Effects of Proteinase Inhibitors and Plant Lectins on the Adult Alfalfa Weevil (Coleoptera: Curculionidae)[1]

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Abstract The effects of selected proteinase inhibitors and plant lectins of alfalfa weevil, *Hypera postica* (Gyllenhal), adult foliar feeding and fecundity were significantly inhibited by the cysteine proteinase inhibitors E-64, pHMB, and leupeptin at a concentration of 0.1%. Pepstatin (aspartic inhibitor) at 0.5% and soybean Bowman-Birk trypsin inhibitor (serine) at 1.0% had no significant effect on adult foliar feeding, survival, or fecundity. Three of the four lectins tested significantly inhibited adult foliar feeding and fecundity at a concentration of 0.5%. A lectin from wheat and one from pea were the only two protein inhibitors tested to significantly inhibit adult survival. Results support a previous study that indicates the alfalfa weevil uses cysteine proteinases as major digestive enzymes. This study is one of few which demonstrates the effects of specific protein inhibitors on the adult stage of a foliar feeding insect species.

Key Words *Hypera postica,* alfalfa weevil, proteinase inhibitors, lectins, adults

Genetically-engineered plants expressing insecticidal proteins offer a novel method of insect control. DNA sequences encoding foreign proteins can now be integrated into the genome of plants. Expression of the insect-control protein genes of *Bacillus thuringiensis* Berliner in cotton, corn and potatoes have led to the development and release of insect resistant cultivars (Grooms 1992). Transgenic plants expressing proteins of proteinase inhibitors (Leple et al. 1995), alpha-amylase inhibitors (Shade et al. 1994), and lectins (Gatehouse et al. 1997) have been developed and point out the potential of these plant defensive genes for insect control.

Proteinases are enzymes involved in the degradation of natural proteins. The digestive proteinases of insects catalyze the release of free amino-acids from dietary protein and provide a supply of nutrients essential for normal growth and development (Broadway and Duffey 1986). There are four known classes of proteinases: serine, cysteine (thiol), aspartic (carboxyl), and metallo-proteinases (Barrett 1986). Serine proteinases are the most studied and have been found in Coleoptera, Lepidoptera, Orthoptera, and Diptera; cysteine proteinases have been in Coleoptera and Hemiptera; and aspartic proteinases are known from Coleoptera, Hemiptera, and Diptera (Oppert et al. 1993, Coppedge et al. 1994). These reports indicate a wide range of diversity in midgut proteolytic activity among insect species.

Plant lectins are carbohydrate-binding proteins commonly referred to as agglutinins, which may play a role in the plant's defense against fungal and insect attack (Chrispeels and Raikhel 1991, Peumans and Van Damme 1995). Lectins from dif-

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ferent plant species often differ with respect to their molecular structure and specificity. The mode of action exerted by lectins is unknown, but may involve binding to glycoproteins present on insect midgut epithelial cells resulting in a disruption of cellular function (Harper et al. 1995). Insecticidal activity of plant lectins has been reported in laboratory bioassays on Coleoptera and Lepidoptera (Czapla and Lang 1990, Harper et al. 1995), Diptera (Eisemann et al. 1994), and Homoptera (Powell et al. 1993, Rahbe et al. 1995).

The effects of specific protein inhibitors on insects have been determined primarily by either their *in vitro* activity on the insect's gut homogenates, or by measuring insect growth and development on artificial diets containing the inhibitors (Murdock et al. 1987, Oppert et al. 1993). Discrepancies in levels and types of inhibition between feeding studies using artificial diets and *in vitro* midgut analyses have been demonstrated (Broadway and Duffey 1986, Purcell et al. 1992). Limited *in vivo* bioassays for phytophagous insects, using an insect preferred-host, have been conducted (Murdock et al. 1987, Wolfson and Murdock 1995, Elden 1995a). *In vivo* bioassays can determine if there is a good association with studies using artificial diets or *in vitro* biochemical assays and provide a more realistic estimation of inhibitor concentrations needed to be effective in transgenic plants.

