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Trypsin inhibitors from *Capsicum baccatum* var. *pendulum* leaves involved in Pepper yellow mosaic virus resistance

M.M. Moulin[1], R. Rodrigues[1], S.F.F. Ribeiro[2], L.S.A. Gonçalves[3], C.S. Bento[1], C.P. Sudré[1], I.M. Vasconcelos[4] and V.M. Gomes[2]

[1]Laboratório de Melhoramento Genético Vegetal, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, RJ, Brasil
[2]Laboratório de Fisiologia e Bioquímica de Microorganismos, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, RJ, Brasil
[3]Laboratório de Melhoramento Genético Vegetal, Universidade Estadual de Londrina, Londrina, PR, Brasil
[4]Laboratório de Toxinas Vegetais, Universidade Federal do Ceará, Fortaleza, CE, Brasil

Corresponding author: M.M. Moulin
E-mail: moniquemoulin@gmail.com

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ABSTRACT. Several plant organs contain proteinase inhibitors, which are produced during normal plant development or are induced upon pathogen attack to suppress the enzymatic activity of phytopathogenic microorganisms. In this study, we examined the presence of proteinase inhibitors, specifically trypsin inhibitors, in the leaf extract of *Capsicum baccatum* var. *pendulum* inoculated with PepYMV (Pepper yellow mosaic virus). Leaf extract from plants with the accession number UENF 1624, which is resistant to PepYMV, was collected at 7 different times (0, 24, 48, 72, 96, 120, and 144 h). Seedlings inoculated with

PepYMV and control seedlings were grown in a growth chamber. Protein extract from leaf samples was partially purified by reversed-phase chromatography using a C2/C18 column. Residual trypsin activity was assayed to detect inhibitors followed by Tricine-SDS-PAGE analysis to determine the N-terminal peptide sequence. Based on trypsin inhibitor assays, trypsin inhibitors are likely constitutively synthesized in *C. baccatum* var. *pendulum* leaf tissue. These inhibitors are likely a defense mechanism for the *C. baccatum* var. *pendulum*-PepYMV pathosystem.

Key words: Biochemical mechanisms; Genetic resistance; Plant-pathogen interaction; Proteinase inhibitor

INTRODUCTION

All living organisms develop different constitutive and inducible mechanisms to defend against pathogens. Numerous compounds that confer resistance to viruses, pathogenic bacteria, and fungi have been identified in plants, including proteins and peptides with antimicrobial activity (Benko-Iseppon et al., 2010). The most widely known proteins involved in plant defense are proteinase inhibitors, lectins, hydroxyproline-rich glycoproteins, and ribosome-inactivating proteins (Macedo et al., 2004; Jamal et al., 2013).

Most plants produce proteinase inhibitors that defend against phytopathogenic organisms and insects. They are the most widely studied in the plant families Solanaceae, Fabaceae, and Poaceae and have been detected in vegetative, reproductive, and storage organs (Macedo et al., 2011).

Proteinase inhibitors are small molecules that inhibit the activity of a pathogen-derived proteinase by binding to and, thus, blocking its active site, suppressing enzymatic activity in phytopathogenic microorganisms. Such inhibitors may be constitutive (*i.e.*, produced during normal plant development) or induced upon a pathogen attack (Joshi et al., 2013).

Among the several groups of proteinase inhibitors, serine proteinase inhibitors are the most well-understood and have been isolated from numerous species, including *Capsicum annuum* (Antcheva et al., 1996; Moura and Ryan, 2001; Tamhane et al., 2005, 2007, 2009; Mishra et al., 2012; Ribeiro et al., 2012, 2013) and *Capsicum chinense* (Dias et al., 2013). Trypsin inhibitors are serine proteinase inhibitors, and high concentrations of such inhibitors are associated with plant resistance to insects, fungi, bacteria, and viruses (Kim et al., 2009, Macedo et al., 2010, Oliva et al., 2011). A successful strategy employing defense proteins in plant breeding requires the investigation of *in vitro* and *in vivo* activity against pathogens, determining the partial or full amino acid sequence, and purifying and characterizing the protein of interest (Oliveira and Macedo, 2011).

Plants have developed a series of resistance mechanisms in response to diseases, including viral infections. Among viral diseases, yellow mosaic, which is caused by the Pepper yellow mosaic virus (PepYMV, *Potyvirus*, Potyviridae), is considered to be the most important viral disease in pepper crops (Moura et al., 2011; Lucinda et al., 2012). Symptoms caused by the disease include reduced fruit and plant size, crinkled leaves, and a green-yellowish mosaic. Yellow mosaic significantly increases crop loss in south-central Brazil (Zambolim et al., 2004; Bento et al., 2009).

Improved cultivars that combine genetic resistance to PepYMV, high-quality fruit, and increased yield are the most efficient alternatives to controlling yellow mosaic. Consequently, national breeding programs for *Capsicum* spp have conducted studies to search for resistance sources in germplasm banks (Bento et al., 2009). Genetic resources that are resistant to PepYMV in *Capsicum* have been reported for peppers in Brazil (Nascimento et al., 2007; Gioria et al., 2009; Bento et al., 2009; Gonçalves et al., 2011). However, in the literature, little information is available regarding *Capsicum baccatum* resistance to PepYMV. Bento et al. (2013) showed that PepYMV resistance in a segregating population is due to several genes by crossing the *C. baccatum* accessions UENF 1629 x UENF 1732, which characterized polygenic inheritance for this trait.

