PLANT ANIMAL INTERACTIONS

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Bioassay versus chemical assay: measuring the impact of induced and constitutive resistance on herbivores in the field

Abstract Studies of induced plant resistance usually either examine physiological/chemical mechanisms or explore the ecological and evolutionary role of induced resistance. To connect these two methods of study, data are needed that address the relationships between plant chemistry and effects of induced resistance on herbivores under field conditions. In this paper we combine the results of a greenhouse experiment and a field experiment to try to make such a connection. Levels of induced and constitutive resistance to Mexican bean beetles in several soybean genotypes were measured in a greenhouse experiment using a behavioral bioassay. In a field experiment, beetle performance and induced and constitutive levels of cystein proteinase inhibitor activity were measured for these same genotypes. Greenhouse bioassay ratings of induced and constitutive resistance and induced and constitutive levels of proteinase inhibitor activity agree closely for individual genotypes, suggesting that proteinase inhibitors (PIs) are involved in induced resistance. However, while greenhouse bioassay measures of constitutive and induced resistance were good predictors of beetle performance in the field, proteinase inhibitor activity did not predict beetle performance well. While PIs appear to be involved in induced resistance, they were not strongly correlated to Mexican bean beetle performance in the field in this study. These results suggest that measurement of single aspects of plant chemistry may not encompass all ecologically important aspects of plant resistance.

Keywords *Epilachna verivestis* · *Glycine max* · Herbivore performance · Induced resistance · Proteinase inhibitors

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Introduction

There are two approaches to the study of induced plant resistance that are largely independent. One body of literature addresses the mechanisms that may be involved in induced resistance, while another addresses possible ecological and evolutionary interactions of induced resistance with herbivores and their predators. Much is now understood, especially for a few species such as tobacco, tomato and soybeans, about how plant chemistry can change after herbivore attack [see Karban and Baldwin (1997) for review]. Many studies have also demonstrated that previously damaged plants can lower insect preference and performance in the laboratory and field (e.g., Edwards et al. 1985; Haukioja and Neuvonen 1985; Karban 1986; Lin and Kogan 1990; Bi et al. 1994; Underwood 2000), and that chemicals that change after damage can inhibit insect digestion or lower performance in the laboratory (e.g., Johnson et al. 1989; Bi et al. 1994; Zhao et al. 1996). A smaller number of theoretical and field studies indicate conditions that should favor the evolution of induced versus constitutive resistance (Adler and Karban 1994; Astrom and Lundberg 1994; Zangerl and Rutledge 1996), and suggest that induced resistance should have important consequences for herbivore populations (Rhoades 1985; Edelstein-Keshet and Rausher 1989; Underwood 1999), and for the predators of herbivores (Thaler 1999). These studies taken together raise the exciting prospect that specific physiological mechanisms in plants can be linked to population-level consequences for plants and herbivores through the study of induced resistance. However, in most cases little is known about the connection between specific chemical changes measurable in the laboratory or greenhouse, and the effects of induced resistance occurring under field conditions. It is only by making this connection that we will be able to determine the significance of particular chemical changes for broader ecological and evolutionary patterns, and know the full consequences of employing plant compounds for agricultural pest control.

Laboratory demonstrations of impacts of plant compounds on herbivores may not translate into significant ecological or evolutionary effects in the field. For example, proteinase inhibitors (PIs) have repeatedly been shown to inhibit insect digestive enzymes in vitro, suggesting that PIs should reduce herbivore performance (Hines et al. 1991; Orr et al. 1994; Zhao et al. 1996). However, when PIs are incorporated into insect diets, effects on performance are mixed, in part because some insects can change their enzyme production to avoid the effects of specific PIs (Broadway 1995). It has also been shown in some systems that many different aspects of plant physiology change when herbivores damage a plant (e.g., Felton et al. 1994; Karban and Baldwin 1997). While individual compounds may have clear effects on herbivores or herbivore enzymes in isolation, if different compounds in the plant interact, especially in non-additive ways, effects of whole plant responses on herbivores may not mimic effects of individual compounds (Duffey and Stout 1996). Finally, plants and herbivores in the field are subject not only to a variety of environmental stresses that differ from those in the laboratory or greenhouse, but also to attack by a variety of other herbivores, predators and pathogens. Biotic and abiotic stresses may limit or increase induced responses or the ability of herbivores to deal with those responses. Thus, to know the consequences of a particular compound in the field, the relationship of that compound with herbivore performance or success needs to be examined under field conditions. Some field studies of induced resistance in trees suggest that correlative connections can be made between plant chemistry and herbivore performance or behavior (e.g., Rossiter et al. 1988; Hunter and Schultz 1993; Seldal et al. 1994; Hunter and Schultz 1995), although others find either no correlation between induced changes in secondary compounds and herbivore performance (Osier and Lindroth 2001) or complex relationships that make effects of particular induced compounds difficult to pinpoint (Kaitaniemi et al. 1998).

Induced resistance in soybeans has been studied a great deal in the laboratory and to a lesser extent in the field. Many changes in soybean physiology are known to be provoked by herbivore damage, including increases in isoflavones (glyceolin), lipoxygenases, peroxidases and PIs (e.g., Kogan and Fischer 1991; Felton et al. 1994). PIs in particular have received a large amount of attention as a potential mechanism for induced resistance in plants (Ryan 1990). It is well documented that damage to soybeans results in increased resistance to herbivores, in the form of insect preference for undamaged plants, and reduced growth and increased development time of insects on damaged plants in the laboratory (Lin and Kogan 1990; Kogan and Fischer 1991; Underwood 2000). However, much less is known about whether induced resistance in soybeans has consequences for herbivores in the field (but see Iannone 1989), and more particularly, how specific compounds such as PIs might be related to any such consequences. Here we report on a study examining how PIs in soybeans may be related to induced resistance to Mexican bean beetles in the field. To address this issue we draw on the results of two experiments. The first is a previously published study (Underwood et al. 2000) that used greenhouse behavioral bioassays to rate six genotypes of soybeans on their induced and constitutive resistance levels. The second study is a new analysis of cystein proteinase inhibitor activity in six genotypes of plants used in a field experiment with Mexican bean beetles and other herbivores. Five of the six genotypes used in these two studies overlap, thus allowing comparison of their results to address the following specific questions:

1. First, do greenhouse bioassays correlate significantly with the level and pattern of PI expression in the field? A positive answer to this question would support the ideas that PIs are involved in induced resistance, and that induced resistance is expressed in the field.
2. Second, do PI expression in the field and behavioral bioassay measures of induced and constitutive resistance each explain significant amounts of variation in herbivore performance in the field? If PIs are an important part of resistance to Mexican bean beetles they should be related to herbivore performance in the field. Likewise, if behavioral bioassays in the greenhouse are related to herbivore performance in the field, this would suggest that the resistance measured in the greenhouse can predict effects of resistance on herbivores in the field.

