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PEST MANAGEMENT

Enzymatic Response of the Eucalypt Defoliator *Thyrinteina arnobia* (Stoll) (Lepidoptera: Geometridae) to a Bis-Benzamidine Proteinase Inhibitor

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Abstract
Ingestion of proteinase inhibitors leads to hyperproduction of digestive proteinases, limiting the bioavailability of essential amino acids for protein synthesis, which affects insect growth and development. However, the effects of proteinase inhibitors on digestive enzymes can lead to an adaptive response by the insect. In here, we assessed the biochemical response of midgut proteinases from the eucalypt defoliator *Thyrinteina arnobia* (Stoll) to different concentrations of berenil, a bis-benzamidine proteinase inhibitor, on eucalyptus. Eucalyptus leaves were immersed in berenil solutions at different concentrations and fed to larvae of *T. arnobia*. Mortality was assessed daily. The proteolytic activity in the midgut of *T. arnobia* was assessed after feeding on plants sprayed with aqueous solutions of berenil, fed to fifth instars of *T. arnobia* for 48 h before midgut removal for enzymatic assays. Larvae of *T. arnobia* were able to overcome the effects of the lowest berenil concentrations by increasing their trypsin-like activity, but not as berenil concentration increased, despite the fact that the highest berenil concentration resulted in overproduction of trypsin-like proteinases. Berenil also prevented the increase of the cysteine proteinases activity in response to trypsin inhibition.

Introduction

Most animals require proteolysis to degrade the proteins and use the amino acids available in their food sources, which is primarily accomplished extracellularly in their food canal (Ryan 1990). Larvae of insect herbivores voraciously feed on plant parts to obtain nutrients for their optimum growth, development, metamorphosis, and adult reproduction (for a review, see Srinivasan *et al* 2006). As a protective response to insect feeding, plants may produce proteinase inhibitors (PIs) (Ferreira *et al* 2005, Fortunato *et al* 2004, 2007). PIs are small proteins described as occurring in reproductive and vegetative tissues of more than 100 plant species (Ryan 1990, Fortunato *et al* 2004, Marinho *et al* 2008). The activity of PIs in the midgut of insect larvae causes amino acid deficiencies and, consequently, developmental arrest or mortality (Bazok *et al* 2005, Telang *et al* 2005, Vila *et al* 2005, Bayés *et al* 2006, Pilon *et al* 2006).

Ingestion of PIs does not eliminate proteolytic digestion in the midgut; instead, it results in the hyperproduction of proteolytic enzymes by the insect herbivore, which in turn will limit the availability of essential amino acids for protein synthesis, and consequently lead to reduced larval growth

and development (Broadway & Duffey 1986, Ryan, 1990). However, herbivorous insects have the potential to become resistant to PIs (Broadway 1995, Vila *et al* 2005, Pilon *et al* 2006, Volpicella *et al* 2006, Marinho *et al* 2008, Moreira *et al* 2011).

Because of the variability in proteinases between insects and the limited spectrum of activity of their inhibitors (Ortego *et al* 1998), the expression of a particular proteinase inhibitor in plants may not result in a broad-spectrum control. Therefore, it is necessary to select the appropriate inhibitors for the digestive proteinases of each particular pest species. Insects are physiologically adapted to circumvent plant protease inhibitors by the secretion of inhibitor-resistant enzymes and by the proteolysis of proteinase inhibitors by nontarget digestive proteases (Pilon *et al* 2006, Volpicella *et al* 2006, Visôtto *et al* 2009a, b).

The selection of appropriate inhibitors requires knowledge about the proteinases present in the insect midgut and the way they interact with different inhibitors. Serine proteinase activity has been found in a wide variety of lepidopteran pests (Broadway & Duffey 1986, Broadway 1995, Pompermayer *et al* 2001, Telang *et al* 2005, Pilon *et al* 2006, Volpicella *et al* 2006), including larvae of *Thyrinteina* (Marinho *et al* 2008, 2011), an important eucalyptus defoliator in Brazil (Holtz *et al* 2003).

