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Bioactivity of trypsin inhibitors from sesame seeds to control *Plodia interpunctella* larvae (Hübner) (Lepidoptera: Pyralidae)

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Biochemical and feeding assays were performed in order to assess the bioactivity of trypsin inhibitors from sesame seeds to control *Plodia interpunctella* larvae. The biochemical assays were based on the ability of protease inhibitors (PI) present in sesame seeds to inhibit bovine pancreatic trypsin and digestive enzymes of insect larvae. The stability of the inhibitors was also assessed at different pH and temperature. The feeding bioassays were carried out in plates containing second instar larvae fed on diet with sesame protein extract. It was observed that all genotypes presented inhibition when tested with BPT, ranging from 51 to 90%, among them BRS Seda, CNPA G3 and CNPA G4 showed average of 69% of inhibitory activity for intestinal homogenate. The FII-fractions in these three genotypes showed thermal and pH stabilities at 40°C and 8.5, respectively. In feeding bioassay, *P. interpunctella* larvae were susceptible to SPCE of all three genotypes, revealing average mortality of 74% to SPCE and 45% to FII-fractions. Among all genotypes, BRS Seda was more effective to inhibition of digestive enzymes of insects and considered the more suitable genotype for further using in sesame breeding program to resistance to *P. interpunctella*.

Key words: *Sesamum indicum* L., serine protease, stored grain pest, Lepidoptera.

INTRODUCTION

Sesame (*Sesamum indicum* L.) is an oilseed grown in many parts of the world and is widely used in food and cosmetics industries (Sharma et al., 2014). The annual worldwide grain production is around 4 million tonnes (FAO, 2014). Sesame is a short cycle crop with broad adaptation to semiarid conditions. The main problem in management is the occurrence of insect-pests, especially at post-harvest season, including *Sitophilus* spp.,

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Abbreviations: CNPA, Centro Nacional de Pesquisa do Algodão; **PIs**, protease inhibitors; **SPCE**, seed protein crude extract.

Table 1. Inhibitory rate of seed protein crude extracts on bovine pancreatic trypsin in different sesame genotypes.

Genotypes	Inhibition (%)
CNPA G4	90.76[a]
CNPA G3	87.57[ab]
BRS Seda	87.02[ab]
ECGSG01	79.45[c]
ECGSG02	77.43[c]
ECGSG03	80.08[c]
ECGSG04	76.54[c]
ECGSG05	64.40[d]
ECGSG07	66.26[d]
ECGSG06	51.85[e]

Means followed by the same letter do not differ by Tukey test ($p < 0.05$).

Tribolium spp. and *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) (Arriel et al., 2009). The extent of damage depends on the degree of infestation in warehouses.

P. interpunctella is a harmful grain storage pest it affects the quality of the grain (Michereff Filho et al., 2013). The control is based on chemical pesticides, but it found limitation due to level of infestation and also resistance of inset to some chemical insecticides, including classes of organophosphates and pyrethroids (Arthur and Phillips, 2003). Resistance via transgenesis could be an alternative; however, Herrero et al. (2001) have found high level of resistance to Cry1 Ab and Cry1 Ac toxins, from *Bacillus thuringiensis*, in insect populations.

Considering these limitations, several researches have focused on identification of natural metabolites with potential to control insect-pests. Massive information is available in literature reporting about effective plant bioactives with toxicity to lepidoptera and coleopteran insects, which are the more harmful to commercial crops.

Several leguminous and oleaginous seeds contain proteins with insecticidal property, such as protease inhibitors (PIs), that can inhibit the activity of proteolytic enzymes present in insect guts, causing severe physiological disorders, such as malnutrition, delayed in development and even death (Martins et al., 2014; Lima et al., 2004). PIs are widely distributed in the plant kingdom; they are proteins able to inhibit the activities of trypsin, chymotrypsin, amylase, carboxipetidase, among others.

