CONTROL OF STORED-PRODUCT INSECTS WITH PROTEIN-RICH PEA FLOUR AND ITS EXTRACT

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Dedicated to my wife

Hongwen Tan

my sons

Jue and Albert

and my relatives in China

ABSTRACT

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Stored-product insects cause billions of dollars of losses every year throughout the world. Synthetic insecticides are widely used to control stored-product insects. However, their use is decreasing, because of concerns about insecticide residues in grain, damage to the environment, worker safety, insecticide resistance and increasing costs. To reduce the use of synthetic insecticides, many natural products have been investigated for the control of stored-product insects. Laboratory studies indicated that protein-rich pea flour, a food additive, was repellant, toxic and reduced offspring of three common stored-product insects, *Sitophilus oryzae* (L.) (rice weevil), *Cryptolestes ferrugineus* (Stephens) (rusty grain beetle) and *Tribolium castaneum* (Herbst) (red flour beetle). *Sitophilus oryzae* was the most sensitive species, followed by *C. ferrugineus* and *T. castaneum*. Insects held on wheat and barley showed similar sensitivities to protein-rich pea flour. Higher doses were required to control insects held on maize. Protein-rich pea flour was more toxic at high temperature and low moisture. Although protein-rich pea flour reduced the penetration of package material by *S. oryzae*, it did not prevent insects from entering perforated packaging.

A granary trial conducted in six 30-tonne bins and each filled with 11 tonnes of barley showed that fewer insects moved into, and more insects moved out of the barley treated with protein-rich pea flour. Treating the entire grain mass with 0.1% protein-rich pea flour had a similar effect to treating the top-half with 0.5% protein-rich pea flour. *Sitophilus oryzae* populations were reduced by approximate 90%. The populations of *C. ferrugineus* and *T. castaneum* were reduced by 50-70% in both treatments. Similar reductions were seen in offspring adults.

Unlike other insecticides, protein-rich pea flour was neither repellent and nor toxic to *Anisopteromalus calandrae* (Howard), a parasitoid of *S. oryzae*, or *Cephalonomia waterstoni* (Gahan), a parasitoid of *C. ferrugineus*. Protein-rich pea flour did not increase the mortality of the two parasitic wasps. It did not reduce the searching ability of parasitoids, and nor did it reduce the production of parasitoid offspring. A large-scale trial combining 0.1% pea protein with parasitoids showed that the population of *S. oryzae* was reduced by 98% and that of *C. ferrugineus* by 75%, which is greater than treatments of parasitoids alone (46% for *S. oryzae*, 50% for *C. ferrugineus*).

Neem and protein-rich pea flour acted synergistically against *T. castaneum*. Malathion and protein-rich pea flour acted synergistically against *S. oryzae*. In contrast, combination of protein-rich pea flour with diatomaceous earth or with pyrethrum acted additively against *S. oryzae*. All other combinations acted antagonistically. A mixed function oxidase inhibitor, piperonyl butoxide, increased the effectiveness of a pea extract as an antifeedant, and increased the mortality of *S. oryzae*. However, a gluthion-S-transferase inhibitor, diethy meleate, did not enhance the antifeedant effect or toxicity of the pea extract.

A large volume of gas was observed in the midgut of *S. oryzae* fed on flour disks treated with protein-rich pea flour, pea extract or pea peptide. Vital staining with fluorescent dyes, calcein AM and propidium iodide, showed that the tissue of the midgut of the insects was damaged by the pea peptides.

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FOREWARD

This thesis is written in manuscript style according to the Journal of Economic Entomology except for the following chapters:

Chapter 3 followed the format of Entomologia Experimentalis et Applicata;

Chapter 5 and 7 followed Journal of Stored Products Research and;

Chapter 6 followed Environmental Entomology.

CHAPTER 1

General Introduction

Stored-product insect pests cause losses by reducing dry weight, germination, nutritional value, or the grade of harvested grain (Semple et al. 1992). The Food and Agricultural Organisation of The United Nations estimates that 5 to 10% of harvested grain is lost in storage, with losses being higher in some developing countries (Hall 1970).

There are more than 200 insect species that infest stored products. *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae) are among the most common and destructive stored-product insect pests, and were used for evaluating the efficacy of commercialized protein-rich pea flour in this study. *Sitophilus oryzae* is one of the most serious cosmopolitan insects, and ranks as 8th commonest insect in grain elevators in Canada (Sinha and Watters 1985). It damages grain cereals, such as wheat, barley and maize and prefers feeding on grain kernels. Females lay eggs within the kernels. Larvae feed inside the kernels until they emerge as adults. The life cycle of *S. oryzae* takes approximate 25 days at 25°C, 70% relative humidity (RH). *Cryptolestes ferrugineus* and *T. castaneum* have a cosmopolitan distribution and are also dominant stored-product insect species in Canada (Sinha and Watters 1985, Madrid et al. 1990). Both species prefer broken kernels or flour of cereals. Unlike *S. oryzae*, neither species can infest whole unbroken kernels, and the larvae and

adults feed on damaged kernels or flour. *Cryptolestes ferrugineus* also feeds preferentially on the germ, resulting in losses in quality and seed germination. Both species reproduce rapidly. The complete life cycle at optimum conditions of 33°C, 70% RH takes 20 days for *T. castaneum* and 23 days for *C. ferrugineus* (Sinha and Watters 1985, Hill 1990).

Synthetic insecticides, such as deltamethrin, malathion, chlorpyrifos-methyl, phosphine and methyl bromide, are the main means to control stored-product insects (Harein and Davis 1992, Arthur 1996). However, increased concerns by consumers over insecticide residues, the occurrence of insecticide-resistant strains and the precautions necessary for the application of these traditional chemical insecticides have increased the interest in alternative control methods. Re-evaluation of insecticides has resulted in de-registration of several synthetic insecticides. In the United States, the use of chlorpyrifos-methyl on stored wheat may be discontinued because of a voluntary cancellation caused by the high cost of updating its registration data (Anonymous 2000). Methyl bromide is an atmosphere ozone depletor, and may be banned after 2005 in most industrialized countries (Fields and White 2002). Hence, there is a pressing need to develop new methods to protect stored products that are effective, and safe for humans and the environment.

Higher plants are a rich source of novel insecticides (Prakash and Rao 1997). Many plants have been investigated for the control of stored-product insects (Jacobson 1989a, Weaver and Subramanyam 2000). Most effective plants are found to be either spices or medicinal plants (Golob et al. 1999). Azadirachtin from the Indian neem tree (*Azadirachta indica* A. Juss., Meliaceae) (Saxena et al. 1988, Jilani and Saxena 1990),

and pyrethrum from chrysanthemums (Prakash and Rao 1997) have received the most attention. However, because of the structural complexity of azadirachtin, the instability of pyrethrum, and the limited availability and the high cost of both, the search for other natural insecticides continues.

Legume seeds contain a wide range of allelochemicals with toxic and deterrent effects against insects (Harborne et al. 1971, Bell 1977). Most stored-product insects are unable to develop on legumes (Singh and Wilbur 1966, Sinha and Watters 1985). In Africa, mixing legumes with the grain is a technique used to protect maize from stored-product insect attack (Coombs et al. 1977). Coombs et al. (1977) suggest admixing yellow split peas *(Pisum sativum* L.) with grain to control *S. oryzae*. Yellow split pea mixed with wheat reduces the survival and reproduction rate of *S. oryzae* (Coombs et al. 1977, Holloway 1986). Protein-rich pea flour (Progress Protein, Parrheim Food, Saskatoon, SK; 60% protein, 30% starch, 7% moisture content) is commercially produced by grinding peas and separating the particles by air an classification method. Protein-rich pea flour is used as an additive in breakfast cereals, dry mixes, snacks and baby foods. (Parrheim Foods 2003). Protein-rich pea flour made from yellow split peas is repellent (Fields et al. 2001) and toxic, and reduces the reproduction of many stored-product insect pests (Bodnaryk et al. 1997). Bodnaryk et al. (1997) produced an extract from protein-rich pea flour that is 20 to 100 times more toxic than protein-rich pea flour itself. A polypeptide from peas is toxic to stored-product insects (Delobel et al. 1998).

Many factors affect the efficacy of grain protectants. The temperature and moisture content of stored grain are affected by seasonal climate change, and the efficacy of grain protectants is affected by grain temperature and moisture content (Ioradanou and

Watters 1969, Snelson 1987, Samson et al. 1988, 1990). Malathion and pirimiphos-methyl have positive temperature coefficients for control of stored-product insects (Iordanou and Watters 1969, Snelson 1987). However, pyrethrins show a negative relationship with temperature for *S. oryzae* (Longstaff and Desmarchelier 1983). The toxicity of chlorpyrifos increases with higher moisture content, whereas the toxicity of malathion and diatomaceous earth decreases with higher moisture contents (Barson 1983, Fields and Korunic 2000a). Pyrethrins, malathion and pirimiphos-methyl breakdown faster in grain with higher moisture contents (Wilbur 1952, Quinlan et al. 1980). In addition, the efficacy of insect growth regulators, such as methoprene and fenoxycarb (Samson and Parker 1989, Samson et al. 1990), also varies among grain species. No studies have investigated the effect of the size of grain kernels on the efficacy of the insecticides. These factors that may affect the efficacy of protein-rich pea flour were investigated (Chapter 3).

All studies on the insecticidal properties of pea products were conducted in the laboratory on a small scale. The field environment is much more complex than a laboratory environment. Any prospective grain protectant must be tested in commercial granaries. Although protein-rich pea flour has been shown effective in the laboratory (Bodnaryk et al. 1997), until this study, it had not been tested under commercial conditions (Chapter 4).

Combining two or more insecticides or using synergists is one way to increase efficacy or broaden the spectrum of activity. For example, piperonyl butoxide is often mixed with pyrethrum to increase the efficacy of pyrethrins. Malathion and deltamethrin are mixed together to control both *Sitophilus spp.* and *Rhyzopertha dominica* (F.)

(Desmarchelier 1977). Laboratory and granary studies indicated that 0.1% of protein-rich pea flour on cereals was required to effectively control insect populations. To investigate the possibility of reducing the amount or enhancing the efficacy of protein-rich pea flour needed to control stored-product insects, protein-rich pea flour was mixed with currently-used grain protectants or natural products in this study (Chapter 5), including diatomaceous earth, neem, *Bacillus thuringiensis*, malathion and pyrethrum. An extract from protein-rich pea flour was isolated and combined with the general enzyme inhibitors, piperonyl butoxide (a mixed function oxidase inhibitor), profenofo (a hydrolase inhibitor), and diethyl meleate (a glutathion-S-transferase inhibitor), to study their effects on the feeding and mortality in *S. oryzae*.

Parasitoids can be used to control stored-product insects (Schöller and Flinn 2000). *Anisopteromalus calandrae* (Howard) (Hymenoptera: Pteromalidae) is an ectoparasitoid of *S. oryzae*. *Cephalonomia waterstoni* (Gahan) (Hymenoptera: Bethylidae) is an ectoparasitoid of *C. ferrugineus*. The population dynamics of *C. waterstoni* on *C. ferrugineus* has been studied by Flinn and Hagstrum (1995). Both parasitoids effectively suppressed the populations of their hosts (Press et al. 1983, Cline et al. 1985, Flinn et al. 1996). Botanicals are usually less toxic to parasitoids than synthetic insecticides (Schöller and Flinn 2000). The effect of protein-rich pea flour on parasitoids was examined to explore the possibility of combining parasitoids and protein-rich pea flour (Chapter 6).

Stored-product insects cause losses not only to bulk grain but also to finished products by penetrating through packages materials or invading through existing holes. Packaging of products is the last line of defense for processors against insects infesting their finished products. In addition to improving the packaging material and design,

insect repellents are used to prevent insects from entering packages by modifying the behavior of insects (Highland 1984, Mullen 1994, Watson and Barson 1996, Mullen and Mowery 2000). Pyrethrins synergized with piperonyl butoxide have been approved as a treatment for insect-resistance packaging on the outer layer of packages or with adhesive in the USA (Highland 1991). Protein-rich pea flour is a food-based repellent. It might be effective in preventing insects from penetrating or invading food packages (Chapter 7).

Some strains of *S. oryzae* survive in the kernels of yellow split peas (Grenier et al. 1997, Holloway and Smith 1985). Various detoxification enzymes are induced to protect insects from plant defense compounds (Lindroth 1991, Terriere 1984). These enzymes include oxidases, glutathion transferases, and hydrolases. Holloway and Mackness (1988) reported that mixed function oxidases, gluthion-S-transferase and hydrolases may be involved in the detoxification of insecticidal compounds in peas in a resistant *S. oryzae* strain. Some general inhibitors of the above enzymes were tested to determine their involvement in the toxicity of the pea extract to susceptible *S. oryzae* (Chapter 5). The mode of action of protein-rich pea flour and its extract has not been determined. Insecticides have three means to enter an insect: ingestion, absorption across the cuticle and as a gas through the respiratory system. A test was conducted to determine if the gut of insects was affected by protein-rich pea flour and its extract, and if the toxins in peas could enter *S. oryzae* as a gas or through the abdominal cuticle of the adults (Chapter 8).

In summary, the purpose of my thesis was to explore protein-rich pea flour as a grain protectant. I investigated:

- the effect of biotic and abotic factors on the efficacy of protein-rich pea flour (Chapter 3);

- the effect of protein-rich pea flour in a field trial (Chapter 4);
- the efficacy of protein-rich pea flour combined with other insecticides, enzyme inhibitors (Chapter 5) and parasitoids (Chapter 6);
- the efficacy of protein-rich pea flour on packaging (Chapter 7) and;
- the mode of action of protein-rich pea flour and its extract (Chapter 8).

CHAPTER 2

Literature Review

Stored-product insects and their damage

The infestation of stored products by insects is a worldwide problem. For nearly 10, 000 years, people have been storing grain as seed, and for consumption by humans and livestock (Jin 1984). Many insects have adapted to the grain storage habitat. Stored-product insects were found in grain stored in Egyptian tombs that were sealed in 2,500 BC (Levinson and Levinson 1998). A survey of stored-product insects in China in 1995 listed insects belonging to 10 orders, 52 families, and 143 genera and 242 species. Ninety-five arthropod species were found in flour mills, grain elevators and feed mills in survey from 1969 to 1981 in Canada, and insects were found in 69% of the samples. Most of the damage was caused by approximately 20 insect species in the world. These insects include Coleoptera: *Sitophilus oryzae* (L.), *Sitophilus granarius* (L.), *Sitophilus zeamais* (Motschulsky), *Tribolium castaneum* (Herbst), *Cryptolestes ferrugineus* (Stephens), *Rhyzopertha dominica* (F.), *Oryzaephilus surinamensis* (L.), *Lasioderma serricorne* (F.) and *Stegobium paniceum* (L.), and Lepidoptera: *Plodia interpunctella* (Hübner) and *Ephestia cautella* (Walker), (Sinha and Watters 1985). *Sitophilus oryzae, T. castaneum* and *C. ferrugineus* are cosmopolitan insects, and rank in the top ten most common insects in grain elevators in Canada (Sinha and Watters 1985).

Stored-product insects cause tremendous losses by lowering seed weight, germination rate, nutritional value and grain grade (BSTIDC 1978). Although there has

been no recent estimate, The Food and Agricultural Organization of The United Nations estimates that losses of grain are about 5 to 10% from harvest to consumption, and the losses are greater in developing countries (Hall 1970). In the United States of America, stored-product insects result in the loss of eight to16 million tonnes of stored grain annually, which cost approximately $465 million in 1965 US dollar per year due to the reduction in grain quality and quantity (USDA 1965). One *Sitophilus oryzae,* one of the major stored-product insects, causes 20% weight loss of wheat kernels during its five-week development from the egg to the adult (White 1953). Caliboso et al. (1985) reported that without proper protection measures, stored-product insects cause weight losses of 35% in maize in the Philippines during eight months of storage.

Methods to control stored-product insects

Due to the tremendous losses caused by stored-product insects, there has been considerable research conducted on methods to control these insects. These methods include the use of synthetic insecticides, botanical insecticides, biological control and physical control.

Synthetic insecticides

Synthetic insecticides are the most effective means in many situations to control stored-product insects (Harein and Davis 1992). They will play a major role in stored-product pest management in the years to come (Arthur 1996). However, as part of integrated pest management programs for stored-product insects, synthetic insecticides should only be used when there are no other effective or safer methods available, because of the concerns over chemical residues in the grain, worker safety and the development of

insecticide-resistant insect populations (Harein and Davis 1992). Synthetic insecticides used for protection of grain in storage are classed as protectants or fumigants.

Grain protectants have been used as the primary means for grain protection in storage since the 1960s. They are applied to the grain as a liquid or a powder, and they are absorbed through the gut or cuticle of insects. Some effective insecticides, such as DDT and dieldrin, are no longer used due to potential hazards to human health or to the environment. Snelson (1987) provides a comprehensive review of the efficacy and toxicity of grain protectants. Arthur (1996) illustrates the development of grain protectants for the future. The most commonly used protectants are organophosphates, such as malathion, pirimiphos-methyl and fenitrothion; and the synthetic pyrethroids, such as deltamethrin. These conventional protectants generally are more effective than biological control or physical controls in warm climates. They are usually safe, easy to apply to the grain and provide long-term protection. However such protectants are becoming less available because of deregistration of protectants and the increased costs for the development of new protectants.

Fumigants are applied as a liquid, solid (pellets) or as a gas. From various formulations, gases are generated, and disperse into interstitial spaces of the grain and kill stored-product insects. The fumigants do not provide long-term protection of grain. Globally, methyl bromide and phosphine are the most commonly used grain fumigants. Both fumigants have broad-spectrum of insecticidal activity. Methyl bromide acts rapidly, and is often used for space and quarantine treatments in the grain storage system. However, methyl bromide depletes stratospheric ozone and is scheduled to be phased out

by 2005 in developed countries and 2015 in developing countries (Fields and White 2002).

Phosphine gas has been used worldwide to disinfest stored grains and other commodities for more than four decades. It is the dominant fumigant for grain storage in most countries. In some situations, it can be used as a replacement for methyl bromide fumigation (Fields and White 2002). However, compared with methyl bromide, it needs longer exposure times (three- to five-day exposures), and warmer temperatures (more than 20°C) to kill insects, and it is corrosive to copper and many other metals (Bond et al. 1984). Phosphine kills insects by altering cellular redox balance, and disrupting normal metabolism in the mitochondrial electron transport chain. Oxygen is essential for uptake of phosphine and its action (Chaudhry 1997).

Carbon dioxide (Mann et al. 1999) is registered as a fumigant in several countries, but it has only been used to a limited extent because it requires longer exposure times and higher doses than phosphine, and must be used in air-tight circumstance. Sulfuryl fluoride (Bell and Savvidou 1999) has been used for decades to control wood-boring insects, and recently additional studies have been conducted to expand its use to grain and flour mills (Fields and White 2002). Carbonyl sulfide (Tan et al. 1998, Weller and Morton 2001) is another promising fumigant, although it is not yet registered for use on grain anywhere in the world.

Problems with synthetic insecticides

At one time, synthetic pesticides were thought to be the "silver bullet" with the ability to solve all pest control problems (Pimentel 1985). However, several problems with synthetic insecticides have come to light with their wide-spread use. These include:

environmental contamination, control failure due to insect resistance, resurgence of pests, secondary pest outbreaks, as well as the killing of wildlife, predators, and beneficial insects at the same time as pests (Mandava 1985, Berenbaum 1989, Prakash and Rao 1997).

Rising resistance of insects to synthetic insecticides could result in some insecticides being eliminated in the future (Arthur 1996). Many stored-product insects around the world are resistant to malathion (Subramanyam and Hagstrum 1995) and phosphine (Zettler et al. 1989, Bengston et al. 1999). A global survey of insect resistance conducted by the Food and Agriculture Organization in 1972 found that 10% of the tested stored-product insect populations had phosphine-resistant individuals (Champ and Dyte 1976). Nine out of ten *R. dominica* strains are resistant to phosphine (Anonymous 1997). Resistant *R. dominica, T. castaneum* and *C. ferrugineus* reduce the uptake of phosphine (Chaudhry 1997). The gene for phosphine resistance in *T. castaneum* is autosomal and semidominant (Bengston et al. 1999). Some cases of phosphine resistance are so severe that resistance causes control failure. In addition, some strains that are resistant to phosphine are also resistant to other synthetic insecticides (Cochran 1995).

There are increasing concerns about environmental and health hazards of pesticides, especially when they are used in and around food products. Pimentel (1985) reported that The United States used 200 million kg of insecticide annually for pest control in the 1980's. These insecticides enter the environment as residues in the air, water, and food chains, and result in environmental pollution and danger to human beings (Crosby 1995). Fumigant applicators exposed to phosphine have significantly increased stable chromosome rearrangements, and chromatid deletions and gaps are significantly

increased during the application season (Garry et al. 1989). Very high doses of phosphine also can reduce the germination of legume seeds (Ahmad 1976).

The increased concern over pesticides by the public has resulted in more stringent standards for reviewing registered pesticides, which may result in the deregistration of available insecticides. Chlorpyrifos-methyl, the most widely used grain protectant in the USA, may be deregistered because registrants may not submit the additional and costly data required to maintain the registration (Anonymous 1997, Anonymous 2000). Thus there is an urgent need to discover alternatives to the synthetic grain protectants and fumigants. However, increased safety requirements and the need for greater selectivity has resulted in fewer new synthetic insecticides being registered in recent years (Arnason et al. 1989, Arthur 1996).

Biological control

Insect predators, parasitoids and pathogens have been studied for potential biological control of stored-product insects. Fifty-eight species of predators and parasitoids have been studied experimentally, mainly in the laboratory (Schöller and Flinn 2000). Unlike chemicals that must be applied to a wide area, predators and parasitoids can be released at a few points because they can seek out their prey, which are hidden in crevices or hard-to-reach places. In some cases their populations can become established, and provide long-term protection of stored grain. *Anisopteromalus calandrae* (Howard) (Hymenoptera: Pteromalidae) is a generalist parasitoid. Its hosts include a wide range of stored-product insects in both the Coleoptera and Lepidoptera (Schöller and Flinn 2000): *Rhyzopertha dominica* (Ahmed 1996), *Sitophilus* spp. (Schöller and Flinn 2000), *Callosobruchus chinensis* L., *Bruchus analis* F., *Sitotroga cerealella* (Olivier) and

several other insects (Chatterji 1955). It has high fecundity, and is a strong flyer (Cline et al. 1985, Smith 1994). *Anisopteromalus calandrae* parasitizes older larvae or pupae of hosts inside grain kernels. Naturally occurring *A. calandrae* effectively controlled a population of *S. cerealella* (Schöller and Flinn 2000). *Anisopteromalus calandrae* reduced *S. oryzae* populations by 99% (Press and Mullen 1992) and *R. dominica* by 59% (Ahmed 1996). *Cephalonomia waterstoni* (Gahan) (Hymenoptera: Bethylidae) is an ectoparasitoid of *C. ferrugineus,* and a population dynamics model has been described by Flinn and Hagstrum (1995). *Cephalonomia waterstoni* can suppress populations of *C. ferrugineus* (Flinn et al. 1996). However, parasitoids have often been found to be more susceptible to insecticides than their hosts (Schöller and Flinn 2000). Diatomaceous earth, a natural grain protectant used in the grain storage, is very toxic to *A. calandrae* (Perez-Mendoza et al. 1999). To incorporate parasitoids and predators into the pest management program, conventional control procedures and the environment need to be modified to meet the requirements for the growth of biological agents (Haines 1984). Timing of the release and age of target insects also need to be considered to insure the control of pest populations (Flinn and Hagstrum 1995, Schöller and Flinn 2000).

Bacillus thuringiensis var. kurstaki is a commercially-produced insecticidal bacterium. The vegetative cells of the bacterium contain crystals that are aggregates of a large protein (protoxin, 130-140 kD). It is solubilised and activated in the midgut of insects with a high midgut pH value by a gut protease that cleaves the protein into a smaller toxic protein (60 kD). This protein binds to the gut epithelial cells, creating pores in the cell membranes and leading to equilibration of ions. As a result, the gut is rapidly immobilised, digestion stops, the larva stops feeding, and the epithelial cells lyse. Insects

are killed due to the influx of water into the epithelial cells (Knowles 1994). The efficacy of *B. thuringiensis* for the control of stored-product insects has been extensively studied. It is much more effective against Lepidoptera than Coleoptera except for a variety of tenebrionsis. Treatment of the top 10 cm layer of wheat in a bin with *B. thuringiensis* reduced the population of *P. interpunctella* and *E. cautella* by 81% and reduced feeding by 92% (MacGaughey 1980, 1985). However, some strains of *P. interpunctella* (MacGaughey 1985) and *E. cautella* (MacGaughey and Beeman 1988) have developed resistance to *B. thuringiensis.* This resistance is due to the altered receptor site of the protein toxin (Van Rie et al. 1990), and lack of a major gut proteinase that activates the protoxin (Oppert et al. 1997). This rapid development of resistance could prevent *B. thuringiensis* from becoming a major tool for control in stored-product insects (Moore et al. 2000).

Spinosad, a fermentation product of the bacterium *Saccharopolyapora spinisa* Mertz and Yao, has a broad-spectrum of toxicity to stored-product insects (Fang et al. 2002a). At 1 ppm, it kills 100% of *R. dominica* adults, 99% of *P. interpunctella* larvae and over 80% of *S. oryzae* adults. It is stable in stored wheat for over one year in granary trials (Fang et al. 2002b).

The bacterium, *Pseudomonas syringae* Van Hall, and the fungus *Fusarium avenaceum* Saccardo, are ice-nucleators, and can raise the supercooling point of stored-product insects. *Pseudomonas syringae* increased the mortality of eight stored-product insects at sub-zero temperatures (Lee et al. 1992). *Pseudomonas syringae* increases the supercooling point of *C. ferrugineus* from –17°C to –6°C, and kills all *C. ferrugineus* at

-9 °C after 24 h (Fields et al. 1995). *Pseudomonas syringae* is more effective than *F. avenaceum* because the density of ice nuclei of *P. syringae* is higher than *F. avenaceum*.

Physical control

Insects need suitable temperatures and moisture levels to develop. The optimum relative humidity for stored-product insects is between 60 and 70% (Sinha and Watters 1985). Lowering the moisture content of grain reduces the rate of increase of the insect populations. Stored-grain insects are unlikely to develop in grain with a moisture content less that 10% (wet weight). Aeration with ambient air is extensively used to reduce the grain moisture content by forcing large volumes of drier air through the grain mass. Grain driers force heated dry air through the grain mass to lower the grain moisture content quickly. The optimum temperature for development of stored-product insects is between 25 and 33°C (Banks and Fields 1995). Reducing the temperature below 20°C will slow the growth of insects. Insects eventually die at temperature below 14°C, and will be rapidly killed at below –10°C. On the other hand, insects die when temperatures are over 40°C and die in 1 min at temperatures over 62°C (Fields 1992).

Good packaging material provides an effective barrier to prevent insects entering packaged products. However, no single package material can protect all products from insect attack while maintaining the quality of preserved products (Mullen and Mowery 2000). Insects attacking packaged products can be divided into two categories: "penetrators", insects that can bore holes through packaging materials; and "invaders", insects that enter packages through existing holes, such as folds, seams and air vents (Highland 1984, Newton 1988). Some insects that are classified as penetrators are *Sitophilus* spp., *R. dominica*, *P. interpunctella*, *L. serricorne* and *S. paniceum*. *Tribolium*

spp., *C. ferrugineus* and *Oryzaephilus* spp., which cannot penetrate intact packages and must enter through existing holes in the package, are classed as invaders (Highland 1991). Most insects enter finished products through openings caused by sewing, folding, or damage, not by chewing through packaging (Mullen and Mowery 2000). Some adult insects can pass through holes less than 1 mm in diameter, and their larvae can enter through smaller holes (Cline and Highland, 1981). Therefore, control of invaders is more important than that of penetrators.

In addition to improvement in packaging material and design, insect repellents are used to prevent insects from entering packages by modifying the behavior of insects (Highland 1984, Mullen 1994, Watson and Barson 1996, Mullen and Mowery 2000). In the USA, pyrethrins synergized with piperonyl butoxide have been approved for use as a treatment for insect-resistance packaging on the outer layer of packages or with adhesive (Highland 1991). The repellency of pyrethrins is the primary mode of action against insect penetration and invasion (Laudani and Davis 1955). Methyl salicylate, an insect repellent, has been registered for use in food packaging to reduce infestation of stored-product insects in the USA (Radwan and Allin 1997).

Botanical insecticides

Higher plants have developed a diverse and effective array of mechanisms to protect against herbivores during their 400 million years of evolution. Physical barriers, such as thick bark, trichomes, and waxes are obvious defences against insect herbivores. Chemical defence mechanisms are much more subtle. These defence compounds may affect the biochemical and physiological functions of insect herbivores (Dev and Koul 1997, Prakash and Rao 1997).

Plants have been used as pesticides since ancient times (Levinson and Levinson 1998), although ancient people did not know their modes of action. Botanical insecticides are natural products of plants that belong to the groups of secondary metabolites, which include thousands of alkaloids, terpenoids, phenolics, and minor secondary chemicals (Arnason et al. 1989).

Plant-derived insecticides, because of their diverse chemistry, novel properties, and natural sources, have attracted the attention of many scientists. However, interest in botanical insecticides has waxed and waned over the years. From 1941 to 1953 over 3000 plant species were studied (Jacboson 1958). However, from 1953 to 1970 less than half that number of plants was studied (Jacboson 1975), probably due to the attention paid to synthetic insecticides during this period. There has been a renewed interest in botanical insecticides to reduce the use of synthetic insecticides. Most of the plants examined originate from tropical or sub-tropical habitats (Berenbaum 1989, Weaver and Subramanyam 2000), and are part of an enthno-botanical tradition of insecticide use (Berenbaum 1989). The neem tree is often used to protect grain from insect attacks in India (Saxena et al. 1989). Rotenone is used in China (Elliott 1995).

Although plants with insecticidal properties have been found in over 60 families (Dev and Koul 1997), the ones that show the most promise come from following plant families: Meliaceae, Asteraceae, Leguminosae, Rutaceae, Malvaceae, Labiatae and Canellaceae (Jacobson 1989a, 1989b). Pyrethrum, isolated from *Tanacetum cineraraefolium* (Treviranus) (Asteraceae), and neem products, isolated from *Azardirachta indica* A. Juss (Meliaceae) have been extensively studied and are used commercially to control stored-product insects. They can serve as examples for

evaluating the potential grain protectants of other plants, which have shown insecticidal properties against stored-product insects, for example, *Pisum sativum* L. (Bodnaryk et al. 1997, Delobel et al. 1998). Many other plant extracts are also toxic to stored-product insects (Weaver and Subramanyam 2000). To investigate the potential of pea products for the control of stored-product insects, I have provided a detailed review of *T. cineraraefolium,* neem, and peas.

Important or promising plants

Tanacetum cineraraefolium

Pyrethrum is the oily extract or resin of the pyrethrum flower, *C. cinerariaefolium* (Snelson 1987). It has been the most commonly used botanical insecticide in grain storage (Munro 1966). The *Chrysanthemum* genus contains more than a hundred species, but only *T. cineraraefolium* has commercial value as an insecticide source. Pyrethrins are the active components of the pyrethrum. Pyrethrins have been registered in many countries for stored-product insect control because they immobilise insects quickly, have a broad spectrum of activity and leave few residues.

Production. Commercial cultivation of *T. cineraraefolium* started in 1840. Today, the major countries producing *T. cineraraefolium* are Kenya and Australia (Tasmania). Other producer countries include Tanzania, Rwanda and Papua New Guinea. The total production of *T. cineraraefolium* in 1992 was 18,100 tonnes in the world of which 69% was from Kenya (Wainaina 1995). To replace the traditional production or to increase the production of pyrethrum, tissue culture of *T. cineraraefolium* has been demonstrated, but

the yield of pyrethrins needs to be increased before this production method becomes commercially viable (Bhat 1995).

Chemistry. The active ingredients, pyrethrins, in pyrethrum are pleasant-smelling esters. Six related esters in pyrethrum together account for the toxic and knockdown properties. Pyrethrin I, cinerin I, and jasmolin I are ester of chrysanthemic acid, and are known collectively as the pyrethins I fraction. Pyrethrin II, cinerin II, and jasmolin II are ester of pyrethric acid, and are known collectively as the pyrethins II fraction (Head 1973). Both the pyrethrins I fraction and the pyrethrins II fraction are designated pyrethrins which constitute 20-30% (w/w) of crude pyrethrum, and 50-60% of refined pyrethrum (Casida and Quistad 1995). Pyrethrin content varies from 0.8% to 2% (W/W) of the *T. cineraraefolium* flower (Jones 1973), and the ratio of pyrethrins I to pyrethrins II varies from 0.8 to 2.8, depending on the plant clones (Maciver 1995). In addition to pyrethrins, pyrethrum also contains 20-25% light isoparaffins, some hydrocarbons, terpenes and high-molecular-weight compounds (Head 1973, Maciver 1995). Light at 290-320 nm quickly reduces the insecticidal activity of the esters in either the absence or presence of oxygen by changing their chemical structure (Elliott and Janes 1973, Crosby 1995). Photooxidation is the principal transformation process of pyrethrins, which can be effectively inhibited by UV-absorbing substances, and antioxidants. The commonly used piperonyl butoxide does not protect pyrethrins against photodegradation, but functions as a synergist by inhibiting insects' mixed function oxidases that are responsible for biodegradation of pyrethrins (Crosby 1995).

Uses. Pyrethrum is used extensively to control stored-product insects (Silcox and Roth 1995). One of the reasons is that pyrethrins are relatively stable when protected

from sunlight. The half-life of pyrethrum is only 1-2 hours outside in soil (Gabriel and Mark 1995), whereas, it is about two months in stored grain (Snelson 1987). Commercially, pyrethrum is applied as a liquid, aerosol or dust to disinfest granaries, warehouses, homes and food processing plants (Gillenwater and Burden 1973, Silcox and Roth 1995). It can also be applied directly to grain. For most of these commercial formulations, piperonyl butoxide is mixed with pyrethrins at ratios of 10:1 to 20:1 (piperonyl butoxide : pyrethrins). Highland (1984) took advantage of the repellant property of the pyrethrins and developed an insect-resistant multiwalled paper bag that is used by the United States Armed Forces.

Mode of action. There has been little direct research on the mode of action of pyrethrum. It has been inferred based on the extensive studies on the pyrethroids, which are synthetic analogs of natural pyrethrins (Soderlund 1995). Pyrethrins affect the nervous system of insects, rapidly paralyzing them. As is the case for DDT, pyrethrins bind to the nerve axon membrane, affecting the sodium channel, and disrupting the normal action potential by prolonging the period of depolarization of the membrane.

Resistance. At one time, it was thought that insects could not develop resistance to pyrethrum (Mark 1973, Prakash and Rao 1997). Unfortunately, resistance to pyrethrum has been reported. Six stored-product insects are resistant to pyrethrins (Cochran 1995). The ratio of LD_{50}s between resistant and susceptible strains of *S. granarius* is greater than 100-fold (Lloyd 1969) and 13-fold in a wild strain of *T. castaneum* (Speirs and Zettler 1969). Use patterns of insecticides are related in part to the development of resistance (Cochran, 1995). Under intense selection pressure, resistance of *S. granarius* increased 52-fold after 29 generations (Lloyd and Parkin 1963). Pyrethrins, some pyrethroids and

DDT share a common mode of action. This may affect the continued use of pyrethrins (Cochran 1995), since resistance to one of these insecticides is likely to produce cross-resistance to the others.

Non-target toxicity. Compared to synthetic insecticides, pyrethrins degrade rapidly in grain. After 3 months, only approximately 20% of pyrethrins remain in shelled corn (Quinlan and Miller 1958). The oral LD_{50} of pyrethrum against rats is 200-400 mg/kg, and varies depending on the degree of purification. The pyrethrate methoxycarbonyl group in the pyrethrins is readily cleaved into non-toxic compounds by an esterase present in the rat and the mouse. Comprehensive reviews of the toxicity, metabolism and degradation of pyrethrins are provided by Schoenig (1995), Casida and Quistad (1995) and Leahey (1985).

Synthesis. Chemical synthesis is one method to overcome some disadvantages of botanical insecticides, such as limited supply, instability of active ingredients and high costs of extraction. The development of successful chemical synthetics is based on the understanding of the biological function of chemical structures and massive screening. Synthetic pyrethroids are more photostable than natural pyrethrins, because of structural change (Elliott 1995). The first synthetic pyrethroids were manufactured and marketed in the 1960s (Gullickson 1995). Bioresmethrin was the first pyrethroid that possessed both higher insecticidal activity and lower mammalian toxicity than its naturally occurring counterparts, the pyrethrins.

