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Salicylic acid treatments induce resistance to *Tuta absoluta* and *Tetranychus urticae* on tomato plants

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ABSTRACT

This study was performed to evaluate the effect of exogenous application of salicylic acid as a resistance inducer to two-spotted spider mite and tomato pinworm on tomato genotypes. Bioassays were carried out in three commercial genotypes ('Redenção', 'Giuliana', and 'Alambra') and one wild genotype (*Solanum habrochaites* var. *hirsutum*, accession PI-127826). Average distance travelled by the two-spotted spider mite and number of eggs deposited over the leaflets of the genotypes were investigated. Concerning pinworm bioassay, number of eggs, leafminers on leaflet surface and percentage of leaf area consumed by leafminers were evaluated using a scanner. Furthermore, severity of attack to plants was evaluated by score scale for damages, types of lesions and attacked leaflets. Salicylic acid application induced resistance in the commercial genotypes against both pests, reducing the two-spotted spider mite and pinworm oviposition. The spider mites travelled shorter distance on leaflets of genotypes treated with salicylic acid at intermediate concentrations, indicating a positive effect of substance as inducing resistance. Attack severity by pinworm was also reduced when intermediate doses of salicylic acid were applied. Also, higher resistance was observed on accession PI-127826 as compared to other genotypes. The obtained results indicate that the resistance of PI-127826 was due to constitutive defense, whereas on commercial genotypes defense was dependent on responses mediated by salicylic acid. Hence, salicylic acid treatment, as resistance inducer, seems to be efficient against two-spotted spider mite and pinworm, being a promising tool within an integrated pest management on tomato plants.

Keywords: *Solanum habrochaites* var. *hirsutum*, *Tuta absoluta*, *Tetranychus urticae*, methyl salicylate, induced resistance.

RESUMO

Ácido salicílico induz resistência à *Tuta absoluta* e *Tetranychus urticae* em tomateiro

Objetivou-se avaliar os efeitos da aplicação exógena de ácido salicílico como indutor de resistência ao ácaro-rajado e à traça em genótipos de tomateiro. Para isso, realizaram-se bioensaios com três genótipos comerciais ('Redenção', 'Giuliana' e 'Alambra') e um silvestre (*Solanum habrochaites* var. *hirsutum* acesso PI-127826). Determinou-se a distância média percorrida pelo ácaro-rajado e o número de ovos depositados sobre os folíolos dos genótipos. Com relação ao bioensaio da traça-do-tomateiro, foram avaliados o número de ovos e lagartas na superfície do folíolo e a porcentagem de área foliar consumida pelas lagartas por meio de digitalização dos folíolos. Além disso, avaliou-se a severidade do ataque na planta utilizando uma escala de notas referente a danos, tipos de lesões e folíolos atacados. A aplicação de ácido salicílico induziu resistência nos genótipos comerciais para ambas as pragas, diminuindo a oviposição do ácaro e da traça do tomateiro. Os ácaros percorreram menor distância nos folíolos dos genótipos que receberam tratamento com ácido salicílico nas concentrações intermediárias, indicando efeito benéfico da substância na indução de resistência. A severidade do ataque da traça do tomateiro, também foi coibida quando aplicadas doses intermediárias de ácido salicílico. Entretanto, observou-se resistência superior do acesso PI-127826 em relação aos demais genótipos. Isso indica que a resistência de PI-127826 se deve à defesa constitutiva, enquanto que nos genótipos comerciais as defesas são dependentes, em parte, de respostas de indução mediadas pelo ácido salicílico. Dessa forma, pode se inferir que o ácido salicílico é eficiente como indutor de resistência ao ácaro rajado e à traça-do-tomateiro, sendo bastante promissor dentro de um manejo integrado de pragas em cultivos comerciais de tomateiro.

Palavras-chave: *Solanum habrochaites* var. *hirsutum*, *Tuta absoluta*, *Tetranychus urticae*, metil salicilato, indução de resistência.