PIs are classified according to the specificity of interaction with amino acids groups that compound the proteolytic enzymes: Serine, cysteine, aspartic, metallo, threonine and glutamic proteases (Ryan, 1990; Powers et al., 2002; Rawlings et al., 2012). The serine and cysteine proteases have been the most studied, with proven interference in the life cycle of the insect, weight reduction, decreased rate of posture and mortality (Aghaali et al., 2013).

Several works are available in literature involving inhibitory activity of seed-PIs from oleaginous crops, such soybean and peanut (Martins et al., 2014; Oliva et al., 2011). In sesame, El-Bramawy (2011) studied antinutritional factors of seeds, including tannins, phytates and trypsin inhibitors (TI), in order to further use them as selection criteria to disease resistance. Despite the little information available, it is suggested that PI expression in sesame is genotype-dependent, similarly to the findings in other species as *Arachis hypogaea*, and also that the bioactivity varies according to the target insects (Martins et al., 2014).

The Brazilian Company of Agricultural Research (Embrapa) holds a Sesame Germplasm Collection, with over 1500 national and international accessions, some of which are used in breeding program for development of cultivars and top lines to semiarid environment (Arriel et al., 2009). Some traits such as grain yield, earliness, oil quality and tolerant to pest and disease are quite useful in selection procedures (Arriel et al., 2009). The identification of high-PIs accessions may be an additional activity to contribute with the sesame breeding to tolerance to stored grain-pests. Then, the present study aimed to investigate the bioactivity of seed sesame-trypsin inhibitors (TI) on gut enzymes of *P. interpunctella*, based on biochemical and feeding experiments.

MATERIALS AND METHODS

Protein extraction and fractioning

The sesame seeds used in this study were harvested in 2013 in Barbalha, CE, (07° 18'18 "S, 39° 18'07" W, 414 m), in a semiarid region of Brazilian northeastern. Seeds (3 g) of ten sesame genotypes (Table 1) were used for total protein extraction, starting with delipidation (Bland and Lax, 2000). Delipidated samples (100

mg) were homogeneized in 1 ml Tris-HCl 0.05 M buffer, pH 8.5 and centrifuged at 12.000 x g for 20 min at 4°C. The supernatant was recovered and incubated at 4°C for 16 h. On the next day, it was performed a new centrifugation and the supernatant was collected and stored at -20°C. The seed protein crude extracts (SPCE) were fractionated by sequential precipitation with ammonium sulfate at 0-30%, 30-60% and 60-90% saturation levels, according to the Green and Hughes method (1955). These fractions were dialyzed in Tris-HCl 0.05 M buffer, pH 8.5 and termed FI (0-30%), FII (30-60%) and FIII (60-90%), according to their respective degrees of saturation, and were kept at -20°C. The partially purified extracts and SPCE were quantified using Bovine Serum Albumin (BSA) as standard (Bradford, 1976), in a spectrophotometer (Femto, Model 700S) at 595 nm.

Determination of antitrypsin activity in *P. interpunctella*

Midguts collected from fifty 5th instar (10 days) larvae of *P. interpunctella* were sectioned and transferred to microtubes (1.5 mL) containing 200 µL of Tris-HCl 0,05 $M.L^{-1}$ buffer, pH 8.5 and homogenized. The homogenate was centrifuged at 12.000 x g, for 30 min, at 4°C, and the supernatant was quantified (Bradford, 1976) and used in inhibition enzyme assays. Briefly, the antitryptic activity was based on the following steps (Martins et al., 2014): |(a) Bovine pancreatic trypsin assay with SPCE; (b) Bovine trypsin assay with trypsin inhibitor partially purified from sesame seeds; (c) in vitro assays with digestive enzyme of insects and SPCE, and (d) in vitro assays with digestive enzyme of insects with trypsin inhibitor partially purified from sesame seeds.

