5

Effects of Dietary Soybean Trypsin Inhibitors on Detection of Resistance to Pyrethroid and Spinosad Insecticides in *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae)

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1. Introduction

During the last 50 years, worldwide use of synthetic insecticides to control insect pests has led to both insecticide resistance and environmental problems (Roush and Tabashnik, 1990). *Helicoverpa armigera* (Hübner) is arguably Australia's most important agricultural pest and insecticide resistance remains an enduring threat. Throughout its history, Australian *H. armigera* has been shown to evolve a range of mechanisms to confer resistance to insecticides (Gunning et al., 1991; Gunning et al., 1996a; Gunning et al. 1996b). Therefore it is vital that any resistance monitoring programme must not only be able to detect resistance at low frequencies but also be able to detect resistance resulting from any number of potential mechanisms. Pyrethroid and spinosad resistance in Australia are known to be caused by sequestration and or hydrolysis of non-specific esterases (Gunning et al., 1996b; Gunning et al., 2007; Gunning & Balfe 2002).

Soybean based diets are common artificial diets for *Heliothine* species. To avoid chronic exposure to artificially high levels of protease inhibitors in artificial insect diets, it is usual to heat-treat legume components to denature the inhibitors (Shorey & Hale, 1965; Teakle and Jensen, 1985). However, not all laboratories follow this regime, and while some laboratories heat-treat legume components of diets, other laboratories involved in *Heliothine* rearing, use or have used, raw soybean flour. There are also a variety of procedures used to heat treat soybean trypsin inhibitors in artificial diet preparation, which may result in varying degrees of protease inhibitor degradation.

Leguminous seeds, such as those from soybean, are protected against herbivores, including insects, by anti-nutritional defence proteins- trypsin inhibitors, which are found in the seeds and raw flour made from the seeds. Levels of these plant protease inhibitors are high in legume seeds, comprising 1-5% of total protein (Macintosh et al., 1990; Sastry et al., 1987) Trypsin inhibitors of the Kunitz type (Kunnitz, 1945a; Kunnitz, 1945b) are single chain polypeptides (~ 20 kDa) that act on target serine proteases in the gut forming a 1:1 complex. Perhaps the best known of these inhibitors is Kunitz soybean trypsin inhibitor (KSTI).

Midgut serine protease activity has been found in a wide variety of Lepidopteran pests, including *H. armigera,* where trypsin is a major protease in the *H. armigera* midgut (Macintosh et al., 1990). In *H. armigera*, KSTI acts against gut serine proteases and are larval growth retardants and may cause death through the prevention of protein digestion (Johnston et al., 1993; Wang et al., 1995).

KSTI and other potent trypsin inhibitors are also known to have synergistic effects with delta endotoxins of *Bacillus thuringiensis*. This has been shown in Lepidoptera (*H. armigera*, *Manduca sexta, Heliothis virescens, Trichoplusia ni* and *H. zea*), as well as *Leptinotarsa decemlineata* (Colorado potato beetle) (Macintosh et al., 1990; Shao et al., 1998; Zhang et al., 2000, Zhu et al., 2007; Christtellier et al., 1992; Hubert et al., 2008). A recent study has shown that raw soybean diets can prevent the detection of non-specific esterase mediated resistance to *Bacillus thuringensies* toxins in *H. armigera* (Gunning & Moores, 2010).

In addition to Bt toxin, non-specific esterases in Australian *H. armigera* are also known to confer resistance to pyrethroids and spinosad by sequestration and/or hydrolysis (Gunning et al., 1996b; Gunning et al., 2007; Gunning & Balfe 2002). This work therefore examines the effects of raw or heat denatured soybean flour artificial diets on the detection of spinosad and pyrethroid resistance in *H. armigera*. The efficacy of two commonly used methods of denaturing soybean trypsin inhibitors (dry roasting soybean flour and boiling soybean flour with water prior to diet incorporation) were also compared.

We conclude that detection of spinosad and pyrethroid (bifenthrin) resistance in *H. armigera* can be masked if an artificial diet gives chronic exposure to potent trypsin inhibitors present in raw soybean flour and that boiling soybean flour does not achieve the temperature required to effectively denature soybean trypsin inhibitors.

2. Materials and methods

2.1 Insects

The *H. armigera* strains used were a susceptible strain (susceptible), and pyrethroid and spinosad resistant strains. The resistant strains Spin-R and Pyr-R (derived from survivors of resistance monitoring) were known have non-specific esterase mediated resistance to spinosad and pyrethroids respectively. A multi-resistant field strain (Breeza Field) collected off cotton at Breeza, NSW was also used.