Berenil is an example of a synthetic trypsin inhibitor (Oliveira *et al* 1993), whose potential as insecticide, repellent, and antifeedant against *Thyrinteina arnobia* (Stoll, 1782) has been early demonstrated (Marinho-Prado *et al* 2011). In here, we demonstrate the effects of berenil on the survivorship and proteolytic activity of enzymes of the midgut of *T. arnobia* larvae exposed to increasing concentrations of berenil applied on eucalyptus leaves to better understand the adaptive mechanism involved in larval resistance to berenil ingestion.

Material and Methods

Eggs of *T. arnobia* obtained from the laboratory colony maintained at the "Universidade do Estado de São Paulo" (UNESP, Botucatu, São Paulo, Brazil) were used to establish a colony at the "Instituto Agronômico de Campinas" (Campinas, Brazil), which was maintained under controlled conditions (26±3°C; 60±15% RH; 12-h photophase). Plants of *Eucalyptus grandis* (70 cm high) free of pesticide residues were offered to the insects in wooden cages (1.0×1.0×1.0 m). The sides and top of the cage were covered with organza to allow for ventilation. Berenil (diminazene aceturate) was purchased from Sigma-Aldrich Química Brasil (São Paulo, Brazil), and the agricultural dispersant Gotafix ($C_{35}H_{64}N_{11}$), obtained from Milenia Agro-Ciências S.A. (Paraná, Brazil), was used to improve adherence and penetration of berenil. The experiments were carried out from January to September 2009.

The experiment was established in three blocks, using six concentrations of the trypsin inhibitor berenil (0, 0.06, 0.12, 0.25, 0.5, and 0.75% *w*/*v*) in water, containing 0.06% (*v*/*v*) Gotafix. Thirteen newly eclosed larvae were individualized in plastic dishes (9 cm diameter) and used for each treatment in each block (i.e., 39 larvae were used per berenil concentration). Plants of *E. grandis* (50 cm high) were used, and leaves were immersed in one of the berenil solutions. Treated leaves were allowed to dry at room temperature and offered to larvae of *T. arnobia*. The leaf petiole was involved in wet cotton when harvested to retain humidity. Upon reaching the fifth instar, larvae of *T. arnobia* were transferred to plastic cups (500 mL) for pupation and adult emergence. Adults were placed in cylindrical cages (8.5 cm diameter and 19 cm height) with the inner wall covered with filter paper for mating. Mortality was daily assessed.

The concentrations of berenil that prevented adult development were not used for the biochemical assays, and intermediate concentrations were added to the others. The tests were carried out under controlled conditions (25±1°C; 75±10% RH; 12-h photophase). Biochemical tests were designed to assess the response of proteolytic activity in the larval midgut to the ingestion of eucalyptus leaves sprayed with berenil at increasing concentrations (0, 0.03, 0.06, 0.09, and 0.12% *w*/*v*, using water as solvent containing 0.06% Gotafix). For this purpose, three eucalyptus plants (70 cm high) were used for each berenil concentration. These plants were placed in cages as those earlier described and maintained at controlled conditions (26±3°C; 60±15% RH, 12-h photophase). Different berenil concentrations were uniformly applied on the plants using a manual plastic spray. Nine fifth instars of *T. arnobia* were placed on each plant for a 48-h feeding period before the larvae were recollected for midgut dissection and enzyme assays.

Larvae were ice-chilled and dissected in 1 mM HCl at 4°C. The midguts obtained were used as an enzyme source for determination of general proteolytic activity, besides amylolytic and esterolytic serine proteinase activity. Cysteine proteinase activity was also determined in the midgut extracts. The midguts removed from the caterpillars were subjected to cell lyses through submission to nine cycles of nitrogen freezing and thawing at 37°C in a water bath. Aliquots of midgut extract obtained (150 mg of midgut to 1.0 mL of 1 mM HCl) were centrifuged (100,000×*g*×30 min×4°C) (Oliveira *et al* 2005, Silva *et al* 2010a, b), and the resulting supernatant was collected and stored at −18°C for subsequent use.

