Combination of protein-rich pea flour and pea extract with insecticides and enzyme inhibitors for control of stored-product beetles

Xingwei Hou

Department of Entomology, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2

Paul Fields[1]

Cereal Research Centre, Agriculture and Agri-Food Canada,
Winnipeg, Manitoba, Canada R3T 2M9

Wes Taylor

Saskatoon Research Centre, Agriculture and Agri-Food Canada,
Saskatoon, Saskatchewan, Canada S7N 0X2

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Abstract—Protein-rich pea flour and its extract are toxic to stored-product beetles and, at a concentration of 0.1%, can control these insects in a granary. To reduce the concentration of protein-rich pea flour needed to control stored-product beetles, natural products or currently used grain protectants (diatomaceous earth, neem, *Bacillus thuringiensis* (Berliner), malathion, and pyrethrum) were mixed with protein-rich pea flour in wheat. Mixtures were tested against the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), and the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Cucujidae). Neem and protein-rich pea flour acted synergistically against *T. castaneum*. Malathion and protein-rich pea flour acted synergistically against *S. oryzae*. Protein-rich pea flour combined with diatomaceous earth or pyrethrum acted additively against *S. oryzae*. All other combinations acted antagonistically. An extract from protein-rich pea flour reduced feeding of *S. oryzae*, and three enzyme inhibitors, piperonyl butoxide, profenofos, and diethyl maleate, were tested for their possible synergistic effects on feeding deterrence and mortality. Piperonyl butoxide and pea extract had additive effects, and diethyl maleate had no effect on the feeding and mortality of insects. Profenofos alone killed all insects in 3 days. The flour consumption of *S. oryzae* was positively correlated with LT_{50} (time to 50% mortality) in flour disks treated with pea extract.

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Résumé—La farine de pois riche en protéines et l'extrait de pois sont toxiques pour les coléoptères ravageurs des produits entreposés et permettent, à une concentration de 0,1 %, de lutter efficacement contre ces insectes dans un grenier à céréales. Afin de réduire la concentration de farine de pois riche en protéines nécessaire pour réprimer les coléoptères ravageurs des produits entreposés, nous avons ajouté à de la farine de pois riche en protéines mêlée à du blé divers produits naturels ou d'autres substances servant couramment à protéger les céréales, soit de la terre de diatomées, du neem, des bactéries *Bacillus thuringiensis* (Berliner), du malathion et de la poudre de pyrèthre. Nous avons évalué ces mélanges pour la lutte contre le charançon du riz, *Sitoplilus oryzae* (L.) (Coleoptera : Curculionidae), le tribolium

[1] Corresponding author (e-mail: pfields@agr.gc.ca).

rouge de la farine, *Tribolium castaneum* (Herbst) (Coleoptera : Tenebrionidae) et le cucujide roux, *Cryptolestes ferrugineus* (Stephens) (Coleoptera : Cucujidae). Le neem et la farine de pois riche en protéines ont une action synergique contre *T. castaneum*. Le malathion et la farine de pois riche en protéines agissent en synergie contre *S. oryzae*. La farine de pois riche en protéines additionnée de terre de diatomées ou de poudre de pyrèthre a une action additive contre *S. oryzae*. Toutes les autres combinaisons ont une action antagoniste. Un extrait de farine de pois riche en protéines réduit l'alimentation de *S. oryzae*. Nous avons évalué les effets synergiques possibles de trois inhibiteurs d'enzymes, le butoxyde de pipéronyle, le profenofos et le maléate de dyéthyle, sur la réduction de l'alimentation et sur la mortalité. Le butoxyde de pipéronyle et l'extrait de pois ont des effets additifs, mais le maléate de diéthyle reste sans effet sur l'alimentation et la mortalité des insectes. Le profenofos seul cause la mort de tous les insectes en 3 jours. Il y a une corrélation positive entre la consommation de farine par *S. oryzae* et le LT_{50} (temps nécessaire pour que le taux de mortalité atteigne 50 %) sur des disques de farine traités à l'extrait de pois.