2.2 Rearing methods

Helicoverpa armigera larvae were reared on a diet modified from that of Shorey and Hale (Shorey & Hale, 1965). The diet was altered by the substitution of dry roasted soybean flour for pinto beans and the addition of wheat germ and propionic acid. The artificial diet comprised (A) soybean flour (Allied Mills) (450 g), roasted in a microwave oven on full power for 5 minutes (temperatures in excess of 200^{o}C are achieved), wheat germ (Allied Mills) (120g), brewers yeast (Phytofoods) (105 g), ascorbic acid (Phytofoods) (10.1 g) and nipagen (3.3g), sorbic acid (3.3g), thiabendazole (0.8 g) and streptomycin sulfate (0.2g) (all obtained from Sigma); (B) agar grade J3 (Gelita) (45 g) and H_20 (1200 ml), (C) formaldehyde (40%) (BDH) (6 ml) and H_20 (1500 ml); (D) propionic acid mix (propionic acid (42%) / 4% phosphoric acid (BDH) (8 ml). Ingredients (A), (C) and (D) were blended together, the agar and cold water of (B) were mixed and brought to the boil, cooled to 70^0 C and then blended with the other ingredients till smooth. The diet was poured into a shallow tray and allowed to set.

Helicoverpa armigera were reared in an insectary at 25^0 C, 70% relative humidity, in natural light. Adults were kept in tall plastic cages with shredded paper at the base and cloth lids (nappy liners), on which the eggs were collected. The moths were fed on a honey solution (5%). The eggs were surface sterilised in a sodium hypochlorite (Coles Supermarkets) solution (0.1%) and allowed to dry. Eggs were sealed into plastic, ventilated containers with a small amount of rearing diet, allowed to hatch and develop to 2nd instar. Second instar larvae were transferred to a block of diet (~2g) in 32 well trays (CD International), sealed with vented, adhesive lids (CD International) and reared to pupation. Pupae were removed from the diet, sterilised in sodium hypochlorite solution (0.1%), dried and transferred to moth cages.

2.3 Diets for bioassays

For pyrethroid and spinosad bioassays, three forms of the artificial diet were prepared. The standard diet (described above) incorporating dry roasted soybean flour, a second diet incorporating raw soybean flour or a third diet in which 450 g soybean flour was first boiled for 4 min in 1500 ml of water before being incorporated with rest of diet ingredients (A, C and D) above.

2.4 Insecticides and bioassay

Insecticides used were technical grade bifenthrin (Crop Care) and spinosad (Dow), dissolved and serially diluted in acetone.

The larval bioassay procedure utilised topical application, similar to that recommended by the Entomological Society of America (Anon, 1970). Technical-grade material was dissolved in acetone and 5 - 6 serially diluted concentrations were prepared. Three replicates of 10, 30-40 mg third instar larva were treated at each dose by applying 1 µl of solution to the dorsal thorax of each with a microapplicator (Hamilton).

Following treatment, the larvae were maintained individually at 25 ± 1^0C in bio-assay trays (Bio-BA-1280©, C-D International, Inc., Pittman, N.J. USA, 609-5832-2392) and supplied with adequate diet. Mortality was assessed at 48 hours and 72 hours after treatment for bifenthrin and spinosad respectively. Larvae were considered dead if they were unable to move in a coordinated manner when prodded with a blunt probe. There was no control mortality. Data were analysed by Probit Analysis (Finney, 1970) and resistance factors calculated from the ratio of the resistant strain LD_{50} and the LD_{50s} / susceptible strain LD_{50} and the $LD_{99.9s}$.

3. Results

3.1 Spinosad

Log dose probit data for spinosad bioassays are shown in Table 1. The treatment of soybean flour in the three rearing diets made no significance to spinosad toxicity in the spinosad susceptible strain. On a diet made from dry roasted soybean flour, the spinosad resistant strain was found to be 62 and 378 fold resistant to spinosad at the LD_{50} and the $LD_{99.9}$ levels respectively. The field strain was 7 and 131 fold resistant at the LD_{50} and the $LD_{99.9}$ levels respectively. Bioassays on the raw soybean flour diet and boiled soybean flour, however, failed to detect resistance, with the LD_{50} and $LD_{99.9}$ levels in the resistant and susceptible strains not being significantly different.