Here we compare the ability of these two measures of induced resistance to predict beetle performance in the field.

Materials and methods

The system

Soybean (*Glycine max*: Fabaceae) genotypes are known to vary in both constitutive and systemic induced resistance to a variety of insect herbivores, including lepidopterans, hemipterans, aphids and beetles including the Mexican bean beetle (Van Duyn et al. 1971; Kraemer et al. 1988; Liu et al. 1993; Powell and Lambert 1993; Underwood et al. 2000). Induced resistance in soybeans has been measured in previous studies both by bioassay and by tracking increases in various substances, including PIs, after damage (e.g., Kraemer et al. 1987; Lin and Kogan 1990; Bi et al. 1994; Botella et al. 1996; Underwood 1998). In this study we focus on cystein PIs because they are known to inhibit the digestive enzymes of several beetle species (Hines et al. 1991; Zhao et al. 1996), and thus might be likely to affect Mexican bean beetles. Soybean seeds were obtained from T. Carter and J. Burton (North Carolina State University, Raleigh, N.C.), L. Lambert (USDA-ARS-SIML, Miss.) and R. Nelson (USDA Soybean Germplasm Collection, Urbana, Ill.).

Mexican bean beetles (*Epilachna varivestis*: Coccinelidae) are specialists on legumes and economic pests of soybeans in many areas. The beetles lay their eggs on the host plant and both larvae and adults feed on foliage. Beetles used in these experiments were originally obtained from T. Dorsey (New Jersey Department of Agriculture, Trenton, N.J.) and were reared in an environmental chamber with a 14-h day length at 28°C. Beetles used in these

experiments were reared on common snap bean plants (*Phaseolus vulgaris*) to prevent them from developing preferences for particular soybean genotypes.

Greenhouse bioassays for induced and constitutive resistance

In a previous study (Underwood et al. 2000), six genotypes of soybeans were tested for both induced and constitutive resistance using bioassays. The methods and results for these experiments are briefly described below. For details see Underwood et al. (2000). Soybeans for this study were grown in the Duke University greenhouses under a 14-h day length, which prevented the plants from becoming reproductive, and were fertilized approximately weekly.

Induced resistance

When the plants had one fully expanded trifoliolate leaf and a partially expanded second trifoliolate, half the plants of each genotype were randomly chosen to receive damage to their first trifoliolate by Mexican bean beetle larvae. When the entire first trifoliolate had been damaged (skeletonized, within 48 h) the larvae were removed. Three days later, 12 pairs of damaged and control plants from each genotype were randomly chosen, and leaf disks were cut with a cork borer from their most recently expanded undamaged trifoliolates for use in a bioassay. Cutting disks from soybean leaves does not cause induced resistance in the disks (Kogan and Fischer 1991). Because leaves collected from the damaged plants did not receive any direct damage, the bioassay tested for systemic induced resistance.

Induced resistance was measured as beetle feeding preference for leaf tissue from a control plant over tissue from a damaged plant of the same genotype (method derived from Kogan 1972). Two leaf disks from control and damaged plants were placed in a Petri dish lined with damp filter paper. One adult female beetle was allowed to feed in the dish until approximately 25% of the total leaf area in the dish was consumed. The consumed area of each disk was measured using a computer image analysis program (Image 1, Universal Imaging Corporation 1991). To describe the degree of induced resistance, a preference index $[2(c/(c+d)]$ was calculated where c and d indicate the amount of damage to the control and damaged disks in each dish. An index of 1 indicates no preference (no difference in feeding between damaged and undamaged plants) and an index >1 indicates induced resistance (preference for undamaged plants). The mean preference index for a genotype indicates the degree of induced resistance for that genotype.

Constitutive resistance

For consistency with the measure of induced resistance, constitutive resistance was measured as beetle feeding preference for undamaged plants of one genotype over another. When the majority of plants had fully expanded their second trifoliolate, the most recently expanded trifoliolate was collected for use in a bioassay. The bioassays for constitutive resistance were the same as the assays for induced resistance, with the following exception: beetles were presented with discs from undamaged plants of each of two different genotypes, rather than discs from damaged and control individuals of the same genotype. All 15 possible pairings of the six genotypes were tested. Constitutive resistance was described by a preference index analogous to the one used to describe induced resistance. For each pair of two genotypes, the genotype whose name came first alphabetically was used as the "reference" genotype (x). The preference index for each dish was then calculated as the amount of tissue of the non-reference genotype (y) as a proportion of the total tissue eaten in that dish ($x+y$): $2[y/(x+y)]$. A mean preference index was calculated for each pairing of genotypes. Values of this index <1 indicate that the beetle preferred the reference genotype and values >1 indicate discrimination against the reference genotype. The five mean index values involving a

Table 1 Design of the field experiment where beetle performance and proteinase inhibitors were measured. Each cell represents one population of 20 soybean plants and the indicated initial density of beetles

Resistance type	Genotype	Initial beetle population size				
		2	6	10	14	20
No induced, no constitutive	Bragg	a		a		a
	Gasoy	a		a		a
Induced, no constitutive	Stonewall	a		a		a
	Davis	a		a		a
No induced, constitutive	PI 416937	a		a		a
	Lamar	a		a		a

[a] Population sampled for plant damage and proteinase inhibitors

particular genotype were then averaged to yield an overall measure of preference for (or against) that genotype. Genotypes that had a high average preference index when measured against the other genotypes used in the experiment were considered to have a relatively high level of constitutive resistance.

Field experiment

A field experiment was conducted to examine the effect of induced and constitutive plant resistance on herbivore population dynamics, using five of the six genotypes tested for induced and constitutive resistance plus one additional genotype. Results regarding the effects of resistance on herbivore dynamics will be reported elsewhere (Underwood and Rausher, in press). Here we report on the effects of cystein PIs on Mexican bean beetle performance in this experiment.

