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Toxicity to cotton boll weevil *Anthonomus grandis* of a trypsin inhibitor from chickpea seeds

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Abstract

Cotton (*Gossypium hirsutum* L.) is an important agricultural commodity, which is attacked by several pests such as the cotton boll weevil *Anthonomus grandis*. Adult *A. grandis* feed on fruits and leaf petioles, reducing drastically the crop production. The predominance of boll weevil digestive serine proteinases has motivated inhibitor screenings in order to discover new ones with the capability to reduce the digestion process. The present study describes a novel proteinase inhibitor from chickpea seeds (*Cicer arietinum* L.) and its effects against *A. grandis*. This inhibitor, named CaTI, was purified by using affinity Red-Sepharose Cl-6B chromatography, followed by reversed-phase HPLC (Vydac C18-TP). SDS-PAGE and MALDI-TOF analyses, showed a unique monomeric protein with a mass of 12,877 Da. Purified CaTI showed significant inhibitory activity against larval cotton boll weevil serine proteinases (78%) and against bovine pancreatic trypsin (73%), when analyzed by fluorimetric assays. Although the molecular mass of CaTI corresponded to α-amylase/trypsin bifunctional inhibitors masses, no inhibitory activity against insect and mammalian α-amylases was observed. In order to observe CaTI in vivo effects, an inhibitor rich fraction was added to an artificial diet at different concentrations. At 1.5% (w/w), CaTI caused severe development delay, several deformities and a mortality rate of approximately 45%. These results suggested that CaTI could be useful in the production of transgenic cotton plants with enhanced resistance toward cotton boll weevil.

Keywords: Serine proteinase inhibitor; *Cicer arietinum*; *Anthonomus grandis*; Plant defense; Cotton; Mass spectrometry; Trypsin; Digestive enzymes

1. Introduction

Cotton crops (*Gossypium hirsutum* L.) are extremely important in providing income to millions of farmers in tropical and subtropical areas of the New World. Cotton serves as an engine of economic growth and thus contributes to food security worldwide. Cotton boll weevil *Anthonomus grandis* is an important insect pest responsible for severe cotton crop damage in several countries of America. Adults feed on fruit and leaf petioles, reducing crop production and damaging cotton fibers (Haynes and Smith, 1992; Alves et al., 1993). The larval phase is endophytic and is not efficiently controlled by pesticides and adult insects are

Abbreviations: AgPL, *Anthonomus grandis* proteinase larvae; AoPL, *Acanthoscelides obtectus* proteinase larvae; BBI, Bowman-Birk inhibitor; BPC, Bovine pancreatic chymotrypsin; BPT, Bovine pancreatic trypsin; BTCI, Black-eyed pea trypsin chymotrypsin inhibitor; CaRP, *Cicer arietinum* Red-sepharose retained peak; CaTI, *Cicer arietinum* trypsin inhibitor; CmPL, *Callosobruchus maculatus* proteinase larvae; CpTI, Cowpea trypsin inhibitor; HPLC, High performance liquid chromatography; HPT, Human pancreatic trypsin; MALDI-TOF, Matrix assisted laser desorption ionizated-time of flight; OCI, Oryzacystatin inhibitor; PPA, porcine pancreatic α-amylase; RASI, Rice α-amylase/subtilisin inhibitor; SDS-PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis; SKTI, soybean Kunitz trypsin inhibitor; SfPL, *Spodoptera frugiperda* proteinase larvae.

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able to move to safe areas, escaping from toxic chemicals. Under favorable conditions, insects move back to the crop area to complete their life cycle (França, 1993).