[Traduit par la Rédaction]

Introduction

Many plants and their extracts have been investigated for the control of stored-product insects (Jacobson 1989; Weaver and Subramanyam 2000), with the most effective plants being spices or medicinal plants (Golob *et al.* 1999). Some foods, such as legume seeds, contain a wide range of allelochemicals with toxic and deterrent effects against insect pests (Harborne *et al.* 1971; Bell 1977). Yellow split peas *(Pisum sativum* L.) (Fabaceae) mixed with wheat are particularly effective in reducing the survival and reproductive output of *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), a major pest of stored cereals around the world (Coombs *et al.* 1977; Holloway 1986). Protein-rich pea flour causes adult mortality, interferes with reproduction (Bodnaryk *et al.* 1997), and is repellant to many stored-product insects (Bodnaryk *et al.* 1997; Fields *et al.* 2001). Protein-rich pea flour at a concentration of 0.1% effectively depresses beetle population levels in granary tests (Hou and Fields 2003*a*); however, this concentration may be too high for practical applications. To address this problem, an extract of protein-rich pea flour that is more toxic than protein-rich pea flour was developed (Bodnaryk *et al.* 1997). In separate studies (Delobel *et al.* 1998), a polypeptide isolated from peas was shown to be toxic to stored-product insects; however, neither of these isolates from peas is available in quantities sufficient to conduct granary trials.

Insects produce various detoxification enzymes that enable them to overcome the toxic effects of natural products from plants (Terriere 1984; Lindroth 1991). These enzymes include oxidases, glutathione *S*-transferases, and hydrolases. Some strains of *S. oryzae* can survive on yellow split peas (Holloway and Smith 1985; Grenier *et al.* 1997). In one resistant strain, mixed function oxidases, glutathione *S*-transferases, and hydrolases are involved in the detoxification of insecticidal compounds from peas (Holloway and Mackness 1988).

The goal of this study was to reduce the amount of protein-rich pea flour needed to control stored-product insects. We mixed protein-rich pea flour with natural products or currently used grain protectants, including diatomaceous earth, neem, *Bacillus thuringiensis* (Berliner), malathion, and pyrethrum, and measured the survival of three stored-product beetles in whole grain. On a smaller scale, we combined a pea extract with piperonyl butoxide (a mixed function oxidase inhibitor) (Holloway and Mackness 1988), profenofos (a hydrolase inhibitor) (Laecke and Degheele 1991), or diethyl maleate (a glutathione *S*-transferase inhibitor) (Welling and De Vries 1985) to study their potential synergistic effects on feeding deterrence and mortality of *S. oryzae*.

Materials and methods

Insects

Three insect species, *S. oryzae*, *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Cucujidae), and *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), were tested. All insects had been cultured in the laboratory at 30 °C and 70% RH for over 5 years. *Sitophilus oryzae* was reared on whole kernels of wheat, *C. ferrugineus* was reared on wheat kernels with 5% wheat germ and 5% brewer's yeast by mass, and *T. castaneum* was reared on wheat flour mixed with 5% brewer's yeast.

Preparation of mixtures of protein-rich pea flour and insecticides

Protein-rich pea flour (60% protein, 30% starch, and 7% moisture content (dry mass basis), Progress Protein, Parrheim Foods, Saskatoon, Saskatchewan) was used in this study. It is produced commercially by grinding peas and isolating a protein-rich fraction by air classification. Protein-rich pea flour was mixed with other compounds at the following ratios (protein-rich pea flour : other compound): Protect-It® (diatomaceous earth, Hedley Technologies Inc, Mississauga, Ontario), 1:1 (*w*/*w*); Novodor (*Bacillus thuringiensis* subsp. *tenebrioniz*, 3% active ingredient, Abbott Laboratories, North Chicago, Illinois) and Amazin™ (3% active ingredient, neem, Amvac Chemical Co, Los Angeles, California), 1:1 (*w*/*v*, g/mL); Premium Pyrocide® 175 (20% pyrethrins without piperonyl butoxide, McLaughlin Gormley King Co, Minneapolis, Minnesota), 40:1 (*w*/*v*, g/mL); and malathion (91% technical grade, Chipman Inc, Winnipeg, Manitoba), 400:1 (*w*/*v*, g/mL). Diatomaceous earth and protein-rich pea flour are powders and were blended in a mixer (American Hospital Supply Corporation, Evanston, Illinois) for 1 min before treating wheat. For treatments with *B. thuringiensis* and neem, wheat was treated with 1 mL of an aqueous solution of *B. thuringiensis* or neem at various concentrations, dried at room temperature for 24 h, and then mixed with the appropriate amount of protein-rich pea flour. For mixtures of protein-rich pea flour and pyrethrum or malathion, 250 µL pyrethrum or 25 µL malathion was dissolved in 15 mL acetone and then 10 g of protein-rich pea flour was added to the acetone. Protein-rich pea flour without other insecticides was mixed with acetone to test the effect of acetone on the toxicity of protein-rich pea flour, as a control. These mixtures were thoroughly blended and then freeze-dried for 24 h to remove the acetone. The dried powders were ground with a mortar and pestle and stored at –20 °C.