The design of the field experiment was as follows (summarized in Table 1). Populations of six genotypes of soybeans with differing resistance characteristics were placed in field cages. Based on greenhouse bioassays, two of the genotypes used in this experiment (Bragg and Gasoy) had no resistance (no induced resistance and low constitutive resistance). Two genotypes (PI 416937 and Lamar) had constitutive resistance (no induced resistance and high constitutive resistance), and two genotypes (Davis and Stonewall) had induced resistance (high induced resistance and low constitutive resistance). Resistance characteristics of Lamar were determined by literature reports of strong constitutive resistance (e.g., USDA/ARS Germplasm Resources Information Network, http://www.ars-grin.gov/npgs/) and greenhouse bioassays for induced resistance (N. Underwood, unpublished data). Populations of Mexican bean beetles were released into each soybean population and followed for one generation. Each genotype of soybean had five densities of beetle populations initiated on it (for a total of 30 soybean/beetle population types). Each population type was replicated once in each of three spatial blocks, and the entire experiment was run twice during the summer of 1998 (first run from May to July, second run from July to September). Soybeans flowered and set seed in all populations during both runs of the experiment.

Before each run of the experiment, the soybean plants were grown in the greenhouse to approximately the eight-leaf stage in 15.24-cm-diameter pots filled with sterile potting mix. Plants were fertilized once when they had one fully expanded trifoliolate and once again later in the experiment, both times with 4.9 ml Osmocote (The Scotts Company, Columbus, Ohio) time-release fertilizer. At the beginning of each run, potted plants were placed in 2-m^2 field cages (20 plants per cage) that had been cleared of weeds. Adult Mexican bean beetles (in a 1:1 sex ratio) were released into the cages and allowed to mate and lay eggs for 1 week. At the end of the week the adults were removed and cages were left open to the field through egg hatching and larval development. This allowed natural predators, parasites and herbivores access to the popula-

tions in the cages. When the Mexican bean beetle larvae pupated, the cages were closed until all adults had emerged. Beetle populations were censused weekly throughout the experiment and adults emerging at the end were counted and weighed. Although predators were observed in the cages during the experiment (spiders and hemipteran larvae), they appeared to be relatively rare. There was no sign of parasitism of beetle larvae during this experiment.

Measuring beetle performance and plant damage

Four measures of beetle performance were examined: number of eggs laid by first generation adults, development time (time from the first observation of first instar larvae in each population to the first observation of adults of the next generation), weight of adults in the second generation and per-capita reproduction (number of adults produced per adult). At the end of each run, damage to six randomly chosen plants in each cage was measured by scoring each leaf on a plant on a scale of 0–4 [where 0 indicated no herbivore damage and 4 indicated that the leaf had been completely eaten (100% damage)]. These scores were then averaged to form a single damage score for each plant. Note that this measure of leaf damage includes damage by Mexican bean beetles and damage by other herbivores present in the cages (primarily lepidopterans). Damage measurements at the end of the experiment were made on a subsample of the soybean populations including the lowest, middle and highest density beetle populations for each genotype in each block (Table 1).

Laboratory assays for cystein PIs

Leaf samples for analysis of proteinase inhibitor activity were taken from a subset of the soybean populations at 2 times during each run of the experiment: at the beginning of the run before beetles were released onto the plants, and at the end of the run after beetles had damaged the plants. At both times, samples were taken from the soybean populations with the lowest, middle and highest density beetle population for each genotype in each block (a total of 18 populations, see Table 1). At the second sample date an additional seven (for the first run) and five (for the second run) populations were also sampled. Within each sampled population, leaf disks were cut from four randomly chosen plants. The disks were cut from the most recently fully expanded leaf available to standardize for leaf age. Because not all plants were actively growing at the end of the experiment, samples were taken from either the most recent leaf, or leaves with little or no damage. Most disks sampled at the end of the experiment did not contain areas of herbivore damage. Leaves to be sampled were cut from the plant at the petiole, and disks were immediately cut with a 1-cm-diameter cork borer [which has been shown not to cause induced resistance in leaf disks (Kogan and Fischer 1991)]. Two disks were cut from each leaf, placed in 1.5-ml microcentrifuge tubes, and put on dry ice. Samples were transferred to storage at –80°C no more than 4 h after being cut.

To measure cystein proteinase inhibitor activity, a modification of the technique of Koiwa et al. (1998) was used to determine papain inhibitory activity in the soybean leaves. Leaf disks were taken from the freezer and ground to a fine powder using liquid nitrogen in 1.5-ml microcentrifuge tubes. To extract the inhibitor, 1 ml of 50 mM phosphate buffer, pH 7.2, with 150 mM NaCl and 2 mM EDTA:Na$_2$ was added to the leaf powder, at approximately 10 ml per gram leaf tissue (fresh weight). This mixture was vortexed for 10 s, then centrifuged at 12,000 g for 15 min. To preactivate the papain (Sigma, St. Louis, Mo.), one volume of 20 µg/ml papain in 25 mM NaPi, pH 7.0, and 20 mM 2-mercaptoethanol was incubated for 10 min at 40°C, combined with two volumes of 0.25 M NaPi, pH 6.0, 2.5 mM EDTA:2Na, and then kept on ice. Next, 0.1 ml of the inhibitor solution and 0.3 ml of the preactivated papain solution were combined and incubated for 5 min at 40°C. The reaction was started by adding 0.2 ml of 7 mM Na-benzoyl-DL-arginine-β-naphthylamide (BANA), resulting in a final concentration of 2.3 mM BANA. This was incubated for 10 min at 40°C,

then 1 ml of 2% HCl in ethanol was added to stop the reaction. Finally, 1 ml of 0.06% p-dimethylaminocinnamaldehyde in ethanol was added to develop color. The samples were left to stand for at least 30 min at room temperature for full color development. The absorbance of the final mixture was read in a spectrophotometer (Perkin-Elmer Lambda 3B) at 540 nm. Inhibitory activity was expressed as the percentage decrease in absorbance relative to the reaction with no inhibitor.

