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Trypsin Inhibitors in Passion Fruit (*Passiflora* f. *edulis flavicarpa*) Leaves: Accumulation in Response to Methyl Jasmonate, Mechanical Wounding, and Herbivory

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This work investigates the effect of methyl jasmonte (MeJa), mechanical wounding, and herbivory caused by larval feeding of a specialist insect (*Agraulis vanillae vanillae*) upon trypsin inhibitory activity in passion fruit leaves. Despite the fact that all treatments caused accumulation of trypsin inhibitors (TIs), higher levels were observed in MeJa treated leaves when plants were assayed 24 and 48 h after stimulus. Concerning both mechanically injured plants and attacked ones, a systemic induction was observed. Partially purified inhibitors from MeJa exposed plants were further characterized by X-ray film contact print technique and N-terminal sequence. Such analysis indicated that the TIs identified belong to the Kunitz family. Moreover, the partially purified inhibitors strongly inhibited trypsin-like digestive enzymes from sugar cane stalk borer (*Diatraea saccharalis*) *in vitro*. Our results further support the protective function of wound-inducible trypsin inhibitors and their potential as tools to improve important crop species against insect predation through genetic engineering.

KEYWORDS: Trypsin inhibitor; Kunitz; herbivory; methyl jasmonate; wound response; passion fruit

INTRODUCTION

Plant protease inhibitors (PIs) are diverse in number and in specificity toward various proteolytic enzymes. Such features place them among some of the most important classes of antiherbivore defense proteins in plants. The accumulation of PIs is elicited in response to various stimuli, such as mechanical wounding and insect pest and pathogen attacks. To coordinate responses to environmental stresses, plants have evolved complex signaling networks. Among signaling molecules, the plant hormone jasmonic acid (JA) plays a very important function in triggering defense responses (*1*). JA and related compounds (collectively called jasmonates) are products of the oxylipin pathway of fatty acid metabolism (*2*). The wound response triggered by insect attack or mechanical injury activates the biosynthesis of a number of jasmonate signaling molecules and thus provides defense against a myriad of predators (*3, 4*).

Inhibitors of different digestive proteases, including serine, cysteine, aspartic, and metalloproteases have been identified in plants (*5*). Serine PIs are the most common and best characterized, and this group comprises several protein families including potato inhibitors I and II, Bowman-Birk inhibitors, pumpkin trypsin inhibitors, trypsin and α-amylase inhibitors from cereal grains, mustard trypsin inhibitors, serpins, and Kunitz trypsin inhibitors (*6*−*8*). Most of the serine PIs characterized are competitive inhibitors, and their reactive site interacts with the active center of the target enzyme as a highly specific substrate. However, such strong affinity renders the inhibitor−protease complex virtually inactive (*9*). Plant PIs are thought to play a variety of physiological functions since they are also developmentally regulated, as has been observed during leaf aging, flower decay, seed development, and germination (*8*).

Wound-inducible PIs were originally studied in Solanaceous species quite long ago (*10*). The extensive studies by C. A. Ryan and co-workers led to the discovery of systemin, the first polypeptide hormone described in plants that is involved in the systemic wound response of tomato plants (*11*). Moreover, they

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indicated the pivotal role of the octadecanoid pathway for plant defense signaling (*12, 13*). Since these findings have been revealed, wound-inducible PIs have been widely used as a model system to study plant defense mechanisms.

Plant PIs are direct defense compounds that act to reduce the activity of digestive proteases of insect pests, and that is considered to lead to a reduction in nutrient absorption and growth of phytophagous insects (*14*). Therefore, it is currently accepted that these inhibitors are involved in the protection of plants against chewing insects. The potential of plant PIs as bioinsecticides has been demonstrated in transgenic plants (*15*−*17*). For example, the development pattern of the light-brown apple moth, *Epiphyas postvittana*, decreased when larvae were fed with leaves of a transgenic apple plant overexpressing PIs precursors from tobacco, *Nicotiana alata* (*18*).

Passion fruit is an economically important tropical crop in Brazil, and the country's passion fruit production is one of the largest in the world (*19*). In spite of the fact that passion fruit is susceptible to insect herbivores, little is known about the defense mechanisms of this plant. In this background, we studied the effect of MeJa, mechanical wounding and herbivory in the protein-based defense system focusing on the induction of TIs. To our knowledge, this is the first report addressing defensive proteins from passion fruit with biotechnological properties.

