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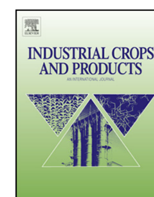

Castor bean cake contains a trypsin inhibitor that displays antifungal activity against *Colletotrichum gloeosporioides* and inhibits the midgut proteases of the dengue mosquito larvae

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ABSTRACT

A novel trypsin inhibitor, named RcTI, was purified from castor bean cake (*Ricinus communis* L.) by heat treatment followed by chromatography on anhydrotrypsin-Sepharose 4B and Resource Q. RcTI is a 14 kDa competitive inhibitor with pI 5.2 and a dissociation constant (Ki) of 1.9×10^{-5} mM. The amino-terminal sequence showed similarity with a 2S sulfur-rich seed storage protein (83%) and napin-like protein (48%). RcTI was stable over a broad pH range and is exceptionally resistant to heating as it retained high inhibitory activity toward trypsin after incubation at 100 °C for 2 h. RcTI (13 μg) inhibited the spore germination of the phytopatogenic fungus *Colletotrichum gloeosporioides* and promoted 91% inhibition of the proteases from the midgut of *Aedes aegypti* larvae. The results of the present study indicate that RcTI has biotechnological potential as an alternative agent to combat the important phytopathogen *C. gloeosporioides* and the larvae of *A. aegypti*.

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1. Introduction

Protease inhibitors are proteins or peptides that form complexes with proteases hindering catalysis (Fan and Wu, 2005). Protease inhibitors are ubiquitously distributed in animals, plants and microorganisms (Katunuma et al., 2003; Ishihara et al., 2006; Bijina et al., 2011) and are classified according to the type of proteases they inhibit; serine protease, cysteine protease, aspartic protease and metalloprotease inhibitors (Laskowski and Kato, 1980; Richardson, 1991). Plant serine proteases inhibitors are grouped in Kunitz, Bowman-Birk, potato I and II and squash families of inhibitors according to their primary and tridimensional structures, molecular masses and disulfide bond contents (Bhattacharyya and Babu, 2009; Rawlings et al., 2010). Additionally, there are 2S albumins (seed storage proteins) that behave as trypsin inhibitors (Mandal et al., 2002; Maleki et al., 2003).

In plant tissues, protease inhibitors, besides being considered storage proteins (Mandal et al., 2002) are associated with regulation and control of endogenous proteases during seed development and germination (Xavier-Filho and Campos, 1889; Richardson, 1991), regulation of programmed cell death (Solomon et al., 1999), response to abiotic stresses (Franco and Melo, 2000) and protection against pathogens and insects (Ryan, 2000; Carrillo et al., 2011).

Many previously studied trypsin inhibitors from plants belong to the Leguminosae, Solanaceae, Cucurbitaceae and Poaceae families (Garcia-Olmedo et al., 1987). However, few protease inhibitors from other families such Rutaceae (Shee and Sharma, 2007; Shee et al., 2007a,b) and Euphorbiaceae (Sritanyarat et al., 2006; Chaudhary et al., 2008) have been purified and characterized.

Within the Euphorbiaceae family, castor bean (*Ricinus communis* L.) is of great socioeconomic importance because its seeds are used mainly for biodiesel production. The oil extraction from castor bean seeds generates a protein-rich by-product known as the castor bean cake, but the presence of toxic and allergenic compounds hinders the use of this residue as a feeding source (Dubois et al., 2013). Nevertheless, a way to add value to the side product castor cake is to discover, purify and characterize bioactive molecules, such as protease inhibitors, that could have applications in agriculture and human health and use as biotechnological tools. To the best of our knowledge there are no reports to date on the biological properties of trypsin inhibitors purified from castor beans.

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In the present study a trypsin inhibitor was purified from castor bean cake and was biochemically characterized and tested against the phytopathogenic fungi *Fusarium oxysporum*, *Rhizoctonia solani* and *Colletotrichum gloeosporioides* and against the midgut proteases from *Aedes aegypti* larvae.

2. Materials and methods

2.1. Materials

Castor bean (*R. communis*) cake was obtained from OLVEQ – Indústria e Comércio de Óleos Vegetais Ltda., Ceara-Brazil. Sepharose 4B and Resource Q were purchased from GE Healthcare. Bovine pancreatic trypsin, Nα-benzoyl-L-arginine p-nitroanilide (BAPNA) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich Ltd. Reagents for SDS-PAGE were purchased from Sigma–Aldrich Ltd. and GE Healthcare. All other chemicals were of analytical grade for biochemical use. The filamentous fungi *F. oxysporum*, *R. solani*, and *C. gloeosporioides* were obtained from the Biochemistry and Molecular Biology Department, Federal University of Ceara, CE, Brazil. *A. aegypti* larvae (3rd stage of development) were obtained from the Department of Biology, Federal University of Ceara, CE, Brazil.

2.2. Purification of a castor bean cake protease inhibitor (RcTI)

Castor bean cake was defatted with 3 volumes (m/v) of petroleum ether. The defatted castor cake was air-dried at room temperature (23 ± 2 °C) until petroleum ether evaporates to obtain a fine dried powder.

A crude extract was obtained by extraction of the defatted cake with 50 mM Tris–HCl, pH 7.5, (1:10, m/v), for 30 min, at 4 °C, followed by centrifugation at 12,000 × g, 30 min, at 4 °C. The supernatant (crude extract) obtained was heated at 100 °C for 30 min and cooled under ice water. To remove coagulated debris, the heat-treated extract was centrifuged at 12,000 × g for 15 min at 4 °C.

The heat-treated extract was subjected to affinity chromatography on anhydrotrypsin-Sepharose 4B column (6.5 × 2.1 cm) pre-equilibrated with 50 mM Tris–HCl, pH 7.5, buffer containing 50 mM galactose (to avoid ricin interaction) and 500 mM NaCl. The retained proteins were eluted with 50 mM glycine–HCl, pH 2.2, containing 500 mM NaCl at 45 mL h^{-1} flow rate. Fractions of 2 mL were collected and read at 280 nm. The fractions containing antitrypsin activity were pooled, dialyzed against distilled water at 4 °C, freeze-dried, and dissolved in 50 mM Tris–HCl, pH 8.5. This sample was applied to a Resource Q column pre-equilibrated with 50 mM Tris–HCl, pH 8.5. The column was washed extensively with the equilibration buffer to remove unbound proteins and those retained were desorbed by stepwise elution with increasing concentrations of NaCl (25, 50, 100, 200, 400, 500 and 1000 mM), prepared in 50 mM Tris–HCl, pH 8.5. Fractions of 2 mL were collected at 60 mL h^{-1} flow rate, read at 280 nm, analyzed for antitrypsin activity, and the purified inhibitor, named RcTI, subjected to further analysis.