Most studies on phytophagous insect dietary proteins and their inhibition have dealt with the larval stage of major pests. This is understandable, because the larva is generally the major consumer of plant protein and the target stage when developing transgenic plants with resistance based on protein inhibition. In some cases, the adult stage of an insect species may be equally as destructive to the plant or make a significant contribution to its demise. Because the use of protein inhibitors as a tool to control insect pests is still in its infancy, studies investigating adult insect proteins and possible inhibitors are limited and included in the following paragraph.

Murdock et al. (1988) demonstrated that the fecundity of the cowpea weevil, *Callosobruchus maculatus* (F.), emerging from artificial seeds treated with E-64, a cysteine proteinase inhibitor, was significantly decreased. E-64 also was shown to have a negative effect of the reproduction potential of the Mexican bean beetle, *Epilacna varivestis* Mulsant, when fed treated lima bean leaves (Wolfson and Murdock 1995). General midgut proteinase activity of the adult southern pine beetle, *Dendroctonus frontalis* Zimmermann, was significantly inhibited *in vitro* by cysteine and serine proteinase inhibitors (Coppedge et al. 1994). Murdock et al. (1987) reported cysteine proteinase activity from the adult midguts of two species of Coleoptera as well as from the adult midguts of two species of plant sucking bugs (Hemiptera). A cysteine proteinase (cathepsin) was identified in the adult midgut of six species of Hemiptera (Houseman and Downe 1983). In Homoptera, several plant lectins acted as antifeedants when fed to the adult rice brown planthopper, *Nilaparvata lugens* (Stal), in artificial diets (Powell et al. 1995) and adult peach-potato aphid, *Myzus persicae* (Sulzer), fecundity was significantly reduced on lectin-containing artificial diets (Sauvion et al. 1996). Fecundity of two species of adult horn flies, blood-feeding Diptera, was significantly reduced when fed artificial diets containing the proteinase inhibitors leupeptin (cysteine) or soybean trypsin (serine) (Spates and Harris 1984). Trypsin activity was detected in midguts of sexually-developed adults from four species of *Anopheles* mosquitoes (Horler and Briegel 1995).

As with the larval insect stage, adult midgut proteolytic activity also appears to vary from species to species which again emphasizes the necessity of individually characterizing protease activity in the specific insect of interest if the development of

transgenic insect resistant germplasm based on protein inhibitors is the targeted control strategy.

In a previous study Elden (1995a) investigated the effects of selected proteinase inhibitors on alfalfa weevil larval feeding, growth, and development when fed treated alfalfa leaves. The objective of the current study was to determine the effects of selected proteinase inhibitor and plant lectins on alfalfa weevil adult foliar feeding, survival, and fecundity.

Materials and Methods

Alfalfa weevil adults used in this study were first-generation, laboratory-reared, derived from eggs of nondiapausing field-collected adults as described by Ratcliffe and Elgin (1987). To assure test adults were not in a reproductive diapause, newly-emerged adults were fed alfalfa foliage for ≈8 d, placed in cold storage at 5°C on 2% sugar water for >8 wk, removed from storage and fed alfalfa foliage for >3 wk prior to the beginning of a test (Elden 1995b). All insects were reared and tested in a walk-in environmental growth chamber maintained under a photoperiod of 8:16 (L:D) h at 24 ± 1°C and 50-90° RH.

Proteinase inhibitors and lectins were incorporated into a 6.5% gelatin solution and applied to excised alfalfa trifoliolate leaves as described by Elden (1995a). Alfalfa stems ('Saranac AR') of the same size and age were excised from greenhouse grown plants in the vegetative stage of development (≈3 wk after cutting) and placed in beakers of tap water to prevent wilting. Alfalfa trifoliolates with ≈2 cm of petiole were excised from the stems and the petiole inserted through a 1.5-mm hole in the polyethylene cap of a 1-dram shell vial filled with water. Two 8-cm length sections were cut from the base of each stem and individually inserted through a 3-mm hole in the cap of a 1-dram shell vial filled with water, leaving 4 cm of stem exposed for alfalfa weevil females in which to oviposit.