Neem

The neem tree, *A. indica*, is native to the Indian subcontinent. Its insecticidal properties have been extensively studied. More than 400 insect species, including stored-

product insects, can be controlled by neem (Lale and Abdulrahman 1999). It has been demonstrated to possess insecticidal, repellent, antifeedant and growth regulatory properties. Several commercial formulations have been developed. The main active component of neem is azardirachtin (Prakash and Rao 1997, Saxena 1989).

Production. Native to India and Pakistan, the neem tree can be found in semi-arid and arid zones from tropical to sub-tropical regions. It completes 60% of its height during the first three years. It begins to bear seed in three to five years. Neem trees grow relatively quickly. Each tree can produce 21 kg seeds per year (Benge 1989). A plantation of neem trees in Indian yields 10-100 tonne of neem products per ha annually. Leaves provide 50% of this production, with seed providing 25% and bark the remaining 25%.

In 1997, there were eighteen commercial formulations of neem insecticides (Prakash and Rao 1997), most of them using neem oil. The oil is a semi-solid with a bitter taste and garlic-like odor, and composed of triglycerides and triterpenoids (Jones 1973). Some products have been registered for the control of stored-product insects, such as Margosan-O in the USA in 1985 (Larson 1989). Neem oil has been commercialized in India with annual production of 83,000 tonnes (Saxena 1989).

Uses. Thirty to sixty percent of farmers use neem products to protect their grain in storage in India (Ahmed and Koppel 1987). Many traditional methods are used: mixing neem leaves into grain, mixing leaf paste with mud to make earthen containers for grain storage, soaking bags in a 2-10% leaf decoction, rubbing crushed leaves on the inner surface of mud bins, sprinkling neem extract on the straw and packing it at the bottom of grain bins, and burning neem leaves to generate smoke for fumigation (Saxena et al.

1989). Neem leaves are often added at 2-5% (w:w) to grain for storage in India and Pakistan.

Neem has been tested against many important stored-product pests (Prakash and Rao 1997). Different parts of the neem tree have different effects against insects. Seed extracts are more effective than leaf extracts for the protection of cowpea and maize (Makanjuol 1989). Neem leaves mixed with wheat protects grain from infestation of *S. oryzae, S. cerealella* and *R. dominica* (Pruthy and Singh 1950). Neem kernel powder at 2% protects rice paddy from these insects under actual storage conditions (Prakash et al. 1982). Neem extract at 0.5% provides significant protection for rice from damage by *R. dominica* and *S. cerealella* (Prakash et al. 1982). The difference may be due to the different composition of the active components among seeds, leaves and extracts.

Active components. The active components in the bark, heartwood, leaves and seeds of the neem tree have been examined. More than 70 compounds have been isolated. Some are found to be active against insects (Prakash and Rao 1997). The most effective compounds are azadirachtin, melantriol, salannin, and nimbin (Saxena 1989, Prakash and Rao 1997). Azadirachtin is considered to be the principal active component. The structures of the active components are too complex to synthesize in large quantities. There are also possible interactions among the active components, but the mechanism is unknown (Jones et al. 1989). Piperonyl butoxide, a mixed function oxidase inhibitor, does not increase the effectiveness of azadirachtin (Saxena 1989).

Mode of action. Neem has many detrimental effects on insects. It acts as a direct toxin, repellent, antifeedant, growth regulator, oviposition suppressant and ovicide (Jacobson 1989b, Makanjuola 1989, Prakash and Rao 1997). The repellent and

antifeedant effects were first described in 1962 (Saxena 1989). Jilani and Saxena (1990), and Xie et al. (1995) reported that it has repellent and antifeedant effects on stored-product insects. Neem extract reduces oviposition, egg hatch and adult emergence of *Callosobruchus maculates* (F.) (Makanjuola 1989). Jilani et al. (1988) reported that neem oil interferes with the development of *T. castaneum*. Wheat flour treated with neem oil results in few, small and abnormal larvae, pupae and adults of *T. castaneum.* The ethanolic extract of the neem kernel does not kill adult *R. dominica,* but 5 mg/kg of azadirachtin effectively blocks reproduction (Rahim 1998).

Detailed mode of action studies have found that the gut of insects poses a physical or physiological barrier to the bioavailability of azadirachtin (Champagne et al. 1989, Mordue and Blackwell 1993). Its effect on eggs of *Sitophilus spp.* is dependant on the absorption of neem extract into the grain kernel (Makanjuola 1989). The growth inhibition of azadirachtin-treated insects may be due to the accumulation of neurosecretory protein in the corpus cardiacum (Rembold 1989).

Non-target toxicity. Jacobson (1989b) provided a review of the toxicity of neem products to non-target organisms. In general, neem oil has low toxicity to mammals, and is safe to the environment because it is biodegradable. The LD_{50} of neem oil in rats is 700-1000 mg/kg and the LD_{50} of azadirachtin in pigs is 6000 mg/kg (Golob et al. 1999). Neem possesses medicinal properties. Toothpastes containing neem products are sold in India and Canada (Jacobson 1989b). The aqueous extract of neem leaves is used for treating constipation, diabetes, indigestion, itch, pyorrhea, sleeplessness and stomach ache (Jacobson 1989b). However, a syndrome similar to Reye's syndrome has been found in children after receiving large doses of neem oil (Sinniah et al. 1982, 1985). Some

studies show that neem oil is toxic to guinea pigs and rabbits (Sadre et al. 1983), tadpoles and fish (Jacobson 1989b). In addition, neem seeds can carry the fungus *Aspergillus flavus* Link that can produce aflatoxin that is harmful to humans and the environment (Pereira and Wohlgemuth 1982).

Peas

Peas, *P. sativum,* are human food and are used as an animal feed additive. Legumes and cereal seeds have very different chemical composition (Harborne et al 1971). In general, insects that are pests of stored legumes are not pests of stored cereals. In Africa, this separation of hosts has been used to reduce the infestation of *S. oryzae,* by storing cereals and legumes mixed together (Ryder 1972). More recent studies have determined the insecticidal components in peas (Bodnaryk et al. 1997, Delobel et al. 1998).

Production. Peas are grown around the world. If a natural insecticide is isolated from peas, there would be an abundant supply of peas for extraction, More than 440,000 ha of field peas are planted annually in Australia, and 80,000 ha are planted in the U.S.A. (Harein and Davis 1992). In Canada, the growing area of peas in 1995 was 819,500 ha, producing 1,454,700 tonnes of peas (Slinkard 1997). The acreage planted in peas has increased by 5% a year in 2001 and 2002, and a similar increase is forecasted for 2003 (Novelli 2002).

Uses. There has been no commercial application of a pea product to control stored-product insects, although a maize-legume mixture is used in Africa to protect maize from the attack of insects (Coombs et al. 1977). Coombs et al. (1977) showed that adults of *S. oryzae* die within a week when placed on yellow split peas, and found that fecundity of *S.*

oryzae is markedly reduced in wheat when yellow split peas were mixed with wheat at a ratio of 1:1.

In Western Canada, some peas are processed by milling, and the fractions are separated by using an air classification procedure depending on the mass of ground particles. The main fractions of pea production are protein-rich, fibre-rich, and starch-rich pea flour. A manager of a pea mill noted that no insects were found in the mill or the pea flours. Subsequent studies found that the pea flours were toxic and repellent to stored-product insects (Bodnaryk et al. 1997, Fields et al. 2001). The protein-rich pea flour, made by the air classification method by Parrheim Foods at Saskatoon, Canada, is commercially available. It contains 60% protein, 30% starch, and 7% moisture content. Protein-rich pea flour is more effective than fibre-rich and starch-rich fractions against insect pests, and there is considerable variation of the efficacy between pea varieties (Bodnaryk et al. 1997). Bodnaryk et al. (1997) produced an extract that was 20-100 times more effective than the protein-rich pea flour, and the extract also reduced the feeding of migratory grasshoppers, *Melanoplus sanguinipes* (F.), and diamondback moth, *Plutella xylostella* (L.). This extract was heat-sensitive, soluble in alcohol, insensitive to protease, and had a molecular weight less than 4000 Da. Delobel et al. (1998) isolated a polypeptide from peas that contains 37 amino acids with a molecular weight of 3741.4 Da. This polypeptide is toxic to *S. oryzae*.

Resistance. There are some strains of *S. oryzae* that are capable of breeding on yellow split peas (Coombs et al. 1977, Grenier et al. 1997). These strains are all tropical in origin (Trinidad, Callao, Singapore, China and Jamaica). Genetic studies have found that this resistance is controlled by a single recessive, autosomal gene (Thind and

Muggleton 1981, Holloway and Smith 1985, Holloway 1986, Grenier et al. 1997). A few studies have examined how these strains can survive on pea kernels. Glutathione-S-transferases and mixed function oxidases may be involved in the detoxification process, and esterases may also play a role (Holloway and Mackness 1988). Symbionts are not directly responsible for the ability to detoxify pea toxins, but may play a role in supplying additional energy to the insects that can be used for detoxification (Grenier et al. 1997).

Other plants

In addition to the *Tanacetum* flower, neem tree and peas, many other plants have been tested for their insecticidal properties (Jacobson 1958, 1975, Arnason et al. 1989, Dev and Koul 1997, Golob et al. 1999, Weaver and Subramanyam 2000). Most of these products are from tropical and subtropical regions. Tropical plants are under greater insect herbivore pressure, and have evolved a wide variety of toxins (Berenbaum 1989). Some of the plants are chosen because they are available locally (Jilani and Su 1983, Hassanali and Lwande 1989, Cunat et al. 1990, Niber et al. 1992, Roger and Hamraoui 1992). Some plants tested are used as spices or medicines (Hassanali and Lwande 1989, Golob et al. 1999). Jacobson (1958, 1975), Prakash and Rao (1997), Golob 1999 and Weaver and Subramanyam (2000) provide extensive lists of plants with insecticidal activity.

Uses. Almost all studies of other plants or plant extracts have been conducted using small-scale laboratory trials. Hassanali and Lwande (1989) found that flower buds of cloves, *Eugenia aromatica (*Zingiberaceae) repel *S. zeamais*. The active component eugenol, is much more repellent than its isomer isoeugenol (II). The oils of corn, ground nut, sunflower and sesame reduce the oviposition and longevity of adult of

Callosobruchus chinensis L. and *C. maculates* (F.) in wheat (Rajapakse and Van-Emden 1997), and *Dermestes maculatus* Degeer on dried fish (Don-Pedro 1989). Tomato leaves contain 2-tridecanone that reduces the population of *S. oryzae* (Kramer et al 1985) and *C. chinensis* (Hou et al. 1989).

The powder of many legumes inhibits the growth of insects (Srivastava et al. 1973). The legumes studied include black gram (*Phaseolus mungo* L.), green gram (*Phaseolus aureus* Roxb.), French bean (*Phaseolus vulgaris* L.), moth bean (*Phaseolu aconitifolius* Jacq.), garden pea (*Pisum sativum* L.), pigeon pea (*Cajanus cajan*), lentil (*Lens culinaris* Medic.), hyacinth bean (*Dolichos lablab* L.), cowpea (*Vigna unguiculata* L.) (Baker et al. 1989), soybean (Applebaum et al. 1969, Srivastava et al. 1973, Gatehouse and Boulter 1983, Hines et al. 1991) and many other legumes (Bodnaryk et al. 1997). Soybeans contain a cysteine proteinase inhibitor, a protein of approximate 12,000 Da, that is effective against *C. maculatus* and *T. castaneum.* The inhibitory activity disappears after heating to 100°C for 30 min (Hines et al. 1991). Gatehouse and Boulter (1983) reported that cowpea trypsin inhibitor also reduces infestations of *C. maculatus.* Applebaum et al. (1969) stated that saponins in legumes inhibit the growth of insects, although Bodnaryk et al. (1997) found that saponins were not insecticidal factor in a pea extract.

Many beans contain α-amylase inhibitors which inhibit the digestion of α-amylase in *T. castaneum, S. oryzae* and *Tenebrio molitor* L. and slow the development of *R. dominica* and *Cryptolestes spp.* (Pueyo et al. 1995). The gene sequence of α-amylase inhibitors from *P. vulgaris* has been described (Shade et al. 1994) and transferred to peas (Schroeder et al. 1995) to protect peas from bruchid insects.

Mode of action. Botanical insecticides cause a number of effects: toxicity, repellency, antifeeding activity, chemosterility and growth inhibition (Prakrash and Rao 1997). However, few studies have been conducted on the mode of action of plant extracts. Only a few active compounds have been isolated and tested for their insecticidal properties. Uncinatone and pectolinarigenin, which are isolated from *Clerodendron siphonenthus* L. (Verbenaceae) reduce feeding of *S. oryzae* (Pal et al. 1989). Shaaya et al. (1991, 1997) reported that the fumigant toxicity of plants that contain the compounds linalool, α-terpineol, carvacrol, terpinenol or 1,8-cineole is stronger against some stored-product insects than that of those which contain β-caryophyllene, ρ-cymene, α-terpinene or mycene. A plant extract containing monoterpenes is highly inhibitory to cholinesterase activity of *R. dominica* (Greenburg-Levy et al. 1993).

Discussion

An ideal insecticide should effectively control insects under a wide range of conditions, be simple to apply, inexpensive, and have minimal effects on non-target organisms, such as insect parasitoids and predators, wildlife and humans. However, no insecticide fully satisfies all these criteria. Each insecticide has its own advantages and disadvantages. Although synthetic insecticides are the major means to control stored-product insects, the concerns and the disadvantages presented above show that there is a pressing need to reduce the use of synthetic insecticides and to develop new methods, which are effective, and simple to use, yet safe for humans and the environment.

Advantages of botanical insecticides

Botanical insecticides or plant extracts are used in grain storage, and there is an increasing number of studies on novel botanical insecticides. There are several reasons why botanical insecticides have been investigated as possible alternatives to reduce the use of synthetic insecticides. They have numerous effects on insects including: reducing oviposition, feeding or survival, preventing normal growth, or repelling insects. In general, botanical insecticides possess low mammalian toxicity with a few exceptions (Ware 2000). Most of the botanical compounds are considered only moderately toxic (Table 2.1). Active ingredients of botanical insecticides are generally sensitive to sunlight or heat. This property makes them degrade very quickly in field applications and leave little or no residue. In addition, Prakash and Rao (1997) indicated that unlike some synthetic insecticides, botanical insecticides might be less hazardous to non-target organisms, discourage pest resurgence, and have no adverse effect on plant growth or cooking quality of grains.

Prakash and Rao (1997) suggest that it may be more difficult for insects to develop resistance to botanical insecticides. Plants have developed, during millions of years of co-evolution with insects, a wide variety of compounds to prevent the herbivores. Usually, plant extracts contain several compounds. Resistance to pyrethrum is still not very widespread, even though it has been used for hundreds of years (Cochran 1995). The matter of concern is the cross-resistance that may occur between botanical insecticides and synthetic insecticides that share the same mode of action or resistance mechanism. In such a situation, tolerance to the botanical insecticide resulting from cross resistance with other insecticides might already exist before the use of the botanical insecticide.

Depending upon the compound and area of use, botanical insecticides can offer a low cost alternative to synthetic insecticides. In developed countries, the regulatory costs for the registration of a botanical insecticide can be considerably less than for a synthetic insecticide (Woodhead et al. 1990). In developing countries, the botanical insecticides produced by farmers or local companies should be less expensive than imported synthetic insecticides.

Disadvantages of botanical insecticides

Although there are several advantages to use of botanical insecticides, there are several negative factors that affect the wide-scale adoption of these compounds. One major limitation is the supply of plants for extraction. Many of the plants that have insecticidal compounds are rare, with limited geographical distributions, or are grown in small quantities to be used for medicines or spices. Neem, one of the two most used botanical insecticides, only grows in arid or semi-arid, tropical or sub-tropical areas, and takes at least 3 years to bear seeds. *Chrysanthemum cinerariaefolium,* the plant that produces pyrethrum, originates from a small area, Persia, and its cultivation has spread to other countries. However, planting insecticidal plants occupies valuable land, which could be used to produce food grains.

Another drawback to botanical insecticides is that with the exception of refined pyrethrum, high doses of crude extracts are generally required for the control of stored-product insects (Golob et al. 1999) (Table 2.2). For example, it was necessary to use powdered neem at 12% to completely inhibit the oviposition of *S. oryzae* (Saxena et al. 1989). When large amounts are added to the grain, they can increase the amount of dockage, reduce the grain grade (Korunic et al. 1998) and require cleaning of the grain.

Refined extract could reduce the application dose, but would be more expensive to produce.

The property of rapid degradation of botanical insecticides is an advantage in regard to environmental safety. However, it is a disadvantage for providing long-term protection of stored grain. Grain usually is stored for more than three months, sometimes for several years. Botanicals, such as pyrethrum, rapidly degrade, and may not last long enough to protect products in long-term storage.

Although there are some advantages in registering a natural insecticide, there are also some disadvantages. Plant extracts are often a cocktail of many compounds that contribute to overall effectiveness of the botanical insecticides. However, the regulatory authorities in Western countries are used to evaluating a single compound or simple mixtures of well-defined synthetic pesticides. In the USA, since 1988, applicants must ensure that the product being registered has all active components identified, guarantee a given concentration and formulation, define the fate of active ingredients, and conduct full toxicological and environmental studies (Casida and Quistad 1995).

Finally, not all botanical insecticides have low toxicity (Table 2.1). It is important to be aware that not all botanicals are safe for human beings and the environment. Ergosterol isolated from *Citrus* spp. and nicotine isolated from *Nicotiana spp.* (LD_{50} below 5 mg/kg) are more toxic than all currently used synthesized grain protectants and fumigants (Golob et al. 1999) (Table 2.1).

Further research on peas

A pea-based insecticide has a number of interesting features. For example it is toxic and repellent to several insect species (Bodnaryk et al. 1997, Delobol et al. 1998). It is a

cultivated and food-based product and with less stringent requirements for registration (Woodhead et al. 1990). It has potential to be used to control stored-product insects. However, there are a number of questions that must be addressed before a pea-based product can be used by producers and grain elevator managers for insect control.

Factors that affect the toxicity and stability of protein-rich pea flour. Many biotic and abiotic factors, such as grain temperature, moisture content and grain species, affect the toxicity and stability of grain protectants, such as pyrethrins, malathion and pirimiphos-methyl (Longstaff and Desmarchelier 1983, Snelson 1987, Samson et al. 1988, 1990). The toxicity of protein-rich pea flour has been reported (Bodnaryk et al. 1997, Delobol et al. 1998). However, this toxicity may also be affected by biotic and abiotic factors.

Efficacy of protein-rich pea flour for packaging. After grain is harvested, grain is stored in granaries, transferred to elevators and processed at mills or food processing plants. Finished grain products are packaged for transportation and distribution to consumers. Infestation of insects can occur at any point in this chain. One way to protect packaged products from insect attack is to modify packages with insecticides. Pyrethrins have been used in an insect-resistant bag (Highland 1984). Some other plant extracts have been tested for packaging purposes (Bloszyk et al. 1990). Protein-rich pea flour, as a food-based product and repellent to many insects, has potential to be used in insect-resistant food packaging.

Enhancing the toxicity of protein-rich pea flour. To control stored-product insects, botanical insecticides usually are applied at high doses. In large-scale commercial storage, it is not practical to store wheat with yellow split peas at a 1:1 ratio, although

there may be applications for this method in small-scale granaries (Coombs et al. 1977). To increase the efficacy of peas, the classic approach would be to identify and concentrate the active components. Bodnaryk et al. (1997) have increased the activity by 20-100 fold by isolating an active extract. Another way is to find synergists, such as other insecticides, enzyme inhibitors or parasitoids, and to combine them with protein-rich pea flour to improve its efficacy. Many parasitoids are sensitive to insecticides. The toxicity of protein-rich pea flour for parasitoids should be tested to determine if parasitoids could be combined, and increase the efficacy of protein-rich pea flour.

Large-scale evaluation. The ultimate purpose of this study of protein-rich pea flour is to develop a product that can be used in farm granaries to control of stored-product insects. However, no field trials have been conducted with protein-rich pea flour. Field conditions are much more complex than those in the laboratory. Laboratory effectiveness of protein-rich pea flour needs to be verified in farm granaries.

Mode of action. Protein-rich pea flour is toxic to several insect species. However, the mode of action of the pea-based insecticides is unknown. Understanding the mode of action is useful on several levels. It would help determine what synergist could increase the efficacy of the pea-based insecticides.

Resistant insect strains. Some researchers think that insects are unlikely to become resistance to botanical pesticides (Prakash and Rao 1997). However, resistant strains of *S. oryzae* already exist (Coombs et al. 1977). Some research into the possibility of habituation to protein-rich pea flour has been conducted (Fields et al. 2001). *Cryptolestes ferrugineus* is repelled by protein-rich pea flour after being held on it for four weeks. However, when the insects feed on wheat treated with a low dose of pea

fiber-rich fraction, the repellent action is lost. The development of insect resistance and its effect on the use of pea-based products to control stored-product pests needs study.

Mammalian toxicity and environmental safety. Information on toxicity to non-target organisms is an essential requirement for the registration of a new pesticide. Consumers are very concerned about chemical residues in food and in the environment. Some insecticides that had been of great benefit to humans, such as DDT and methyl bromide (Elliott 1995, Fields and White 2002), have been or will banned mainly because of their accumulation in the environment and negative effect on wildlife. As a food-based product, protein-rich pea flour or extracts from peas should be safe. However, the toxicity and environmental fate of the main components in the final product would have to be determined.

Conclusion

Chemical protectants and fumigants are often the primary means to control stored-product insects. However, due to insect resistance and concerns over chemical residues and environmental safety, fewer conventional insecticides are available for use in grain storage. This has led to an interest in developing botanical insecticides (Arthur 1996, Prakash and Rao 1997, Weaver and Subramanyam 2000). Thousands of plants have been shown to have insecticidal activity. Pyrethrum and neem are the only botanical insecticides that have been commercialised for the control of stored-product insects, and can be used as benchmarks to estimate the potential of pea products as grain protectants. Our current knowledge of pea-based products shows there is promise for using this food product to control stored-product insects.

Table 2.1. Mammalian toxicity of 109 botanical compounds isolated from medicinal plants and spices (modified from Golob et al. 1999, Ware 2000).

Toxic Rating[1]	Oral LD_{50}[2] (mg/kg) (Rat or Mouse)	Number of compounds[3]
Super toxic	<5	2
Extremely toxic	5 - 50	6
Very toxic	50 - 500	26
Moderately toxic	500 - 5 000	73
Slightly toxic	5 000 - 15 000	2

[1] Rating is adapted from Ware (2000).

[2] LD_{50} indicates the dose lethal to 50% of the test population.

[3] Collected from Golob et al. (1999).

Table 2.2. Common stored-grain protectants used for grain storage in various parts of the world.

Insecticide	Class	Oral LD_{50}[1] (Rat or Mouse) (mg/kg)	Recommended application rate (ppm)	Reference
Malathion	Organophosphate	5400	8-20	Ware 2000, Snelson 1987
Pirimiphos-methyl	Organophosphate	2050	5-15	Ware 2000, Snelson 1987
Deltamethrin	Pyrethroid	128-5000	1-5	EXTOXNET 2003, Snelson 1987
Pyrethrum	Plant extract	1030-2370	5-15	Schoenig 1995, Snelson 1987
Neem	Plant extract	700-1000	1000-5000	Golob et al. 1999, Parmar 1986
Diatomaceous earth	Mineral deposit	Not toxic at 3000	100-500	Fields and Korunic 2000a

[1] LD_{50} indicates the dose lethal to 50% of the test population.

CHAPTER 3

Effectiveness of Protein-Rich Pea Flour for the Control of Stored-Product Beetles

Abstract

Protein-rich pea flour is toxic to many stored-product insects. I investigated several factors that may affect the efficacy of protein-rich pea flour: insect species, insect population densities, grain species, temperature and moisture. Adult *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) was more susceptible to protein-rich pea flour than *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae). Protein-rich pea flour did not increase the mortality of adult *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). However, it reduced the number of offspring of all three species. The toxicity of protein-rich pea flour was not reduced after 9 months when stored at –15°C or at room temperature as flour or mixed with grain. Its toxicity in wheat increased at higher temperatures and at lower grain moisture contents. Protein-rich pea flour was more toxic in wheat and barley than in maize. This difference among grain species was not due to the kernel size of the grain, as ground wheat or maize with the same particle size still had different LD_{50}s.

Key words: peas, protein, *Sitophilus oryzae,* toxicity, particle size, temperature, moisture

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Introduction

Insect pests cause extensive damage to stored grains qualitatively and quantitatively (Hall, 1970; Semple et al., 1992). Many managers of grain storage facilities use synthetic insecticides to reduce the losses of stored grain by insect pests (Arthur, 1996). However, there are several reasons to search for alternatives to synthetic insecticides: consumer preference for food without insecticide residues, worker safety concerns, resistant insect populations, and de-registration of current synthetic insecticides. Higher plants are a good source of novel insecticides (Prakash & Rao, 1997). Thousands of plants have been investigated for their ability to control insect pests (Jacobson, 1989a; Golob et al., 1999; Weaver & Subramanyam, 2000). Legume seeds contain a wide range of allelochemicals with toxic and deterrent effects against insect pests (Harborne et al., 1971; Bell, 1977).

Yellow split pea *(Pisum sativum* L.) mixed with wheat reduces the survival and reproduction rate of *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) (Coombs et al., 1977; Holloway, 1986). As an animal feed and human food, protein-rich pea flour made from yellow split pea has potential to be a good grain protectant (Bodnaryk et al., 1997; Fields et al., 2001; Hou & Fields, 2003a (Chapter 4)). It is repellent (Fields et al., 2001) and toxic to, and reduces the reproduction of many stored-product insect pests (Bodnaryk et al., 1997). Bodnaryk et al. (1997) produced an extract from protein-rich pea flour that is 20 to 100 times more toxic than protein-rich pea flour itself. Delobel et al. (1998) isolated a polypeptide from peas that is toxic to stored-product insects.

There are a number of factors that may affect the toxicity of stored-grain insecticides. Temperature and moisture content of stored-grain are the two most important factors (Ioradanou & Watters, 1969; Snelson, 1987; Samson et al., 1988). Grain is stored under a wide range of temperature and moisture conditions due to differences in geography, seasons and storage practices. It is important to understand how temperature and moisture influence the efficacy of insecticides. Malathion and pirimiphos-methyl have positive temperature coefficients for stored-product insects (Ioradanou & Watters, 1969; Snelson, 1987). However, some pyrethroids show a negative relationship with temperature for *S. oryzae* (Longstaff & Desmarchelier, 1983). Both positive and negative coefficients to temperature have been reported for lindane (De Vries & Georghious, 1979) and carbamates (Reichenbach & Collins, 1984). The degradation of malathion and other insecticides increases with temperature (Strong & Sbur, 1960). The toxicity of dichlorvos, chlorpyrifos and carbaryl increases with moisture content of grain, whereas the toxicity of malathion (Barson, 1983) and diatomaceous earth (Fields & Korunic, 2000a) decreases with moisture content. Pyrethrins, malathion and pirimiphos-methyl break down faster in grain with higher moisture content (Quinlan et al., 1980; Snelson, 1987). Other factors can also affect efficacy. The toxicity of methoprene and fenoxycarb (Samson & Parker, 1989; Samson et al., 1990) varies with grain species.

The purpose of this study was to determine how temperature and moisture content, as well as insect density, grain species, size of grain kernel, and relative humidity affect the efficacy of protein-rich pea flour as a grain storage insecticide.

Materials and Methods

Three species of beetles, *S. oryzae*, *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae) and *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) had been cultured in the laboratory at 30°C, 70% relative humidity (RH) for over 5 years. *Sitophilus oryzae* was reared on whole kernels of wheat. *Cryptolestes ferrugineus* was reared on wheat kernels with 5% wheat germ and 5% brewer's yeast by weight. *Tribolium castaneum* was reared on wheat flour mixed with 5% brewer's yeast.

Protein-rich pea flour (Progress Protein; 60% protein, 30% starch, and 7% moisture content, Parrheim Food, Saskatoon, SK) was used in this study. It is produced commercially by grinding peas and isolating a protein-rich fraction by air classification. Except when specified, the LD_{50} of protein-rich pea flour was tested by the following procedures. Grain with a 14% moisture content (wet weight based) was treated with protein-rich pea flour, previously stored at –15°C, at concentrations of 0, 0.001, 0.01, 0.1, 1.0, and 10.0% (wt:wt). Wheat, barley or maize were mixed with protein-rich pea flour in a half-litre jar by shaking by hand for 2 min. Twenty grams of treated grain and 20 1- to 2-week-old adult insects were placed in glass vials (29 mm diameter, 88 mm height) with screened caps. The vials and insects were held at 30°C, 70% RH for 2 weeks, and the number of live and dead insects at each concentration was counted to estimate the LD_{50}. Each treatment had 5 replications. To examine the effect of protein-rich pea flour on the reproduction of insects inside grain kernels, after adults had been removed, the grain in the vials was incubated for another 5 weeks at 30°C, 70% RH, and the number of

emerged adults of *S. oryzae*, *C. ferrugineus* and *T. castaneum* in wheat and barley and *S. oryzae* in maize was counted.

To produce different particle sizes, wheat and maize with 14% moisture content were ground with a Stein laboratory mill (model M-2, Fred Stein Laboratory, Inc. Achison, KS). The ground particles were sieved with No. 10-, 14- and 20-mesh sieves (2.0, 1.4, and 0.85 mm openings) respectively. The grain particles in the 2.0-1.4 mm and 1.4-0.85 mm range were collected. Grain kernels or particles were mixed with protein-rich pea flour at different concentrations. The LD_{50}s of protein-rich pea flour against *S. oryzae* were compared among particle sizes with the same grain species or between and wheat and maize at the same size.

To determine if population density affects the efficacy of protein-rich pea flour against *S. oryzae*, two population densities were prepared by putting 20 adult insects in 20 g wheat kernel vials (equivalent to 1000 insects per kg) and by putting 25 adult insects in 2500 g wheat kernels in 4-liter jars (equivalent to 10 insects per kg), the latter density being closer to that found in commercial grain stores (Sinha & Watters, 1985). For each density, wheat kernels were treated with protein-rich pea flour at various concentrations as described above, with five replicates per concentration.

To test for stability, the toxicity of protein-rich pea flour against *S. oryzae* was tested immediately upon delivery from the manufacturer (fresh), or after 9 months under the following conditions: held at –15°C, held in a room where temperature ranged from 20 to 30°C and relative humidity ranged from 25 to 70% RH, or held in the same room but on wheat, barley and maize at various concentrations.

Toxicity of protein-rich pea flour was tested on wheat against *S. oryzae* in growth cabinets at temperatures of 20, 25, 30, and 35 ± 1°C. Vials containing 20 g wheat and 20 adult *S. oryzae* were placed in desiccators with a constant relative humidity of 75% maintained by a saturated NaCl solution (Loveridge, 1980) and verified with a psychrometer (Cole-Parmer Instruments, Chicago, IL).

To study the effect of moisture against *S. oryzae*, wheat was conditioned to moisture content of 18, 16, 14, and 10%, and was placed, respectively, in sealed containers with 300 ml saturated salt solution of KCl, NaCl, $NaNO_2$ and K_2CO_3 (Loveridge, 1980) at 30°C in a growth cabinet. The relative humidities were approximately 85, 75, 65 and 45%, and the moisture content of wheat after 3 weeks was 17.4, 16.7, 13.0 and 10.2%, respectively. The wheat was then treated with protein-rich pea flour at different concentrations. Vials containing 20 g wheat and 20 adult *S. oryzae* were immediately placed in their respective containers sealed with a cotton plug to maintain the grain moisture content, yet allow some air movement. Mortality was noted as above.

LD_{50}s, LD_{90}s, and slopes were analyzed by probit analysis (Finney 1971) using POLO-PC (LeOra Software, 1994). The confidence limits of LD_{50} or LD_{90} were estimated only when g value, the index of heterogeneity, was less than 0.5. LD_{50} values and the slopes of the regression lines were compared to determine the equality or parallelism of the regression lines by using the chi-square likelihood ratio test ($\alpha = 0.05$). Numbers of offspring in grain treated at different concentrations of protein-rich pea flour

were compared with the SAS GLM (General Linear Models) procedure with CONTRAST with linear functions ($\alpha = 0.05$) (SAS Institute Inc., 2000).

Results

Grain species and kernel size

Sitophilus oryzae was less sensitive to fresh protein-rich pea flour when held on maize ($LD_{50} = 0.17\%$) than on wheat or barley ($LD_{50} = 0.04\%$) ($\chi^2 = 21.9518$, df = 2, $P < 0.001$) (Table 3.1). When the wheat and maize were ground and the particle size standardized between grains, response of *S. oryzae* to protein-rich pea flour did not change (Figure 3.1). The LD_{50}s for *S. oryzae* held on whole kernels of wheat and 2.0-1.4 mm granules were 0.03% (95% C.L.: 0.02-0.05) and 0.04% (95% C.L.: 0.03-0.05), respectively. The LD_{50} for 1.4-0.85 mm granules in wheat was not available because the data did not fit the model of the probit analysis. The LD_{50}s for *S. oryzae* held on whole kernels of maize and granules of 2.0-1.4 and 1.4-0.85 mm were 0.13% (95% C.L.: 0.10-0.16), 0.15 % (95% C.L.: 0.11-0.19), and 0.11% (95% C.L.: 0.09-0.14), respectively. No difference was found within the same grain ($\chi^2 = 0.8959$, df = 2, $P = 0.639$).

Stability

For wheat, barley and maize, no difference in LD_{50} was detected among fresh protein-rich pea flour, flour stored for 9 months at -15°C, flour stored at room temperature, or flour stored at room temperature on grain for 9 months ($\chi^2 = 0.4815$, df = 3, $P = 0.923$). (Table 3.1).

Insect species and density

Sitophilus oryzae (LD_{50} = 0.03%, 95% C.L.: 0.02-0.04) was more sensitive to protein-rich pea flour stored at -15°C than *C. ferrugineus* (= 6.0%, 95% C.L.: 3.2-12.6) at a population density of 1000 insects per kg wheat ($\chi^2 = 28.0585$, df = 1, $P < 0.001$). The mortality of *T. castaneum* was less than 20% at all concentrations; hence the LD_{50} could not be estimated. No significant difference in the LD_{50} of *S. oryzae* was detected between a population density of 1000 insects per kg and 10 insects per kg ($\chi^2 = 0.8116$, df = 1, P = 0.368). At 10 insects per kg, *S. oryzae* had a LD_{50} of 0.04% (95% C.L.: 0.03-0.06) and a slope of the mortality against doses of 2.83 ± 0.39 (mean ± SEM) (g = 0.072). Data for the test using 1000 insects per kg are given in Table 3.1.

Offspring

Protein-rich pea flour reduced the number of *S. oryzae, C. ferrugineus* and *T. castaneum* emerging from wheat, barley and maize (Table 3.2). The higher the dose of protein-rich pea flour, the greater the reduction in the number of emerged adults from grain. Treating

with protein-rich pea flour at 1%, the number of *S. oryzae* emerging from wheat, barley and maize were reduced by 99.8, 99.7, and 63.8%, respectively. Numbers of *C. ferrugineus* and *T. castaneum* emerging from the grain were reduced by 74% and 52% in wheat, and 85% and 74% in barley, respectively.

Temperature and moisture

Temperature affected the toxicity of protein-rich pea flour. The LD_{50} decreased with higher temperatures ($\chi^2 = 52.7430$, df = 2, $P < 0.001$) (Table 3.3). All *S. oryzae* died at 35°C, 70% RH in all treatments including the controls, and this treatment was excluded from the statistical analysis. The LD_{50} of protein-rich pea flour decreased as relative humidity increased ($\chi^2 = 16.6295$, df = 2, $P < 0.001$) (Table 3.4). The mortality of *S. oryzae* exceeded 80% in the controls when the relative humidity was 45% (moisture content of wheat was 10.2%).

Discussion

Protein-rich pea flour is toxic to many stored-product insects (Bodnaryk et al., 1997). I found that the toxicity of protein-rich pea flour was species specific. Interspecific variations in susceptibility of stored-product insect species are known for other insecticides. For deltamethrin, *T. castaneum* is approximately 10 times, and *Rhyzopertha dominica* (F.) approximately 100 times more susceptible than *S. oryzae* (Snelson, 1987).

For malathion, *S. oryzae* is 2 times more susceptible than *T. castaneum* (Snelson, 1987). Diatomaceous earth is approximately 3 times more effective against *Sitophilus granarius* (L.) than against *Tribolium confusum* Duval. (Aldryhim, 1990). For protein-rich pea flour, *S. oryzae* adults were approximately 20 times more susceptible than *C. ferrugineus* adults, and it reduced numbers of *S. oryzae* offspring more than *C. ferrugineus* offspring. It reduced number of *T. castaneum* offspring, but did not kill *T. castaneum* adults in the 2-week test. Therefore, it is important to identify which insect pests are present before applying protein-rich pea flour to control infestations in commercial grain stores.