MATERIALS AND METHODS

Plant Material and Treatments. Passion fruits (*Passiflora edulis* f. *flavicarpa*) were purchased at the local market of Campos dos Goytacazes, a city located in the northern region of Rio de Janeiro state, Brazil. The seeds were collected, dried at room temperature, and stored at 4 °C in the dark upon use. Plants were grown in peat pots and maintained in environmental chambers for 17 h under 300 mE m^{-2} s^{-1} light at 28 °C and for 7 h in the dark at 18 °C.

Four-week-old plants (with 3−4 developed leaves) were used for all treatments. The lower leaves were wounded multiple times (3−4) with a hemostat. Four hours later, the same leaves were wounded again, as repeated mechanical wounding causes an increase in the defense response. The next upper leaves were assayed as systemic ones. Intact plants were continuously exposed to MeJa vapor in closed Plexiglas boxes as described by Farmer and Ryan (*20*). Plants were maintained in environmental chambers under constant light for the first 24 h. Plants that would be assayed 48 h following treatment were exposed again to normal daylight periods. Herbivore experiments were carried out as follows: eggs of the specialist insect pest *Agraulis vanillae vanillae* were collected in passion fruit fields from local growers and kept in the laboratory in order to obtain larvae according to Silva et al. (*21*). The feeding experiments were performed in 20-cm-high insect cages within the environmental chambers. For each plant, 2 starved fourth-instar larvae were placed on leaves and allowed to feed for 4 h, and samples were collected 24 and 48 h after the beginning of the experiment. These experimental plants were kept in the same conditions as described above.

Crude Leaf Extract. Leaf tissue was ground to a fine powder in liquid N_2, 10% (w/w) insoluble polyvinylpolypyrolidone (PVPP) was added, and protein was extracted by adding 3 mL of ice-cold extraction buffer (50 mM sodium phosphate, pH 6.5) to 1 g of powdered tissue. The homogenate was centrifuged at 10,000*g* for 20 min at 4 °C, and the supernatant was used as inhibitor source. Protein concentration was determined using the Bradford assay kit and bovine serum albumin as standard protein according to the manufacturer's instructions (BioRad).

Assay of Inhibitory Activity against Trypsin. The inhibition of trypsin was determined by measuring the residual enzymatic activity toward the substrate BAPNA (*N*-benzoyl-DL-arginyl-*p*-nitroanilide) after contact with crude leaf extract (*22*). The crude leaf extract (5−10 μL) was preincubated for 5 min at 37 °C with 1 μg of trypsin prior to substrate addition (20 mM) in 50 mM Tris-HCl buffer, $CaCl_2$ 20 mM, pH 8.0, for 30 min at 37 °C in a 200-μL final volume. The reaction was interrupted by adding 100 μL of 30% acetic acid (v/v). Substrate hydrolysis was followed by measuring the absorbance of released *p*-nitroaniline at 405 nm.

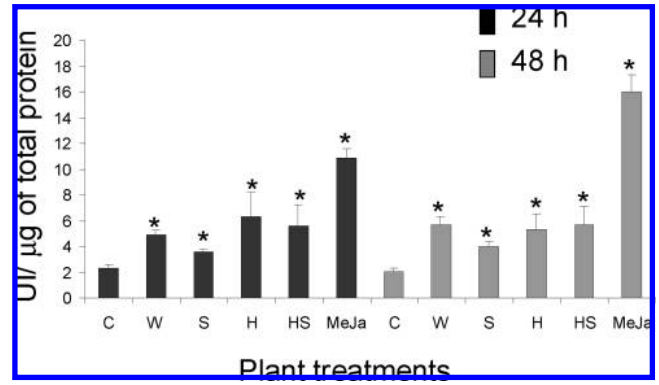

Figure 1. Induction of trypsin inhibitors in passion fruit leaves. C, crude leaf extract from control plants; W, crude leaf extract from mechanically wounded leaves; S, crude leaf extract from unwounded (systemic) leaves of mechanically wounded plants; H, crude leaf extract from damaged leaves by herbivory; HS, crude leaf extract from unwounded (systemic) leaves of attacked plants; MeJA, crude leaf extract from MeJA treated plants. At 24 and 48 h after stimuli, samples were collected for analysis. In all cases, three plants were pooled for each protein extraction to obtain an average response. One unit of inhibitor (UI) was defined as the amount of inhibitor that reduces the optical density reading of BApNA digestion by trypsin by 0.01. Data are the mean of three independent experiments (±S.D.). Asterisks denote significant difference at $P < 0.05$ (Student's *t*-test) from control leaves.