2.3. Protein quantification

Protein contents were determined by the dye-binding method of Bradford (1976), using bovine serum albumin as the standard.

2.4. Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the methodology described by Laemmli (1970) adapted for use in plates (10.5 × 10 cm). The samples were prepared by dilution (1:3, v/v) in sample buffer (62.5 mM Tris–HCl, pH 6.8, containing 1% [m/v] SDS and 0.1% [m/v] bromphenol blue) in the presence or absence of 0.5% 2-β-mercaptoethanol and boiled for 3 min. The samples (20 μg) were loaded on the 3.5% stacking gel and fractionated in the 12.5% separating gel at a constant current of 20 mA per gel using a Hoefer miniVE vertical electrophoresis system (Amersham Pharmacia Biotech, San Francisco CA, USA). Proteins were visualized by incubation of the gel after electrophoresis with Coomassie Brilliant Blue G-250 (Candiano et al., 2004). Phosphorylase B (97.0 kDa), bovine serum albumin (67.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (29.0 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa) were used as molecular mass markers.

2.5. Assessment of the glycoprotein nature of RcTI

The presence of covalently bound carbohydrate to the RcTI structure was assessed after SDS–PAGE and specific staining by the periodic acid-Schiff (PAS) method (Kapitany and Zebrowski, 1973). SDS–PAGE (Laemmli, 1970) of RcTI (10 μg) was carried out using the Hoefer SE 600 Series electrophoresis unit (GE Healthcare). Fetuin (10 μg), a glycoprotein, and soybean trypsin inhibitor (SBTI) (10 μg) were used as positive and negative control, respectively. The proteins were loaded (10 μg) concomitantly on the gel and run as above. After electrophoresis, the gel was immersed in a fixing solution of 12% (v/v) trichloroacetic acid (TCA) for 30 min, transferred and kept incubated for 60 min in 1% (v/v) periodic acid and stained with the Schiff's reagent for 50 min in the dark. The gel was washed repeatedly with a solution of 0.5% (m/v) sodium metabisulphite and documented using a digital camera.

2.6. Amino-terminal sequencing

This was done in a Shimadzu Co® PPSQ-10 Automated Protein Sequencer performing Edman degradation. Phenylthiohydantoin amino acid derivatives were detected at 269 nm after separation on a RP-HPLC C18 column (4.6 × 2.5 mm) eluted at isocratic conditions according to the supplier's instructions. The amino-terminal sequence of RcTI was subjected to search for similar sequences in the database of the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) using the BLASTP search tool (Altschul et al., 1990). Those with the highest percentage of identity were selected and aligned using the ClustalW program.

2.7. Two-dimensional gel electrophoresis

To determine the isoelectric point and confirm the molecular mass of RcTI, 45 μg were solubilized in 250 μl of rehydration buffer (8 × 103 mM urea, 1.0 × 103 mM thiourea, 10% [v/v] glycerol, 2% [m/v] CHAPS, IPG buffer pH 3–10 and 0.001% [m/v] bromophenol blue) and placed into contact with immobilized pH gradient polyacrylamide gel strip (IPG), 13 cm, pH 3–10 (GE Healthcare) for 12 h. Isoelectric focusing (IEF) was performed in Ettan IPGphor-II system (Amersham Bioscience®) using the schedule: 200 V for 1 h; 500 V for 1 h, 1000 V for 1 h and a gradient from 4000 V up to 18,000 V/h total, exposed to 50 μA continuous electric current, at 20 °C. After IEF, the strip was incubated in the equilibrium and reducing solution (50 mM Tris–HCl, pH 8.8, containing 30% [v/v] glycerol, 6 × 103 mM urea, 2% [m/v] SDS, 2% [m/v] DTT, 0.1% [m/v] bromophenol blue) for 15 min under gentle stirring. Next, the strip was washed with an alkylation solution (50 mM Tris–HCl, pH 8.8, containing 30% [v/v] glycerol, 6 × 103 mM urea, 2% [m/v] SDS, 2.5% [m/v] iodoacetamide, 0.1% [m/v] bromophenol blue) for 15 min under gentle orbital shaking. For electrophoresis in the second dimension, the strip was placed on the top of a 12.5% polyacrylamide gel that was submitted to 30 mA constant current and 250 V as maximum voltage. This procedure was performed on a

vertical electrophoresis system (Hoefer SE 600 Ruby, Amersham Bioscience®). After the run, the gel was staining with Coomassie Brilliant Blue G-250 (Candiano et al., 2004), washed and incubated in Milli-Q grade water. The gel image was acquired using a scanner (HP-1200, 600 dpi) and analyzed by Marter II software (Amersham Bioscience®).

2.8. Inhibitory activity

Trypsin inhibitory activity was measured according to Erlanger et al., (1961) using BAPNA as substrate. Trypsin (2.25 μg) solubilized in 1 mM HCl (15 μL) was pre-incubated with 50 mM Tris–HCl, pH 7.5, containing 20 mM $CaCl_2$ (685 μL) and 100 μL of the sample for 10 min at 37 °C. The reaction was started by addition of 500 μL of 1.25 mM BAPNA solution (previously solubilized in dimethyl sulfoxide and diluted with 50 mM Tris–HCl buffer, pH 7.5). After 15 min at 37 °C, the reaction was stopped by addition of 120 μL of 30% (v/v) acetic acid solution. The enzymatic hydrolysis of BAPNA was evaluated by recording the absorbance at 410 nm. One inhibitory activity was defined as a decrease in 0.01 absorbance per 15 min at 37 °C.

2.9. Kinetic analyses

To determine both the inhibition mechanism and dissociation constant (Ki) of RcTI, the Lineweaver–Burk double-reciprocal and Dixon plots were used, respectively. Assays were performed using increasing RcTI (14.3, 21.4, 28.6 and 31.7×10^{-5} mM) and BAPNA (0.5, 0.75, 1.0, 1.25 and 2.0 mM) concentrations, but with 0.15 mg mL^{-1} fixed bovine trypsin concentration. Reaction was performed as previously described. The reciprocal of the rate of the enzyme reaction was expressed as 1/v (OD 410 h^{-1} mL^{-1}) and the Ki value was calculated from the intersection of two lines plotted for two different BAPNA concentrations (0.5 and 1.25 mM) using the Dixon plot (Dixon, 1953). The Lineweaver–Burk plot was obtained by the reciprocal of the rate of the enzyme reaction (1/v) vs the reciprocal of substrate concentrations (1/S), in the absence and presence of RcTI.