Data analysis

Constitutive and induced proteinase inhibitor activities were characterized for each soybean genotype in the field. Constitutive PI level was defined as the average amount of PI activity in plants of each genotype at the beginning of the experiment, before any insect damage had occurred. Induced PI level was defined as the difference between the initial level of PI activity and the level of activity for each genotype at the end of the experiment (after damage by Mexican bean beetles and other naturally occurring herbivores). Analyses were also done on the final levels of PIs in each genotype at the end of the experiment. ANOVA was used to determine if the levels of induced, constitutive, and final PI activity varied among genotypes in the field experiment (Proc GLM, SAS Institute 2000). The correlation between induced and constitutive PI activity in the field and levels of induced and constitutive resistance measured by bioassay in the greenhouse was also examined. Because the measures of resistance used in the bioassays are expressed as ratios and are not normally distributed, the non-parametric Spearman's rank correlation statistic was used (Proc CORR, SAS Institute 2000).

ANOVA was used to ask how greenhouse bioassay measures of resistance and PI activity levels contributed to beetle performance and plant damage in the field experiment. Because our goal was to compare how bioassay and PI level data each relate to beetle performance, and because bioassay and PI level results were strongly correlated, greenhouse bioassay and PI activity level data were examined in separate analyses. Proteinase inhibitor levels, constitutive, induced and final, were included in ANOVA models as continuous covariates. The results of the bioassay indicated that the genotypes could be divided into three distinct resistance classes (induced resistance, constitutive resistance and no resistance), so bioassay measures of resistance were included in ANOVA models as a class variable. ANOVA models for both PI activity and bioassay results included run (first versus second runs of the experiment), block, genotype and initial beetle density on each plant population as fixed effects (Proc GLM, SAS Institute 2000). The exception was an ANOVA examining patterns of PI activity at the beginning of the experiment, which did not include initial beetle density because beetles were not yet present when this analysis was done. In models including bioassay resistance classes, genotype was nested within resistance class. Residuals from all models were approximately normal. One strong outlier was removed from analyses involving per-capita recruitment. Removal of this data point did not affect results for any other response variable.

Results

Pattern of proteinase inhibitor activity in the field

At the beginning of the field experiment, proteinase inhibitor activity differed significantly among plant genotypes (significant effect of genotype on PI activity, $F_{3,166}=24.35$, $P<0.001$), and among greenhouse-bioassay-defined resistance classes ($F_{2,166}=58.27$, $P<0.001$, Fig. 1). Plant genotypes classified as constitutively resistant by bioassay had significantly greater PI activity at the beginning of the experiment than plants in the other
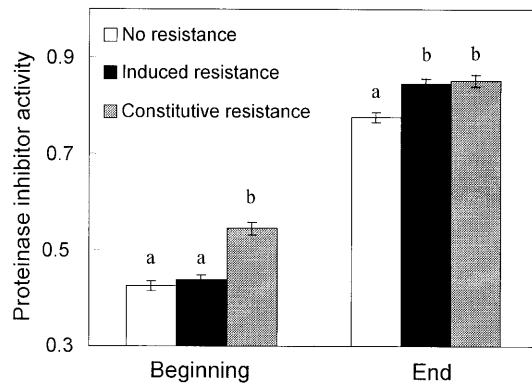
Fig. 1 Change in proteinase inhibitor (PI) activity between the beginning of the experiment (when plants were undamaged) and the end of the experiment (when most plants were heavily damaged). The three resistance classes were defined on the basis of greenhouse bioassays, and each resistance class includes two soybean genotypes. For each bar $n \approx 36$, *error bars* indicate 1 SE. *Bars with different letters* are significantly different at the 0.05 level, significance not corrected for multiple comparisons
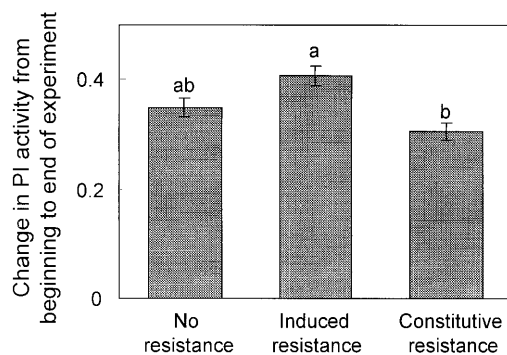
Fig. 2 Induced resistance measured as the change in PI activity between the beginning and end of the experiment, as a function of resistance type of soybean genotype and initial beetle density. For each bar $n \approx 36$, *error bars* indicate 1 SE. *Bars with different letters* are significantly different at the 0.05 level, significance not corrected for multiple comparisons

Fig. 3 Bioassay (beetle feeding preference) and PI measures of induced (**A**) and constitutive (**B**) resistance for five soybean genotypes are significantly correlated. Spearman correlation coefficient for induced resistance, $r^2 = 1$, $P < 0.001$, and for constitutive resistance $r^2 = 0.872$, $P < 0.05$. *G* Gasoy, *B* Bragg, *P* PI 416937, *S* Stonewall, *D* Davis, *beg.* beginning

two resistance classes according to post hoc contrasts. By the end of the experiment, all plants had increased PI levels (perhaps due to ontogenetic effects), and plants classified as having constitutive and induced resistance (based on greenhouse bioassays) both had significantly greater PI activity than plants classified as having no resistance (Fig. 1). Plants classified as having induced resistance had the largest change in PI activity between the beginning and the end of the experiment (Fig. 2), (significant effect of resistance class, $F_{2,154} = 4.96$, $P = 0.008$). The degree of change in PI activity increased very slightly with initial beetle density ($F_{1,154} = 6.59$, $P = 0.01$, parameter estimate $= 0.004$). Block and run were significant in all models.