Among the few studies related to biochemical defense mechanisms against PepYMV in peppers, Gonçalves et al. (2013) reported an increase in peroxidase expression in *C. baccatum* when it is inoculated with the virus, indicating that pathogenesis-related (PR)-9 is involved in the defense mechanism for such plants. Proteinase inhibitors related to *C. baccatum* defense against PepYMV have not been studied. However, trypsin inhibitor characterization and isolation studies have been performed for other chili pepper species, such as *C. annuum* and *C. chinense* proteinase-inhibitory properties using PR-6 (Ribeiro et al., 2012, 2013; Dias et al., 2013) although no previous plant-pathogen contact was reported in such biochemical studies. Thus, the objective of this study was to detect proteinase inhibitors, specifically trypsin inhibitors, in the leaf extract from *C. baccatum* var. *pendulum* (accession UENF 1624) plants inoculated with PepYMV.

MATERIAL AND METHODS

Plant material

C. baccatum var. *pendulum* plants from accession UENF 1624, which is resistant to PepYMV (Bento et al., 2009), were grown in a growth chamber at 22°C, 80% humidity, and a 16-h photoperiod. After 2 pairs of permanent leaves had developed, 112 seedlings were transferred to 1-L plastic pots containing a mixture of soil and substrate (2:1). After 45 days of cultivation, the plants were inoculated with PepYMV.

Inoculation procedure

Nicotiana debneyi plants infected with PepYMV isolate 3 were used as the inoculum source. The virus isolate was collected from a bell pepper plant in a field in Igarapé, Minas Gerais, and kindly provided by Professor Murilo Zerbini from Universidade Federal de Viçosa (UFV).

C. baccatum seedlings were inoculated using a plant extract buffered in 0.05 M potassium phosphate, pH 7.2, containing 0.01% sodium sulfite and the abrasive carborundum (600 mesh) (Truta et al., 2004). The inoculation procedure was conducted when seedlings had 3 or 4 definitive leaves; the youngest, fully expanded leaves were inoculated. As a control, 14 seedlings were inoculated only with the buffer solution and abrasive.

Control and PepYMV-infected seedlings were grown for 7 different time periods: 0, 24, 48, 72, 96, 120, and 144 h. At each time point, the leaves were collected and the seedlings were weighed; a 10-g sample was collected at each time point and subsequently ground at

78°C with a mortar and pestle in liquid nitrogen. The ground material was used for the protease inhibitor assay.

Protein extraction and quantification

Approximately 1 g fresh ground tissue (inoculated and control) was transferred to 15-mL tubes and immersed in 4 mL buffer containing 10 mM sodium borate and 0.125 M sodium chloride according to Leon et al. (2002) using the modifications described below. Tubes containing the sample in 4 mL buffer but not supplemented with phenylmethylsulfonyl fluoride were incubated in a shaker at 4°C for 3 h. The material was centrifuged at 10,000 *g* for 10 min at 4°C. The precipitate was discarded and the supernatant was collected in 1.5-mL tubes. A solution composed of 14% polyethylene glycol and 8.5% K_2HPO_4 was then added to the supernatant. After 30 min, the 2 phases were separated and the bottom phase containing the soluble proteins was collected. Protein levels were quantified using the bicinchoninic acid protein assay as described by Smith et al. (1985), with modifications; ovalbumin (Sigma, St. Louis, MO, USA) was used as the protein standard.

Reversed-phase high-performance liquid chromatography

Reversed-phase chromatography using a C2/C18 column equilibrated with 0.1% trifluoroacetic acid (TFA) was performed sequentially to isolate proteins with low molecular masses. Total extract obtained after extraction of leaves collected at 120 h (inoculated and control) were solubilized in 0.1% TFA, and 200 µL (150 µL + 50 µL TFA) of this mixture was injected into the column. Chromatography was performed at a flow rate 0.5 mL/min and 33°C in a high-performance liquid chromatography system. The protein was eluted from the column using an increasing acetonitrile (ACN) gradient (0-80%). The column was washed with Solution A (0.1% TFA in ultrapure water) for 10 min; next, a gradient was applied by adding Solution B (80% ACN in 0.1% TFA) for approximately 80 min. The column was washed with 100% Solution B for 60 min. The elution profile was monitored using a diode array detector at an absorbance wavelength of 220 nm. The non-retained peak corresponding to time of 120 h was injected into the same column and the chromatography was developed using the same method described above.

Assay for residual trypsin activity

Proteinase inhibitory activity was determined by measuring residual hydrolytic activity for trypsin using the BA*p*NA substrate (5 mM stock solution). Proteolytic activity was measured by incubating a synthetic peptide derived from *p*-nitroanilide in Tris-HCl buffer (50 mM, pH 8.0) for 30 min at 37°C in 200 µL. Triplicate controls were used for each inoculation time, which only included the BA*p*NA substrate and Tris-HCl buffer. The reaction was stopped by adding 100 µL 30% (v/v) acetic acid, and substrate hydrolysis was monitored using a spectrophotometer to measure *p*-nitroanilide absorbance at 405 nm (adapted from Macedo et al., 2007). The results were expressed as the mean for 3 assays as described by Macedo et al. (2007). The percent trypsin inhibition was calculated for the control enzyme and sample measurements. The plots show the percent inhibition.

Statistical analysis

One-way analysis of variance was performed for statistical analysis. The means were compared using the Student *t*-test with 1% probability.