The reactions were performed in a microtube (1.5 mL) containing the following components (Kakade et al., 1969): 5 µL of bovine pancreatic trypsin (BPT, 1 µg/µL) or 5 µL of insect digestive enzyme preparations (4 µg/µL), 20 µL of seed protein crude extract or partial purified seed trypsin inhibitor (3.5 µg protein/µL), 125 µL of 50 mM Tris-HCl buffer pH 8.5. The reaction was pre-incubated at 37ºC for 20 min and 200 µL azocasein (1.5%, m/v) was added, after which it was incubated again at the same temperature and period. The reaction was discontinued with 300 µL of 20% trichloroacetic acid. After 5 min at room temperature, the samples were centrifuged at 12.000 x g for 10 min. An aliquot of 250 µL of supernatant was collected and added to 250 µL of 2 mM NaOH; the reading was performed in a spectrophotometer at 440 nm. All assays were performed in triplicate with three biological repetitions and with blanks. Reagents from Sigma Aldrich (USA) were used in these assays.

Thermal and pH stability of the inhibitory extract

These tests were conducted with partially purified protein extract (fraction FII) from seeds of the genotypes BRS Seda, CNPA G3 and CNPA G4, following the methodology described by Gomes et al. (2005). To estimate the thermal stability, the samples (1 mL) were previously incubated for 30 minutes at temperatures of 40, 60, 80 and 100°C; and to determine the pH stability, the samples (1 mL) were previously dialyzed at different pHs using the following buffers: 50 mM sodium phosphate, pHs 6.5 to 7.5, and 50 mM Tris-HCl, pHs 8.5 to 10.5. Then, the samples were incubated at 37°C and dialyzed again for 4 h in 50 mM Tris-HCl, pH 8.5. The antitrypsin activities were determined according to Kakade et al. (1969), using the preparation of digestive enzymes of *P. interpunctella* and BPT. All assays were performed in triplicate with three biological replicates.

In vivo bioassays using *P. interpunctella*

Fraction FII and SPCE from genotypes BRS Seda, CNPA G3 and CNPA G4 were used in feeding assay with 2nd larvae (4 days) of *P. interpunctella*. Both extracts were lyophilized (Liotop, L10L model) for 24 hours and added to the artificial diet (Amorim et al., 2008) at 0.1, 0.3, 1, 1.5, 3 and 6%. Bioassays were performed in 24-cell plates (1 larva/cell, with 12 larvae/treatment) containing 200 mg of diet. The plates were incubated in Biochemical Oxygen Demand (BOD) chamber at 25°C, 65 to 70% relative humidity and photoperiod of 12:12 h. The assay was completely randomized with 4 replications. The mortality rate of the larvae was estimated until 30 days.

Statistical analyses

The data were statistically analyzed using the Sisvar software system (Version 5.1). The means were compared by Tukey test (p<0.05). The regression curves were generated and the lethal concentrations were defined (LC 50%) for bioassays.

RESULTS

Inhibitory activity of SPCE on BPT and digestive enzymes of *P. interpunctella*

The inhibitory activity of SPCE on BPT from all genotypes assessed ranged among 51 to 90%, revealing average rate of 88% to cvs. BRS Seda, CNPA G3 and CNPA G4 (Table 1). In order to estimate the inhibitory activity of SPCE on digestive enzymes of *P. interpunctella* larvae, the extracts of these three genotypes were used, showing inhibition rate of 77, 60 and 67%, respectively (Figure 1).

Inhibitory activity of fractions FI, FII and FIII on BPT and digestive enzymes of *P. interpunctella*

Figure 2 shows the inhibition rates obtained from FI, FII and FIII protein fractions from BRS Seda, CNPA G3 and CNPA G4 seeds on BPT and digestive enzymes of *P. interpunctella.* The FII fraction provided high rate of inhibitory activity as tested on BPT and digestive enzymes of larvae, in all genotypes with averages of 78, 60 and 70% (Figure 2a) and 68, 55 and 59% (Figure 2b), respectively.

Thermal stability of FII fraction on bovine pancreatic trypsin and digestive enzymes of *P. interpunctella*

The thermal stability of FII fractions on bovine pancreatic trypsin was quite similar in both BRS Seda and CNPA G3, with inhibition rate between 58 and 38% at 40 to 100ºC. CNPA G4 was more stable showing rates of 63 and 40% at 40 to 100ºC (Figure 3a). On digestive enzymes of *P. interpunctella*, the thermal stability was similar in all three cultivars, with inhibition rate of 55% at 40ºC, with further gradual reduction and keeping in about 45 at 100ºC (Figure 3b).