There was a significant positive correlation between induced bioassay resistance and induced changes in proteinase inhibitor levels (Spearman correlation coefficient $= 1$, $P < 0.001$) and between constitutive bioassay resistance and constitutive proteinase inhibitor levels
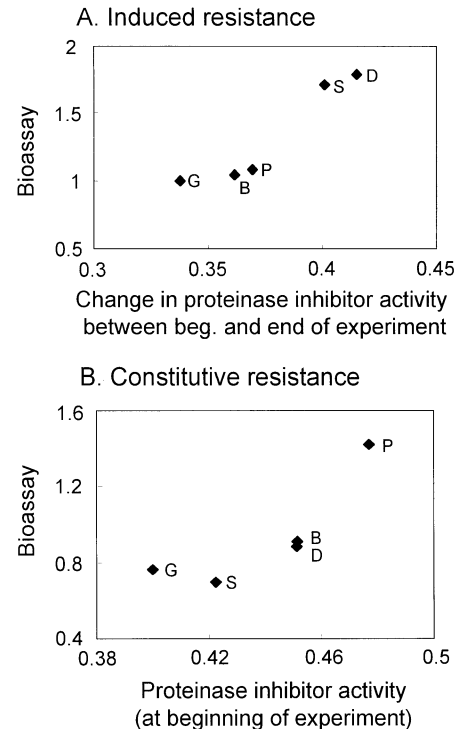
(Spearman correlation coefficient $= 0.872$, $P < 0.05$) across the five soybean genotypes (Fig. 3A, B).

Effects of PIs on beetle performance and plant damage

There was no clear or consistent relationship between proteinase inhibitor levels and beetle performance in the field. The development time, number, and weight of adults emerging at the end of the experiment were not significantly correlated with constitutive levels of PIs, final levels of PIs or induced changes in PIs (ANOVA). For number of adults emerging and adult weight PIs had little explanatory power; individual regressions of constitutive PIs, final PIs and induced changes in PIs on per-capita recruitment and adult weight for each run of the experiment were all not significantly different from zero ($P > 0.3$ in each case). Finally, there was a marginally significant correlation between induced changes in PIs and the number of eggs laid at the beginning of the experiment ($F_{1, 115} = 3.45$, $P = 0.066$, Fig. 4), with higher values of induced resistance associated with fewer eggs laid. However, this relationship has an r^2 of only 0.0003, suggesting that any effect of induced resistance on egg production is very weak.

PIs were related to the amount of damage to plants. Constitutive, final and induced changes in PIs could not all be included in the same model due to multicolinearity,
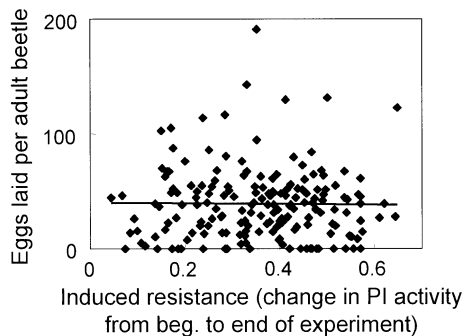
Fig. 4 Relationship between induced resistance (measured as change in PI activity between beginning and end of the experiment) and per-capita egg production of Mexican bean beetles in field cages. *Each point* indicates a single beetle/soybean population. Equation for the best-fit line is per-capita egg production=−3.7766induced resistance+40.722, R^2=0.0003

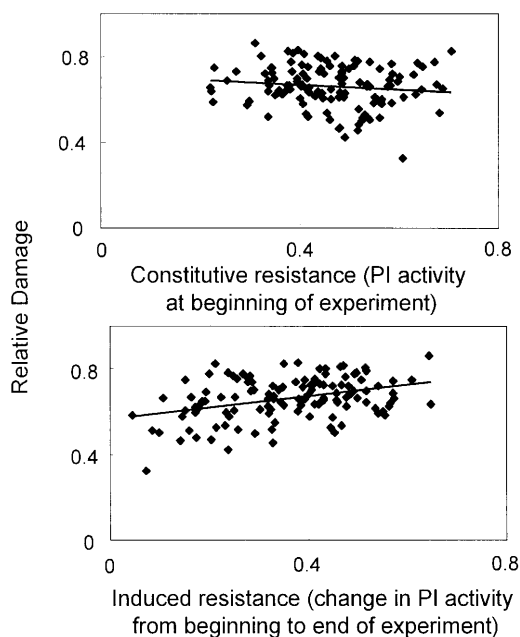**Fig. 5** Relationship of constitutive and induced resistance with amount of damage plant received. Resistance is measured by PI activity, and damage is based on scoring each leaf on a plant on a scale of 1–4, with 4 indicating a completely eaten leaf. *Points* indicate mean values for individual beetle/soybean populations
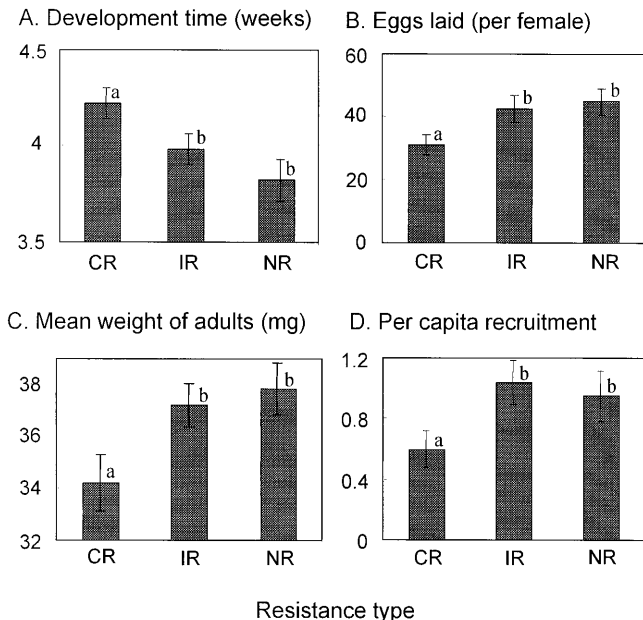

Fig. 6 Soybean genotypes in different resistance classes (based on bioassays) have different effects on beetle performance in the field: **A** development time, **B** eggs laid per female, **C** mean weight of adults, **D** per-capita recruitment. In each case *error bars* indicate 1 SE, for each bar $n\approx60$ (less for weight, since populations where no adults emerged had missing values for this measure of performance). *Bars with different letters* are significantly different at the 0.05 level [except in **D** where constitutive resistance (*CR*) and no resistance (*NR*) differ at the 0.07 level], significance not corrected for multiple comparisons. *IR* Induced resistance

but models with pairs of factors could be run. With constitutive and induced PIs in the model, there were significant relationships of both constitutive PIs and induced changes in PIs with plant damage (constitutive: $F_{1, 120}$=12.76, $P<0.001$, induced: $F_{1,120}$=36.18, $P<0.001$, Fig. 5). However, when the final level of PIs was included in the model, final PIs were significant (in model with induced PIs: $F_{1, 120}$=9.17, P=0.003, in model with constitutive PIs: $F_{1, 120}$=28.0, $P<0.001$), and neither induced nor constitutive PIs were significant. Regressions of damage on resistance indicated that increasing levels of constitutive resistance resulted in decreased plant damage (slope=0.11, r^2=0.015), while induced resistance and final PI level were positively related to plant damage (induced change in PIs, slope=0.26, r^2=0.136; final PIs, slope=0.41, r^2=0.16).