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Among the different biotic stresses to which tomato plants are exposed during cultivation, pest arthropods are the most damaging (Silva *et al.*, 2019).

In particular, pinworm (*Tuta absoluta*), a chewing herbivore that causes large damages due to galleries created on the leaf mesophyll by its leafminers on

the leaf mesophyll, which also affects petioles, stems, and fruits, causing high production losses (Dias *et al.*, 2019). Another arthropod pest that causes

large losses in tomato plants is the two-spotted spider mite [*Tetranychus urticae*, (Acari: Tetranychidae)], a generalist sucking pest that feeds by punching leaf mesophyll cells with its stylets (Zanin *et al.*, 2018). The large proportion of damages caused by those species is related to the presence of overpopulations in cultivated areas.

Generally, the intensive use of pesticides can lead to increased costs and environmental damage, as well as potential harm towards consumer and producer health (Youssef *et al.*, 2019). In this scenario, the use of pest-resistant tomato genotypes appears as an adequate alternative to agrochemicals (Zanin *et al.*, 2018). In particular, control of arthropod-pests on crop plants is performed mainly with agrochemical applications, which have resulted in problems such as high production costs, the development of genetic resistance and residual damages to the environment (Ilias *et al.*, 2017). In this sense, the elucidation of defense mechanisms of tomato plants can be a way to assist the pest control and, thus, to reduce the use of pesticide applications.

Plants respond to stresses by triggering a series of events, including the transcription of genes involved in the biosynthesis of plant hormones involved in defense responses (Bostock *et al.*, 2014). Among these plant hormones, the salicylates (SA), jasmonates (JA) and ethylene (Et) are known to modulate plant defense responses (Brodersen *et al.*, 2006; Aljibory & Chen, 2017).

The signaling pathway induction of those plant hormones is triggered according to the type of feeding habit of the arthropods. The SA pathways are associated mainly with hypersensitivity responses and acquired systemic resistance (Yan & Dong, 2014), while herbivory defenses have been related to JA pathways (Howe & Jander, 2008; Ojha & Chatterjee, 2012). However, studies have shown that SA may also be associated with defense against herbivory (Schweiger *et al.*, 2014; Aljibory & Chen, 2017; Favaro *et al.*, 2019).

Therefore, in the present study we aimed to evaluate the effect of exogenous application of salicylic acid

as a resistance inducer against the two-spotted spider mite and pinworm on tomato genotypes.

MATERIAL AND METHODS

Pests bioassays were conducted at the Universidade Estadual do Centro-Oeste do Paraná (UNICENTRO) from October 2015 to May 2016. The tomato plants were grown in pots and kept in a greenhouse with controlled environment. Different salicylic acid concentrations (25, 50, 75, and 100 mg L⁻¹) were applied to evaluate the influence on tomato resistance against attack of spider mite (*T. urticae*, sucking pest) and pinworm (*T. absoluta*, chewing pest).

Plant material and treatment application

For bioassay with two-spotted spider mite (*T. urticae*), three commercial tomato genotypes were used, including *Solanum lycopersicum* F₁ hybrid Alambra (shelf life), F₁ hybrid Giuliana (salad) and Redenção cultivar (for industrial processing), in addition to a wild species resistant to pests *S. habrochaites* var. *hirsutum* accession PI-127826 (Maluf *et al.*, 2007). Bioassay with pinworm (*T. absoluta*) was carried out with 'Alambra' and 'Redenção' and accession PI-127826.

Genotypes were seeded on polypropylene boxes containing biostabilized pine bark-based commercial substrates. About 30 days after sowing, seedlings were transplanted to polypropylene pots (7 dm³) filled with subsurface soil corrected according to chemical analysis and commercial substrate (1:1). Each pot was considered as an experimental plot.

