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ORIGINAL CONTRIBUTION

Survival and feeding avoidance of the eucalyptus defoliator *Thyrinteina arnobia* exposed to the proteinase inhibitor berenil

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Abstract

Proteinase inhibitors are recognized as potential plant protection agents against pest insects and their use is an alternative for integrated pest management. Berenil is an example of a synthetic trypsin inhibitor and its potential for use as insecticide was assessed against *Thyrinteina arnobia* (Stoll) (Lepidoptera: Geometridae), one of the main eucalypt defoliators in Brazil. Insect survival and its life history traits including developmental time, pupa weight and viability, and life table parameters of *T. arnobia* were assessed in larvae reared on eucalyptus leaves containing 0.00%, 0.06%, 0.12%, 0.25%, 0.50% and 0.75% (w/v) of the synthetic trypsin inhibitor berenil. In addition, food preference and leaf consumption of *T. arnobia* caterpillars were also assessed. Berenil delayed larval development. Larva survival was severely affected by berenil, which also delayed larval development. Sub-lethal concentrations of berenil compromised life table parameters of *T. arnobia* reducing its net reproductive rate and population growth rate, while extending generation time. Berenil was also deterrent to *T. arnobia* and did not elicit larva compensatory feeding. The berenil activity as insecticide, repellent and antifeedant against *T. arnobia* suggests its potential use against eucalyptus defoliating caterpillars.

Introduction

Plant proteinase inhibitors (PIs) are small proteins generally found as constitutive components in storage tissues such as seeds and tubers, and are synthesized in response to pest and pathogen attack (Ryan 1990). The insect chronic ingestion of PIs leads to the inhibition of proteolytic digestion consequently interfering with the bioavailability of essential amino acids required for insect growth, development and reproduction (Broadway 1995; Oliveira et al. 2005).

Alternative or biorational control methods against pest insects using PIs may be exploited in two different ways (Oliveira et al. 2005; Xavier et al. 2005). First, by plant genome transformation for increased expression of potent PIs (Boulter 1993; Jouanin et al. 1998). Second, by applying peptides or synthetic compounds (e.g. mimetic peptides), which are potent inhibitors of insect midgut proteinases, as insecticides (Xavier et al. 2005; Pilon et al. 2006; Nicholson, 2007).

Proteinase inhibitors which target insect digestive proteins can decrease insect growth, survival and fertility (Carlini and Grossi-de-Sá 2002). However, several insects are known to counter the effects of ingested PI by overexpressing and desensitizing existing proteinases, producing PI-insensitive proteinases, proteolytically inactivating PI, or increasing food consumption (Jongsma and Bolter 1997; Winterer and Bergelson 2001; Zhu-Salzman et al. 2003).

Production of PI can even become a liability for the plant, if the compensatory response requires the herbivore to eat more leaf material to complete development.

The selection of suitable inhibitors for the pool digestive proteinases prevailing in a given pest species is paramount for their use as control agents. 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 proteinases are the main digestive proteinases reported in a wide variety of lepidopteran pests, including *Thyrinteina leucoceraea*, a species closely related to *T. arnobia,* and another key-pest of eucalyptus in Brazil (Reeck et al. 1999; Holtz et al. 2003; Terra and Ferreira 2005; Marinho et al. 2008). Caterpillars of *T. arnobia* have been reported in most areas where *Eucalyptus* spp. is cultivated in Brazil (Holtz et al. 2003). This species' outbreaks are believed to be favoured by the implementation of extensive areas of eucalypt plantations established in the country affording abundant and stable shelter and food supply (Santos et al. 1996). These conditions have allowed *T. arnobia* to cause serious damage to these plants requiring insecticide use for their control (Anjos et al. 1987).

Among serine proteinases, trypsins and chymotrypsins are the most commonly reported enzymes acting in a wide range of physiological processes, playing essential role in protein digestion and absorption in insects (Reeck et al. 1999; Terra and Ferreira 2005; Pilon et al. 2006; Zhu et al. 2007). Berenil is an example of a synthetic trypsin inhibitor that may have potential for use as insecticide and may be useful in understanding the insect response to dietary PIs (Oliveira et al. 1993). In the present article we report the effect of berenil application on eucalyptus leaves in the life history traits and food preference of *T. arnobia*.

