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RESEARCH REPORT

Foraging and oviposition of *Thyrinteina leucoceraea* (Lepidoptera: Geometridae) on introduced and native hosts in Brazil sprayed with the protease inhibitor benzamidine

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Abstract

The protease inhibitor (PI) benzamidine may be an option for protecting introduced Myrtaceae plants from insect pests. The foraging behavior of the larvae (number of larvae per plant) and oviposition (number of egg masses per plant and eggs per mass) of *Thyrinteina leucoceraea* (Lepidoptera: Geometridae) females were evaluated on an introduced Myrtaceae (*Eucalyptus grandis*) and a native one (*Psidium guajava*), both sprayed by the PI benzamidine in aqueous concentrations of 0.000, 0.125, 0.250, 0.375, and 0.500 $mol \cdot L^{-1}$ and with the adhesive spreader Triton X-100 at 0.01 % ($mg \cdot mL^{-1}$) in water as a control. The foraging preference by *T. leucoceraea* was similar between the different concentrations of the PI benzamidine on the treated host plants and the control. The numbers of egg masses per plant and eggs per mass of *T. leucoceraea* were similar between the treatments, but this insect showed slighter oviposition preference on non-sprayed *E. grandis* plants than on those of *P. guajava* sprayed with different concentrations of the inhibitor PI benzamidine. Similar foraging of larvae among treated plants and the lower reproduction of *T. leucoceraea* on treated *E. grandis* plants, show possibilities of using the PI benzamidine in the management programs of this herbivore, in this culture.

Key Words: benzamidine; *Eucalyptus grandis*; geometrid moth; protease inhibitor; *Psidium guajava*

Introduction

Insect herbivores need free amino acids (organic molecules with an amino group, a carboxyl group, and a specific lateral chain) and nitrogen, which can be obtained from the degradation of peptide bonds between the amino acids forming proteins, by extracellular enzymes named as proteases, in process called proteolytic cleavage (Bazok *et al*., 2005). Plants can express specific protease inhibitors (PIs) in response to adverse abiotic or biotic factors, such as herbivory by insects (Moreira *et al*., 2011). Proteolytic enzymes in the gut of the insect herbivores (digestive system) can reduce the availability of amino acids when inhibited by PIs from plants, which may affect the physiological processes, such as lengthening the life cycle and reducing the fecundity and the fertility of insects (Oliveira *et al*., 2005).

Natural (isolated from plants) or synthetic (produced in the laboratory and commercially available) PIs can be used for the protection of plants against insect herbivores (Xavier *et al*., 2005) with specific genes introduced via genetic manipulation to encode the PIs, which induce resistance (Delledonne *et al*., 2001), or with synthetic PIs in food (artificial diets in the laboratory). These PIs can lengthen development, reduce the reproductive parameters, and cause insect mortality (population control) (Pilon *et al*., 2006).

Benzamidine is a competitive, reversible inhibitor of the trypsin (enzyme that acts upon proteins of the chyme), trypsin-like, and of serine proteases (primary amino acid, non-essential, which

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comprises of a majority of glycolipids in animal cells) (Macedo and Freire, 2011; Ranjbar *et al*., 2011). The insecticidal potential of the synthetic form of PI benzamidine was tested to evaluate the response of the eucalypt defoliator, *Thyrinteina arnobia arnobia* (Lepidoptera: Geometridae) to this inhibitor when pulverized on plants or mixed in food (Marinho-Prado *et al*., 2011). The weight of larvae and reproductive parameters of sugarcane borer, *Diatraea saccharalis* (Lepidoptera: Pyralidae) were lower with food (artificial diet) treated with PIs from seeds of soybean, *Glycine max* (Fabaceae) (soybean proteinase inhibitor, SPI) (Pompermayer *et al*., 2001). The larvae survival of Australian bollworm, *Helicoverpa punctigera* (Lepidoptera: Noctuidae) was lower with food (artificial diet) treated with a common inhibitor of the Solanaceae family (winged tobacco, *Nicotiana alata* proteinase inhibitor, NaPI) (Dunse *et al*., 2010).