Plants were treated either with four concentrations of salicylic acid (25, 50, 75, and 100 mg L⁻¹) or with water as check control treatment. Treatments were applied weekly after plants presented four fully expanded leaflets. For the two-spotted spider mite test, treatments were applied eight times (up to 50 days after transplant). For pinworm, two applications were carried out, at 15 and 30 days after infestation. Treatments were applied

with a backpack sprayer adapted with a constant pressure valve (Jacto®) with 2 kgf cm⁻² pressure, cone nozzle X2 (2/110), 1.05 m s⁻¹ speed and plastic curtains to prevent drift in the neighboring plots. Randomized blocks design in a factorial arrangement with four replicates was used.

Bioassay - *Tetranychus urticae*

Spider mites used in the bioassay were obtained from a stock rearing maintained in common bean plants (*Phaseolus vulgaris*) on the Laboratory of Agricultural Entomology (UNICENTRO). Spider mite resistance test was performed at the pre-flowering stage (50 days after transplanting) following the methodology proposed by Weston & Snyder (1990). Young leaflets, fully expanded and with similar sizes, were taken from the upper third of plants and maintained in a controlled environment room located at the Laboratory of Agricultural Entomology (67.0±3% relative humidity and 15±2°C temperature).

After that, each leaflet was fixed with a 9 mm diameter brass thumbtack in its center on a white sulphite paper sheet, A4 size (212 x 297 mm), placed on a polystyrene board. The adaxial surface of the leaves was kept facing upwards. Then, ten female mites were transferred to the thumbtack center using a brush. Subsequently, the distance travelled (WD) by mites (mm) on the surface of each leaflet from the center of thumbtack was determined with a ruler 20, 40 and 60 minutes after pest released. When mites remained on thumbtack, distance was considered zero. When they left leaflet surface towards the surface of the paper sheet, the travelled distance was considered that from the center of thumbtack to the end of the leaflet. The distance covered by each mite at 20, 40 and 60 minutes was measured for each time to obtain the final average of the distances for each treatment (Zanin *et al.*, 2018). Experimental design was completely randomized with five replications. Treatments were four genotypes [Alambra, Giuliana, Redenção and *S. habrochaites* var. *hirsutum* (PI-127826)] and four concentrations of salicylic acid (25, 50, 75 and 100 mg L⁻¹) besides witness

(application with water), totalizing 100 leaflets.

Bioassay - *Tuta absoluta*

Pinworm bioassay was carried out in a greenhouse with a controlled environment (Dias *et al.*, 2019). The temperature was set to $29\pm 4^{\circ}\text{C}$ during the day and to $22\pm 4^{\circ}\text{C}$ during the night, with 12 hours daylength and $78\pm 3\%$ relative humidity. Plants were infested with pinworm 50 days after transplanting in the pre-flowering stage. The experimental design was completely randomized, with four replications. Treatments were four genotypes [Alambra, Giuliana, Redenção and *S. habrochaites* var. *hirsutum* (PI-127826)] and four concentrations of salicylic acid (25, 50, 75 and 100 mg L^{-1}) in addition to the witness treatment (application with water). Each replicate consisted of 15 plants totalizing 60 plants for the entire experiment. Pinworms were created on susceptible tomato plants (cultivar Santa Cruz). Plants were grown in pots placed inside voile cages ($100 \times 100\text{ cm}$) of PVC structure that were kept inside a greenhouse under controlled temperature ($27\pm 3^{\circ}\text{C}$). To standardize the infestation, deposited eggs were transferred every three days to a new set of susceptible 'Santa Clara' plants (not infested) that were also placed in voile cages. After 18 days, two pots of the new set of 'Santa Cruz' plants, infested with eggs and leafminers, were randomly placed inside each block.

Number of eggs (NE) and leafminers (NL) of pinworm was determined in three leaflets on the upper third of plants at 15, 25, 35, 45, and 55 days after infestation. The eggs were counted with a stereoscopic microscope on the abaxial and adaxial surface of the leaflet in an area of 10 cm^2 . Leafminers were counted within an area of 10 cm^2 of the sampled leaflet surface. Number of eggs and leafminers correspond to the average of the three sampled leaflets obtained during the five evaluations (15, 25, 35, 45, and 55 days after infestation).