3.2 Bifenthrin

Data for bifenthrin are shown in Table 2. The treatment of soybean flour in the three rearing diets again resulted it no significant effect to bifenthrin toxicity in the pyrethroid susceptible

strain. In the pyrethroid resistant strain, high levels of resistance to bifenthrin were recorded from larvae reared and bioassayed on the diet prepared with dry roasted soybean flour. Resistance factors were 284 and 320 fold at the LD_{50} and the $LD_{99.9}$ levels respectively. Larvae from the field strain were 37 and 154 fold at the LD_{50} and the $LD_{99.9}$ levels respectively. Low levels of bifenthrin resistance (13.3 and 10.4 fold at the LD_{50} and the $LD_{99.9}$ levels respectively) were detected in the bifenthrin resistant strain on a raw soybean flour diet, but significant resistance was not detected in the field strain. Significant bifenthrin resistance was not detected in either the field or pyrethroid resistant strain when soyflour was boiled prior to incorporation into the *Helicoverpa* diet mix.

Strain	Soybean flour treatment	χ^2	Slope	LD_{50} (95 %fiducial limits)	Resistance factor	$LD_{99.9}$ (95 %fiducial limits)	Resistance factor
Susceptible	Dry roasted	0.5	3.1	0.29 (0.16 – 0.34)	1.0	1.6 (0.17 – 3.1)	1.0.
Spin-R	Dry roasted	0.43	2.1	10.0 (13.0– 21.0)	62	605 (153 – 2390)	378
Breeza Field	Dry roasted	5.8	1.5	1.9 (1.1- 3.2)	7	210 (45 – 910)	131
Susceptible	Raw	0.45	3.0	0.28 (0.15 – 0.34)	1.0	1.5 (0.19 – 3.2)	1
Spin-R	Raw	0.5	3.2	0.29 (0.16 – 0.33)	1.0	1.7 (0.14 – 3.2	1.1
Breeza Field	Raw	1.2	3.1	0.29 (0.06 – 0.14)	1.0	1.8 (0.19 – 3.2)	1.2
Susceptible	Boiled	0.4	3.2	0.28 (0.16 -0.33)	1.0	1.5 (0.2 - 3.3	1
Spin-R	Boiled	1.1	3.0	0.29 (0.14 – 0.37)	1.0	1.8 (0.25 – 4.0)	1
Breeza Field	Boiled	0.6	3.0	0.3 (0.13 – 0.39)	1.1	1.7 (0.16 – 3.2)	1.1

Table 1. The effects of differing dietary soybean flour treatments on response of third instar (30 – 40 mg) *Helicoverpa armigera* larvae to topically applied spinosad.

Strain	Soybean flour treatment	χ^2	Slope	LD_{50} (95 %fiducial limits)	Resistance factor	$LD_{99.9}$ (95 %fiducial limits)	Resistance factor
Susceptible	Dry roasted	1.5	3.8	0.03 (0.01 – 0.05)	1	0.125 (0.08 – 1.6)	1
Pyr-Resistant	Dry roasted	0.62	4.6	8.5 (6.7 – 10.1)	284	40.0 (31 -66)	320
Breeza Field	Dry roasted	1.9	1.9	1.1 (0.7 – 1.9)	37	19.2 (6.6 – 56)	154
Susceptible	Raw	1.5	3.8	0.03 (0.01 – 0.05)	1	0.11 (0.07 – 1.6)	1
Pyr-Resistant	Raw	0.8	3.6	0.4 (0.1 – 0.6)	13.3	1.30 (0.5 – 4.2)	10.4
Breeza Field	Raw	1.1	3.5	0.06 (0.01 – 0.07)	2	0.15 (0.05 – 1.6)	1.2
Susceptible	Boiled	1.5	3.6	0.03 (0.01 – 0.05)	1	0.120 (0.06 – 1.9)	1
Pyr-Resistant	Boiled	1.1	3.0	0.12 (0.08 – 0.47)	4	0.5 (0.20 – 3.5)	4.3
Breeza Field	Boiled	0.7	3.3	0.04 (0.01 - 0.07)	1	0.14 (0.07 – 1.9)	1.1

Table 2. The effects of differing dietary soybean flour treatments on response of third instar (30 – 40 mg) *Helicoverpa armigera* larvae to topically applied bifenthrin.

4. Conclusion

The data show that treatment of soybean flour in the preparation of *Helicoverpa* rearing diet greatly influenced the observed mortality following topical application of spinosad and bifenthrin in the *H. armigera* resistant strains. Diet prepared from raw soybean flour and soybean flour that had been boiled prior to diet incorporation, hindered detection of spinosad and pyrethroid resistance in strains known to be highly resistant. In particular, these diets interfered in the detection of non-specific esterase mediated resistance to spinosad and pyrethroids (bifenthrin).