Grain species affected the toxicity of protein-rich pea flour. To achieve the same level of mortality, a higher dose was required in maize than in wheat and barley. The difference in efficacy was not due to the differences in the kernel size, because ground particles of the same sizes still showed the difference between maize and wheat. Grain species also affects the toxicity of insect growth regulators and other grain protectants (Samson & Parker, 1989; Samson et al., 1990). For example, methoprene and fenoxycarb are more effective against *R. dominica* in wheat and paddy (unmilled rice) than in maize (Samson et al., 1990). Diatomaceous earth used against *S. oryzae* is 3-4 times more effective in wheat than in maize (Paul Fields, Cereal Research Centre, Agriculture and Agriculture and Agri-Food Canada, Winnipeg, MB, unpublished data). However, with barley and wheat, grain species did not affect the toxicity of either malathion or fenitrothion against *C. ferrugineus* (Tyler & Green, 1968). Baker (1988) found that *S. oryzae* produces more progeny, and develops faster in barley or wheat than in maize. Therefore, the lower effectiveness of protein-rich pea flour in maize was not likely due to

the differences in fitness of insects on different nutritional value of the grains. Insects may feed less in maize, which may result in less uptake of protein-rich pea flour, thereby making it less toxic in maize. More studies are needed to determine the reasons for the difference observed between grain species.

The toxicity of protein-rich pea flour was not affected by storing it at low or room temperatures or on treated grain for 9 months. This property enables protein-rich pea flour to be used as a grain protectant, especially for long-term grain storage. However, warmer and moister conditions should be avoided for storage of protein-rich pea flour, because there is a reduction of toxicity after storing protein-rich pea flour at 30°C, 70% RH for 8 months (Bodnaryk et al., 1997).

The effects of temperature and moisture on the toxicity of stored-grain insecticides has been extensively studied (Snelson, 1987; Samson et al., 1988, 1990). Usually, increasing the moisture of grain decreases the effectiveness of organophosphate insecticides (Samson et al., 1988). Protein-rich pea flour was more toxic when grain temperature was high or moisture content low. The effect of environmental factors on the insecticidal efficacy can be subdivided into: (i) effects on insect biology, such as development of insects, metabolism of insecticides, and storage and excretion of insecticides; (ii) effects on insecticide toxicity, such as the penetration, movement within the insects and decomposition; and (iii) effects on the persistence of the insecticides, such as rate of degradation (Snelson, 1987). Each factor has its own relationship to temperature and moisture. Protein-rich pea flour was stable under normal storage conditions. The phenomenon of lower toxicity of protein-rich pea flour at higher

moistures might be related to detoxification of pea protein inside *S. oryzae.* The water content of a resistant strain of *S. oryzae* is higher than that of a susceptible strain (Hou and Fields, unpubl.). The stress of low moisture content may make the insects more susceptible to a second stress, such as a toxin. Low moisture content increases the toxicity of synthetic insecticides, such as fenitrothion and pirimiphos-methyl (Samson et al., 1988).

Granaries in Western Canada with high grain temperature immediately after harvest have the most insects after 2 -months of storage (Loschiavo, 1985). Therefore, to reduce the risk of infestation of grain, it would be best to treat grain immediately after harvest when grain is warm and protein-rich pea flour would be more effective in reducing the risk of infestation of grain.

The development of resistance to insecticides has been observed in many insects (Subramanyam & Hagstrum, 1995; Cochran, 1995; Ware, 2000). There are several tropical strains of *S. oryzae* that are capable of breeding on yellow split peas (Coombs et al., 1977; Grenier et al., 1997). The resistance is controlled by a single recessive, autosomal gene (Thind & Muggleton, 1981; Holloway 1986; Grenier et al., 1997). Glutathione-S-transferases and oxygenases in insects are involved in the detoxification of allelochemicals from peas (Holloway & Smith, 1985; Holloway & Mackness, 1988, Grenier et al., 1997). The possibility of adaptation of insects to peas should be seriously considered in the development of protein-rich pea flour for the control of stored-product insects, as well as the effect of the grain species, insect species, temperature and moisture content of the grain.

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Table 3.1. LD_{50} (lethal dose for 50% of the population) of protein-rich pea flour to *S. oryzae* held on wheat, barley or maize for two weeks, treated with protein-rich pea flour that was stored by various methods. There were six concentrations, and each concentration had five replications, with 20 adults per replicate.

Protein-rich pea flour storage conditions	Wheat			Barley			Maize		
	LD_{50} (%) (95% C.L.)	Slope	g value[a]	LD_{50} (%) (95% C.L.)	Slope	g value	LD_{50} (%) (95% C.L.)	Slope	g value
Fresh	0.04 (0.03-0.05)	3.3 ± 0.3	0.034	0.04 (0.03-0.05)	3.4 ± 0.4	0.048	0.17 (0.12-0.23)	1.9 ± 0.2	0.064
Flour at –15°C for 9 months	0.03 (0.02-0.04)	3.0 ± 0.4	0.059	0.05 (0.02-0.06)	3.4 ± 0.8	0.028	0.21 (0.14-0.28)	1.6 ± 0.2	0.062
Flour in a room for 9 months	0.03 (0.02-0.04)	3.0 ± 0.06	0.137	0.04 (0.03-0.05)	3.0 ± 0.3	0.034	0.17 (0.12-0.23)	1.8 ± 0.2	0.057
Treated grain in a room for 9 months	0.03 (0.02-0.04)	3.2 ± 0.5	0.098	0.03 (0.02-0.04)	3.6 ± 0.6	0.107	0.11 (0.08-0.15)	1.6 ± 0.2	0.043

[a] Index of heterogeneity

Table 3. 2. Mean number (± SEM) of live adult offspring emerged from grain treated with various concentrations of protein-rich pea flour at 30°C, 70% RH after 7-week incubation. Parent adults were removed two weeks after being released. There were five replications each concentration, with 20 adults per replicate.

Concentration of protein-rich pea flour (%)	Number of insects (mean ± SEM)[a]						
	Wheat			Barley			Maize
	S. oryzae	*C. ferrugineus*	*T. castaneum*	*S. oryzae*	*C. ferrugineus*	*T. castaneum*	*S. oryzae*
0	357 ± 46 a	10.8 ± 4.9 a	20.4 ± 2.3 a	234 ± 53 a	20.2 ± 3.7 a	40.4 ± 6.3 a	7.2 ± 1.7 a
0.01	247 ± 33 b	7.6 ± 3.4 a	23.8 ± 2.4 a	205 ± 10 a	26.6 ± 5.2 a	39.4 ± 1.0 a	6.0 ± 1.1 a
0.1	146 ± 25 b	8.6 ± 2.3 a	25.4 ± 2.2 a	77 ± 9 b	24.6 ± 10.4 a	32.4 ± 2.9 a	6.0 ± 1.1 a
1	0.8 ± 0.6 c	2.8 ± 1.4 b	9.6 ± 2.2 b	0.8 ± 0.4 b	3.0 ± 1.8 b	10.6 ± 2.0 b	2.6 ± 0.6 b
10	0 ± 0 c	0.2 ± 0.2 b	9.8 ± 2.5 b	0.2 ± 0.2 b	3.8 ± 3.1 b	3.2 ± 1.5 b	0 ± 0 b

[a] Different letters indicate that the number of insects in a column were significantly different (PROC GLM, CONTRAST; df = 4, 20; $P < 0.05$).

Table 3.3. LD_{50} (lethal dose for 50% of the population) and LD_{90} of protein-rich pea flour (%) for *S. oryzae* in wheat at various temperatures at 70% RH after two weeks. There were six concentrations, and each concentration had five replications, with 20 adults per replicate.

Temperature (°C)	LD_{50} (%)[a] (95% C.L.)	LD_{90} (%) (95% C.L.)	Slope[a]	g value[b]
20	0.043 (0.034-0.052) a	0.247 (0.175-0.415)	1.68 ± 0.14 a	0.054
25	0.038 (0.033-0.042) a	0.099 (0.086-0.12)	3.07 ± 0.20 b	0.025
30	0.028 (0.024-0.031) b	0.067 (0.067-0.077)	3.32 ± 0.27 b	0.025

[a] Different letters in a column indicate that the LD_{50}s or slopes were significantly different (POLO-PC, chi-square likelihood ratio test, $P < 0.05$).

[b] Index of heterogeneity.

Table 3.4. LD_{50} (lethal dose for 50% of the population) and LD_{90} of protein-rich pea flour (%) for *S. oryzae* in wheat at 3 relative humidities and at 30°C for two weeks. There were six concentrations, and each concentration had five replications, with 20 adults per replicate.

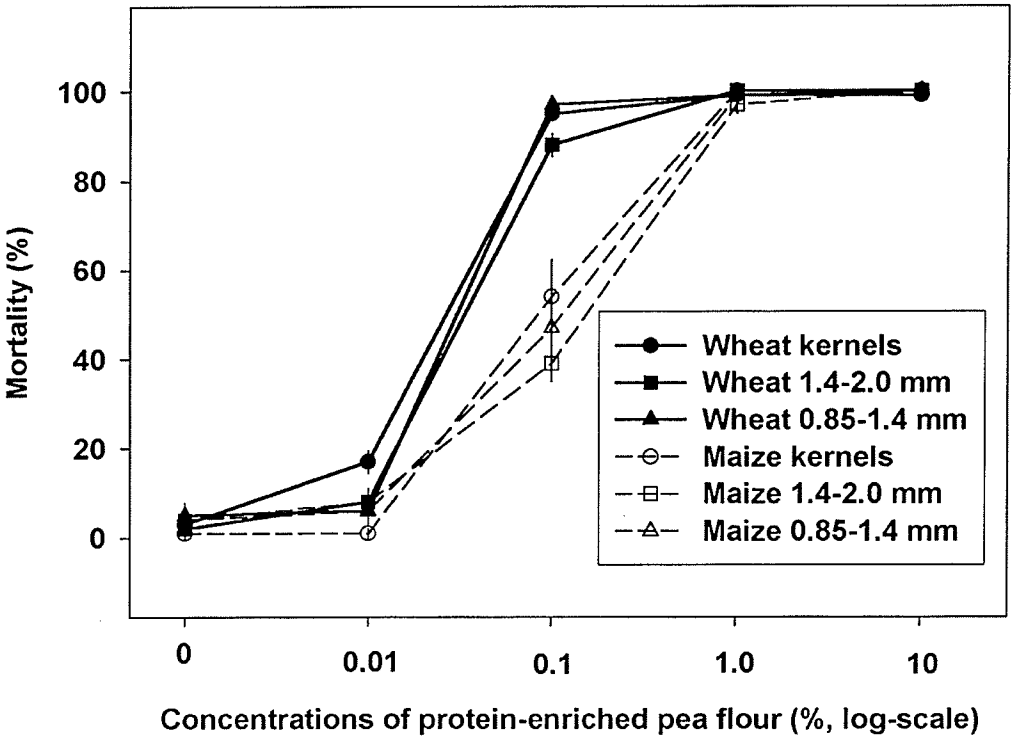
Relative humidity (%)	Moisture content (%)	LD_{50} (%)[a] (95% C.L.)	LD_{90} (%) (95% C.L.)	Slope[a]	g value[b]
85	17.4	0.085 (0.073-0.098) a	0.224 (0.176-0.33)	3.027 ± 0.289 a	0.072
75	16.7	0.055 (0.046-0.063) b	0.119 (0.10-0.158)	3.776 ± 0.325 a	0.085
65	13.0	0.035 (0.025-0.044) c	0.117 (0.09-0.175)	2.417 ± 0.191 b	0.083

[a] Different letters indicate that the number of insects that the LD_{50}s or slopes were significantly different (POLO-PC, chi-square likelihood ratio test, $P < 0.05$).

[b] Index of heterogeneity.

Figure 3. 1. Mortality of *Sitophilus oryzae* (mean ± SEM) in whole and ground grain at various particle sizes and treated with various concentrations of protein-rich pea flour. Insects and grain were held at 30°C, 70% RH for two weeks.

Figure 3-1


Mortality (%)
100
80
60
40
20
0
0
0.01
0.1
1.0
10
Concentrations of protein-enriched pea flour (%, log-scale)
Wheat kernels
Wheat 1.4-2.0 mm
Wheat 0.85-1.4 mm
Maize kernels
Maize 1.4-2.0 mm
Maize 0.85-1.4 mm

CHAPTER 4

Granary Trial of Protein-Rich Pea Flour for the Control of Three Stored-Product Insects in Barley

ABSTRACT

A granary trial was conducted to evaluate the efficacy of protein-rich pea flour against three common stored-grain insects, *Sitophilus oryzae* (L.), *Tribolium castaneum* (Herbst), and *Cryptolestes ferrugineus* (Stephens). Six 30-t farm granaries were filled with approximately 11 t of barley. The barley was either not treated, treated with protein-rich pea flour at 0.1% throughout the entire grain mass, or treated at 0.5% throughout the top half of the grain mass. Adult insects were released in screened boxes (2 insects per kg barley for *S. oryzae* and *T. castaneum*; 1.4 insects per kg barley for *C. ferrugineus*). Barley was sampled four times during the 70-d trial. The number and mortality of released adults and newly emerged adults in the samples were noted. Four kinds of traps: flight, surface-pitfall, probe-pitfall, and sticky-bar, were placed at different locations in the granaries to estimate the movement of insects. The 0.1% protein-rich pea flour treatment reduced released adult numbers of *S. oryzae* by 93%, *T. castaneum* by 66%, and *C. ferrugineus* by 58%, and reduced the newly emerged adults by 87, 77, and 77%, respectively. Treating the top half of the barley at 0.5% protein-rich pea flour had effects similar to treating the entire grain mass with 0.1% pea-protein flour. However, the top-half treatment failed to prevent insects from penetrating into the untreated lower layer. Differences between traps are discussed.

KEYWORDS: *Sitophilus, Cryptolestes, Tribolium*, botanical insecticide, peas, traps

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Stored-product insect pests cause losses by directly reducing dry weight, germination, nutritional value, or the grade of harvested grain. The Food and Agricultural Organization of The United Nations estimates that 5 to 10% of harvested grain is lost in storage with losses being higher in some developing countries (Hall 1970).

Synthetic insecticides, such as deltamethrin, malathion, chlorpyrifos-methyl, phosphine, and methyl bromide, are the main means to control stored-product insects (Harein and Davis 1992, Arthur 1996). However, chemical residues, insect resistance, and worker safety concerns have increased the interest in alternative control methods. Resistance to malathion, phosphine and deltamethrin has been reported in several stored-product insects (Zettler and Cuperus 1990, Subramanyam and Hagstrum 1995). In the United States, the use of chlorpyrifos-methyl may be discontinued because of a voluntary cancellation caused by the high cost of updating its registration data (Anonymous 2000). Methyl bromide is an ozone depletor, and may be banned after 2005 in most industrialized countries (Fields and White 2002). Hence, there is a pressing need to develop new insecticides to protect stored products that are effective, and safe for humans and the environment.

Most stored-product insects are unable to develop on legumes (Singh and Wilbur 1966, Sinha and Watters 1985). In Africa, mixing legumes with the grain is used as a strategy to protect maize from stored-product insect attack. Admixing yellow split peas with grain was suggested by Coombs et al. (1977) to control *Sitophilus oryzae* (L). Delobel et al. (1998) reported that a small polypeptide from peas is insecticidal. Protein-rich pea flour is toxic (Bodnaryk et al. 1997) and repellent (Fields et al. 2001) to many stored-product insects. *Sitophilus* spp. are the most sensitive insect pests, followed by the

rusty grain beetle, *Cryptolestes ferrugineus* (Stephens), and the red flour beetle, *Tribolium castaneum* (Herbst).

As a human food and as a protein additive for animal feed, pea protein is well suited as a natural stored-grain protectant (Fields et al. 2001). The goal of this study was to determine if the repellent and toxic properties of protein-rich pea flour are sufficient to reduce the populations of *S. oryzae, C. ferrugineus*, and *T. castaneum* in barley under farm storage conditions.

Materials and Methods

Granaries

Trials were conducted at Agriculture and Agri-Food Canada's experimental farm at Glenlea, Manitoba (49° 53' N, 97° 08' W) from 17 August to 2 November 1999. Each of six 30-t capacity galvanized bolted-steel farm granaries (Fig. 4.1), with fully perforated floors and a 25 cm high subfloor plenum, were filled with approximately 11 t of barley (2 truck-loads) with 12.9 ± 0.02% moisture content, 629.2 ± 0.8 kg/m^3 bulk density and 0.69 ± 0.07% dockage on 17-19 August 1999. The barley (6-row) was harvested on 15 August and temporarily stored in other granaries. Protein-rich pea flour (Progress Protein; 60% protein, 30% starch, and 7% moisture content, Parrheim Food, Saskatoon, SK) was used in this study. It is produced commercially by grinding peas and isolating a protein-rich faction by air classification. The protein-rich pea flour was manually shaken into the barley at the base of an 18-cm-diameter auger running at a rate of 0.5 t per min. There were three treatments: untreated barley (control), all barley in a granary treated at 0.1% (w/w) protein-rich pea flour, and only the top half of the barley in

the granary treated with 0.5% (w/w) protein-rich pea flour with the bottom half untreated. There were two granaries for each treatment.

Insects

Three insect species, *S. oryzae*, *T. castaneum* and *C. ferrugineus*, were reared in the laboratory at 30°C, 70% relative humidity (RH). All three species had been cultured in the laboratory for over five years. *Sitophilus oryzae* was reared on whole kernels of wheat, *T. castaneum* on wheat flour mixed with 5% brewer's yeast, and *C. ferrugineus* on wheat kernels with 5% wheat germ and 5% brewer's yeast. To get sufficient *C. ferrugineus*, a field strain of this species was collected in April 1999 at Glenlea, Manitoba, and cultured in the laboratory. The released *C. ferrugineus* population was a mixture of laboratory strain and the newly collected strain.

Sitophilus oryzae and *T. castaneum* were released at the rate of approximately 2 insects per species per kg of barley (22,000 insects per species per granary), and *C. ferrugineus* at approximately 1.4 insects per kg (15,000 insects per granary). Before their release, all insects were sieved out of their rearing medium and 4,400 *S. oryzae*, 4,400 *T. castaneum*, and 3,000 *C. ferrugineus* were placed together in 4-liter jars containing 2.5 kg of untreated barley taken from the control granaries. In each granary, insects were released into five boxes (30 × 30 × 30 cm) with a screened floor (2 mm^2 openings) and an open top. These five boxes were buried in the top layer of grain at the sampling points (Fig. 4.1) so that the top edge of the box was level with the top surface of the levelled grain mass. The screened bottom and open top allowed insects to move freely between the release boxes and the grain mass. Insects were released into the boxes on 23 August 1999. After emptying one jar of insects, the box was filled with untreated barley to the

top edge of the box. The boxes were removed 10 d after insects had been released. The mortality and number of insects remaining in the boxes was assessed after the grain was sieved with a Carter® dockage tester (Simon-Day Ltd. Winnipeg, Manitoba, Canada) with a No. 8 sieve.

Temperature

A data logger (CR10, Campbell Scientific Corp., Edmonton, Alberta, Canada) was used to record temperature. In each granary, 12 thermocouples were placed in the grain mass at three depths and four locations per depth (Fig. 4.1). A thermocouple was located 5 cm above the center of the grain surface to measure the air temperature of the head space. Temperatures were measured every 12 min, and the hourly means were recorded. The average daily temperatures were used for the data analysis.

Grain Sampling

Grain samples of about 1 kg per sampling location were taken from each of 10 points (Fig. 4.1) in each granary, five at the surface and five at 1 m below the surface which was below the mid depth of the grain bulk, on 9 and 23 September and 7 and 21 October 1999 (17, 31, 45, and 59 d after insect release). A 1-liter cup was used for the surface layer samples, and a 0.3-liter torpedo probe sampler (Seedburo, Chicago, Illinois) was used for the 1 m samples. In the laboratory, adult insects were removed from each sample with a sieve (2 mm^2 openings). Species and number of live and dead insects were recorded. After adult insects were removed, grain samples and sieved dust and broken kernels were placed in a 4-liter jar and held at 30°C, 70% RH for 5 wk. The grain was sieved a second time and the number of live and dead emerged adults was counted to estimate the mortality of second-generation adults.

Physical Characteristics

Moisture content, dockage and bulk density of barley samples were measured three times during trials. The first sampling was from truckloads before treatment with protein-rich pea flour and augering into granaries. Four 1-kg barley samples were taken from each truckload at four corners approximate 40 cm away from the edges. The second sampling was on 19 August 1999 in granaries after treatment with protein-rich pea flour, but before the release of insects. Finally, the third sampling was on 2 November 1999 in granaries at the end of the trial. In the granaries, sampling was done at ten locations (Fig. 4.1).

Traps

Insect movement was monitored with surface-pitfall, flight, probe-pitfall, and sticky-bar traps that were placed around the grain mass (Fig. 4.1). Flight traps were made with 25 cm diameter paper plates covered with TangleFoot® (The Tanglefoot Company, Grand Rapids, MI, USA) and vertically placed 20 cm above the grain surface. Surface-pitfall traps (Storgard Flit-Trak M^2, Trécé Inc., Salinas, CA) were filled with 10 ml corn oil and placed on the surface of the grain. The flight traps and surface-pitfall traps were used to estimate the movement of insects in the head space and on the surface of the grain. Probe-pitfall traps (Storgard WB Probe II, Trécé Inc., Salinas, CA) were inserted into the grain just below the top surface of the grain and to depth of 1 m from the top surface at each sampling point to measure the movement of insects within the grain mass. To measure the tendency of insects to leave granaries through the bottom, a wooden bar (9 cm width × 330 cm length × 4.5 cm height) covered with TangleFoot® was placed underneath the perforated floor of the granaries through the aeration duct. The length of

wood was marked into five equal sections, each with an area of 594 cm^2 (Fig. 4.1). Insects in all traps were recorded and traps emptied once a week. For probe-pitfall traps, the number of live and dead insects was recorded.

Data Analysis

Means of daily temperatures were used to compare the temperature differences in granaries and in layers by using ANOVA with PROC GLM procedure (General Linear Models) (SAS Institute 2000). A split-plot ANOVA analysis was used to analyses the variation in number of insects, mortality of insects, bulk density, moisture content and dockage among treatments with PROC MIXED procedure (General Linear Models) (SAS Institute 2000). All data for the number of insects in grain samples, release boxes and traps were expressed as number of insects per kg or per trap before being transformed with logarithm of x + 0.0001, except for the number of *S. oryzae* emerged adults, which was transformed with the square root of x + 1. Mortality data were transformed using arcsine square root. The means of treatments were linearly tested with SAS CONTRAST ($\alpha = 0.05$).

Results

Grain Temperature

Grain temperature was the same in all granaries ($F = 0.19$; df = 5, 432; $P = 0.9664$) and the same for all treatments ($F = 0.04$; df = 2, 216; $P = 0.9603$). Therefore, the grain temperatures of the six granaries were pooled. Middle layer temperatures (15.9 ± 0.7°C) were significantly different from top layer (13.3 ± 0.6°C) ($F = 7.55$; df = 1, 216; $P = 0.0065$) and bottom layer temperatures (13.6 ± 0.7°C) ($F = 5.53$; df = 1, 216; $P =$

0.0196). There was no difference between top layer and bottom layer ($P > 0.05$) ($F = 0.16$; df = 1, 216; $P = 0.6932$) (Fig. 4.2). Grain temperatures declined during the trials from about 25 °C to 5 °C. Average grain temperature of all granaries was 14.3 ± 0.7 °C during the tria. The maximum and minimum grain temperatures were 34.8 and -1.1 °C. Before 30 September, the average head-space daily maximum temperature was 23.6 ± 1.0 °C, ranging from to 35.7 to 13.4 °C, and the minimum temperature was 11.5 ± 0.7 °C, ranging from 22.3 to 0.5 °C. After 30 September, the average daily head-space maximum temperature was 12.1 ± 0.6 °C, and the minimum temperature was 1.7 ± 0.5 °C (Fig. 4.2).

Grain Samples

Numbers of Live Insects. The numbers of live *S. oryzae* in the grain samples treated with 0.1% protein-rich pea flour (0.12 ± 0.07 insects per kg) or with the top-half treated with 0.5% protein-rich pea flour (0.17 ± 0.07 insects per kg) was significantly lower than in untreated granaries (1.67 ± 0.36 insects per kg) ($F = 63.72$; df = 2, 3; $P = 0.0035$), but there was no difference between the two pea flour treatments ($F = 2.62$; df = 1, 3; $P = 0.2042$) (Fig. 4.3A). Similar differences were seen in the number of live emerged adults of *S. oryzae* ($F = 51.51$; df = 2, 3; $P = 0.0048$) (Fig. 4.4A).

Numbers of live adult *T. castaneum* in the 0.1% pea flour treated granaries (0.28 ± 0.08 insects per kg) were less than those in the 0.5% pea flour top-half treated granaries (0.42 ± 0.13 insects per kg) ($F = 34.54$; df = 1, 3; $P = 0.0098$), and the numbers in both treatments were significantly lower than in untreated granaries (0.83 ± 0.17 insects per kg) ($P < 0.05$) (Fig. 4.3B). The number of live *T. castaneum* emerged adults in the 0.1% pea flour treatment (0.87 ± 0.6 insects per kg) and 0.5% pea flour top-half treatment (1.24 ± 0.48 insects per kg) was lower than in the untreated granaries (3.71 ± 2.09 insects per kg)

($P < 0.05$), but no difference was seen between the two protein-rich pea flour treatments ($F = 0.20$, df = 1, 3; $P = 0.6830$) (Fig. 4.4B). Neither protein-rich pea flour treatments significantly reduced the number of live *C. ferrugineus* adults ($F = 0.50$; df = 2, 3; $P = 0.6511$), nor did in reduction of number of emerged *C. ferrugineus* adults ($F = 1.97$; df = 2, 30; $P = 0.2864$) (Figs. 4.3C and 4.4C).

Protein-rich pea flour changed the distribution of *S. oryzae* and *C. ferrugineus* in the granaries. More live adult *S. oryzae* were found in the middle layer (2.9 ± 0.8 insects per kg) than in the top layer in untreated granaries (0.4 ± 0.2 insects per kg) ($F = 10.97$; df = 1, 2; $P = 0.0453$). However, there was no difference between layers in either protein-rich pea flour treated granaries ($P > 0.05$). There was no difference in the number of adult *C. ferrugineus* between the top layer and the middle layer in the untreated granaries ($F = 0.21$; df = 1, 2; $P = 0.6886$). However, more *C. ferrugineus* were found in the 0.1% protein-rich pea flour treated top layer (2.5 ± 0.6 insects per kg) than in the middle layer (0.4 ± 0.1 insects per kg) ($F = 325.50$; df = 1, 2; $P = 0.0031$). The emerged adults of all three species were found in the middle layer in both protein-rich pea flour treated granaries (Table 4.1).

Mortality. Mortality of adult *S. oryzae, T. castaneum* and emerged adults of *S. oryzae* in grain samples treated with 0.1% or 0.5% protein-rich pea flour was significantly higher than in the untreated control. There was no difference between the two protein-rich pea flour treatments (Table 4.2). Treatments of protein-rich pea flour did not increase the mortality of adult *C. ferrugineus* ($P > 0.05$), or the emerged adults of *T. castaneum.*

Mortality of emerged adults of *S. oryzae* was high in both the top (97 ± 9%) and middle layers (95 ± 7%) in the 0.1% pea flour treatment. However, the mortality of emerged adults of *S. oryzae* was higher in the top layer (93 ± 16%) than in the middle layer (6 ± 5%) in the 0.5% pea flour treatment ($F = 23.41$; df = 1, 6; $P = 0.0029$). Mortalities of adults and emerged adults of *T. castaneum* and *C. ferrugineus* in the top and middle layers were not different in any treatment ($P > 0.05$).

Physical Characteristics

There was no difference in the bulk density of the barley throughout the trial before treatment (629 ± 0.8 kg/m^3), after the treatment (632 ± 1.1 kg/m^3) and at the end of the trial (630 ± 1.5 kg/m^3) ($F = 2.63$; df = 2, 160; $P = 0.0754$). There was no difference in the bulk density between untreated, 0.1% all grain treated and 0.5% half treated before treatment ($F = 1.61$; df = 2, 54; $P = 0.2128$), after treatment ($F = 0.66$; df = 2, 54; $P = 0.5194$) and at the end of trial ($F = 0.25$; df = 2, 54; $P = 0.7824$). No difference in the moisture content was found among all treatments within each of the three periods of the trial ($P > 0.05$). For three sampling dates, the dockage was higher in the 0.5% top-half treatment (0.87 ± 0.07%) than in untreated (0.55 ± 0.03%) and 0.1% treatment (0.53 ± 0.03%) ($P < 0.05$).

Insects Remaining in Release Boxes

For all three insect species, more insects remained in the boxes in pea flour treated granaries than in the controls (Table 4.3). Mortality of *S. oryzae* (27 ± 4%) in boxes in granaries top-half treated with 0.5% pea flour was significantly higher than in the 0.1% treatment (12 ± 2%) and untreated granaries (11 ± 2%) ($F = 4.75$; df = 2, 15; P

= 0.0117). The mortality of *T. castaneum* and *C. ferrugineus* did not differ among untreated and the two pea flour treatments ($P > 0.05$).

Traps

Surface-Pitfall Traps. Surface-pitfall traps in both pea flour treated granaries caught more *S. oryzae, T. castaneum*, and *C. ferrugineus* than in untreated controls (Table 4.3). No *S. oryzae* were trapped in the surface-pitfall traps after 45 d in the 0.1% pea flour treated granaries, but a few were found in traps placed in the untreated granaries. Most *T. castaneum* were caught during the first 10 d. *Cryptolestes ferrugineus* was found in the traps during the entire trial period, even when grain temperatures were below 10°C.

Flight Traps. Flight traps caught more *T. castaneum* than *S. oryzae* and *C. ferrugineus* (Table 4.3). Over 90% of all trapped *T. castaneum* were caught during the first 4 d (each trap caught more than 30 adult *T. castaneum*) when grain temperatures were over 25°C in the top layer. Few were caught after 2 September, when the top layer was below 20°C. Traps in the granaries with the top-half treated with 0.5% pea flour caught few but significantly more *S. oryzae* than in the 0.1% pea flour treatment or in the untreated granaries (Table 4.3). There were no differences in the number of *C. ferrugineus* on the traps among all treatments.

Probe-Pitfall Traps. Probe-pitfall traps caught more insects than each of other traps combined (Table 4.3). The traps in the granaries treated with 0.5% pea flour in the top half of the barley and treated with 0.1% pea flour in all the barley caught more *S. oryzae* than those in the untreated controls. Traps in the 0.1% and the 0.5% pea flour treatment caught more *S. oryzae* at the beginning of the trial than in the untreated controls, and then caught less than those in the controls (Fig. 4.5). There were no

differences in the number of *T. castaneum* and *C. ferrugineus* caught in the traps among treatments. More insects were caught in the top layer (3.7 ± 0.2) than in the middle layer for *S. oryzae* (1.9 ± 0.4) ($F = 14.29$; df = 1, 3; $P = 0.0325$), *T. castaneum* (top, 7.6 ± 0.2; middle, 2.0 ± 0.7) ($F = 82.20$; df = 1, 3; $P = 0.0027$), and *C. ferrugineus* (top, 6.8 ± 0.2; middle, 1.6 ±0.8) ($F = 68.94$; df = 1, 3; $P = 0.0037$). The mortality of *S. oryzae* in traps in the 0.1% and 0.5% top-half pea flour treatments was 74 ± 9% and 62 ± 11%, respectively, which were higher than in the untreated granaries 38 ± 8% ($P < 0.05$).

Floor Sticky Traps. Traps underneath the perforated floor caught more *C. ferrugineus* than *S. oryzae* or *T. castaneum* (Table 4.3). More *S. oryzae* were caught in the 0.1% pea flour and 0.5% pea flour treated granaries than in the untreated granaries. *Sitophilus oryzae* moved down quickly in 0.1% pea flour treated granaries; the sticky trap caught 0.9 insects per section per day during the first 2 d of the trial in the 0.1% pea flour treatment while none were found in the 0.5% protein-rich pea flour treatment or the controls. When grain temperatures dropped below 10°C in the last 2 wk of the trial, no *S. oryzae* were caught on the sticky traps. Traps in both protein-rich pea flour treated granaries caught more *T. castaneum* and *C. ferrugineus* than those in untreated granaries. No *T. castaneum* were found on the sticky traps in untreated granaries during the trials. *Cryptolestes ferrugineus* were found on the traps during the entire trial in all treatments. Middle sections of the sticky trap (section 3 and 4) caught more *C. ferrugineus* than outer sections ($F = 9.87$; df = 4, 12; $P < 0.0009$).

Discussion

Laboratory tests (Bodnaryk et al. 1997, Fields et al. 2001) demonstrate that protein-rich pea flour is repellent and toxic to *S. oryzae, T. castaneum* and *C. ferrugineus*. My goal was to determine if these properties of protein-rich pea flour are sufficient to reduce stored-grain pests in commercial granaries. Compared to the number of live insects found in the untreated barley, the 0.1% protein-rich pea flour treatment reduced the parent population of *S. oryzae* by 93%, *T. castaneum* by 66% and *C. ferrugineus* by 58%. Similar reductions were shown in the 0.5% top-half treatment. The efficacy of protein-rich pea flour at 0.1% against *S. oryzae* in this granary trial was similar to the 95% mortality reported in a laboratory test (Bodnaryk et al. 1997). However, the reduction in the populations of *T. castaneum* and *C. ferrugineus* in the granary trial was higher than in the laboratory tests, in which there was about 20% and 15% mortality respectively (Bodnaryk et al. 1997). In the laboratory trials of Bodnaryk et al. (1997), insects were not allowed to leave the test vials. The higher efficacy in the granary trial could be due to an additional effect of fewer insects moving into the grain mass and more insects leaving the granary due to the repellency of the protein-rich pea flour.

I believe that the reduction of *S. oryzae* in treated granaries was primarily due to increased mortality. Few *S. oryzae* were found in traps above or below the granaries, and many dead and few live *S. oryzae* were found within the treated granaries. On the other hand, I believe that the reduction of *T. castaneum* populations was due to a combination of repellency and toxicity of the protein-rich pea flour. More *T. castaneum* were caught on traps and suffered higher mortality in the treated granaries.

Many *C. ferrugineus* left the barley bulks through the perforated floor. By using the number of *C. ferrugineus* caught on the floor sticky trap and extrapolating for the entire floor area, I estimated that 6,475, 11,045, and 9,501 adults (untreated, 0.1% and 0.5% pea flour treated granaries, respectively) left the granaries through the perforated floor. This is significant considering that only 15,000 *C. ferrugineus* were released into each granary. Therefore, for protein-rich pea flour to be effective against *C. ferrugineus,* it should be used with granaries that allow *C. ferrugineus* to leave the granary, such as granaries with perforated floors. I believe that there were very few insects immigrating into the granaries other than the ones released. White et al. (1995) showed that at this test location, a Johnson-Taylor insect suction trap operated from 1987 to 1993 caught no *S. oryzae*, 6 *T. castaneum*, and 14 *C. ferrugineus* during the entire period of operation, whereas hundreds of stored-grain fungus feeding species were caught.

The higher number of insects remaining in the release boxes, and the higher mortality of insects in the boxes in treated granaries indicated that protein-rich pea flour has the potential of reducing the initial population of insects that immigrate into stored grain. Insects can infest stored grain through perforated floors. The repellency of protein-rich pea flour could reduce this type of immigration. Mohan (1997) developed an insect removal granary by using a screened wall instead of a solid wall. This granary, used in conjunction with protein-rich pea flour, could protect grain from stored-product insects (Mohan and Fields 2002).

The live emerged adults in the 0.1% protein-rich pea flour treatment were reduced by 87% for *S. oryzae*, 77% for *T. castaneum* and 77% for *C. ferrugineus*, compared to the untreated granaries. For *S. oryzae,* the reduction is due in part to the toxicity of protein-

rich pea flour to emerged adults. Also, the reduction of emerged adults could be due to fewer parent insects in treated grain. The reduction in the parent populations could be due to reduced immigration, increased mortality or increased emigration. Other possible factors are the reduction of adult fecundity and reduced larval survival in treated barley. Further tests are needed to determine the relative importance of each factor.

Protein-rich pea flour shows repellency in the laboratory (Fields et al. 2001). I hypothesised that control could be possible by treating only the top layer of the grain, reducing the number of insects immigrating into the grain. This method would reduce labour costs and the amount of pea protein required in large commercial granaries. Top-half treatment with protein-rich pea flour had a similar effect to treating the entire grain mass on the reduction of insect populations in this trial. However, top-half treatment failed to prevent insects from moving into the untreated barley. Unlike treating the entire grain mass, in which *S. oryzae* offspring in the middle layer were killed by protein-rich pea flour, offspring of *S. oryzae* survived in the middle layer in the top-half only treatment. The *S. oryzae* population could increase unhindered in the untreated middle layer in top-half treatment. Therefore, I would not recommend this method of application.