Materials and Methods

Insects, plants and products

The insect colony used was established from eggs of *T. arnobia* obtained from a laboratory colony maintained at the São Paulo State University (UNESP, Botucatu, São Paulo, Brazil). The insects were maintained under controlled conditions (26 ± 3°C; 12 h/12 h L/D; 60 ± 15% r.h.). Plants of *Eucalyptus grandis* free of pesticide residues were used in the experiments. Berenil (diminazene aceturate) was purchased from Sigma-Aldrich Química Brasil (São Paulo, Brazil) and the agricultural dispersant Gotafix ($C_{35}H_{64}N_{11}$), which was used to improve adherence and penetration of the PI in the leaf surface, was obtained from Milênia Agro-Ciências S.A. (Londrina, PR, Brazil).

Life table experiment

The experiment was established in three blocks, each one containing 13 replicates of each concentration of the trypsin inhibitor berenil (0%, 0.06%, 0.12%, 0.25%, 0.5% and 0.75% w/v) using water as solvent and containing 0.06% (v/v) Gotafix. Each replicate encompassed a newly emerged caterpillar individualized in plastic dish (9 cm diameter). Thirty-nine larvae were used per concentration of berenil. Plants of *E. grandis* (50 cm high) were used; the plant leaves were immersed in berenil solution and placed within the Petri dish, where the caterpillars were subsequently released. The leaf petiole was involved in wet cotton when harvested to retain humidity. When the larvae reached the fifth instar, they were transferred to plastic cups (500 ml). Larval mortality was assessed daily; pupa weight and pupa viability were also recorded. During the adult stage, the moths were paired and placed in a cylindrical cage (8.5 cm diameter and 19 cm height), closed with a Petri dish (9 cm diameter), and with its inner walls covered with filter paper. Each couple was considered as one replicate. To obtain enough larvae for studies of the pupa and adult stages (due to possible mortality), 120 insects were maintained under the same conditions of each treatment for eventual replacement, if necessary.

Behavioral studies

The berenil concentrations that prevented adult formation were not used for the behavioural assays and intermediate concentrations were added to the others. The free-choice and no-choice bioassays were all carried out in climate rearing chamber under controlled conditions (25 ± 1°C; 12 : 12 h L : D; 75 ± 10% r.h.).

Free-choice bioassays

Free-choice tests were designed to assess whether *T. arnobia* will exhibit any preference for eucalyptus plants sprayed with aqueous solution containing different berenil concentrations (0%, 0.03%, 0.06%, 0.09% and 0.12% w/v), in addition to 0.06% of the dispersant Gotafix. For this purpose, five eucalyptus plants (40 cm height), one from each berenil concentration, were placed at equidistant distances in a

styrofoam platform lined with a layer of filter paper within a glass arena (26 cm diameter and 9.5 cm height) (fig. 1). Thirty-six caterpillars (fourth and fifth instars) of *T. arnobia* were starved for 12 h (for a quicker response) and introduced into the individual arenas, divided in three blocks of 12 caterpillars each (i.e. the caterpillars were released in groups of 12 on the arena). The observations were carried out at 15, 30 and 60 min later to register the first choice. Thereafter, the caterpillars were released at the centre of the arena again and the choice was registered 24 h after. Un-injured leaves were recorded before and after the 24-h test. The variables recorded were: (i) caterpillar's first choice, (ii) choice after 24 h, and (iii) number of leaves injured by caterpillars during 24 h. A negative control bioassay was previously carried out where all of the plants within the arena were sprayed with water + Gotafix and there were no differences in caterpillar preference (i.e. no position effect within the arena).

