

Bioactivity of *Piper* extracts on *Tuta absoluta* (Lepidoptera: Gelechiidae) in tomato

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Abstract – The objective of this work was to evaluate the bioactivity of ethanolic leaf extracts from four species of the genus *Piper* against the tomato leafminer *Tuta absoluta* (Lepidoptera: Gelechiidae) under laboratory conditions. The evaluated species were: *P. amalago* var. *medium*, *P. glabratum*, *P. mikanianum*, and *P. mollicomum*. In the initial screening assay (extract concentration of 2,000 mg L⁻¹), all tested extracts caused significant larval mortality, particularly the extract of *P. amalago* var. *medium*; however, no extracts reduced the weight of the surviving larvae. The extract from *P. amalago* var. *medium* at the concentration of 1,011 mg L⁻¹ caused a significant lengthening of the larval and pupal stages. The ethanolic leaf extract of *P. amalago* var. *medium* is promising for the control of *T. absoluta* larvae in tomato, since it exhibits acute toxicity toward these caterpillars at the concentration of 2,000 mg L⁻¹ and affects the insect's development by reducing its survival and lengthening the larval and pupal stages.

Index terms: *Piper amalago* var. *medium*, botanical extracts, piperamides, tomato leafminer.

Bioatividade de extratos de *Piper* sobre *Tuta absoluta* (Lepidoptera: Gelechiidae) em tomateiro

Resumo – O objetivo deste trabalho foi avaliar a bioatividade de extratos etanólicos de folhas de quatro espécies do gênero *Piper* sobre a traça-do-tomateiro *Tuta absoluta* (Lepidoptera: Gelechiidae), em condições de laboratório. As espécies avaliadas foram: *P. amalago* var. *medium*, *P. glabratum*, *P. mikanianum* e *P. mollicomum*. Na triagem inicial (concentração do extrato de 2.000 mg L⁻¹), todos os extratos testados causaram significativa mortalidade larval, principalmente o extrato de *P. amalago* var. *medium*; entretanto, nenhum extrato ocasionou redução do peso das lagartas sobreviventes. O extrato de *P. amalago* var. *medium* na concentração de 1.011 mg L⁻¹ promoveu alongamento significativo da fase larval e pupal. O extrato etanólico de folhas de *P. amalago* var. *medium* é promissor no controle de lagartas de *T. absoluta* em tomateiro, pois exibe toxicidade aguda a lagartas na concentração de 2.000 mg L⁻¹ e afeta o desenvolvimento do inseto, ao reduzir a sua sobrevivência e alongar as fases larval e pupal.

Termos para indexação: *Piper amalago* var. *medium*, extratos botânicos, piperamidas, traça-do-tomateiro.

Introduction

The tomato leafminer *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is native to South America. The species is present in the main producing countries of tomato [*Solanum lycopersicum* (L.)] (Solanaceae) in Latin America and is currently gaining status as a major threat to the worldwide production of this fruit due to the recent occurrence of tomato leafminer in European production centers (European and Mediterranean Plant Protection Organization, 2005; Desneux et al., 2010).

In Brazil, *T. absoluta* is one of the key insect pests of the tomato crop, reducing productivity up to 100% (Souza & Reis, 2000). The damage is caused by the larvae of the insect, which feed and grow on soft tissues, such as leaves, shoots, and fruits from the aerial part of the plant, at any stage of tomato growth.

Although chemical control has been the main method used against *T. absoluta* in South America, the low efficiency of the active ingredients against this insect has been reported since the 1990s (Campos et al., 2014). Furthermore, the intensive use of synthetic

chemicals as the sole strategy for insect control together with insect traits, such as high reproductive potential (10–12 generations per year) and mining habit, has favored the selection of insects resistant to the different active ingredients, especially pyrethroids, avermectins, cartap, and diamides (Lietti et al., 2005; Silva et al., 2011; Roditakis et al., 2015). Although the Brazilian Ministry of Agriculture, Livestock and Food Supply requires an efficiency higher than 80% for the registration of a new formulation, the continued use of certain active ingredient leads to the selection of resistant populations (selection pressure), which has already been shown in some studies developed in the field, with populations of *T. absoluta* resistant to avermectin, pyrethroids, and cartap (Lietti et al., 2005; Silva et al., 2011). The high biotic potential of this species (10–12 generations per year) leads to an increase in spraying frequency, reaching 40 applications per crop cycle in growing conditions in Brazil, which accelerates the evolution of resistance. In Europe, where *T. absoluta* was recently introduced, there have been reports of populations of this insect with high frequency of individuals containing alleles that confer resistance to pyrethroids (Haddi et al., 2012; Roditakis et al., 2013). Therefore, researches on alternative management and new active compounds are necessary.