Many factors such as grain temperature, the behavior of insects and population size affect the number of insects collected in traps (White and Loschiavo 1986, Buchelos and Athanassiou 1999, Wakefield and Cogan 1999). Buchelos and Athanassiou (1999) found the ranking of species collected in probe-pitfall traps differs from that of insects in the grain samples taken from the same granary. In this trial, protein-rich pea flour increased the movement of insects, which may be due to the repellency of protein-rich pea flour (Fields et al. 2001). I assume all granaries had similar densities of insects 2 d

after releasing insects. My laboratory tests have shown that the mortality of *S. oryzae* in 2 d in 100% protein-rich pea flour is very low (Chapter 8). However, the number of insects collected in probe-pitfall traps was significantly different among treatments. Thus, the use of traps to evaluate insecticides that affect insect behavior should be analyzed with caution.

Some tropical strains of *S. oryzae* can reproduce on yellow split pea (Coombs et al. 1977). The inheritance of this ability is controlled by a single recessive, autosomal gene (Thind and Muggleton 1981, Holloway 1986, Grenier et al. 1997). Symbionts, glutathione-S-transferases, and oxygenases in insects may be involved in the detoxification of allelochemicals from peas (Holloway and Smith 1985; Holloway and Mackness 1988, Grenier et al. 1997). The adaptation of insects to peas should be seriously considered in the development of pea extract for the control of stored-product insects.

Protein-rich pea flour shows potential as a grain protectant with both toxic and repellant properties (Bodnaryk et al. 1997). Stored-product insects are affected by many plant extracts (Jacobson 1989a). Most of them are medicinal plants or spices (Golob et al. 1999) with limited availability, because they are either wild or grown on a small scale. In addition, some extracts are toxic to mammals (Golob et al. 1999, Ware 2000). Peas are consumed by humans, and grown around the world with an annual production of about 12 million tonnes (Skrypetz 2001). Protein-rich pea flour is also available to many farmers who may be too poor to afford chemical insecticides. Unlike diatomaceous earth (Korunic et al. 1998, Fields and Korunic 2000b), protein-rich pea flour did not reduce bulk density. Also, most grain protectants harm parasitoids (Perez-Mendoza et al. 1999).

Protein-rich pea flour is not toxic to *Anisopteromalus calandrae* (Howard), an ectoparasitoid of several stored-product insects, and *Cephalonomia waterstoni* (Gahan), an ectoparasitoid of *C. ferrugineus* (Hou et al. 2002). Powdered grain protectants are difficult to apply. Further research is required to determine if protein-rich pea flour or purified extract (Bodnaryk et al. 1997) can be applied in a liquid form.

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Table 4.1. The number (mean ± SEM) of live emerged adults of three insect species in the samples taken from middle layer of granaries filled with 11 t barley treated with protein-rich pea flour at 0.1%, 0.5% (only top half of the bulk treated), or untreated as controls on 9 and 23 September and 7 and 21 October 1999. Each treatment had two replicates. The number of emerged adults was recorded when barley samples had been cultured for 5 weeks at 30°C, 70% RH, after parent adults were removed.

Insect	Number of Emerged Adults (mean ± SEM)[a]			*F*	*P*
	Untreated	0.1% All treated	0.5% Top-half treated		
S. oryzae	11.0 ± 3.2 a	0.5 ± 0.3 b	4.8 ± 1.8 b	51.10	0.0048
T. castaneum	3.2 ± 1.5 a	0.5 ± 0.2 b	0.7 ± 0.3 b	7.21	0.0714
C. ferrugineus	2.5 ± 0.4 a	0.7 ± 0.2 a	1.4 ± 0.5 a	1.97	0.2846

[a] different letters in the same row indicate significant differences between treatments (PROC MIXED, CONTRAST; df = 2, 3; experimentwise error = 0.14).

Table 4.2. The mortality (mean ± SEM) of three insect species found in the samples taken from granaries filled with 11 t barley treated with protein-rich pea flour at 0.1%, 0.5% (only top half of the bulk treated), or untreated as controls on 9 and 23 September and 7 and 21 October 1999. Each treatment had two replicates. The mortality of emerged adults was recorded when barley samples were cultured 5 weeks at 30°C, 70% RH, after parent adults were removed.

Stage	Insect	Mortality (%) (mean ± SEM) [a]			*F*	*P*
		Untreated	0.1% All treated	0.5% Top-half treated		
Adult	*S. oryzae*	44 ± 10 a	96 ± 3 b	91 ± 5 c	133.03	0.0091
	T. castaneum	26 ± 8 a	63 ± 15 b	48 ± 10 b	7.18	0.0715
	C. ferrugineus	12 ± 5 a	14 ± 12 a	3 ± 2 a	1.10	0.4385
Emerged adults	*S. oryzae*	2 ± 1 a	67 ± 10 b	50 ± 16 b	3.12	0.0849
	T. castaneum	4 ± 4 a	8 ± 4 a	15 ± 9 a	1.95	0.2866
	C. ferrugineus	2 ± 1 a	0 ± 0 a	14 ± 8 a	2.00	0.2803

[a] different letters in the same row indicate significant differences between treatments (PROC MIXED, CONTRAST; df = 2, 3; experimentwise error = 0.14).

Table 4.3. The number (mean ± SEM) of insects (live and dead) remaining in release boxes 10 days after being placed in granaries filled with 11 t barley treated with protein-rich pea flour at 0.1%, 0.5% (only top half of the bulk treated), or untreated as controls. Each treatment contained five boxes, five surface-pitfall traps, five flight traps, ten probe-pitfall traps and 1 sticky trap. Each treatment had two replicates. The number of insects caught in various traps was counted every wk for 12 wk.

Traps	Insects	Number of Insects (mean ± SEM) [a]			*F*	*P*
		Untreated	0.1% All treated	0.5% Top-half treated		
Insect remaining in release boxes (per box)	*S. oryzae*	177 ± 32 a	419 ± 40 b	350 ± 66 b	16.08	0.0249
	T. castaneum	30 ± 8 a	53 ± 4 b	59 ± 11 b	5.46	0.0165
	C. ferrugineus	21 ± 7 a	254 ± 74 b	116 ± 32 b	9.90	0.0477
Surface-pitfall traps (per trap per week)	*S. oryzae*	0.8 ± 0.3 a	1.6 ± 0.3 b	3.8 ± 0.9 b	8.20	0.0608
	T. castaneum	1.5 ± 0.4 a	2.5 ± 0.8 b	3.4 ± 0.7 c	25.16	0.0133
	C. ferrugineus	0.1 ± 0.0 a	0.2 ± 0.1 b	0.3 ± 0.1 b	21.99	0.0161
Flight traps (per trap per week)	*S. oryzae*	0.04 ± 0.03 a	0.2 ± 0.1 a	1.3 ± 0.4 b	15.28	0.0267
	T. castaneum	5.8 ± 1.8 a	6.2 ± 1.8 a	4.0 ± 1.1 a	0.02	0.9856
	C. ferrugineus	0.1 ± 0.1 a	0.1 ± 0.1 a	0.04 ± 0.02 a	4.39	0.1285
Probe-pitfall traps (per trap per week)	*S. oryzae*	13.9 ± 1.2 a	19.2 ± 2.3 b	26.2 ± 3.8 b	8.56	0.0575
	T. castaneum	29.5 ± 3.8 a	35.9 ± 4.5 a	35.6 ± 4.7 a	0.07	0.9318
	C. ferrugineus	29.4 ± 6.1 a	35.2 ± 5.2 a	24.2 ± 3.6 a	5.00	0.1108
Floor sticky traps (per section per week)	*S. oryzae*	0.02 ± 0.01 a	0.2 ± 0.1 b	0.1 ± 0.04 b	15.24	0.0268
	T. castaneum	0 ± 0 a	0.1 ± 0.03 b	0.1 ± 0.03 b	24.84	0.0136
	C. ferrugineus	2.9 ± 0.3 a	5.0 ± 0.5 a	4.3 ± 0.5 a	0.98	0.6493

[a] different letters in the same row indicate significant differences between treatments (PROC MIXED, CONTRAST; df = 2, 3; experimentwise error = 0.14).

Figure 4.1. Diagrams of the arrangement of thermcouple wires, sampling points and traps in the granaries. Insects were released at the five sampling points in the top layer of grain.

Figure 4-1

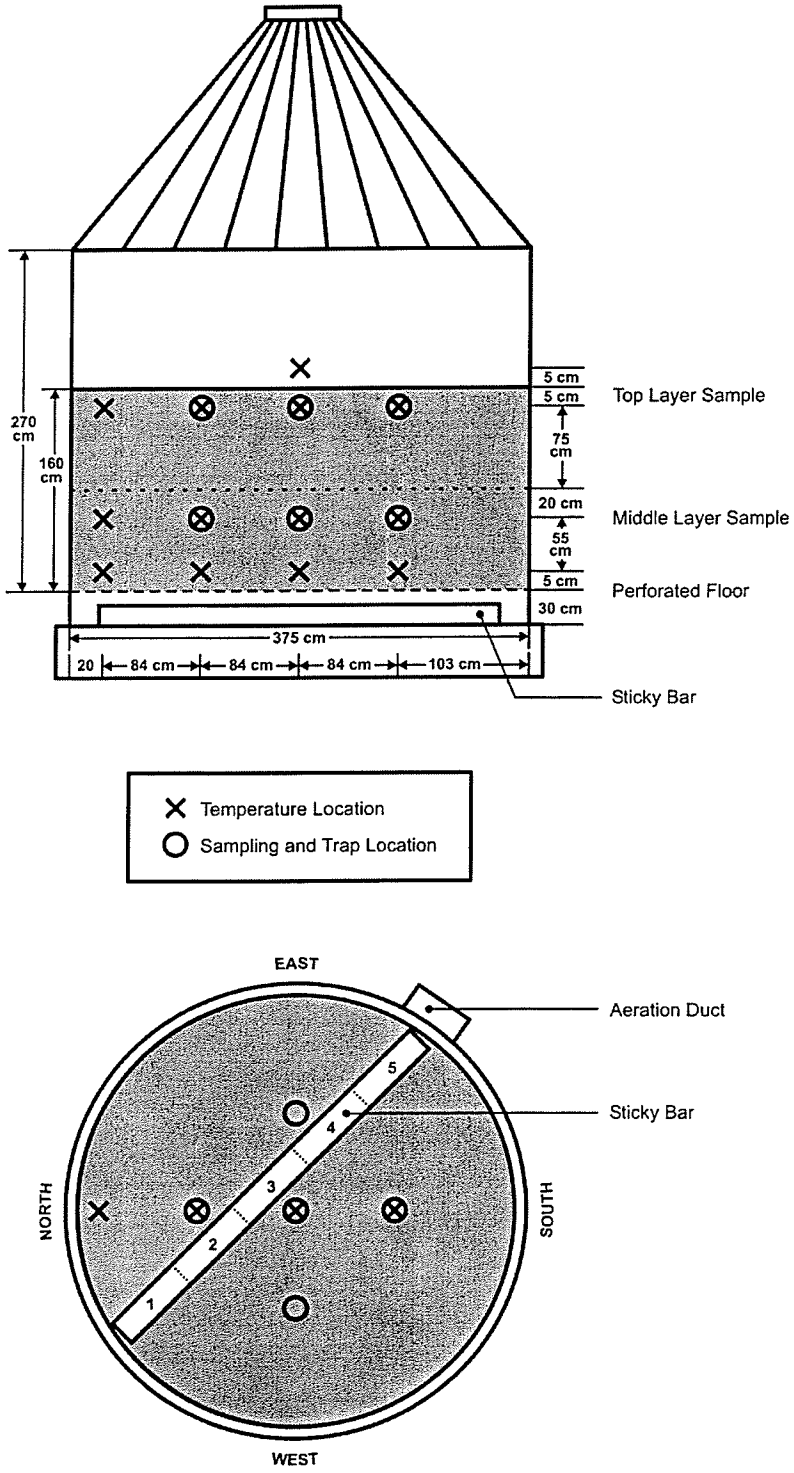

270 cm
160 cm
5 cm
5 cm
75 cm
20 cm
55 cm
5 cm
30 cm
Top Layer Sample
Middle Layer Sample
Perforated Floor
375 cm
20
84 cm
84 cm
84 cm
103 cm
Sticky Bar
Temperature Location
Sampling and Trap Location
EAST
WEST
NORTH
SOUTH
Aeration Duct
Sticky Bar
1
2
3
4
5

Figure 4.2. Grain temperatures in the top layer (5 cm below grain surface), middle layer (100 cm below grain surface), bottom layer (5 cm above the granary floor) and maximum and minimum head space air temperatures (5 cm above grain surface) in the granaries filled with 11 t barley.

Figure 4-2

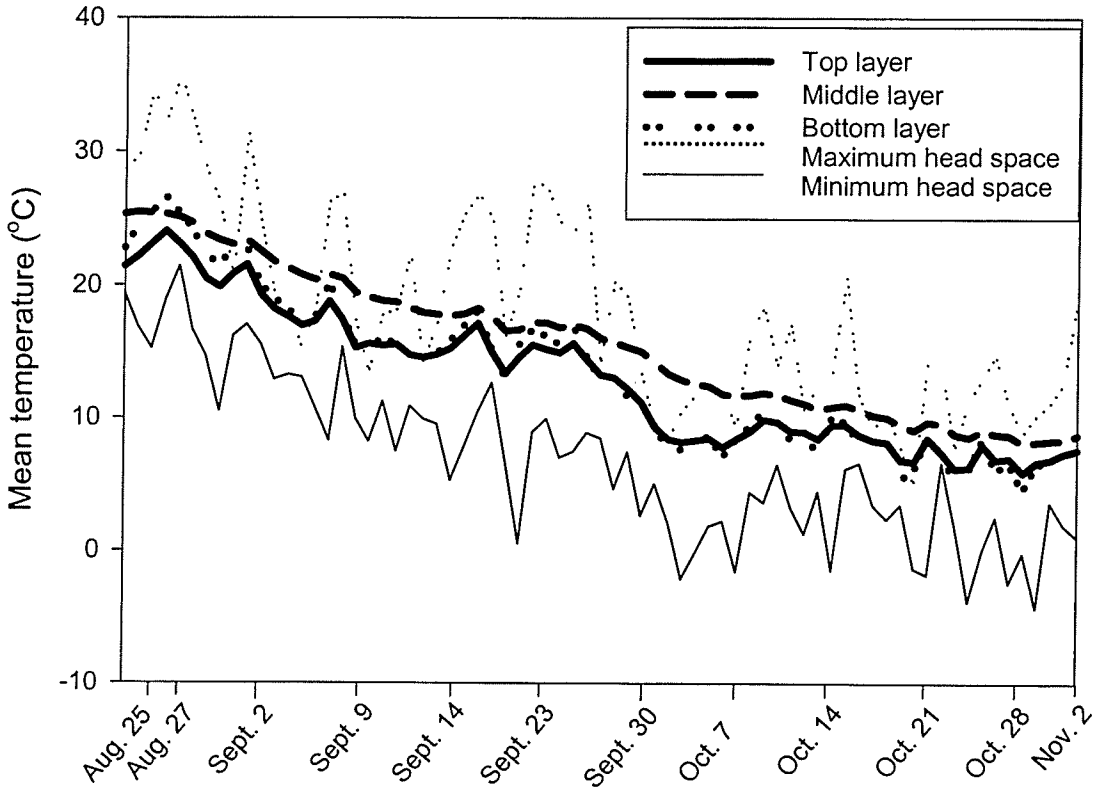
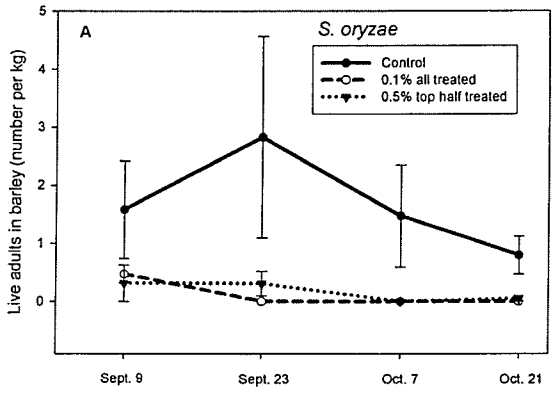
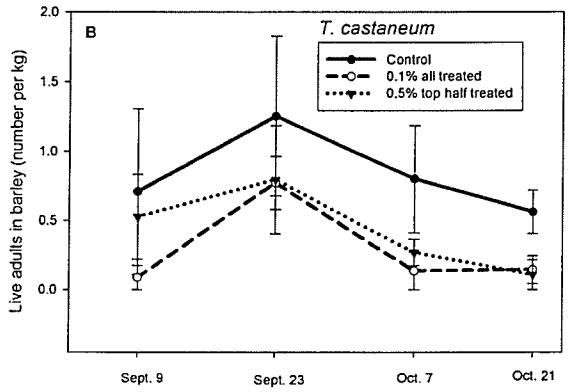

Mean temperature (°C)
40
30
20
10
0
-10
Top layer
Middle layer
Bottom layer
Maximum head space
Minimum head space
Aug. 25
Aug. 27
Sept. 2
Sept. 9
Sept. 14
Sept. 23
Sept. 30
Oct. 7
Oct. 14
Oct. 21
Oct. 28
Nov. 2

Figure 4.3. The number of live adults in barley on different sampling dates from granaries untreated or treated with protein-rich pea flour at 0.1% with all the grain treated or 0.5% with the top half of the bulk treated. A: *S. oryzae*; B: *T. castaneum;* C: *C. ferrugineus*.

Figure 4-3


A
S. oryzae
Control
0.1% all treated
0.5% top half treated
Live adults in barley (number per kg)
0
1
2
3
4
5
Sept. 9
Sept. 23
Oct. 7
Oct. 21


B
T. castaneum
Control
0.1% all treated
0.5% top half treated
Live adults in barley (number per kg)
0.0
0.5
1.0
1.5
2.0
Sept. 9
Sept. 23
Oct. 7
Oct. 21

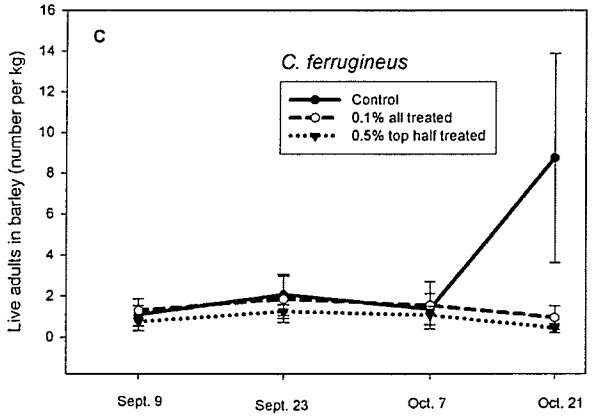
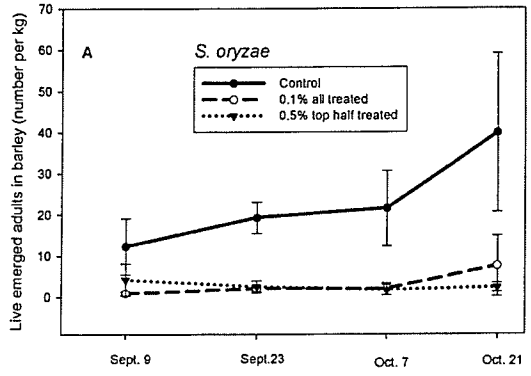
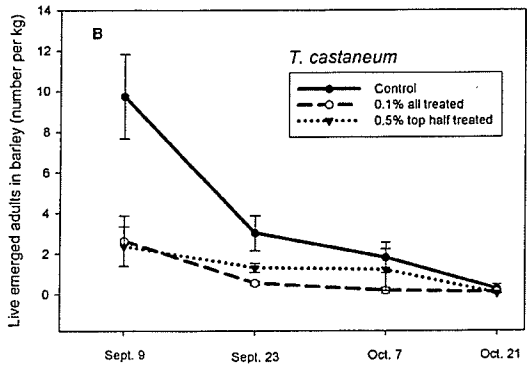

C
C. ferrugineus
Control
0.1% all treated
0.5% top half treated
Live adults in barley (number per kg)
0
2
4
6
8
10
12
14
16
Sept. 9
Sept. 23
Oct. 7
Oct. 21

Figure 4.4. The number of live emerged adults in barley samples taken from granaries untreated or treated with protein-rich pea flour at 0.1% with all the grain treated or 0.5% with the top half of the bulk treated at different sampling dates. Grain samples with adults removed were cultured 5 weeks at 30°C, 70% RH. A: *S. oryzae*; B: *T. castaneum;* C: *C. ferrugineus.*

Figure 4-4


A
S. oryzae
Control
0.1% all treated
0.5% top half treated
Live emerged adults in barley (number per kg)
70
60
50
40
30
20
10
0
Sept. 9
Sept.23
Oct. 7
Oct. 21


B
T. castaneum
Control
0.1% all treated
0.5% top half treated
Live emerged adults in barley (number per kg)
14
12
10
8
6
4
2
0
Sept. 9
Sept. 23
Oct. 7
Oct. 21

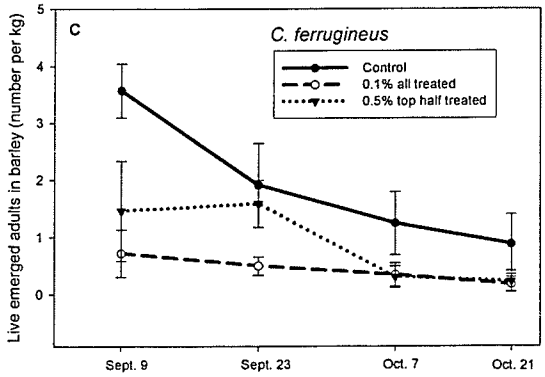
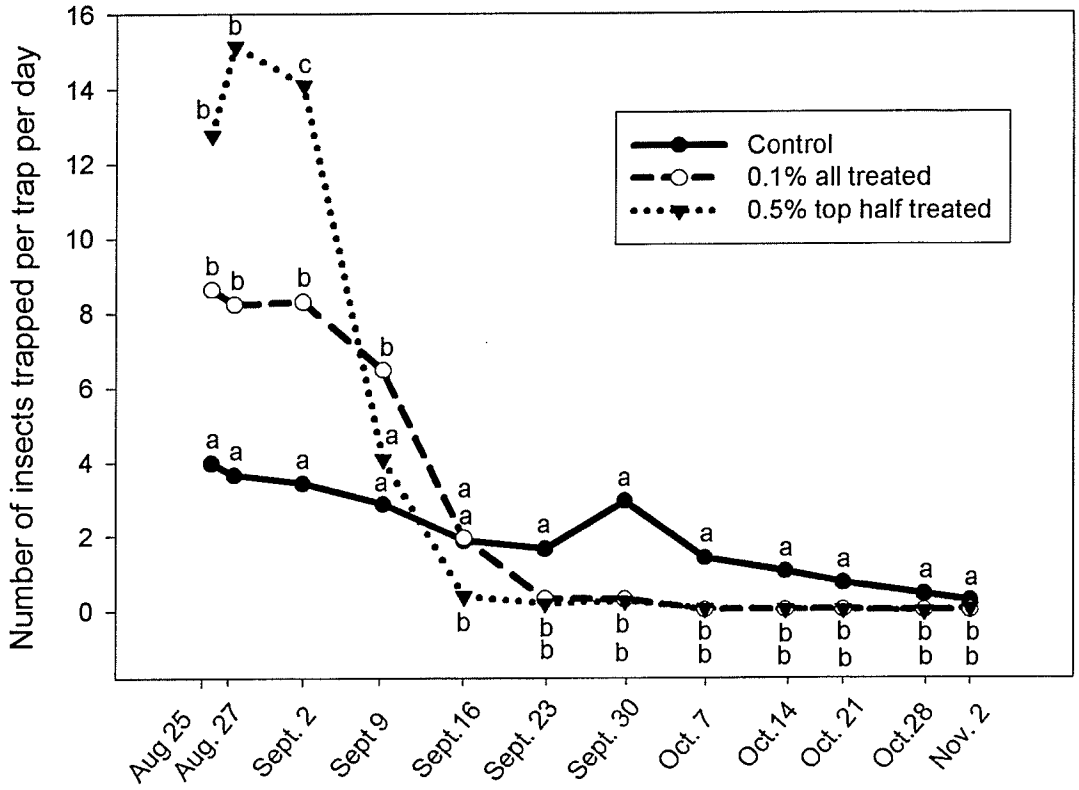

C
C. ferrugineus
Control
0.1% all treated
0.5% top half treated
Live emerged adults in barley (number per kg)
5
4
3
2
1
0
Sept. 9
Sept. 23
Oct. 7
Oct. 21

Figure 4.5. The number of *S. oryzae* adults caught in probe pitfall traps inserted in granaries untreated or treated with protein-rich pea flour at 0.1% with all the grain treated or 0.5% with the top half of the bulk treated on different sampling dates (Different letters vertically on the same day indicate significant differences ($P < 0.05$)).

Figure 4-5


Number of insects trapped per trap per day
16
14
12
10
8
6
4
2
0
Control
0.1% all treated
0.5% top half treated
Aug 25
Aug. 27
Sept. 2
Sept 9
Sept.16
Sept. 23
Sept. 30
Oct. 7
Oct.14
Oct. 21
Oct.28
Nov. 2

CHAPTER 5

Combination of Protein-Rich Pea Flour and Pea Extract with Insecticides and Enzyme Inhibitors for Control of Stored-Product Insects

Abstract

Protein-rich pea flour and its extract are toxic to stored-product insects. Protein-rich pea flour at 0.1% controlled stored-product insects in a granary trial. To reduce the concentration of protein-rich pea flour needed to control stored-product insects, natural products or currently used grain protectants, diatomaceous earth, neem, *Bacillus thuringiensis*, malathion and pyrethrum, were mixed with protein-rich pea flour in wheat. Mixtures were tested against the rice weevil, *Sitophilus oryzae* (L.), the red flour beetle, *Tribolium castaneum* (Herbst) and the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens). 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 with pyrethrum acted additively against *S. oryzae*. All other combinations acted antagonistically. Pea extract reduced the feeding of *S. oryzae*. Enzyme inhibitors, piperonyl butoxide, profenofos and diethyl maleate, at 3000 ppm, were mixed with pea extract in wheat flour to test for possible synergistic effects on the feeding deterrence and mortality of *S. oryzae*. The effects of piperonyl butoxide and pea extract were additive. Diethyl maleate did not change the effect of pea extract on feeding and mortality. Profenofos alone killed all insects in 3 days. The

reduction of feeding of *S. oryzae* had a positive correlation with LT_{50} in flour disks treated with pea extract and its combinations with enzyme inhibitors.

Keywords: pea protein; stored-product insects; antifeeding; combination; synergism; protectant; enzyme inhibitors

1. Introduction

Many plants or their extracts have been investigated for the control of stored-product insects (Jacobson, 1989a; Weaver and Subramanyam, 2000). Most effective plants are spices or medicinal plants (Golob et al., 1999). 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.) mixed with wheat cause a marked reduction in survival and reproduction of *Sitophilus oryzae* (L.) (Coombs et al., 1977; Holloway, 1986). Protein-rich pea flour causes adult mortality and reduces reproduction in several stored-product insect pests (Bodnaryk et al., 1997), and is repellant to many stored-product insects (Bodnaryk et al., 1997; Fields et al., 2001). Granary tests (Hou and Fields, 2003a, (Chapter 4)) show that 0.1% protein-rich pea flour effectively depresses insect populations, a concentration that may be too high for practical applications. An extract of protein-rich pea flour is more toxic than protein-rich pea flour itself (Bodnaryk et al., 1997). Delobel et al. (1998) isolated a polypeptide from peas that is toxic to stored-product insects.

Insects produce various detoxification enzymes that enable them to overcome the toxic effect 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). Mixed function oxidases, gluthion-s-transferases and hydrolases are involved in the detoxification of insecticidal compounds from peas in a resistant *S. oryzae* strain (Holloway and Mackness, 1988).

To investigate the possibility of reducing the amount of protein-rich pea flour needed to control stored-product insects, protein-rich pea flour was mixed with natural products or currently used grain protectants, including diatomaceous earth, neem, *Bacillus thuringiensis*, malathion and pyrethrum. An extract from protein-rich pea flour was isolated and combined 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 effect on the feeding and mortality of *S. oryzae*.

2. Materials and Methods

2.1. Insects

Three insect species, *S. oryzae*, *Cryptolestes ferrugineus* (Stephens), and *Tribolium castaneum* (Herbst) were tested. All insects had been cultured in the laboratory at 30°C, 70% RH for over five years. *Sitophilus oryzae* was reared on whole kernels of wheat. *Cryptolestes ferrugineus* was reared on wheat kernels, with 5% wheat germ and 5% brewer's yeast by weight. *Tribolium castaneum* was reared on wheat flour mixed with 5% brewer's yeast.

2.2. Preparation of mixtures of protein-rich pea flour with insecticides

Protein-rich pea flour (Progress Protein; 60% protein, 30% starch, and 7% moisture content, Parrheim Food, Saskatoon, SK) 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 applied with other toxins by different methods at the following ratios: Protect-It™ (diatomaceous earth, Hedley Technologies Inc. Mississauga, ON, Canada) at the ratio of 1:1 (w/w), Novodor (*Bacillus thuringiensis* subsp. *tenebrionis,* 3% EC, Abbott Laboratories, North Chicago, IL, USA) and Amazin™ (3% EC, neem, AMVAC Chemical Co., Los Angeles, CA, USA) at the ratio of 1:1(w/v, g:ml), Premium Pyrocide®175 (20% pyrethrins without piperonyl butoxide, McLaughlin Gormley King Co., Minneapolis, MN, USA) at the ratio of 40:1 (w/v, g : ml), and malathion (91% technical grade, Chipman Inc. Winnipeg, MB, Canada) at the ratio of 400:1 (w/v, g : ml). Diatomaceous earth and protein-rich pea flour are powders, and were mixed with a mixer (American Hospital Supply Corporation, Evanston, IL) for 1 min before treating wheat. For treatments with *B. thuringiensis* and neem, wheat was treated with *B. thuringiensis* or neem at various doses, which were diluted with deionized water and dried at room temperature for 24 h, and then treated with protein-rich pea flour at the same doses by weight. For making mixtures of protein-rich pea flour with pyrethrum or malathion, 250 µl pyrethrum or 25 µl malathion was dissolved in 15 ml acetone and then 10 g 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 control. These mixtures were thoroughly mixed and freeze dried for 24 h to remove the acetone. The dried powders were ground with a mortar and stored at –20°C.

2.3. Toxicity of mixtures with insecticides

Toxicity was evaluated on Canadian hard red spring wheat at 14% moisture content. Protein-rich pea flour, and its combinations with neem, diatomaceous earth, or *B.*

thuringiensis were evaluated against *S. oryzae, T. castaneum,* and *C. ferrugineus*. Protein-rich pea flour treated with acetone, mixture of Protein-rich pea flour with malathion or pyrethrum were tested against *S. oryzae*. Pre-tests were conducted to find a suitable range that caused from five to 95% mortality of the three insects species. Mixtures were tested at different doses (Table 5.1). Protein-rich pea flour at the doses of 0, 100, 200, 400, 600, 800 and 1000 ppm (w:w) was tested against *S. oryzae,* and 0, 100, 200, 400, 600, 800, 1600 and 3200 ppm was tested against *T. castaneum* and *C. ferrugineus.* Insecticide powders were weighted and added to 100 g wheat in a 500 ml jar. For liquid forms, 1 ml of solution at various doses of insecticides was pipetted into 100 g wheat in the jar. Jars were sealed after insecticides were added, and were shaken by hand for 2 min. Jars treated with liquid were opened to allow solvents to evaporate in a fume hood for 24 h. Treated grain was then divided into vials (29 mm diameter × 88 mm length) with 20 g each, five vials per dose. Twenty, one to two week-old adult insects of the three species were placed in each vial, and the vial was covered with a screened lid. The number of live and dead insects was counted after 2 wk at 30°C, 70% relative humidity (RH). Either acetone or water was tested as control when it was used as a solvent.

2.4. Isolation of pea extract

To obtain pea extract, the method described by Bodnoraky et al. (1997) was followed with the following modifications. One hundred and twenty g of protein-rich pea flour was defatted by stirring in one-liter chloroform for one h and filtered with a Büchner funnel with No. 1 Whatman filter paper at reduced pressure. Defatted protein-

rich pea flour was dried at room temperature. One hundred g of defatted dry protein-rich pea flour was mixed with 2 liter of 80% methanol and refluxed 5 minutes at boiling temperature (70°C) with stirring. The mixture was filtered while hot using a Büchner funnel with No. 1 Whatman filter paper. The filtrate was evaporated (Evaporator BÜCHI EL 131) at 37-40°C under reduced pressure until it contained less than 30% methanol. The evaporated residual solution was stirred with 10 g of DIAION HP 20 AG (Biotage, 250-600μm 300-600Å) beads for 24 h. The beads were removed and washed with 500 ml 30% methanol once, 200 ml 100% methanol twice, and 100 ml 100% methanol once. The filtrate of the last three washes was collected and evaporated to dryness under reduced pressure. The brown and waxy material was scraped with a spatula off the round-bottomed flask, and freeze dried for 24 h and weighed. There was no difference in the antifeedant activity and insect mortality in five batches of the dried pea extract. Therefore, all batches of pea extract were pooled for bioassay.

2.5. Combination of pea extract with enzyme inhibitors

Pea extract was dissolved in 70% ethanol. All-purpose wheat flour was mixed with the enzyme inhibitors profenofos, diethyl maleate or piperonyl butoxide at 3000 ppm, mixed with pea extract at the doses of 0, 500, 1000, 2000, 4000, 8000, and 16000 ppm, and also mixed with each inhibitor at 3000 ppm combined with pea extract at the above serial doses. The flour was made into disks and the feeding rate of *S. oryzae* on flour disks in Petri dishes was measured after three days at 30°C, 70% RH (Xie et al., 1996). Feeding rate was expressed as the percentage of the amount of flour consumed in the treated disks divided by the amount consumed in the untreated control, and listed with

standard error of the mean in the text. Insects were returned to the flour disks and the mortality was noted daily. There were five flour disks and 25 *S. oryzae* adults at one-two-week old per Petri dish, and three Petri dishes per treatment.

2.6. Data analysis

The co-toxicity coefficient of mixtures (Sun and Johnson, 1960) was used to evaluate synergistic, additive or antagonistic responses. A co-toxicity coefficient less than 80 is considered to indicate antagonism, between 80-120 is considered additive, and higher than 120 is evidence of synergism. If a mixture (M) is made of two components (A and B), and both components have known LD_{50} (dose lethal to 50% of the test population (Finney 1971)), then the following formulas would be used (using A as standard):

Toxicity index (TI) of A =100,

$$\text{Toxicity index (TI) of B} = \frac{LD_{50}\text{ of A}}{LD_{50}\text{ of B}} \times 100,$$

$$\text{Actual TI of M} = \frac{LD_{50}\text{ of A}}{LD_{50}\text{ of M}} \times 100,$$

Theoretical TI of M =TI of A × % of A in M + TI of B × % of B in M,

$$\text{Co - toxicity coefficient} = \frac{\text{actual TI of M}}{\text{theoretical TI of M}} \times 100.$$

If one component of the mixture alone (for example B) is not toxic, causing low mortality (< 20%) at all doses. No LD_{50} will be available. In this case, the co-toxicity

coefficient of the mixture was calculated by the formula:

$$\text{Co - toxicity coefficient} = \frac{LD_{50} \text{ of A alone}}{LD_{50} \text{ of A in the mixture}} \times 100$$

The LD_{50}s of protein-rich pea flour, pea extract and their mixtures were calculated with probit analysis by using POLO-PC (LeOra Software 1994). Regression lines of the mortality against doses were compared to determine the equality or parallelism of the regression lines by using likelihood ratio test ($\alpha = 0.05$). LT_{50}s (lethal time to 50% mortality) of pea extract were estimated with survival analysis (Robertson and Preisler 1992) by using SigmaStat (SPSS Inc. 2003). The relationship of LT_{50}s and feeding rates of *S. oryzae* was tested with SAS REG procedure (SAS Institute Inc., 2000).

3. Results

3.1. Mixtures with insecticides

Acetone did not affect the toxicity of protein-rich pea flour against *S. oryzae*. The LD_{50} and slope of protein-rich pea flour suspended in acetone (288 ppm, 4.79 ± 0.32) was the same as protein-rich pea flour without acetone (281 ppm, 3.97 ± 0.35) ($\chi^2 = 3.1145$, df = 2, $P = 0.211$). Protein-rich pea flour and neem at 1:1 acted synergistically against *T. castaneum,* and antagonistically against *S. oryzae* and *C. ferrugineus* (Table 5.2). Protein-rich pea flour and diatomaceous earth had additive effects against *S. oryzae,* and were antagonistic against *T. castaneum* and *C. ferrugineus* (Table 5.3). *Bacillus thuringiensis* did not increase toxicity of protein-rich pea flour in any of the three insect species (Table 5.4). The combination of protein-rich pea flour with malathion had a synergistic effect,

and the combination of protein-rich with pyrethrum was additive against *S. oryzae* (Table 5.5).