Assay of Inhibitory Activity against α-Amylase. The inhibition of α-amylase was determined by measuring the residual enzymatic activity toward the substrate (starch 1%) after contact with partially purified inhibitors (*23*). The inhibitors (5−50 μL) were preincubated for 15 min at 37 °C with 1.3 μg of porcine pancreatic α-amylase prior to substrate addition in reaction buffer (1 mM sodium phosphate, pH 7.3, and 30 mM $CaCl_2$), for 15 min at 37 °C in a 100-μL final volume. The reaction was interrupted by the addition of 400 μL of 3,5-dinitrosalicylic acid reagent and boiling for 30 min in a water bath. To each tube, 400 μL of water was added. Absorbance was measured at 540 nm. The α-amylase inhibitory activity was expressed as a relative α-amylase activity without preincubation with the partial purified inhibitors.

Polyacrylamide Gel Electrophoresis. Proteins were analyzed in 12.5% SDS−polyacrylamide gel according to Laemmli (*24*). Protein bands were stained with Coomassie Brilliant Blue. For sequencing purposes, samples were separated by prolonged gel electrophoresis in order to better separate the major protein bands and then transferred to PVDF membranes as described by Towbin et al. (*25*).

Partial Trypsin Inhibitors Purification. Leaf tissue (10 g) was ground to a fine powder in liquid N_2, 10% (w/w) insoluble PVPP was added, and protein was extracted by the addition of 30 mL of ice-cold extraction buffer (50 mM sodium phosphate, pH 6.5). The homogenate was centrifuged at 10,000*g* for 20 min at 4 °C, and the supernatant was used as enzyme source. Powdered $(NH_4)_2SO_4$ was added to the crude extract to achieve 20% of saturation. The solution was gently stirred for 3 h at 4 °C and then centrifuged as described above. $(NH_4)_2SO_4$ was added to the resulting supernatant to achieve 55% saturation. The solution was gently stirred for 3 h at 4 °C and then centrifuged as described above. The precipitate was collected and suspended in a minimum volume of 50 mM Tris, pH 8.0. The suspension was subjected to chromatography through a Sephadex G-100 column (1.5−45 cm) previously equilibrated with 50 mM Tris, pH 8.0, at a flow rate of 30 mL h^{-1}. Fractions of 0.5 mL were collected. The fractions with inhibitory activity were combined, dialyzed against distilled water, and lyophilized.

Visualization of Trypsin Proteinase Inhibitors. Partially purified inhibitors were separated on a 12.5% SDS−PAGE gel according to the method described by Laemmli (*24*) with some modifications: lack

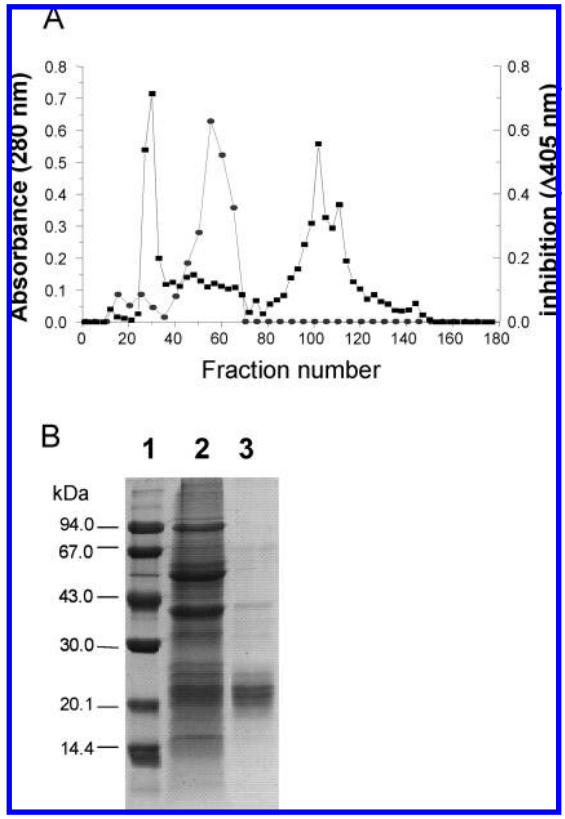

Figure 2. Partial purification of trypsin inhibitors from MeJa treated plants. (**A**) Gel filtration chromatography. Proteins were applied to a Sephadex G−100 column. The column was equilibrated in 50 mM Tris-HCl buffer, pH 8.0, and eluted with the same buffer. ■, absorbance at 280 nm; ●, inhibitory activity. (**B**) Electrophoretic analysis. (12.5%) SDS−PAGE, lane 1. molecular weight markers; lane 2, 45 μg of crude leaf extract; lane 3, 5 μg of partially purified inhibitors.

