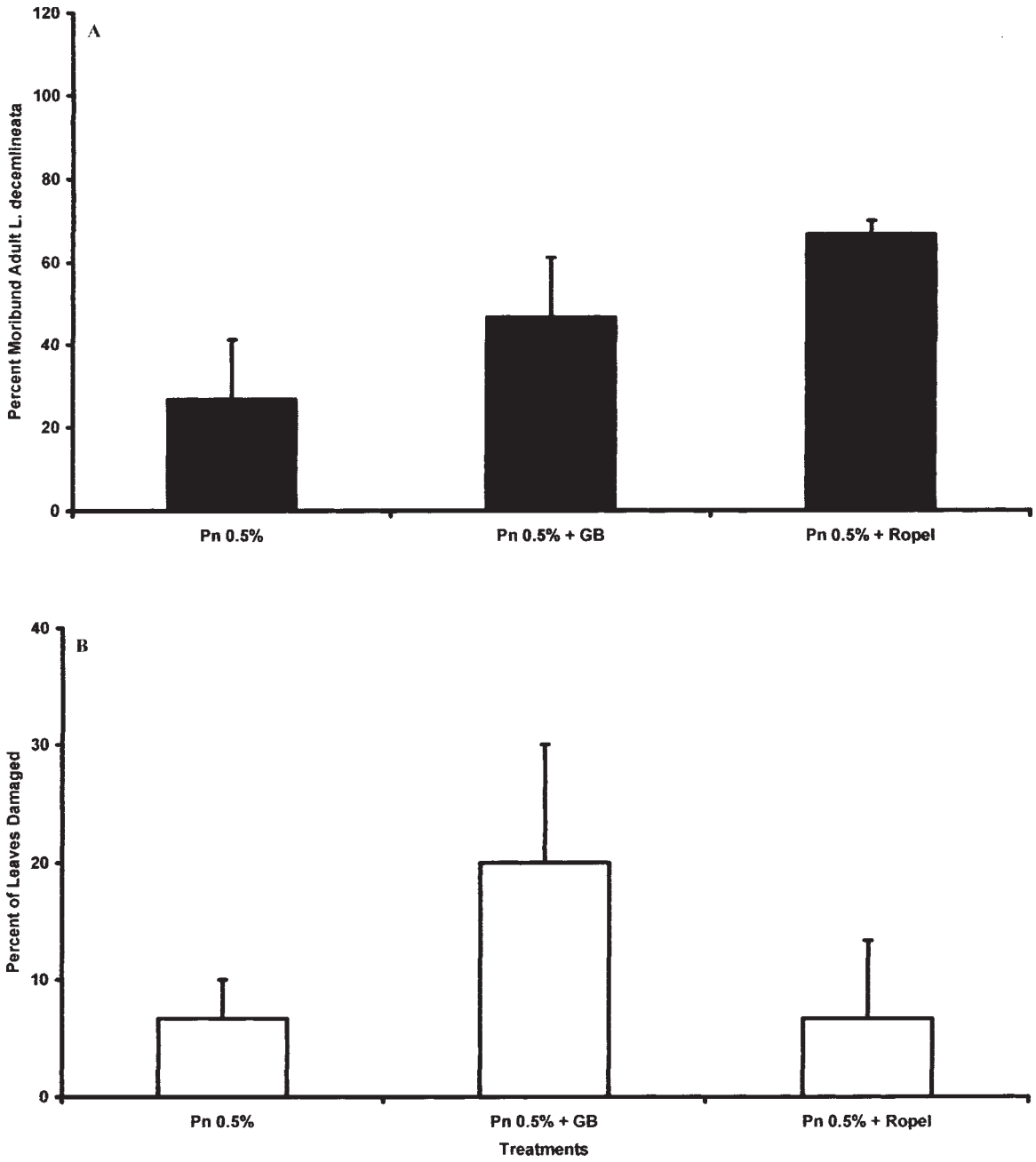

Fig. 7. Mean percent adult *L. decemlineata* survival and morbidity ± standard error (**A**) and percent damage to leaves ± standard error (**B**) 24 h after potato plants treated with 0.5% *P. nigrum* only, or in combination with either Garlic Barrier® (GB) or Ropel® commercial insect repellents.

was supported by the relatively low tolerance ratio between resistant and susceptible strain *L. decemlineata* larvae and the ability of piperine to inhibit PSMO activity.

The potential benefit, as demonstrated in this study, for extracts of *P. nigrum* and other *Piper* species is the control of *L. decemlineata* larvae and adults due to the novel activity of piperamides, their relative safety for use and storage, and from universal availability of the seed and leaf material.