Alternative pest management, aiming at the sustainability of production systems, has been investigated, resulting in new perspectives for studies with allelochemicals and their application in the formulation of botanical insecticides (Cantrell et al., 2012). Various derivatives of vegetable origin, especially in the form of extracts or essential oils, have been suggested as alternatives to synthetic pesticides (Philogène & Vincent, 2005). One of the most studied species in recent decades is neem (*Azadirachta indica* A. Juss) (Meliaceae) (Morgan, 2004), and several commercial formulations with it are now available worldwide.

Under tropical and subtropical conditions, Piperaceae also constitute a promising source of botanical insecticides due to their great abundance (Dyer & Palmer, 2004) and diversity of allelochemicals with insecticidal/insectistatic properties (Scott et al., 2008). In Brazil, there are approximately 450 species of Piperaceae, mostly belonging to the genus *Piper* (Yuncker, 1975), and bioassay-guided Piperaceae studies have been conducted to identify species

with insecticidal potential (Scott et al., 2005, 2008). Moreover, there are formulations of Piperaceae derivatives already in registration process in Brazil (Brasil, 2009). Piperamides are among the major classes of compounds found in species of the genus *Piper*, and these compounds offer dual biological activity on insects, including neurotoxic effects and effects on lipid metabolism (Dyer & Palmer, 2004), an important characteristic for the management of resistant populations (Scott et al., 2004).

The objective of this work was to evaluate the bioactivity of ethanolic leaf extracts from four species of the genus *Piper* against the tomato leafminer *T. absoluta* under laboratory conditions.

Materials and Methods

A stock rearing of *T. absoluta* was established using larvae collected in commercial tomato fields, in the municipality of Botucatu, in the state of São Paulo, Brazil (22°85'S, 48°26'W). The stock was maintained in laboratory conditions (25±2°C, 65±10% relative humidity, and 14-hour photophase). Leaves of 'Santa Clara' commercial tomato were used to feed the insect during the larval stage, and a solution of 10% (p/v) honey during the adult stage. The insects were maintained in aluminum cages (40x40x60 cm) with an antiaphid mesh on the sides and acrylic at the front and on the top.

The Piperaceae plants used were collected in the campus of Escola Superior de Agricultura Luiz de Queiroz (Esalq) of Universidade de São Paulo in the municipality of Piracicaba, in the state of São Paulo, Brazil (22°42'52,7"S, 47°37'46"W), on November 15th 2012. A voucher of each studied specimen was deposited in the herbarium of Esalq, under the following reference numbers: *Piper amalago* var. *medium* (121200); *P. glabratum* (121206); *P. mikanianum* (121201); and *P. mollicomum* (121202).

To prepare the extracts, the leaves collected from adult plants were oven-dried at 40°C for 48–72 hours. Subsequently, the leaves were separately milled in a knife mill to obtain a powder and then stored in a refrigerator at -20°C in sealed glass containers until use; storage time was less than 14 days. The extractions were carried out by cold maceration using 99.5% ethanol as a solvent in a 1:5 (v/w) ratio. For this, the macerated material was kept for 3 days in the solvent and then filtered through filter paper. Any remaining

residue was resuspended in the solvent, and the process was repeated four times. The solvent present in the filtered solution was eliminated using a rotary evaporator at a temperature of 40°C and a pressure of 600 mm Hg. After total evaporation of the solvent in an air-flow chamber, the obtained residue was weighed to determine the yield of extraction. After that, the extracts were kept under refrigeration at -20°C until use in the bioassays.

All assays were conducted in a completely randomized design in an acclimated room at 25±2°C, 65±10% relative humidity, and 14-hour photophase.

For the selection of the most promising treatment, leaf extracts from *P. amalago* var. *medium*, *P. glabratum*, *P. mikanianum*, and *P. mollicomum* were tested at the concentration of 2,000 mg L⁻¹, with the negative control consisting of the acetone solvent used to solubilize the extracts. Seven replicates were used per treatment, and each replicate (experimental unit) consisted of four plates, each containing four newly-hatched larvae for a total of 112 larvae per treatment.