of β-mercaptoethanol in the protein sample and heat treatment for 3 min at 65 °C. Visualization of trypsin proteinase inhibitors after SDS−PAGE was carried out using the gel-X-ray film contact print technique (*26, 27*). After electrophoresis, the gel was washed three times to remove SDS, with 2.5% Triton X-100 in 0.1 M Tris-HCl (pH 8.0) for 20 min. The gel was incubated in 0.1% trypsin solution for 10 min, and excess trypsin was rinsed off, and the gel was overlaid on an X-ray film for 10 and 15 min. The film was then washed with tap water, and inhibitor activity bands were visualized as unhydrolyzed gelatin. The same gel was then washed and stained by Coomassie Brilliant Blue.

Extraction of Larval Digestive Enzymes and *in Vitro* Enzyme Assays of *D. saccharalis*. Ten fourth-instar larvae of *D. saccharalis*, originally supplied by Universidade Federal Rural do Rio de Janeiro, Campos dos Goytacazes, Brazil, were cold-immobilized, and midgut contents were removed in cold 150 mM NaCl. The midguts were subsequently homogenized in 150 mM NaCl, centrifuged at 6000*g* for 5 min, and supernatants cooled. The effect of partially purified inhibitors on the proteolytic activity of whole midgut extracts was measured by using BApNA (20 mM) as substrate. The assays were run at pH 8.0 in 50 mM Tris-HCl. Various concentrations of partially purified inhibitors were incubated with 80-μg aliquots of midgut extracts at 37 °C for 5 min prior to substrate addition. After 30 min of incubation, the reaction was stopped by adding 30% acid acetic (v/v). The resulting absorbance was read at 405 nm.

The effect of partially purified inhibitors on the α-amylase activity of whole midgut extracts was performed on the basis of the method of Bernfeld (*23*). The assays were run at pH 7.3 in 1 mM sodium phosphate and 30 mM $CaCl_2$. Various concentrations of partially purified inhibitors were incubated with 20 μg of aliquot of midgut extracts at 37 °C for 15 min before adding the substrate (starch 1%). After 15 min of incubation, the reaction was stopped by adding 400 μL of 3,5-dinitrosalicylic acid reagent and boiling for 30 min in a water bath. To each tube, 400 μL of water was added. The resulting absorbance was read at 540 nm.

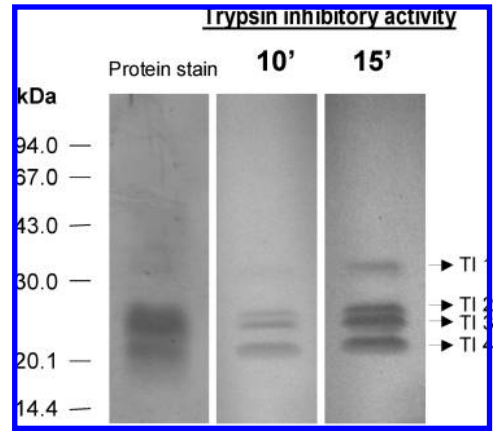

Figure 3. Visualization of passion fruit trypsin inhibitors. Partially purified inhibitors (8 μg) were separated on a 12.5% SDS−PAGE. The gel was subsequently incubated in trypsin solution and overlaid on an X-ray film for 10 and 15 min (for details see Materials and Methods). The gel was removed, and the X-ray film was washed with water to visualize the inhibitor bands. The same gel was then stained for total protein. Arrows indicate TI bands in the molecular mass range of 20−35 kDa.

Protein Sequencing. The N-terminal sequence was determined on a Shimadzu PPSQ-10 automated protein sequencer using Edman degradation. Phenylthiolhydantoin amino acids (PTH-AA) were detected at 269 nm after separation on a reverse-phase C_{18} Wakopack Wakosil HPLC column (4.6 mm × 25 cm) from Shimadzu, under isocratic conditions, using 40% acetonitrile, 20 mM acetic acid, and 0.014% sodium dodecyl sulfate as the mobile phase at a flow rate of 1.0 mL/min at 40 °C. The sequence obtained was utilized for a protein blast search using the NCBI system as follows: an initial blast using only the 12 amino acid sequence and a second blast also using the word trypsin inhibitor as an entrez query.