The protease inhibitors present in soybean seeds, including the KSTI-like inhibitors, are reversibly denatured and require prolonged heating at high temperatures to bring about irreversible denaturation (Kunitz, 1948). KSTI is highly resistant to thermal and acidic denaturation (Roychaudhuri, 2003). Thus any resistance to xenobiotics that is conferred by enhanced serine hydrolases (i.e. esterases) could be compromised by the presence of KSTI-like inhibitors in the diet.

A previous study (Gunning & Moores, 2010), showed that a raw soybean flour diet prevented detection of non-specific esterase mediated resistance to *Bacillus thuringensis* toxins in *H. armigera*, whereas resistance was detectable in larvae reared on a roasted soybean flour diet. The difference was attributed to synergism by dietary KSTI or other inhibitors in the raw flour diet. These data are consistent with that of the present study, where there were observed differences between ability to detect an esterase mediated

resistances (spinosad and pyrethroid) in *H. armigera* on raw soybean flour and roasted soybean flour diets.

Given that KSTI is remarkably resistant to thermal denaturation and that denaturation is readily reversible at lower temperatures, it is likely that failure to detect resistance on a diet made from boiled flour is due inadequate denaturation of KSTI and/or other trypsin inhibitors. We observed that a soybean flour/ water mix boiled at 40^{o}C, which is far below the temperature required to permanently denature KSTI (Kunitz, 1948). It is also possible that boiling the soybean flour in water solubilises the tyrpsin inhibitors.

KSTI and other trypsin inhibitors are known to have synergistic effects with delta endotoxins of *Bacillus thuringiensis* against *H. armigera* and other insects (*Manduca sextai, Heliothis virescens, Trichoplusia ni, Helicoverpa zea*) and *Leptinotarsa decemlineata* (Colorado potato beetle) (Macintosh et al., 1990; Shao et al., 1998; Zhang et al., 2000, Zhu et al., 2007; Christtellier et al., 1992; Hubert et al., 2008). It was suggested that the mechanism of synergism might be prevention of activation of the toxin by gut serine proteases due to KSTI action (Zhu et al., 2007). In Australian *H. armigera,* differences in detection of resistance to Bt toxin between raw and dry roasted soybean flour were attributed to synergism by dietary KSTI in the raw flour diet. It was also suggested that greater susceptibility of cotton populations of *H. armigera* to Cry1Ac, compared to *Helicoverpa punctigera*, might have been due to dietary STI inhibition of detoxificative enzymes in *H. armigera* (Bird & Akhurst, 2007). The present data suggest that KSTI and/or other trypsin inhibitors in soybean flour may also act as spinosad and pyrethroid synergists against non-specific esterase mediated resistance in *H. armigera*.

Non-specific esterases, derived from cell adhesion proteins, are serine hydrolases with an ability to sequester, pyrethroids, spinosad and Bt toxins in Australian *H. armigera* (Gunning et al., 1996b; Gunning et al., 2007; Gunning & Balfe, 2002, Gunning et al., 2005;). These resistances can be synergised by esterase inhibitors (Gunning et al., 1999, Young et al., 2006, Gunning et al., 2007). It is possible that these non-specific esterases, found in the *H. armigera* midgut, are inhibited by dietary protease inhibitors such as KSTI, found in a raw or incompletely denatured soybean flour diet.

This study has indicated that rearing and bioassay of *H. armigera* on artificial diets that have been inadequately treated and thus give chronic exposure to abnormally high levels of active soybean protease inhibitors may be unsuitable for detection of metabolic-based resistance to spinosad or pyrethroids (bifenthrin).

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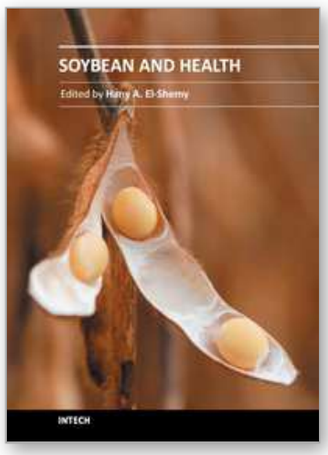

Soybean and Health

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Worldwide, soybean seed proteins represent a major source of amino acids for human and animal nutrition. Soybean seeds are an important and economical source of protein in the diet of many developed and developing countries. Soy is a complete protein, and soy-foods are rich in vitamins and minerals. Soybean protein provides all the essential amino acids in the amounts needed for human health. Recent research suggests that soy may also lower risk of prostate, colon and breast cancers as well as osteoporosis and other bone health problems, and alleviate hot flashes associated with menopause. This volume is expected to be useful for student, researchers and public who are interested in soybean.

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