Effects of bioassay resistance categories on beetle performance and plant damage

Resistance categories derived from the greenhouse bioassays were significantly related to all measures of beetle performance in the field. Beetle development time differed among resistance categories (ANOVA $F_{2, 90}$=7.51, P=0.001) with development time being the slowest on plants with constitutive resistance and fastest on plants with no resistance (Fig. 6A). Beetles also laid fewer eggs on constitutively resistant plants (significant main effect of resistance on per capita eggs laid in ANOVA $F_{2,149}$=3.49, P=0.03, Fig. 6B). The weight of adult beetles varied among resistance classes (ANOVA, $F_{2,86}$=9.37, $P<0.002$, Fig. 6C) with constitutive resistance having the strongest negative effect on weight. Finally, per-capita beetle recruitment was significantly affected by resistance class (ANOVA $F_{2,137}$=3.04, P=0.05, Fig. 6D), with lower recruitment on constitutively resistant plants than on the other two resistance types. Resistance classes were not related to the amount of damage accrued by plants in the field (no significant effect of resistance class on damage, $P>0.8$).

Discussion

In this study, the levels of cystein PIs in soybean genotypes in the field corresponded closely with greenhouse bioassay measures of both induced and constitutive resistance to Mexican bean beetles. At the beginning of the field experiment, when plants were undamaged, genotypes classed as constitutively resistant in the greenhouse had higher levels of PI activity than genotypes classed as having low levels of constitutive resistance. By the end of the experiment, after plants had been damaged, genotypes classed as inducible in the greenhouse had PI levels indistinguishable from constitutively resistant genotypes, while genotypes classed as "no resistance" had significantly lower levels of PI activity. Thus, after being damaged the inducible plants caught up to the level of PIs in constitutively resistant plants (Fig. 1). The correspondence between greenhouse bioassay and field proteinase inhibitor measures of resistance was also confirmed by significant correlations between greenhouse and PI rankings of the levels of induced and constitutive resistance across genotypes of soybeans (Fig. 3). These results suggest that cystein PIs are involved in induced and constitutive resistance in soybeans, and that at least part of this resistance affects Mexican bean beetles. This conclusion is consistent with studies that find that trypsin PIs increase in soybeans after herbivore damage (Kraemer et al. 1987; Bi et al. 1994), in vitro demonstrations that cystein PIs inhibit coleopteran digestive enzymes (Hines et al. 1991; Zhao et al. 1996), and studies showing that beetle preference and performance are lower on damaged versus undamaged plants (Kogan and Fischer 1991; Bi et al. 1994; Underwood et al. 2000).

It is also clear that the resistance classes defined by greenhouse bioassays based on beetle behavior (constitutive resistance, no resistance, and induced resistance) differ in their effects on Mexican bean beetle performance in the field (Fig. 6). The number of eggs laid per female beetle, development time from first instars to adults, adult beetle weight and number of adult beetles produced per female were all significantly affected by resistance class. For three out of these four measures of beetle performance, the pattern of effects of resistance classes is consistent: constitutively resistant genotypes have the strongest negative effect on beetle performance, and induced resistance genotypes have a weaker (and often not significant) negative effect on performance relative to no resistance genotypes. Although for per-capita beetle recruitment performance is slightly higher on plants with induced resistance than on plants with no resistance, this difference is not statistically significant. Moreover, as with the other three measures, constitutive resistance leads to significantly lower performance than no resistance. These data suggest that constitutive resistance has a stronger impact on herbivore performance than induced resistance, although the consistent direction of the effects of induced resistance suggests that induced resistance has some impact as well. This result is intuitively appealing, since plants with high constitutive resistance should have negative effects on herbivores all the time, while plants with induced resistance may reach high levels of resistance only later in the herbivore's development. It has been suggested that the variability inherent to induced resistance could have a negative effect on herbivores (if, for instance, herbivore physiology cannot adjust to changing levels of plant resistance compounds) (Adler and Karban 1994). If variability itself were important, however, we would expect plants with induced resistance to be similar to or more resistant than plants with high constitutive resistance, a pattern that was not seen in this experiment.

Given that resistance classes based on behavioral bioassays explain significant variation in beetle performance, and that PIs are significantly correlated with resistance classes, it would be reasonable to expect that PIs would also be correlated with performance, especially since PIs have been shown to influence beetle digestive enzymes in the laboratory (Hines et al. 1991; Zhao et al. 1996). However, in this study there was no consistent relationship between PI activity and beetle performance. There was a marginally significant and very small effect of induced levels of PIs on numbers of eggs laid (Fig. 4), and we could detect no effect at all of PI activity on beetle weight, development time or per-capita recruitment. These results suggest that while PIs may affect some aspects of beetle performance in the complex field environment, those effects are neither consistent nor large. Compared to measures of resistance based on greenhouse bioassays, PI levels were a relatively poor predictor of beetle performance in this field experiment. This result is counterintuitive because PIs were measured in the same plants on which the beetles fed in the field experiment, whereas the bioassays were done in a greenhouse environment, using different plants and beetles.