3.2. Combination with enzyme inhibitors

The cumulative mortality of *S. oryzae* fed on wheat flour disks without treatment was less than 4%. Piperonyl butoxide alone caused a cumulative mortality of *S. oryzae* of 17, 31, 56, 59 and 64% after 6, 8, 10, 12, and 14 days, respectively. Diethyl maleate alone did not cause any mortality within 14 days. The pea extract alone caused more than 50% mortality after 14 days at all doses. Therefore the data on 10 days were used for estimating the LD_{50}. The LD_{50} of the mixture of pea extract with piperonyl butoxide (957 ppm; 95% C.L., 574-1374) was significantly lower than those of pea extract alone (1728 ppm; 95% C.L., 1295-2214) ($\chi^2 = 31.0460$, df = 1, $P < 0.001$) and the mixture of pea extract with diethyl maleate (1700 ppm; 95% C.L., 1443-1996) ($\chi^2 = 1.1989$, df = 1, $P = 0.274$). No difference in LD_{50}s was detected between the pea extract alone and the mixture of pea extract with diethyl maleate. The regression lines of the pea extract alone, the mixture of pea extract with piperonyl butoxide and the mixture of pea extract with diethyl maleate were parallel ($\chi^2 = 2.1409$, df = 2, $P = 0.343$).

After three days, all *S. oryzae* on flour disks treated with piperonyl butoxide and diethyl maleate and their combination with pea extract were still alive. The feeding rate by insects on flour disks treated with profenofos or piperonyl butoxide alone was reduced to 20 ± 1% (mean ± SEM) and 80 ± 5% respectively. Diethyl maleate alone did not reduce feeding rate (107 ± 8%) of *S. oryzae.* The feeding rate of *S. oryzae* was reduced with increasing concentrations of pea extract (Fig. 5.1). The feeding rate of *S. oryzae* on

the combination of pea extract and piperonyl butoxide was constantly lower than with the pea extract alone at all concentrations. Diethyl maleate did not reduce the feeding rate in the mixture of pea extract and diethyl maleate compared with that in the pea extract alone. The feeding rate of *S. oryzae* on the combination of pea extract and profenofos was not related to the dose of pea extract (Fig. 5.1).

The feeding rate of *S. oryzae* was positively related with the LT_{50} of pea extract (Fig. 5.2). It was described by the formula: $LT_{50} = 3.6852 + 0.1377 \times$ (3-day feeding rate) ($r^2 = 0.9388$).

3. Discussion

Protein-rich pea flour shows good potential as a grain protectant (Bodnaryk et al., 1997; Delobel, 1998; Fields et al., 2001; Hou and Fields, 2003a, (Chapter 4)). This study was designed to find ways to enhance the effectiveness of protein-rich pea flour by mixing it with other natural products or currently used grain protectants. However, the effect of combinations 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 for control of *S. oryzae.* One advantage of mixing diatomaceous earth with protein-rich pea flour is that both doses can be reduced. Thus the undesirable effects of diatomaceous earth, such as reducing bulk density and grain rheological properties (Korunic et al., 1998), can be partially mitigated. I speculate that this additive effect is because *S. oryzae* picked up more diatomaceous earth because protein-rich pea flour increased the movement of *S. oryzae* (Hou and Fields, 2003a, (Chapter 4)). In addition,

food is an important water source for *S. oryzae* (Arlian, 1979). Protein-rich pea flour would reduce the intake of water by reducing the feeding; Diatomaceous earth damages the wax layer of cuticle and kills *S. oryzae* through desiccation (Carlson and Ball, 1962); and protein-rich pea flour is more toxic under dry conditions (Hou and Fields, 2003b, (Chapter 3)).

As protein-rich pea flour is a powder, but neem and *B. thuringiensis* are in liquid form, the different formulations may have interacted and affected the combined toxicity of the mixtures. Treating neem or *B. thuringiensis* before treating grain with protein-rich pea flour may have affected the distribution of pea protein on the kernels and reduced its efficacy. The pea protein may have covered the neem and *B. thuringiensis*, and made them inaccessible to insects. In the mixture of protein-rich pea flour with pyrethrum or malathion, pyrethrum and malathion had been adsorbed to the protein-rich pea flour, which coated the insects, and may have increased the exposure of *S. oryzae* to pyrethrum and malathion, thus resulting in higher mortalities.

Insects produce various enzymes, such as mixed function oxidases, gluthione-S-transferase and hydrolases, to protect themselves from toxic compounds in plants (Krieger et al., 1971; Ahmad 1982; Dowd et al., 1983). Inhibition of detoxifying enzymes weakens the insect defense system, thereby increasing the toxicity of the target insecticide (Ishaaya, 1993). The mixed function oxidase inhibitor, piperonyl butoxide and the esterase inhibitor tributylphosphorotrithioate, when mixed with yellow split peas, increase the mortality of a *S. oryzae* strain that is normally able to reproduce on peas (Holloway and Mackness 1988). The glutathione-S-transferase specific 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 the 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 piperonyl butoxide was toxic and acted as an antifeedant against *S. oryzae*, and had an additive effect on both the antifeedant activity and toxicity of pea extract. It could be used as an additive to increase the effectiveness of pea extract. However, it is not known if mixed function oxidases are involved in the detoxification of pea extract. I cannot comment on profenofos, since it was very toxic, and it killed all insects in three days.

Protein-rich pea flour and pea extract may cause *S. oryzae* to die from starvation. The greater the dose of pea extract, the less *S. oryzae* ate, and the faster they died. One mechanism that regulates food intake is mechanoreceptors located in the gut wall. When the gut wall is distended, feeding is inhibitated via the central nervous system (Bernays and Simpson, 1982). *Sitophilus oryzae* fed on flour disks treated with pea extract produced large bubbles in their midgut (Chapter 8), which may fire signals to the brain that cause cessation of feeding. The relationship of feeding rate and time to death of insects treated with pea extract could be used as a criterion of efficacy or to predict the toxicity of pea extract from the three-day feeding rate.

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Table 5.1. The concentrations (ppm) of various insecticides used in combination with protein-rich pea flour tested except for the untreated control. Each range contained six to eight doses for each test.

Insects	Minimum and maximum dose range (ppm)									
	Diatomaceous Earth	Protein-rich pea flour and diatomaceous earth (1:1)	*B. thuringiensis*	Protein-rich pea flour and *B. thuringiensis* (1:1)	Neem	Protein-rich pea flour and neem (1:1)	Malathion	Protein-rich pea flour and malathion (400:1)	Pyrethrum	Protein-rich pea flour and pyrethrum (40:1)
S. oryzae	100 – 2000	50 - 2000	250 - 16000	100 - 64000	100 - 20000	1000 - 2000	0.1 - 2	25 - 800	0.5 - 64	25 - 800
T. castaneum	50 – 1000	50 - 1000	300 - 16000	400 - 64000	100 - 10000	100 - 10000				
C. ferrugineus	10 – 600	0 - 2000	300 - 16000	300 - 64000	50 - 10000	50 - 10000				

Table 5.2. LD_{50} (lethal dose to kill 50% of the population) with 95% C.L.(ppm) and slope (mean ± SEM) of protein-rich pea flour and its mixtures with neem at a ratio of 1: 1 against three insect species at 30°C, 70% RH after two wk.

Insect	Statistical index	Protein-rich pea flour	Neem	Protein-rich pea flour with neem	Co-toxicity coefficient
S. oryzae	LD_{50} (ppm)	307 (190–430)	6752 (6047–7397)	2691 (2123-3772)	22
	slope	2.15 ± 0.15	6.59 ± 0.55	1.89 ± 0.20	
	g^2	0.146	0.071	0.081	
T. castaneum	LD_{50} (ppm)	na[1]	1024 (484–1710)	601 (92-1589)	170
	slope	na	1.11 ± 0.12	0.59 ± 0.10	
	g	na	0.102	0.251	
C. ferrugineus	LD_{50} (ppm)	3368 (2327–4587)	5087 (3260–6418)	5590 (3482–7328)	73
	slope	1.75 ± 0.16	5.27 ± 0.58	2.57 ± 0.43	
	g	0.5	0.182	0.381	

[1] na means not available: LD_{50} or slope was not calculated because the mortality was less than 20%.
[2] Index of heterogeneity.

Table 5.3. LD_{50} (lethal dose to kill 50% of the population) with 95% C.L.(ppm) and slope (mean ± SEM) of protein-rich pea flour and its mixtures with diatomaceous earth at a ratio of 1: 1 against three insect species at 30°C, 70% RH after two wk.

Insects	Statistical index	Protein-rich pea flour	Diatomaceous earth	Protein-rich pea flour with diatomaceous earth	Co-toxicity coefficient
S. oryzae	LD_{50} (ppm)	307 (190 – 430)	145 (122 – 166)	205 (182 – 226)	96
	slope	2.15 ± 0.15	3.97 ± 0.40	4.60 ± 0.43	
	g^2	0.146	0.058	0.034	
T. castaneum	LD_{50} (ppm)	na[1]	280 (226 – 325)	497 (438 – 543)	56
	slope	na	7.04 ± 1.07	7.13 ± 0.81	
	g	na	0.138	0.083	
C. ferrugineus	LD_{50} (ppm)	3368 (2327 –4587)	18 (16 – 19)	85 (72 – 96)	42
	slope	1.75 ± 0.16	5.81 ± 0.60	5.60 ± 0.84	
	g	0.5	0.041	0.179	

[1] na means not available: LD_{50} or slope was not calculated because the mortality was less than 20%.
[2] Index of heterogeneity.

Table 5.4. LD_{50} (lethal dose to kill 50% of the population) with 95% C.L.(ppm) and slope (mean ± SEM) of protein-rich pea flour and its mixtures with *B. thuringiensis* at a ratio of 1: 1 against three insect species at 30°C, 70% RH after two wk.

Insect	Statistical index	Protein-rich pea flour	*B. thuringiensis*	Protein-rich pea flour with *B. thuringiensis*	Co-toxicity coefficient
S. oryzae	LD_{50} (ppm)	307 (190–430)	na[1]	817 (723–920)	37
	slope	2.15 ± 0.15	0.12 ± 0.23	3.01 ± 0.23	
	g[2]	0.146	13.814	0.037	
T. castaneum	LD_{50} (ppm)	na	na	na	na
	slope	na	na	na	
	g	na	na	na	
C. ferrugineus	LD_{50} (ppm)	3368 (2327–4587)	na	na	na
	slope	1.75 ± 0.16	na	0.16 ± 0.09	
	g	0.5	382.6	3.003	

[1] na means not available: LD_{50}, slope or co-toxicity coefficient was not calculated because the mortality was less than 20%.

[2] Index of heterogeneity.

Table 5.5. LD_{50} (lethal dose to kill 50% of the population) with 95% C.L.(ppm) and slope (mean ± SEM) of protein-rich pea flour, pyrethrum, malathion and its mixtures with pyrethrum (40:1) or with malathion (400:1) against *S. oryzae* at 30°C, 70% RH after two wk.

Statistical index	Protein-rich pea flour	Pyrethrum	Protein-rich pea flour with pyrethrum (40:1)	Malathion	Protein-rich pea flour + malathion (400:1)
LD_{50} (ppm)	289 (256 – 321)	37 (34 – 40)	221 (202 – 239)	1.5 (1.4 – 1.6)	136 (126 – 147)
slope	4.79 ± 0.32	7.69 ± 0.912	6.29 ± 0.64	8.04 ± 0.78	7.24 ± 0.78
g^2	0.034	0.067	0.04	0.036	0.044
Co-toxicity coefficient			111		143

[2] Index of heterogeneity.

Figure 5.1. Feeding rate (percentage of untreated) by 25 adult *S. oryzae* on wheat flour disks treated with pea extract at various doses, or combined with piperonyl butoxide (PBO), diethyl maleate (DEM), or profenofos (PFF) at 3000 ppm after three days. The feeding rate (% of untreated) with PBO alone was 80%, DEM alone was 107% and PFF alone was 20%, all at 3000 ppm.

Figure 5.1

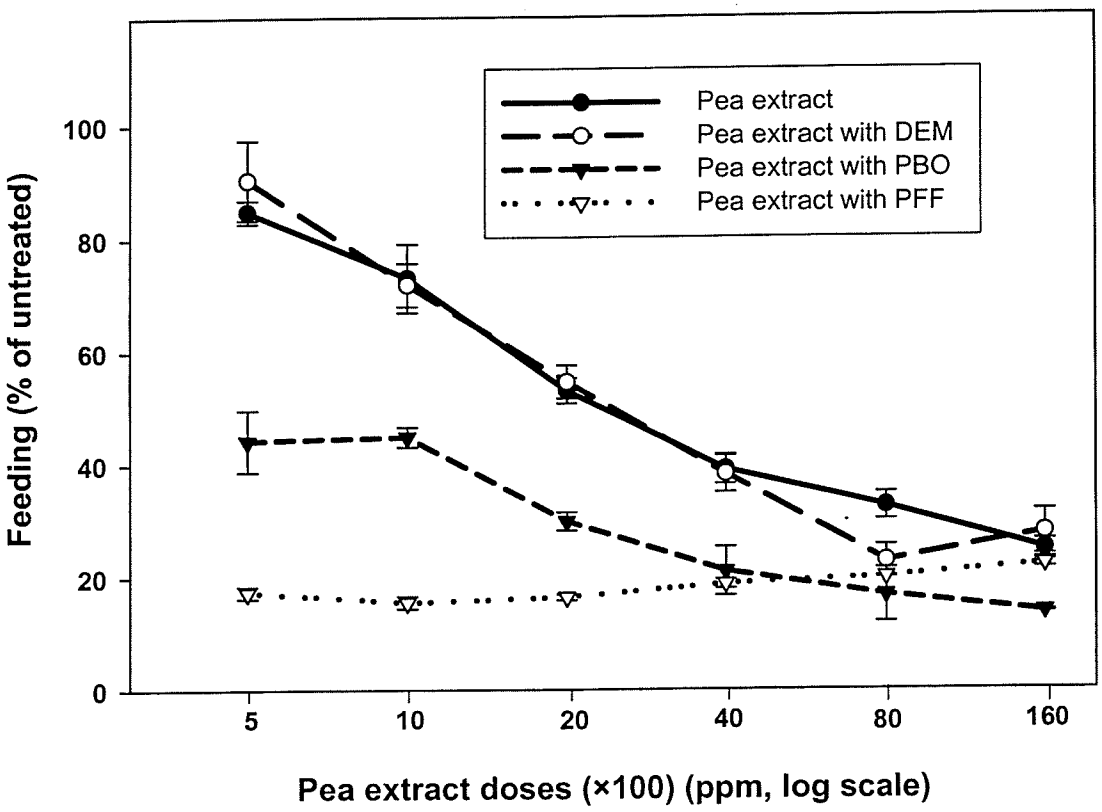
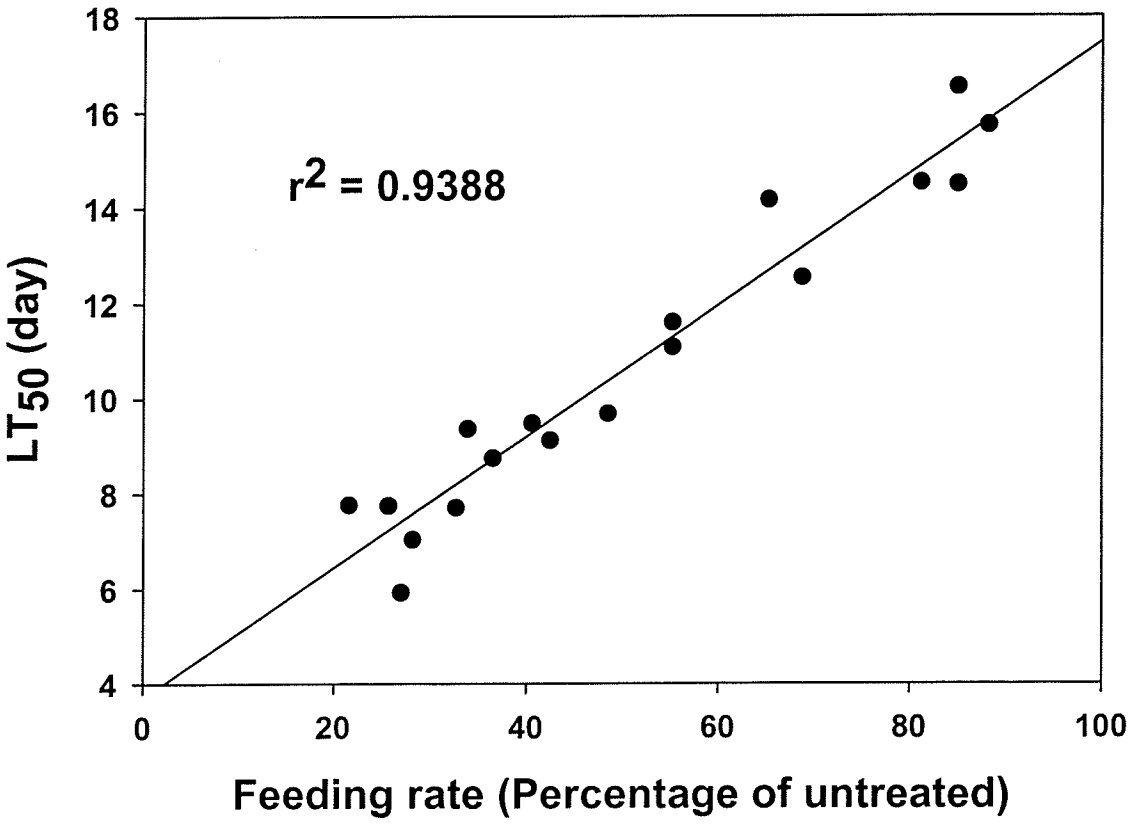

Pea extract
Pea extract with DEM
Pea extract with PBO
Pea extract with PFF
Feeding (% of untreated)
100
80
60
40
20
0
5
10
20
40
80
160
Pea extract doses (×100) (ppm, log scale)

Figure 5.2. Relationship of LT_{50} (d) and feeding rate of *S. oryzae* over three d in wheat flour disks treated with pea extract at various concentrations.

Figure 5.2


18
16
14
12
10
8
6
4
0
20
40
60
80
100
r^2 = 0.9388
LT_{50} (day)
Feeding rate (Percentage of untreated)

CHAPTER 6

Control of Stored-Product Insects with Combinations of Protein-Rich Pea Flour and Parasitoids

ABSTRACT

Protein-rich pea flour is toxic and repellent to three major stored-grain pests, the rice weevil, *Sitophilus oryzae* (L.) red flour beetle, *Tribolium castaneum* (Herbst), and rusty grain beetle, *Cryptolestes ferrugineus* (Stephens). This study found that protein-rich pea flour was not toxic to, and did not reduce the offspring production of *Anisopteromalus calandrae* (Howard), a parasitoid of *S. oryzae*, nor did it reduce offspring production of *Cephalonomia waterstoni* (Gahan), a parasitoid of *C. ferrugineus*. Protein-rich pea flour was also not repellent to *A. calandrae.*

Small-scale and large-scale tests of a combination of protein-rich pea flour and parasitoids were conducted in 2-liter jars and in barrels containing 330 kg wheat. In small-scale test, a larger population of *A. calandrae* was found at a high host infestation rate (24 *S. oryzae* adults/kg for 25 d), but the parasitoid did not become established at middle and low host infestation rates (2.4, or 0.24 *S. oryzae* adults/kg for 25 d). The combinations of protein-rich pea flour and parasitoids reduced populations of *S. oryzae* in both small-scale and large-scale tests. Additional effects of protein-rich pea flour and parasitoids were found in the large-scale test. Releasing parasitoids alone reduced the

populations of *S. oryzae* by 46% and *C. ferrugineus* by 49%. Treating wheat at 0.04% or 0.1% protein-rich pea flour reduced the population of *S. oryzae* by 26 and 79%, and *C. ferrugineus* by 27 and 43%, respectively. Combining parasitoids with 0.04% or 0.1% protein-rich pea flour reduced *S. oryzae* populations by 76 and 98%, and *C. ferrugineus* populations by 42 and 75%, respectively. Pest insect infestation increased dockage and reduced bulk density of wheat in untreated and all single treatments of parasitoids or protein-rich pea flour. However, combinations of parasitoids with 0.1% protein-rich pea flour maintained the grain quality with no change in grain bulk density.

KEY WORDS: Pea protein, biological control, stored wheat, *Anisopteromalus calandrae, Cephalonomia waterstoni, Sitophilus, Cryptolestes*

Introduction

Stored-grain insect pests cause damage to grain by reducing its dry weight, nutritional value and seed viability (Semple et al. 1992). As a result, protecting grain from insect attack during the storage period is of prime importance to the food industry. Plants are a rich and promising source of new and alternative chemicals that may help replace synthetic insecticides for grain protection (Prakash and Rao 1997). Many plant-derived chemicals are insecticidal to stored-product pests (Jacobson 1989a, Golob et al. 1999, Weaver and Subramanyam 2000). Azadirachtin from the Indian neem tree (*Azadirachta indica* A. Juss., Meliaceae) (Saxena et al. 1988, Jilani and Saxena 1990), and pyrethrum from chrysanthemums (Prakash and Rao 1997) have received the most attention. However, because of the structural complexity of azadirachtin and the instability of pyrethrum, combined with the restricted availability and the high cost of both, the search for other natural insecticides is on-going.

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.) mixed with wheat reduce the survival and reproduction of *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) (Coombs et al. 1977, Holloway 1986). Recently, Delobel et al. (1998) isolated a polypeptide from yellow split pea that is toxic to stored-product insects. Additional studies have demonstrated that protein-rich pea flour obtained from these peas is repellant (Fields et al. 2001), as well as toxic, to many stored-grain insects (Bodnaryk et al. 1997, Hou and Fields 2003a, 2003b (Chapter 3, 4)). However,

nothing is known of the effects of this allelochemical on the interaction of parasitoids with their insect hosts.

Sitophilus oryzae, Cryptolestes ferrugineus (Stephens) and *Tribolium castaneum* (Herbst) are cosmopolitan stored-product insect pests. Protein-rich pea flour at 0.1% reduces populations of these insects in farm granaries (Hou and Fields 2003a, Chapter 4). *Anisopteromalus calandrae* (Howard) (Hymenoptera: Pteromalidae) is an ectoparasitoid of the immature stages of *S. oryzae*. *Cephalonomia waterstoni* (Gahan) (Hymenoptera: Bethylidae) is an ectoparasitoid of late instar larvae of *C. ferrugineus* (Flinn and Hagstrum 1995). Both parasitoids can suppress the populations of *S. oryzae* and *C. ferrugineus,* respectively (Press et al. 1983, Cline et al. 1985, Flinn et al. 1996). I hypothesized that these parasitoids could be used in combination with protein-rich pea flour, if there were no negative effects on the parasitoids, because the parasitoids attack the immature stages and the protein-rich pea flour is toxic to the adults. This hypothesis was tested by exposing these two parasitoids to protein-rich pea flour and evaluating the efficacy of the combination on the reduction of insect populations in a small-scale and a large-scale experiment.

Materials and Methods

Insects

Sitophilus oryzae was reared on whole kernels of wheat, *T. castaneum* on wheat flour mixed with brewer's yeast (5% by weight), and *C. ferrugineus* on wheat kernels with wheat germ (5% by weight) and brewer's yeast (5% by weight).

Anisopteromalus calandrae and *C. waterstoni* were obtained from the Grain Marketing Production and Research Center, USDA-ARS, Manhattan, KS. For culturing *A. calandrae,* 2-3 wk old *S. oryzae* larvae were obtained by allowing approximately 1000 2-wk old *S. oryzae* adults to oviposit in 2500 g of 14% moisture content wheat for 7 d. Approximately 500 g of infested wheat that contained 2-3 wk-old larvae was placed in a 1 liter jar with 100 newly emerged *A. calandrae* adults, moisturized raisins and filter paper soaked with honey solution. *Anisopteromalus calandrae* adults were removed after 7 d. Adult *A. calandrae* emerged after two wk. For culturing *C. waterstoni,* 3-4 wk old *C. ferrugineus* larvae were prepared by placing approximately 2000 *C. ferrugineus* adults on 2 kg of a 2 cm thick mixture of all-purpose wheat flour, ground wheat germ (5% by weight) and ground brewer's yeast (5 % by weight) and removing them after 7 d. Approximately 500 larvae were placed in a half-liter jar containing 16% moisture content wheat, wheat germ, yeast, moisturized raisins and filter paper soaked with honey along with 100 newly emerged *C. waterstoni* adults. *Cephalonomia waterstoni* adults were removed after 1 wk. All rearing was carried out at 30°C and 70% relative humidity. The parasitoids were reared with a 12 h photophase and 12 h scotophase. The beetles were reared in the dark.

Effect of protein-rich pea flour on parasitoids

Protein-rich pea flour (Progress Protein; 60% protein, 30% starch, and 7% moisture content, Parrheim Food, Saskatoon, SK) was used in this study. It is produced commercially by grinding peas and isolating a protein-rich fraction by air classification.

Studies were conducted at 30 ± 1°C, 70 ± 5% relative humidity, unless specified otherwise. There were five replicates in each treatment.

Direct contact toxicity. Without provision of honey, approximately 25 1-d-old *A. calandrae* adults were placed in 15 × 100 mm plastic Petri dishes containing 30 mg protein-rich pea flour, 30 mg wheat flour, or 5 g wheat containing 2-3 wk-old *S. oryzae* larvae. Dishes without wheat or flour served as controls. The number of dead *A. calandrae* was recorded each day until all adult *A. calandrae* died. In another similar set of experiments, which were conducted at the same time, honey was provided as food for *A. calandrae* with a filter paper soaked with honey. The paper was taped underneath the cover of each Petri dish. The mortality of *C. waterstoni* with protein-rich pea flour or wheat flour was observed using the same methods as for *A. calandrae.*

Choice test. A Petri dish was divided into two equal sections. One section was filled with 10 g of untreated wheat, and the other was filled with 10 g of wheat treated with 0.1% protein-rich pea flour, which had been shaken by hand in a jar with the protein-rich pea flour for 2 min. The wheat in both sections contained 2-3 wk-old *S. oryzae* larvae. A single 1-d-old *A. calandrae* female was placed in the middle of each Petri dish, so that the parasitoid could choose to parasitize larvae in either treated or untreated wheat. *Anisopteromalus calandrae* was removed after 0, 24, 48, 72, or 96 h. Untreated and treated wheat were collected separately, placed in vials (29 mm diameter, 80 mm high) with screened tops and the number of emerged *A. calandrae* adults was recorded after four wk.

No choice test. Twenty 1-d-old *A. calandrae* females were placed in each vial that contained 20 g of wheat treated at 0, 0.04, or 0.1% protein-rich pea flour. Wheat contained 2 to 3 wk-old *S. oryzae* larvae. The *A. calandrae* were removed after 0, 1, 3, 5 or 7 d. The numbers of emerged *A. calandrae* and *S. oryzae* were counted after 4 wk. For testing *C. waterstoni*, 100 3 to 4 wk-old *C. ferrugineus* larvae were placed in 20 g 16% moisture content wheat that had been either untreated or treated with 0.1% protein-rich pea flour and covered with a screened lids and a piece of filter paper. Five *C. waterstoni* females per vial were left in the vials for 24, 48, 72, or 96 h. The numbers of emerged *C. waterstoni* and *C. ferrugineus* were counted after four wk.

Combination of protein-rich pea flour and parasitoids

Small-scale test. The small-scale test was conducted at 25 ± 1°C, and 60 ± 5% relative humidity. Wheat was disinfested by placing it in a room at –15°C for more than three wk. The wheat was then moisturized in open bags at 25 ± 1°C, and 60 ± 5% relative humidity for three wk. Wheat (2.5 kg) was placed in each of 22 4-liter jars, and infested with 60 unsexed 1-2 wk-old *S. oryzae* adults for 25 d. Infested wheat from different jars was thoroughly mixed with a modified cement mixer for 30 min at 20 rotations per min. To obtain different densities of *S. oryzae,* infested wheat was diluted with uninfested wheat in the mixer at the following ratios (infested wheat : uninfested wheat): 1 : 0 (high infestation rate), 1: 9 (moderate infestation rate), and 1: 99 (low infestation rate). Three 500 g samples were taken from each infestation level as initial untreated controls before treatment with protein-rich pea flour with the mixer to estimate the effect of mixing. Wheat of each infestation level was divided into two equal samples using a sample

divider (Humboldt MFG, IL, USA). One half of the wheat was combined with protein-rich pea flour at 0.04% and mixed in the mixer for 30 min. Another half was mixed with nothing in the mixer for 30 min. The wheat was then divided into 2-liter jars with 500 g of wheat each. There were total of 279 jars: nine jars as initial untreated control, 45 jars with wheat treated with protein-rich pea flour and 45 jars with no protein-rich pea flour in each of the infestation levels.

Twenty-nine days after the initial infestation, 12 newly emerged female *A. calandrae* were added to the jars once (single release), or 4 parasitoids were added in each of three consecutive wk for a total of 12 parasitoids (multiple release). Controls contained no parasitoids and no protein-rich pea flour. Jars were sealed with a metal screen and filter paper to prevent cross contamination. There were six treatments for each infestation rate: no parasitoids and no pea flour as untreated controls, 0.04% protein-rich pea flour with no parasitoids, parasitoids single release and no pea flour, parasitoids multiple release with no pea flour, parasitoids single release with 0.04% pea flour, and parasitoids multiple release with 0.04% pea flour. Three jars were randomly taken from each treatment every three wk for five times until the end of the experiment at 15 wk. The grain was sifted and the number of live and dead adults of *A. calandrae* and *S. oryzae* was counted. Numbers of insects in the initial untreated controls were checked at the same time, and all insects were placed back to the jars.

Large-scale test. Twenty-one steel barrels (168 cm high, 58 cm diameter) (White and Jayas 1991) were used to evaluate the combination of parasitoids and protein-rich pea flour. Because parasitoids are very active and can easily move between treatments

(Flinn et al. 1996), different locations for the separate treatments were used. Nine barrels were set up at the Cereal Research Centre with no parasitoids. Twelve barrels were set up nearby at the Department of Biosystem Engineering at the University of Manitoba. The rooms were heated, and the temperature and relative humidity were monitored with HOBO® data loggers (Onset computer Corporation, Bourne, MA). Each barrel was filled with 330 kg of hard red spring wheat with 13.5% moisture content. Wheat was treated with protein-rich pea flour at 0, 0.04% or 0.1% by dusting on the grain, and augured into each barrel on 22-24 May 2001. On 18 June 2001, 660 of each of *S. oryzae, C. ferrugineus* and *T. castaneum* were released together on the top surface of the wheat in all barrels. After 29 d, on 17 July, a single release of 660 *C. waterstoni* and 660 *A. calandrae* (1-7 d old) was conducted at the University of Manitoba site. Fluoropolymer resin (TeFlon®, Du Pont) was brushed on the top inside of the metal wall to prevent insects from climbing, and the barrels were sealed with fine cloth to prevent insects from escaping by flying, and to protect the wheat from external contamination. Three grain samples (approximate 1 kg each) were gently vacuumed every three wk (7 and 28 August, 18 September, 9 and 29 October) through small holes on the side of the barrel located at the top layer (20 cm from the grain surface), the middle layer (68 cm from the top), and the bottom layer (24 cm from the bottom) of the barrel. The samples were weighed, the grain was sifted, and the number of live and dead adult insects of each species was counted.

A set of samples was taken on 11 July, after release of pest insects but before release of parasitoids, for measuring the efficacy of protein-rich pea flour before

parasitoids were released. These samples were cultured separately at 30°C, and 70% relative humidity for four wk, and the number of emerged *S. oryzae* adults was counted to estimate the number of immature insects within the wheat available for the parasitoids and their distributions at the time of sampling. The change in dockage, moisture and bulk density of wheat was measured with samples taken on 11 July before the release of insects and on 29 October at the end of the experiment. There were four treatments at the University of Manitoba site: untreated controls, parasitoids alone, parasitoids with 0.04% protein-rich pea flour and parasitoids with 0.1 % protein-rich pea flour. There were three treatments at the Cereal Research Centre site: untreated controls, 0.04% protein-rich pea flour and 0.1 % protein-rich pea flour. There were three barrels per treatment.

Data analysis

The numbers of parasitoids and host insects in the small-scale tests were transformed with log (X + 1). The numbers of insects in large-scale combination tests were calculated as insects per kg wheat before using the same transformation. The percentage mortality of insects and dockage and moisture content of wheat were transformed with arcsine square root. The bulk density of wheat was transformed with square root. In the small-scale test, treatments without parasitoids were excluded from the analyses to compare the number of parasitoids reproduced among treatments that parasitoids had been released. Effects of treatments on the number of insects, mortality and the physical characteristics of wheat were compared by analysis of variance with SAS PROC GLM (General Linear Models, SAS Institute Inc. 2000) followed by the pair-wise CONTAST test ($\alpha = 0.05$) to discriminate means for the small-scale test. SAS

PROC MIXED split-plot variance analysis was used for the large-scale test. Sampling time, layers within the barrels and treatments were factors in the model. Means were followed by standard error of the means.

Results

Effect of protein-rich pea flour on parasitoids

Direct contact. The daily mortality of *A. calandrae* between 24 h and 168 h, when all parasitoids had died, was used for the analysis. There were no differences in the mortality of adult *A. calandrae* in protein-rich pea flour (35 ± 6%), in wheat flour (34 ± 6%) or in the control (37 ± 6%) in the presence of honey ($F = 1.03$; df = 3, 16; $P = 0.4070$). Similar results were seen in the experiment run without honey with no differences in the mortality in protein-rich pea flour (38 ± 6%), in wheat flour (40 ± 6%) or in the control (40 ± 6%) ($F = 1.45$; df = 3, 16; $P = 0.2668$) (Fig. 6.1A). Honey significantly reduced mortality of *A. calandrae* in all treatments (36 ± 3%) when compared with wasps that received no honey (41 ± 3%) ($F = 11.17$; df =1, 32; $P = 0.0021$). The daily mortality of *C. waterstoni* between 24 h and 120 h was used for the analysis, because all parasitoids died after 120 h. The mortality of *C. waterstoni* without access to honey was the same in protein-rich pea flour (55 ± 8%) as it was in wheat flour (55 ± 7%) ($F = 0.04$; df =1, 8; $P = 0.8481$) (Fig. 6.1B).

Choice test. Parasitism by *A. calandrae* was not affected by protein-rich pea flour. The longer *A. calandrae* had access to wheat infested with *S. oryzae* larvae, the more *A. calandrae* progeny emerged from the wheat (Fig. 6.2). Protein-rich pea flour did

not reduce the number of *A. calandrae* emerging from the wheat (2.8 ± 0.8 insects per section) compared with the number emerging from untreated wheat (3.6 ± 0.8 insects per section) ($F = 1.19$; df =1, 32; $P = 0.2842$).

No choice test. No difference in the number of emerged *C. waterstoni* was detected between untreated wheat (6.4 ± 1.5 insects per vial) and wheat treated with 0.1% protein-rich pea flour (5.4 ± 1.1 insects per vial) ($F = 0.04$; df =1, 35; $P = 0.8366$). There was no difference in the number of emerged *C. ferrugineus* adults between untreated wheat (5.2 ± 0.7 insects per vial) and wheat treated with 0.1% protein-rich pea flour (4.3 ± 0.6 insects per vial) ($F = 0.40$; df =1, 35; $P = 0.5296$). There were no differences in the total number of emerged *A. calandrae* (71 ± 3, 73 ± 4 and 76 ±3 insects per vial, respectively) ($F = 0.66$; df =1, 48; $P = 0.5197$), and in the total number of emerged *S. oryzae* (21 ± 1, 22 ± 2, and 21 ± 2 insects per vial, respectively) ($F = 0.91$; df =1, 48; $P = 0.4087$) among wheat treated at 0, 0.04, or 0.1% of protein-rich pea flour. However, the mortality of emerged *S. oryzae* adults in the 0.1% treatment (20 ± 2%) was higher than that in the 0.04% treatment (6 ± 1%) ($F = 5.80$, df = 1,48; $P = 0.0199$) or in the 0% treatment (3 ± 1%) ($F = 8.12$, df = 1,48; $P = 0.064$). This mortality resulted in fewer live *S. oryzae* adults in the 0.1% treatment (16 ± 1 insects per vial) than in the 0.04% treatment (21 ± 1 insects per vial) ($F = 15.19$; df = 1, 48; $P = 0.0003$) or in the 0% treatment (20 ± 1 insects per vial) ($F = 14.62$; df = 1, 48; $P = 0.0004$).