Tricine gel electrophoresis

Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) was performed following the method described by Schaggner and Vou Jagow (1987). Glass plates (8 x 10 cm and 7 x 10 cm) with 0.5-mm spacers were used. A 17-µL fraction from each sample with a known protein concentration was added to 5.5 µL water, 1.25 µL 5% β-mercaptoethanol, and 6.25 µL sample buffer (10% glycerol, 2.3% SDS, and 0.0625 M Tris-HCl, pH 6.8), boiled for 5 min, centrifuged for 5 min, and loaded onto the gel. The gel was removed from the plates and silver stained using the method described by Morrissey (1981) with the modifications described below.

Silver staining after Tricine-SDS-PAGE

The gel was carefully removed from the glass plates. Next, the gel was incubated for 40 min in 0.26 M 10% acetic acid, 0.96 M ethanol (absolute), and 1.2 M ultrapure water. Next, the gel was washed in ultrapure water for 5 min. Subsequently, the gel was incubated in a solution with 0.48 M 25% glutaraldehyde and 1.2 M ultrapure water for 20 min. The gel was then washed 2 times in ultrapure water for 10 min each and placed in a solution of 1.44 M ethanol (absolute) and 1.2 M ultrapure water for 20 min. The gel was then incubated in a mixture of 1.2 M ethanol (absolute), 0.06 M silver nitrate, 0.3 M ammonium hydroxide, and 4.38 M ultrapure water for 20 min. After incubation, the gel was placed in developing solution composed of 1.2 M ethanol (absolute), 100 µL formaldehyde, 25 µL 2.3 M acetic acid, and 1.2 M ultrapure water until the desired stain intensity was produced (visible bands). To fix the gel, it was immersed in a solution composed of 0.3 M 10% acetic acid, 0.03 M glycerol, and 1.2 M ultrapure water for 10 min.

Amino acid sequence analysis

Peptides were separated using reversed-phase chromatography with a C2/C18 column. The samples obtained were collected and stored at 4°C and later applied for gel electrophoresis. Visualized bands were subjected to amino acid sequence analysis. A Shimadzu PSQ-23A protein sequencer (Shimadzu, Kyoto, Japan) was used to determine the N-terminal sequence of the respective bands.

RESULTS AND DISCUSSION

The residual trypsin activity assay revealed serine protease inhibitors in leaf extract from *C. baccatum* var. *pendulum* collected at different times. Trypsin activity inhibition was observed both in samples inoculated with PepYMV and in the control samples (Figure 1), suggesting that an inhibitor proteinase is present as a constitutive defense. Prasad et al. (2010) indicated that proteinase inhibitors are typically synthesized constitutively during normal

plant development or in response to a pathogen attack. By examining the proteinase inhibitor profile of *Solanum nigrum*, a Solanaceae with phytotherapeutic potential, Hartl et al. (2010) reported high constitutive levels of trypsin inhibitors in both the control and plants exposed to *Spodoptera exigua*. Intrinsic inhibitors from this plant may affect larval pest growth, indicating a potential natural defense mechanism against herbivore insects.