In order to explain these results we must reconcile the observations that: (1) beetle behavioral measures of resistance and PI activity levels are correlated across soybean genotypes, and (2) beetle performance differs among genotypes in a way that parallels the behavioral bioassay but not PI activity levels. One explanation consistent with these observations is that both beetle behavior and performance are influenced primarily by resistance factors other than PIs, but that levels of PI activity are correlated with levels of these other resistance factors. This explanation is supported by the observation of several workers that the induction responses of soybeans appear to involve changes in many aspects of plant physiology in addition to PIs (Bi et al. 1994; Felton et al. 1994). An alternative explanation is that Mexican bean beetles respond behaviorally but not physiologically to PIs. Under this explanation, resistance measured by bioassay reflects an aversion of adult beetles to feeding on plants with elevated levels of PIs (antifeedent resistance), while the performance differences among genotypes are due primarily to differences in levels of resistance factors other than PIs. Although we cannot distinguish between these explanations, both explanations imply that PIs contribute relatively little, compared with other

unidentified factors, to antibiotic resistance to Mexican bean beetles in soybeans. PIs could, of course, still be effective in reducing the performance of other types of herbivores. A final possibility is that sampling PI activity at the beginning and end of the field experiment did not adequately represent PI activity during the experiment and thus could not detect effects of PIs on herbivore performance. It is not possible to rule out this option, and more frequent sampling of PIs would certainly have been preferable if logistically possible. However, positive correlations between PIs and plant damage (which was accumulated over the experiment) suggest that PIs on the dates we measured them did represent at least some aspect of plant resistance through the experiment.

Results of this study do offer support for the idea that PIs are involved in plant defense against other herbivores. While PIs were not particularly strongly associated with beetle performance, they were significantly associated with the amount of damage that plants received in the field from a combination of Mexican bean beetles and other herbivores (Fig. 5). Plants with higher constitutive levels of PIs had somewhat lower levels of damage. Higher levels of induced resistance (change in PIs from the beginning of the study to the end) and final levels of PIs were significantly associated with increased damage. This pattern seems more likely to be due to the effect of damage on induced resistance, rather than to induced resistance or final PI levels causing increased damage. In this study, plants were damaged by a variety of herbivores including Mexican bean beetles, several lepidopterans, and grasshoppers. Over all soybean populations in the experiment, lepidopterans appeared to do the largest amount of damage, followed by Mexican bean beetles. Thus the negative effect of constitutive levels of PIs on plant damage might represent primarily a negative effect on lepidopterans, rather than an effect on Mexican bean beetles. These results are in agreement with many studies that have suggested that PIs should be an important resistance factor in plants (reviewed in Ryan 1990). Although cystein PIs have not previously been shown to affect lepidopterans, it is also possible that cystein PI production might be correlated with other aspects of plant physiology that do affect lepidopterans.

Several kinds of evidence suggested that cystein PIs were likely to be involved in induced resistance to Mexican bean beetles in soybeans. PIs have been shown to affect the performance of herbivores in several other systems [e.g., tomatoes and *Spodoptera* (Broadway et al. 1986), potatoes and *Diabrotica* (Orr et al. 1994)], and Mexican bean beetles are known to have cystein proteinases that are inhibited by cystein PIs in the laboratory (Ryan 1990). Given this evidence, why might cystein PIs not have affected beetle performance in the field? One possibility is that Mexican bean beetles may be able to adjust their production of digestive enzymes to favor enzymes not affected by cystein PIs. Bolter and Jongsma (1995) found that Colorado potato beetles (*Leptinotarsa decemlineata*) induced production of PI-insensitive proteases when reared on potato plants containing high levels of PIs, and (Broadway 1995) found that some lepidopteran larvae responded to exposure to cabbage PIs by shifting their production of digestive enzymes to favor enzymes that were less susceptible to inhibition. Another possible explanation is that phytochemicals may act differently in mixtures than they do in isolation, as elegantly argued by Duffey and Stout (1996). Thus measures of individual compounds may not be good predictors of whole plant effects on herbivores in some cases.

Conclusion

Understanding how plant resistance affects herbivores is important both for the improvement of agricultural practices and for building our understanding of ecological and evolutionary interactions between plants and insect herbivores. There is a great deal of work being done to identify substances produced by plants that are likely to be involved in plant resistance, particularly induced resistance. This study suggests that particular compounds such as PIs can have effects in the field, but that predicting the full effects of constitutive or induced resistance based on studies of PIs in isolation may be difficult. To determine the actual importance of a particular substance, studies are needed that experimentally manipulate that substance in plants growing in the field and examine effects on herbivores subject to field conditions. Likewise, determining the full effects of induced or constitutive resistance will require comparing whole plant responses. Studies that compare herbivore performance in the field with measures of plant chemistry in woody plants (e.g., Rossiter et al. 1988; Hunter and Schultz 1995; Kaitaniemi et al. 1998) are a step in the right direction. However, several of these studies (Rossiter et al. 1988; Kaitaniemi et al. 1998) reinforce the conclusion that the complexity and interaction of induced responses with other factors will make isolating the effects of individual compounds very difficult. Determining how the effects of individual compounds interact to form whole plant responses that are important to herbivores will be a very interesting challenge for plant and insect physiological ecologists. Only by putting future laboratory and field studies together will we be able to achieve the goal of linking the mechanistic building blocks of plant chemistry with the ecological functioning of plant-insect systems in the field.

References

Adler FR, Karban R (1994) Defended fortresses or moving targets? Another model of inducible defenses inspired by military metaphors. Am Nat 144:813–832

Astrom M, Lundberg P (1994) Plant defence and stochastic risk of herbivory. Evol Ecol 8:288–298

Bi JL, Felton GW, Mueller AJ (1994) Induced resistance in soybean to *Helicoverpa zea*: role of plant protein quality. J Chem Ecol 20:183–198

Bolter CJ, Jongsma MA (1995) Colorado potato beetles (*Leptinotarsa decemlineata*) adapt to proteinase inhibitors induced in potato leaves by methyl jasmonate. J Insect Physiol 41:1071–1078

Botella MA, Xu Y, Prabha TN, Zhao Y, Narasimhan ML, Wilson KA, Nielsen SS, Bressan RA, Hasegawa PM (1996) Differential expression of soybean cystein proteinase inhibitor genes during development and in response to wounding and methyl jasmonate. Plant Physiol 112:1201–1210

Broadway RM (1995) Are insects resistant to plant proteinase inhibitors? J Insect Physiol 41:107–116

Broadway RM, Duffey SS, Pearce G, Ryan CA (1986) Plant proteinase inhibitors: a defense against herbivorous insects? Entomol Exp Appl 41:33–38

Duffey SS, Stout MJ (1996) Antinutritive and toxic components of plant defense against insects. Arch Insect Biochem 32:3–37

Edelstein-Keshet L, Rausher MD (1989) The effects of inducible plant defenses on herbivore populations. 1. Mobile herbivores in continuous time. Am Nat 133:787–810