Insect digestive proteinases are promising targets in the control of several insect pests since insects obtain essential amino acids by using extra-cellular proteinases secreted into the midgut lumen, digesting soluble and structural proteins. Despite the predominance of digestive cysteine proteinase-like activity in some coleopterans as the bean bruchids *A. obtectus*, *Z. subfasciatus* and *C. maculatus* (Wielman and Nielsen, 1988; Zhu-Salzman et al., 2003; Melo et al., 2003), the Colorado potato beetle *Leptinotarsa decemlineata*, the black vine weevil *Otiorynchus sulcatus* (Michaud et al., 1996) and phytophagous beetle, *Phaedon cochleariae* (Girard and Jouanin, 1999); the cotton boll weevil *A. grandis* and others pests including the coleopteran *Tenebrio molitor* (Lee et al., 2002), *Holotrichia diomphalia* (Kwon et al., 2000), the lepidopterans *Manduca sexta* (Johnson et al., 1989), *Heliothis zea* (Broadway and Duffey, 1986), *Spodoptera litura* (Yeh et al., 1997) and the dipteran *Lucilia cuprina* (Reed et al., 1999) have large quantities of digestive serine proteinases. For this reason several plant serine proteinase inhibitor screenings have been undertaken in order to identify inhibitors highly effective against digestive enzymes (Leplé et al., 1995; Gatehouse and Gatehouse, 1998; Bode and Huber, 2000; Franco et al., 2004).

The serine proteinase inhibitor family is composed of abundant, small and stable molecules found in plant storage tissues such as seeds, tubers, leaves and fruits (Richardson, 1977, 1991; Haq and Khan, 2003). Various functions have been suggested for these plant proteinase inhibitors, including their action as storage proteins (Xavier-Filho, 1992), regulators of endogenous proteolytic activity (Ryan, 1991) and components of programmed plant cell death mechanisms (Solomon et al., 1999). Mostly serine proteinase inhibitors are known for their function in response to abiotic stresses (Franco and Melo, 2000) and in plant defense processes against pests and pathogens (Gatehouse and Gatehouse, 1998) reacting with cognate enzymes, binding to catalytic sites in a canonical fashion (Laskowski and Kato, 1980; Grütter et al., 1990). All canonical inhibitors possess an exposed loop that simulates the substrate, with a rapid binding and a slow dissociation mechanism (Laskowski and Kato, 1980; Grütter et al., 1990; Richardson, 1992). Despite strong efforts dedicated to understanding the structure and specificity of canonical serine proteinase inhibitors, there are still unanswered questions awaiting detailed investigation concerning mechanistic aspects of inhibitors in plant defense. Due to the complexity of enzyme/inhibitor interactions, the choice of an efficient inhibitor will determine the success of pest a control strategy. Our main goals in this study were the purification and characterization of a novel serine-proteinase inhibitor (CaTI) from chickpea seeds (*C. arietinum*), and evaluation of the in vitro and in vivo activity of this inhibitor against the cotton boll weevil *A. grandis*.

2. Materials and methods

2.1. Purification of C. arietinum trypsin inhibitor (CaTI)

Chickpea seeds (*C. arietinum*) were triturated into a fine textured flour. The flour was defatted with acetone 50% and was extracted with a solution of 0.5 M Tris–HCl pH 7.0 containing 100 mM NaCl in the proportion of 1:3 (w/v) for 4 h under agitation at 4 °C. This extract was centrifuged at 5000×*g* for 40 min at 4 °C. Precipitate was discarded and the supernatant was dialyzed against distilled water. Crude extract was precipitated with ammonium sulphate (100%), following further centrifugation. Precipitate was dissolved and dialyzed against distilled water. This fraction was applied onto a Red-Sepharose Cl-6B affinity column equilibrated to 0.1 M Tris–HCl buffer, pH 7.0 containing 5.0 mM $CaCl_2$. The retained peak (CaRP) was eluted using a single step of 0.1 M Tris–HCl buffer, pH 7.0 containing 3.0 M NaCl. Fractions of 3.0 mL were collected and the optical density was measured at wavelength of 280 nm. After dialysis and lyophilization, 4.0 mg of CaRP fraction was dissolved in 250 μL of 0.1% trifluoroacetic acid and applied onto a semi-preparative reversed phase HPLC column (Vydac C18-TP) and eluted with a liner acetonitrile gradient (0–100%) generating several peaks. The purified *Cicer arietnum* trypsin inhibitor, which was named CaTI, composed one of them.