No-choice bioassays

Assessment of the potential impact of berenil on leave consumption by caterpillars of *T. arnobia* was carried out using five different concentrations (0%, 0.03%, 0.06%, 0.09% and 0.12% w/v) in aqueous solution, again containing the dispersant Gotafix (0.06% v/v). The amount of leaf surface consumed was determined using a leaf area meter LI-COR (LI-3100A; LI-COR Biosciences, Lincoln, NE, USA) before and after the leaf exposure to the caterpillars. The leaf petiole was involved in wet cotton when harvested to retain humidity. The fifth instar caterpillars of *T. arnobia* were starved for 5 h and 10 caterpillars were used per berenil concentration. Each replicate encompassed a single caterpillar individualized in a Petri dish (14 cm diameter) containing one eucalyptus leaf previously immersed in the solution of the respective berenil concentration. The exposure time was 24 h.

Statistical analysis

The survival curves were obtained through Kaplan–Meier estimators generated from the proportion of caterpillars surviving each day from the start until the conclusion of the experiment using the procedure LIFETEST from SAS (SAS Institute 2001). The

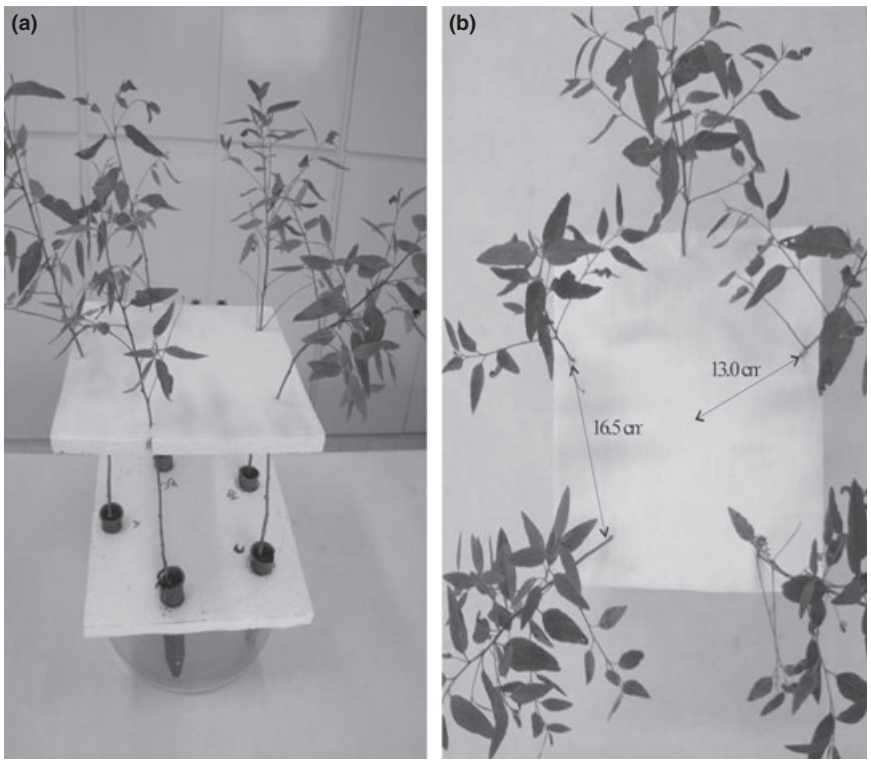

Fig. 1 Photos of the experimental set-up used in the free-choice bioassays with eucalyptus plants impregnated with different concentrations of berenil. A lateral (a) and an aerial (b) view of the arena are presented indicating its radius (13.0 cm) and distance between neighbour eucalyptus seedlings (16.5 cm).

insects surviving until adult emergence were treated as censored data (Allison 1998). The survival curves of each berenil concentration were compared using the Cox's regression method (PHREG procedure from SAS; SAS Institute 2001).