Toxicity of mixtures with insecticides

Toxicity studies were carried out on Canadian hard red spring wheat (*Triticum aestivum* L., Poaceae) with 14% moisture content (wet mass basis). Pretests were conducted to find suitable ranges of protein-rich pea flour and insecticide concentrations that caused 5% to 95% mortality of the three insect species (Table 1). Protein-rich pea flour at concentrations of 0, 100, 200, 400, 600, 800, and 1000 ppm (*w*/*w*) was tested against *S. oryzae*, and concentrations of 0, 100, 200, 400, 600, 800, 1600, and 3200 ppm were tested against *T. castaneum* and *C. ferrugineus*. Insecticide powders were weighed and added to 100 g of wheat in a 500-mL jar. For liquid forms, 1 mL of solution was pipetted into 100 g of wheat in the jar. Jars were sealed after insecticides were added, shaken by hand for 2 min, and then opened, if treated with liquid, to allow solvents to evaporate in a fume hood for 24 h. For each concentration, the treated grain was subdivided into five vials (29 mm diameter × 88 mm length; 20 g/vial) to give five replicates. Twenty 1- to 2-week-old adults of each of the three species were placed in separate vials, which were covered with a screen lid. The numbers of live and dead

TABLE 1. The range of concentrations (ppm) of various insecticides tested in combination with protein-rich pea flour against three species of stored-product beetles.

Treatment	*Sitophilus oryzae*	*Tribolium castaneum*	*Cryptolestes ferrugineus*
Diatomaceous earth	100 – 2 000	50 – 1 000	10 – 600
Protein-rich pea flour and diatomaceous earth (1:1)	50 – 2 000	50 – 1 000	10 – 2 000
Bacillus thuringiensis	250 – 16 000	300 – 16 000	300 – 16 000
Protein-rich pea flour and *B. thuringiensis* (1:1)	100 – 64 000	400 – 64 000	300 – 64 000
Neem	100 – 20 000	100 – 10 000	50 – 10 000
Protein-rich pea flour and neem (1:1)	1 000 – 2 000	100 – 10 000	50 – 10 000
Malathion	0.1 – 2		
Protein-rich pea flour and malathion (400:1)	25 – 800		
Pyrethrum	0.5 – 64		
Protein-rich pea flour and pyrethrum (40:1)	25 – 800		

NOTE: Each range contained six to eight concentrations for each test.

insects were counted after a 2-week incubation at 30 ± 1 °C and 70 ± 5% RH. Either acetone or water was tested as a control when each was used as a solvent.

Isolation of pea extract

To obtain insecticidal pea extracts, a method described by Bodnaryk *et al.* (1997) was followed with minor modifications. Protein-rich pea flour (120 g) was defatted by stirring in chloroform (1 L) for 1 h and then filtered with a Büchner funnel (Grade 1 Whatman paper). The defatted pea flour was dried at room temperature. A portion (100 g) of this flour was extracted with 80% methanol (2 L) under reflux (70 °C) for 5 min with stirring. The hot mixture was filtered and the filtrate was concentrated by rotary evaporation (bath temperature 37–40 °C) until less than 30% methanol remained. The concentrated solution was purified by stirring for 24 h with 10 g of Mitsubishi DIAION® HP20 beads (particle size, 250–600 µm; porosity, 300–600 Å). The beads were collected by filtration and washed with 30% methanol (500 mL). This wash was discarded. The beads were rewashed with 100% methanol (500 mL). The methanol was removed by rotary evaporation and the residue remaining in the flask was dried for 24 h. The yield of pea extract, isolated as a brown, waxy solid, was approximately 1%. All batches of pea extract were pooled for bioassay.