2.2. Isolation of fluid from larval pest midgut

A. grandis and *Spodoptera frugiperda* larvae were obtained from the Biological Control Department of EMBRAPA/Cenargen (Brasília-DF, Brasil). Larvae were reared on an artificial diet (Monnerat et al., 1999) at 25 °C and 55% relative humidity. Furthermore, *Callosobruchus maculatus* and *Acanthoscelides obtectus* were reared in cowpea and common bean seeds, respectively. The guts were dissected from larvae and placed into an iso-osmotic saline (0.15 M NaCl). Midgut tissues were homogenized and centrifuged for 10 min at 16,000×*g* at 4 °C. The supernatant was removed and used for enzymatic assays.

2.3. Proteinase inhibitory assays

Bovine and human pancreatic trypsin (BPT and HPT), as well bovine pancreatic chymotrypsin were purchased from Sigma, St. Louis, MO, USA, and proteinase extracted from larvae of *A. grandis* (AgPL), *S. frugiperda* (SfPL), *A. obtectus* (AoPL) and *C. maculatus* (CmPL) were used for enzymatic assays. Proteolytic inhibitory activities were tested against AgPL, SfPL, CmPL, AoPL and BPT using 10 mM of fluorogenic peptide Z-CBZ-Phe-Arg-7-MCA (Sigma) and against bovine pancreatic chymotrypsin (BPC) using 10 mM of fluorogenic peptide Ala-Ala-Pro-Phe-MCA. Assays were performed in 25 mM Tris–HCl, pH 6.5 and 20 mM DMSO according to Solomon et al. (1999).

The reaction was stopped with 1.9 mL of 0.2 M Na_2CO_3. The endpoint reaction was measured after 30 min in a DyNA Quant 500 fluorescence reader (Amersham Pharmacia), with excitation at 365 nm and emission at 460 nm. Crude chickpea extract, CaRP and CaTI were tested at a standard concentration of 75 μg mL^{-1}. Inhibitory activities were calculated using the fluorescence reduction. The inhibitory activities were direct compared to free MCA produced by trypsin-like enzymes. One relative unit corresponds to 0.5 mM of free MCA produced for 30 μg mL^{-1} of proteinases utilized, after 30 min of reaction. The blank fluorescence readings (minus substrate) were subtracted. Assays were carried out in triplicate, with variability in endpoint fluorescence values not exceeding 10%.

2.4. Molecular mass analysis

Peaks obtained in HPLC were analyzed by 15% SDS-PAGE (Laemmli, 1970) and by mass spectrometry using the method of Franco et al. (2000). Freeze-dried samples of the peaks from HPLC were prepared for Matrix-Assisted Laser Desorption Time of Flight Analysis (MALDI-TOF) on a Voyager-DE STR Bioworkstation (PerSeptive Biosystems, Framingham, MA, USA). Samples were dissolved in trifluoracetic acid (TFA) 1.0% and α-cyan (a saturated solution dissolved in acetonitrile/0.1% TFA 1:1, v/v) from Sigma. The solution was then vortex mixed and 1 mL aliquots were applied onto the Voyager Bioworkstation sample plate. Samples were air-dried at room temperature. The spectrometer, equipped with a delayed-extraction system, was operated in linear mode. Sample ions were evaporated by irradiation with a N_2 laser at a wavelength of 337 nm, and accelerated at 23 kV potential in the ion source with a delay of 150 ns. Samples were ionized with 100–200 shots of a 3 ns pulse width laser light. The signal was digitalized at a rate of 500 MHz and averaged data was presented to a standard Voyager data system for manipulation. MALDI-ToF was calibrated using a Saquazyme calibration mixture (Applied Biosystems) consisting of bovine insulin (5734 Da), *E. coli* thioredoxin (11,674 Da) and horse apomyoglobin (16,952 Da).