2.10. Thermal and pH stability of RcTI

To assess thermal stability, a 500 μL aliquot of a 20 μg mL^{-1} RcTI solution in 50 mM Tris–HCl (pH 7.6) were incubated at 100 °C (boiling temperature) for 10, 20, 30, 40, 50, 60, 90 and 120 min in a water bath. After incubation, samples were cooled to 4 °C. Subsequently, 100 μL (2 μg) of the heat-treated RcTI solutions were assayed for remaining anti-tryptic activity (Erlanger et al., 1961), performed in triplicate, as described above.

For pH stability, RcTI solutions (20 μg mL^{-1}) were prepared in 50 mM buffers at various pHs: glycine–HCl, pH 2 and 3; sodium acetate, pH 4 and 5; potassium phosphate, pH 6; Tris–HCl, pH 7 and 8; and glycine–NaOH, pH 9, 10, and 11. After incubation for 60 min at 37 °C, the inhibition assay against trypsin was performed as previously described (Erlanger et al., 1961), in triplicate, using 100 μL of RcTI at 20 μg mL^{-1} concentration.

2.11. Effect of RcTI on the spore germination and vegetative growth of filamentous fungi

The filamentous fungi *F. oxysporum*, *R. solani* and *C. gloeosporioides* were grown in Petri dishes containing potato dextrose agar for 15 days at room temperature (24 ± 2 °C). Spores were obtained by gently rinsing with sterile water and scraping the surface of 15-day-old sporulated cultures with a triangular Drigalsky rod, and remaining hyphae were removed by filtering through cheesecloth under sterile conditions. The spore suspension was counted using a Neubauer chamber with the aid of an optical microscope (Olympus BX 60 Microscope System) and adjusted to 2.0×10^5 spores mL^{-1}. The inhibitory effect on fungus germination was evaluated in contact plates, where 10 μL of the spore suspension and 10 μL (13 μg protein) of RcTI were incubated. Sterile water and hydrogen peroxide (100 mM) were used as negative and positive controls, respectively. The contact plates were maintained at 22 ± 2 °C in the dark, for 24 h, inside a box under near 100% relative humidity maintained by sterile water–saturated filter papers. The plates were analyzed by light microscopy (Olympus BX 60 Microscope System) and spores were considered germinated when the length of the germ-tube was twice the spore diameter (Paul et al., 1992).

To evaluate the activity of RcTI on the vegetative growth of the studied fungi the spore suspension was incubated for 12 h with 100 μL of yeast potato dextrose in 96-well microtiter plates, at 37 °C, in the absence of light, to allow spore germination. Then, 100 μL RcTI solution (1 mg mL^{-1} in ultrapure water), previously filtered through a Millex GV (0.22 μm) filter, were incubated with each fungus tested. Optical readings at 630 nm were taken at zero time and every 12 h up to 96 h after incubation. Sterile water and 100 mM hydrogen peroxide were used as negative and positive controls, respectively. All experiments were carried out in triplicate.

2.12. Effect of RcTI on the midgut proteases of *A. aegypti* larvae

2.12.1. Larval gut proteases

A. aegypti larvae were maintained at 27 ± 2 °C, 70 ± 10% relative humidity, 12-h light and 12-h dark photoperiod and fed a diet for turtles (ReptoLife Alcon Club®). To obtain larval gut proteases, third instar larvae were immobilized on ice, the midguts removed with the use of tweezers under a stereomicroscope and homogenized in an ice bath for about 5 min in 50 mM Tris–HCl, pH 7.5. Subsequently, the homogenate was centrifuged at 12,000 × g for 30 min at 4 °C, and the supernatant obtained used immediately as a source of proteases for inhibition assays.

2.12.2. Inhibition assay of *A. aegypti* larval gut proteases

This assay was conducted according to Erlanger et al., (1961). The intestinal larvae homogenate (60 μL) prepared in 50 mM Tris–HCl, pH 7.5, buffer and 100 μL of RcTI (100 μg mL^{-1}) were incubated and evaluated for inhibition in comparison with the larval homogenate incubated with the buffer in the absence of RcTI. Soybean trypsin inhibitor (SBTI) was used as reference standard at the same RcTI concentration (100 μg mL^{-1}).

3. Results and discussion

3.1. Purification of RcTI

The crude extract of castor bean cake prepared with 50 mM Tris–HCl buffer, pH 7.5, was subjected to heat treatment at 100 °C toward eliminating unwanted proteins based on the known thermostability of protease inhibitors (Fang et al., 2010; Klomklao et al., 2010, 2011). Such procedure depleted labile proteins resulting in a high specific trypsin inhibitory activity in the heat-treated extract (Table 1). Heat treatment as a purification step is usually employed when the protein of interest is thermostable. Indeed, the first step for purification of the *Vigna angularis* trypsin inhibitor was the treatment of the crude extract at 90 °C for 10 min, resulting in a six-fold inhibitory specific activity against trypsin over that of the crude extract (Klomklao et al., 2010). Likewise, heat treatment was also employed for purification of the *Vigna radiata* trypsin inhibitor, which led to 3-times increase in the specific trypsin inhibitory activity compared to that of the starting protein extract (Klomklao et al., 2011).

Table 1
Purification of RcTI.

Purification steps	Total activity (IU)	Protein (mg)	Specific activity (IU/mg)	Purification (fold)	Yield (%)
Crude extract	214293.3	392	546.7	1	100
Heat-treated extract	121589.4	210.1	578.7	1.1	53.6
Anhydrotrypsin-Sepharose 4B fraction (AII)	18919.11	0.8	23648.9	43.3	0.2
Ion exchange fraction (RcTI)	11980	0.45	26622.2	48.7	0.1

Affinity chromatography of the heat-treated (100 °C, 30 min) castor bean cake crude extract on anhydrotrypsin-Sepharose 4B column (Fig. 1A) gave rise to a retained protein fraction (AII). This affinity chromatography is especially advantageous because it is highly selective. Therefore, various protocols for purification of trypsin inhibitors include the use of affinity columns where trypsin is covalently attached (Macedo et al., 2003; Oliveira et al., 2007, 2012; Yoshizaki et al., 2007). However, according to Macedo et al., (2003), the possibility of breakage during chromatography of these inhibitors should not be neglected as trypsin immobilized on the matrix is catalytically active. Consequently, trypsin inhibitors can be proteolytically modified at the enzyme reactive site during chromatography. For this reason inactive anhydrotrypsin coupled to Sepharose 4B matrix was chosen in this present study for RcTI purification.