The *T. arnobia arnobia* and the *Thyrinteina leucoceraea* (Lepidoptera: Geometridae) (native species to Brazil) are the main defoliators of eucalyptus, *Eucalyptus* spp. (Myrtaceae) plants in some regions in Brazil (Grosman *et al*., 2005; Oliveira *et al*., 2010). Monocultures (large-scale of a single culture in one area) of the native plants of Myrtaceae in Brazil, as those of the genera *Campomanesia*, *Eugenia*, and *Psidium*, can be damaged by native Lepidoptera (Wessels *et al*., 2007). Its larvae can feed and develop on introduced or native hosts of this family, such as *Eucalyptus* spp. and guava, *Psidium guajava*, respectively (Holtz *et al*., 2003).

Lepidoptera defoliators of *Eucalyptus* spp. can be controlled with biological and chemical methods, but their deficiencies depends on monitoring programs such as the use of light or pheromone traps, to observe their population levels (Zanuncio *et al*., 2003, 2012; Freitas *et al*., 2005). Damage on Tasmanian blue gum, *Eucalyptus globulus* (Myrtaceae) plants caused by insect pests is higher in susceptible materials than in resistant ones, although those susceptible have a higher concentration of secondary metabolic compounds that could be toxic, such as, phenols, essential oils, and tannins (Rapley *et al*., 2004).

The aim of this study is to evaluate the foraging behavior of larvae and of oviposition site selection by *T. leucoceraea* females on the rose gum, *Eucalyptus grandis* (Myrtaceae) (introduced host) and *P. guajava* (native host) plants, sprayed with different concentrations of synthetic PI benzamidine compared with Triton X-100 as control.

Material and Methods

Obtaining insects

The larvae of *T. leucoceraea* were obtained from the mass rearing of this insect, which originated from individuals that were collected from *E. grandis* and *P. guajava* plants in the field and preserved in the original hosts at 25 ± 1 ºC, 12-h photoperiod and 70 ± 10 % relative humidity in rearing chamber in the Department of Animal Biology (DBA) of the "Universidade Federal de Viçosa (UFV)" in Viçosa, Minas Gerais State, Brazil. *Thyrinteina leucoceraea* pupae were collected with

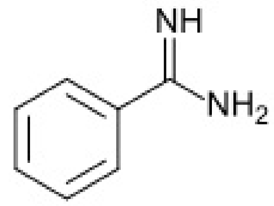

Fig. 1 Chemical structure of benzamidine. International Union of Pure and Applied Chemistry (IUPAC) name: benzenecarboximidamide. Molecular formula: $C_7H_8N_2$. Molar mass: 120.15 g.mol^{-1}.

a forceps when they were attached to the *E. grandis* and *P. guajava* leaves and placed in plastic Petri dishes (9 cm diameter × 1.5 cm height) lined with cotton wool. The adults obtained from these pupae were reared in wooden cages (0.60 m width × 0.60 m length × 1.00 m height) closed with a thin organza-type fabric. Egg masses obtained from these adults were removed from the cages with a brush and placed on cotton strips inside the plastic Petri dishes with a cotton swab moistened with water, per dish. Soon after hatching, the larvae were placed in cages similar to those described, and they received leaves of the *E. grandis* or *P. guajava* plants, about 90 days old, kept in vases (one plant per vase), with a capacity of 20 L, with a vermiculite-type substrate (hydrated basaltic minerals) and without synthetic chemicals. The substrate of the vessels was moistened daily in the morning with 2 L of water irrigated uniformly on it. Fourth-instar larvae were used for feeding preference and oviposition tests because they had higher food consumption rates and mobility on the host plants (Marinho-Prado *et al*., 2011).