2.5. Chickpea rich fraction feeding tests on cotton boll weevil

Bioassays were performed in 40 ml sterilized artificial diet (Monnerat et al., 1999). CaRP was incorporated in the artificial diet at standard concentrations of 0.5%, 1.0% and 1.5% (w/w). Purified CaTI was also used for feeding tests, being incorporated in the artificial diet at the concentration of 0.5%. Protein concentration was calculated according to Bradford (1976). The artificial diet was added to Petri dishes and larvae 48 h post-hatch were placed in pits created in solid diet. After 7 days, the dead larvae were counted and the size of surviving larvae was measured. In negative control treatment, bovine serum albumin (BSA) was added to the artificial diet at the same concentration of inhibitor. Each treatment was repeated three times and each replicate used 15 larvae. The bioassay was maintained under controlled conditions at 28 °C and 55% stable humidity.

3. Results and discussion

3.1. Cicer arietinum trypsin inhibitor (CaTI) purification

Lyophilized chickpea crude extract was precipitated with ammonium sulphate 100%, dialyzed and subjected to a Red-Sepharose CL-6B affinity column, originating a single retained peak (CaRP) (Fig. 1A). Proteins with positive charge surface, generally generated by residues of lysine and/or arginine, bind to Red-Sepharose resin (Melo et al., 2002). This resin, when associated to HPLC reversed phase columns, has been shown to be an efficient means of purification of plant defense proteins, such as thionins (Bloch and Richardson, 1991), α-amylase inhibitors (Melo et al., 1999) and proteinase inhibitors (Melo et al., 2002). CaRP was applied onto an HPLC column, generating several peaks, in which only three of them demonstrated proteolytic inhibitory activity (1, 2 and 3) (Fig. 1B). Fraction 3 was selected due to its higher activity against our main target AgPL.

In order to analyze the purification degree and molecular masses, SDS-PAGE of crude extract, CaRP fraction and HPLC fractions 1 and 2 were undertaken (Fig. 1C). CaRP analyses indicated the presence of a strong band of molecular mass of approximately 13 kDa and some minor contaminants. When this fraction was applied onto HPLC, fractions 1 and 2 showed several bands with different molecular masses (Fig. 1C). Although both fractions are able to inhibit AgPL, the presence of different contaminants and their lower inhibitory activity (Fig. 1B) led to the choice of fraction 3. Other inhibitor fractions from chickpea seeds warrant future study. In fraction 3, contaminants were eliminated using reversed-phase HPLC and a single band of approximately 12 kDa was observed by SDS-PAGE (Fig. 2B). The protein purified in peak 3 was named *C. arietinum* trypsin inhibitor (CaTI) and its molecular mass was calculated using mass spectrometry (MALDI-TOF) (Fig. 2A). This method showed a unique protein of 12,877.44 Da observed in monomeric form. A lower presence of dimeric form was also detected (Fig. 2A). Wheat α-amylase inhibitors (Feng et al., 1996; Franco et al., 2000) and rye bifunctional (α-amylase/proteinase) inhibitors (Iulek et al., 2000) are known to be multimeric hydrolytic enzyme inhibitors. Proteinase inhibitor from the Kunitz family with different molecular masses from CaTI occurs in soybean (19.0 kDa) and mustard seeds (20.0 kDa) (Mandal et al., 2002) when compared to CaTI (Fig. 2). Only *Cajanus cajan* trypsin inhibitor from the Kunitz family (Haq and Khan, 2003), with approximately 14 kDa, showed a similar molecular mass to CaTI. Furthermore, bifunctional inhib-

