Effects of soybean proteinase inhibitor on development, survival and reproductive potential of the sugarcane borer, *Diatraea saccharalis*

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Abstract

One approach that can be employed in integrated pest management is the use of proteins with antinutritional effects on insect metabolism and development. The antitoxic properties of soybean proteinase inhibitor (SPI) on growth of neonate larvae of the sugarcane borer, *Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae) have been evaluated. When incorporated into an artificial diet at 0.5% (w/w), SPI retarded growth rate and development of larvae when compared with larvae fed on artificial diet alone. However, larval survival was not significantly affected. The purpose of our research was to calculate demographic statistics for the sugarcane borer reared on diet either with or without semi-purified extract of SPI. Net reproductive rate (*R₀*), instantaneous rate of increase (*rₘ*), combined age-specific survivorship (*lₓ*) and age specific fecundity (*mₓ*) provide information about population growth potential. These parameters were measured in order to determine the effects of the proteinase inhibitor on the insect’s population dynamics. The observed differences would potentially translate into large reductions in population growth, indicating a potential value of using SPI for protecting sugarcane plants against damage by the sugarcane borer.

Introduction

*Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera, Crambidae), the sugarcane borer, is the major insect pest of sugarcane in Brazil and other South American countries, and is responsible for significant economic damage. The larvae feed, pupate, and emerge as second generation moths over an extended period in the sugarcane crop. The increasing pressure to use non-hazardous, environmentally-compatible pest control measures has spurred an interest in the use of natural insecticides such as *Bacillus thuringiensis* (*Bt*) and plant-derived proteinase inhibitors (Duan et al., 1996).

Biological control agents such as *Cotesia flavipes*, a gregarious larval endoparasitoid native to the Indo-Australian region (Sallam et al., 1999) offer an important control method for sugarcane borer. It was introduced into Brazil in 1974 (Degaspari et al., 1987), but there was a residual and remaining pest population which caused yield loss. The evaluation of proteaseous inhibitors of proteolytic enzymes as candidates to provide resistance to economically-important crops against insect pests is currently attracting much research interest (Jouanin et al., 1998). The potential utility of these proteins has been enhanced by the demonstration that the introduction of genes that encode proteinase inhibitors into crop plants can confer significant insect pest resistance to the transformants (McManus & Burgess, 1995). Although the techniques for introducing foreign genes into many crop plants are now becoming routine, the identification
of useful genes to be transferred remains a difficult problem. It is necessary to select the appropriate inhibitors for the digestive proteinases of each particular pest species. This requires knowledge of the proteinases present in the insect gut and the way they interact with the various inhibitors (Ortego et al., 1998). Serine proteinase activity has been found in a wide variety of lepidopteran pests, including *D. saccharalis* (Pompermayer, 2000). Several reports have been able to show directly that proteinase inhibitors can inhibit the activity of insect digestive proteinases in *vivo*. Proteinase inhibitors purified from different plant sources have shown deleterious effects in *in vivo* artificial diet bioassays and in *in vitro* assays with insect gut proteinases (Jouanin et al., 1998). These molecules interfere with the growth and development of the larvae and in some cases lead to insect death (Jongsma & Bolter, 1997). These observations have been extended by the demonstration that these proteinase inhibitors can retard insect larval development when incorporated into artificial diets. Soybean seeds contain two forms of serine proteinases inhibitors, called Bowman–Birk and Kunitz. These inhibitors have been incorporated into artificial diets and their effectiveness against distinct insect proteinases including some found in Lepidoptera (Broadway & Duffey, 1986; McManus & Burgess, 1995), Coleoptera (Ortego et al., 1998) and Orthoptera digestive systems (Burgess et al., 1991), have been successfully demonstrated. However in some cases they showed relatively poor efficacy (Jongsma & Bolter, 1997; Paulillo et al., 2000). One way to obtain more efficient inhibitors against insect proteinases is to survey plant species for proteinase inhibitor families that do not exist in the host plant (Jongsma et al., 1996).