Combination of pea extract with enzyme inhibitors

Pea extract was dissolved in 70% ethanol and mixed with all-purpose wheat flour at concentrations of 0, 500, 1000, 2000, 4000, 8000, and 16,000 ppm. For mixtures of pea extract and enzyme inhibitors, profenofos, diethyl maleate, or piperonyl butoxide were mixed with all-purpose flour at 3000 ppm (Holloway and Mackness 1988) before the pea extract in ethanol was added. The flour was made into disks, and twenty 1- to 2-week-old *S. oryzae* adults were held on five disks in a petri dish at 30 °C and 70% RH (Xie *et al.* 1996). After 3 days, insects were removed and the remains of the flour disks weighed. Insects were then returned to the flour disks and mortality was noted daily. There were three petri dishes per treatment. Flour consumption was expressed as the amount of flour consumed in the treated disks divided by the amount consumed in the untreated control.

Data analysis

The LC_{50}s (concentration lethal to 50% of the population, Finney 1971) of protein-rich pea flour, pea extract, and the mixtures were calculated by probit analysis of all insects responding to each concentration using POLO-PC (LeOra Software 1994). The regression lines were compared to determine their equality or parallelism using a likelihood ratio test ($\alpha = 0.05$). LT_{50}s (time to 50% mortality) of pea extract were estimated with survival analysis (Glantz 2001) using SigmaPlot (SPSS Inc 2003). The correlation (Steel *et al.* 1997) between LT_{50} and flour consumption of *S. oryzae* was tested with the SAS REG procedure (SAS Institute Inc 2000).

The co-toxicity coefficient of each mixture (Sun and Johnson 1960), which is based on the LC_{50} and the proportion of each component in the mixture, was used to evaluate synergistic, additive, or antagonistic responses. A co-toxicity coefficient <80 is considered as indicating antagonism, whereas a coefficient between 80 and 120 or >120 is considered as indicating additiveness or synergism, respectively.

Results

Mixtures with insecticides

Acetone did not affect the toxicity of protein-rich pea flour against *S. oryzae*. The LC_{50} and the slope of the regression line of protein-rich pea flour suspended in acetone (288 ppm, 4.79 ± 0.32) were the same as those of protein-rich pea flour without acetone (281 ppm, 3.97 ± 0.35) ($\chi^2 = 3.1145$, $P = 0.211$). Protein-rich pea flour and neem at a ratio of 1:1 acted synergistically against *T. castaneum* and antagonistically against *S. oryzae* and *C. ferrugineus* (Table 2). Protein-rich pea flour and diatomaceous earth had additive effects against *S. oryzae* and were antagonistic against *T. castaneum* and *C. ferrugineus* (Table 2). *Bacillus thuringiensis* did not increase the toxicity of protein-rich pea flour against the three insect species examined. The co-toxicity coefficient of the mixture of *B. thuringiensis* and protein-rich pea flour was 37 against *S. oryzae* but could not be calculated for *T. castaneum* and *C. ferrugineus* because of the low mortality. Protein-rich pea flour had little effect on *T. castaneum*, and only high concentrations were active against *C. ferrugineus* (Table 2). The combination of protein-rich pea flour and malathion had a synergistic effect against *S. oryzae*, whereas the combination of protein-rich pea flour and pyrethrum had an additive effect (Table 3).