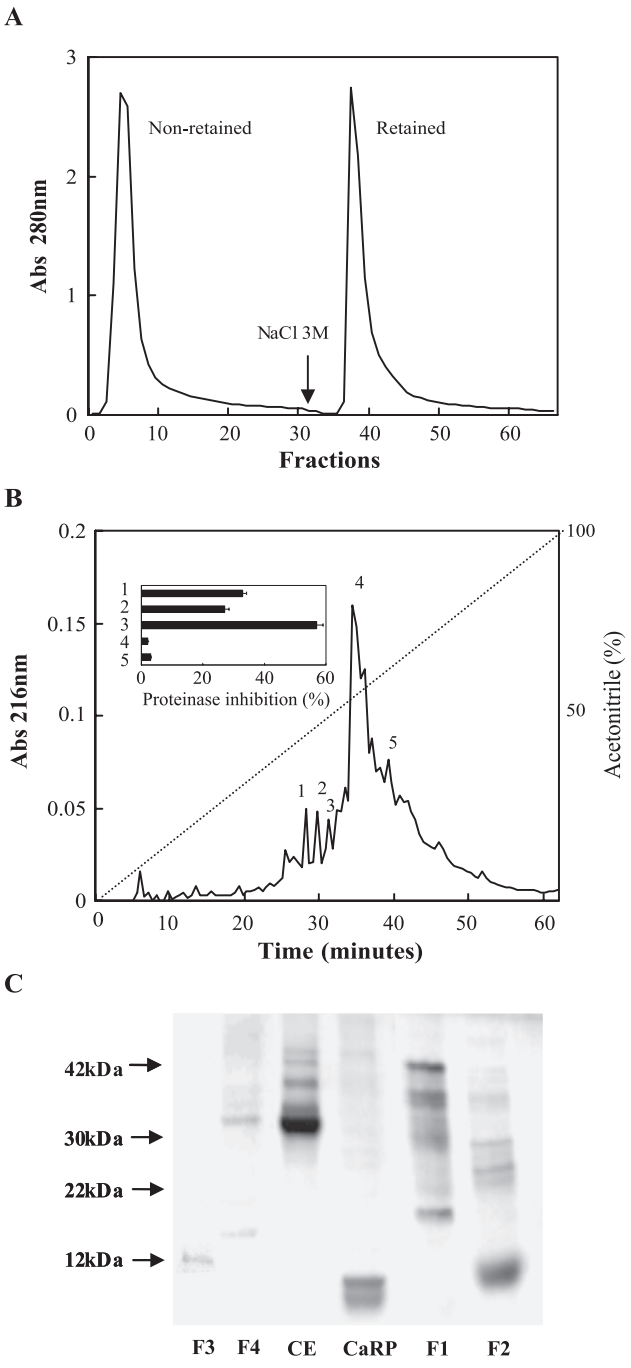

Fig. 1. (A) Elution profile on Red-Sepharose CL-6B of trypsin-like serine proteinase inhibitor from *C. arietinum* seeds. Approximately 25 mg of protein was applied onto the column equilibrated with 0.1 M Tris–HCl buffer pH 7.0 containing 5.0 mM $CaCl_2$. Fractions were eluted with a single step of 3.0 M NaCl (black arrow) and they were monitored at 280 nm. (B) Analytical reverse-phase HPLC of the retained peak. The separation was carried out on a Vydac C18-TP analytical column using a flow rate of 1 ml min^{-1}. TFA (0.1%) was used as an ion-pairing agent and the dashed line indicates the acetonitrile gradient. The sample applied contained 1.0 mg of CaRP. Fractions I, II, III, IV and V were tested against AgPL. (C) SDS-PAGE analysis of crude extract (CE), *C. arietinum* Red-Sepharose Retained-peak (CaRP) and fractions 1 (F1), 2 (F2), 3 (F3) and 4 (F4). Gel was stained with silver.