Protein concentration was measured following Bradford (1976), using a solution of 0.2 mg/ml of bovine serum albumin as standard. Total protease concentration was

determined using azocasein as substrate (2% *w*/*v*) at 37°C, following Tomarelli *et al* (1949). The reaction mixture encompassed 25 µL substrate and 30 µL enzyme extract, which was incubated at 37°C for 30 min. The reaction was stopped by adding 120 µL of 10% trichloroacetic acid and then rested on ice for 15 min, after which 140 µL of 1.0 M NaOH was added before reading the absorbance at 440 nm. The activity of serine proteinases was assessed using two substrates: *N*-a-benzoyl-L-Arg-*p*-nitroanilide (L-BApNA) as a substrate for determination of amylolytic activity, and *N*-a-*p*-tosyl-L-Arg methyl ester (L-TAME) as a substrate for determination of esterolytic activity. The amylolytic activity was determined following Erlanger *et al* (1961), using 1.2 mM L-BApNA in 0.1 M Tris–HCl buffer (pH 8.2) containing 20 mM $CaCl_2$. The reaction mixture encompassed 0.5 mL substrate and 10 µL enzyme extract, which was incubated at 25°C for 2.5 min. The absorbance was read at 410 nm, and the extinction coefficient 8,800 M^{-1} cm^{-1} was used to calculate the enzymatic activity. The esterolytic activity was determined following Hummel (1959) using 0.1 mM L-TAME in the same buffer system used for amylolytic activity. The reaction mixture encompassed 0.5 mL substrate and 10 µL enzyme extract, which was incubated at 25°C for 2.5 min. The absorbance was read at 247 nm, and the extinction coefficient of 540 M^{-1} cm^{-1} was used to calculate the enzyme activity. As previously described, cysteine proteinase activity was assessed using L-BApNA as substrate, but adding 5 mM dithiothreitol (reducer agent of sulfur bonds) and 0.1 mL of 10 mM benzamidine, a serine-proteinase inhibitor, to the buffer Tris–HCl 0.1 M (pH 8.0) to constitute the reaction mixture (Araújo *et al* 2008).

The data obtained were subjected to regression analyses using the software TableCurve 2D to fit the regression lines (SPSS 2000). The regression models were tested from the simplest (linear and quadratic) models to the alternative models of increasing complexity (nonlinear peak models). The model selection was carried out based on relative adj. R^2 (rel. adj. R^2), parsimony, and high *F* values (and mean squares). The rel. adj. R^2 was estimated by dividing the adj. R^2 of the selected model by the maximum possible adj. R^2 of the alternative models to obtain an indication of the goodness-of-fit of the selected model compared to the alternative ones.

Results and Discussion

Increased berenil concentrations compromised the survivorship of *T. arnobia* larvae (Fig 1). No caterpillar reached the adult stage at berenil concentrations above 0.5%, and 0.25% berenil allowed only 5% survivorship to the adult stage.

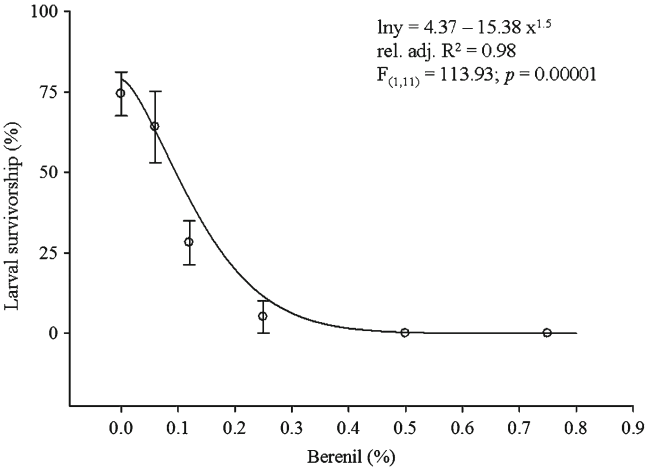

Fig 1 Larval survivorship of *Thyrinteina arnobia* (percentage reaching adulthood) when reared on eucalyptus leaves treated with different concentrations of berenil.