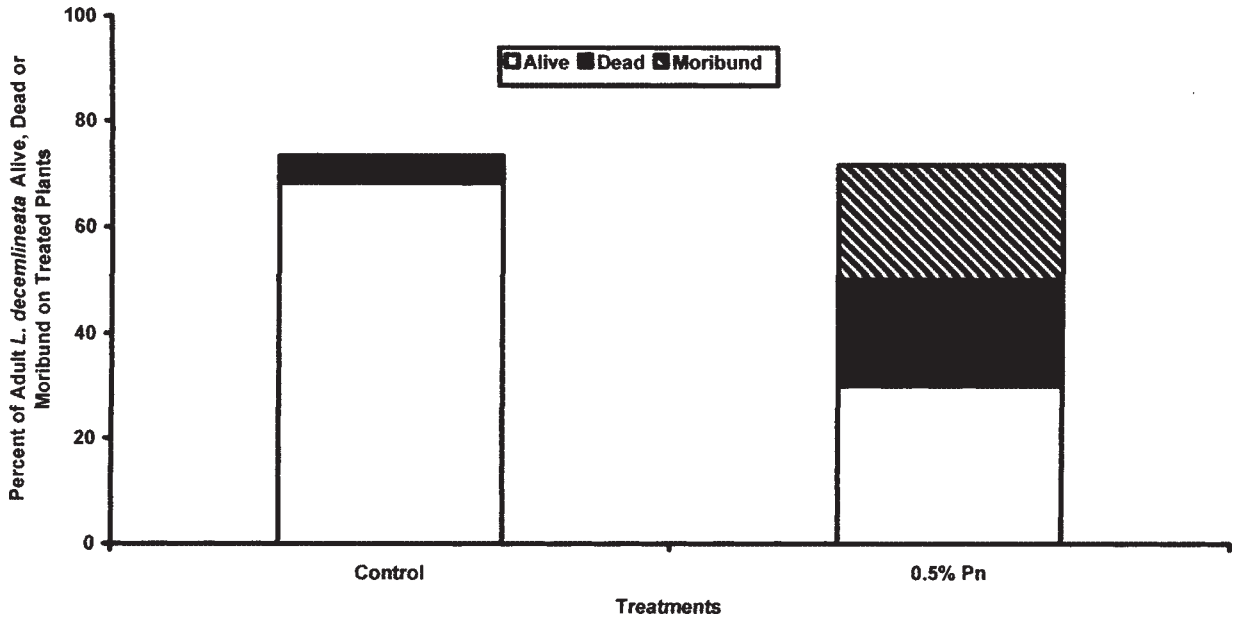

Fig. 8. Mean percent adult *L. decemlineata* survival and morbidity 24 h after treatment with 0.5% *P. nigrum* extract while feeding on field grown potato plants exposed to full sunlight.

In the case of black pepper, it is one of the world's most traded spices and is grown in several equatorial regions of the globe (Simpson and Ogorzaly, 1995). Extracts of black pepper contain high proportions of piperine and other active piperamides, which already have been tested and applied for use in several developing regions (Arnason et al., 2002). Black pepper is considered a food grade spice and categorized by the U.S. F.D.A. as "Generally Regarded as Safe" or GRAS (CITE:21CFR182.10). In terms of the health risks, piperine is the only piperamide thus far listed on the E.P.A.s Toxic Substances Control Act (TSCA) inventory. It is a category R22, harmful if swallowed, and has precautions S22-25, not inhaling the dust, fumes, or vapours, as well as avoiding contact with skin and eyes (Sigma-Aldrich 2002). The LD_{50}s for piperine in male adult mice were determined to be 330 and 200 mg/kg for single intra gastric (i.g.) or subcutaneous (s.c.) injections, respectively, and in subacute studies piperine at 100 mg/kg body weight /day for 7 days was not toxic (Piyachaturawat et al., 1983). The relatively low mammalian toxicity of these food grade spices should preclude many of the necessary tests required for registration of conventional insecticides. Lowered health risks imply safer use of these extracts by applicators. However, the "hot taste" associated with pepper suggests that the precautions taken when applying other chemicals should be equally observed with these extracts. The greatest risk will be from irritation of the skin and eyes, therefore applicators should take steps to avoid such exposure when spraying.

A great deal of research to date suggests that *P. nigrum* formulations can effectively protect against stored product pests. Both *Sitophilus oryzae* (L.) and *Rhyzopertha dominica* (F.) were controlled at concentrations above 100 mg/L for up to 30 days (Sighamony et al., 1986) while stored beans were protected from the bruchid *Acanthoscelides obtectus* Say by ground black pepper for up to 18 weeks (Baier and Webster, 1992). Early investigations with *P. nigrum* indicated that amides were responsible for the toxicity of the extracts to the adzuki bean weevil *Callosobruchus chinensis* L. (Miyakado et al., 1979, 1980). Three of the isobutyl amides isolated from *P. nigrum*, pipercide, pellitorine, and piperine were toxic at 0.15, 2, and 20 µg/male *C. chinensis*, respectively (Dev and Koul, 1997).