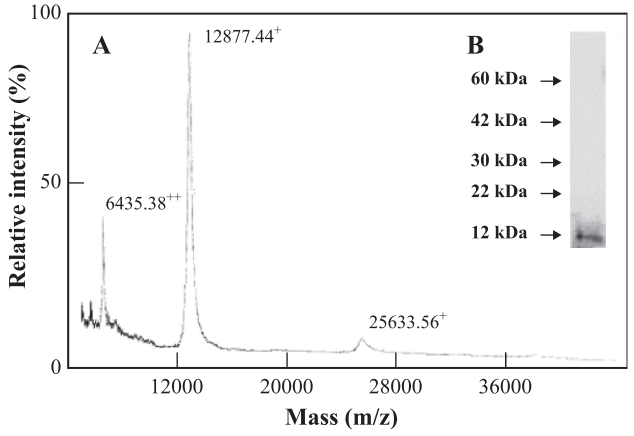

Fig. 2. (A) Mass spectrum (MALDI-TOF) and (B) SDS-PAGE analyses of fraction 3 (CaTI inhibitor). Electrophoresis was stained with silver.

itors that act against α-amylases and trypsin (Xavier-Filho, 1992; Iulek et al., 2000; Franco et al., 2002) also have molecular masses near 13 kDa. Additionally, a peptide mass fingerprint (data not shown) indicated, by two peptides produced by trypsin cleavage that matched to rice amylase/subtilisin inhibitor (RASI), that this inhibitor probably belongs to α-amylase/trypsin bifunctional class. No peptide produced by trypsin cleavage matched to Kunitz inhibitors. The similarities between CaTI and bifunctional inhibitors prompted us to test CaTI against α-amylases. We first looked for inhibitory activity against porcine pancreatic α-amylase (PPA) and found that CaTI did not inhibit PPA (data not shown). CaTI was also assayed against insect α-amylases from *A. grandis* and bean bruchids and no inhibitory activity was observed (data not shown). While it is possible that CaTI inhibits other α-amylases, our results suggested that this inhibitor is only capable to inhibit proteinases.

3.2. Enzymatic assays of CaTI against digestive proteinases from A. grandis larvae

Two trypsin-like serine proteinases (AgPL and BPT) were used to monitor the chickpea inhibitory activity (Fig. 1B, data not shown). Purified CaTI inhibited approximately 73% of AgPL and 51% of SpPL, strongly suggesting that this inhibitor is a trypsin-like serine proteinase inhibitor since both pests synthesize enhanced quantities of digestive trypsin-like enzymes (Franco et al., 2004). This result was confirmed by inhibition of BPT (78%) and HPT (82%) (Fig. 3). CaTI also did not show any inhibitory activity against AoPL and CmPL (Fig. 3). This may be due a low level of trypsin-like serine proteinase and a higher rate of cysteine-like proteinases in bruchids midguts (Melo et al., 2003) suggesting that CaTI was unable to inhibit this proteolytic enzyme class. Furthermore, CaTI was incapable of inhibiting BPC, confirming that this inhibitor does not pertain to Bowman-Birk family. Although cotton boll weevil is capable of synthesizing a wide range of digestive protei-

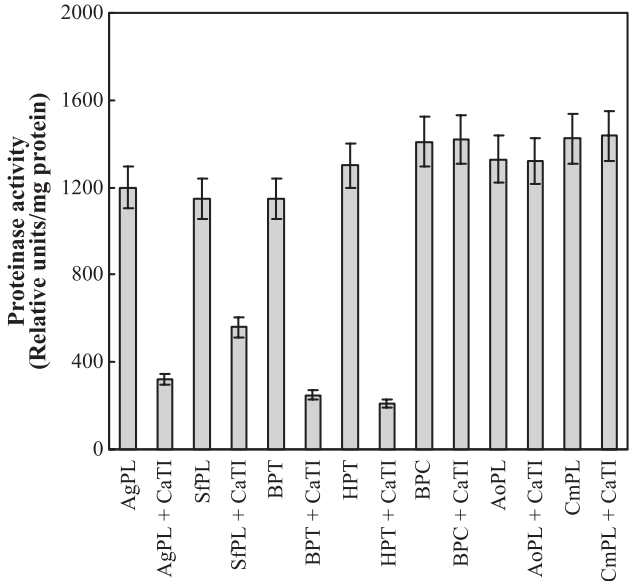

Fig. 3. Inhibitory activities of CaTI toward *A. grandis* and *S. frugiperda* proteinase larvae (AgPL and SfPL), bovine and human pancreatic trypsin (BPT and HPT), bovine pabcreatic chymotrypsin (BPC) and proteinases from *A. obtectus* and *C. maculatus* larvae (AoPL and CmPL). Each measurement was done in triplicate and each replicate does not differ by more than 10%.

