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Survival and developmental impairment induced by the trypsin inhibitor bis-benzamidine in the velvetbean caterpillar (*Anticarsia gemmatalis*)

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ABSTRACT

Proteinase inhibitors are potential insect control agents, but insect adaptation to these compounds is one of the main limitations for their potential use as such. The velvetbean caterpillar (*Anticarsia gemmatalis* Hübner) (Lepidoptera: Noctuidae) is a key soybean pest species well-adapted to its prevailing (serine-) proteinase inhibitors, particularly trypsin-like inhibitors. The recognition of proteinase inhibitors with insecticidal activity towards such pest species is therefore challenging and important as a basis for the development of mimetic peptides with potential use as biorational insecticides. Thus, bis-benzamidine was tested against the velvetbean caterpillar with the expectation of greater insecticidal activity of this more potent trypsin inhibitor than the negligible effects observed with other natural and synthetic trypsin inhibitors. Bis-benzamidine ingestion by the caterpillars led to higher survival time with increased doses up to 76 ppm, but drastically reduced survival time at higher doses (over 150 ppm). Insects exposed to bis-benzamidine doses of up to 76 ppm exhibited extended larval development and decreased pupa weight. Increased doses of this trypsin inhibitor led to increased diet consumption and protein digestibility during the larval phase, but drastically compromised the proteolytic activity in the caterpillar gut. These results indicate that, unlike benzamidine, another related synthetic trypsin inhibitor, bis-benzamidine exhibits insecticidal activity towards the velvetbean caterpillars at doses as low as 9.5 ppm in the insect diet due to suppression of gut proteinase activity despite the compensatory feeding. Such compensatory feeding may however increase insect damage in the field by more tolerant individuals and should be the object of further study.

1. Introduction

Insecticides have been prevailing insect control agents for the last few decades, but the ever increasing demands of human and environmental safety resulted in the development of novel compounds (Nauen and Bretschneider, 2002; Matsumura, 2004; Cooper and Dobson, 2007; Matthews, 2008). Among these novel compounds, bioinsecticides or biorational insecticides re-emerged as an object of growing attention (Carlini and Grossi-De-Sá, 2002; Isman, 2006; Rosell et al., 2008).

Toxic compounds from plants are recognized as bioinsecticides, but they are also valuable as new chemical backbones for the development of novel insecticide molecules (Kidd, 2000; Rosell et al., 2008). Plant toxic proteins, such as proteinase inhibitors, are among such compounds, which were initially explored for generating genetically modified plants resistant to insect pests (Hilder et al., 1987; Hilder and Boulter, 1999; Abdeen et al., 2005). Proteinase inhibitors have recently been the object of attention for the development of mimetic peptides with insecticidal activity (Pompermayer et al., 2001; Pilon et al., 2006; Nicholson, 2007). The chronic ingestion of proteinase inhibitors limits availability of essential amino acids for insect growth, development and reproduction while leading to a hyperproduction of digestive proteinases (Broadway and Duffey, 1986; Koiwa et al., 1997).

The efficacy of proteinase inhibitors against insects depends on the target species and their prevailing classes of digestive proteinases, a trait which limits their spectrum of activity (Pompermayer et al., 2001; Pilon et al., 2006). In addition, insect adaptation to proteinase inhibitors may compromise their use for insect pest control and has been reported in some insect species (Jongsma et al., 1995; Winterer and Bergelson, 2001; Yang et al.,

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2009). The key soybean insect pest *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae), known as the velvetbean caterpillar, is well-adapted to its proteinase-rich host (Gallo et al., 2001; Fortunato et al., 2007). Such adaptation also extends to other natural and even synthetic serine-proteinase inhibitors, particularly trypsin-like proteinase inhibitors (Oliveira et al., 2005; Xavier et al., 2005; Pilon et al., 2006). The inducible expression of proteinases insensible to these inhibitors either by the insect itself or its associated gut symbionts may allow such adaptation (Jongsma et al., 1995; Visôtto et al., 2009a,b). This ability of the velvetbean caterpillar to circumvent serine-proteinase inhibition, making it a very tolerant species to such inhibitors, makes it a good model to test the efficacy and effects of promising proteinase inhibitors for future development of mimetic peptides for use as biorational insecticides.

Serine-proteinase is the main class of digestive proteinases reported in Lepidoptera at large, and in the velvetbean caterpillar in particular (Reeck et al., 1999; Terra and Ferreira, 2005). However, the velvetbean caterpillar is able to circumvent the potentially harmful effects of high concentrations of benzamidine, a serine-proteinase inhibitor (Pilon et al., 2006). Here we tested a more potent parabolic competitive inhibitor of trypsin-like (serine) proteinases against the velvetbean caterpillar – bis-benzamidine. Because of its higher potency against trypsin-like proteinases than benzamidine (Junqueira et al., 1992), we expected bis-benzamidine to significantly impair protein digestion in the velvetbean caterpillar, compromising the insect's post-embryonic development and increasing its mortality.