Assessments of damage severity were carried out 20 and 60 days after infestation, according to a score scale proposed by Labori *et al.* (1999) described as follows: A) Intensity of damage to plants (IDP) [0= no damage;

1= small injuries (0.1 to 5% damage); 2= small, non-coalescent lesions (5.1 to 20% damage); 3= medium to large injuries (20.1 to 50% damage); 4= large and coalescent lesions (50.1 to 80% of damage); and 5= plants completely destroyed (over 80% damage)]. B) Types of lesions in the leaflets (TLL) (0= no lesions; 1= few and minor injuries; 2= small and medium lesions; 3= medium-sized lesions, without coalescence; 4= large and coalescent lesions, deformed leaflets; and 5= leaflet completely destroyed). C) Percentage of attacked leaflets (PAL) (0= without attacked leaflets; 1= 0.1 to 5% attacked leaflets; 2= 5.1 to 20% attacked leaflets; 3= 20.1 to 50% attacked leaflets; 4= 50.1 to 80% attacked leaflets; and 5= more than 80% attacked leaflets).

The intensity of damage to plants (IDP), types of lesions in the leaflets (TLL) and percentage of attacked leaflets (PAL) were determined by 5 previously trained evaluators. Extreme score values were discarded from the final weighting average.

To analyze the percentage of consumed leaf area, three whole leaves were harvested from each third of the plant (upper, middle and lower), from which, the six most distal leaflets were evaluated. Leaflets were scanned using an HP j4550 multifunction printer connected to a microcomputer. The images (300 dpi resolution) were analyzed using the Quant 1.0.1 software and the percentage of consumed leaf area by the leafminers was assigned according to the score of the damage scale.

Statistical analysis

The results were submitted to the normality (Shapiro-Wilk) and homogeneity tests (Kolmogorov-Smirnov). The analysis of variance (ANOVA) was performed when the assumptions were met. When statistical differences were found, the Tukey's test ($p\leq 0.05$) and polynomial regression analyses were performed using the software SISVAR (Ferreira, 2011) to estimate the adjustments of equations related to salicylic acid concentrations. Equations were derived for determining maximum and minimum points

whenever necessary.

RESULTS AND DISCUSSION

Analysis of variance indicated significant interaction between variation factors (concentrations of salicylic acid and genotypes) for the travelled distance (WD) by mites on leaflet surface at 40 minutes after female release. On the other hand, the variation factors had significant effect on the travelled distance at 20 and 60 minutes only when taken individually. Concerning the leafminer's bioassay, the analysis of variance showed that the variation factors individually influenced the number of eggs (NE), number of leafminers (NL) and types of leaflet lesions (TLL). However, there was significant interaction between the variation factors on plant damage intensity (PDI), percentage of attacked leaflets (PAL) and percentage of consumed leaf area (PCL) by the leafminers.

Average travelled distances by spider mites on leaflets were plotted as a function of different concentrations of salicylic acid, presenting adjustment to the quadratic model. Reductions in spider mite travelled distance were observed for all concentrations tested, regardless the genotype (Figure 1). At 20 minutes, the quadratic model estimated the shortest travelled distance to occur at the SA concentration of 115.5 mg L^{-1} , with an average travelled distance of 14.41 mm. In addition, the model indicated that the shortest average travelled distance by spider mites at 40 minutes (13.34 mm) and 60 minutes (17.08 mm) was expected to occur with SA concentrations of 97.25 mg L^{-1} and 109 mg L^{-1} , respectively (Figure 1).

Regression analysis showed that the results of pinworm bioassay had quadratic adjustment when plotted as a function of salicylic acid concentrations, except for the number of leafminers (NL). Significant reductions were observed on the number of eggs of *T. absoluta* (2.5 eggs) at 51.0 mg L^{-1} of SA and on leaflet lesions at 43.5 mg L^{-1} of SA, according to the estimated model (Figure 2).