The following fertility life table parameters were calculated for each berenil concentration using standard procedures and formulae (Pompermayer et al. 2001): probability of surviving to age x ($l_x = N_x/N_0$); number of female offspring produced per age interval (m_x); remaining life expectancy at age interval x ($e_x = T_x/l_x$); reproductive value at age x ($V_x = l_x \times m_x$); net reproductive rate ($R_0 = \sum V_x$); intrinsic rate of increase [r_m, $1 = \sum e^{(-rm \times x)} \times V_x$]; mean generation time [$T = (\ln R_0)/r_m$] and doubling time [$DT = (\ln 2/r_m)$]. The life table parameters were estimated by the jackknife method (PROC GLM; SAS Institute 2001).

Analyses of covariance were carried out to assess the effect of berenil concentration on the developmental time and pupa weight of males and females of *T. arnobia* (PROC GLM; SAS Institute 2001); such analyses were subsequently complemented by (linear) regression analyses, if necessary (PROC REG; SAS Institute 2001). The data of the behavioural tests 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) to the alternative models of increasing complexity (non-linear peak models). The model selection was carried out based on the increase of adjusted coefficient of determination (adj. R^2) relative to the model complexity, relative adjusted R^2 (rel.adj. R^2), simplicity, 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 with the alternative ones.

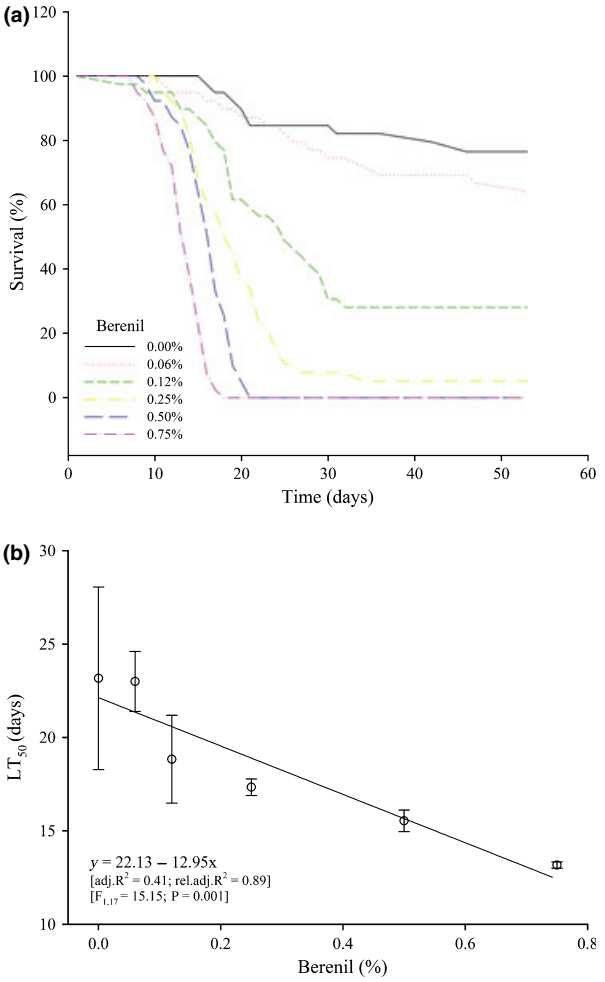

Fig. 2 Survival plots (a) and mean survival time (LT_{50}) (b) of the eucalyptus defoliator *Thyrinteina arnobia* exposed to leaves impregnated with the proteinase inhibitor berenil. Each symbol of the LT_{50} plot represents the mean of three replicated determinations and the vertical bars indicate standard errors of the mean.

Results

Effect of berenil on insect survival and development

Berenil affected caterpillar survival, whose plots are shown in fig. 2a. The survival data submitted to Cox's regression indicated significant differences in caterpillar survival among berenil concentrations (d.f. = 1; $\chi^2 = 18.71$; $P < 0.001$). The survival curves obtained using Kaplan–Meier estimators allowed the estimation of the mean survival time (LT_{50}) for caterpillars exposed to the different inhibitor concentrations. These mean survival times (LT_{50}'s) exhibited a linear decrease with increased concentrations of berenil (adj. $R^2 = 0.41$; rel.adj. $R^2 = 0.89$; $F_{1,17} = 15.15$; $P < 0.001$) (fig. 2b). The analysis of covariant testing the effect of sex on larval development and pupa weight, with berenil concentrations as covariate, was significant only for pupa weight ($P < 0.05$). However, berenil delayed larva development (fig. 3), exhibiting opposite trend to survival time (i.e. the longer the development, the shorter the survival time). Although female pupae were heavier than male pupae ($F_{1,63} = 184.58$, $P < 0.0001$), there was no influence of berenil on pupae weight ($F_{1,63} = 2.05$, $P = 0.11$). Mean pupal weight of females and males were 0.36 (±0.010) and 0.13 (±0.007) mg respectively.