The novel structure of the piperamides and their

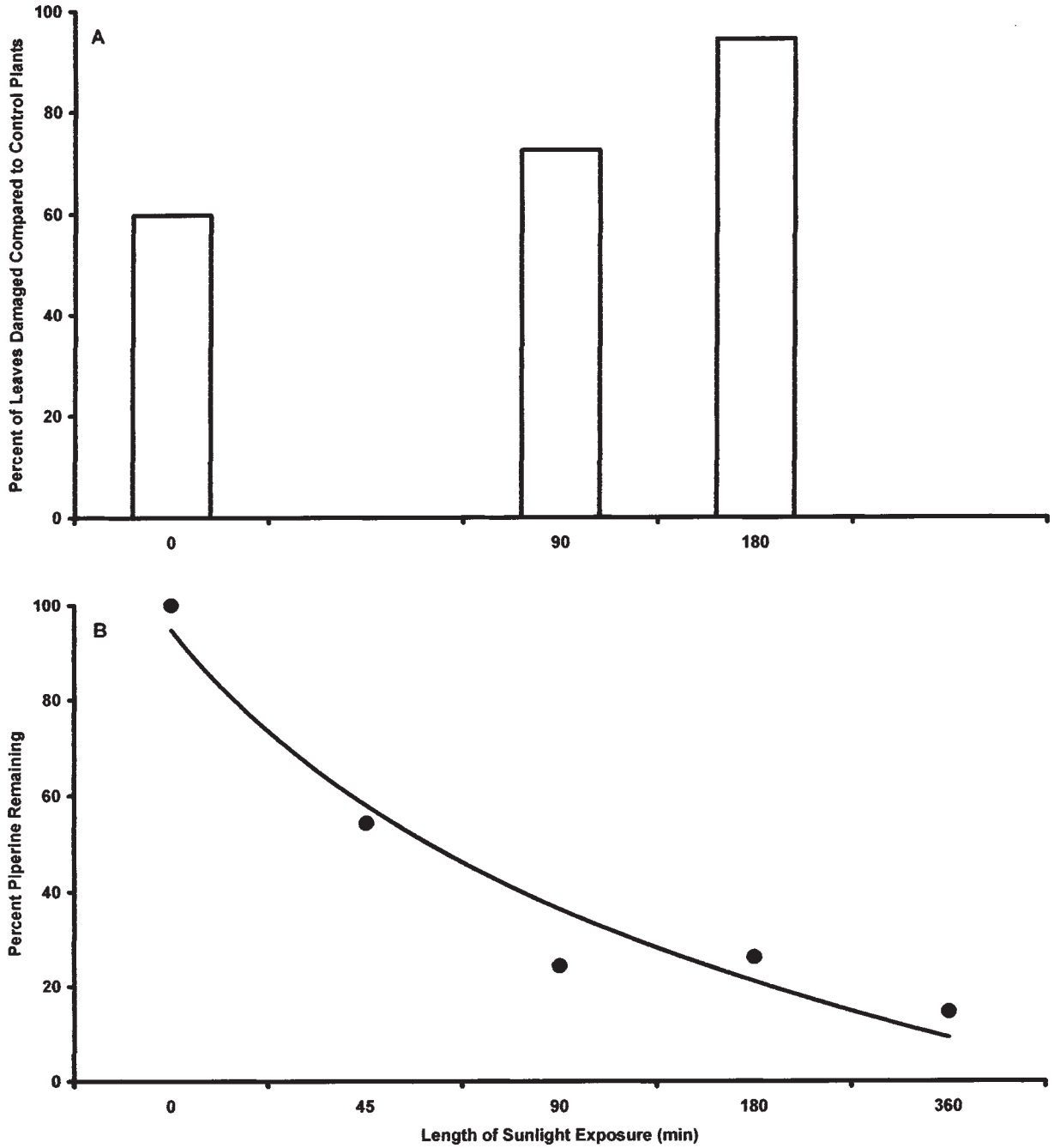

Fig. 9. Mean percent damage to potato plant leaves on plants previously treated with 0.5% *P. nigrum* and then exposed to full sunlight for either 0, 90, or 180 min before the addition of adult *L. decemlineata* (**A**) and the mean percent piperine remaining in *P. nigrum* extract samples exposed to full sunlight for 0, 45, 90, 180, and 360 min (**B**).