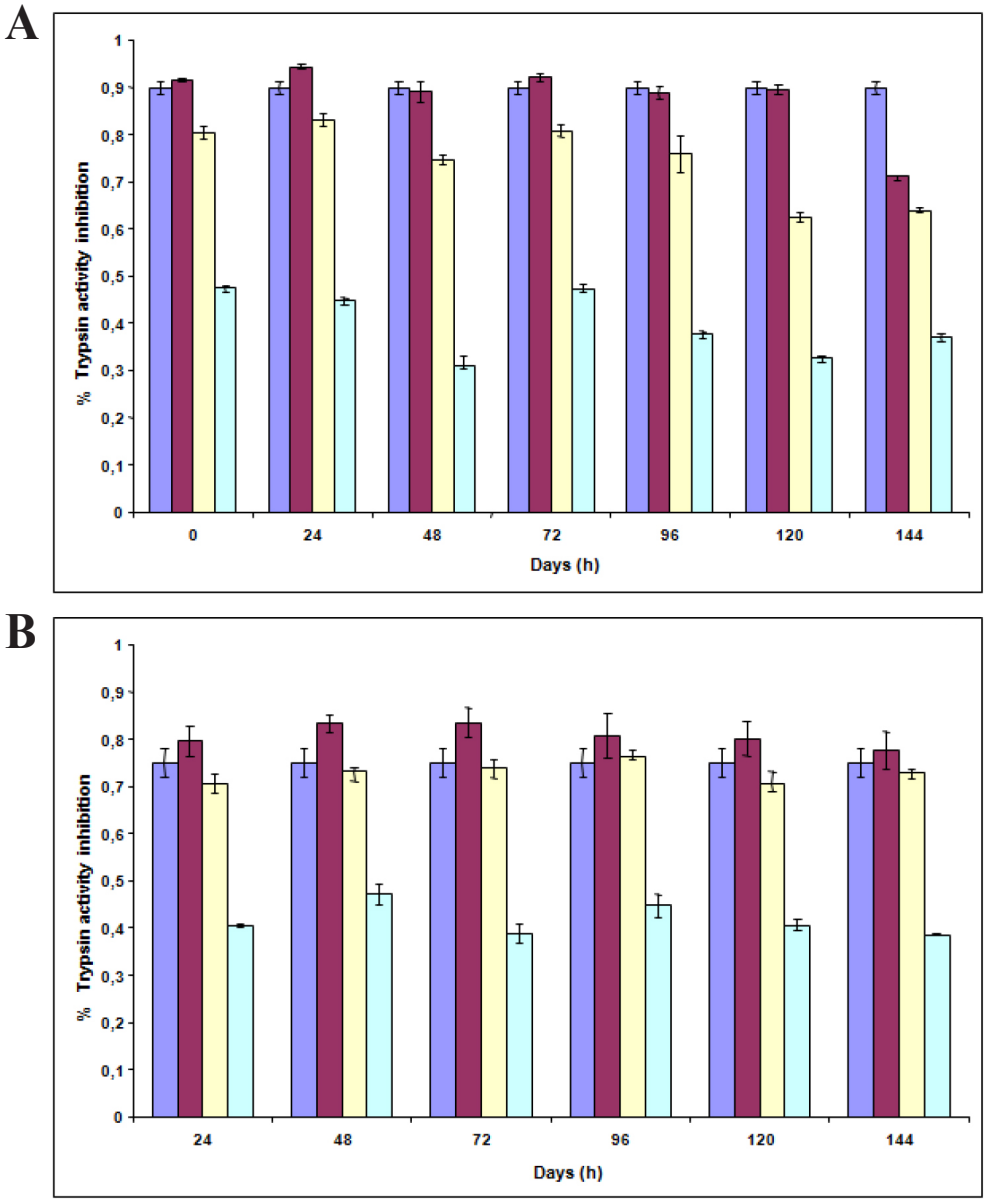

Figure 1. Trypsin activity in plant extracts from *Capsicum baccatum* var. *pendulum*.

Kim et al. (2005) observed antifungal and antibacterial activity for a trypsin inhibitor isolated from *S. tuberosum*. High concentrations of this inhibitor are constitutively expressed for potato tubers (*i.e*., even without pathogen exposure). Different results were reported by Bar-Ziv et al. (2012), who reported that proteinase inhibitor synthesis was induced in tomatoes as a defense mechanism against numerous pathogens, including Tomato yellow leaf curl virus (TYLCV). TYLCV is considered to be the most destructive viral disease for this crop. In the absence of pathogen exposure, the inhibitor level detected was minimal.

Interestingly, in the residual trypsin activity assay, trypsin inhibition increased with increasing leaf extract levels (1, 5, and 25 μL). In the analyses performed by Bariani et al. (2012), trypsin inhibition did not significantly differ for different leaf extract concentrations from *Caesalpinia ferrea* and *Swartzia polyphylla* (1, 9, and 12 μg).

In this study, leaf extract was used for each assay. Few studies have investigated proteinase inhibitors using leaf tissue from *Capsicum* because most studies use the storage organs, particularly seeds. However, the leaves are the most exposed to the environment and thus are the most susceptible organs to pathogen and pest attacks. According to Macedo et al. (2011), most studies on serine protease inhibitors use seed isolates from numerous species, primarily Leguminosae, Cucurbitaceae, Solanaceae, and Graminae. Chevreuil et al. (2011) reported the importance of investigating expression for different trypsin and chymotrypsin inhibitors in leaf tissue to better understand the plant-pathogen interaction.

Both control and PepYMV-inoculated protein extracts from *C. baccatum* var. *pendulum* leaves collected at the different times from the growth chamber were loaded onto Tricine-SDS-PAGE gels. Inoculated and control plants generated the same electrophoretic pattern (Figure 2), supporting the residual trypsin activity assay results and indicating constitutive resistance. Therefore, SDS-PAGE analysis showed similar protein patterns in PepYMV-infected and control plants. Similar results were reported by Gonçalves et al. (2013), who evaluated the SDS-PAGE gel profile for chitinases and lipid transfer proteins in both control and PepYMV-inoculated *C. baccatum* seedlings. These data showed similar protein profiles for the different inoculation times.

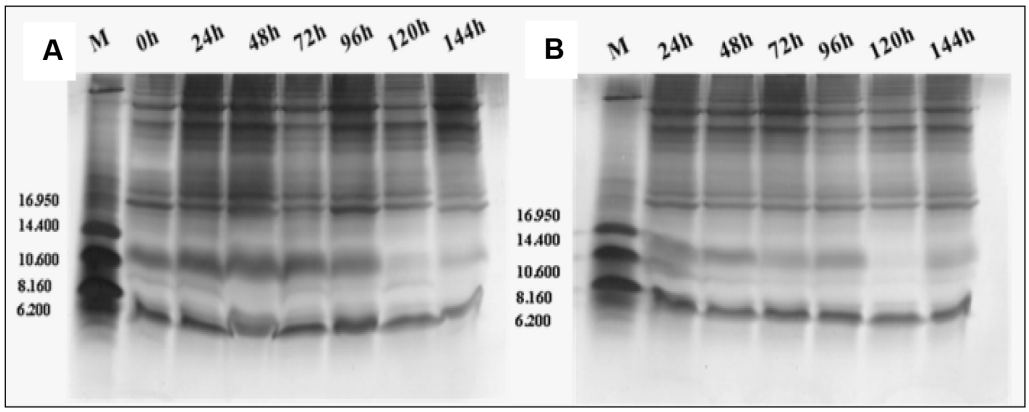

Figure 2. Tricine-SDS-PAGE gel electrophoresis of *Capsicum baccatum* var. *pendulum* leaf extracts at different sampling times.