2. Material and methods

2.1. Insects and chemicals

A colony of the velvetbean caterpillar was established from eggs obtained from the Soybean National Research Center of the Brazilian Agricultural Research Corporation (EMBRAPA Soja, Londrina, PR, Brazil), where field-collected insects from soybean crops were periodically added to the laboratory colony. The insect eggs were surface-sterilized with UV light (20 W lamps placed 25 cm away) and subsequently transferred to sterile glass containers at 25 ± 5 °C until hatching. The newly hatched larvae were transferred to plastic containers in a laminar flow chamber and reared on the artificial diet following the methods given by Hoffman-Campo et al. (1985), under controlled conditions of 26 ± 2 °C, 70 ± 5% r.h. and 14:10 (L:D) photoperiod. All reagents were purchased from Sigma–Aldrich Química Brasil Ltda (São Paulo, SP, Brazil), except ascorbic acid and nipagin, which were obtained from Synth-LabSynth (São Paulo, SP, Brazil). Beans, wheat germ, soybean protein and vitamins were all obtained from the local market.

2.2. Exposure to bis-benzamidine

The experiment was established using doses of the synthetic trypsin inhibitor bis-benzamidine (berenil) mixed to the artificial diet during its preparation (i.e., 0, 9, 19, 38, 76, 152, 304, 608, 912, 1250, and 2500 ppm). Forty replicates were used and each replicate was constituted of one newly emerged caterpillar individualized in a plastic container at the same rearing conditions described above. Diet, containing bis-benzamidine or not, was provided *ad libitum* to insects according to each established treatment. The post-embryonic development of each insect was followed daily until its eventual death. Diet consumption and faeces production were recorded daily using an analytical balance (10 μg readability; Sartorius BP210D, Göttingen, Germany). The larvae weight was also recorded daily after the fifth day of egg hatching until the pupal stage. Separate groups of insects were maintained under the same conditions for midgut extraction from fifth instar caterpillars for protein determination and assessment of digestive proteinase activity; three replicates each of three insects were used in these determinations.

2.3. Protein concentration and digestibility

Weight of diet consumed and faeces produced per insect were used for determining protein concentration so that protein digestibility could be calculated. Protein determination in the non-consumed diet and in the faeces produced per insect was determined using the micro-Kjeldahl method to quantify total nitrogen, following the methods described by Pilon et al. (2006). Nitrogen from bis-benzamidine does not interfere with the determination not only because of its low concentration in the diet, but also because this element constitutes a highly stable functional group in the synthetic inhibitor which is stabilized by resonance between its amino groups linked to the non-aromatic carbon, preventing its release during the Kjeldahl (Instituto Adolf Lutz, 1985). Nitrogen concentration was converted to protein concentration using the factor 6.25. The digestibility was calculated using the formulae: % digestibility = [(ingested protein − excreted protein) × 100]/ ingested protein. Triplicate readings were made for determining the protein of diets and duplicate readings were made for the faeces produced for each replicate.

2.4. Preparation of midgut extracts

The fifth instar larvae from each treatment were water rinsed and ice-chilled for dissection in 10^{-3} M HCl at 4 °C. The extracted midguts were used as the enzyme source for determination of general proteolytic, besides amydolytic and esterolytic activities after their removal from the insects and cellular lyses through submission to nine cycles of nitrogen freezing and thawing at 37 °C in a water bath (Oliveira et al., 2005; Pilon et al., 2006). Aliquots of 1 ml of midgut extract were centrifuged at 100,000 g for 30 min at 4 °C. The resulting supernatant was collected and stored at − 18 °C for later use as enzyme source.

2.5. Protein quantification and enzyme assays

Protein concentration was measured following Bradford (1976). Bovine serum albumin (BSA) solutions of 0–0.2 mg/ml were used as standard. General proteolytic activity was determined using azocasein as substrate at the final concentration of 2% (w/v) following a 30 min incubation, when the reaction was interrupted by adding trichloroacetic acid (10%, w/v) to the mixture (Kunitz, 1947), as modified by Oliveira et al. (2005). Serine-proteinase amidolytic activity was determined as described by Erlanger et al. (1961) using N-α-benzoyl-L-Arg-*p*-nitroanilide (L-BApNA) as substrate at a final concentration of 1.2 mM in 0.1 M Tris–HCl buffer (pH 8.2) containing 20 mM $CaCl_2$ and 1% dimethyl formamidine (DMF, w/v). The resulting absorbance was read at 410 nm. Serine-proteinase esterolytic activity was determined using N-α-*p*-tosyl-L-Arg methyl ester (L-TAME; 0.1 mM) as substrate in 0.1 M Tris–HCl buffer (pH 8.2) (Hummel, 1959). The absorbance was read at 247 nm. Determinations were always performed in triplicate (for each replicate) and appropriate controls were included.

2.6. Data analyses

Survival analysis was carried out to recognize potential differences in caterpillar mortality during development under the different bis-benzamidine doses. The survival curves were obtained