Combination of protein-rich pea flour and parasitoids

Small-scale test. The three infestation rates of *S. oryzae* were successfully established. In the first sample, the total number of emerged *S. oryzae* adults jar at the

high, moderate and low infestation rates, was 82 ± 11, 7 ± 1, and 1 ± 0.6 insects per jar respectively in the initial untreated controls, 95 ± 9, 7 ± 0.6 and 1 ± 0.6 insects per jar respectively in the untreated control which had been mixed in the mixer for 30 min, and 81 ± 10, 7 ± 1, 1 ± 1 respectively in the treatment of 0.4% protein-rich pea flour. The mixing did not affect the total number of emerged *S. oryzae* ($F = 0.06$; df = 1, 42, $P = 0.8080$), nor did the treatment with 0.4% protein-rich pea flour ($F = 0.14$; df = 1, 42, $P = 0.7133$).

Anisopteromalus calandrae did not establish a population (12 ± 0.1 insects per jar) at the low *S. oryzae* infestation rate. At the moderate infestation rate, the total numbers of live and dead *A. calandrae* (14 ± 0.4 insects per jar) increased by approximately two parasitoids in the first three wk. However, no live *A. calandrae* were found in later samples. A large population of *A. calandrae* became established in all treatments with high infestation rates of *S. oryzae* (Table 6.1). Compared to the high number of *S. oryzae* (Table 6.2), the ratios of *A. calandrae* to *S. oryzae* at the end of the experiment were 0.38 in single release, 0.58 in multiple releases, 0.43 in single release with pea flour, and 0.15 in multiple releases with pea flour. There were no differences in the overall means of the number of *A. calandrae* among treatments with a single release multiple release, protein-rich pea flour with single release, or protein-rich pea flour with multiple release ($F = 0.56$; df = 3, 40; $P = 0.6468$). The population of *S. oryzae* was reduced after six wk in all treatments compared to the control (Table 2). Compared to the untreated control, both the single release of parasitoids (2563 ± 912 insects per jar) ($F = 17.52$; df = 1, 70, $P < 0.0001$), and the multiple releases (1451 ± 404 insects per jar) ($F =$

19.98; df = 1, 70, $P < 0.0001$) reduced the number of live *S. oryzae*. The combinations of protein-rich pea flour with the single release (1655 ± 481) or multiple releases (1775 ± 597) reduced more live *S. oryzae* than 0.4% protein-rich pea flour treatment alone (3264 ± 1110) ($F = 17.02$; df = 1, 70, $P = 0.0001$ and $F = 10.15$; df = 1, 70, $P = 0.0023$, respectively). The combinations of protein-rich pea flour with the single release also reduced more live *S. oryzae* than single release alone ($F = 4.70$; df = 1, 70, $P = 0.0342$).

Large-scale test. Although there was no significant difference in the ambient mean daily temperature between the Cereal Research Centre and University of Manitoba sites ($F = 2.18$, df = 1, 166, $P = 0.1413$), the relative humidity was significantly different between the two locations ($F = 39.51$, df = 1, 166, $P < 0.0001$). At the Cereal Research Centre, the mean temperature was 24.03 ± 0.04°C with a range of 12.9-30.7°C, and the mean relative humidity was 50 ± 0.3% with a range of 24-79%. At the University of Manitoba, the mean temperature was 23.7 ± 0.03°C with a range of 19.0-27.9°C, and the relative humidity was 37 ± 0.3% with a range of 23-84%. In addition, the numbers of total emerged *S. oryzae* (86 ± 31 insects per kg, Cereal Research Centre; 37 ± 13 insects per kg, University of Manitoba) ($F = 5.73$; df = 1, 12, $P = 0.0339$) and *T. castaneum* (15 ± 3 insects per kg, Cereal Research Centre; 6 ± 1 insects per kg, University of Manitoba) ($F = 47.80$; df = 1, 12, $P < 0.0001$) in the untreated barrels before the release of parasitoids (11 July) were different between the Cereal Research Centre and the University of Manitoba except for the number of emerged *C. ferrugineus* (12 ± 2 insects

per kg, Cereal Research Centre; 17 ± 2 insects per kg, University of Manitoba) ($F = 0.43$; df = 1,12, $P = 0.5222$). Therefore, data from the two locations were analyzed separately.

The number of adults emerging from grain after four wk at 30°C gives an estimate of the immature stages present at sampling. Before parasitoid release, treatments with 0.1% protein-rich pea flour alone reduced the number of emerged *S. oryzae* at both CRC ($F = 13.55$; df = 2, 18; $P = 0.0003$) and UM ($F = 19.59$; df = 3, 24; $P < 0.0001$), *C. ferrugineus* at UM ($F = 4.02$; df = 3, 24; $P = 0.0160$) and *T. castaneum* at the CRC ($F = 3.55$; df = 2, 18; $P = 0.0501$) (Table 6.3). More *S. oryzae* were in the top layer at the Cereal Research Centre ($F = 622.07$; df = 2, 18; $P < 0.0001$) and the University of Manitoba ($F = 309.88$; df = 2, 24; $P < 0.0001$), and more *T. castaneum* were also in the top layer at the Cereal Research Centre ($F = 11.90$; df = 2, 18; $P = 0.0005$) and the University of Manitoba ($F = 70.21$; df = 2, 24; $P < 0.0001$). However, more *C. ferrugineus* emerged from samples taken from the middle and bottom layers than in the top layer at both the Cereal Research Centre ($F = 11.79$; df = 2, 18; $P < 0.0005$) and the University of Manitoba ($F = 72.86$; df = 2, 24; $P < 0.0160$) (Table 6.4).

At the Cereal Research Centre, compared with the means of insects in the untreated control (762 ± 271 insects per kg) in all samples, the population of live *S. oryzae* in the treatment with 0.04% protein-rich pea flour, was reduced by 26% (562 ± 194 insects per kg) and further reduced by 79% in the 0.1% treatment (160 ± 64 insects per kg) ($F = 16.28$; df = 2, 6; $P < 0.0035$) (Fig. 6.3A). Protein-rich pea flour did not significantly reduce the population of *C. ferrugineus* ($F = 2.28$; df = 2, 6; $P = 0.1836$) or *T. castaneum* ($F = 3.74$; df = 2, 6; $P = 0.0881$) (Fig. 6.3B, C).

At the University of Manitoba, no *A. calandrae* or *C. waterstoni* was found in the control barrels. Compared with the number in the untreated control (473 ± 167 insects per kg), the number of live *S. oryzae* with only parasitoids (254 ± 95 insects per kg) was not significantly reduced, but the number was reduced by 76% in the combination of parasitoids and 0.04% protein-rich pea flour (113 ± 35 insects per kg), and further reduced by 98% in the combination of parasitoids with 0.1% protein-rich pea flour (10 ± 3 insects per kg) ($F = 92.25$; df = 3, 8; $P < 0.0001$) (Fig. 6.4A). The number of live *C. ferrugineus* was significantly lower in all three treatments (6 ± 2 insects per kg with parasitoid alone, 7 ± 2 insects per kg with parasitoid with 0.04% protein-rich pea flour, 3 ± 0.6 insects per kg with parasitoids and 0.1% protein-rich pea flour) than in the untreated control (12 ± 4 insects per kg) ($F = 16.19$; df = 3, 8; $P = 0.0009$, Fig. 6.4B). There were no differences among the treatments of parasitoids alone and the combination of parasitoids with 0.04% ($F = 0.36$; df = 1, 8; $P = 0.5650$). However, the combination of parasitoids and 0.1% protein-rich pea flour reduced more live *C. ferrugineus* than treatments of parasitoids alone ($F = 5.76$; df = 1, 8; $P = 0.0432$). There was no difference in the number of *T. castaneum* among treatments ($F = 0.81$; df = 3, 8; $P = 0.5253$) (Fig. 6.4C).

The population of *A. calandrae* was greater than that of *C. waterstoni* (Figs. 6.5A, B). The mean of the numbers of live and dead *A. calandrae* in the treatment of parasitoids without protein-rich pea flour (96 ± 36 insects per kg) and parasitoids with 0.04% protein-rich pea flour (18 ± 8 insects per kg) and parasitoids with 0.1% protein-rich pea

flour (2 ± 0.7 insects per kg) were significantly different ($F = 36.08$; df = 2, 6; $P = 0.0005$) (Fig. 6.5A). At the end of the test, the ratios of the number of *A. calandrae* to *S. oryzae* adults were 1.0 (parasitoids alone), 0.8 (combination of parasitoids with 0.04% protein-rich pea flour), and 3.3 (combination of parasitoids with 0.1% protein-rich pea flour). There were more *A. calandrae* in the top layer (84 ± 27 insects per kg) than in the middle (3 ± 0.8 insects per kg) and bottom (0.5 ± 0.2 insects per kg) ($F = 331.34$; df = 2, 12; $P < 0.0001$). The total *C. waterstoni* populations were the same in all treatments (parasitoids alone, 0.3 ± 0.1 insects per kg; parasitoids with 0.04% protein-rich pea flour, 0.2 ± 0.1 insects per kg; and parasitoids with 0.1% protein-rich pea flour, 0.5 ± 0.4 insects per kg) ($F = 1.72$; df = 2, 12; $P < 0.2573$) (Fig. 6.5B). There were no differences in the numbers of *C. waterstoni* in the top (0.5 ± 0.3 insects per kg), middle (0.10 ± 0.04 insects per kg), or bottom layers (0.08 ± 0.05 insects per kg) ($F = 2.73$; df = 2, 12; $P = 0.1053$) of wheat.

In the untreated control, the dockage of the wheat increased and the bulk density decreased at both the Cereal Research Centre and the University of Manitoba over the course of the experiment (Tables 6.5, 6.6). Similar changes occurred in 0.04% and 0.1% protein-rich pea flour treatments at the Cereal Research Centre, and the treatment of parasitoids without protein-rich pea flour at the University of Manitoba. However, the bulk density of wheat was not reduced in the combination with parasitoids and 0.04% protein-rich pea flour, or with parasitoids with 0.1% protein-rich pea flour.

Discussion

Usually, parasitoids and insecticides are incompatible, because beneficial insects are often more susceptible to insecticides than their hosts (Schöller and Flinn 2000). For example, diatomaceous earth is more toxic to *A. calandrae* than to *S. oryzae* (Perez-Mendoza et al. 1999). By direct contact, diatomaceous earth has an LT_{50} of 49 min for *A. calandrae*, and approximately 24 h for its host *S. oryzae* (Perez-Mendoza et al. 1999). My studies demonstrated that protein-rich pea flour and parasitoids were compatible for reducing populations of *S. oryzae* and *C. ferrugineus*. The flour had no direct contact toxicity, and did not affect the parasitism of *A. calandrae* and *C. waterstoni.* Protein-rich pea flour had no effect on the emergence of *A. calandrae, C. waterstoni* and their hosts. Assuming this was also true in the large-scale test, the low number of emerged *A. calandrae* in the protein-rich pea flour treatment in the large-scale test was probably not due to any adverse effect of protein-rich pea flour, but to the low number of available immature hosts, *S. oryzae*, as a result of the treatment with protein-rich pea flour. *Sitophilus oryzae* and *A. calandrae* have a very different diet. *Sitophilus oryzae* adults may take in protein-rich pea flour while they feed on grain treated with protein-rich pea flour, and may be killed because insects stop feeding or as a result of midgut injury caused by protein-rich pea flour (Chapter 8). *Anisopteromalus calandrae* adults do not feed on wheat and the larvae develop on *S. oryzae* larvae inside the wheat kernel. Therefore, *A. calandrae* may be less directly affected by protein-rich pea flour. This may explain why the protein-rich pea flour did not affect the parasitoids.

Because parasitoids target the immature stages, and the protein-rich pea flour is effective against adults and has no effect on parasitoids, I hypothesized that parasitoids and protein-rich pea flour are compatible for the control of *S. oryzae.* The large-scale test demonstrated an additive effect of this combination. The combination treatment reduced insect populations further that the treatment with parasitoids alone. In addition, the combination maintained grain quality, whereas neither of the individual treatments did. However, the additive effect was not observed in the small-scale test.

A high ratio of parasitoids to hosts may be required to control the pest population. In the large-scale test, a higher ratio of *A. calandrae* to *S. oryzae* was observed in the protein-rich pea flour treatment. The population of *C. waterstoni* did not become firmly established, and this may explain the insignificant additional reduction of *C. ferrugineus* populations. In the small-scale test, the low ratio of the number of *A. calandrae* to *S. oryzae* might explain the failure of pest control in the combination treatment. The failure might be also due to high moisture levels in the grain produced by insect metabolic activity, which decreased the efficacy of protein-rich pea flour (Hou and Fields (2003b, Chapter 3).

Sufficient numbers of hosts at the appropriate stages were required to establish the parasitoid populations. In the small-scale test, *A. calandrae* established at the high pest infestation rate, but not at the moderate and low infestation rates, apparently because of the low numbers of available hosts. *Anisopteromalus calandrae* adults live approximately 10 d, and they prefer parasitizing late instar larvae or prepupae (Rilett 1949, Burks et al. 1999). Therefore, only certain stages of *S. oryzae* were parasitized by *A. calandrae,*

although continuous stages of host *S. oryzae* were provided by allowing host adults to lay eggs continuously for 25 d, to simulate population age structures in commercial bins. Based on the number of *S. oryzae* adults emerged in the untreated control during the first 3 wk, I estimated that there were approximately 27, 2.7, 0.27 suitable hosts per wk for *A. calandrae* at the three infestation levels, respectively. Parasitoids failed to persist probably because few suitable hosts were available at moderate and low infestation rates. Unsuitable host stages, such as eggs and very young larvae, developed without the impact of the parasitoids, and the treatments failed to control the pests.

Differences in the distribution of *S. oryzae* and *C. ferrugineus* in the bulk grain may have affected the establishment of *A. calandrae* and *C. waterstoni* in the large-scale test. Most immature *S. oryzae* were in the top layer, and would be easily located and parasitized by *A. calandrae*. The few *S. oryzae* in the lower layer that survived the exposure to the protein-rich pea flour may have acted as a reservoir to maintain a low density of host population, allowing some *A. calandrae* to survive. However, most immature stages of *C. ferrugineus* were found in the middle and bottom layer. *Cephalonomia waterstoni* adults find *C. ferrugineus* by following a kairomone secreted by *C. ferrugineus* (Howard and Flinn 1990). It is not known if the failure of *C. waterstoni* to establish a population is because of its inability to penetrate the grain to the necessary depth.

Release time has a greater effect than the number of *C. waterstoni* on the dynamics of *C. ferrugineus* populations (Flinn and Hagstrum 1995). Augmentative release of parasitoids has been tested in a granary trial (Flinn et al. 1996). Both the single

and multiple releases of *A. calandrae* had equal efficacy on the reduction of populations of *S. oryzae* in the small-scale test. More studies on the optimum release time and effect of other environmental factors are required to determine the proper ratios of the parasitoids to hosts.

In addition to the failure of the establishment of *C. waterstoni* populations, the lack of a significant reduction of *C. ferrugineus* in the large-scale test may also be related to the lower efficacy of protein-rich pea flour against this species (Hou and Fields 2003b, Chapter 3). The repellence of protein-rich pea flour, a major factor in reducing *C. ferrugineus* in granary trials (Fields et al. 2001, Hou and Fields 2003a, (Chapter 4)), was precluded in this test, because the insects could not leave the bulk.

Populations of *T. castaneum* were not significantly reduced in any of the treatments in the large-scale test. Of the three species tested, *T. castaneum* is least affected by the protein-rich pea flour (Bodnaryk et al. 1997, Hou and Fields 2003a and 2003b, (Chapter 3, 4)). Also, *T. castaneum* was unable to leave the grain mass in this experiment. This is different from a granary trial, where *T. castaneum* is able to leave the grain, and a lower population is observed in grain treated with protein-rich pea flour, compared to untreated grain (Hou and Fields 2003a, (Chapter 4)).

One limitation of using protein-rich pea flour to control stored-product insects in commercial granaries is that *Rhyzopertha dominica* (F.), the lesser grain borer, is not controlled with a 0.1% concentration of protein-rich pea flour (Bodnaryk et al. 1997). However, *A. calandrae* is a generalist parasitoid, and parasitizes a number of stored-product Coleoptera and Lepidoptera (Schöller and Flinn 2000), including *R. dominica*

(Chatterji 1955, Ahmed 1996). As *A. calandrae* is not adversely affected by the pea flour treatments, the combination of this parasitoid and protein-rich pea flour may help suppress mixed populations of stored product insects including *R. dominca*.

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Table 6.1. Number of *A. calandrae* (mean ± SEM) in wheat with a high initial *S. oryzae* population in a small-scale test. Treatment with protein-rich pea flour was at 0.04%. Twelve *A. calandrae* were released once (single release) or four parasitoids each wk for three wk (multiple release).

Weeks after first release of parasitoids	Total number of *A. calandrae* (per jar) (mean ± SEM) [1]			
	Parasitoid single release	Parasitoid Multiple release	Protein-rich pea flour with parasitoid single release	Protein-rich pea flour with parasitoid multiple release
3	24 ± 3	24 ± 3	15 ± 2	16 ± 3
6	32 ± 6	48 ± 3	37 ± 2	28 ± 4
9	60 ± 32	81 ± 3	55 ± 20	41 ± 6
12	728 ± 377	350 ± 215	225 ± 44	449 ± 299
15	3413 ± 526	2046 ± 885	1608 ± 271	786 ± 428
Overall mean	851 ± 366	510 ± 259	388 ± 171	261 ± 121

[1] There is no significant difference between treatments in the same row (PRO GLM; CONTRAST; df = 3, 8; $P < 0.05$).

Table 6.2. Number of live *S. oryzae* (mean ± SEM) in wheat with a high initial *S. oryzae* population in a small-scale test. Treatment with protein-rich pea flour was at 0.04%. Twelve *A. calandrae* were released once (single release) or four parasitoids each wk for three wk (multiple release).

Weeks after first release of parasitoids	Number of live *S. oryzae* (per jar) (mean ± SEM)[1]					
	Untreated	Parasitoid single release	Parasitoid multiple release	Protein-rich pea flour	Protein-rich pea flour with parasitoid single release	Protein-rich pea flour with parasitoid multiple release
3	81 ± 10 a	61 ± 5 a	76 ± 12 a	81 ± 10 a	75 ± 5 a	82 ± 9 a
6	199 ± 3 a	149 ± 10 b	154 ± 4 b	146 ± 7 b	128 ± 9 b	141 ± 6 ab
9	1083 ± 40 a	872 ± 59 ab	756 ± 35 ab	754 ± 77 ab	616 ± 58 b	834 ± 134 ab
12	4799 ± 264 a	2833 ± 670 b	2752 ± 502 b	4564 ± 845 a	3734 ± 763 a	2630 ± 255 b
15	12305 ±802 a	8902 ± 1034 a	3522 ± 659 b	10777 ± 479 ab	3721 ± 495 b	5189 ± 1731 b
Overall mean	3488 ± 1165 a	2563 ± 912 b	1451 ± 404 b	3264 ± 1110 a	1655 ± 481 b	1775 ± 597 b

[1] Means in a row followed by different letters are different (PRO GLM; CONTRAST; df = 5, 12; $P < 0.05$).

Table 6.3. Number of emerged adults (mean ± SEM) in wheat treated with protein-rich pea flour and parasitoids, and sampled on July 11 before release of parasitoids in a large-scale test at the Cereal Research Centre and the University of Manitoba. Wheat was incubated at 30°C, 70% relative humidity for four wk.

Species	Total number of insects (per kg) (mean ± SEM)[1]						
	Cereal Research Centre			University of Manitoba			
	Untreated	0.04% protein-rich pea flour	0.1% protein-rich pea flour	Untreated	Parasitoid	Parasitoid + 0.04% protein-rich pea flour	Parasitoids + 0.1% protein-rich pea flour
S. oryzae[2]	159 ± 82a	75 ± 38a	23 ± 13b	48 ± 27a	78 ± 39a	20 ± 12b	3 ± 2c
C. ferrugineus	16 ± 4a	12 ± 3a	8 ± 2a	21 ± 5a	22 ± 5a	14 ± 3b	12 ± 2b
T. castaneum	23 ± 8a	14 ± 4ab	7 ± 1b	5 ± 2a	5 ± 2a	11 ± 4b	5 ± 2a

[1] Means in a row at same location followed by different letters are different (PROC MIXED, CONTRAST, $P < 0.05$; df = 2, 18 for the Cereal Research Centre; df = 3, 24 for the University of Manitoba).

[2] The percentage of dead *S. oryzae* adults in wheat samples in 0.1% and 0.04% were 83% and 24% at Cereal Research Centre, 67% and 10% at University of Manitoba. In all other samples, there were fewer than 10%.

Table 6.4. Number of emerged adults in wheat sampled at different layers of the barrels on July 11, before the release of parasitoids in a large-scale test at the Cereal Research Centre and the University of Manitoba. Wheat was incubated at 30°C, 70% relative humidity for four wk.

Species	Total number of insects (per kg)					
	Cereal Research Centre			University of Manitoba		
	S. oryzae	*C. ferrugineus*	*T. castaneum*	*S. oryzae*	*C. ferrugineus*	*T. castaneum*
Top[1]	257 ± 64 a	5 ± 2 a	28 ± 8 a	111 ± 28 a	4 ± 1 a	16 ± 3 a
Middle	0.7 ± 0.4 b	11 ± 2 b	12 ± 2 b	0.6 ± 0.3 b	20 ± 2 b	3 ± 1 b
Bottom	0 ± 0 c	21 ± 2 b	5 ± 1 b	0.2 ± 0.1 b	27 ± 3 b	1 ± 1 c
F	622.07	11.79	11.90	309.88	72.86	70.21
P	< 0.0001	0.0005	0.0005	< 0.0001	< 0.0001	< 0.0001

[1] Means in a column followed by different letters are different (PROC MIXED, CONTRAST; df = 2, 18 for Cereal Research Center, and 2, 24 for university of Manitoba; $P < 0.05$).

Table 6.5. Changes in physical characteristics of wheat before release of insects and at the end of a large-scale test at the Cereal Research Centre.

Grain quality	Untreated		0.04% protein-rich pea flour		0.1% protein-rich pea flour	
	Start	Finish	Start	Finish	Start	Finish
Dockage (%)	4.7 ± 0.6	16.3 ± 5.0 *	4.8 ± 0.7	16.8 ± 5.2 *	4.6 ± 0.4	8.3 ± 0.6 *
Moisture content (%)	13.6 ± 0.01	13.0 ± 0.4 *	13.7 ± 0.1	13.1 ± 0.3 *	13.7 ± 0.1	12.9 ± 0.3 *
Bulk density (kg/m^3)	717 ± 2	649 ± 36 *	718 ± 1	654 ± 35 *	716 ± 1	694 ± 11 *

*Indicates a significant difference (PROC MIXED, CONTRAST; df = 1, 6; $P < 0.05$).

Table 6.6. Changes of physical characteristics of wheat before release of insects and at the end of the test in a large-scale test at the University of Manitoba.

Grain quality	Untreated		Parasitoids		Parasitoids with 0.04% protein-rich pea flour		Parasitoids with 0.1% protein-rich pea flour	
	Start	Finish	Start	Finish	Start	Finish	Start	Finish
Dockage (%)	7.6 ± 0.9	15.2 ± 3.2 *	6.7 ± 0.4	11.9 ± 1.2 *	6.7 ± 0.5	9.2 ± 0.8 *	5.0 ± 0.7	7.0 ± 0.7 *
Moisture content (%)	13.4 ± 0.1	12.9 ± 0.5 *	13.5 ± 0.1	12.7 ± 0.5 *	13.4 ± 0.1	12.4 ± 0.6 *	13.6 ± 0.1	12.5 ± 0.7 *
Bulk density (kg/m^3)	716 ± 1	687 ± 15 *	714 ± 1	702 ± 7 *	712 ± 2	713 ± 2	715 ± 2	717 ± 2

*Indicates a significant difference between start and finish (PROC MIXED, CONTRAST; df = 1, 6; $P < 0.05$).

Figure 6.1. Mortality of 25 parasitoids in direct contact with 30 mg protein-rich pea flour, or wheat flour without honey in Petri dishes for various durations. *Anisopteromalus calandrae* was also exposed to 5 g wheat kernels containing 2-3 wk-old *S. oryzae* larvae, or in empty Petri dishes as control (n=5). A: *A. calandrae*; B: *C. waterstoni*.

Figure 6.1

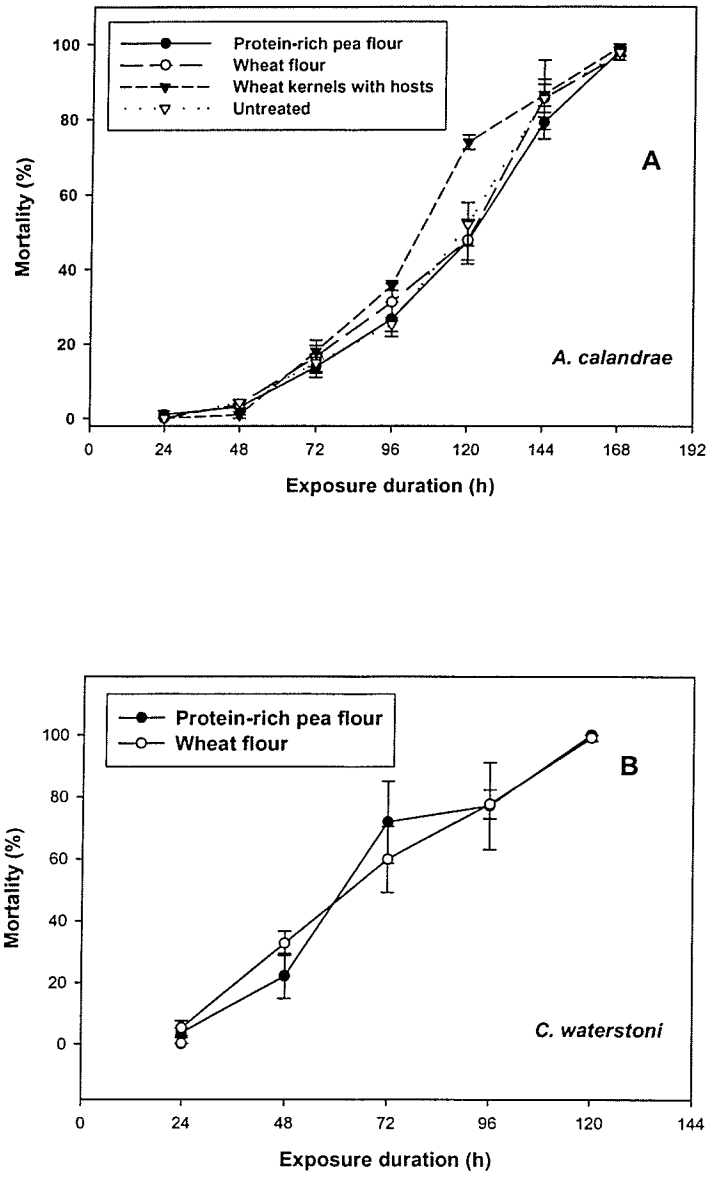

Protein-rich pea flour
Wheat flour
Wheat kernels with hosts
Untreated
A
A. calandrae
Mortality (%)
Exposure duration (h)
0
20
40
60
80
100
0
24
48
72
96
120
144
168
192
Protein-rich pea flour
Wheat flour
B
C. waterstoni
Mortality (%)
Exposure duration (h)
0
20
40
60
80
100
0
24
48
72
96
120
144

Figure 6.2. Number of emerged *A. calandrae* from 10 g untreated wheat or wheat treated with 0.1% protein-rich pea flour and placed in a Petri dish divided into two equal sections. A single female *A. calandrae* was placed in the Petri dish for various time intervals. Wheat contained 2-3 wk old *S. oryzae*, and was maintained at 30°C, 70% relative humidity for four wk after removal of the parent *A. calandrae* female (n=5).

Figure 6.2

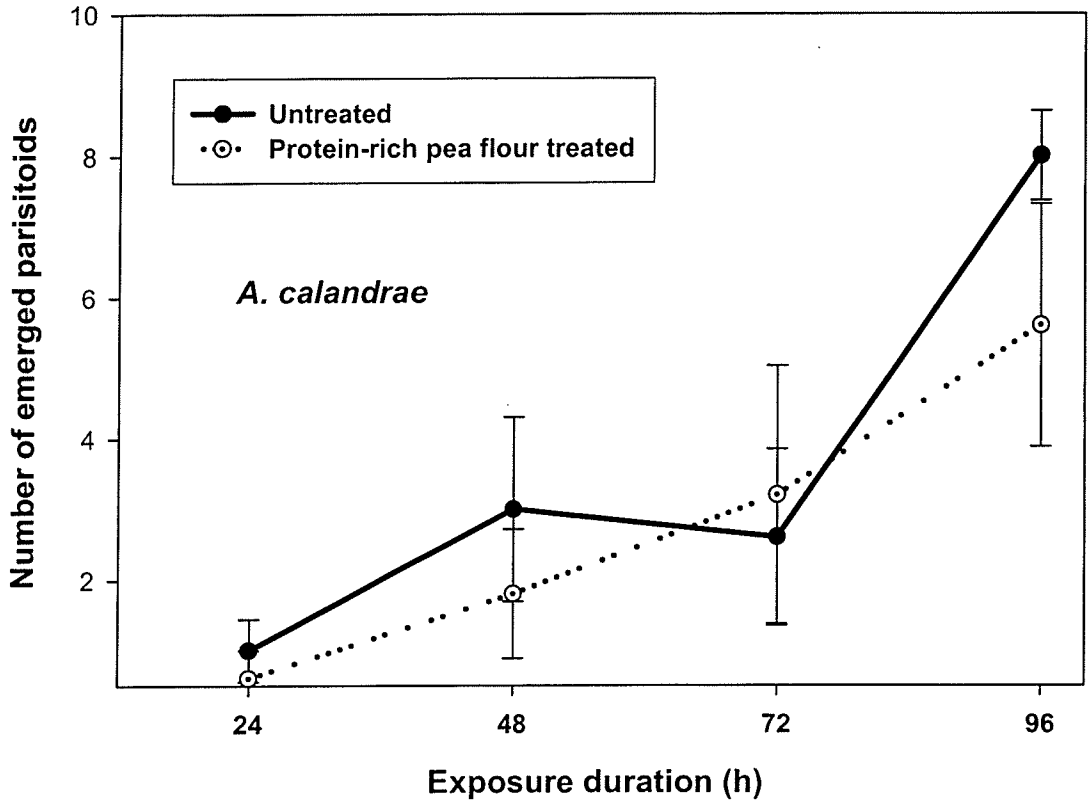

10
8
6
4
2
Number of emerged parisitoids
Untreated
Protein-rich pea flour treated
A. calandrae
24
48
72
96
Exposure duration (h)

Figure 6.3. Number of live pest insects in barrels containing wheat treated with protein-rich pea flour at 0, 0.04% and 0.1% at the Cereal Research Centre (n=3). A: *S. oryzae,* B: *C. ferrugineus.* C: *T. castaneum.* Lines followed by different letters were different ($P < 0.05$).

Figure 6.3

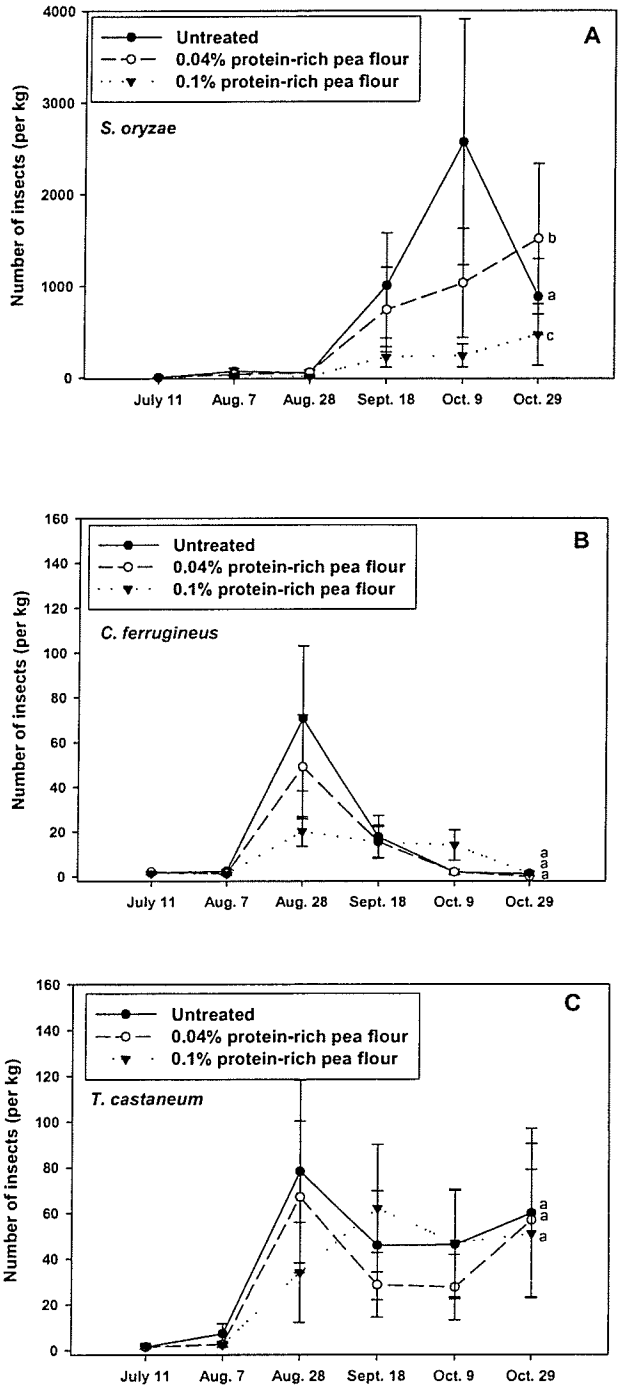

A
Untreated
0.04% protein-rich pea flour
0.1% protein-rich pea flour
S. oryzae
Number of insects (per kg)
4000
3000
2000
1000
0
b
a
c
July 11
Aug. 7
Aug. 28
Sept. 18
Oct. 9
Oct. 29
B
Untreated
0.04% protein-rich pea flour
0.1% protein-rich pea flour
C. ferrugineus
Number of insects (per kg)
160
140
120
100
80
60
40
20
0
a
a
a
July 11
Aug. 7
Aug. 28
Sept. 18
Oct. 9
Oct. 29
C
Untreated
0.04% protein-rich pea flour
0.1% protein-rich pea flour
T. castaneum
Number of insects (per kg)
160
140
120
100
80
60
40
20
0
a
a
a
July 11
Aug. 7
Aug. 28
Sept. 18
Oct. 9
Oct. 29

Figure 6.4. Number of live pest insects in barrels containing wheat treated with parasitoids alone, parasitoids with 0.04% protein-rich pea flour, parasitoids with 0.1% protein-rich pea flour, and no parasitoids and no protein-rich pea flour (control) at the University of Manitoba (n=3). *Anisopteromalus calandrae* and *C. waterstoni* were released at two insects per kg of wheat 29 d after *S. oryzae, C. ferrugineus* and *T. castaneum* were released at 2 insects per kg wheat. A: *S. oryzae,* B: *C. ferrugineus;* C: *T. castaneum.* Lines followed by different letters were different ($P < 0.05$).