The Student *t*-test (Figure 3) indicated that trypsin activity inhibition was the same for inoculated and control plants at the 0-h time point. However, inoculated plants showed a lower trypsin inhibition rate compared with the control plants after 24 h. At subsequent times, the enzymatic activity inhibition rates were similar for inoculated and control plants; the rates changed at 144 h for case A (1 µL extract) and at 120 h for B and C (5 and 25 µL, respectively). At the latter time points, the highest enzymatic activity inhibition rate was observed for inoculated plant extracts, suggesting that trypsin inhibitors are involved in the response to PepYMV. This response is consistent with the expectations for the plant-pathogen interaction because the plant requires a particular amount of time to mediate the biochemical defense response. The most obvious enzyme inhibition changes were observed for the last 2 culture time points. Therefore, protein extract from PepYMV-infected and control *C. baccatum* var. *pendulum* plants at the 120-h time point was used in reversed-phase chromatography with the C2/C18 column (Figure 4). This experiment produced a peak, referred to as Cb-R2 for the control extract, and 2 peaks, referred to as Cb-R2' and Cb-R3', for the PepYMV-inoculated extract. In both chromatograms, a non-binding peak with high absorbance at 220 nm was observed (Cb-R1 and Cb-R1') before the ACN gradient began.

Cb-R2, Cb-R2', and Cb-R3' peaks from leaf extracts harvested at the 120-h time point were run on Tricine-SDS-PAGE (Figure 5). The protein profile for Cb-R2 corresponded to the proteins from Cb-R2' and Cb-R3'; thus, the trypsin inhibitor was not present in the latter fractions. Bariani et al. (2012) investigated trypsin inhibitors from *C. ferrea* and *S. polyphylla* protein extracts and also observed 3 different peaks (PI, PII, and PIII) after gel filtration chromatography using a Sephadex G-100 column, which yielded fractions with low trypsin inhibitory activity.

To identify the fraction with trypsin inhibitory activity, the peaks collected before the ACN gradient began were assayed for residual trypsin activity, showing trypsin inhibitor activity in the non-binding fractions (Figure 6). Trypsin inhibition activity was not observed in the 3 binding peaks. Fractions from the non-binding peaks from plants inoculated with PepYMV and control plants were again submitted to chromatography using a C2/C18 column (Figure 7) for protein purification.

Tricine-SDS-PAGE chromatography of non-binding peaks (Figure 8) showed a completely different profile between the binding peak (Cb-2 and Cb-2') and non-binding peak (Cb-1 and Cb-1'), indicating that the non-binding fraction included a trypsin inhibitor. The low-molecular weight bands in the lanes for the non-binding peaks may indicate trypsin inhibitors because the molecular weights corresponded to the gel results.

N-terminal sequences were assessed for the Cb-1 and Cb-1' bands with approximate molecular masses of 10 and 14 kDa from the Tricine-SDS-PAGE gel. The Cb-1 and Cb-1' bands yielded the following sequences: GFPFLLNGPDQDQGDFIMFG and GFKGEQGVPQEMQNEQATIP, respectively. Analysis of the N-terminal amino acid sequence of Cb-1 and Cb-1' bands revealed no sequence homology with any other known protein, even those isolated from plant seeds. Studying *C. chinense*, Dias et al. (2013) isolated a similar inhibitor with molecular masses of 5 and 8 kDa and N-terminal sequences of GICTNCCAGRKGCNYFSAD and QICTNCCAGRKGCNYYSAD, respectively. Ribeiro et al. (2007) isolated a trypsin inhibitor from *C. annuum* with an approximate 6-kDa molecular mass and N-terminal amino acid sequence of SEPRNEPTEISYSVAPSVS.

Macedo et al. (2011) purified, characterized, and determined the partial primary structure for a trypsin inhibitor isolated from Sapindaceae. Through Tricine-SDS-PAGE gel