The results obtained herein showed that SA application was effective in defense induction of tomato genotypes against herbivory, corroborating with Schweiger *et al.* (2014) who found a significant effect of both SA and JA application on defense induction against herbivory of sucking and chewing insects in *Plantago lanceolata*. Previously, most studies indicate that defense against herbivory is associated with jasmonate (JA) (Howe & Jander,

2008) and that salicylate (SA) is mainly involved in defense against biotrophic pathogens (Ojha & Chatterjee, 2012).

It's important to mention that, defense induction responses triggered by different classes of herbivores are conflicting. Aljbory & Chen (2017) suggested that SA is associated with defense against sucking and puncturing insects, such as mites, and that protection against chewing insects, such as pinworm, is associated with

jasmonate. On the other hand, Ament *et al.* (2004) found a specific response of tomato to attack by *T. urticae*, which seems to induce simultaneously both JA and SA dependent resistance. This may explain the reason why higher SA concentrations were necessary to cause a significant effect on two-spotted spider mite management as compared to pinworm. JA and SA, when applied simultaneously, reduced defense induction against herbivores (sucking and chewing) due to a negative crosstalk between some pathways of these hormones (Schweiger *et al.*, 2014). This crosstalk is characterized by an antagonism between the response to SA and JA, the latter being co-activated by ethylene, which inhibits SA-mediated responses (Brodersen *et al.*, 2006).

As expected, the wild genotype (*S. habrochaites* var. *hirsutum* accession PI-127826) showed greater resistance to the two-spotted spider mite and tomato pinworm as compared to the commercial genotypes. In addition, the accession PI-127826 was less responsive to the salicylic acid treatments, having a significant influence only on the percentage of consumed leaf area by pinworm, with 10.24% of leaf area consumed with the application of 67.7 mg L⁻¹ concentration. The low influence of SA application was perhaps due to the high level of constitutive defense of PI-127826 provided by the presence of allelochemicals produced in glandular trichomes, mainly type IV and VI, which were found in a high quantity in this genotype (Lima *et al.*, 2015; Bergau *et al.*, 2015).

The density of leaf trichomes and, consequently, allelochemical production are possibly dependent on jasmonate-related immune responses (Escobar-Bravo *et al.*, 2017). On the other hand, salicylate acts on other tissues promoting responses such as induction of genes related to pathogenesis-related (PR) proteins, which have antimicrobial activity (Van Loon *et al.*, 2006), accumulation of reactive oxygen species (ROS) associated with programmed cell death, and methylation of salicylic acid into methyl salicylate (MeSA) (Van Schie *et al.*, 2007), a volatile compound that induces immune response in non-attacked leaves and neighboring plants

Table 1. Average travelled distance (WD) by spider mite (*Tetranychus urticae*) at 20, 40 and 60 minutes after exposure and number of eggs (NE), number of leafminers (NL), and types of leaflet lesions (TLL) caused by pinworm (*Tuta absoluta*) in tomato genotypes treated with salicylic acid. Guarapuava, UNICENTRO, 2016.

Genotype	WD (mm)			NE (4 cm ²)	NL (10 cm ²)	TLL** (grades)
	20 min	40 min	60 min			
Alambra	30.16* a	31.02 a	33.18 A	0.47 A	2.97 a	1.99 a
Giuliana	30.52 a	32.47 a	34.20 A	-	-	-
Redenção	27.06 a	27.49 b	27.72 B	0.39 A	3.24 a	1.92 a
PI-127826	0.43 b	0.56 c	0.62 C	0.26 B	1.39 b	1.27 b
CV%	10.38	9.47	7.58	29.58	33.2	7.89

*Means followed by equal letters belong to the same group by the Tukey's test at 5% probability; (-) Not evaluated; **Measured using a score scale ranging from 1 to 5 according to the type of lesion in the leaflet (-1 corresponds to absence of lesion and 5 corresponds to leaflets totally destroyed).