Obtaining solutions

Aqueous solutions were prepared with PI benzamidine (Fig. 1) in different concentrations of 0.000, 0.125, 0.250, 0.375, and 0.500 M (or mol.L^{-1}) and at 0.01 % (mg·mL^{-1}) of the adhesive spreader Triton X-100 (chemical product that increases the efficiency of PI benzamidine by reducing the surface tension of the inhibitor sprayed on leaves, due to the increased power of wetting and adhesion) (Fig. 2). These solutions were sprayed on *E. grandis* or *P. guajava* leaves as treatments: *E. grandis* + 0.000 M of PI; *E. grandis* + 0.125 M of PI + 0.01 % (mg·mL^{-1}) of Triton X-100; *E. grandis* + 0.250 M of PI + 0.01 % (mg·mL^{-1}) of Triton X-100; *E. grandis* + 0.375 M of PI + 0.01 % (mg·mL^{-1}) of Triton X-100; *E. grandis* + 0.500 M of PI + 0.01 % (mg·mL^{-1}) of Triton X-100; *P. guajava* + 0.000 M of PI; *P. guajava* + 0.125 M of PI + 0.01 % (mg·mL^{-1}) of Triton X-100; *P. guajava* + 0.250 M of PI + 0.01 % (mg·mL^{-1}) of Triton X-100; *P. guajava* + 0.375 M of PI + 0.01 % (mg·mL^{-1}) of Triton X-100; *P. guajava* + 0.500 M of PI + 0.01 % (mg·mL^{-1}) of Triton X-100, and the control with 0.01 % Triton X-100. Each plant was sprayed with 20.0

mL of PI benzamidine solution and compared with the control (without PI benzamidine). Benzamidine is economic to be sprayed on plants (Silva *et al*., 2010) and has no known negative effect on non-target organisms (Oliveira *et al*., 2006).

Food preference test

Each treatment had four replications, with one plant each. A total of 120 thirty-fourth-instar *T. leucoceraea* larvae, without food for 12 h, were used per treatment. The feeding preference test was conducted in wood cages closed with organza-type fabric representing arenas, with six plants arranged in a hexagon and alternated by treatment and replication. The *T. leucoceraea* larvae were released in the center of the hexagon (arena), free to choose their hosts, who had the same chance of being colonized. The presence of larvae on the plants was recorded according to the position (layout) of the plants in the arena, time of observation, and treatment. Nine evaluations were performed every hour after the start of the test and the last after 24 h. The larvae were removed from the plants after each evaluation.

Oviposition preference test

T. leucoceraea females, from mass rearing in cages, were used in the oviposition preference test. The solutions with PI benzamidine and the control (water and Triton X-100) were prepared similarly to the food preference test. The cages with a wooden structure, as described, were closed with organza-type fabric, with equidistant plants in a hexagon and alternated to arena formation. *Eucalyptus grandis* and *P. guajava* plants had about 40 cm height and the same number of developed leaves. Each plant was sprayed with 30 mL of one of the concentrations of PI benzamidine solution, according to the treatment, with a plastic spray bottle with a capacity of 500 mL and the control with only water and Triton X-100 solution in the same quantity. *T. leucoceraea* females were mated in the rearing cages and released in the center of the hexagon forming arenas. The egg masses of this Lepidoptera were removed, recording their position on the plant, reading time, and treatment. Readings were taken 12, 24, and 36 h after release of the insects.

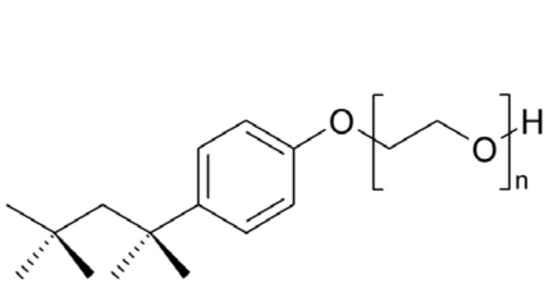

Fig. 2 Chemical structure of Triton X-100. Molecular formula: $C_{14}H_{22}O(C_2H_4O)_n$, with n = 9 or 10. Molar mass: 647 g·mol^{-1}.

Statistical analysis

The experimental design was completely randomized (DCR). Data obtained from the tests of feeding and oviposition preferences were subjected to variance analysis (one-way, ANOVA). A regression was adjusted, to explore and infer the relationship between the concentrations of the products and the plant species used as a model in the tests, and the R^2 value was calculated by the equation obtained. The means of the number of *T. leucoceraea* eggs by mass were analyzed by the Tukey test ($p \leq 0.05$) using the software 'R' version 2.2.1 (Ritz and Streibig, 2005) provided by the Federal University of Viçosa (UFV).