Figure 6.4

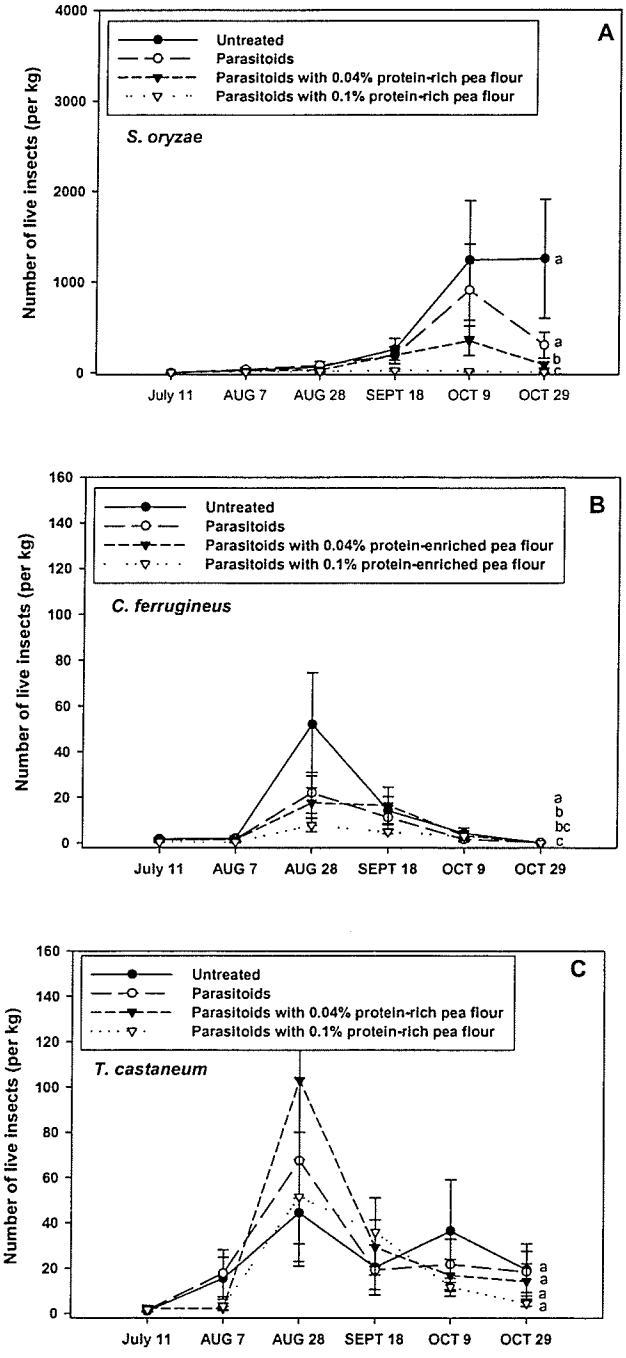
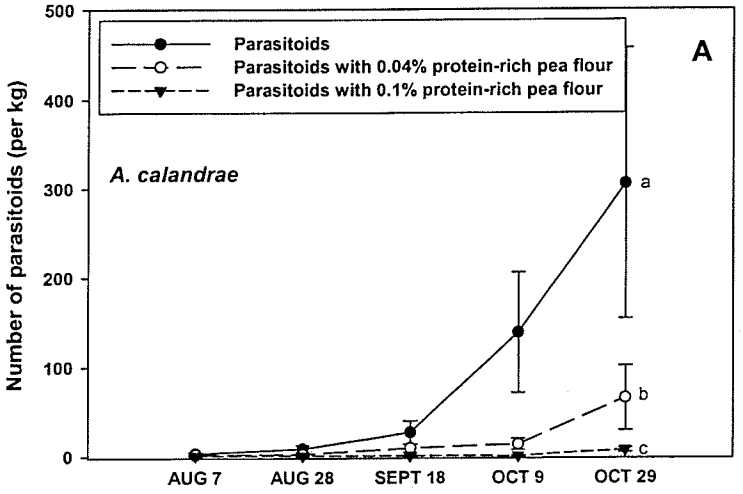
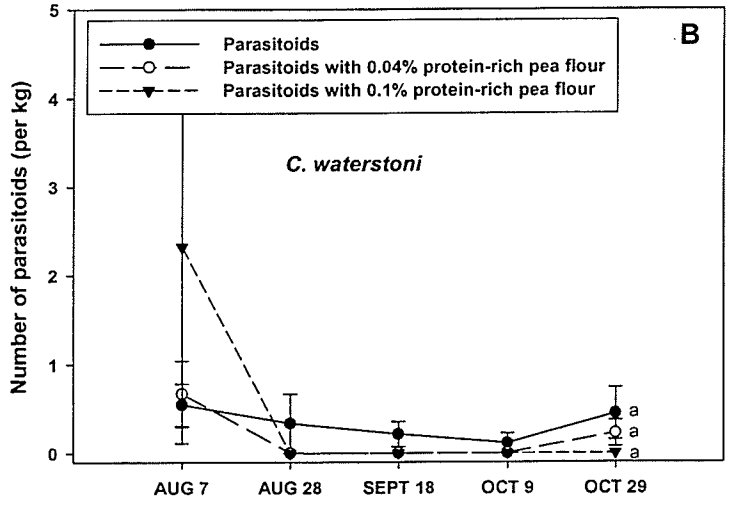

A
Untreated
Parasitoids
Parasitoids with 0.04% protein-rich pea flour
Parasitoids with 0.1% protein-rich pea flour
S. oryzae
Number of live insects (per kg)
4000
3000
2000
1000
0
a
a
b
c
July 11
AUG 7
AUG 28
SEPT 18
OCT 9
OCT 29
B
Untreated
Parasitoids
Parasitoids with 0.04% protein-enriched pea flour
Parasitoids with 0.1% protein-enriched pea flour
C. ferrugineus
Number of live insects (per kg)
160
140
120
100
80
60
40
20
0
a
b
bc
c
July 11
AUG 7
AUG 28
SEPT 18
OCT 9
OCT 29
C
Untreated
Parasitoids
Parasitoids with 0.04% protein-rich pea flour
Parasitoids with 0.1% protein-rich pea flour
T. castaneum
Number of live insects (per kg)
160
140
120
100
80
60
40
20
0
a
a
a
a
July 11
AUG 7
AUG 28
SEPT 18
OCT 9
OCT 29

Figure 6.5. Number of live parasitoids in barrels containing wheat treated with parasitoids alone, parasitoids with 0.04% protein-rich pea flour, parasitoids with 0.1% protein-rich pea flour, and no parasitoids and no protein-rich pea flour (control) at the University of Manitoba (n=3). *Anisopteromalus calandrae* and *C. waterstoni* were released at two insects per kg wheat 29 d after *S. oryzae, C. ferrugineus* and *T. castaneum* were released at two insects per kg wheat. A: *A. calandrae*; B: *C. waterstoni.* Lines followed by different letters were different ($P < 0.05$).

Figure 6.5


Parasitoids
Parasitoids with 0.04% protein-rich pea flour
Parasitoids with 0.1% protein-rich pea flour
A
A. calandrae
Number of parasitoids (per kg)
500
400
300
200
100
0
a
b
c
AUG 7
AUG 28
SEPT 18
OCT 9
OCT 29


Parasitoids
Parasitoids with 0.04% protein-rich pea flour
Parasitoids with 0.1% protein-rich pea flour
B
C. waterstoni
Number of parasitoids (per kg)
5
4
3
2
1
0
a
a
a
AUG 7
AUG 28
SEPT 18
OCT 9
OCT 29

CHAPTER 7

The Effect of Repellents on the Penetration into Packaging by Stored-Product Insects

Abstract

Two known repellents of stored-product insects, DEET and neem, were compared to protein-rich pea flour, defatted protein-rich pea flour and pea protein extract for their efficacy at reducing penetration and invasion by four common stored-product insects: *Sitophilus oryzae* (L.), *Tribolium castaneum* (Herbst), *Cryptolestes ferrugineus* (Stephens) and *Oryzaephilus surinamensis* (L.). The methods of preparation of pea extract affected the penetration of *S. oryzae*. Protein-rich pea flour, DEET and neem reduced the penetration of *S. oryzae,* but defatted protein-rich pea flour and pea protein extract did not. The number of *S. oryzae, T. castaneum, C. ferrugineus* and *O. surinamensis* entering pierced paper envelopes that contained wheat and were treated with DEET was reduced by 99, 86, 97 and 91%, respectively. Neem was less effective than DEET in reducing penetration and invasion of insects. Protein-rich pea flour did not prevent insects entering pierced envelopes.

Keywords: DEET; Neem; Pea extracts; Repellency; Invasion

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1. Introduction

Although finished products can be shipped from production facilities uninfested, stored-product insects can enter packaged goods during transportation, storage in the warehouse or in retail stores. Ultimately, the consumer of the products holds the manufacturer responsible for any insect infestation, even if the cause of the problem is poor storage by a third party. The packaging of products is the last line of defense for processors against insect infestation of their finished products. There are two types of insects that attack packaged products: "penetrators", which are insects that can bore holes through packaging materials; and "invaders", which are insects that enter packages through existing holes, such as folds, seams and air vents (Highland, 1984; Newton, 1988). *Sitophilus* spp., *Rhyzopertha dominica* (F.), *Plodia interpunctella* (Hübner), *Lasioderma serricorne* (F.) and *Stegobium paniceum* (L.) are some of the stored-product insects that are capable of penetrating food packaging. *Tribolium* spp., *Cryptolestes ferrugineus* (Stephens) and *Oryzaephilus surinamensis* (L.) cannot penetrate intact packages and must invade through existing holes in the package (Highland, 1991). Penetrators are also able to enter packages through existing holes.

In addition to improving the packaging material and design, manufacturers and scientists use insect repellents to prevent insects from entering packages by modifying the behavior of insects (Highland, 1984; Mullen, 1994; Watson and Barson, 1996; Mullen and Mowery, 2000). Pyrethrins synergized with piperonyl butoxide on the outer layer of packages or with glues have been approved for use as a treatment for insect -resistant

packaging in the USA (Highland, 1991). The repellency of pyrethrins is their primary mode of action against insect penetration and invasion (Laudani and Davis, 1955). Methyl salicylate, an insect repellent, has been registered for use in food packaging to control stored-product insects in the USA (Radwan and Allin, 1997). DEET, neem and protein-rich pea flour are repellent to many stored-product insects when tested by exposure on filter paper or in preference chambers (Khan and Wohlgemuth, 1980; Xie et al., 1995; Fields et al., 2001). The purpose of this work was to explore the possibility of using these insect repellents to prevent insects from penetrating or invading food packages.

2. Materials and methods

2.1. Insects

Four insect species, *Sitophilus oryzae* (L.), a penetrator and *Tribolium castaneum* (Herbst), *C. ferrugineus,* and *Oryzaephilus surinamensis* (L.), all invaders, were reared in the laboratory at 30°C, and 70% relative humidity (r.h.). All species had been cultured in the laboratory for over 5 years. One- to two-week-old adults were used in all experiments. *Sitophilus oryzae* was reared on whole kernels of wheat, and *T. castaneum* was reared on wheat flour mixed with 5% brewer's yeast. *Cryptolestes ferrugineus* and *O. surinamensis* were reared on wheat kernels, with 5% wheat germ and 5% brewer's yeast, by weight.

2.2. *Compounds*

Protein-rich pea (*Pisum sativum* L.) flour, defatted protein-rich pea flour, pea protein extract, DEET (diethyl-m-toluamide, >95%, Record 100, Recochem Inc., Vancouver, Canada) and neem (Amazin™ with 3% azadirachtin, AMVAC Inc., Los Angeles, USA) were used to estimate their effect on the penetrating ability of *S. oryzae.* Protein-rich pea flour (Progress Protein; 60% protein, 30% starch, and 7% moisture content, Parrheim Food, Saskatoon, SK) is produced commercially by grinding peas and isolating a protein-rich fraction by air classification. Defatted protein-rich pea flour was prepared from protein-rich pea flour by defatting with chloroform for 1 h at room temperature. Pea protein extract was prepared from defatted protein-rich pea flour by a batch process with a copolymeric resin of styrene and divinylbenzene (Bodnaryk et al., 1997). One hundred mg of protein-rich pea flour produced about 1 mg of pea protein extract. Test materials were dissolved in water, except pea protein extract and DEET which were dissolved in 70% ethanol.

2.3. *Paper selection*

To select suitable paper for the penetration test, the following papers were tested: filter paper (Whatman number 1, 4, and 5), waxed paper, rough tissue paper, napkin paper, and coffee filter paper. The weights by area and the thickness of paper (measured with a Manostat 15-100-500 calliper) are listed in Table 7.1. The test apparatus was

similar to that described by Newton (1988) (Fig. 7.1). Test paper was clamped between two steel plates (0.5 cm thick). Each plate had 10 holes (1.0 cm diameter). For each hole, the top plate had a small notch close to the paper to help the insect penetrate the paper. One adult *S. oryzae* was placed in each hole, and confined with a metal screen and covered with another metal plate on the top. The assembled blocks were placed at 30°C, and 70% r.h. The number of insects penetrating the paper was noted after 12, 24, 48, 72, and 96 h. The tests were repeated three times.

2.4. Penetration test

The apparatus for penetration tests consisted of four metal plates the same as those used in the paper selection test (Fig. 7.1). Napkin paper was clamped between two steel blocks. Neem, protein-rich pea flour, and defatted protein-rich pea flour were mixed with water. DEET and pea protein extract were mixed with 70% ethanol. The doses used in this test were: 31.25 g/m^2 for protein-rich pea flour and defatted protein-rich pea flour, 12.5 g/m^2 for pea protein extract and neem and 0.02 g/m^2 for DEET. Fifty µl of each mixture was placed on the napkin paper within each well. The paper clamped in the plates was allowed to dry at room temperature in a fume hood for 24 h. A similar set of plates with napkin paper was treated with either 50 µl of water to serve as the control for neem, protein-rich pea flour and defatted protein-rich pea flour, or 50 µl of 70% ethanol to serve as the control for DEET and pea protein extract. One adult *S. oryzae* was placed in each hole. Plates with the control paper were placed on the top and the two sets of plates were bolted together. The plate with a small notch was placed close

to the treated paper. The bolted plates were shaken gently and placed vertically in incubators at 30°C, 70% r.h. The number of insects penetrating the treated paper after 24 h was noted. The test was repeated four times.

2.5. Invasion test

Protein-rich pea flour (50 mg protein-rich pea flour, mixed with 200 µl water), neem (200 µl of Amazin), or DEET (50 µl DEET mixed with 150 µl 70% ethanol) were pipetted on to each paper envelope (9×15 cm). The suspensions or solutions containing repellents were evenly pipetted on a 1 cm wide strip near the bottom of the envelopes (short edge). The envelopes were allowed to dry overnight at room temperature, and then within the treated strip, six holes (2 mm diameter) on each side of the envelope were punched. The holes simulated the stitching, damaged packaging, or poor sealing of packages. Water was applied to the envelopes as controls for neem and protein-rich pea flour. Seventy percent ethanol was used as the control for DEET. The envelope was filled with 80 g hard red spring wheat (15% moisture content, wet weight-based) and placed in a 30 × 30 × 30 cm screened cube box. Six treated envelopes were split into two groups of three and placed on the bottom of the box at opposite corners. Three control envelopes were placed at each of the two remaining corners. The box was placed at 30°C, and 70% r.h.. Two hundred adults each of *S. oryzae*, *T. castaneum*, *C. ferrugineus*, and *O. surinamensis* were placed at the middle of the bottom of the box, and confined for 1 h with a 2 l jar to allow them to acclimate before being released into the cage. The

number and species of insects inside each envelope were noted after 1 week. Tests were repeated three times.

2.6. Data analysis

The correlation (Steel et al. 1997) of paper weight and the number of insects that penetrated the paper was estimated by using using PROC CORR (SAS Institute Inc., 2000). Data for penetration were tested with contingency table anylsis (Steel et al. 1997) using the SAS GENMOD (Generalized Linear Models) procedure by comparing by the numbers of each insect species that penetrated through the treated paper. For envelope tests, the sum of insects caught in six treated and untreated envelopes was compared using a contingency table by the PROC GENMOD procedure with type 3 contrast with the expectation that insects were evenly distributed between treated and untreated bags. To compare the effectiveness of the three repellents, the proportion of the total number of insects in treated envelopes out of all insects found in the screen boxes was transformed with the arcsine function and compared pairwise with Studentized Maximum Modulus (GT2) in the GLM (General Linear Models) procedure (Steel et al. 1997).

3. Results and discussion

The heavier the paper, the fewer *S. oryzae* that penetrated the paper (Table 7.1). The correlation coefficients of paper weight to the penetration of *S. oryzae* at 12, 24, 48, 76, and 96 h were -0.79, -0.93, -0.87, -0.85, and -0.82, respectively. Most of the *S. oryzae*

penetrated tissue paper and coffee filter paper within 12 h, and all insects escaped after 48 h. Whatman filter papers were more resistant than all other papers. Most of the *S. oryzae* penetrated waxed paper after 96 h. The correlation coefficients of the thickness of the paper to the penetration at 12, 24, 48, 76, and 96 h were -0.37, -0.70, -0.65, -0.67, and -0.63, respectively. I chose napkin paper for further experiments for two reasons. First, most of the *S. oryzae* could penetrate it within 24 h thus reducing the adverse effects of starvation. Second, it absorbed the test solutions well.

DEET and neem reduced the penetration of *S. oryzae* adults (Table 7.2). No insects penetrated through napkin paper treated with DEET at 0.02 g/m^2. Neem has antifeedant effects (Saxena et al., 1989). The reduction of penetration by neem may be due to either or both of its repellent and antifeedant properties. Protein-rich pea flour significantly reduced the number of insects penetrating the treated paper. However, defatted protein-rich pea flour and pea protein extract did not reduce the penetration of *S. oryzae*. This suggested that repellent compounds had been removed by chloroform, or the defatting procedure destroyed the repellent compounds in the protein-rich pea flour.

DEET reduced the number of insects entering the envelopes (Table 7.3). Based on the total number of insects invading in both treated and untreated envelopes, DEET repelled *S. oryzae, T. castaneum, C. ferrugineus* and *O. surinamensis* by 99, 86, 97 and 90%, respectively. The total number of all insect species in the DEET-treated envelopes was only 6% of insects found in all envelopes. Neem also was repellent (Table 7.4), but it was less effective than DEET ($F = 248.59$, df = 1, 6; $P < 0.0001$). The number of *S. oryzae, T. castaneum, C. ferrugineus* and *O. surinamensis* in neem-treated envelopes was

37, 21, 44 and 39% of the same insects species invading in both treated and untreated envelopes, respectively. The total number of all insect species in neem-treated envelopes was 38% of all insects found in envelopes. Protein-rich pea flour did not stop insects from entering the envelopes (Table 7.5). Protein-rich pea flour treated envelopes attracted more *S. oryzae* and *O. surinamensis* than untreated envelopes. The total number of insects in pea flour-treated envelopes was 60% of all insects invading in both treated and untreated envelopes. As a natural product, protein-rich pea flour showed a repellent effect to many insects when it was tested on grain in chambers (Fields et al., 2001). It repelled *S. oryzae* in penetration tests but not in the invasion test. This suggests that the repellency of pea flour is weak, or there might be an interaction between the chemicals in pea flour and wheat.

Mullen and Mowery (2000) stated that most insects enter into finished products through openings caused by sewing, folding, or damage, not by chewing through packaging. Some adult insects can pass through holes less than 1 mm in diameter, and their larvae can enter through smaller holes (Cline and Highland, 1981). Therefore, the ability of chemical barriers to prevent insects from invading is more important than to prevent penetration. Although protein-rich pea flour showed repellency in the penetration test, it attracted insects into packages so it would not provide added protection to packaging. Neem is repellent to many insects (Xie et al., 1995). My data showed that neem was repellent enough to reduce insect immigration into packages. Further work is needed to determine if this has commercial potential.

Hundreds of materials, such as synthetic pyrethroids, natural botanical antifeedants, and silica gel (Laudani and Davis, 1955; Watters, 1966; Highland et al., 1984; Bloszyk et al., 1990), have been investigated as insect-resistant packaging. Highland et al. (1984) showed that insects do not infest cereal grains packed in bags that have been treated with permethrin. A barrier layer was included in the construction of the multiple-wall bags to prevent the migration of permethrin into the cereals. Both the penetration and the envelope tests in this study suggested that DEET has a great potential for preventing the infestation of packaged goods. This repellent is mainly used for protecting humans from biting flies. It has a low acute oral toxicity to rats (2.0 g/kg) (Ware, 2000). There is no evidence showing DEET to be acutely toxic, carcinogenic, developmentally toxic or mutagenic (Anonymous, 1998). DEET also is used on clothing and mosquito netting. My data suggest it might also be effective on jute bags, which are commonly used in many developing countries for grain storage. However, DEET has a plasticizing action and is not compatible with wax paper and plastic sheet, which are currently used in many food packages. The packaging would have to be modified to prevent the contamination of the finished product by DEET, and the contamination of neighboring products by the volatile action of DEET. Barriers developed for preventing the migration of pyrethrins into packaging (Highland, 1984), or similar barriers may prevent the contamination of food by DEET.

Acknowledgments

I would like to thank Tannis Mayert for technical assistance, Ken Fulcher for providing protein-rich pea flour, Agri-Food Research and Development Initiative and University of Manitoba for financial support and Michael Mullen at USDA for providing a copy of the chamber used for the penetration tests.

Table 7.1. Percentage of *S. oryzae* that penetrated through various types of paper. Ten insects were used in each replicate (n=3).

Paper	Paper weight ($mg/cm^2 \pm$ SEM)	Thickness ($\mu m \pm$ SEM)	Number of insects penetrating paper (% ± SEM) time after insect release (h)				
			12	24	48	72	96
Tissue Paper	1.6 ± 0.1	6.7 ± 0.1	77 ± 12	90 ± 6	100 ± 0	100 ± 0	100 ± 0
Coffee Filter Paper	3.2 ± 0.1	10.1 ± 0.1	57 ± 3	93 ± 3	100 ± 0	100 ± 0	100 ± 0
Napkin Paper	3.5 ± 0.02	15.9 ± 0.2	83 ± 3	83 ± 3	90 ± 3	93 ± 3	100 ± 0
Waxed Paper	6.8 ± 0.1	3.3 ± 0.1	0 ± 0	43 ± 12	47 ± 3	63 ± 3	83 ± 3
Whatman No. 1	8.5 ± 0.3	17.3 ± 0.1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Whatman No. 4	9.8 ± 0.1	21.7 ± 0.1	0 ± 0	0 ± 0	3 ± 3	10 ± 6	10 ± 6
Whatman No. 5	9.9 ± 0.1	19.7 ± 0.1	0 ± 0	0 ± 0	3 ± 3	13 ± 3	13 ± 3

Table 7.2. Number of *S.oryzae* (mean ± SEM) out of ten that penetrated through napkin paper treated with different materials after 24 h. Ten insects were used in each replicate (n=4).

Materials	Dose (g/m^2)	Number of insects that penetrated through treated paper [a]
Water treated control	0	7.5 ± 0.5 a
70% ethanol treated control	0	7.5 ± 0.6 a
Pea protein extract	12.5	7.3 ± 0.5 a
Defatted protein-rich pea flour	31.25	7.3 ± 0.6 a
Protein-rich pea flour	31.25	3.5 ± 0.3 b
Neem	12.5	4.3 ± 0.3 b
DEET	0.02	0 ± 0 c

[a] Different letters indicate that the materials were significantly different (PROC GENMOD, df = 5, 12; $P < 0.05$).

Table 7.3. Number of insects (mean ± SEM) in envelopes treated with DEET at 50 µl/envelope, 1 week after insects were released (n=3).

Insect	Number of insects		Likelihood ratio χ^2	*P*
	Treated	Untreated		
Sitophilus oryzae	2 ± 0.3	189 ± 3	735.82	<0.0001
Tribolium castaneum	17 ± 3	101 ± 8	197.88	<0.0001
Cryptolestes ferrugineus	4 ± 0.7	117 ± 28	354.1	<0.0001
Oryzaephilus surinamensis	11 ± 3	100 ± 8	246.77	<0.0001
All insects	34 ± 5	507 ± 31	1481.56	<0.0001

Table 7.4. Number of insects (mean ± SEM) in envelopes treated with neem at 200 µl/envelope, 1 week after insects were released (n=3).

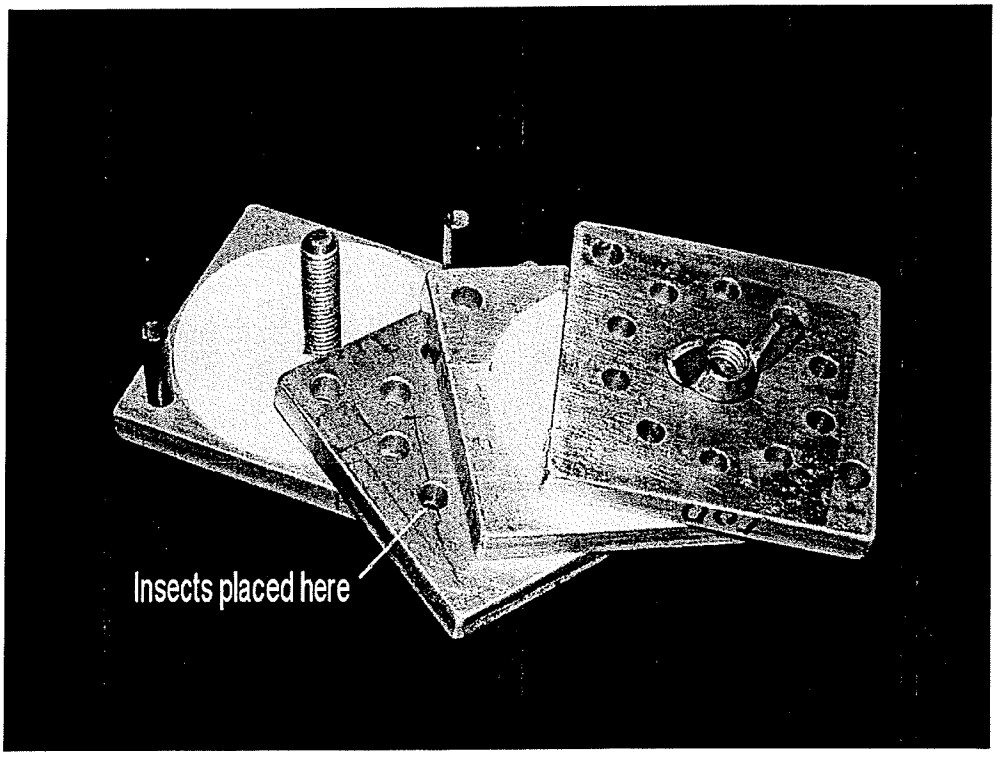
Insect	Number of insects		Likelihood ratio χ^2	*P*
	Treated	Untreated		
Sitophilus oryzae	70 ± 9	121 ± 12	41.63	<0.0001
Tribolium castaneum	20 ± 4	74 ± 10	95.26	<0.0001
Cryptolestes ferrugineus	60 ± 10	76 ± 1	7.06	0.0079
Oryzaephilus surinamensis	57 ± 4	89 ± 8	20.24	<0.0001
All insects	220 ± 16	361 ± 12	104.3	<0.0001

Table 7.5. Number of insects (mean ± SEM) in envelopes treated with protein-rich pea flour in at 50 mg/envelope, 1 week after insects were released (n=3).

Insect	Number of insects		Likelihood ratio χ^2	*P*
	Treated	Untreated		
Sitophilus oryzae	116 ± 2	67 ± 3	40.19	<0.0001
Tribolium castaneum	70 ± 4	59 ± 9	3.4	0.0653
Cryptolestes ferrugineus	73 ± 16	69 ± 12	0.13	0.7178
Oryzaephilus surinamensis	75 ± 10	42 ± 2	26.81	<0.0001
All insects	337 ± 24	229 ± 12	61.54	<0.0001

Fig. 7.1. The unstacked apparatus for penetration tests. One adult *S.oryzae* was confined in the hole in the second plate.

Figure 7.1



CHAPTER 8

The Site of Action of Protein-Rich Pea Flour and Its Extract

ABSTRACT

Protein-rich pea flour is antifeedant, repellent and toxic to *Sitophilus oryzae* (L.) Tests were conducted to investigate the mode of action of the protein-rich pea flour. Protein-rich pea flour had no fumigation effect on adult survival or offspring production of *S. oryzae*. To test if pea toxins could enter *S. oryzae* through the cuticle, slurries of protein-rich pea flour and wheat flour were placed on the abdomens of immobilized starved insects. The insects treated with protein-rich pea flour did not have reduced longevity compared with wheat-flour-treated insects, but insects in both treatments died faster than in the untreated controls. In a similar experiment, immobilized insects were every other day, and had their abdomens brushed with protein-rich pea flour or wheat flour on the alternate days. Insects treated with protein-rich pea flour had an average longevity of 9.6 d, which was shorter than for wheat flour (11.3 d), which was shorter than insects brushed with nothing (17.6 d). This suggests that toxins from the protein-rich pea flour may cross the insect cuticle. Protein-rich pea flour, pea extract and pea peptides caused the production of gases in the midgut of *S. oryzae,* and the tissues of the midgut were injured, as shown by dual staining with fluorescent dyes, calcein AM and propidium iodide. The volume of the gases increased rapidly when insects were fed on protein-rich pea flour and pea extract. There was no gas found in the *S. oryzae* fed on wheat kernels or wheat flour.

KEY WORDS pea protein, *Sitophilus oryzae*, midgut, gas, dual fluorescent staining

Introduction

Higher plants are a good source of novel insecticides (Prakash & Rao 1997). Thousands of plants or extracts from plants have insecticidal properties (Jacobson 1989a, Golob et al. 1999, Weaver & Subramanyam 2000). They control insects by repellency, contact toxicity or fumigation (Dev and Koul 1997, Shaaya et al. 1997, Golob et al. 1999). Legume seeds contain a wide range of allelochemicals with toxic and deterrent effects against insect pests (Harborne et al. 1971, Bell 1977).

Yellow split pea *(Pisum sativum* L.) mixed with wheat reduces the survival and reproduction of *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) (Coombs et al. 1977, Holloway 1986). Protein-rich pea flour made from yellow split pea has the potential to be a good grain protectant (Bodnaryk et al. 1997, Fields et al. 2001, Hou & Fields 2003a (Chapter 4)). It is repellent (Fields et al. 2001), toxic, and reduces the reproduction of many stored-product insect pests (Bodnaryk et al. 1997). Since it is used as an animal feed and human food, it is less likely to have negative effects on mammals. Bodnaryk et al. (1997) produced an extract from protein-rich pea flour that is 20 to 100 times more toxic than protein-rich pea flour itself. Delobel et al. (1998) isolated a peptide from peas that is toxic to stored-product insects. However, the mode of action of protein-rich pea flour and its extract has not been determined. Like synthetic insecticides, botanical insecticides have three means to enter an insect: ingestion, absorption across the cuticle and through the respiratory system. The purpose of this study was to determine if the gut of insects is affected by protein-rich pea flour and its extracts, and test if the toxins in peas enter *S. oryzae* as a gas or through the abdominal cuticle of the adults.

Two fluorescent dyes, calcein AM and propidium iodide, are commonly used to detect the viability of cells in insects (Collins and Donoghue 1999), bacteria (Bunthof et al. 2001) and plants (Haugland 2002). Calcein AM probe is a fluorogenic chemical that can penetrate through live cell membranes, and it is cleaved intracellularly by esterases of viable cells. Calcein, the hydrolyzed product of calcein AM, is produced only in viable cells. Its absorption maximum is 492 nm, and the fluorescence emission maximum is 517 nm. Therefore, live cells fluoresce green. Propidium iodide penetrates only dead cells and binds to nucleic acids. The absorption maximum for propidium iodide bound to nucleic acids is 535 nm, and the fluorescence emission maximum is 617 nm. Hence dead cells fluoresce red.

Materials and Methods

Sitophilus oryzae, maintained in the laboratory for over five years, was reared on whole kernels of wheat at 30°C, 70% relative humidity (RH). Protein-rich pea flour (Progress Protein; 60% protein, 30% starch, and 7% moisture content, Parrheim Food, Saskatoon, SK) was used in this study. It is produced commercially by grinding peas and isolating a protein-rich fraction by air classification. Pea extract was obtained from protein-rich pea flour with the method described in Chapter Five. Purified pea peptides were provided by Dr. Taylor from Saskatoon Research Centre, Agriculture and Agri-Food Canada.

Fumigant toxicity

Twenty g of wheat (14% moisture content) was placed in a small cloth bag (10 × 10 cm) with 30 one to two week-old adults of *S. oryzae*. A Petri dish containing two g of

protein-rich pea flour was placed at the bottom of a one liter jar. The cloth bag with insects was hung five cm above the Petri dish. The jar lid was sealed with tape. After one wk, the adults were removed from the wheat. The number of live and dead insects was noted. The wheat, without the adults, was held separately in vials (ten cm high, 3.3 cm diameter) at 30°C, and 70% RH for five wk without exposure to protein-rich pea flour, and the number of emerged adults was noted. The original exposed adults were placed on fresh wheat and allowed to lay eggs for one wk, and the wheat was cultured for five wk, before counting the number of emerged adults. A control was set up with no protein-rich pea flour. There were five replicates per treatment.

Contact toxicity

Seven-day-old insects were placed for ten min at 4°C to reduce their activity, and glued (Lepage® five min Epoxy, Henkel Canada Corp., Brampton, ON) ventral surface up in a Petri dish. The insects' legs were glued to their thorax to prevent the legs from moving material placed on the abdomen to the mouth. Slurries of each of protein-rich pea flour and wheat flour at the concentrations of 0, 0.01, 0.1, and 0.5% were prepared by mixing them in water for one min. One µl of the slurry was placed on the abdomen of *S. oryzae* with a micro-manipulator. Insects were placed at 25°C, and 70% RH. The number of dead and live insects was noted every 24 h. There were three replicates for each treatment, with 20 insects per replicate.

In another experiment, insects were fed or treated on alternate days to reduce the stress due to starvation. Insects were glued to Petri dishes as above, and the dry powder of protein-rich pea flour or wheat flour was brushed on to the abdomen of insects. The insect abdomen was brushed three times with a small soft brush dipped in one of the

powders, so that the abdomen was covered with a thin layer of protein-rich pea flour or wheat flour. To feed the insects, every other day wheat flour was added to the Petri dish, deep enough to cover the mouths of insects. Insects were thoroughly cleaned with a micro-vacuum and blower between each feeding or treatment. Number of live and dead insects was noted every 24 h. There were four treatments: brushing with protein-rich pea flour and fed on alternate days, brushing with wheat flour and fed on alternate days, brushing with nothing but fed every other day, and no brushing with feeding every day. Each treatment had three replicates, with 20 insects per replicate.

Dissection and vital staining

Twenty seven-day-old *S. oryzae* adults were held at 30°C, and 70% RH on one of seven treatment diets: no food, two g of wheat kernels, two g wheat flour, two g protein-rich pea flour, five wheat flour disks (Xie et al. 1996) made from 0.2 g wheat flour, five disks made with 0.2 g wheat flour mixed with 0.8 mg of pea extract, or five disks made with 0.2 g wheat flour mixed with 0.8 mg of pea peptides. The number of dead and live insects was noted every 24 h. There were three replicates for each treatment. Five *S. oryzae* treated as above were dissected in physiological saline (Maddrell 1969) every day for three d at approximately 4°C. A pretest showed that the isolated guts of *S. oryzae* still moved after 19 h in the saline solution at room temperature. The guts of three insects were video-taped using a dissecting microscope during dissection, and length and width of each gas bubble were measured on the TV screen and calibrated with a micro-ruler.

Three guts were stained immediately after dissection. Guts were placed on glass slides for 5 min with 25 µl calcein AM (20 µM) (Molecular Probes Inc., Eugene, OR, USA), and 25 µl propidium iodide (15 µM) (Molecular Probes Inc.) diluted from stock

solution with physiological saline just before use. Calcein AM stock solution was prepared by dissolving 20 μg of calcein AM in 500 μl anhydrous dimethylsulfoxide on the day of use. Propidium iodide stock solution was prepared by dissolving 1 mg propidium iodide in 1 ml deionized water. Stock solutions were stored at 4°C, and protected from light.

Stained guts in saline were covered with cover slips raised by four fine copper wires. A fluorescent microscope (Leica DMRB, Germany) with a red-blue filter at a magnification of 100 was used to observe and photograph the guts.

Data analysis

Numbers of insects were transformed with log (X+1) and the mortality of insects was transformed with arcsine square root of X+1. SAS PROC GLM (General Linear Models) with Studentized Maximum Modulus (GT2) test (SAS Institute Inc. 2000) was used to compared the effect of treatments ($\alpha = 0.05$). LT_{50}s, time to the death of 50% of insect samples, were calculated with Kaplan-Meier survival analysis and pairwise compared with Log-Rank by using SigmaStat (SPSS Inc. 2003). Most of the bubbles were sphere-shaped. The gas volume in the midgut was derived from the sum of all bubbles calculated by $1/6\ \pi d^3$, where d equals the average width and length of each bubble.

Results

Fumigant toxicity

A seven-day fumigation with protein-rich pea flour did not cause any mortality in *S. oryzae* adults. No difference in the mortality ($F = 2.67$; $df = 1,8$; $P = 0.141$) or the

number of emerged adults (F = 0.78; df = 1,8; $P = 0.4021$) was detected between protein-rich pea flour (10 ± 0.5%, 257 ± 18) and the control (12 ± 1%, 241 ± 13). There were no differences in the mortality (F = 2.69; df = 1,8; $P = 0.1396$) or number of emerged adults (F = 0.5; df = 1,8; $P = 0.4988$) produced in fresh wheat by fumigated *S. oryzae* adults between protein-rich pea flour (15 ± 2%, 204 ± 27) and the control (9 ± 2%, 188 ± 30) ($P > 0.05$).

Contact toxicity

Without food, 50% of *S. oryzae* died in six d (Table 8.1). Placing protein-rich pea flour or wheat flour as a slurry on the abdomen of *S. oryzae* slightly reduced longevity. No differences in LT_{50}s were detected between protein-rich pea flour and wheat flour at the same doses.

When food was provided, the mortality of glued *S. oryzae* in the control was less than 15% after 15 d (Fig. 8.1). The LT_{50} of *S. oryzae* treated with protein-rich pea flour (9.2 d, 95% C.L.: 8.7-9.7) was significantly shorter than when treated with wheat flour (10.2 d, 95% C.L: 9.1-11.2) ($P < 0.05$), which was shorter than when brushed with nothing (12.5 d, 95% C.L.: 11.5-13.5) ($\chi^2 = 39.521$, df = 3, $P < 0.01$).