Mixtures with enzyme inhibitors

Cumulative mortality of *S. oryzae* fed on wheat flour disks without pea extract or enzyme inhibitors was less than 4%. Piperonyl butoxide alone caused cumulative mortality of *S. oryzae* of 17%, 31%, 56%, 59%, and 64% after 6, 8, 10, 12, and 14 days, respectively. Diethyl maleate alone caused no mortality within 14 days. The pea extract alone caused more than 50% mortality after 14 days at all concentrations. Therefore, we compared LC_{50}s using data from day 10, when mortalities were between 5% and 95%. The LC_{50} of the mixture of pea extract and piperonyl butoxide (1020 ppm; 95% CI, 414–1712) was significantly lower than that of pea extract alone (1728 ppm; 95% CI, 1060–2536) ($\chi^2 = 20.1761$, $P < 0.001$). No difference in LC_{50}s was detected between the pea extract alone and the mixture of pea extract and diethyl maleate (1700 ppm; 95% CI, 1412–2342) ($\chi^2 = 2.0566$, $P = 0.358$). The regression lines of pea extract with piperonyl butoxide ($\chi^2 = 1.3862$, $P = 0.239$) and pea extract with diethyl maleate ($\chi^2 = 1.928$, $P = 0.165$) were parallel to that of pea extract alone.

After 3 days, all *S. oryzae* on flour disks treated with piperonyl butoxide, diethyl maleate, or combinations with pea extract had survived. Flour consumption by insects

TABLE 2. LC_{50} (95% CI) (ppm), slope of the regession line (mean ± SEM), and index of heterogeneity (*g*) of protein-rich pea flour and its mixtures with neem and diatomaceous earth at ratios of 1:1 against three beetle species after a 2-week incubation at 30 °C and 70% RH.

	Sitophilus oryzae			*Tribolium castaneum*			*Cryptolestes ferrugineus*		
Treatment	LC_{50}	Slope	*g*	LC_{50}	Slope	*g*	LC_{50}	Slope	*g*
Protein-rich pea flour alone	263 (175–348)	3.12±0.22	0.140	na*	na	65.919	3753 (1252–8743)	1.27±0.08	0.243
Neem alone	6752 (4957–8210)	6.59±0.55	0.512	1024 (264–2147)	1.11±0.12	0.230	4982 (na)	5.27±0.58	0.828
Protein-rich pea flour with neem	2767 (1862–5555)	1.89±0.20	0.081	601 (22–2099)	0.59±0.10	0.431	5590 (na)	2.57±0.43	1.994
Co-toxicity coefficient	18			170			77		
Diatomaceous earth alone	145 (104–180)	3.97±0.40	0.155	284 (222–327)	7.04±1.07	0.152	18 (16–19)	5.81±0.60	0.041
Protein-rich pea flour with diatomaceous earth	205 (172–235)	4.60±0.43	0.068	497 (453–533)	7.13±0.81	0.049	85 (72–96)	5.60±0.84	0.179
Co-toxicity coefficient	91			57			42		

* na, not available because mortality was <20%.

TABLE 3. LC_{50} (95% CI) (ppm), slope of the regression line (mean ± SEM), and index of heterogeneity (*g*) of protein-rich pea flour, pyrethrum, malathion, and mixtures of protein-rich pea flour with pyrethrum (40:1) or malathion (400:1) against *Sitophilus oryzae* after a 2-week incubation at 30 °C and 70% RH.

Treatment	LC_{50}	Slope	*g*
Protein-rich pea flour alone	289 (232–346)	4.79±0.32	0.099
Pyrethrum alone	37 (32–41)	7.69±0.92	0.160
Protein-rich pea flour with pyrethrum	221 (202–239)	6.29±0.64	0.04
Co-toxicity coefficient	111		
Malathion alone	1.5 (1.3–1.7)	8.04±0.78	0.087
Protein-rich pea flour with malathion	136 (126–147)	7.24±0.78	0.044
Co-toxicity coefficient	143		