Results and Discussion

The foraging (number of larvae per plant) of *T. leucoceraea* was similar between the hosts (introduced and native) with different concentrations of PI benzamidine and the control (water and Triton X-100) (Table 1). The positions of the plants and reading times also did not show effect on the foraging by larvae of this species (Table 1). The similar foraging (number of larvae per plant) by *T. leucoceraea* on either 'sprayed' or 'not sprayed' plants (control), with different concentrations of PI benzamidine, suggests lack of detection of this compound by the chemoreception system of this insect on the hosts. These chemoreceptors are normally in the ovipositor and antennas of adults and in the mouthparts of immature (Kim and Mullin, 2003). Protease inhibitors can be used in pest control, in agriculture and forestry, but can be recognized by larvae and nymphs, which can avoid plants with these substances (Pereira *et al*., 2005). *T. leucoceraea* was collected from native plants of the family Myrtaceae, grown in the vegetation belts, in Brazil, where their populations could increase, and colonize *Eucalyptus* spp. plants that were grown near them. However, treated plants or expressing PIs may reduce the development, reproduction, and survival of this pest (Pilon *et al*., 2006). The choice of the host for egg laying is made by the female (Stadler *et al*., 2002) and the success of their offspring depends on suitable oviposition locations (site). However, *T. leucoceraea* larvae from the fourth-instar can have higher mobility and avoid contact with plants expressing or sprayed with PIs, as observed for those of the fourth-instar of glanville fritillary, *Melitaea cinxia* (Lepidoptera: Nymphalidae) and the European corn worm, *Ostrinia nubilalis* (Lepidoptera: Crambidae), avoiding plants expressing toxic compounds (Reudler Talsma *et al*., 2008; Suverkropp *et al*., 2008). Compounds from plants, especially those produced after herbivore damage can affect development and cause mortality, especially of first-instar larvae, which are more susceptible (Scott *et al*., 2010). This was shown by the synthesis in higher quantity of jasmonic acid (vegetable endogenous regulator that can be extracted from the volatile portion of essential oils from Oleaceae plants after herbivory). This compound becomes more concentrated in the plant and activates the expression of genes, to codify for the production of

Table 1 Number of *T. leucoceraea* (Lepidoptera: Geometridae) larvae recaptured on *P. guajava* (native host) or *E. grandis* (introduced host) (Myrtaceae) plants to Brazil in five concentrations (M or mol·L^{-1}) of the protease inhibitor (PI) benzamidine

	P. guajava	
IP benzamidina	OPI	WPI
0.000	16.00 ± 0.82	16.75 ± 2.17
0.125	19.00 ± 1.87	18.25 ± 1.38
0.250	17.00 ± 2.27	18.50 ± 1.85
0.375	18.25 ± 0.63	17.25 ± 2.53
0.500	16.50 ± 2.18	17.25 ± 1.70
	E. grandis	
IP benzamidina	OPI	WPI
0.000	16.50 ± 2.39	17.00 ± 1.96
0.125	18.25 ± 2.36	17.50 ± 1.85
0.250	18.50 ± 2.33	17.25 ± 1.75
0.375	17.50 ± 0.87	18.50 ± 1.71
0.500	17.50 ± 1.19	16.50 ± 1.85

Values (mean ± standard error) per column per species do not differ by Tukey test ($p \leq 0.05$). Control without IP benzamidine (OPI) (0.01 % mg·mL-1 of aqueous solution of the adhesive spreader Triton X-100) and treatment with PI benzamidine (WPI).

PI, which have great toxic potential, mainly for the early instar Lepidoptera (Fortunato *et al*., 2007).