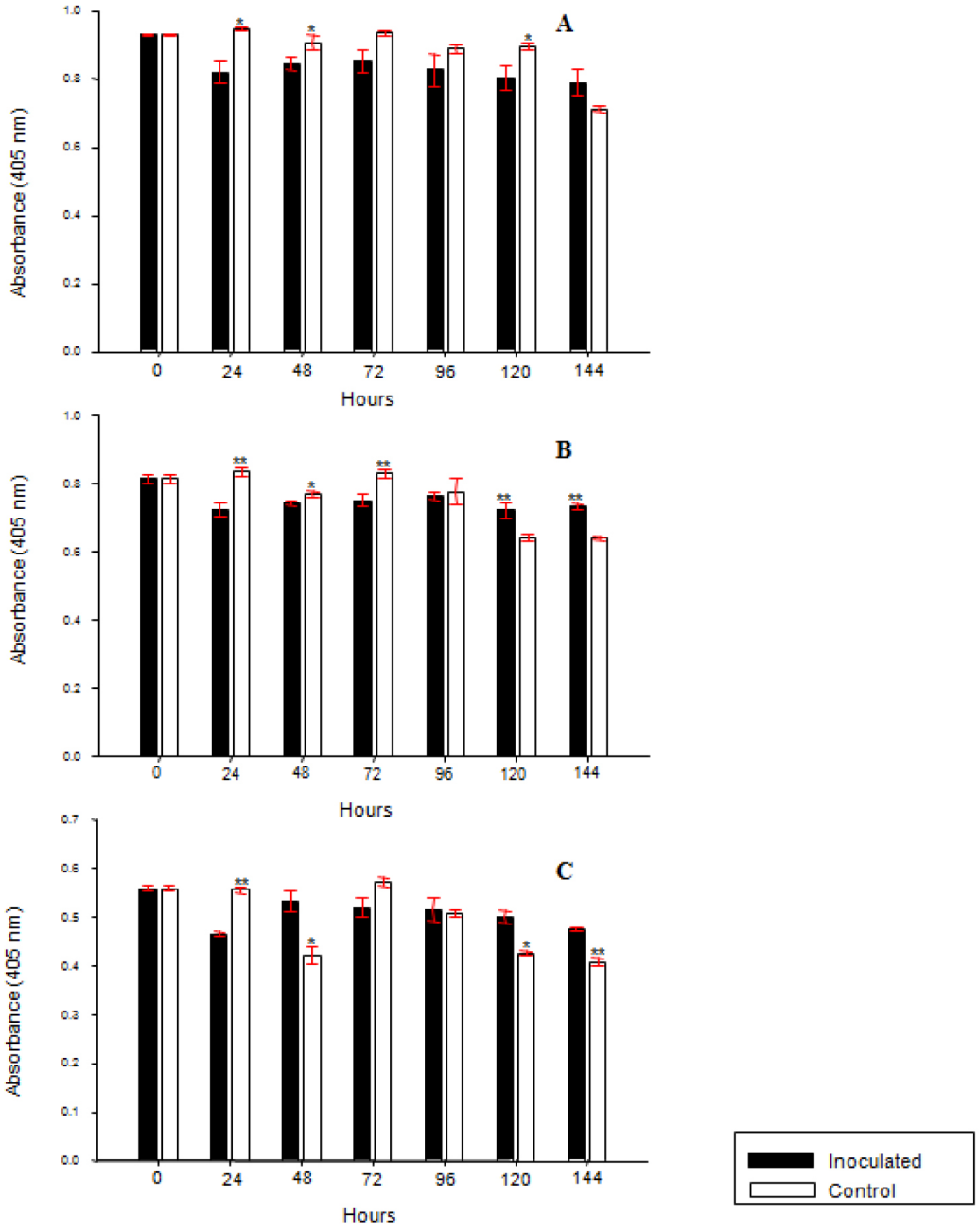

Figure 3. Student *t*-test applied to the 1 and 5% levels to assess inhibition of enzyme activity at different concentrations of plant extracts (1, 5, and 25 µL/mL). *5% probability; **1% probability.

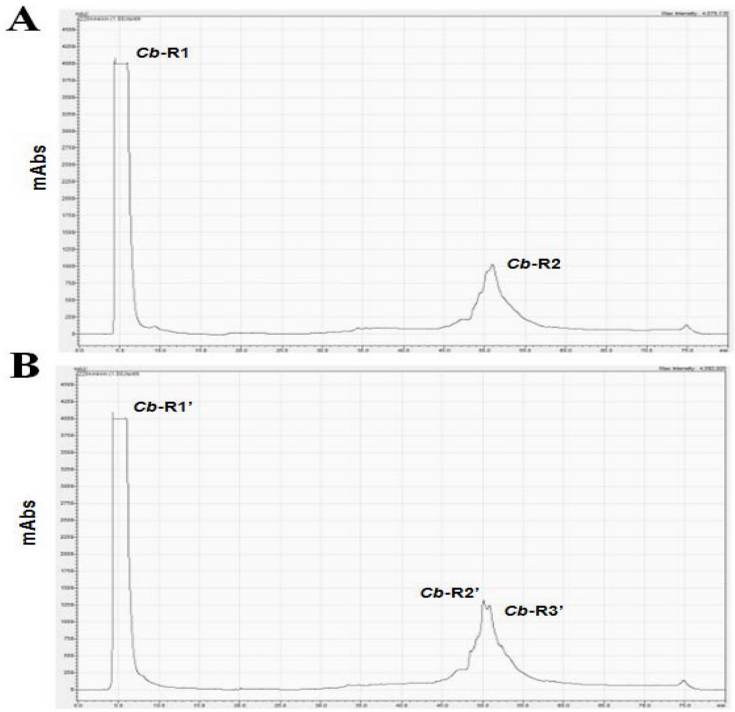

Figure 4. Reverse-phase chromatography in C2/C18 column of total extract obtained from *Capsicum baccatum* var. *pendulum* leaves collected at 120 h after PepYMV inoculation.

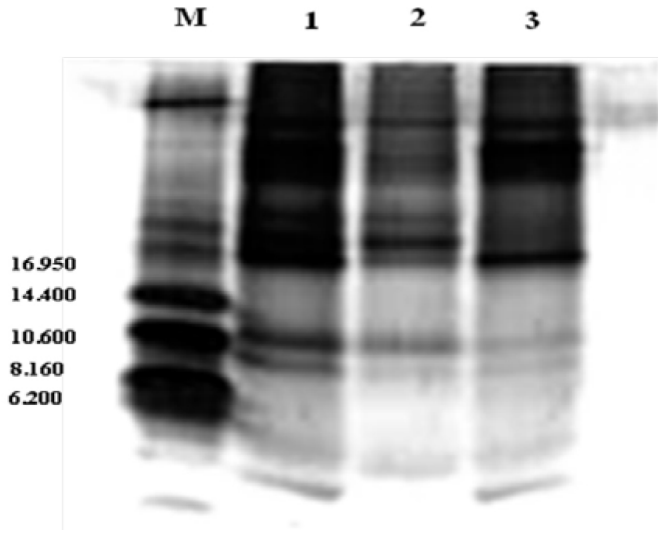

Figure 5. Tricine-SDS-PAGE of fractions obtained from reverse-phase chromatography in C2/C18 column of leaves total extract obtained after 120 h of growth. *Lane M* = marker; *lane 1* = peak Cb-R2 of the uninoculated extract; *lane 2* = peak Cb-R2' of the inoculated extract; *lane 3* = peak Cb-R3' of the inoculated extract.