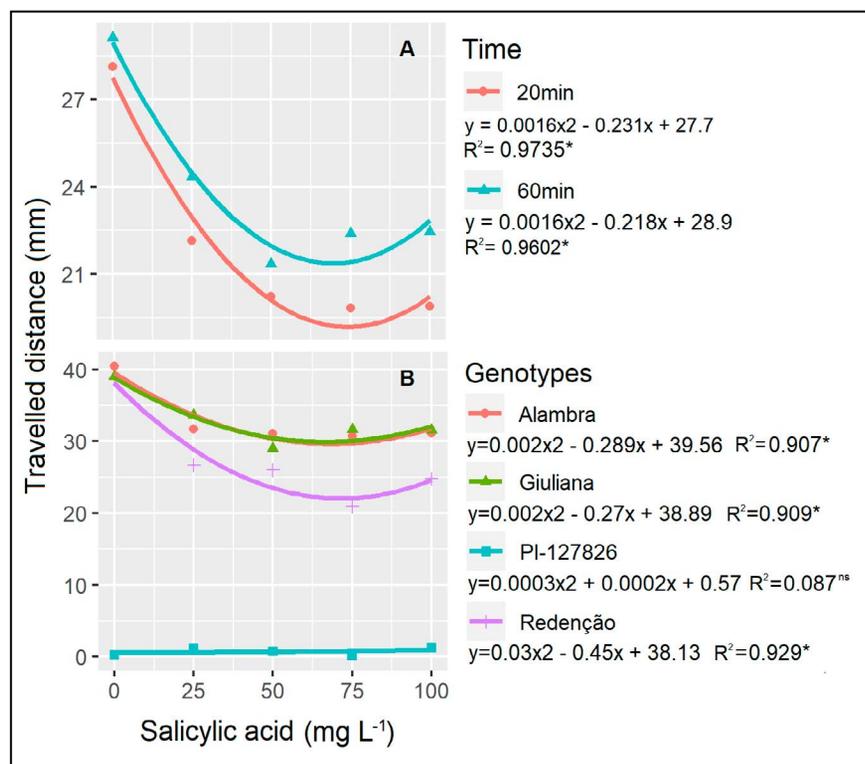


Figure 1. Average travelled distance in mm (DT) by the two-spotted spider mite (*Tetranychus urticae*) on tomato leaflets treated with different concentrations of salicylic acid (mg L⁻¹) at 20 and 60 minutes (A) and 40 minutes (B). Guarapuava, UNICENTRO, 2016.

and attracts natural predators of the arthropod-pest (Aljbory & Chen, 2017).

In consequence, the insensitivity of PI-127826 genotype to SA found in the present study suggests that the allelochemical production is independent of the SA signaling pathway. On the other hand, the influence of SA application on the percentage of consumed leaf area in PI-127826 suggests that this type of damage may be associated with the acquired systemic resistance (ASR), mediated by salicylate, instead of the constitutive resistance present in the genotype. These results are in agreement

with Kawazu *et al.* (2012) who observed an increase in the expression of SA-induced PR-genes after the beginning of tomato leaf blade consumption by the leafminers *Liriomyza sativae*, although the expression of SA-related genes was low during oviposition. Those authors pointed out that SA-induced response seems to be more associated with JA-mediated suppression than with SA accumulation. This may be associated with plant plasticity in terms of resource allocation (trade-off). Since resources destined to a particular process become unavailable for others, such as on dichotomy between defense and growth,

plants can allocate energy to certain pathways over others to ensure effective defenses to specific types of attacks (Vries *et al.*, 2017).