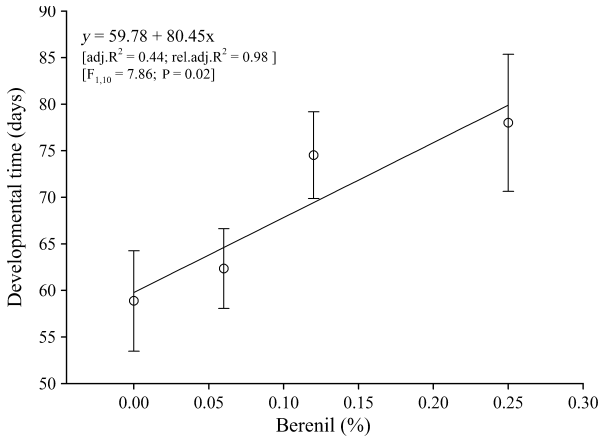

Fig. 3 Developmental time (days from egg hatching to death) of *Thyrinteina arnobia* reared on eucalyptus leaves impregnated with the proteinase inhibitor berenil. Each symbol represents the mean of three blocks of replicates, each containing 13 individualized insects. Vertical bars indicate standard errors of the mean.

Demographic effects of berenil on *T. arnobia*

Berenil significantly reduced *T. arnobia* reproductive potential (table 1). The net reproductive rate (R_0) was significantly lower for insects reared on both 0.06% and 0.12% of berenil, which also led to significant delay in generation time (T). The consequence of these effects of berenil were a lower intrinsic rate of population growth (r_m) leading to extended doubling time (DT) under exposure to higher concentrations of this PI (table 1). Insects reared on berenil at 0.12% had significantly lower intrinsic rates of increase (r_m) and mean duration of a generation (T).

Effects of berenil on *T. arnobia* preference and leaf consumption

Free-choice bioassays with eucalyptus plants impregnated with berenil resulted in significant larvae avoidance in the first choice of feeding substrate (which took place at varying time intervals from 15 to 60 min) and also after a 24-h exposure (fig. 4). Decrease in larva feeding preference was observed as berenil concentrations increased, which was also observed for the number of leaves injured after 24 h in free-choice tests (fig. 5a).

No-choice bioassays were also carried out and a significant reduction in leaf consumption by *T. arnobia* was observed with increased concentrations of berenil (fig. 5b).

Discussion

This is the first report on the insecticidal activity of a plant-applied synthetic PI and on its influence on insect behaviour. Ingestion of PIs by insects does not eliminate protein digestion in the midgut, but leads to a hyperproduction of digestive proteinases that limits the bioavailability of essential amino acids for protein synthesis, impairing insect growth and development (Broadway 1995; Koiwa et al. 1997). Indeed, this work shows that *T. arnobia* development was affected by berenil applied on eucalyptus leaves. Both lethal and sub-lethal consequences were observed in larvae exposed to berenil. Furthermore, berenil does not increase leaf consumption by *T. arnobia* caterpillars, but actually reduces leaf ingestion when applied at high concentrations of 0.06% and higher.

Berenil-contamined plants increased mortality of *T. arnobia*, especially during the early larval development. Concentrations above 0.25% of berenil severely reduced the survival of *T. arnobia* larvae. Berenil concentrations of 0.12% and lower allowed adult formation, but sub-lethal effects of berenil were also apparent. Berenil delayed the development of *T. arnobia* larvae surviving the exposure. 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 and to the risk of consuming harmful levels of toxins (Raubenheimer 1992; Mochizuki et al. 1999). In addition, the regular larval development pattern may not be possible under conditions of nutritional stress (Pompermayer et al. 2001).