The extracts were applied on tomato leaves detached from the upper third of approximately 30-day-old potted plants cultivated in greenhouse. The extracts were applied with the aid of an Arprex microatomizer, model 5A (Arprex, Mogi das Cruzes, SP, Brazil), coupled to a pneumatic pump and adjusted to provide a pressure of 0.5 kgf cm⁻²; the extract was sprayed until the excess started dripping off the leaves. After drying the waste (~30 min), the leaflets were placed separately in plastic Petri dishes (96 mm in diameter and 21 mm in height) and infested with five newly-hatched larvae. To maintain turgor, the petiole of each leaflet was wrapped in moistened cotton wool. Larval mortality was assessed daily for six days with the aid of a binocular stereoscopic microscope. After this period, the weight of the surviving larvae was also evaluated.

The extract that caused the highest average mortality was selected for further studies, and concentration-response curves were evaluated to estimate the concentrations necessary to cause 50 and 90% mortality (LC₅₀ and LC₉₀, respectively) of the individuals of the exposed population. Preliminary tests were conducted to estimate the concentrations that could cause mortality levels of 95% and close to the control. Based on this, six concentrations to be tested, ranging from 500–7,000 mg L⁻¹, were established using the procedure proposed by Finney (1971). The

experimental procedures, sample units, number of replicates, and exposure times were the same as those used for initial screening.

The sublethal effects of the extract of *P. amalago* var. *medium* at the estimated concentration (LC₅₀) were evaluated on *T. absoluta*. Tomato leaves detached from the upper third of approximately 30-day-old plants were used. The extracts were sprayed following the same procedures described for initial screening. The acetone solvent used for the solubilization of the extract was used as a control. After drying the residue (~30 min), the petiole of each leaf was placed in a 50-mL glass tube containing distilled water. The leaves were then infested with six newly-hatched caterpillars and placed inside cages consisting of a rectangular polystyrene base (20x10x3 cm) and a transparent cylindrical plastic arena (11 cm in diameter and 27 cm in height); the arena was covered with voile to prevent the caterpillars from escaping. Twenty replicates for each treatment were used, totalizing 120 larvae per treatment. Larval mortality was assessed daily until the pre-pupal stage; subsequently, the larvae were placed separately in Petri dishes. The insects were monitored throughout their lifecycle to obtain the following variables: duration (days) of larval and pupal stages, larval mortality, pupal weight, and sex ratio.

The estimates of lethal concentrations were analyzed by a binomial function with the complementary log-log link (gompit model) using the Probit procedure of the SAS software, version 9.2 (SAS Institute, Cary, NC, USA). For the analysis of the other bioassays, generalized linear models (GLMs) belonging to the exponential family of distributions (Nelder & Wedderburn, 1972) were used. The goodness of fit of the data to the models was verified through the use of a half-normal probability plot with a simulation envelope (Hinde & Demétrio, 1998). When significant differences were found between the treatments, multiple comparisons were performed by Tukey's post hoc test, at 5% probability, using the general linear hypothesis test (glht) function from the multcomp package with adjustment for p values. All analyses were performed using the R statistical software, version 2.15.1 (R Development Core Team, 2012).

Results and Discussion

The yield of the obtained extracts varied with the plant species, and the highest yield was obtained from

the extract of *P. mikanianum* (7.97%), followed by the extracts of *P. mollicomum* (6.98%), *P. glabratum* (6.46%), and *P. amalago* var. *medium* (6.10%). Although the yield of the leaf extracts can be considered high using ethanol (bipolar solvent), further studies are required to optimize the extraction method of the promising extracts, taking into account the economic aspect of the process and its adequacy to the concepts of environmental sustainability and green chemistry (Ribeiro et al., 2014).

With the exception of the ethanolic leaf extract of *P. mollicomum*, the other extracts caused significant mortality in *T. absoluta* larvae (Table 1). However, although showing acute toxicity, neither of the extracts caused a significant reduction in the weight of the surviving larvae by the sixth day (144 hours) of exposure. Other studies have also shown the potential of *Piper* species as a source of botanical insecticides, with bioactivity on other pests of agricultural importance, such as: *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) (Alves et al., 2014; Murillo

et al., 2014); *Spodoptera litura* (F.) (Lepidoptera: Noctuidae) (Kitayama et al., 2013); *Mythimna separata* (Walker) (Lepidoptera: Noctuidae) (Qu et al., 2013); *Diatraea saccharalis* (Fabr.) (Lepidoptera: Pyralidae) (Debonsi et al., 2008); *Malacosoma disstria* Hübner (Lepidoptera: Lasiocampidae); *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) (Scott et al., 2007); and *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae) (Navickiene et al., 2007). However, this is the first study to report the bioactivity of *P. amalago* var. *medium* derivatives on insects and of piperaceous derivatives on *T. absoluta*.