The general proteolytic activity profile of the midgut extract from larvae fed on leaves treated with different berenil concentrations peaked between 0.03% and 0.06% berenil (Fig 2). At 0.09% berenil, a decreased activity was observed. The highest proteolytic activity in the larval midgut of *T. arnobia* was detected with the ingestion of eucalyptus leaves treated with 0.12% berenil. It is important to note that the proteolytic activity of the midgut from larvae exposed to 0.12% berenil was higher than that observed by larvae fed on leaves treated with 0.06% of berenil (first peak) or those fed on control leaves, free of berenil.

Amidolytic activity was detected using the substrate L-BApNA (Fig 3a), while esterolytic activity was detected with L-TAME (Fig 3b). Both the amidolytic and esterolytic profiles for larvae exposed to different berenil concentrations were similar to the general proteolytic activity profile, and a peak corresponding to larvae that fed on leaves containing 0.03% and 0.06% of berenil. The poorest results of protease activity were obtained for larvae fed on leaves treated with 0.09% berenil, while the highest enzymatic activity was detected in midgut extract from larvae fed on leaves treated with 0.12% berenil. The specific activity of cysteine proteases in the larval midgut of *T. arnobia* was shown to decrease as berenil concentration increased (Fig 4).

Berenil showed a concentration-dependent inhibition of protease activity in the midgut of *T. arnobia* larvae. Larvae that ingested berenil at lower concentrations were able to overcome the negative effects of PI by increasing the trypsin activity in their midgut. Berenil at 0.09%, which would negatively affect *T. arnobia* larval development, actually reduced larval enzymatic activity. The increased activity of trypsin-like enzymes in the midgut of larvae fed on leaves treated with 0.12% of berenil, as an attempt

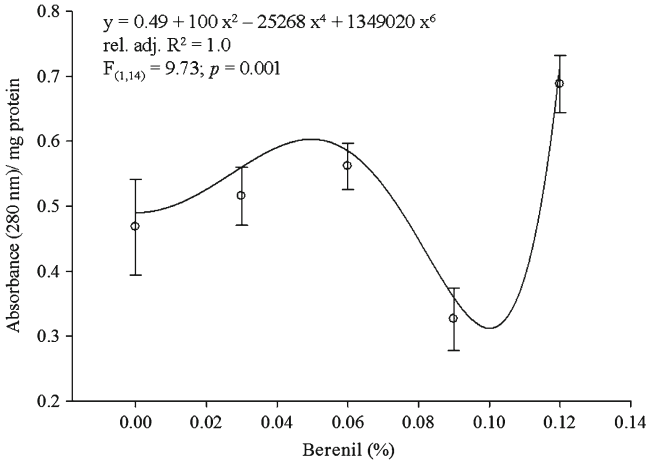

Fig 2 Profile of (general) proteolytic activity from larval midgut extracts of *Thyrinteina arnobia* reared on eucalyptus leaves treated with increasing concentrations of berenil. *Vertical bars* indicate standard errors of the mean.

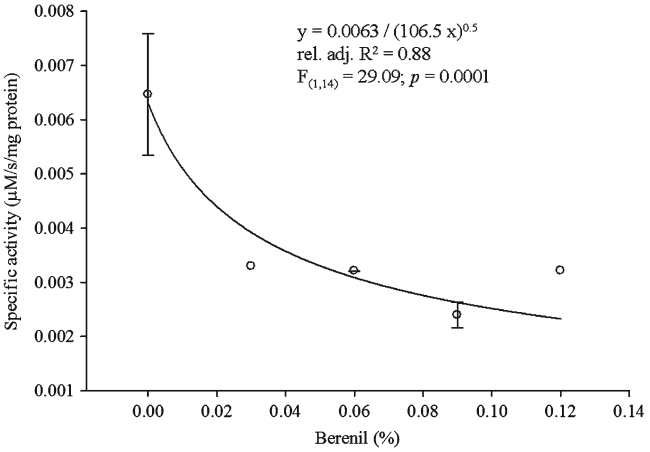

Fig 4 Cysteine proteinase activity from larval midgut extracts of *Thyrinteina arnobia* reared on eucalyptus leaves treated with increasing concentrations of berenil. *Vertical bars* indicate standard errors of the mean.

to overcome the inhibitory effects of beronil, severely affected *T. arnobia* survivorship.