The AII fraction was subjected to anion exchange chromatography on a Resource Q column (Fig. 1B) and the unbound protein fraction, named RcTI, showed a single band after SDS–PAGE under reducing (not shown) and non-reducing conditions (Fig. 1B, inset), corresponding to a molecular mass of approximately 14 kDa compatible with the majority of the protease inhibitors described, which have low molecular mass (Macedo et al., 2000; Klomklao et al., 2010, 2011). RcTI represented 0.1% of the crude extract proteins and showed inhibitory specific activity of 26622.2 IU/mg (Table 1).

3.2. Assessment of the glycoprotein nature and amino-terminal analysis of RcTI

RcTI has no covalently linked carbohydrate to its structure and, therefore, it is not a glycoprotein (Fig. 1B, inset). However, there are reports of trypsin inhibitors with covalently linked carbohydrates to their structures. For example, the trypsin inhibitor from *Acacia victoriae* (AvTI) has a glycosylation degree of 2.06% (Ee et al., 2011). The protease inhibitors of *Echinodorus paniculatus* (Paiva et al., 2003) and *Peltophorum dubium* (Macedo et al., 2003) are all glycosylated proteins.

An N-terminal sequence of 32 amino acid residues was achieved for RcTI by automated sequencing (Fig. 2). The comparative analysis with other proteins deposited in databases of National Center for Biotecnology Information (NCBI) (www.ncbi.nlm.nih.gov) showed that the amino-terminal sequence of RcTI has 83% similarity with an internal sequence of a 2S sulfur-rich seed storage protein from *R. communis* and 48% with an internal sequence of a napin-like protein (2S albumin) from *R. communis*. Some protease inhibitors are members of the storage protein families as, for example, the trypsin inhibitor purified from *Brassica juncea* (BjTI) (Mandal et al., 2002), and Ara h 2, a peanut allergen protein (Maleki et al., 2003).

3.3. Isoeletric point of RcTI

Two-dimensional electrophoresis analysis of RcTI revealed the presence of a protein spot of 14 kDa molecular mass and isoelectric point (pI) of 5.2 (Fig. 3A). This result is consistent with the majority of the previously studied trypsin inhibitors. The four isoforms of a trypsin inhibitor from *Archidendron ellipticum* seed have isoelectric points of 4.10, 4.55, 5.27 and 5.65 (Bhattacharyya et al., 2006). Likewise, the trypsin inhibitor of *Calliandra selloi* has pI 4.00 (Yoshizaki et al., 2007).

After pI determination it was raised the question why RcTI did not bound to Resource Q, an anionic matrix (Fig. 1B), equilibrated with 50 mM Tris–HCl, pH 8.5, buffer, when the protein fraction AII was loaded in the column (Fig. 1B). To clarify this issue, after purified, RcTI was rechromatographed on Resource Q under the same above conditions employed for its purification. Fig. 3B shows that RcTI in its purified form was able to interact with Resource Q. However, when applied on Resource Q as a mixture with other castor bean cake proteins as in AII (Fig. 1A) RcTI did not bind to the matrix (Fig. 1B). A plausible explanation for this result is the presence of other more negatively charged and abundant proteins in AII that

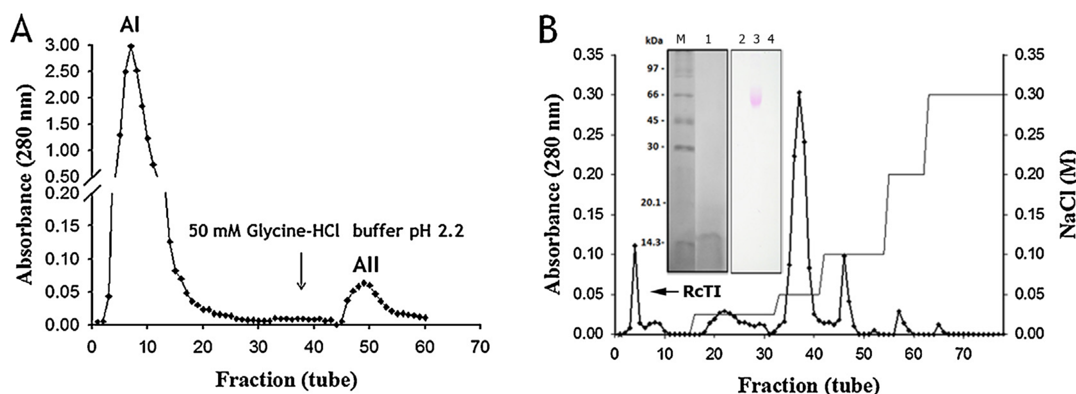

Fig. 1. Chromatographic steps for purification of RcTI. (A) Elution of the heat-treated *R. communis* seed cake extract from anhydrotrypsin-Sepharose 4B column (6.5 × 2.1 cm) previously equilibrated with 50 mM Tris–HCl, pH 7.5, buffer, containing 50 mM galactose. The retained proteins (AII) were eluted with 50 mM glycine–HCl, pH 2.2, containing 500 mM NaCl. Fractions of 2 mL were collected at 45 ml h^{-1} flow rate. (B) The AII fraction obtained was loaded on a Resource Q column (1 mL) equilibrated and initially eluted with 50 mM Tris–HCl, pH 8.5, to yield the purified trypsin inhibitor (RcTI). The bound proteins were desorbed by stepwise elution with increasing concentrations of NaCl (25, 50, 100, 200, 400, 500, and 1000 mM), prepared in 50 mM Tris–HCl, pH 8.5. Fractions of 2 mL were collected at 60 ml h^{-1} flow rate. In both chromatographic steps protein readings were taken at 280 nm. Insert: SDS–PAGE profile: M, molecular mass standards; 1 and 2, RcTI; 3, Fetuin (positive control); 4, SBTI (negative control). M and 1 were stained with coomassie brilliant blue and 2–4 stained by the periodic acid–Schiff method. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

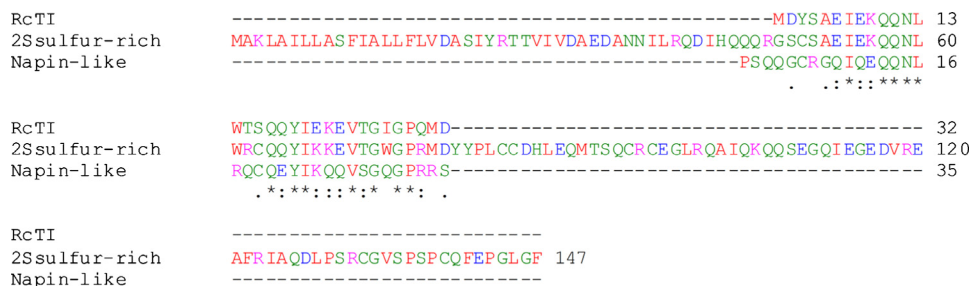

Fig. 2. Alignment of the amino-terminal sequence of RcTI with other 2S albumins from other plant species using ClustalW program. The accession numbers of the sequences used in the comparative analysis are: XP_002522856.1 (2S sulfur-rich seed storage protein) and AAB50870.1 (napin-like protein), both of *R. communis*. (*) Indicates identical amino acids; (.) Indicates semi-conserved amino acids; (:) indicates conserved amino acids. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