mode of action suggest that the mixture of analogues could be useful to mitigate the development of insecticide resistance, unlike many conventional products such as the single entity synthetic pyrethroids. In this case, insecticide-resistant *L. decemlineata* were found to have a tolerance ratio of less than 2 for *P. tuberculatum* while the tolerance ratios for cypermethrin, azinphosmethyl, and endosulfan were 11 to 22, 18 and 80, respectively (Agriculture and Agri-food Canada unpublished

data). One reason for the lower tolerance ratio exhibited by *P. tuberculatum*-treated *L. decemlineata* could be that insect detoxification enzymes have greater difficulty metabolizing mixtures of analogues (Berenbaum and Zangerl, 1996). In relation to Piperaceae chemical defence, it was demonstrated in *Aedes atropalpus* larval acute toxicity bioassays that tertiary and quarternary combinations of piperamides found in *P. tuberculatum* displayed synergistic activity (Scott et al., 2002). The additional significance, as demonstrated by Feng and Isman (1995), is that there is less evidence of resistance development over many generations with multiple actives compared to a single entity insecticide. Individually, the piperamide analogues share a bifunctional nature, both a neurotoxic effect and PSMO inhibition. With respect to the former, Lees and Burt (1988) determined that certain lipid amide activity is much like the effect of pyrethroids; a modification of axonal excitability through an effect on sodium currents. Later Ottea et al. (1990) found that N-alkyl amides act at site 2 of the sodium channel, which in the mouse brain is the alkaloid activator recognition site. Miyakado et al. (1989) suggested that piperamides have a mechanism distinct from that of the pyrethroids since a central nervous system (CNS) preparation from American cockroaches, *Periplaneta americana*, resistant to pyrethroids was affected by the same doses of pipercide which affected susceptible cockroaches. The results of the present study support these findings and further suggest that piperamides singly, or more importantly in combination, have a role to play in replacing contact insecticides for which resistance has developed.

Ours is the first experiment to demonstrate the PSMO inhibition by piperine in insects. Piperine had previously been shown to inhibit the activities of other detoxification enzymes in mammals; arylhydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin deethylase (7ECDE) activities in rat lung microsomes (Reen and Singh, 1991), 7-methoxycoumarin demethylase (MOCD) activity in hepatoma cells with and without phenobarbitol, a substrate that induces monooxygenase activity (Singh and Reen, 1994) and the major drug-metabolizing enzyme CYP3A4 (Bhardwaj et al., 2002). However, further mammalian studies showed that piperine can also induce phase I and II PSMO enzymes such as glutathione S-transferase (GST), cytochrome b_5, cytochrome P-450, acid-soluble sulfhydryl (-SH), and malondialdehyde (MDA) levels (Singh and Rao, 1993).Our bioassay indicates metabolism of methoxyresorufin to resorufin is inhibited in vitro. Whether this occurs in vivo or if PSMO inhibition contributes to the toxicity has yet to be confirmed.

Overall the activity of our *P. nigrum* based botanical suggests that contact toxicity is effective when early instar *L. decemlineata* larvae are targeted. Late instar larvae can be knocked down with concentrations above 0.1 and 50% of the adults can be killed with a 0.5% application. There is an apparent repellent or feeding deterrent action at 0.5% as well. However, oviposition is not deterred and there is little residual activity under full sunlight conditions. Combinations with other natural commercial repellents applied at their recommended rates did not improve the acute toxicity or the repellency of black pepper. We have shown, however, that pepper alone was as effective or more so than the Ropel® or neem oil (*Azadirachta indica*) when tested against another coleopteran pest, the Lily Leaf beetle *Lilioceris lilii* Scop. (Scott, unpublished data). Another positive feature of our pepper extract was the lack of phytotoxicity at the LD_{50} concentration. Burning of plant leaves at higher concentrations is likely a problem of the formulation rather than a consequence of the active ingredients and could be improved. Nontarget and beneficial species such as ladybird beetles (Coleoptera: Coccinellidae) and the stink bug *Perillus bioculatus* Fabricius (Hemiptera: Pentatomidae), both common predators in potato fields, were not tested. However, we assume that these species would likely be susceptible to the contact action of the *Piper* extracts.

One limitation to the use of *Piper* extract formulations is the lack of residual action. The repellent activity of piperine is quickly lost under full sunlight conditions through UV degradation. Activity may be prolonged on shaded surfaces such

as the underside of the leaf. It is apparent from use in stored product insect control applications that pepper residual activity is much greater when it is not exposed to light (Sighamony et al., 1986; Baier and Webster, 1992). Therefore, under field conditions repeated sprays may be required to reduce larval and adult *L. decemlineata* damage to plants. However, Piperaceae extracts in combination with other management tools such as perimeter trenches, mulching, crop rotation, and intercropping could provide better control of *L. decemlineata* and related agricultural pests for organic and small-scale farmers or gardeners in a healthier and environmentally sound manner.

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