Most genes related to immune responses and defense metabolites production were lost throughout the process of domestication and breeding of crop plants. While the wild tomato has a large number of glandular trichomes, this number is very low in commercial genotypes (Lima *et al.*, 2015; Silva *et al.*, 2019). Thus, cultivated tomato requires other defense strategies against herbivorous attacks, such as hormone-mediated defense induction. Thus,

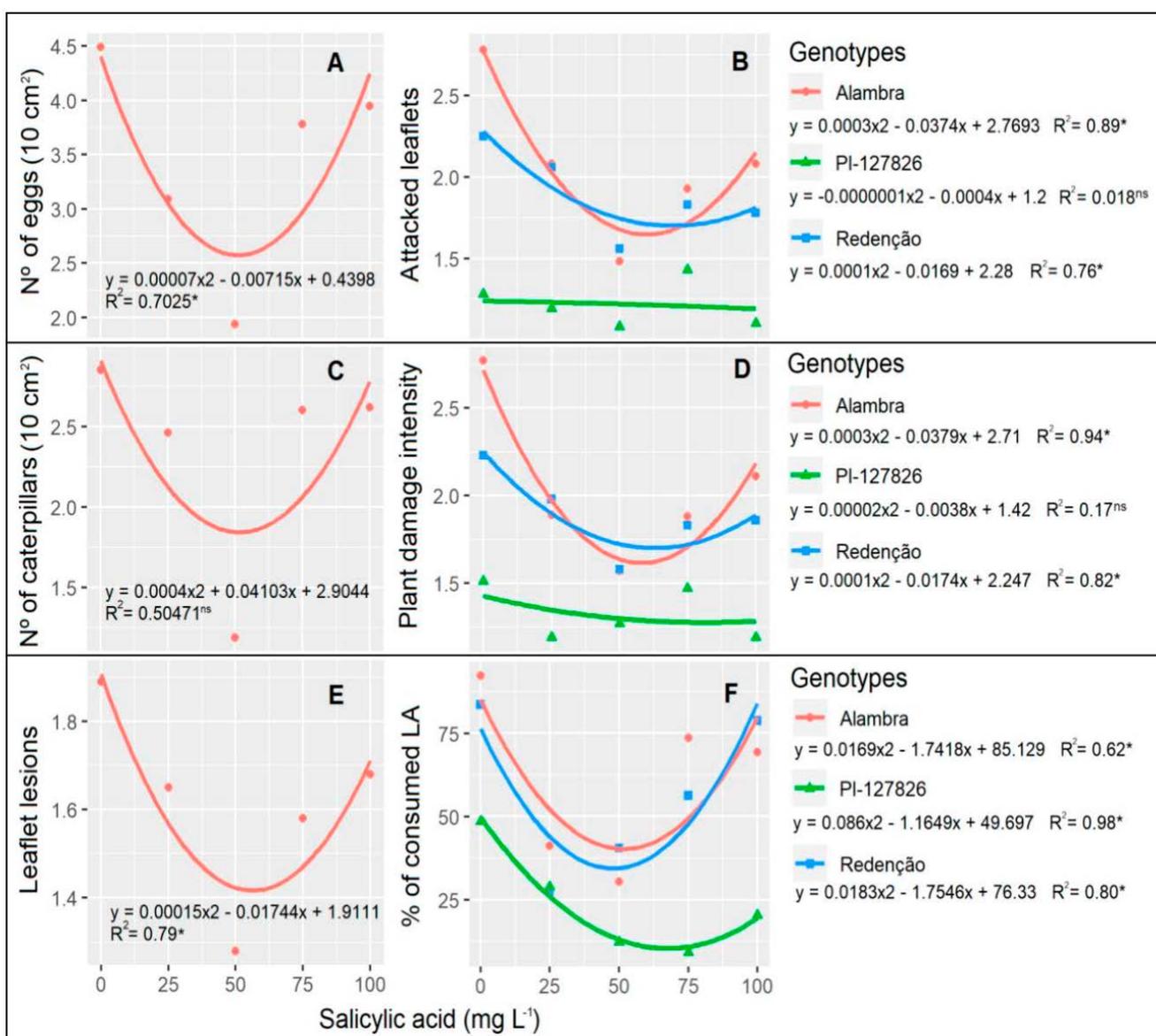


Figure 2. Average number of eggs (NE) 10 cm² of leaf area (A), average number of leafminers (NL) 10 cm² of leaf area (B), average score scale value for plant damage intensity (PDI) (C), average score scale value for percentage of attacked leaflets (PAL) (D), average score scale value for types of leaflet lesions (TLL) (E) and percentage of consumed leaf area (PCL) (F) by pinworm (*Tuta absoluta*) in tomato plants treated with various concentrations of salicylic acid. Guarapuava, UNICENTRO, 2016.

the results obtained in the current study indicate that resistance induction in these plants depends on defense responses mediated by salicylic acid.