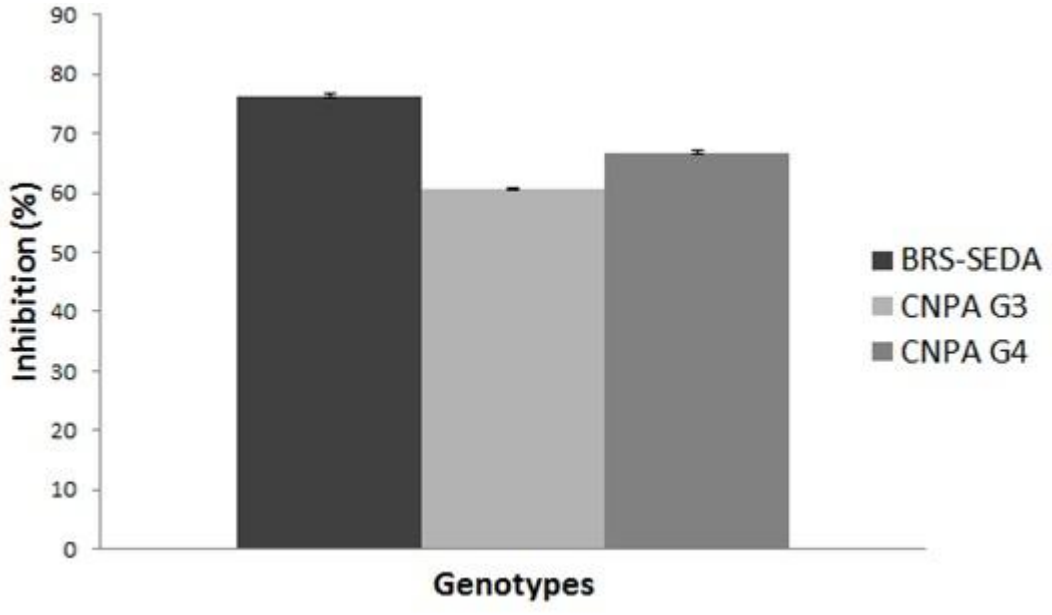

Figure 1. Inhibition rate of digestive enzymes of larvae of *P. interpunctella,* by using seed protein crude extracts of BRS Seda, BRS G3 and BRS G4 sesame genotypes. Data are mean values ± SD of three biological replicates.