To minimize the probability of developing resistant insect biotypes, against a desirable trait, a strategy for pest control would be to interfere with insect pest population growth rates but without causing high insect mortality. Decreasing population growth rates could result from delayed larval development, delayed reproduction, lower fecundity, or a combination of these factors (Wolfson & Murdock, 1995). Sources of resistance that cause high mortality are relatively easy to screen but they have limited longevity because of the development of resistance-breaking biotypes. In the present article we report the effect of consumption of SPI on the life history characteristics of *D. saccharalis*, the sugarcane borer. This paper reports the use of the fertility life table parameters to evaluate the effect of a proteinase inhibitor on larval life cycles.

### Material and methods

**Extraction and partial purification of proteinase inhibitors.** Proteinase inhibitors (Kunitz and Bowman–Birk) were extracted according to Broadway (1993) by homogenizing 100 g of soybean seeds in 1 liter of a 0.15 M NaCl solution, squeezing the homogenate through cheesecloth, centrifuging the filtrate at 3000 × g for 20 min at 4 °C and collecting the supernatant. The supernatant was adjusted to 70% saturation with ice-cold acetone whilst stirring. The solution was then centrifuged at 6000 × g for 20 min at 4 °C. The resultant pellet was lyophilized to remove the acetone and produced a semi-purified proteinase inhibitor (SPI) corresponding to 12% (w/w) of the initial seed protein as calculated using Enzfitter software (Elsevier Biosoft).

**Insects.** A laboratory colony of *D. saccharalis* was maintained on artificial diet (King & Hartley, 1985) under a photoperiod of L14:D10 at 25 °C ± 1 °C and 60% ± 10% rh, and used in all feeding trials.

**Insect feeding trial.** To determine the effect of soybean proteinase inhibitor (SPI) on larval growth and development, *D. saccharalis* larvae were reared on a wheat germ-based meridic diet (King & Hartley, 1985) supplemented either with or without SPI (0.5% w/w). The semi-purified proteinase inhibitor was dissolved in water and heated at 90 °C, and used in all feeding trials. The experiment was initiated using eggs, and was completed when control insects reached the adult stage. Larval weights were recorded at 20 days after eclosion (DAE).

The parameters analyzed included initial mortality, length of the larval and pupal period, larval and pupal viability and average larval weight. The instar number was calculated using Dyar’s method (Dyar, 1890), adapted by Parra & Haddad (1989). Oviposition experiments were carried out with insects reared on the same control or SPI incorporated diets. The following fertility life table parameters were calculated for each diet (treatment) using standard procedures and formulae (Carey, 1982; Brødsgaard, 1994): the probability of surviving to age *x* (*l*<sub>x</sub> = *N*<sub>x</sub>/*N*<sub>0</sub>); number of female offspring produced per age interval (*m*<sub>x</sub>); remaining life expectancy at age *x* (*e*<sub>x</sub> = *T*<sub>x</sub>/*l*<sub>x</sub>); reproductive value at age *x* (*V*<sub>x</sub> = *l*<sub>x</sub> × *m*<sub>x</sub>); net reproductive rate (*R*<sub>0</sub> = ∑ *V*<sub>x</sub>); intrinsic rate of increase (*r*<sub>m</sub>, *l* = ∑ e<sup>(−*r*<sub>m</sub> × *x*)</sup> × *V*<sub>x</sub>); finite rate of increase
\( \lambda = e^\mu \); mean generation time \( T = (\ln R_0)/r_m \) and doubling time \( DT = (\ln 2)/r_m \).

**Statistical analysis.** Overall data on initial mortality, length of larval and pupal period, and larval and pupal average weight were statistically analyzed using general ANOVA. When a difference was found between treatments, it was then tested at the \( P > 0.05 \) level using the Tukey test. The parameters associated with the life table were estimated by the jackknife method (Meyer et al., 1986) using the SAS System (Maia et al., 2000).

**Results**

**Effect of SPI on different stages of insect development.** The weights of larvae fed on SPI incorporated diet were significantly lower than those from control diet fed larvae. However, pupal weight was not significantly different. In addition, larvae fed on control diet weighed more than those fed on SPI diet (Table 1). The size difference between control diet and SPI fed larvae (Figure 1) arose from different growth patterns during the experiment, being significantly higher in the control treatment \( P > 0.05 \). Consumption of SPI diet also delayed the developmental time to pupation and adult emergence, significantly \( P > 0.05 \). About 56% of larvae reared on SPI diet presented six instars whereas insect fed on control diet presented five instars only (Figure 2). Similar results were observed at the pupal and adult stage, when SPI diet increased pupal duration (Table 1) and significantly reduced \( P > 0.05 \) female longevity (Figure 3). There were no statistically detectable mortality differences between treatments.