Dissection and vital staining

No gas was observed in the midguts of *S. oryzae* fed on wheat kernels (Fig. 8.2A), wheat flour or wheat flour disks. However, bubbles were produced in the midgut of *S. oryzae* fed on protein-rich pea flour (Fig. 8.2B), wheat flour disks treated with pea extract or pea peptides and starved insects. Less fat tissue was observed on the anterior midgut of *S. oryzae* that fed on protein-rich pea flour than insects fed on wheat kernels (Fig. 8.2). The midgut of insects fed with the various pea treatments became transparent due to the

distention caused by large bubbles (Figs. 8.2B). The volume of gas bubbles was less in *S. oryzae* fed pea peptides (5.4 ± 0.7 mm^3) than in insects fed pea extract (8.9 ± 3.1 mm^3) or protein-rich pea flour (8.1 ± 3.7 mm^3) ($P < 0.05$). However, all these volumes were larger than starved insects (0.8 ± 0.4 mm^3) ($P < 0.05$). The volume of the gas increased rapidly after two d of treatment with pea extract and protein-rich pea flour (Fig. 8.3). Dissections to measure the size of the bubble in the midgut became impossible after three d, as the guts were too fragile. The mortality of insects fed protein-rich pea flour, and pea extract was higher than those fed pea peptides (Fig. 8.4). There was no mortality of insects held on wheat kernels within 10 d.

There were more red spots, indicating dead tissue (Haugland 2002), in the midgut of *S. oryzae* fed on protein-rich pea flour (Fig 8.5 B), pea extract and pea peptides than in those fed on wheat kernels (Fig 8.5A).

Discussion

Many plants and their extracts act as fumigants (Shaaya et al. 1997). Volatiles from plants by definition have a high vapor pressure. There are many compounds in protein-rich pea flour, and some of them are volatile as protein-rich pea flour has a distinct odor. However, protein-rich pea flour had no fumigant toxicity to *S. oryzae*. Delobel et al. (1998) showed that a 37-amino acid peptide isolated from peas is toxic to *S. oryzae*. Peptides of this size are not volatile.

Wheat flour and protein-rich pea flour placed on the abdomen of *S. oryzae* increased mortality. The higher mortality of fed *S. oryzae* in the treatment using protein-rich pea flour than that using only wheat flour suggests that toxins from protein-rich pea

flour may cross the cuticle. But the difference between protein-rich pea flour and wheat flour may have been masked by rapid death from the starvation in the no food test. Wheat flour itself had no contact or oral toxicity to *S. oryzae* as shown by the controls. I suspect that the increased mortality in the treatment of wheat flour and protein-rich pea flour may be due to one of three possible causes. First, a slurry on the abdomen may be an irritant that causes increased movement and the insects starved more quickly. Second, when the flours were brushed on to the abdomen every other day, there could have been damage to the insect cuticle. Finally, the toxins from the pea protein-rich pea flour may have entered the insect across the cuticle, through damaged sites or via the respiratory system. The polar pea peptides, toxic compounds in the protein-rich flour (Delobel et al. 1998), would not be expected to penetrate the non-polar wax layer of the insect cuticle, but may be able to cross a damaged cuticle or via the lining of the tracheal system. However, non-polar components in the protein-rich pea flour (Wes Taylor, Agriculture & Agri-Food Canada, Saskatoon, unpublished data) may have penetrated the cuticle and increased mortality. Although this work suggests that toxins from the pea protein cross the insect cuticle, given the small difference, additional tests are needed to confirm this hypothesis.

The protein-rich pea flour and the pea extract caused the production of more gas in *S. oryzae* midguts than did the purified pea peptides. There are many other compounds in protein-rich pea flour and pea extracts that might work in conjunction with pea peptides to increase gas production. One mechanism to regulate feeding is the stretch receptors on the gut wall which measure the distention of the gut and inhibit feeding control when the gut is full (Bernays and Simpson 1982). It is possible that the bubble produced in the *S. oryzae* midgut triggered these receptors and caused the inhibition of

feeding. The protein-rich pea flour and pea extract reduced feeding dramatically (Chapter Five).

Pea extract and protein-rich pea flour interfered with the digestive system of *S. oryzae,* and caused tissue death in the midgut. This might be caused by the gas stretching the midgut and causing mechanical damage to the cells; or because the pea peptides were directly toxic to the peritrophic membrane or damaged the microvilli of midgut epithelium cells, s is the case with other plant extracts, such as neem (Nogueira et al. 1997) or *Celastrus angulatus* Max (Liu et al. 1998). Pea peptides have an approximate molecular weight of 4000 Da, which is smaller than the toxic protein of *Bacillus thuringiensis* (Slaney et al. 1992). It is possible that the pea peptides penetrate cell membranes aided by other compounds in the pea extract, thus disrupting the normal physiology of insects.

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Table 8.1. LT_{50} (d) (lethal time to kill 50% of the test population) (95% confidence limit) of 20 *S. oryzae* adults treated with 1µl slurry of protein-rich pea flour or wheat flour at various doses without food.

Dose (%)	LT_{50}[1] (d)	
	Protein-rich pea flour	Wheat flour
0	6.1 (5.4-7.1) a	6.0 (5.0-7.0) a
0.01	5.5 (5.1-6.1) a	5.0 (4.7-5.3) b
0.1	5.2 (4.9-5.6) b	5.3 (4.8-5.9) b
0.5	4.8 (4.6-5.0) c	5.0 (4.6-5.3) b

[1] Different letters following LT_{50} s in the same column indicate significant differences in the regression lines between treatments (Kaplan-Meier Survival Analysis, Log-Rank, df =3, experimentwise error = 0.26).

Figure 8.1. The cumulative mortality of *S. oryzae* glued on Petri dishes and brushed with protein-rich pea flour, brushed with wheat flour, brushed with nothing or not brushed. Wheat flour was provided as food for 24 h every second day in brushing treatment and all continuously in the no-brushing treatment. There were three replicates with 20 insects per replicate.

Figure 8.1

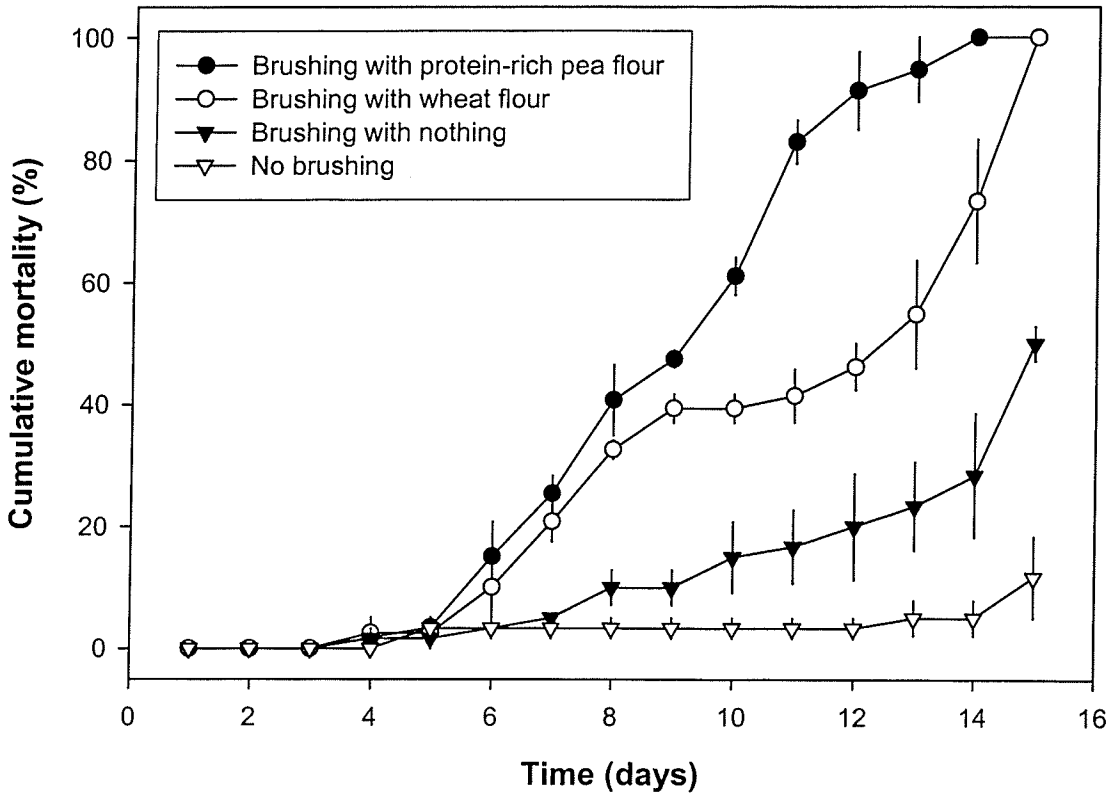
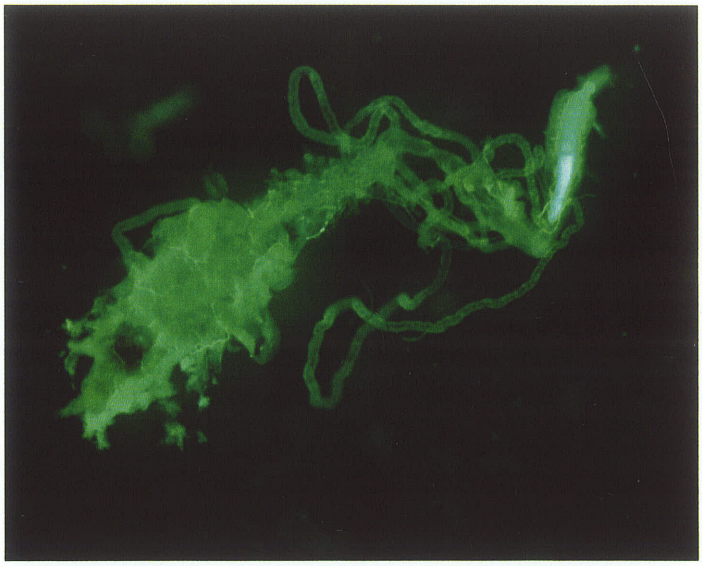
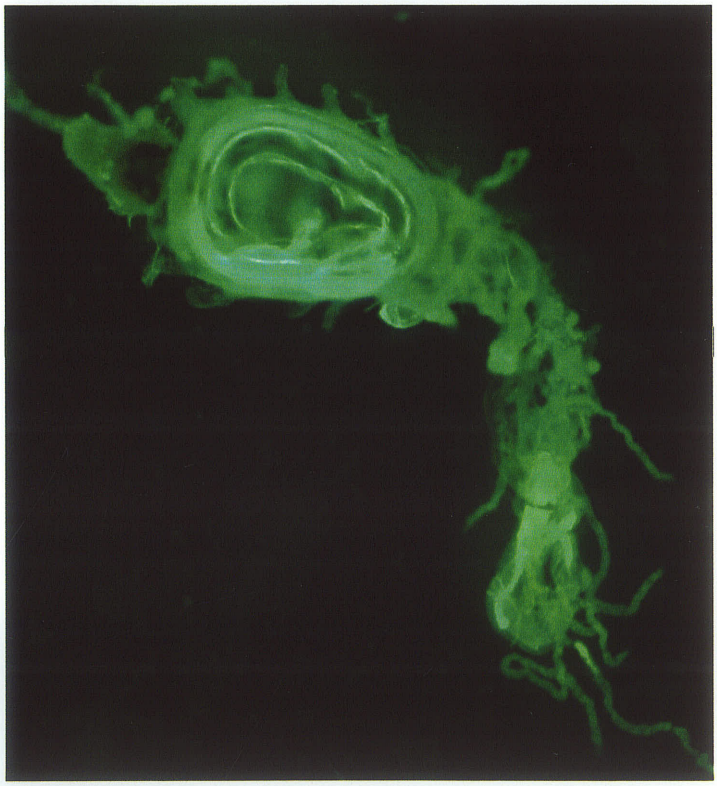

Brushing with protein-rich pea flour
Brushing with wheat flour
Brushing with nothing
No brushing
Cumulative mortality (%)
Time (days)
0
20
40
60
80
100
0
2
4
6
8
10
12
14
16

Figure 8.2. Isolated midguts of *S. oryzae,* showing a gas bubble formed in the midgut in the treatment of protein-rich pea flour. A: fed on wheat kernels; B: fed on protein-rich pea flour for three d.

Figure 8.2



A



B

Figure 8.3. Changes over time of gas volume (mm^3) in the midgut of *S. oryzae* fed on protein-rich pea flour, flour disks mixed with pea extract, purified pea peptides, or unfed.

Figure 8.3

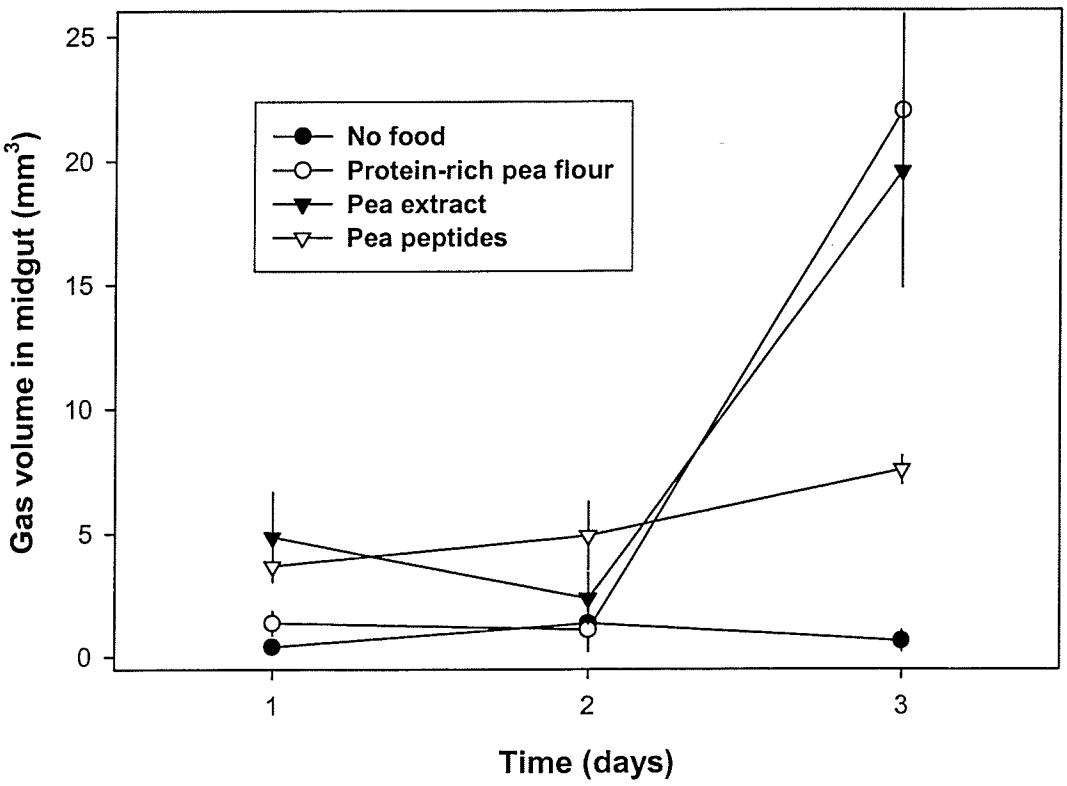

25
20
15
10
5
0
Gas volume in midgut (mm3)
No food
Protein-rich pea flour
Pea extract
Pea peptides
1
2
3
Time (days)

Figure 8.4. The mortality of *S. oryzae* over time when fed on 100% protein-rich pea flour, flour disks mixed with 0.4% pea extract, 0.4% purified pea peptide, or unfed.

Figure 8.4

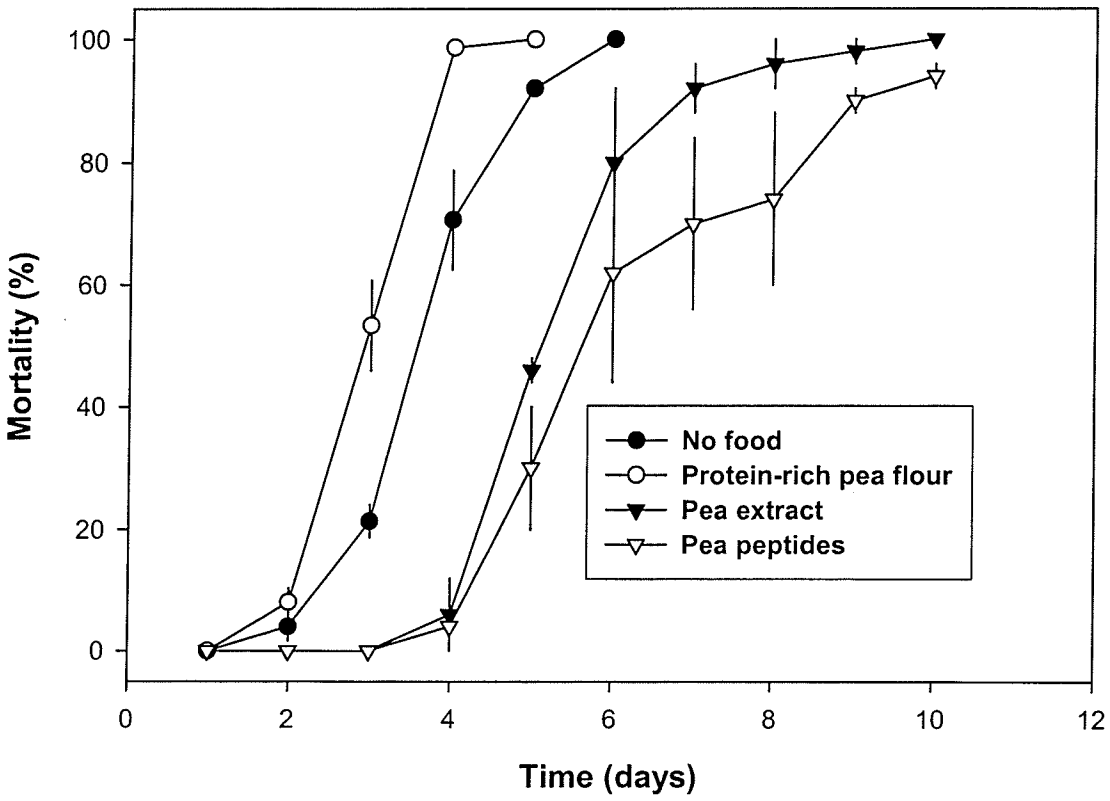
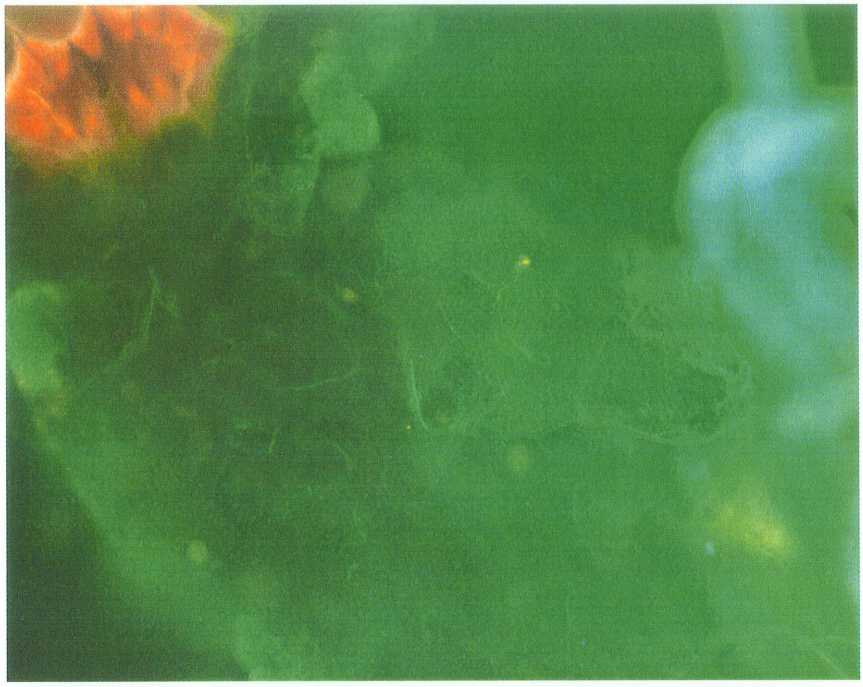
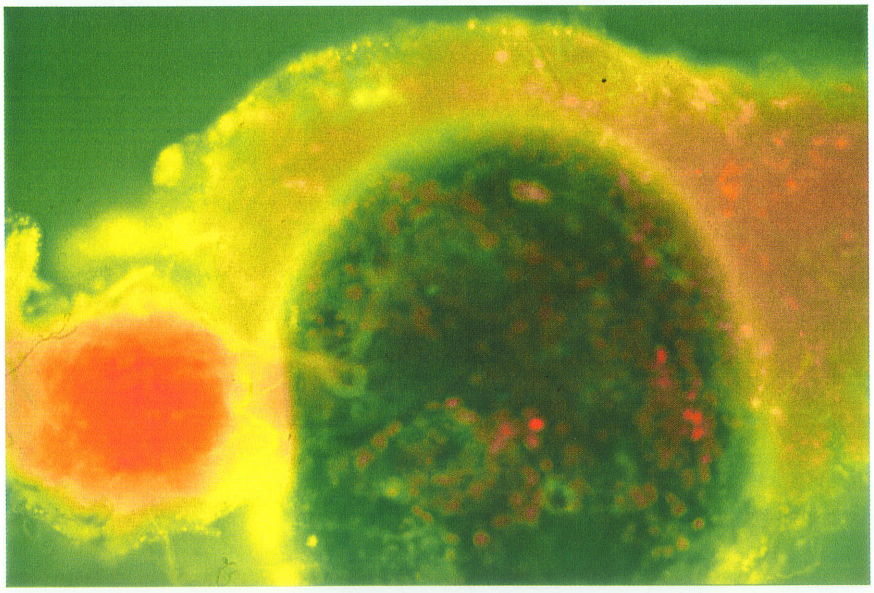

Mortality (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
Time (days)
No food
Protein-rich pea flour
Pea extract
Pea peptides

Figure 8.5 The midgut of *S. oryzae* stained with calcein AM and propidium iodide under a fluorescent microscope. Dead tissue in the midgut fluoresces red color and live tissue fluoresces green color. A: fed on wheat kernel; B: fed on protein-rich pea flour.

Figure 8.5



A



B

CHAPTER 9

General Discussion

The primary purpose of these studies was to evaluate the potential of protein-rich pea flour to control stored-product insects. Protein-rich pea flour is an additive for human food and animal feed. It is also repellent and toxic to many stored-product insects, as well as reducing their offspring production (Bodnaryk et al. 1997, Fields et al. 2001). In this study, the efficacy of protein-rich pea flour was evaluated in laboratory experiments and in a granary trial. It was combined with other chemical and biological agents in an effort to increase its effectiveness. The active components were isolated, and the mode of action was studied.

Factors affecting efficacy

Many factors affected the efficacy of protein-rich pea flour, including insect species, grain species, grain temperature and grain moisture. *Sitophilus oryzae* was the most sensitive insect tested. Its population was reduced approximately 90% in grain treated with 0.1% protein-rich pea flour in both laboratory and granary trials. The main reason for this reduction was death of adult *S. oryzae*. Protein-rich pea flour was less toxic to *C. ferrugineus*. There was no significant reduction of *C. ferrugineus* populations in treatments of 0.1% protein-rich pea flour in sealed containers. But based on the granary trials, a substantial number of insects leave the treated grain. Therefore, I suggest that if insects cannot leave the grain mass, protein-rich pea flour will not be an effective

means to control *C. ferrugineus.* A similar recommendation would hold for *T. castaneum,* which showed very low mortality in treated grain, but was also repelled by protein-rich pea flour. Variations in susceptibility of stored-product insects to insecticides are common for many grain protectants, e.g. deltamethrin, malathion (Snelson 1987) and diatomaceous earth (Fields and Korunic 2000a). Identification of pest species is always important for effective control. Given the wide range in susceptibility, this will be an important factor in any control program using protein-rich pea flour.

A higher dose was required in maize to achieve a similar mortality to that in wheat and barley. This phenomenon was not due to the difference in kernel size of the grain species, because ground particles of the same sizes maintained the difference between maize and wheat. More studies are needed to determine the reasons for the differences observed between grain species.

Unlike pyrethrum which degrades quickly (Quinlan and Miller 1958), protein-rich pea flour was stable in the room environment, which suggests that it can be stored at room temperature and be used as a grain protectant for long-term grain storage. However, storage at high temperature and high moisture should be avoided since reduced efficacy of protein-rich pea flour has been reported after eight months at 30°C, and 70% RH (Bodnaryk et al. 1997).

Protein-rich pea flour was more toxic at high temperature and low grain moisture content. Therefore, I recommend that protein-rich pea flour be used at the beginning of grain storage when grain temperature is high (Loschiavo 1985), and insect population densities are low. Protein-rich pea flour should not be used when moisture content of

grain is higher than 16%, as the dose required to control insects will be significantly greater.

When applied to grain, protein-rich pea flour was repellent to stored-product insects in laboratory experiments and in granary trials. However, insects invaded envelopes containing grain treated with protein-rich pea flour through existing holes (Hou et al. 2003, (Chapter 7)). Insects may have to contact the pea product or feed on it before they are repelled by it.

The repellence of protein-rich pea flour (Fields et al. 2001) cannot be used to protect packaged food (Hou et al. 2003, (Chapter 7)). However, the repellence of protein-rich pea flour was an important attribute for reduction of *C. ferrugineus* and *T. castaneum* in granary trials. The efficacy of protein-rich pea flour in the granary trial with barley was higher than in the barrel experiment with wheat, especially for *C. ferrugineus* and *T. castaneum,* species that were more tolerant to protein-rich pea flour than *S. oryzae*. The populations of these two species were significantly reduced in the granary trial, where emigration was possible. Many *T. castaneum* and *C. ferrugineus* left through the top or bottom of the grain masses treated with protein-rich pea flour. However, their numbers were not reduced in the barrel experiment, where escape was prevented. Although barley was used in the granary test and wheat was used in the barrel test, grain species is not likely to be the factor causing the difference in efficacy as laboratory tests showed that there was no difference between barley and wheat. A storage structures with perforated floors could facilitate the efficacy of protein-rich pea flour by allowing emigration of the insects.

The repellence of protein-rich pea flour could also reduce the chance that insects would immigrate through the perforated floor. Mohan and Fields (2002) reported successful reduction of insect populations by using protein-rich pea flour in a screen-walled granary.

Treating the entire grain mass at 0.1% of protein-rich pea flour was required to achieve a reduction over 90%for *S. oryzae* and over 50% for *T. castaneum* and *C. ferrugineus.* Top-half treatment is not suitable as an application strategy for protein-rich pea flour, although it had a similar effect to treating the entire grain mass on the reduction of insect populations. It failed to prevent insects from moving into the untreated layer of grain, where insects could increase unhindered.

Enhancing efficacy

Compared to neem kernel powder, for which 2-10% is required to control insects (Prakash et al. 1982, Saxena et al 1989), protein-rich pea flour is very effective. However, protein-rich pea flour at 0.1% is still a relatively high dose, if protein-rich pea flour is considered as dockage. Also, at this concentration there is a slight odor, and it is visible on the grain, which may affect grading. Two strategies should be explored to enhance the efficacy of protein-rich pea flour: isolation of the active components or combining protein-rich pea flour with other agents such as insecticides, enzyme inhibitors or biological control agents. Studies on the combination with currently used grain protectants or natural products, including diatomaceous earth, neem, *B. thuringiensis*, malathion and pyrethrum, showed that the effect of combinations was species specific.

None of the combinations was able to control all tested species, and some of the combinations were antagonistic.

Neem had a synergistic effect when combined with protein-rich pea flour against *T. castaneum*. When protein-rich pea flour was combined with diatomaceous earth, it had an additive effect against *S. oryzae*. Before combining neem and protein-rich pea flour, additional work is required to resolve the practical problems of combining liquid formulated neem with powder formulated protein-rich pea flour. There are powdered forms of neem that could be tested to see if similar results can be obtained.

Diatomaceous earth and protein-rich pea flour are both powders and can be mixed easily. Using them as a mixture could reduce concentrations of both products. The main advantages of such a mixture would be the reduction of the negative effects of diatomaceous earth on bulk density and grain handling properties (Korunic et al. 1998).

The mechanism for additive effects when protein-rich pea flour is combined with pyrethrum and for the synergistic effect with malathion is unknown. One possible explanation is that pyrethrum and malathion had been absorbed by the protein-rich pea flour coated the insect body, and may have increased the exposure of *S. oryzae* to pyrethrum and malathion, and thus resulting in higher mortality. Alternatively, protein-rich pea flour is a repellent, and may cause the insect to be exposed to more pyrethrum and malathion by increasing the movement of insects.

Parasitoids can be added to increase the efficacy of protein-rich pea flour, as parasitoids attack the immature stages in the grain kernels, stages that are unaffected by protein-rich pea flour; whereas protein-rich pea flour affected the adults, a stage that is unaffected by parasitoids. In the combination treatment of protein-rich pea flour and

parasitoids, the population of *S. oryzae* adults was reduced by 98%, which was higher than any treatment done in isolation. The combinations also maintained the quality of wheat, whereas neither of the individual treatments did not.

Parasitoids are usually more susceptible to insecticides than their hosts (Schöller and Flinn 2000). This was not the case with protein-rich pea flour. The survival rate, reproduction, and parasitism by the parasitoids, *A. calandrae* and *C. waterstoni,* were not affected by protein-rich pea flour. Why would parasitoids be insensitive to protein-rich pea flour while many stored products insects are adversely affected? The digestive system of *Sitophilus spp.* is adapted to feed almost exclusively on cereals, and may not function properly when exposed to protein-rich pea flour. In addition, *Sitophilus* take protein-rich pea flour directly while feeding on wheat, while the parasitoids feed on insects do not take protein-rich pea flour, and are less affected by the protein-rich pea flour.

Baker (1995) reported a strain of *A. calandrae* that is 2, 800-fold more resistant to malathion than normal susceptible strains, and the inheritance of the resistance is stable in the absence of selection pressure (Baker 1995). Malathion and protein-rich pea flour acted synergistically against *S. oryzae.* This resistant strain of *A. calandrae* could be released together with malathion to further reduce the amount of protein-rich pea flour needed to control *S. oryzae.*

For long-term control of pest populations using parasitoids, it is necessary for the parasitoid populations to become established. This could explain why there was no significant population reduction of *C. ferrugineus* and *T. castaneum* in the large-scale combination treatment. The population of *C. waterstoni* was not well established, and the

two species of parasitoids released do not attack *T. castaneum*. In this study, the abundance, stage and distribution of hosts affected the establishment of parasitoid populations. In the small-scale test, the population of *A. calandrae* was well established in wheat with a high infestation of *S. oryzae,* but not in the moderate and low infestation rate treatments. Correct timing of the release of parasitoids is essential for the establishment of parasitoid populations (Flinn and Hagstrum 1995), because only certain stages of *S. oryzae* and *C. ferrugineus* are suitable for attack by the parasitoids. However, both the single and multiple releases of *A. calandrae* were equally effective at reducing the population of *S. oryzae* in the small-scale test. I also found that the distribution of host may have affected the establishment of parasitoids. More studies are required to determine the optimum release time, release ratio and the effect of environmental factors for the establishment of parasitoids.

One limitation of protein-rich pea flour for controlling stored-product insects in commercial granaries is that *R. dominica*, a major stored-product insect pest, is not controlled by 0.1% pea flour (Bodnaryk et al. 1997). *Anisopteromalus calandrae* is a generalist parasitoid. It parasitizes a wide range of coleopeteran and lepidopertan stored-product insects (Schöller and Flinn 2000). *Anisopteromalus calandrae* depresses the population of *R. dominica* (Ahmed 1996) and other insect pests. Granary trials are required to determined if parasitoids can be combined with protein-rich pea flour to control *R. dominica.*

Mode of action

There has been no research on the mode of action of peas, protein-rich pea flour or its extract in insects. I studied this question using detoxification enzymes, and histology to examine the damage to insect tissues. Mixed function oxidases and hydrolases are usually involved in the detoxification of xenobiotics in insects (Dowd et al. 1983, Krieger et al. 1971, Ahmad 1982). If these enzymes are also involved in the detoxification of protein extract, inhibition of these enzymes would lower the insect defense system thereby increasing the toxicity of the insecticide (Ishaaya 1993). There are a few strains of *S. oryzae* that can develop on yellow split peas (Coombs et al. 1977). The mortality of these strains increase when the mixed function oxidase inhibitor, piperonyl butoxide and the esterase inhibitor tributylphosphorotrithioate are mixed with yellow split pea (Holloway and Mackness 1988). In this study, piperonyl butoxide could be used to increase the efficacy of pea extract by reducing feeding and increasing the mortality of a susceptible laboratory strain of *S. oryzae.* However, we only infer that piperonyl butoxide is involved in the detoxification of pea extract in the susceptible strains, as piperonyl butoxide alone also increased the mortality of *S. oryzae*. The glutathion-S-transferase inhibitor, diethyl maleate, did not enhance the antifeedant effect or toxicity of the pea extract. This implies that glutathion-S-transferase is not involved in the detoxification of pea extract in susceptible *S. oryzae*.

In addition to the direct toxic, repellent and reproductive effects (Bodnaryk et al. 1997, Fields et al. 2001, Hou and Fields 2003a, 2003b (Chapter 3, 4)), pea extract reduced the feeding of *S. oryzae.* Starvation may be involved in the mortality of *S.*

oryzae. My results showed that the higher the dose of pea extract, the less *S. oryzae* ate, and the sooner they died. Pea peptides and other compounds in pea extract and protein-rich pea flour produced gas in the midgut of insects, and the gas volume increased with increased exposure. Fluorescent vital staining showed that the tissue of the gut was injured. The gas in the midgut might affect feeding. One mechanism to regulate feeding is that stretch receptors on the gut wall measure the distention of the gut, and inhibit feeding when the gut is full (Bernays and Simpson 1982). It is possible that the bubble produced in the *S. oryzae* midgut triggered these receptors, and caused the inhibition of feeding.

Potential as a grain protectant

Based on the information I have obtained, protein-rich pea flour could be used for control of *S. oryzae*. Diatomaceous earth, pyrethrum, malathion and *A. calandrae* can be combined to increase the efficacy of protein-rich pea flour. It may also be effective in controlling *Sitophilus granarius* (L.) and *Sitophilus zeamais* (Motschulsky), since laboratory data showed no difference among these three *Sitophilus* species (Bodnaryk et al. 1997). However, granary studies would be needed to verify this prediction. Protein-rich pea flour would have to be used in a storage structure that allows emigration of insects to effectively control *C. ferrugineus* and *T. castaneum*. A combination with neem should greatly increase the efficacy of protein-rich pea flour against *T. castaneum*.

Unlike diatomaceous earth, that reduces the bulk density of grain (Korunic et al. 1998, Fields and Korunic 2000b) and is harmful to parasitoids (Perez-Mendoza et al. 1999), protein-rich pea flour maintained the bulk density, moisture content and dockage

of grain, and was not toxic to parasitoids. Compared with many botanical insecticides, there is an ample supply of peas, which are available to many farmers who may be too poor to afford chemical insecticides. The world annual production of peas is approximately 12 million tons (Skrypetz 2001). The efficacy of protein-rich pea flour was stable at room temperature, which is an important feature for long-term storage of a grain protectant. To date, there are no data on the mammalian toxicity of protein-rich pea flour, pea extract or pea peptides. However, protein-rich pea flour is consumed by humans and animals. It would be reasonable to assume protein-rich pea flour has low mammalian toxicity.

More studies are needed to improve the application of protein-rich pea flour to grain. It is inconvenient to treat stored grain with powders. Additional studies should investigate if a pea-based insecticide could be applied as a solution or a slurry. In addition, the effect of protein-rich pea flour on grain quality, for example grading or baking, should be evaluated. Further studies should explore the loss of repellency during the isolation procedure of pea extract from protein-rich pea flour.

Before a pea extract could be registered for commercial use, mammalian toxicity needs to be determined. Even though it is isolated from a food product, its toxicity may change when used in isolation or used at high concentrations. Although pea extracts are more toxic to insects than protein-rich pea flour (Bodnaryk et al. 1997), granary trials are required to determine if these concentrated extracts will control insects under commercial storage conditions.

The development of resistance to insecticides has been observed in many insects (Ware 2000, Subramanyam and Hagstrum 1995, Cochran 1995). It also poses a problem

for a pea-based insecticide, as there are tropical strains of *S. oryzae* that feed on peas (Coombs et al. 1977). This resistant ability is controlled by a single recessive, autosomal gene (Grenier et al. 1997). A plan to deal with resistant strains must be developed, if pea-based insecticides are to be a useful tool for grain storage.

CHAPTER 10

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