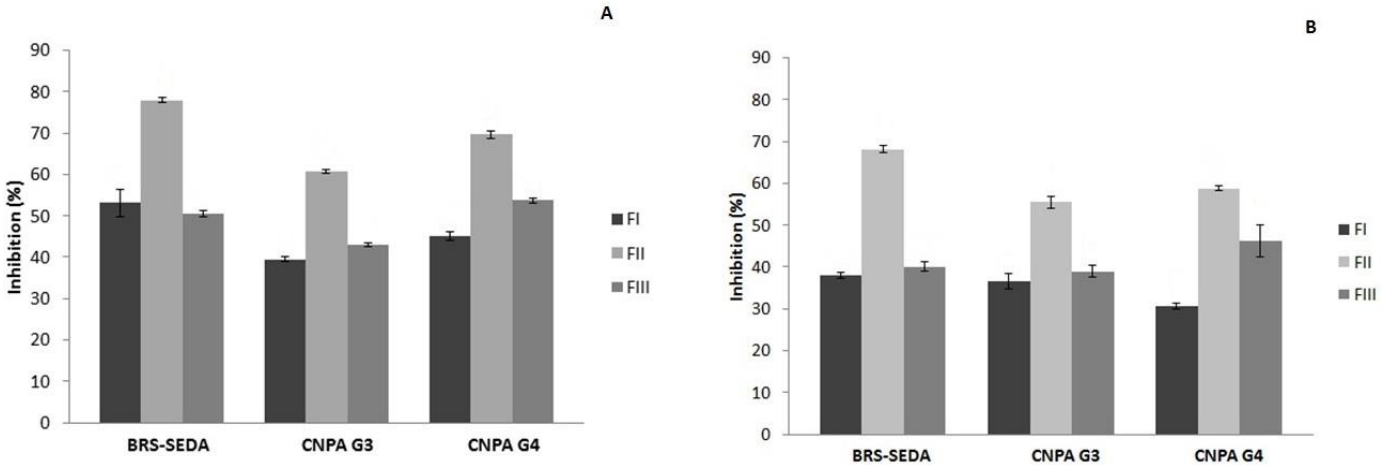

Figure 2. Inhibition of bovine pancreatic trypsin (A) and digestive enzymes of *P. interpunctella* (B) by using FI, FII and FIII fractions from seed protein crude extract in different sesame cultivars. Data are mean values ± SD of three biological replicates.

The inhibition rates on bovine pancreatic trypsin and digestive enzymes of *P. interpunctella* for FII fraction at different pHs are shown in Figure 4. The highest inhibition rates for both bovine pancreatic trypsin (Figure 4a) and digestive enzymes of *P. interpunctella* (Figure 4b) were obtained at pH 8.5, with means of 73 and 63%, respectively, in all three cultivars. The inhibition rate for bovine pancreatic trypsin decreased from pH 9.5, reaching less than 20% at pH 10.5. On the other hand, for digestive enzymes, that rate remained close to 45% at pHs above of 8.5.

Feeding bioassays with *P. interpunctella*

In bioassays with *P. interpunctella*, 2nd instar larvae were fed on SPCE and FII fraction of BRS Seda, CNPA G3 and CNPA G4, at different concentrations. As seen in Table 2, statistically significant difference was found only for concentrations tested. No effect of interactions arising from genotypes was observed.

The average of mortality rates of *P. interpunctella* in the treatments with SPCE and FII fraction of the three cultivars were 74 and 45%, respectively (Tables 3 and 4).

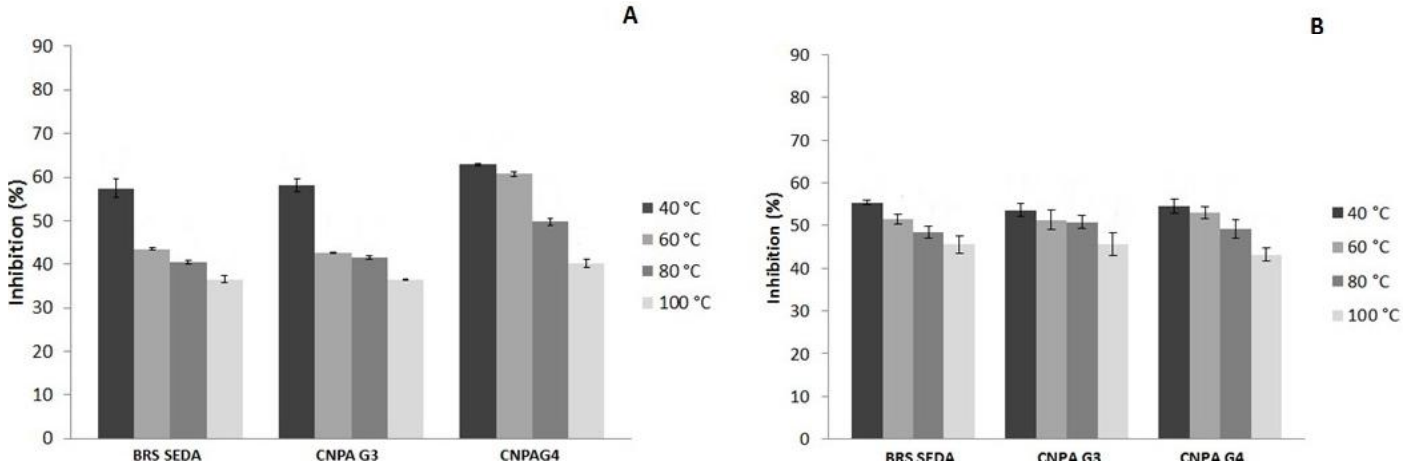

Figure 3. Thermal stability of FII fraction of seed protein crude extract on bovine pancreatic trypsin (A) and digestive enzymes of *P. interpunctela* (B). Data are mean values ± SD of three biological replicates.