Statistical Analysis. Data are presented as the mean ± S.D. of three independent experiments, and the differences from controls were assessed with Student's *t*-test; statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Induction of Inhibitory Activity toward Trypsin by MeJa, Mechanical Wounding, and Herbivory. Mechanical wounding and MeJa treatment were previously shown to induce accumulation of two enzymes involved in JA biosynthesis (lipoxygenase and allene oxide synthase) and cysteine proteinase inhibitors in passion fruit leaves (*28, 29*). These results led us to investigate the induction of TIs in response to MeJa treatment, mechanical wounding, and herbivory.

To study the effect of herbivory upon inhibitor induction, we chose a specialist lepidopteran pest, *Agraulis vanillae vanillae*. **Figure 1** shows the accumulation of TIs in leaf tissue in response to all stimuli. Exogenous MeJa treatment caused higher increase of inhibitory activity for both periods of time (24 and 48 h), when compared with the induction observed in response to mechanical wounding and larval feeding. Plants damaged artificially or by larval attack displayed local and systemic induction since undamaged leaves also accumulated TIs. Nevertheless, there were no clear differences between induction levels observed in response to mechanical wounding and herbivory. These results suggest that the induction of TIs in leaf tissue is part of the protein-based system involved in wound/herbivore defense. However, a further detailed analysis is required to evaluate the possible contribution of elicitor(s)

Table 1. Alignment of the N-Terminal Amino Acid Sequence of the TIs from Passion Fruit with Those Kunitz-Type Members[a]

	Initial position	Sequence/homology	% ID	Ref.	Acession number
TI 3	1	**ELRTGVPYYLAR**			
TI 4	1	**ELRTGVPYYLAR**			
Theobroma mammosum	8	**ELRTGVQYY**	88	(38)	AAL85652
Theobroma subincanum	8	**ELRTGVQYY**	88	(38)	AAL85644
Theobroma sylvestre	8	**ELRTGVEYY**	88	(38)	AAL85657
Solanum melongena	4	**LRTGIDYY**	75	-	BAA82843
Lycopersicon esculentum	39	**LRTGVDYY**	87	(40)	AAC63057
Nicotiana tabacum	39	**LRTGIDYY**	75	(41)	AAC49969
Populus tremula	37	**GVPYYM**	83	(39)	CAI77814

[a] Conserved amino acids are in bold. ID, identities; Ref., reference.

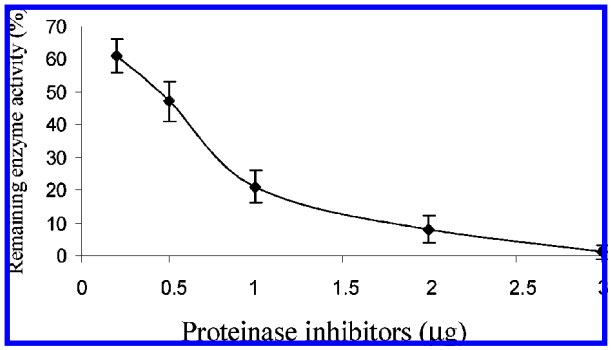

Figure 4. In vitro inhibition of digestive trypsin-like endopeptidases from *D. saccharalis* with partially purified inhibitors. The assay was made using 80 μg of midgut extract and different concentrations of inhibitors. Data are the mean of three independent experiments (±S.D.).

from oral secretion, liberated on the wound sites during insect feeding, to the TI induction observed in our studies.

The higher induction levels obtained in MeJa treated plants could be explained not only because jasmonates are powerful elicitors of defensive proteins (*30*) but also because these plants were exposed to MeJa vapors during the whole experiment. However, wounds caused mechanically or by caterpillars were restricted to the first 4 h of the experiment (see Materials and Methods). Nevertheless, these results suggest that the wound response in passion fruit triggered by mechanical wounding or herbivore attack might be mediated by an octadecanoid-based signaling pathway, as MeJa treatment increased the inhibitory activity against trypsin. The induction of PI expression and/or activity in response to mechanical wounding, herbivory, and/or jasmonates have been reported in hybrid poplar, *S. viminalis*, sweet potato, trembling aspen, and chickpea (*31*−*35*).