Due to the abundance of plant material at the collection site and the higher levels of mortality observed during screening, the leaf extract of *P. amalago* var. *medium* was considered as the main candidate for the development of a botanical insecticide for protection of horticultural crops in Brazil. Therefore, it was selected for further investigation.

Based on the results of the concentration-response curves, in which $LC_{50}=1,011 \text{ mg L}^{-1}$ (Table 2),

Table 1. Means±standard error of *Tuta absoluta* larval weight and mortality after six days of exposure to tomato (*Solanum lycopersicum*) leaflets treated with ethanolic leaf extracts from different Piperaceae species, at the concentration of $2,000 \text{ mg L}^{-1}$ (¹).

Plant species	Mortality (%)						Larval weight (mg)
	24 hours	48 hours	72 hours	96 hours	120 hours	144 hours	
<i>Piper amalago</i> var. <i>medium</i>	66.66±4.36a	67.62±4.22a	67.62±4.22a	67.62±4.22a	67.62±4.22a	67.62±4.22a	0.33±0.05
<i>Piper glabratum</i>	41.90±6.12a	43.81±5.41a	43.81±5.41a	43.81±5.41a	43.81±5.41a	43.81±5.41a	0.36±0.03
<i>Piper mikanianum</i>	50.47±7.81a	50.48±7.82a	50.48±7.82a	50.48±7.82a	50.48±7.82a	50.48±7.82a	0.24±0.04
<i>Piper mollicomum</i>	9.52±2.86b	11.43±2.80b	11.43±2.80b	11.43±2.80b	11.43±2.80b	11.43±2.80b	0.36±0.07
Control	9.40±2.87b	11.25±2.80b	11.25±2.80b	11.25±2.80b	11.25±2.80b	11.25±2.80b	0.32±0.03
F value	25.23**	26.34**	26.34**	26.34**	26.34**	26.34**	0.823 ^{ns}

(¹)Means followed by different letters, in the columns, differ significantly among treatments according to the general linear model with quasi-binomial distribution, followed by Tukey's post hoc test, at 5% probability. ^{ns}Nonsignificant. Temperature, 25±2°C; relative humidity, 60±10%; and photophase, 14 hours.

Table 2. Estimate of the concentrations (mg L^{-1}) necessary to cause 50 and 90% mortality (LC_{50} and LC_{90} , respectively), as well as the confidence interval (CI) of the ethanolic leaf extract from *Piper amalago* var. *medium* against *Tuta absoluta* at different exposure times.

Exposure time (hours)	n(¹)	Slope±standard error	LC_{50} (95% CI)	LC_{90} (95% CI)	χ^2 (²) (df=6)	h(³)
24	744	1.86±0.24**	1,008 (700.24–1,295)	4,464 (3,510–6,281)	70.8	1.77
48	744	1.87±0.25**	1,011 (688.79–1,311)	4,440 (3,475–6,303)	73.91	1.84
72	744	1.87±0.25**	1,011 (688.79–1,311)	4,440 (3,475–6,303)	73.91	1.84
96	744	1.87±0.25**	1,011 (688.79–1,311)	4,440 (3,475–6,303)	73.91	1.84
120	744	1.87±0.25**	1,011 (688.79–1,311)	4,440 (3,475–6,303)	73.91	1.84
144	744	1.87±0.25**	1,011 (688.79–1,311)	4,440 (3,475–6,303)	73.91	1.84

(¹)n, number of insects tested. (²) χ^2 , Pearson's chi-square value. (³)h, heterogeneity factor. Temperature, 25±2°C; relative humidity, 60±10%; and photophase, 14 hours. **Significant at 1% of probability.

there was no increase in mortality after 24 hours of exposure, according to the superimposition of the estimated confidence intervals. These results indicate that *T. absoluta* larval mortality occurs mainly in the period prior to leaf penetration, in particular via contact with the residue of the leaf extract. It was also observed that the caterpillars that had not penetrated the mesophyll after contact with the residue showed symptoms of lethargy and low mobility, which is likely associated with the mode of action of neurotoxins from piperaceous derivatives. The neurotoxic effects of Piperaceae extracts on insects have been described in other studies (Scott et al., 2005, 2007). Among the major classes of secondary compounds often found in species of the genus *Piper*, amides, lignans, alkaloids, and neolignans are highlighted. Amides, such as piperamides, commonly found in the genus *Piper* have dual biological activity on insects, including neurotoxic and lipid metabolism effects (Dyer & Palmer, 2004). Effects of the knockdown type, with paralysis and immediate death of the insect, have also been reported in insects treated with amides isolated from Piperaceae species (Scott et al., 2007).