Among the analyzed commercial genotypes, 'Redenção' showed a reduction of spider mite travelled distance when compared to 'Alambra' and 'Giuliana' at 60 minutes after exposure (Table 1). Similarly, at 40 minutes, the regression analysis indicated a lower spider mite travelled distance on 'Redenção' at all concentrations, with the shortest travelled distance (21.3 mm) estimated at the concentration of 75 mg L⁻¹, while 'Alambra' (28.1 mm at 72.3 mg L⁻¹) and Giuliana (29.8 mm at 67.5 mg L⁻¹) showed a similar behavior (Figure 1). At 20 minutes, commercial genotypes did not differ from each other (Table 1).

In the pinworm bioassay, 'Redenção' showed reduction on plant damage (PDI) of 33.1% at concentration of 87 mg L⁻¹. For 'Alambra', reduction was 45.3% on concentration of 63.1 mg L⁻¹ (Figure 2). Concerning severity of the attacked leaflets, cultivar 'Redenção' reduced the leafminer attack intensity by 43.6%, on concentration of 84.5 mg L⁻¹ and 'Alambra', 29.1% reduced these damages on the concentration of 62.3 mg L⁻¹, as compared to the respective controls. Overall, Redenção cultivar was less damaged by pinworm attack as compared to Alambra hybrid. However, Alambra tomato was more responsive to salicylic acid, since it showed greater control efficiency at lower doses of the compound.

This may be associated with the mechanism of action of SA since when a plant is attacked, salicylate concentration increases drastically, triggering the degradation of the transcription factor NPR1 (NONEXPRESSOR OF PR GENES1), responsible for the induction of resistance genes. NPR1 degradation induces an increase in the level of reactive oxygen species (ROS), especially hydrogen peroxide and superoxide, which induce cell death at the site of injury (Yan & Dong, 2014). This process is called hypersensitivity response and could explain the increase in plant damage intensity (PDI) observed in 'Alambra'

(Herrera-Vásquez *et al.*, 2015). This performance is in agreement with Peng *et al.* (2004) who found increases in the levels of endogenous SA, MeSa, and hydrogen peroxide in tomato plants attacked by leafminers of *Helicoverpa armigera*. In addition, Donovan *et al.* (2013) found an increase in the levels of SA and ROS in tobacco leaves attacked by aphids (*Myzus persicae*). In contrast, in cells adjacent to the injury region, SA concentration remained intermediate, which activates the transcription of acquired systemic resistance genes (induced by NPR1). Thus, 'Alambra' may have acquired systemic resistance, which explains the lower number of leaflets attacked (Wu & Baldwin, 2010) at the maximum dose.

The exogenous application of salicylic acid was effective in reducing the two-spotted spider mite travelled distance and the number of eggs and leafminers, plant damages, and the percentage of leaf area consumed by pinworm in commercial genotypes, thus being an interesting alternative to assist in the integrated pest management on tomato. *S. habrochaites* var. *hirsutum* accession PI-127826 had a constitutive defense independent of SA-mediated induction responses, proving more resistant than commercial genotypes.

PI-127826 genotype, considered as a resistant standard, presented the lowest damage severity due to pest attack and the lowest oviposition on the leaflets for both, spider mite and pinworm. Although intermediate doses of salicylic acid were efficient in inducing resistance on evaluated genotypes, they are still less resistant as compared to the wild genotype. It is important to mention that even constitutive resistance can be transferred through crosses between species. It takes a long time to develop commercial materials with good resistance level. In this regard, the results obtained herein indicate the use of salicylic acid as a valuable alternative to pest control in commercial crops, mainly within integrated management program.

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