Some insects may overcome the presence of protease inhibitors added to artificial diets or produced by natural or transgenic host plants (Broadway 1995, Pilon *et al* 2006) by producing proteinases that are resistant to the inhibitor (Mazumdar-Leighton & Broadway 2001), which may or may not belong to the same family as the proteinases targeted by the ingested PI. Our data indicate that the expression of cysteine proteases does not represent an adaptive response of *T. arnobia* to berenil intake because of the decreased activity of cysteine proteinases observed for all berenil concentrations tested. A reduction in cysteine proteinase expression may have occurred favoring the expression of other proteinases in response to PI intake.

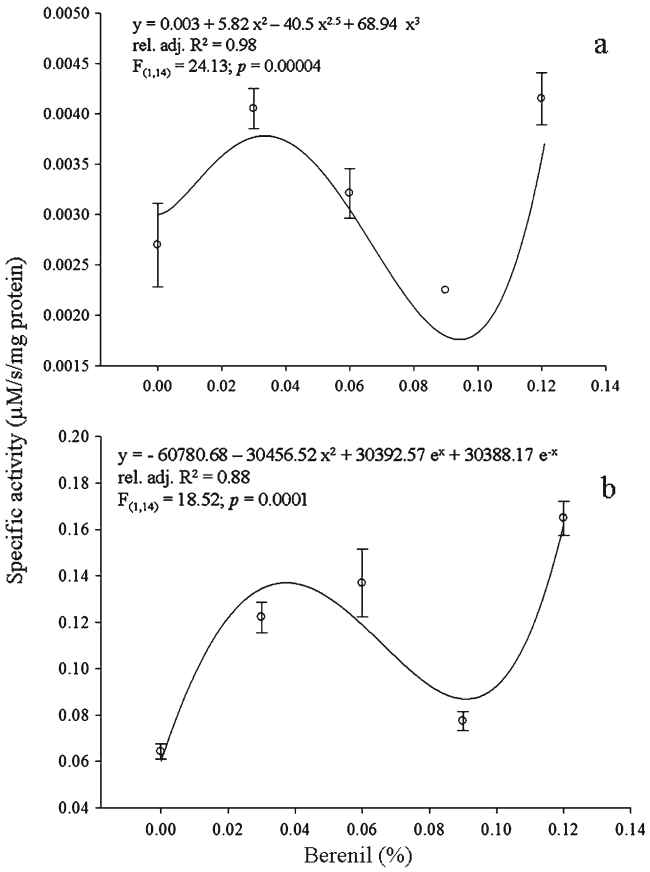

Fig 3 **a** Amidolytic and **b** esterolytic activities of serine proteinases from larval midgut extracts of *Thyrinteina arnobia* reared on eucalyptus leaves treated with increasing concentrations of berenil. *Vertical bars* indicate standard errors of the mean.

Berenil did not affect fertility life table parameters of *T. arnobia* when eucalyptus leaves treated with 0.06% berenil were fed to larvae (Marinho-Prado *et al* 2011). Here, *T. arnobia* larvae fed on leaves containing 0.06% berenil showed higher trypsin activity (amidolytic and esterolytic) and higher total concentration of midgut proteinases if compared to larvae that were not fed with PI. Secretion of proteinases has been attributed to a direct effect of food components (proteins) on the midgut epithelial cells or a hormonal effect triggered by food consumption (Ryan 1990). Thus, the increase in trypsin-like expression might be due to the increased concentration of berenil in the diet.