Several authors have argued that the best approach for evaluation of the total effect of insecticidal compounds is life table analysis (e.g. Stark and

Table 1 Fertility life table parameters (mean) for *Thyrinteina arnobia* reared on eucalyptus leaves containing increase concentrations of berenil

Berenil	n	Net reproductive rate (R_0)	Intrinsic rate of population growth (r_m)	Generation time (T)	Doubling time (DT)
0%	12	226.89 a	0.09 a	57.90 a	7.39 a
0.06%	8	154.48 bc	0.08 a	57.30 a	7.88 a
0.12%	7	107.73 c	0.06 b	66.93 b	9.91 b

Means followed by the same letter within each column are not significantly different ($P \leq 0.05$) based on Student's *t*-test *t* using Jackknife.

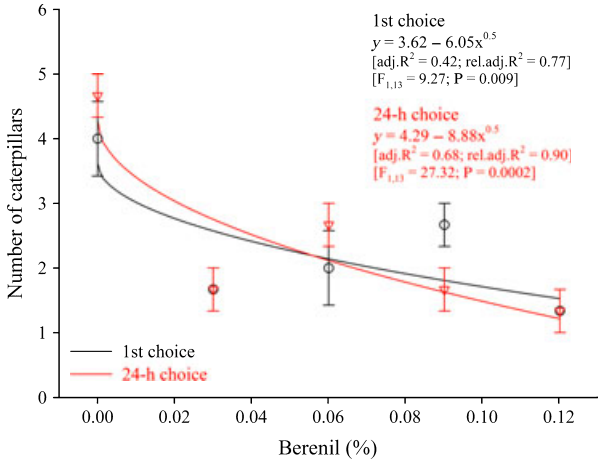

Fig. 4 First choice (within 60 min) and 24-h choice by larvae of *Thyrinteina arnobia* subjected to free-choice bioassays with eucalyptus plants impregnated with different concentrations of berenil. Each symbol represents the mean of three blocks of replicates, each containing 12 individualized insects. Vertical bars indicate standard errors of the mean.

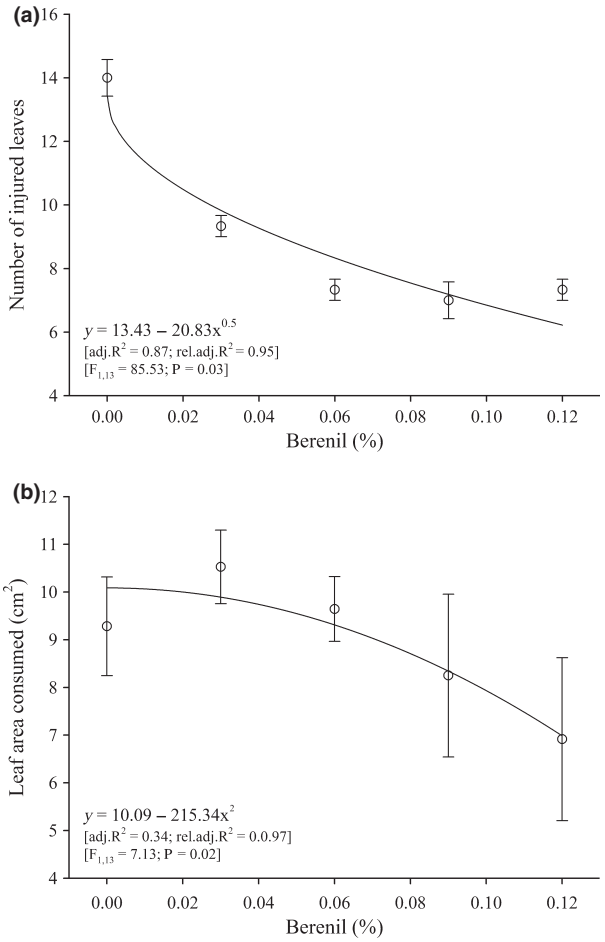

Fig. 5 Number of injured leaves (a) and leaf area consumed (b) by the eucalyptus defoliator caterpillars *Thyrinteina arnobia* subjected respectively to free-choice and no-choice bioassays with eucalyptus plants impregnated with different concentrations of berenil. Each symbol represents the mean of three blocks of replicates, each containing 10–12 individualized insects. Vertical bars indicate standard errors of the mean.