**Reproductive potential of SPI fed insects.** Larval consumption of SPI had a significantly negative effect on adult *D. saccharalis* reproductive potential (Table 2). Oviposition commencement was significantly delayed by 6 days (an 18% increase) compared to the control fed adults (Figure 4). In contrast, the number of eggs laid per female varied greatly in both treatments resulting in no significant differences (not shown).

The life table parameters (Table 2), indicated that both the net reproductive rates \( (R_0) \) and the intrinsic rates of increase \( (r_m) \) were significantly lower for insects reared on SPI-containing diet. The mean duration of a generation \( (T) \), that is the period of time elapsed
Table 1. Fertility life table (mean ± SE) for Diatraea saccharalis reared on diet with semi-purified extract of soybean proteinase inhibitor (SPI)

<table>
<thead>
<tr>
<th>Diet</th>
<th>n</th>
<th>$R_0$</th>
<th>$r_m$</th>
<th>$T$</th>
<th>DT</th>
<th>$\lambda$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>29</td>
<td>350.50 ± 20.98</td>
<td>0.170 ± 0.002</td>
<td>34.55 ± 0.25</td>
<td>4.085 ± 0.042</td>
<td>1.185 ± 0.002</td>
</tr>
<tr>
<td>0.5% (w/w) SPI</td>
<td>29</td>
<td>261.90 ± 15.18</td>
<td>0.134 ± 0.001</td>
<td>41.56 ± 0.25</td>
<td>5.172 ± 0.055</td>
<td>1.143 ± 0.002</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>&lt;0.00062a</td>
<td>&lt;0.000001b</td>
<td>&lt;0.000001b</td>
<td>&lt;0.000001b</td>
<td>&lt;0.00001a</td>
</tr>
</tbody>
</table>

$R_0$ – net reproductive rate (female/female/generation); $r_m$ – intrinsic rate of increase (female/female/day); $T$ – mean generation time, days; DT – doubling time days; $\lambda$ – finite rate of increase (female/female/day).

$a$ P-values compared by upper tailed $t$-test.

$b$ P-value compared by low tailed $t$-test.

Table 2. Effect of semi-purified extract of soybean proteinase inhibitor (SPI) on survival and growth of Diatraea saccharalis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Larval Weight (mg ± SE)</th>
<th>Larval Duration (days ± SE)</th>
<th>Larval Mortality (%)</th>
<th>Pupal Weight (mg ± SE)</th>
<th>Pupal Duration (days ± SE)</th>
<th>Pupal Mortality (%)</th>
<th>Adult Longevity (days ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controla</td>
<td>198 ± 10a</td>
<td>20.80 ± 2.89a</td>
<td>6.77</td>
<td>132 ± 1a</td>
<td>198 ± 4a</td>
<td>9.08 ± 0.58a</td>
<td>2.00</td>
</tr>
<tr>
<td>0.5% (w/w) SPIb</td>
<td>160 ± 5b</td>
<td>25.69 ± 0.31b</td>
<td>10.85</td>
<td>129 ± 5a</td>
<td>199 ± 4a</td>
<td>10.08 ± 0.07b</td>
<td>4.36</td>
</tr>
</tbody>
</table>

Means followed by the same letter within each parameter are not significantly different ($p > 0.05$) according to Tukey test.

$^a$ For larval weight $n = 30$; larval duration $n = 219$; male pupal weight $n = 108$; female pupal weight $n = 96$; pupal duration $n = 204$; adult duration $n = 29$.

$^b$ For larval weight $n = 30$; larval duration $n = 219$; male pupal weight $n = 92$; female pupal weight $n = 62$; pupal duration $n = 154$; adult duration $n = 29$.

Discussion

It has been demonstrated that ingestion of proteinase inhibitors in natural or artificial diets can retard growth and development of several insect pests, suggesting that these inhibitors may serve as an effective mean of insect control. However, mortality was quite low in most studies and protection not complete, even though high level of inhibitors were used (Ortego et al., 1998).

The early effect on growth rates observed in this study resulted in significant differences in larval weight. The mean weight of larvae fed on control diet was significantly greater than the mean weight of larvae fed SPI diet. These results are in agreement with those observed for Spodoptera litura, using the same SPI (McManus & Burgess, 1995).