nases, most of them belong to the trypsin-like serine proteinases class (Purcell et al., 1992). Inhibition caused by CaTI from chickpea seeds (Fig. 3) to midgut extracts of *A. grandis* was similar to results observed with SKTI from soybean seeds (soybean Kunitz trypsin inhibitor) (Purcell et al., 1992; Franco et al., 2004) and BTCI from cowpea seeds (black eyed-pea trypsin/chymotrypsin inhibitor) (Franco et al., 2003). Other proteinases inhibitors shown to have inhibitory activity against pest enzymes have been considered to be natural agents of plant defense (Broadway and Duffey, 1986; Hilder et al., 1987). Among them, rice oryzacystatin (OCI) and soybean Bowman-Birk inhibitors (BBI) showed inhibition of *Phaedon cochleariae* digestive enzymes (Girard et al., 1998). Nevertheless, is important to remember that a substantial in vitro inhibition of digestive proteinases is not enough to determine an efficient resistance factor against an insect-pest. Pests are able to adapt to the presence of inhibitors, modifying the composition of their digestive proteinases, altering their concentrations or inducing the expression of novel proteinases (Jongsma et al., 1995). In order to measure the CaTI inhibitory efficiency, feeding tests were also carried out.

3.3. Feeding tests

Due to the in vitro activity of CaTI against trypsin-like enzymes from cotton boll weevil midgut (Fig. 3), bioassays were conducted in order to demonstrate the potential of chickpea serine proteinase inhibitors as a protective factor. Bioassays revealed an enhanced mortality rate and delayed insect development when *A. grandis* larvae where fed on diet with three different CaRP concentrations (Fig. 4A and B). Using a CaRP concentration of 1.5% we observed a mortality rate of 43.33% (Fig. 4A) and 40% of surviving larvae had retarded development (Fig. 4B). Feeding test using purified CaTI was also carried out, using a single concentration of 0.5%, causing a mortality rate of 48% (data not shown). Increased mortality at 0.5% protein concentration could be caused by the high purity of CaTI used. Furthermore, several larvae that consumed CaRP and CaTI showed severe deformities. The concentration used, 0.5–1.5% (w/w), corresponds to the expressed levels in legume seeds (Ryan, 1991) and was very similar to that used by other researchers (Gatehouse et al., 1999; Franco et al., 2003; Oppert et al., 2003). Pests fed artificial diet containing proteinase inhibitors with specificity to their main class of digestive proteinases at similar concentrations suffered a significant delay in their development and an increase in mortality (McManus and Burgess, 1995). The potential of serine proteinase inhibitors against larvae and adult insects of the cotton boll weevil has been demonstrated previously. Two inhibitors (SKTI and BTCI) showed deleterious effects