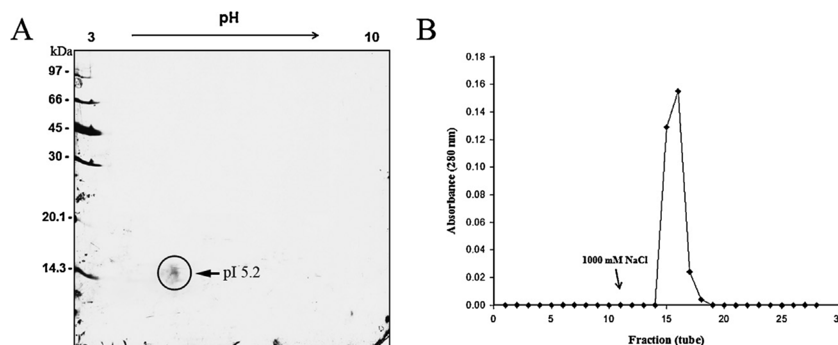

Fig. 3. (A) Two-dimensional electrophoresis of RcTI for determination of pI. The molecular mass markers used are indicated in the left of the polyacrylamide gel (12.5%). The sample was subjected to isoelectric focusing in polyacrylamide gel strips of immobilized pH gradient, in the range of 3–10. (B) Chromatography of the purified RcTI (190 μg 2 mL^{-1}) on Resource Q. The column was previously equilibrated with 50 mM Tris–HCl, pH 8.5. RcTI was eluted with 1000 mM NaCl.

impede RcTI of binding to Resource Q as, in its purified form, RcTI did interact with Resource Q under the above mentioned conditions (Fig. 3B).

3.4. Inhibition mechanism and Ki determination

The Lineweaver–Burk (Fig. 4A) and Dixon (Fig 4B) diagrams revealed that RcTI is a competitive inhibitor with a Ki value for trypsin of 1.9×10^{-5} mM. A much lower Ki value (1.7×10^{-8} mM) was found for a competitive trypsin inhibitor purified from the seeds of *Putranjiva roxburghii* (Chaudhary et al., 2008), a species belonging to the Euphorbiaceae family as *R. communis*. However, the Ki value of RcTI is similar to that (1.5×10^{-5} mM) of a seed trypsin inhibitor from *Piptadenia moniliformis* (Cruz et al., 2013) and lower compared with that (22.1×10^{-5} mM) of a trypsin inhibitor purified from *C. selloi* (Yoshizaki et al., 2007), both plant species of the Fabaceae family. Nevertheless, RcTI has strong affinity for trypsin.

3.5. Stability of inhibitory activity

RcTI remained stable even after heating at 100 °C for 2 h, as its initial inhibitory activity decreased only 7% after treatment (Fig. 5A). Similarly, the trypsin inhibitor of *V. radiata* lost only about 5% of the trypsin inhibitory activity after heating at 90 °C for 1 h (Klomklao et al., 2011). On the other hand, trypsin inhibitors from beans, groundnuts and cereals are sensitive to heat treatment (Liener and Kakade, 1969). Moreover, Vasconcelos et al., (1997) found that the trypsin inhibitors from some Brazilian soybean cultivars (Bays, BR-10, Rio Balsas, Serido and Tropical) are completely eliminated after heat treatment at 92 °C for 5 min. Differences in heat sensitivity observed between protease inhibitors from different origins might be related to structural aspects, such as variation

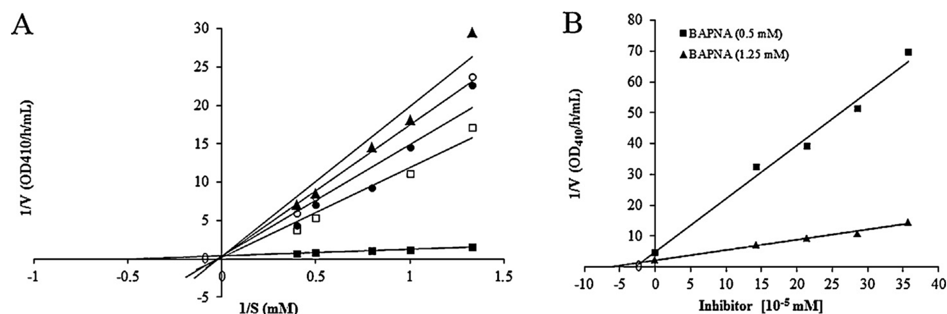

Fig. 4. Kinetic analysis of RcTI. (A) Lineweaver–Burk plot of inhibition of the bovine trypsin with increasing concentrations of RcTI (■, without inhibitor; □, RcTI at 14.3×10^{-5} mM; ●, RcTI at 21.4×10^{-5} mM; ○, RcTI at 28.6×10^{-5} mM; ▲, RcTI at 35.7×10^{-5} mM). (B) Dixon plot for determination of the dissociation constant (Ki) of RcTI at two different concentration of BAPNA (0.5 mM and 1.25 mM).

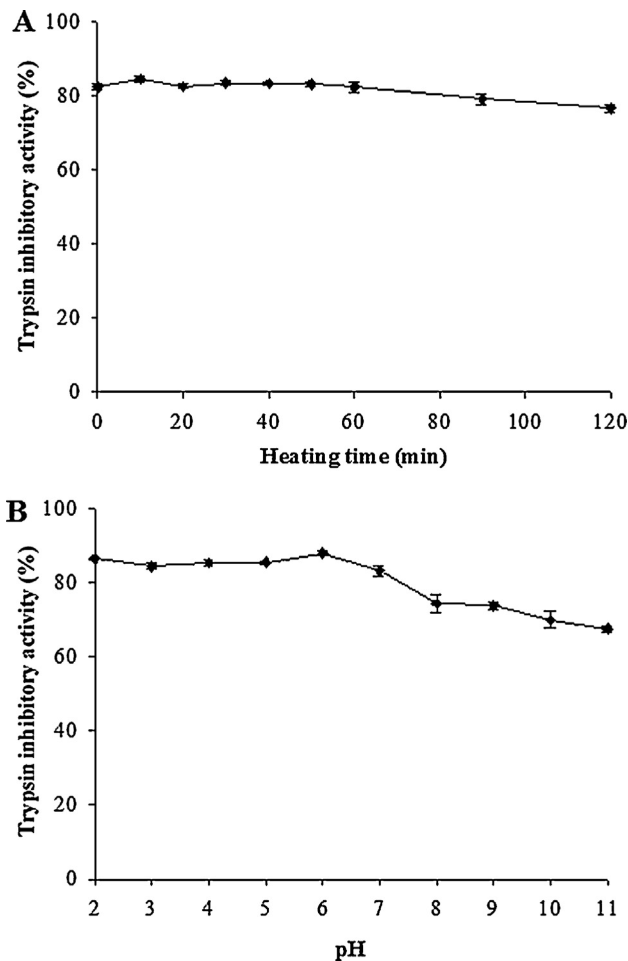

Fig. 5. Thermal (A) and pH stability (B) of RcTI. Bars indicate standard deviation from triplicate determination.