Partial Purification and Characterization of the Trypsin Inhibitors. In order to better characterize the induced inhibitors, a partial purification procedure was performed using crude leaf extract from MeJa treated plants. The inhibitory activity was precipitated with ammonium sulfate (saturation level between 20 and 55%). The protein fraction obtained was subjected to gel filtration chromatography on a Sephadex G-100 column. The A_{280} of eluted fractions was determined, and inhibitory activity against trypsin was assayed. As shown in **Figure 2A**, the inhibitory activity eluted as one peak. SDS−PAGE analysis revealed that this fraction was basically constituted by 3 major proteins ranging from 20 to 25 kDa (**Figure 2B**). Moreover, the difference between the protein profile of the crude extract and the enriched fraction (**Figure 2B**) indicates the efficiency of gel filtration chromatography to partially purify the passion fruit TIs. The inhibitory activity against trypsin of the partially purified proteins was studied using the gel-X-ray film contact print technique (*26, 36*). For that, a sample was prepared in semidenaturing conditions (see Materials and Methods), and after the contact time of the gel to the X-ray film had elapsed, four TI bands were detected (**Figure 3**). These inhibitors were named according to their increased mobility as follows: TI 1, TI 2, TI 3, and TI 4. The size of the stronger ones (TIs 2, 3, and 4) correlated well with the 3 major bands identified by SDS−PAGE analysis (**Figure 2B**), while the TI 1 band with ~35 kDa was better detected after a longer contact period (15 min). It is important to note that due to the semidenaturing condition and several incubation periods (see Materials and Methods), the protein migration pattern in **Figure 3** is slightly different from that in **Figure 2B**. However, concerning the TI 1 band, a protein band stained with coomassie blue that could account for its activity was not identified (**Figure 2B**). The visualization of the TI 1 band can be explained by the high sensitivity of activity detection since it can reveal inhibitors that are bellow the Coomassie blue detection range (*37*). These results reinforce the hypothesis that the partially purified proteins (**Figure 3**) are trypsin inhibitors and that on the basis of size range they could be Kunitz-type inhibitors.

To further characterize these inhibitors, the N-terminal sequence of 12 amino acids from two of them (TIs 3 and 4) were obtained from residue #1. The sequence data revealed that both inhibitors not only present the same partial sequence but also display homology with Kunitz-type inhibitors from *T. mammosum*, *T. subincanum*, *T. sylvestre* (*38*), and *P. tremula* (*39*) or with proteins that are similar to Kunitz-type inhibitors: miraculin (from tomato (*40*) and egg-plant) and tobacco tumor-related protein (*41*) (**Table 1**). Some of these proteins were studied in the context of plant stress response. In the case of the inhibitor TI 5 from *P. tremula* that is wound-inducible (*39, 42*), the tobacco tumor-related protein is able to induce hypersensitive response (*41*), while the miraculin from tomato is root- not nematode- and wound-inducible (*40*).

Wound-inducible inhibitors displaying similar size and high sequence identity with each other have been previously documented in different model plants. For example, 6 inhibitors from potato inhibitor II family in tobacco (*43*), 2 inhibitors from Kunitz inhibitor family in trembling aspen (*34*), and 7 inhibitors from the potato inhibitor II family in pepper (*44*).

***In Vitro* Inhibition of Insect Digestive Enzymes Activity by Partially Purified Trypsin Inhibitors.** To address the insecticide potential of the partially purified TIs against lepidopteran insect pest, initially *A. vanillae vanillae* was considered for utilization as a model insect, mainly due to its negative

impact in passion fruit fields. However, midgut extracts from these larvae were strongly pigmented (greenish), compromising the results of the inhibitory assay. In light of such technical difficulties, we chose another insect pest, sugar cane stalk borer (*Diatraea saccharalis*).

D. saccharalis is a major pest that attacks sugar cane, causing important field losses. Such damage is particularly worrying because Brazil is a pioneer in the use of ethanol obtained from this crop as biofuel. **Figure 4** shows that the trypsin-like activity from midgut homogenate obtained from fourth-instar larvae of *D. saccharalis* is strongly inhibited by TI enriched fractions. This result suggests that TIs from passion fruit may have a potential as transgene proteins for use in engineered sugar cane modified for heightened resistance to *D. saccharalis*.

Some plant PIs can also be bifunctional, for example, they can interact with serine protease and α-amylase. With these thoughts in mind, we verified whether the TIs studied exhibit α-amylase inhibitory activity as well. The TI enriched fraction was tested against α-amylase activity from midgut extracts of *D. saccharalis* and from a pancreatic enzyme. For both experiments, no α-amylase inhibitory activity was detected in our experimental conditions (data not shown). Further experiments will be necessary to investigate and evaluate the effect of these inhibitors in different insect pests.

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