At the concentration of 1,011 mg L⁻¹ (LC₅₀), the ethanolic leaf extract from *P. amalago* var. *medium*

lengthened the larval and pupal stages of *T. absoluta* (Tables 3 and 4) but did not affect survival in this stage or larval weight. Unexpectedly, the level of mortality was lower than in the previous bioassay with concentration-response curves. This reduction may be related, in hypothesis, to variations in the nutritional composition of the tomato leaves (testing substrate) used in the different bioassays or to differences in insect strength over the generations, factors that can affect the tolerance of the exposed insects to xenobiotic compounds (De Bruyn et al., 2002). Dyer et al. (2003) tested the effects of three *Piper* amides isolated from *P. cenocladum* on *S. frugiperda* and also observed a lengthening of the insect's development time. It should be noted that the elongation of a moth's life stages is a desirable effect because it can increase the time of exposure to natural enemies and the average time of each generation, reducing the population growth rate and minimizing crop damage.

The ethanolic leaf extract of *P. amalago* var. *medium*, at the concentration of 2,000 mg L⁻¹, caused mortality in *T. absoluta* larvae of almost 70% and shows promise for the control of tomato leaf miner in small growing areas.

Table 3. Means±standard error of duration of the larval stage and of mortality of *Tuta absoluta* exposed to ethanolic leaf extract from *Piper amalago* var. *medium* at the previously-estimated concentration of 1,011 mg L⁻¹, necessary to cause 50% mortality (LC₅₀).

Treatment	Duration (days) ⁽¹⁾	Mortality (%) ⁽²⁾
<i>Piper amalago</i> var. <i>medium</i>	11.22±0.17a	32.05±4.88
Control (acetone)	10.60±0.12b	19.17±6.09
F value	9.0888**	2.3645 ^{ns}

⁽¹⁾Means followed by different letters, in the column, differ significantly among treatments by the general linear model (GLM) with Gaussian distribution, followed by Tukey's post hoc test, at 5% probability. ⁽²⁾Means followed by different letters, in the column, differ significantly among treatments by the GLM with quasi-binomial distribution, followed by Tukey's post hoc test, at 5% probability. ^{ns}Nonsignificant. Temperature, 25±2°C; relative humidity, 60±10%; and photophase, 14 hours.

Table 4. Means±standard error of larval weight, mortality, and duration of pupal stage, as well as the sex ratio of emerged adults of *Tuta absoluta* exposed to ethanolic leaf extract from *Piper amalago* var. *medium* at the previously-estimated concentration of 1,011 mg L⁻¹, necessary to cause 50% mortality (LC₅₀).

Treatment	Weight (mg) ⁽¹⁾	Mortality (%) ⁽²⁾	Duration (days) ⁽³⁾	Sex ratio ⁽⁴⁾
<i>Piper amalago</i> var. <i>medium</i>	4.47±0.10	5.83±3.30	11.15±0.17a	0.55±0.07
Control	4.42±0.10	12.32±3.46	10.24±0.07b	0.51±0.05
F value	0.0973 ^{ns}	0.304 ^{ns}	23.705**	χ ² =24.751 ^{ns}

⁽¹⁾No significant differences by the general linear model (GLM) with Gaussian distribution, at 5% probability. ⁽²⁾No significant differences by the GLM with a quasi-binomial distribution, at 5% probability. ⁽³⁾Means followed by different letters, in the column, do not differ significantly by the GLM with Gaussian distribution, followed by Tukey's post hoc test, at 5% probability. ⁽⁴⁾No significant differences by the GLM with a binomial distribution, at 5% probability. ^{ns}Nonsignificant. Temperature, 25±2°C; relative humidity, 60±10%; and photophase, 14 hours.

Conclusions

1. Ethanolic leaf extracts, at the concentration of 2,000 mg L⁻¹, from *Piper amalago* var. *medium*, *P. glabratum*, and *P. mikanianum* have insecticidal activity against *Tuta absoluta* larvae, although none of them reduce the weight (growth inhibition) of the surviving larvae.

2. The ethanolic leaf extract from *P. amalago* var. *medium* causes a significant lengthening of the development time of the larval and pupal stages of *T. absoluta*.

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