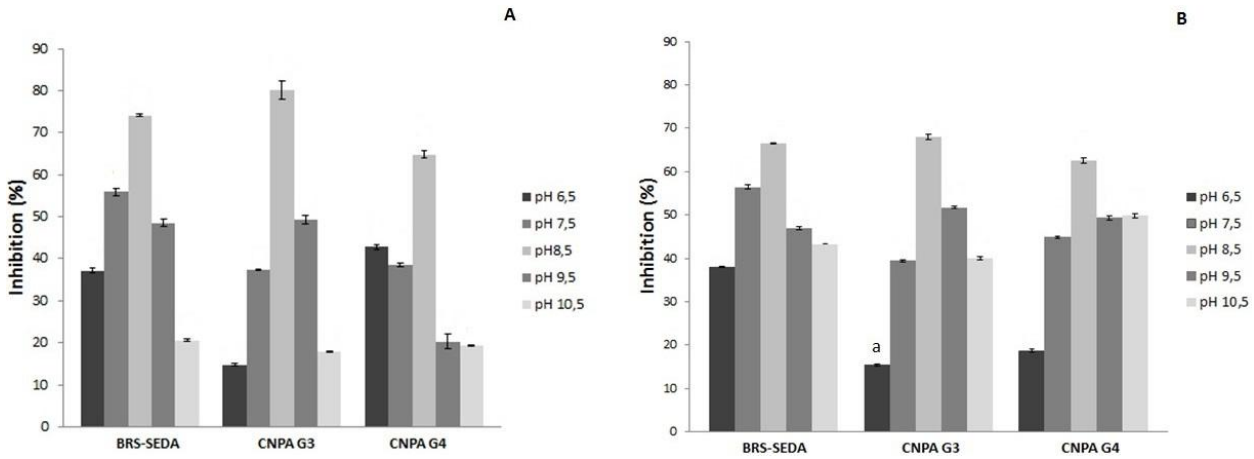

Figure 4. PH stability of FII fraction of seed protein crude extracts on bovine pancreatic trypsin (A) and digestive enzymes of *P. interpunctella* (B). Data are mean values ± SD of three biological replicates.

Figure 5 shows the regression curves related to mortality rate in both treatments. The estimated CL_{50} of FII fraction was 0.55% to BRS Seda and 0.70% to CNPA G3 and CNPA G4. However, in the treatments with SPCE, CL_{50} could not be determined due to high mortality rate.

DISCUSSION

Plant PIs, known as anti-enzymes, are widely studied in various biological processes, including those related to plant defense against pathogens and insect pests. These metabolites could be a strategic alternative to pest control, either by natural or transgenesis means (Bariani et al., 2012; Macedo et al., 2013; Ranjbar et al., 2014).

In this work, PIs of sesame seeds were investigated for their ability to inhibit bovine pancreatic trypsin and digestive enzymes of *P. interpunctella* based on *in vitro* and *in vivo* assays. In *in vitro* assays, it was found that the SPCE of three sesame cultivars, developed by Embrapa, allowed high rate of inhibition both for bovine pancreatic trypsin and digestive enzymes of the insect.

The activity of these extracts on digestive proteases of *P. interpunctella* larvae was verified by feeding bioassays, which demonstrated mortality rate of 74% in larvae fed on diet containing SPCE, and only 45%, when FII fraction were provided. This reduced mortality rate suggests that, although the highest rate of inhibition on digestive enzymes of *P. interpunctella* has been detected in FII fraction, both FI and FIII must have some intrinsic functions that could contribute to raise the mortality rate of insect. As they were absent in the extract, the mortality rate was 39% lower, considering the average observed

Table 2. Analysis of variance for mortality rates of *P. interpunctella* fed on SPCE and FII fraction from different sesame genotypes, at different concentrations.