T. leucoceraea showed a slighter oviposition preference (number of egg masses per plant) on *E. grandis* than on *P. guajava* plants, but this parameter was similar among different concentrations of the IP benzamidine by host-model and control (water and Triton X-100) (Fig. 3). Similar values of the number of egg masses per plant and eggs per mass, per *T. leucoceraea* female, on *E. grandis* and *P. guajava* plants, with different concentrations of the PI benzamidine, and in the control with water and Triton X-100, indicates a higher production of toxic compounds on this herbivore. The selection of the host for oviposition by Lepidoptera females is related to learning by previous contact with them (Paixão *et al*., 2013). *T. leucoceraea* individuals were collected in the field from host plants and kept with those of origin, and therefore, with previous experience potential in the host plant (Solarz and Newman, 2001). The nutritional value of the plant could also affect the oviposition site choice for Monarch butterfly, *Danaus plexippus* (Lepidoptera: Nymphalidae) females as observed, to plants that allowed better development of their larvae with nontoxic proper balance of nutrients (Yeargan and Allard, 2005; Pereira *et al*., 2010). The slighter oviposition preference by *T. leucoceraea* females on non-treated *E. grandis* than on treated *P. guajava* plants indicates that this herbivore can recognize compounds from the introduced species that negatively affects their descendants, after treatment with different concentrations of the PI benzamidine (Oliveira *et al*., 2005), despite the short time of contact of this defoliator with plants of the introduced species. The production of toxic compounds by *E. grandis* plants treated with PI benzamidine can be similar or higher than those of Myrtaceae, native to Brazil. However, *T. leucoceraea* females could recognize these compounds, which would explain their similar reproduction on *E. grandis* and *P. guajava* plants with different concentrations of PIs, and in the control. This should be considered in pest control, because the oviposition preference was slightly higher on *E. grandis* plants and the application of PIs could reduce reproduction of this herbivore.

The number of *T. leucoceraea* eggs per mass was similar between the treatments (different concentrations of PI benzamidine) and the control (water and Triton X-100) (one way, ANOVA, $p \leq 0.05$) (Table 2). This parameter also showed similar values between the position of the plants and reading times (one way, ANOVA, $p \leq 0.05$) (Table 2). The variation between plants in plots with different concentrations of PI benzamidine and the slight number of eggs per mass of *T. leucoceraea* females on plants without this PI, showed a preference for more suitable hosts for the development and reproduction of their offspring. Females of herbivorous-insects could choose hosts for phagostimulant or phagoinhibitor substances, nutrients, and volatile substances expressed by them (Ikeura *et al*., 2010; Larue and Welty, 2010). *T. leucoceraea* females might have identified the PI benzamidine on the leaves of host plants and reduced oviposition, since this compound would affect the development of its offspring. This agreed with the lowest reproduction of *D. saccharalis* after their larvae fed on an artificial diet treated with PI,

Table 2 Number of eggs per mass of *T. leucoceraea* (Lepidoptera: Geometridae) on *P. guajava* (native host) or *E. grandis* (introduced host) (Myrtaceae) plants to Brazil in five concentrations (M or mol.L^{-1}) of the protease inhibitor (PI) benzamidine

	P. guajava	
IP benzamidina	OPI	WPI
0.000	35.76 ± 1.82	37.31 ± 2.70
0.125	30.94 ± 2.55	32.85 ± 2.59
0.250	28.20 ± 2.21	29.20 ± 5.76
0.375	31.71 ± 2.47	28.50 ± 5.48
0.500	32.79 ± 2.59	35.78 ± 2.82
	E. grandis	
IP benzamidina	OPI	WPI
0.000	28.15 ± 3.15	18.47 ± 2.28
0.125	28.56 ± 3.35	18.64 ± 2.70
0.250	27.44 ± 3.26	29.82 ± 4.49
0.375	20.94 ± 3.08	26.20 ± 4.61
0.500	19.00 ± 2.76	27.09 ± 4.45

Values (mean ± standard error) per column per species do not differ by Tukey test ($p \leq 0.05$). Control without PI benzamidine (OPI) (0.01 % mg·mL^{-1} of aqueous solution of the adhesive spreader Triton X-100) and treatment with PI benzamidine (WPI).

from *G. max* grains (Pompermayer *et al*., 2001), with lower weight and larval length, and higher larvae mortality of corn earworm, *Helicoverpa zea* (Lepidoptera: Noctuidae) with three different PIs [benzamidine, phenylmethylsulfonyl fluoride (PMSF), and N-α-tosyl-l-lysine chloromethyl ketone (TLCK)] in cotton, *Gossypium* sp. (Malvaceae) plants (Zhu *et al*., 2007).