in the number of disulfide bonds and conformation (Klomklao et al., 2011).

RcTI was also stable over a wide pH range (Fig. 5B). However, a 20% loss of inhibitory activity was observed at alkaline pH (pH 8–11). Similar result was reported for the trypsin inhibitor purified from *Pithecellobium dumosum* seeds (PdKI) that lost approximately 20% of the inhibitory activity after incubation in alkaline pHs for 30 min at 37 °C (Oliveira et al., 2007). However the trypsin inhibitors purified from *V. angularis* and *V. radiata* remained stable in the pH range of 4–10 (Klomklao et al., 2010) and 5–10 (Klomklao et al., 2011), respectively, and the Kunitz type trypsin inhibitors purified from Korean large black soybeans (KBTI) and *Entada acacifolia* (Benth.) seeds were stable in the pH range of 3–10 (Fang et al., 2010) and 2–10 (Oliveira et al., 2012), respectively. Variation in the disulfide bond number, molecule conformation or atom arrangement (Klomklao et al., 2011) might explain differences in pH stability observed between these protease inhibitors from distinct plant species.

3.6. Effect of RcTI on the spore germination and vegetative growth of filamentous fungi

RcTI (13 μg per total volume of the assay, ≈46.4 μM) inhibited the spore germination of *C. gloeosporioides*, but did not inhibit those of *F. oxysporum* and *R. solani* (Fig. 6). A trypsin inhibitor purified from corn kernels (at 40 μM) was capable of causing ruptures in *Aspergillus flavus* conidia within 1 h (Chen et al., 1998). At 5 μg mL^{-1} concentration (0.31 μM), the trypsin inhibitor from *Helianthus annuus* flower completely inhibited the *Sclerotinia sclerotiorum* ascospore germination (Giudici et al., 2000). Moreover, the barley trypsin inhibitor (BTI-CMe) inhibited the spore germination of the fungi *Magnaporthe grisea*, *Plectosphaerella cucumerina* and *F. oxysporum* at 0.5, 1.0, and 1.5 μM concentrations, respectively (Carrillo et al., 2011). In general, the effects of protease inhibitors against phytopathogenic fungi are associated with inhibition of fungus proteases needed for growth and differentiation.

RcTI was not effective in inhibiting the vegetative growth of the studied fungi (figures not shown). Chen et al., (1999) reported that the recombinant trypsin inhibitor from corn overexpressed in *Escherichia coli* inhibited both the spore germination and vegetative growth of the fungi *A. flavus*, *Aspergillus parasiticus*, and *Fusarium moniliforme*. However, the effect of this recombinant inhibitor on conidial germination did not correlate with the effect on vegeta-

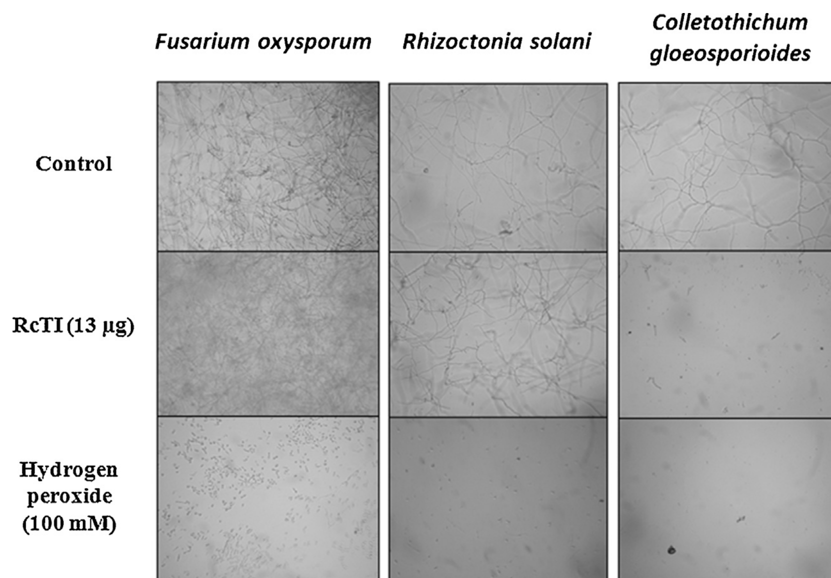

Fig. 6. Effect of RcTI on the phytopatogenic fungi *F. oxysporum*, *R. solani* and *C. gloeosporioides*. Spores were incubated with 46.4 μM of RcTI, sterile water (negative control) and 100 mM hydrogen peroxide (positive control). Magnification: 10×.

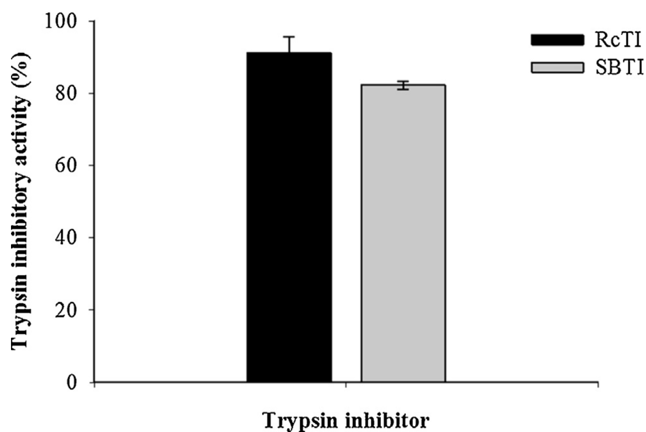

Fig. 7. Effect of RcTI (100 μg mL^{-1}) on the activity of midgut proteases of *A. aegypti* larvae. SBTI (100 μg mL^{-1}) was used as a reference standard. Five replicates were done for each measurement.

tive growth, being more potent in inhibiting conidial germination. Thus, inhibition of spore germination does not imply similar extent in inhibition of vegetative growth, as they are distinct stages of fungus development.