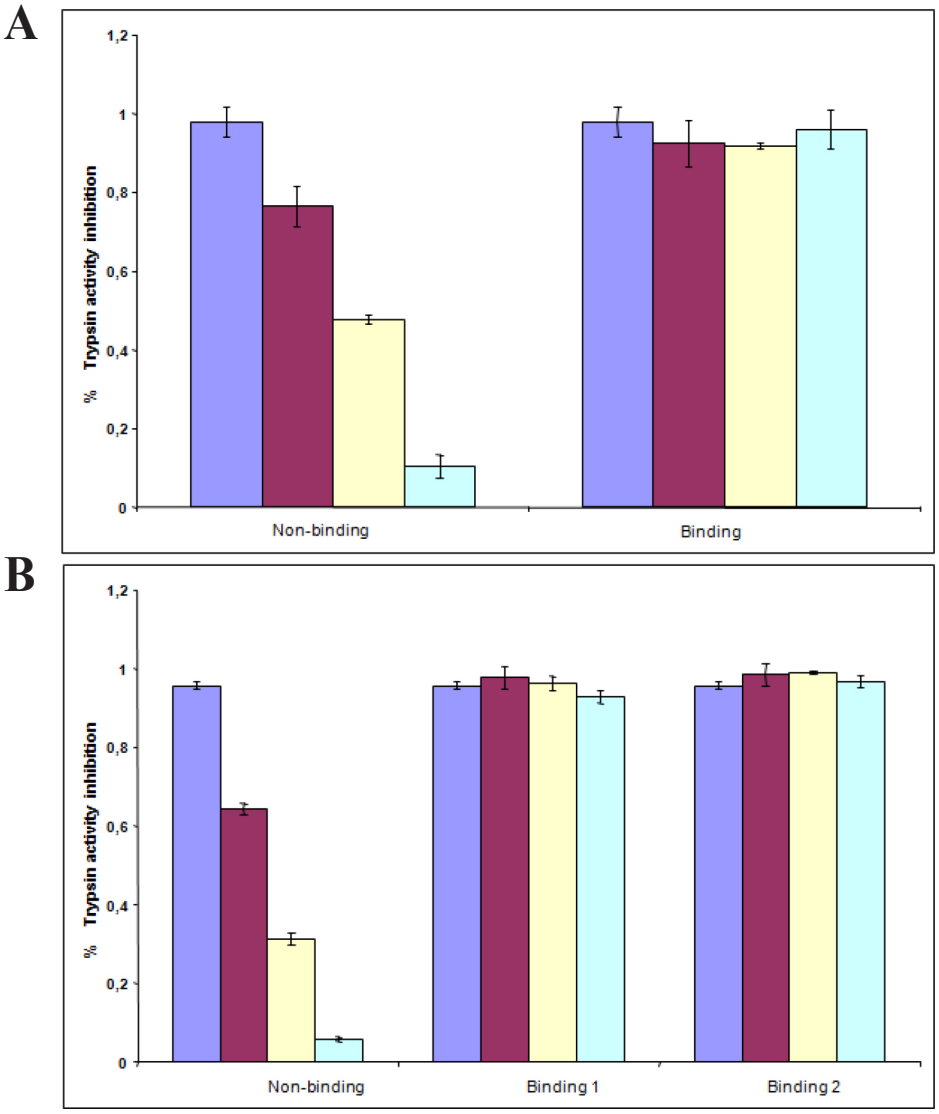

Figure 6. Trypsin activity in plant extracts from *Capsicum baccatum* var. *pendulum* of 120 h after PepYMV inoculation.

analyses, the authors identified a protein with an 18-kDa polypeptide chain. Yang et al. (2006) isolated a protein with a molecular mass 18 kDa from *Psoralea corylifolia* L. and showed, through sequencing the N-terminal region, that this protein was homologous to plant trypsin inhibitors, which also exhibit antifungal activity. Kim et al. (2005) isolated a proteinase inhibitor from *S. tuberosum* with a molecular mass 5.6 kDa.

Two proteinase inhibitors were purified from *C. annuum* leaves (i.e., CapA1 and CapA2) with *in vitro* and *in vivo* inhibitory activity for *Helicoverpa armigera* proteinases. The molecular mass of these inhibitors was 12 kDa and showed inhibitory activity for bovine

trypsin (68-91%) and chymotrypsin (39-85%). Inhibition for pathogen-generated proteinases was estimated to be 60-80% (Tamhane et al., 2005).