The possible adaptation of *T. leucoceraea* to an exotic host (*E. grandis*) was reduced with different concentrations of PI benzamidine. This was shown by foraging (number of larvae per plant) and similar numbers of egg masses per plant and eggs per mass by female on *E. grandis* and *P. guajava* plants, disagreeing with the development and reproduction of *T. arnobia* with *E. grandis* and gympie messmate, *Eucalyptus cloeziana* (Myrtaceae) without PIs (Holtz *et al*., 2003). Native hosts can have a more developed defense system, such as, chemical and physical barriers, which reduce the digestibility and affect the present and future insect population (population control) (Castells and Berenbaum, 2008). These barriers can induce herbivores to seek new hosts, including the *Eucalyptus* species, mainly because of the quantity and availability of this host, which is widely cultivated in Brazil, and because of its lower capacity of defense.

The foraging and reproduction behaviors of *T. leucoceraea* on *E. grandis* (introduced host) and *P. guajava* (native host) shows lack of adaptation of this pest to the introduced host treated with PI benzamidine. This makes it necessary to evaluate the specialization of *T. leucoceraea* to this exotic plant, because it could form new races adapted to certain hosts. Spraying with PI benzamidine can reduce defoliation and oviposition of *T. leucoceraea* on *E. grandis* plants. This shows the possibilities of using this PI in the integrated pest management (IPM) programs in *Eucalyptus* spp. cultures.

Acknowledgments

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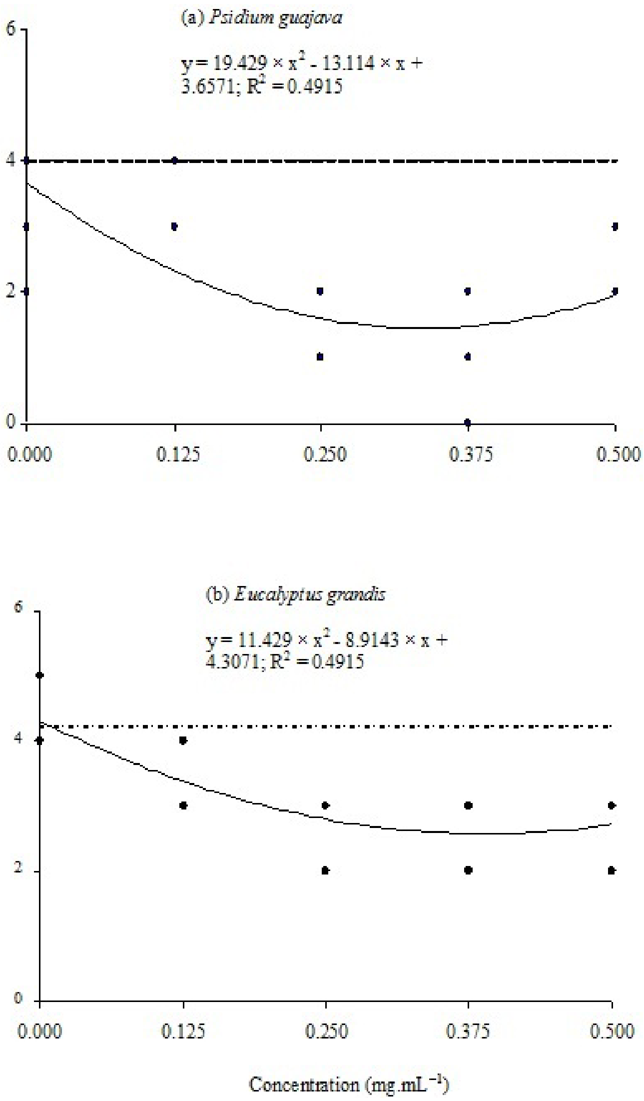

Fig 3 Number of egg masses of *T. leucoceraea* (Lepidoptera: Geometridae) females per concentration of protease inhibitor (PI) benzamidine on *P. guajava* (native host) (a) and *E. grandis* (introduced host) (b) plants, Myrtaceae to Brazil. One way, ANOVA of regression ($p \leq 0.05$). Mean of the control (without spraying PI benzamidine; with 0.01 % mg·mL^{-1} of aqueous solution of the adhesive spreader Triton X-100).

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