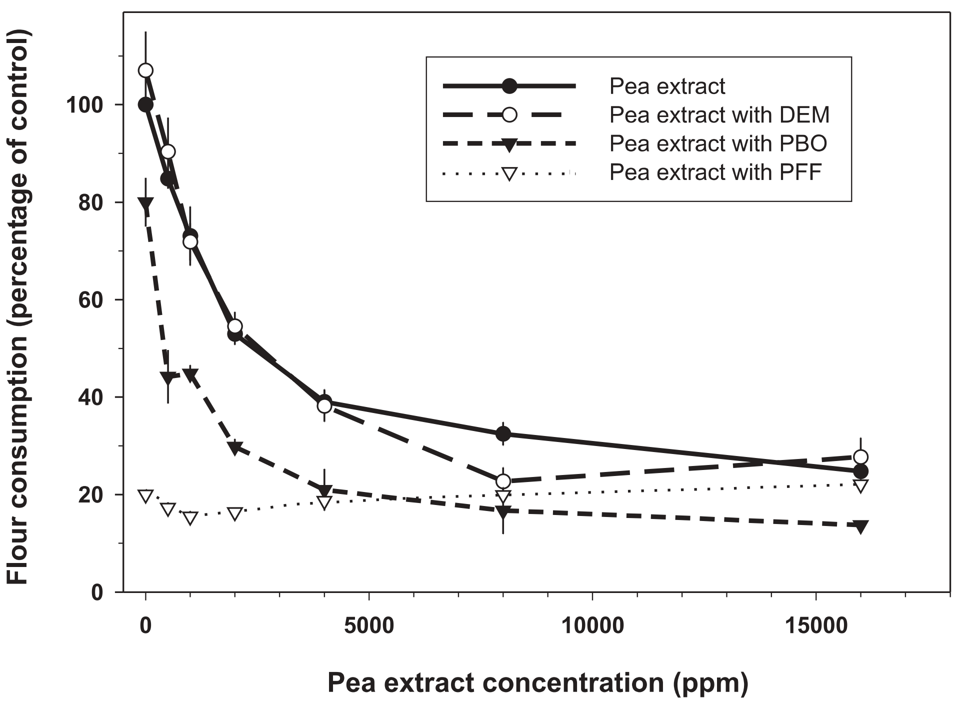

FIGURE 1. Flour consumption (percentage of control) by 25 adult *Sitophilus oryzae* after 3 days on wheat flour disks treated with pea extract at various concentrations or combined with 3000 ppm piperonyl butoxide (PBO), diethyl maleate (DEM), or profenofos (PFF).

on flour disks treated with profenofos or piperonyl butoxide alone was reduced to 20 ± 1% and 80 ± 5%, respectively, of that on untreated control disks (Fig. 1). Diethyl maleate alone did not reduce flour consumption (107 ± 8% of control) of *S. oryzae*. Flour consumption of *S. oryzae* was reduced with increasing concentrations of pea extract (Fig. 1) and was lower with the combination of pea extract and piperonyl butoxide than on the pea extract alone at all concentrations. Diethyl maleate combined with pea

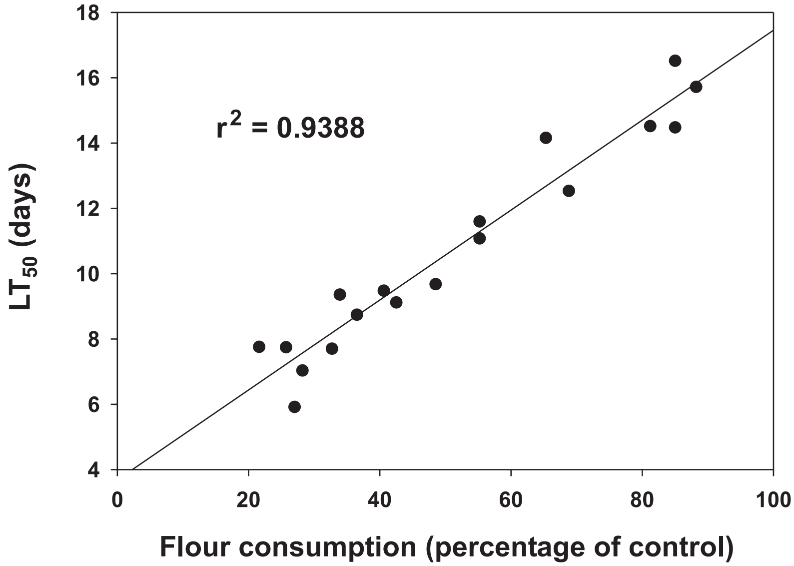

FIGURE 2. Relationship between LT_{50} (d) and flour consumption (percentage of control) of *Sitophilus oryzae* over 3 days on wheat flour disks treated with pea extract at various concentrations.

extract did not reduce flour consumption compared with that on the pea extract alone. Flour consumption of *S. oryzae* on the combination of pea extract and profenofos was not related to the concentration of pea extract (Fig. 1).