3.7. Inhibition assay of RcTI against A. aegypti larvae digestive proteases

RcTI (10 μg protein per total volume of the assay, ≈4.5 μM) promoted 91% inhibition of the intestinal proteases of *A. aegypti* larvae whereas SBTI inhibited approximately 82% (Fig. 7). Most of the intestinal proteases of *A. aegypti* are serine type (Kunz, 1978), which might explain the inhibition observed for both inhibitors tested. Nevertheless, the inhibition effectiveness of protease inhibitors against insect digestive proteases depends on the site specificity of these enzymes of the target organism (Garcia et al., 2004).

4. Conclusion

Our present study indicates that RcTI has biotechnological potentials as an alternative agent to control both the germination of the phytopathogenic fungus *C. gloeosporioides* and the larval development of *A. aegypti*.

Authors' contributions

J.T.A. Oliveira conceived and designed the study, prepares the final version of the article, obtained financial support and performed data interpretation. R.G.G. Silva performed the experiments for RcTI purification and its physicochemical and biological characterization, performed data interpretation and wrote the draft manuscript. I.M. Vasconcelos performed the RcTI N-terminal sequence analyses, critically revised the article and obtained funding. A.J.U.B. Filho participated in RcTI purification. A.F.U. Carvalho and T.M. Souza evaluated experimentally the effect of RcTI on the midgut proteases of *A. aegypti* larvae. DMF Gondim and A.L.N. Varela conducted the experiments to evaluate the effects of RcTI on fungi. All authors read and approved the final version of the manuscript.

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
Bhattacharyya, A., Babu, C.R., 2009. Purification and biochemical characterization of a serine proteinase inhibitor from *Derris trifoliata* Lour. seeds: insight into structural and antimalarial features. Phytochemistry 70, 703–712.
Bhattacharyya, A., Mazumdar, S., Leighton, S.M., Babu, C.R., 2006. A Kunitz proteinase inhibitor from *Archidendron ellipticum* seed: purification, characterization, and kinetic properties. Phytochemistry 67, 232–241.
Bijina, B., Chellappan, S., Basheer, S.M., Elyas, K.K., Bahkali, A.H., Chandrasekaran, M., 2011. Protease inhibitor from *Moringa oleifera* leaves: isolation, purification, and characterization. Process Biochem. 46, 2291–2300.
Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities for proteins utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Candiano, G., Bruschi, M., Musante, L., Santucci, L., Ghiggeri, G.M., Carnemolla, B., Orecchia, P., Zardi, L., Righetti, P.G., 2004. Blue silver: a very sensitive coloidal coomassie G-250 staining for proteome analysis. Electrophoresis 25, 1327–1333.
Carrillo, L., Herrero, I., Cambra, I., Sánchez-Monge, R., Diaz, I., Martinez, M., 2011. Differential in vitro and in vivo effect of barley cysteine and serine protease inhibitors on phytopathogenic microorganisms. Plant Physiol. Biochem. 49, 1191–1200.
Chaudhary, N.S., Shee, C., Islamb, A., Ahmad, F., Yernool, D., Kumar, P., Sharma, A.K., 2008. Purification and characterization of a trypsin inhibitor from *Putranjiva roxburghii* seeds. Phytochemistry 69, 2120–2126.
Chen, Z.Y., Brown, R.L., Lax, A.R., Guo, B.Z., Cleveland, T.E., Russin, J.S., 1998. Resistance to *Aspergillus flavus* in corn kernels is associated with a 14-kDa protein. Phytopathology 88, 276–281.
Chen, Z.Y., Brown, R.L., Lax, A.R., Cleveland, T.E., Russin, J.S., 1999. Inhibition of plant-pathogenic fungi by a corn trypsin inhibitor overexpressed in *Escherichia coli*. Appl. Environ. Microbiol. 65, 1320–1324.
Cruz, A.C.B., Massena, F.S., Migliolo, L., Macedo, L.L.P., Monteiro, N.K.V., Oliveira, A.S., Macedo, F.P., Ucho, A.F., Grossi de Sá, M.F., Vasconcelos, I.M., Murad, A.M., Franco, O.L., Santos, E.A., 2013. Bioinsecticidal activity of a novel Kunitz trypsin inhibitor from Catanduva (*Piptadenia moniliformis*) seeds. Plant Physiol. Biochem. 70, 61–68.
Dixon, M., 1953. The determination of enzyme inhibitor constants. Biochem. J. 55, 170–171.
Dubois, J.L., Piccirilli, A., Magne, J., He, X., 2013. Detoxification of castor meal through reactive seed crushing. Ind. Crop Prod. 43, 194–199.
Ee, K.Y., Zhao, J., Rehman, A., Agboola, S., 2011. Glycosylation, amino acid analysis and kinetic properties of a major Kunitz-type trypsin inhibitor from *Acacia victoriae* Bentham seeds. Food Chem. 129, 1224–1227.
Erlanger, B.F., Kokowsky, N., Cohen, W., 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95, 271–278.
Fan, S.G., Wu, G.J., 2005. Characteristic of plant protease inhibitors and their applications in combating phytophagous insects. Bot. Bull. Acad. Sin. 46, 273–292.
Fang, A.F., Wong, J.H., Ng, T.B., 2010. Thermostable Kunitz trypsin inhibitor with cytokine inducing, antitumor and HIV-1 reverse transcriptase inhibitory activities from Korean large black soybeans. J. Biosci. Bioeng. 109, 211–217.
Franco, O.L., Melo, F.R., 2000. Osmoprotectants – a plant strategy in response to osmotic stress. Russ. J. Plant Physiol. 47, 137–144.
Garcia, V.L., Freire, G.M., Novello, J.C., Marangoni, S., Macedo, M.L.R., 2004. Trypsin inhibitor from *Poecilantha parviflora* seeds.: purification, characterization, and activity against pest protease. Protein J. 23, 343–350.
Garcia-Olmedo, F., Salcedo, G., Sanchez-Monge, R., Gomez, L., Royo, J., Carbonero, P., 1987. Plant proteinaceous inhibitors of proteinases and α-amylases. Plant Mol. Cell. Biol. 4, 275–334.
Giudici, A.M., Regente, M.C., Canal, L., 2000. A potent antifungal protein from *Helianthus annuus* flowers is a trypsin inhibitor. Plant Physiol. Biochem. 38, 881–888.
Ishihara, M., Shiroma, T., Taira, T., Tawata, S., 2006. Purification and characterization of extracellular cysteine protease inhibitor, ECPI-2, from *Chlorella* sp. J. Biosci. Bioeng. 101, 166–171.
Kapitany, R.A., Zebrowski, E.J., 1973. A high resolution PAS stain for polyacrylamide gel electrophoresis. Anal. Biochemistry 56, 361–369.
Katunuma, N., Matsunaga, Y., Himeno, K., Hayashi, Y., 2003. Insights into the roles of cathepsins in antigen processing and presentation revealed by specific inhibitors. Biol. Chem. 384, 883–890.
Klomklao, S., Benjakul, S., Kishimura, H., Osako, K., Tanaka, M., 2010. A heatstable trypsin inhibitor in adzuki bean (*Vigna angularis*): effect of extraction media, purification and biochemical characteristics. Int. J. Food Sci. Technol. 45, 163–169.
Klomklao, S., Benjakul, S., Kishimura, H., Chaijan, M., 2011. Extraction: purification and properties of trypsin inhibitor from Thai mung bean (*Vigna radiata* (L.) R. Wilczek). Food Chem. 129, 1348–1354.
Kunz, P.A., 1978. Resolution and properties of the proteinases in the larva of the mosquito, *Aedes aegypti*. Insect Biochem. 8, 43–51.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
Laskowski, M.J., Kato, I., 1980. Protein inhibitors of proteinases. Annu. Rev. Biochem. 49, 593–626.