Small quantities of ingested PI could cause little effect on larval development and stimulate the secretion of additional midgut trypsin-like proteinases, possibly as ternary complex inhibitor–enzyme–substrate (I-E-S). This may occur because the mechanism of inhibition involves the interaction of inhibitor residues with the active site of the target protease in a canonical (substrate-like) manner through an exposed reactive site loop. For serine proteinase inhibitors, most residues interacting with the proteinases are located on a single loop (see Jongsma & Beekwilder 2011 for review). Berenil is a partially competitive inhibitor of trypsin, and it is able to form binary and

ternary complexes between enzyme, substrate, and modifier, which could also be a substrate (Oliveira *et al* 1993). Ternary complex I-E-S does not prevent the product formation, but the binary complex enzyme–inhibitor, on the other hand, may cause the phenomenon of enzymatic activation, enhancing the rate of trypsin hydrolysis (Oliveira *et al 1993).*

A second possible explanation for the results obtained with berenil at 0.03% and 0.06% is the activation of trypsin-like proteinases by this trypsin inhibitor. Berenil occupies two subsites of the trypsin catalytic center (Mares-Guia & Shaw 1965, Oliveira *et al* 1993). In addition to connecting to the active center at the site specificity (S_1), causing the effect of competitive inhibition, berenil binds to the secondary active site of the enzyme ($S_2^{'}$), causing a parabolic behavior with the substrate. This fact enhances the inhibition of serine proteinases by this compound (Oliveira *et al* 1993).

Eucalyptus leaves treated with 0.09% berenil induced more than 50% mortality in *T. arnobia* larvae according to the survivorship curve obtained. The observed profiles of proteolytic, amidolytic, and esterolytic activities in the larval midgut of *T. arnobia* indicated lower proteolytic levels in the midgut of insects exposed to 0.09% berenil than to in the midgut of insects fed on leaves treated with lower concentrations of berenil. Berenil might have triggered the formation of the ternary complex I-E-I between trypsin enzymes and inhibitors, thereby preventing the binding of these PIs with other midgut proteins. PIs inhibit the activity of proteolytic enzymes in the midgut, reducing the amount of digested protein, and induce an overproduction of digestive enzymes, which results in a deficiency of amino acids to sustain for the basic body physiological processes (Broadway & Duffey 1986, Oliveira *et al* 2005). Inhibition of proteolytic activity was successfully obtained at 0.09% berenil, while 0.12% berenil led to the overproduction of digestive enzymes.

Ingestion of eucalyptus leaves containing 0.12% berenil led to increased activity of trypsin-like (amidolytic and esterolytic) and total proteinase activity in the midgut of *T. arnobia* larvae, while severely affecting insect survivorship, similarly to what has been observed for *Anticarsia gemmatalis* (Hübner) (Pilon *et al* 2006, Moreira *et al* 2011). The increased activity of trypsin-like proteinases in *T. arnobia* caterpillars indicates an attempt of adaptation to the intake of berenil through proteinase overproduction.

Our data indicated that larvae of *T. arnobia* can withstand the effects of berenil at lower concentrations by slightly increasing the activity of trypsin-like proteinases and limiting the potential harmful effects of berenil to insect development at these concentrations. As berenil concentration increased, a decrease in insect survivorship was observed, showing that the larvae failed to maintain their self-protective strategy. Intake of 0.12% berenil caused increase in midgut proteinase activity in *T. arnobia* and larval mortality above 60%, indicating that the self-protective mechanism used was not efficient to overcome the effects of high berenil ingestion.

Therefore, besides inhibiting trypsin-like activity, berenil also inhibits cysteine proteinase activity preventing its alternative production as a self-protective strategy against PI intake. This is important because the effect of berenil spans to both serine- and cysteine proteinases, the two most important groups of proteolytic enzymes in Lepidoptera digestion (Srinivasan *et al* 2006). The apparent effects of berenil on serine- and cysteine proteinases reinforce its potential use as a backbone for the development of mimetic peptides as insect control agents.

In conclusion, berenil compromises the survival of *T. arnobia* larvae by affecting the proteolytic activity in the larval midgut. Additionally, inhibition by berenil also impairs the activity of cysteine proteinases, preventing its alternative production as an adaptation strategy against inhibition of serine proteinase activity in the larval midgut. It is also possible to conclude that *T. arnobia* larvae that ingest berenil at lower concentrations can overcome the effects of PI by increasing their midgut trypsin activity, but such protective response is not effective against the ingestion of higher concentrations of this protease inhibitor.

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