SV	DF	MS	SPCE		FII		
			F	P>F	MS	F	P>F
Genotype (G)	2	0.013	1.024	0.366 ^{ns}	0.001	0.070	0.932^{ns}
Dose (D)	5	0.334	26.23	0.000**	0.166	11.34	0.000**
G*D	10	0.003	0.200	0.995^{ns}	0.001	0.059	1.000^{ns}
Error	54	0.013			0.015		
CV (%)		10.84			13.01		

SV: Source of variation; DF: Degree of freedom; MS: Mean square; F: F test; P: Probability test; CV: Coefficient of Variation. ns- No significant, **significant at 1% probability.

Table 3. Mortality rate (%) of *P. interpunctella* fed on SPCE from different sesame genotypes, at different concentrations.

Genotypes	Concentration (%)					
	0	0.1	0.3	1	1.5	3
BRS Seda	0.0^{Ab}	67^{Aa}	83^{Aa}	83^{Aa}	83^{Aa}	83^{Aa}
CNPA G3	0.0^{Ab}	58^{Aa}	58^{Aa}	67^{Aa}	75^{Aa}	83^{Aa}
CNPA G4	0.0^{Ab}	67^{Aa}	67^{Aa}	75^{Aa}	75^{Aa}	83^{Aa}
Average	0.0	64	69	75	78	83

* Means followed by the same letter in the line and column do not differ statistically by the Tukey test (p <0.05). Capitalized letters compare genotypes and lowercase letters, concentrations.

Table 4. Mortality rate (%) of *P. interpunctella* fed on FII fraction from different sesame genotypes, at different concentrations.

Genotypes	Concentration (%)					
	0	0.1	0.3	1	1.5	3
BRS Seda	0.0^{Ab}	33^{Aa}	50^{Aa}	50^{Aa}	50^{Aa}	58^{Aa}
CNPA G3	0.0^{Ab}	33^{Aa}	42^{Aa}	42^{Aa}	50^{Aa}	58^{Aa}
CNPA G4	0.0^{Ab}	33^{Aa}	42^{Aa}	50^{Aa}	50^{Aa}	58^{Aa}
Average	0.0	33	45	47	50	50

Means followed by the same letter in the row and column do not differ statistically by the Tukey test (p <0.05). Capitalized letters compare genotypes and lowercase letters, concentrations.

with SPCE. These results allow inferring that interactions between these fractions may lead to potential benefits for plant protection against insects, especially because, as a complex of proteins, specific components in the extract may affect the binding and inhibition of proteases associated to digestion (Linser et al., 2009; Vinokurov et al., 2006; Dow, 1992).

The three cultivars evaluated in this study showed potential to control *P. interpunctella,* however, considering the results of inhibitory activity of SPCE and inhibition of digestive enzymes of insect with the fractions of the extract, BRS Seda seems to be the most suitable for further studies involving tolerance to storage grain pests. Moreover, the CL_{50} obtained from FII fraction of this cultivar was only 0.55%, demonstrating that it is an interesting candidate to sesame breeding program.

Another relevant result seen in BRS Seda is that the protein extracts retained the inhibitory activity above 50% at pH ranging between 7.5 and 8.5. It means that the extract from the seeds could be able to fight various species of insects if the intestinal pH is at this range of alkalinity. It must be highlighted that BRS Seda, along with CNPA G4, presented inhibitory activity at pH 6.5, although the rate did not exceed 40%. This confirms one of the characteristics of trypsin inhibitors, which can act in media ranging from neutral to alkaline, and thus become more relevant for plant defense against insects, since the intestinal lumen may also vary in the same proportions (Terra and Ferreira, 1994; Lopes et al., 2006).