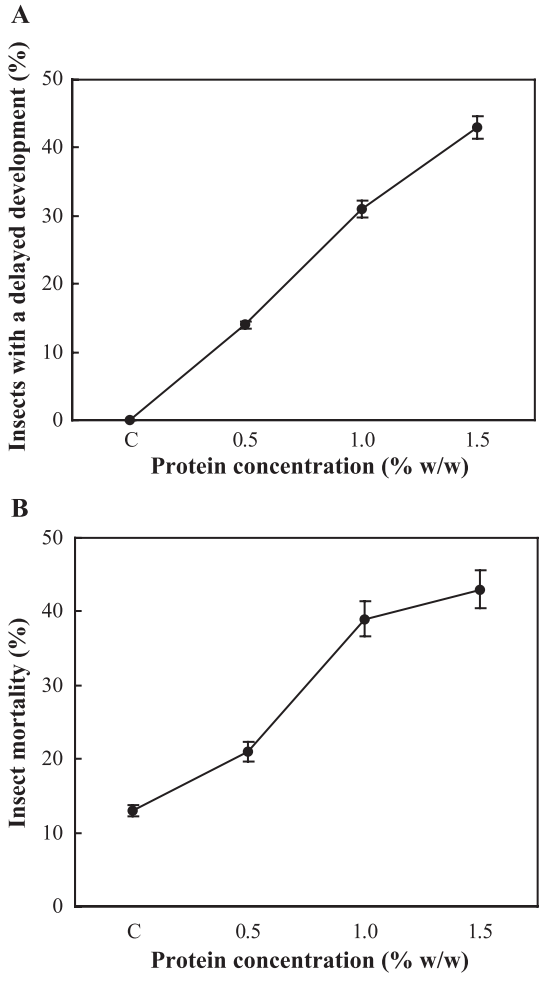

Fig. 4. Effects of CaRP on development (A) and on mortality (B) of cotton boll weevil *A. grandis.* Each measurement was done in triplicate and each replicate does not differ by more than 10%.

against cotton boll weevil (Franco et al., 2003, 2004). A lower BTCI concentration, when compared to SKTI and CaTI, was necessary to cause significant reduction in pest development. This enhanced effectiveness could be explained by the double function of BTCI, where this inhibitor was able to reduce the activity of digestive trypsins and chymotrypsins of *A. grandis* larvae (Franco et al., 2003). Despite of CaTI lower activity, this inhibitor could be used in combination with another inhibitor to produce a greater effect. Several reports have demonstrated that trypsin inhibitors can affect pest development when expressed in a heterologous system (Broadway and Duffey, 1986; Gatehouse et al., 1999). Transgenic tobacco expressing cowpea trypsin inhibitors had enhanced resistance to *H. virescens* (Hilder et al., 1987). Similar results were obtained in *Arabidopsis thaliana* expressing an inhibitor from mustard (MTI-2). The presence of MTI-2 increased the plant resistance against *Plutella xylostella* larvae, when expressed at 0.8% of total soluble protein (De Leo et al., 2001). This same inhibitor was efficacious in protecting transgenic tobacco against *Mamestra brassicae* (L.), when expressed in leaves, and against *S. litoralis*, when expressed in seeds (De Leo et al., 2001).

Another symptom observed in insects fed on CaTI was deformities, such as the absence of wings and thorax. These effects were previously observed in larvae of *A. grandis* consuming SKTI (Franco et al., 2003). A possible explanation for these effects is that CaTI activity may inhibit proteins important in metamorphosis (Chapman, 1982). Production of new adult tissues and enzymes systems are dependent on proteins localized in the insect cuticle (Missios et al., 2000). These proteins are synthesized primarily from free amino acids following a re-organization of peptides from larval proteins without total degradation of amino acids (Hopkins et al., 2000). Proteolytic activity including that of serine and cysteine proteinases is necessary to this re-organization. The mode of action of these inhibitors is still under debate, and it remains unclear whether the deleterious effects of proteinase inhibitors stem from an anti-digestive effect, through proteolysis inhibition (Jongsma et al., 1995), or from a toxic effect by inducing hyper production of proteinase, leading to a shortage in amino acids (Broadway and Duffey, 1986). The deformity and retardation of metamorphoses caused by CaTI suggests a different mode of action of inhibitors against insects.

The purified inhibitor CaTI inhibited insect trypsin-like enzymes from *A. grandis* and *S. frugiperda* and a fraction enriched with CaTI incorporated into artificial diet caused high mortality, delayed development and deformities. Results presented here, confirm previous results demonstrating that the use of serine proteinase inhibitors could be a useful strategy in development of modified plants with enhanced resistance to the cotton boll weevil, *A. grandis*. Furthermore, the expression of CaTI together with other resistance genes could be necessary to achieve effective protection in cotton crops.

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