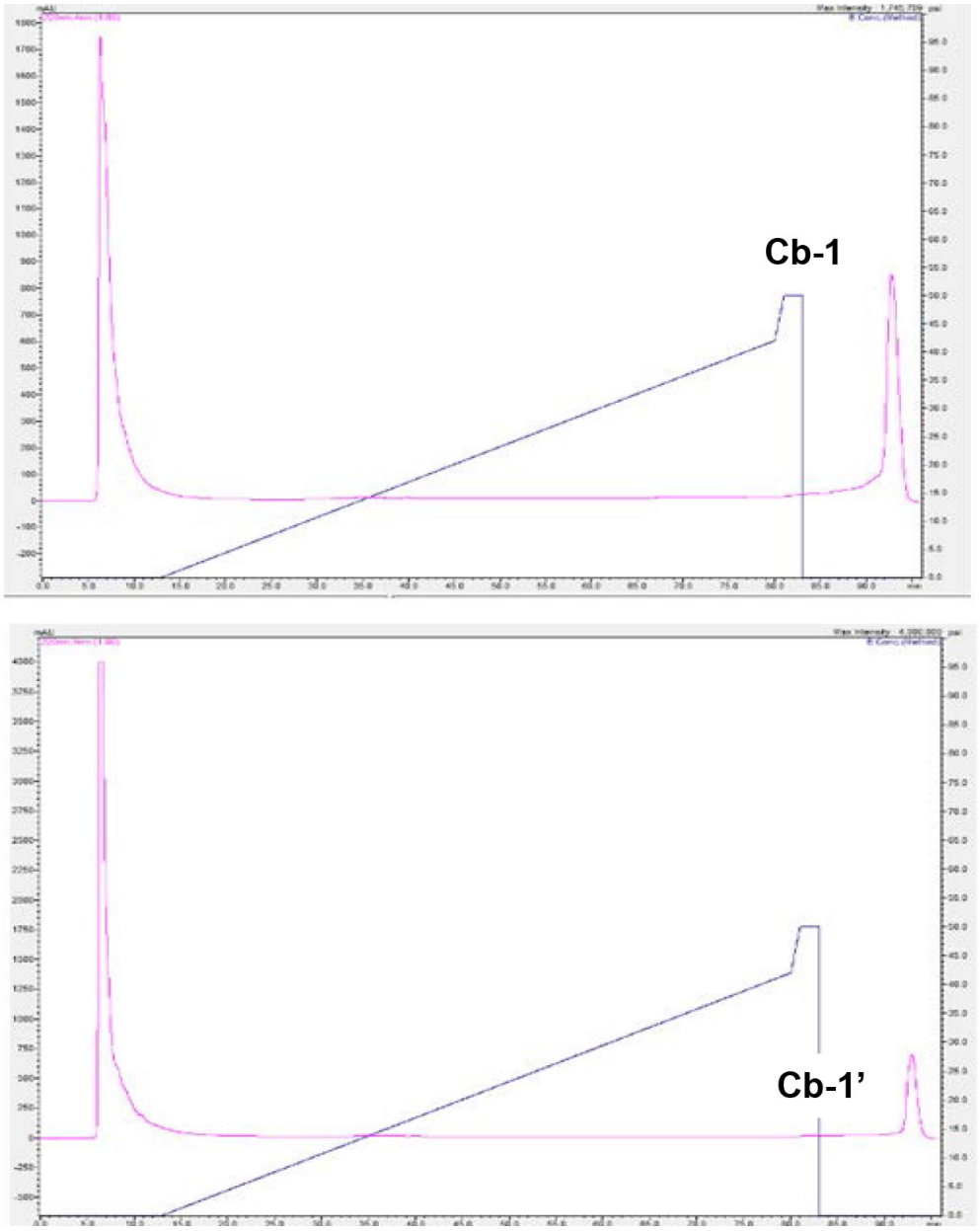

Figure 7. Reverse-phase chromatography in C2/C18 column from the non-retained peaks obtained from *Capsicum baccatum* var. *pendulum* leaves collected at 120 h after PepYMV inoculation.

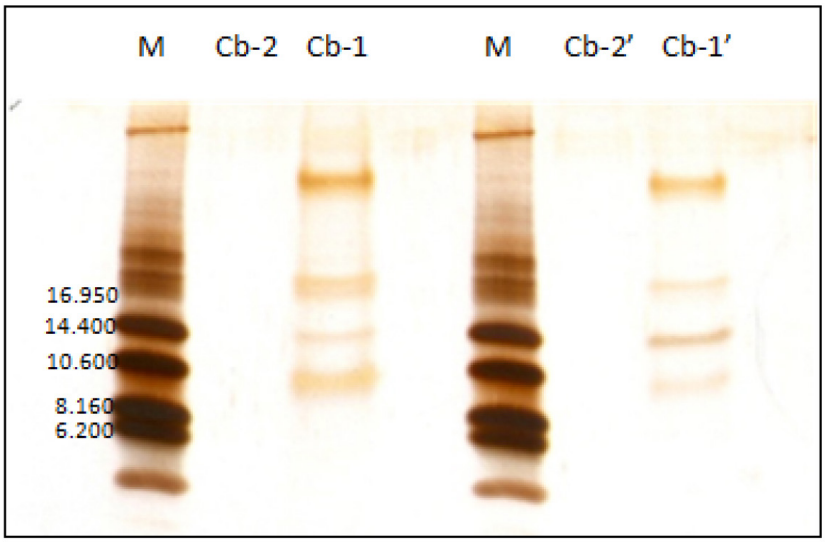

Figure 8. Tricine-SDS-PAGE of fractions obtained from the non-retained peaks time after 120 h of growth. *Lane M* = marker.

Additional studies that have characterized and isolated proteinase inhibitors from the genus *Capsicum* have been reported (Antcheva et al., 1996; Moura and Ryan, 2001; Tamhane et al., 2007, 2009; Mishra et al., 2012). However, a proteinase inhibitor has not been described for *C. baccatum*, which does not possess such inhibitors associated with defense mechanisms for the *C. baccatum*-PepYMV interaction. Thus, this is a pioneer study on the protein patterns associated with such inhibitors.

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