Macedo, M.L.R., Matos, D.G.G., Machado, O.L.T., Marangoni, S., Novello, J.C., 2000. Trypsin inhibitor from *Dimorphandra mollis* seeds: purification and properties. Phytochemistry 54, 553–558.

Macedo, M.L.R., Freire, M.G.M., Cabrini, E.C., Toyama, M.H., Novello, J.C., Marangoni, S., 2003. A trypsin inhibitor from *Peltophorum dubium* seeds active against pest proteases and its effect on the survival of *Anagasta kuehniella* (Lepidoptera: Pyralidae). Biochim. Biophys. Acta 1621, 170–182.

Maleki, S.J., Viquez, O., Jacks, T., Dodo, H., Champagne, E.T., Chung, S., Landry, S.J., 2003. The major peanut allergen, Ara h 2, functions as a trypsin inhibitor, and roasting enhances this function. J. Allergy Clin. Immunol. 112, 190–195.

Mandal, S., Kundu, P., Roy, B., Mandal, R.K., 2002. Precursor of the inactive 2S seed storage protein from the Indian mustard *Brassica juncea* is a novel trypsin inhibitor. J. Biol. Chem. 277, 37161–37168.

Oliveira, A.S., Migliolo, L., Aquino, R.O., Ribeiro, J.K.C., Macedo, L.L.P., Andrade, L.B.S., Bemquerer, M.P., Santos, E.A., Kiyota, S., Sales, M.P., 2007. Identification of a Kunitz-type proteinase inhibitor from *Pithecellobium dumosum* seeds with insecticidal properties and double activity. J. Agric. Food Chem. 55, 7342–7349.

Oliveira, C.F.R., Vasconcelos, I.M., Aparicio, R., Freire, M.G.M., Baldasso, P.A., Marangoni, S., Macedo, M.L.R., 2012. Purification and biochemical properties of a Kunitz-type trypsin inhibitor from *Entada acaciifolia* (Benth.) seeds. Process Biochem. 47, 929–935.

Paiva, P.M.G., Souza, A.F., Oliva, M.L.V., Kennedy, J.F., Cavalcanti, M.S.M., Coelho, L.C.B.B., Sampaio, C.A., 2003. Isolation of a trypsin inhibitor from *Echinodorus paniculatus* seeds by affinity chromatography on immobilized *Cratylia mollis* isolectins. Bioresour. Technol. 88, 75–79.

Paul, G.C., Kent, C., Thomas, C.R., 1992. Viability testing and characterisation of germination of fungal spores by automatic imageanalysis. Biotechnol. Bioeng. 42, 11–23.

Rawlings, N.D., Barrett, A.J., Bateman, A., 2010. Merops: the peptidase database. Nucleic Acids Res. 38, 227–233.

Richardson, M., 1991. Seed storage proteins: the enzyme inhibitors. Methods in Plant Biochemistry, 5. New York Academic Press, New York, pp. 259–305.

Ryan, C.A., 2000. The systemin signaling pathway: differential activation of plant defensive genes. Biochim. Biophys. Acta 1477, 112–121.

Shee, C., Sharma, A.K., 2007. Purification and characterization of a trypsin inhibitor from seeds of *Murraya koenigii*. J. Enzyme Inhib. Med. Chem. 22, 115–120.

Shee, C., Islam, A., Ahmed, F., Sharma, A.K., 2007a. Structure-function studies of *Murraya koenigii* trypsin inhibitor revealed a stable core beta sheet structure surrounded by α-helices with a possible role for α-helix in inhibitory function. Int. J. Biol. Macromol. 41, 410–414.

Shee, C., Singh, T.P., Kumar, P., Sharma, A.K., 2007b. Crystallization and preliminary X-ray diffraction studies of *Murraya koenigii* trypsin inhibitor. Acta Crystallogr. Sect. F: Struct. Biol. Cryst. Commun. 63, 318–319.

Solomon, M., Belenghi, B., Delledonne, M., Menachem, E., Levine, A., 1999. The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. Plant Cell 11, 431–443.

Sritanyarat, W., Pearce, G., Siems, W.F., Ryan, C.A., Wititsuwannakul, R., Wititsuwannakul, D., 2006. Isolation and characterization of isoinhibitors of the potato protease inhibitor I family from the latex of the rubber trees, *Hevea brasiliensis*. Phytochemistry 67, 1644–1650.

Vasconcelos, I.M., Siebra, E.A., Maia, A.A.B., Moreira, R.A., Neto, A.F., Campelo, G.J.A., Oliveira, J.T.A., 1997. Composition, toxic and antinutritional factors of newly developed cultivars of Brazilian soybean (*Glycine max*). J. Sci. Food Agric. 75, 419–426.

Xavier-Filho, J., Campos, F.A.P., 1889. Proteinase inhibitors. In: Cheeke, P.R. (Ed.), Toxicants of Plant Origin, 3. Boca CCR Press, pp. 1–27.

Yoshizaki, L., Troncoso, M.F., Lopes, J.L.S., Hellman, U., Beltramini, L.M., Wolfenstein-Todel, C., 2007. *Calliandra selloi* Macbride trypsin inhibitor: isolation, characterization, stability, spectroscopic analyses. Phytochemistry 68, 2625–2634.