Edwards PJ, Wratten SD, Cox H (1985) Wound-induced changes in the acceptability of tomato to larvae of *Spodoptera littoralis*: a laboratory bioassay. Ecol Entomol 10:155–158

Felton G, Summers CB, Mueller AJ (1994) Oxidative responses in soybean foliage to herbivory by bean leaf beetle and three-cornered alfalfa hopper. J Chem Ecol 20:639–650

Haukioja E, Neuvonen S (1985) Induced long-term resistance of birch foliage against defoliators: defensive or incidental? Ecology 66:1303–1308

Hines ME, Osuala CI, Nielsen SS (1991) Isolation and partial characterization of a soybean cystatin cysteine proteinase inhibitor of coleopteran digestive proteolytic activity. J Agric Food Chem 39:1515–1520

Hunter MD, Schultz JC (1993) Induced plant defenses breached? Phytochemical induction protects an herbivore from disease. Oecologia 94:195–203

Hunter MD, Schultz JC (1995) Fertilization mitigates chemical induction and herbivore responses within damaged oak trees. Ecology 76:1226–1232

Iannone N (1989) Soybean resistance induced by previous herbivory: extent of the response, levels of antibiosis, field evidence and effect on yield. University of Illinois, Urbana-Champaign, Ill.

Johnson R, Narvaez J, Gynheung A, Ryan C (1989) Expression of proteinase inhibitors I and II in transgenic tobacco plants: effects on natural defense against *Manduca sexta* larvae. Proc Natl Acad Sci USA 86:9871–9875

Kaitaniemi P, Ruohomaki K, Ossipov V, Haukioja E, Pihlaja K (1998) Delayed induced changes in the biochemical composition of host plant leaves during an insect outbreak. Oecologia 116:182–190

Karban R (1986) Induced resistance against spider mites in cotton: field verification. Entomol Expl Appl 42:239–242

Karban R, Baldwin IT (1997) Induced responses to herbivory. University of Chicago Press, Chicago, Ill.

Kogan M (1972) Intake and utilization of natural diets by the Mexican bean beetle, *Epilachna verivestis* – a multivariate analysis. In: Rodriguez JG (ed) Insect and mite nutrition. North-Holland, pp 107–126

Kogan M, Fischer DC (1991) Inducible defenses in soybean against herbivorous insects. In: Tallamy DW, Raupp MJ (eds)

Phytochemical induction by herbivores. Wiley, Chichester, pp 347–378

Koiwa H, Shade RE, Zhu-Salzman K, Subramanian L, Murdock LL, Nielsen SS, Bressan RA, Hasegawa PM (1998) Phage display selection can differentiate insecticidal activity of soybean cystatins. Plant J 14:371–379

Kraemer ME, Rngappa M, Gade W, Benepal PS (1987) Induction of trypsin inhibitors in soybean leaves by Mexican bean beetle (Coleoptera: Coccinellidae) defoliation. J Econ Entomol 80:237–241

Kraemer ME, Rangappa M, Benepal PS, Mebrahtu T (1988) Field evaluation of soybeans for Mexican bean beetle resistance. I. Maturity groups VI, VII, and VIII. Crop Sci 28:497–499

Lin H, Kogan M (1990) Influence of induced resistance in soybean on the development and nutrition of the soybean looper and the Mexican bean beetle. Entomol Exp Appl 55:131–138

Liu S, Norris DM, Xu M (1993) Insect resistance and glyceollin concentration in seedling soybeans support resistance ratings of fully developed plants. J Econ Entomol 86:401–406

Orr GL, Strickland JA, Walsh TA (1994) Inhibition of *Diabrotica* larval growth by a multicystatin from potato tubers. J Insect Physiol 40:893–900

Osier TL, Lindroth RL (2001) Effects of genotype, nutrient availability, and defoliation on aspen phytochemistry and insect performance. J Chem Ecol 27:1289–1313

Powell JE, Lambert L (1993) Soybean genotype effects on bigeyed bug feeding on corn earworm in the laboratory. Crop Sci 33:556–559

Rhoades DF (1985) Offensive-defensive interactions between herbivores and plants: their relevance in herbivore population dynamics and ecological theory. Am Nat 125:205–238

Rossiter M, Schultz JC, Baldwin IT (1988) Relationships among defoliation, red oak phenolics, and gypsy moth growth and reproduction. Ecology 69:267–277

Ryan CA (1990) Protease inhibitors in plants: genes for improving defenses against insects and pathogens. Annu Rev Phytopathol 28:425–449

SAS Institute (2000) The SAS system. SAS Institute, Cary, N.C.

Seldal T, Dybwad E, Andersen K.-J, Hogstedt G (1994) Wound-induced proeinase inhibitors in grey alder (*Alnus incana*): a defence mechanism against attacking insects. Oikos 71:239–245

Thaler JS (1999) Jasmonate-inducible plant defences cause increased parasitism of herbivores. Nature 399:686–688

Underwood NC (1998) The timing of induced resistance and induced susceptibility in the soybean Mexican bean beetle system. Oecologia 114:376–381

Underwood N (1999) The influence of plant and herbivore characteristics on the interactions between induced resistance and herbivore population dynamics. Am Nat 153:282–294

Underwood N (2000) Density dependence in induced plant resistance to herbivore damage: threshold, strength and genetic variation. Oikos 89:295–300

Underwood N, Morris W, Gross K, Lockwood JR (2000) Induced resistance in soybeans to Mexican bean beetles: genetic variation and correlation. Oecologia 122:83–89

Underwood N, Rausher MD (in press) Comparing the consequences of induced and constitutive resistance for herbivore population dynamics. Am Nat

Van Duyn JW, Turnipseed SG, Maxwell JD (1971) Resistance in soybeans to the Mexican bean beetle. I. Sources of resistance. Crop Sci 11:572–573

Zangerl AR, Rutledge CE (1996) The probability of attack and patterns of constitutive and induced defense: a test of optimal defense theory. Am Nat 147:599–608

Zhao Y, Botella MA, Subramanian L, Niu X, Nielsen S, Bressan RA, Hasegawa PA (1996) Two wound-inducible soybean cysteine proteinase inhibitors have greater insect digestive proeinase inhibitory activities than a constitutive homolog. Plant Physiol 111:1299–1306