All three leaflets of a trifoliolate were then coated with the designated protein-gelatin solution and allowed to dry at ambient room temperature. Two controls were used in most tests. One control was treated with plain 6.5% gelatin and the other received no treatment. After drying, individual trifoliolates were placed in glass Petri dishes (100 × 20 mm) along with a 4 cm exposed stem section and infested with 2 female alfalfa weevil adults. A total of 8 dishes (replications) per treatment was used in each test.

At 3, 6, and 10 d after the beginning of a test, trifoliolates and stem sections in all treatments were changed, and the amount of leaf material consumed, adult survival, and fecundity recorded. Fourteen days after infestation, tests were terminated and variables again recorded to give data for four dates. Leaf feeding was estimated by rating the amount of foliage consumed from all three leaflets of a trifoliolate on a 0 to 12 leaf feeding index (0, no feeding; 12, completely eaten). The leaf feeding index values were converted to and reported as the percentage of total leaf area consumed per 2 adults. Adult survival is reported as the percentage of living adults and fecundity is reported as eggs per female per day.

The protein inhibitors grouped by class and their weight percentage concentrations (wt:wt) used in this study were as follows: cysteine proteinase inhibitors, *trans*-epoxy-succinyl-L-leucylamido-(4 guanidino)-butane [E-64] (0.1%) and p-hydroxy-mercuribenzoic acid [pHMB] (0.1%); cysteine and serine proteinase inhibitor, leupeptin (0.1%); aspartic proteinase inhibitor, pepstatin (0.5%); aspartic and serine protein-

ase inhibitor, antipain (0.5%); and serine proteinase inhibitor, soybean Bowman-Birk trypsin-chymotrypsin inhibitor (1.0%). Plant lectins included wheat germ agglutinin [WGA] (0.5%), pea agglutinin [PSA] (0.5%), and two bean phytohemagglutinins [PHA-E and PHA-L] (0.5%). All compounds were purchased from Sigma (St. Louis, MO).

Five tests were conducted in this study. E-64 and the untreated control were included in all tests. Other treatments were included in 1 to 4 tests. The experimental design for each test was a randomized complete-block with eight replications. Mean values of each test were combined and data were analyzed as an incomplete-block design. Least-square means and pairwise comparisons were estimated using the SAS general linear model procedure (SAS Institute 1988). Partial correlations between dependent variables, adjusted for model effects, are reported. Significance is reported at the 5% level. The arcsine square-root transformation was used to account for nonadditivity and to stabilize the variance. Data are presented as back transformed least-square means.

Results and Discussion

Percentage of alfalfa weevil adult foliar feeding, survival, and fecundity, as influenced by selected proteinase inhibitors and plant lectins, are presented in Table 1. Data for the untreated control and gelatin control were approximately the same, indicating that the 6.5% gelatin used as a base in all treatments had no influence on alfalfa weevil adult feeding, survival, or fecundity. Comparisons discussed below are between a specific treatment and the controls. Table 1 reports comparisons among all treatments.

The main treatment effect for adult survival was not significant. Main treatment effects for adult foliar feeding ($P < 0.001$) and fecundity ($P = 0.03$) were significant. Adult foliar feeding was positively correlated with adult survival ($r = 0.40$, $P < 0.001$) and fecundity ($r = 0.58$, $P < 0.001$).

Adult foliar feeding and fecundity were significantly inhibited by the cysteine proteinase inhibitors E-64, pHMB, and leupeptin at a concentration of 0.1%. A higher 0.5% concentration of antipain also significantly inhibited adult foliar feeding and fecundity. Pepstatin at 0.5% and the Bowman-Birk trypsin inhibitor at 1.0% had no significant effect on adult feeding, survival, or fecundity.

All lectins were tested at a concentration of 0.5%. The wheat (WGA) and pea (PSA) lectins significantly inhibited adult foliar feeding and fecundity and were the only two compounds tested to significantly inhibit adult survival. One of the bean lectins (PHA-L) significantly inhibited adult feeding and fecundity while the other (PHA-E) did not.

The main effects of dates (days), for the mean of all treatments, for adult feeding, survival, and fecundity were all significant ($P < 0.001$). Adult feeding, survival, and fecundity all decreased over time with values for days 3 and 6 significantly less than days 10 and 14 (Table 2).