Flour consumption of *S. oryzae* was positively correlated with the LT_{50} of pea extract (Fig. 2). It was described by the formula $LT_{50} = 3.6852 + 0.1377 \times$ 3-day flour consumption (%) ($r^2 = 0.9388$).

Discussion

Protein-rich pea flour shows good potential as a grain protectant (Bodnaryk *et al.* 1997; Delobel *et al.* 1998; Fields *et al.* 2001; Hou and Fields 2003*a*, 2003*b*). The objective of this study was to find ways to enhance the effectiveness of protein-rich pea flour by mixing it with other natural products or currently used grain protectants. The effect of combinations, however, was insect-specific, and none of the compounds dramatically increased the efficacy of protein-rich pea flour against all insects.

For an additive effect, diatomaceous earth could be combined with protein-rich pea flour to control *S. oryzae*. One advantage of mixing diatomaceous earth with protein-rich pea flour is that the concentrations of both can be reduced. Thus, the undesirable effects of diatomaceous earth, such as reduction of bulk density (Korunic *et al.* 1998), can be partially mitigated. There are a number of mechanisms by which protein-rich pea flour and diatomaceous earth could work together. Protein-rich pea flour increases movement of *S. oryzae* (Hou and Fields 2003*a*), which may cause more contact with diatomaceous earth. In addition, food provides an important water source for *S. oryzae* (Arlian 1979); protein-rich pea flour reduces *S. oryzae* feeding and thus probably lowers water reserves, and diatomaceous earth absorbs the wax layer of the cuticle and kills *S. oryzae* through desiccation (Carlson and Ball 1962). Finally, protein-rich pea flour is more toxic under dry conditions (Hou and Fields 2003*b*).

Insects produce various enzymes, such as mixed function oxidases, gluthione *S*-transferases, and hydrolases, to protect themselves from toxic plant compounds (Krieger *et* *al.* 1971; Ahmad 1982; Dowd *et al.* 1983). Inhibition of detoxifying enzymes decreases the insect defense system and increases the toxicity of the insecticide (Ishaaya 1993). The mixed function oxidase inhibitor piperonyl butoxide and the esterase inhibitor tributylphosphorotrithioate, mixed with yellow split peas, increased the mortality of an *S. oryzae* strain that is normally able to reproduce on peas (Holloway and Mackness 1988). The glutathione *S*-transferase inhibitor diethyl maleate, the hydrolase inhibitor profenofos, and piperonyl butoxide enhance the toxicity of diflubenzuron, a chitin synthesis inhibitor, against beet armyworm, *Spodoptera exigua* (Hübner) (Laecke and Degheele 1991; Ishaaya 1993). Piperonyl butoxide enhances the toxicity of pyrethrum by inhibiting mixed function oxidases, which degrade pyrethrum (Jones 1998). In this study, diethyl maleate did not enhance the antifeedant activity or toxicity of the pea extract. This suggests that glutathione *S*-transferase is not involved in the detoxification of pea extract in susceptible *S. oryzae*. This study showed that, against *S. oryzae*, piperonyl butoxide was toxic, acted as an antifeedant, and had an additive effect on both the antifeedant activity and toxicity of pea extract; thus, it could be used as an additive to increase the effectiveness of pea extract. It is not known, however, if mixed function oxidases are involved in the detoxification of pea extract. Profenofos was very toxic, killing all insects in 3 days, and therefore no interaction with pea extract was detected.

Protein-rich pea flour and pea extract may cause *S. oryzae* to die from starvation. The greater the concentration of pea extract, the less *S. oryzae* ate and the faster they died. One mechanism that regulates food intake is feedback from mechanoreceptors located in the gut wall. When the gut wall is distended, feeding is inhibited via the central nervous system (Bernays and Simpson 1982). Large bubbles form in the midgut of *S. oryzae* fed on flour disks treated with pea extract (unpublished data), which may cause signals to be sent to the brain and in turn cause cessation of feeding. The close relationship of flour consumption with the toxicity of the pea extract indicates that 3-day flour consumption could be used as a screening tool for insecticides.

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