Wennergren 1995; Pompermayer et al. 2001; Stark and Banks 2003; Guedes et al. 2009, 2010). Insect susceptibility to PIs can be measured in terms of survival, developmental time and fecundity. However, demographic analysis provides a quantitative method of analysing insect populations by assessing survival, fecundity and population growth patterns (Zeng et al. 1993; Pompermayer et al. 2001). Our results showed that sub-lethal concentrations of berenil effectively compromised the population parameters of *T. arnobia*, which may minimize the risk of development of resistant insect biotypes (Wolfson and Murdock 1995). 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 and 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 (Pompermayer et al. 2001).

The identification of compounds or proteins that, in addition to being toxic/antimetabolic, will change the behaviour of an insect by causing it to leave the plant in search for better food substrates is of great interest. Such repellent effect potentially reduces the likelihood of pests developing resistance (Outchkourov et al. 2004). In this study, we have demonstrated that berenil is repellent to *T. arnobia* larvae, and can quickly affect the behaviour of this insect, since the first contact. Larvae of *T. arnobia* are generalist feeders and the repellent effect of berenil could stimulate the larvae to search other plants.

Several studies have documented increased food consumption by herbivores in response to the heterologous expression of PIs in different plants (Cloutier et al. 2000; Winterer and Bergelson 2001; Abdeen et al. 2005). Compensatory feeding is a well-described response in larvae that feed on diets low in nutritional protein (Simpson and Simpson 1990). As PIs are anti-digestive and reduce gut proteinase activity, they can also elicit compensatory consumption. Such strategy would allow the insects to maintain satisfactory absorption of amino acids and peptides allowing them to reach the critical weight

for molting (Pilon et al. 2006). Our results showed that there was no compensatory feeding for *T. arnobia* – larvae consumed slightly less leaf material in the presence of increased berenil concentration. Steppuhn and Baldwin (2007) demonstrate that by limiting consumption, nicotine prevents *Spodptera exigua* from compensating for proteinase inhibition by increasing consumption. There is high concentration of secondary compounds in tissues of eucalyptus plants (Bragança et al. 1998), and it is possible that one or more of these compounds may prevent the compensatory feeding by *T. arnobia* on such plants.

In contrast with berenil, artificial diet with benzamidine, another synthetic PI, negatively affected the development of the velvetbean caterpillar (*Anticarsia gemmatalis*), but caused compensatory feeding by larvae of this species (Pilon et al. 2006). Benzamidine occupies only the sub-site S_1 of the catalytic centre of trypsins (Mares-Guia et al. 1981; Oliveira et al. 1993). The bis-benzamidine berenil (diminazene aceturate) occupies two sub-sites of the catalytic centre of trypsins, which probably increases its efficacy against *T. arnobia* (Mares-Guia and Shaw 1965; Oliveira et al. 1993). In addition to connecting to the active centre of trypsin at the site specificity (S_1), causing the effect of competitive inhibitor, berenil binds to the secondary active site of the enzyme (S'_2) causing a parabolic behaviour with the substrate (Andrade et al. 1990). This fact enhances the inhibition of this compound in serine proteinases (Junqueira et al. 1992; Oliveira et al. 1993).

The effectiveness of berenil as insecticide against *T. arnobia* suggests an important potential use of berenil in anti-herbivore defense on eucalyptus plants. Nonetheless, additional studies should be carried out using berenil against other caterpillar pest species to assess its potential as crop protection agent or, more precisely, as a basic backbone for development of mimetic peptides.

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