Results on the proteinase inhibitors tested in this study against the adult alfalfa weevil are in agreement to those of a previous study that demonstrated that the same cysteine proteinase inhibitors significantly inhibited alfalfa weevil larval foliar feeding, pupation, and adult emergence (Elden 1995a). Pepstatin (aspartic) and soybean trypsin (serine) inhibitors were not active in either study, even at much higher concentrations. These studies demonstrate that selected protein inhibitors may have the

Table 1. Alfalfa weevil adult feeding, survival, and fecundity when fed excised alfalfa trifoliolates treated with selected proteinase inhibitors and lectins*

Treatment**	Concn	*n*	Feeding†	Survival	Fecundity
			Proteinase Inhibitors		
E-64	0.1	5	22.5 e	93.1 abcd	0.9 e
pHMB	0.1	3	31.7 cd	95.0 abc	1.9 cde
Leupeptin	0.1	4	28.7 cde	93.0 abcd	1.9 cde
Antipain	0.5	3	22.9 de	89.5 bcd	1.0 de
Pepstatin	0.5	3	49.9 ab	96.1 ab	4.5 ab
Trypsin	1.0	3	48.8 ab	97.7 a	4.7 ab
			Plant Lectins		
WGA	0.5	2	26.4 cde	87.1 cd	1.7 cde
PSA	0.5	2	35.9 c	84.9 d	2.7 bcd
PHA-E	0.5	1	53.8 ab	92.8 abcd	3.3 abc
PHA-L	0.5	1	37.1 bc	87.6 bcd	2.6 bcde
			Controls		
Gelatin	6.5	4	53.8 a	96.0 ab	6.3 a
Control		5	56.0 a	95.4 ab	6.3 a

* Values are the adjusted mean of *n* tests with 8 replications per test.

** Means within a column not followed by the same letter are significantly different, Fisher protected LSD with $\propto = 0.05$.

† Feeding = percentage of leaf eaten per 2 adults. Survival = percentage of adults living after 14 d. Fecundity = eggs per female per d.

ability to interfere with alfalfa weevil larval growth and development in the late spring when damage occurs in the field and with adult oviposition in the late fall and early spring when sexually mature females lay their eggs. These results also add support to the hypothesis that the alfalfa weevil uses cysteine proteinases as one of its major digestive enzymes.

The present study is aimed at the larger goal of identifying plant defensive proteins, such as proteinase inhibitors, alpha-amylase inhibitors, lectins, and other insecticidal compounds which may interfere with the digestive physiology of the alfalfa weevil. Insecticidal genes which encode plant defensive proteins have been identified in other insect-host relationships as previously cited. A gradual decline in the effectiveness of these transgenic plants can be expected if they will be used on a commercial basis as has been demonstrated both in the field and laboratory with the *B. thuringiensis* toxins (Tabashnik 1994).

To minimize the probability of developing resistant alfalfa weevil biotypes feeding on transgenic insect resistant alfalfa, if and when they are developed, our research

Table 2. Alfalfa weevil feeding, survival, and fecundity over time when fed selected protein inhibitors*

Date**	Days	Feeding†	Survival	Fecundity
1	3	42.4 a	97.9 a	4.5 a
2	6	43.4 a	95.1 a	3.8 a
3	10	34.5 b	88.5 b	2.0 b
4	14	34.2 b	86.9 b	1.8 b

*Values are the adjusted mean of all treatments.

** Means within a column not followed by the same letter are significantly different, Fisher protected LSD with $\propto = 0.05$.

† Feeding = percentage of leaf eaten per 2 adults. Survival = percentage of adults living. Fecundity = eggs per female per d.

group is attempting to determine the complex of proteins present in the alfalfa weevil and identify a number of plant defensive proteins which will inhibit these proteins. This approach may provide a number of insecticidal genes which can be combined or rotated to avoid the build-up of resistant insect populations. Another method proposed to lessen the development of resistant insect biotypes is to use a control system which does not cause acute adult or larval mortality and can be integrated with other IPM tactics (Gould 1988). In the present and a previous study (Elden 1995a) several protein inhibitors were identified which did not cause acute adult or larval alfalfa weevil mortality but did significantly retard foliar feeding, pupation, adult emergence, and fecundity.

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