Larvae fed on SPI diet showed reduced growth rate. Indeed, most SPI fed insects presented an additional instar which significantly extended the developmental time. Within certain species, the number of instars may be quite constant between individuals or it may vary, especially under certain unfavorable conditions (e.g., an inadequate food supply). If environmental conditions become unsuitable, the response of the larval will vary depending on whether it has reached its minimal weight for pupation, on the nature of the environmental change, and on its particular adaptive strategy (Slansky & Scriber, 1985).

The severe delay in growth and development caused by inhibitors, if occurring in a natural setting, would provide a much longer period in which the larvae would be subject to their natural predators and pathogens (Mochizuki et al., 1999). Additionally, the full normal pattern of development of the larvae may not be possible under conditions of nutritional stress, as imposed here.
Figure 4. Age in days to the beginning of oviposition by *D. saccharalis* reared on control diet (A) and on (B) diet containing soybean proteinase inhibitor (SPI) (0.5%, w/w).

Our results lend support to the hypothesis of Broadway & Villani (1995) that generalist feeders are better adapted than specialists to deal with proteinase inhibitors. The significant results observed in this work may be due to the specialized feeding of *D. saccharalis*. Proteinase inhibitor (PI)-insensitive proteinases are probably the result of selection pressures when insects encounter high PI levels in host plants. Such selection for insensitive proteases has not occurred for non-host plant PIs. One way to obtain more efficient inhibitors of insect proteinases is, therefore, to screen plant species unrelated to the host plant for PI families that do not exist in the host plant (Jongsma et al., 1996). Duan et al. (1996) suggested that insects that normally feed on dicots or monocots are not capable of adapting to PIs from the other plant type. This observation is in agreement with the results presented in this study, although the inhibition levels were relatively low. One possible explanation might be the supra-optimal composition of the diet which was designed for mass rearing of the *D. saccharalis*, thus presenting high level of nutrients, not normally found in sugarcane plants. This support previous work showing the importance of the diet composition on the effect of proteinase inhibitors (Broadway & Duffey, 1986; Jongsma & Bolter, 1997).

Several authors have argued that the best approach for evaluation of the total effect of xenobiosis is life table analysis (Stark & Wennergren, 1995). Various types of life table analysis have been used to assess the effects of biotic and abiotic factors on population growth in nature. Laboratory age-specific life tables are used to estimate intrinsic birth and death rate parameters from a cohort of individuals under a given
set of conditions (Gutierrez, 1996), and the effects of different levels of factors on vital rates. Insect susceptibility to proteinase inhibitors can be measured in terms of survival, development time, and fecundity of the pest. However, demographic statistics provide a quantitative method of analyzing insect populations by assessing survival, fecundity, and growth patterns (Zeng et al., 1993). These parameters are appropriate measures of the effects of the proteinase inhibitor on the insects’ population dynamics. Such analysis are particularly useful in the study of host plant resistance when some specific life stage of the target insect is affected (Zeng et al., 1993). Fertility life tables are appropriate to study the dynamics of animal populations, especially arthropods as an intermediate process for estimating parameters related to the population growth potential, also called demographic parameters (Maia et al., 2000).

Proteinase inhibitors that are slower acting and produce sub-lethal effects are being developed and registered. For these products, sub-lethal effects may be as important as lethal effects (Stark & Rangus, 1994). The slow acting nature of these new products and the important sub-lethal effects they produce make their analysis more complex. For products (e.g.; proteinase inhibitors) that manifest a high degree of sub-lethal effects, demographic analysis appears to be an ideal method of evaluation because it combines both lethal and sub-lethal effects.

In this study we have demonstrated that soybean proteinase inhibitors, when incorporated into artificial diets at 0.5% (w/w), could have a significant effect on D. saccharalis populations. Although a thermal treatment has not affected the inhibitory activity of SPI extract (data not presented), we cannot exclude the possibility that thermal-resistant impurities could have contributed to larval mortality or delayed development. Further questions concerning the relationship between these proteinase inhibitors and the growth, development, and survival of the insect can be addressed in transgenic plants, where effects of single gene changes can be observed. This technology allows the possibility to obtain transgenic plants resistant to the sugarcane borer.

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