Some species of coleoptera have acidic pH in the

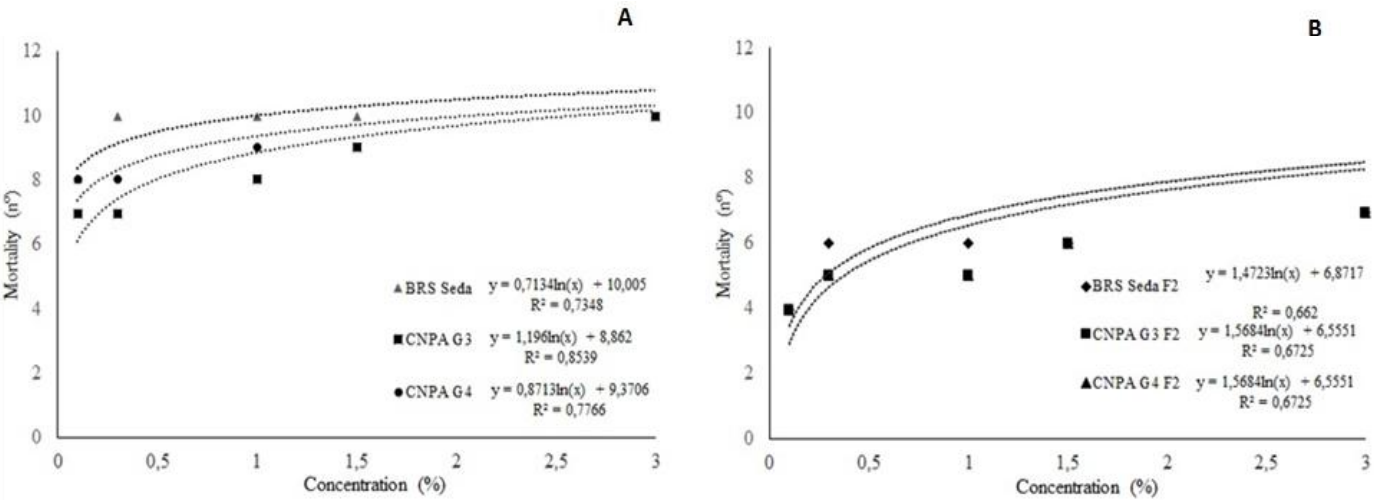

Figure 5. Mortality rate of *P. interpunctella* larvae fed on diet containing different concentrations of SPCE (A) and FII fraction (B) of different sesame genotypes.

anterior midgut (pH 5.2-5.6), where cysteine proteases predominate, and slightly alkaline (pH 7.8-8.2) in the posterior region, where serine proteases are in activity (Vinokurov et al., 2006; Terra and Cristofoletti, 1996). In lepidopteran, most of the contents of the intestinal lumen (endoperitrophic region) has alkaline pH, while the ectoperitrophic portion (outside the membrane) has pH next to neutrality (Rossi et al., 2009; Rossi et al., 2012; Ranjbar et al., 2014). Thus, since the protein extracts of the cultivars investigated in this study showed better inhibitory activity at pH 8.5 for serine proteases, it is suggested that all of them could be used in a sesame program aiming to tolerance to *P. interpunctella* or even other lepidopteran insects. Further studies are necessary to attest these assumptions.

As to temperature, results found here showed that the inhibitory activity of FII fraction of three cultivars maintained a rate above 40% up to 80°C, in both experiments with bovine pancreatic trypsin and digestive enzymes of *P. interpunctella*, revealing a subsequent decreasing of 30% at most, when submitted to 100ºC. This is an interesting result since the inhibitory capacity of proteins depends on temperature. Above 40°C, the activity of some proteins is reduced or even inactivated as result of denaturation. It could be a limiting factor to its use to insect control in semiarid environments where the temperatures of the soil and the air are often high. In thermostability assays using protein extracts from peanut seeds tested on bovine trypsin, Martins et al. (2014) reported maintenance of inhibitory activity above 60%, at 40 to 100ºC. In *Peltophorum dubium* Spreng, however, Macedo et al. (2013) tested the thermostability of the protein extract and found that trypsin inhibitors attenuated the inhibitory capacity from 80ºC. According to Bariani et al. (2012), these different responses can be explained by the three-dimensional structure of each protein, considering the formation of amino acids and peptide bonds that constitute the structure and provide functional stability.

Conflict of Interest

The authors declare no conflict of interest between the partners with the dissemination of results.

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