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Trypsin (serine protease) inhibitors in peanut genotypes aiming for control of stored grain pests

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Peanut seeds from different genotypes were evaluated for activities of trypsin inhibitors (serine protease), based on *in vitro* and *in vivo* assays, aiming to further selection of genitors in breeding programs for tolerance of stored grain pests. The *in vitro* assays were based on inhibition of insect digestive enzymes and also on thermal and pH stabilities of seed protein, while the *in vivo* assays were performed with insects *Alphitobius diaperinus*, *Tribolium castaneum*, *Tenebrio molitor* and *Spodoptera frugiperda*. Seed inhibitors of all genotypes inhibited bovine trypsin at 70 to 94%. The seed extract of BRS Havana inhibited *T. castaneum* and *T. molitor* up to 80% while the extract of BRS 151 L7 inhibited *A. diapennu*s at nearly 20%. The seed inhibitors of both cultivars were stable at 80°C and also at different pH values. The two peanut genotypes are recommended as promising parents for breeding program aiming to selecting lines with tolerance to *Tenebrio* and *Alphitobius* insects.

Key words: Trypsin inhibitor, *Arachis hypogaea*, lepidoptera, coleoptera.

INTRODUCTION

Peanut is an important oleaginous known for its broad environmental adaptation. One of the major bottlenecks in the management is the post harvest phase mainly storage of grains. In this stage, the problems with storage pests are recurrent especially those caused by weevils: *Tribolium castaneum (Horbst, 1797) (Coleoptera: Tenebrionidae), Alphitobius diaperinus (Panzer, 1797) (Coleoptera: Tenebrionidae), Plodia interpunctella (Hübner, 1813) (Lepidoptera: Pyralidae), Tenebrio molitor (Linnaeus, 1758) (Coleoptera: Tenebrionidae), Corcyra cephalonica (Stainton, 1865) (Lepidoptera: Pyralidae) and Tenebroides mauritanicus (Linnaeus, 1758) (Coleoptera: Ostomidae)*. Depending on the level of infestation, the control of weevils can become unfeasible due to high costs with chemical treatment.

Genetic resistance to insect-pests is a desired goal by the most plant breeder. However, the acquisition of this trait is a big challenge due to heavy interactions between genetic and environmental factors. Thus, other natural strategies should be researched to detect tolerant

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Table 1. Main traits of peanut genotypes used in this study.

Genotype	G	SP	BT	GH	C	SS	O	CS	100S
BRS Perola Branca	C	H	Vi	R	110-115	L	50-52	White	55
176 AM	L	F	V	U	90-95	M	46-48	Red	43
173 AM	L	F	V	U	90-95	M	46-48	Tan	51
Florunner	C	H	Vi	R	120-130	L	49-51	Tan	50
IAC Caiapó	C	H	Vi	R	120-130	L	48-50	Tan	51
186 AM	L	F	V	U	90-95	L	46-48	Red	46
BRS Havana	C	F	V	U	88-90	M	43-45	Tan	50
L7 bege	L	F	V	U	88-90	L	45-47	Tan	61
175 AM	L	F	V	U	90-95	M	46-48	Tan	47
LGoPE-06	L	H	Vi	R	120-130	EL	50-52	Tan	72
BRS 151 L7	C	F	V	U	85-87	L	46-48	Red	67

G: Genealogy, C- Cultivar, L- Top line; SP- Subspecies: F- fastigiata, H- hypogaea; BT- Botanic type: V- Valencia, Vi- Virginia; GH- Growth habit: R- Runner. U- Upright; C- Cycle (days); SS- Seed size: M- Medium, L- Large, EL- Extra large; O- Oil content (%); SC- Seed colour and 100S- Average of 100 seed weight.

genotypes.

Focusing on stored grain pests, it is known that several plant species produce proteins with insecticidal property, such as inhibitor of proteases (IPs) which play a key role in plant defense against various orders of insects (Marinho et al., 2008; Pereira et al., 2007). The insecticidal activity of IPs is due to inhibition of proteolytic enzymes in the midgut of insects, leading to malnutrition, delay in larvae development and even death (Mosolov and Valueva, 2008). Among the groups of proteolytic enzymes affected by IPs, serine proteinases are the most investigated (Oliveira et al., 2005; Habib and Fazili, 2007).

Peanut seeds have different levels of IPs but information on using this trait for selecting genotypes tolerant to pests of stored grain is limited (Suzuki et al., 1987; Norioka et al., 1981). Considering the wide genetic base between intraspecific accessions of *A. hypogaea*, it is possible to identify promising materials for further use in hybridization works aiming subsequent selection of top lines with different level of tolerance to these pests.

The present research aimed to estimating the inhibitory activity of trypsin (serine protease) in seeds of different peanut genotypes for further recommendation of parents in breeding programs to tolerance to store grain pests.

MATERIALS AND METHODS

Extraction of proteins and determination of antitryptic activity

The peanut seeds used in this study were collected in January 2011 and January 2012 in Barbalha, CE (07 ° 18'18 "S, 39 ° 18'07" W, 414 m), semiarid region of northeastern Brazil. The study begin when the seeds were 8% moisture. The main traits of peanut genotypes are found in Table 1. Total crude protein of each genotype was extracted using methodology described in Bland and Lax (2000). The proteins were quantified by Bradford method (Bradford, 1976) at 595 nm using bovine serum albumin (BSA) asanalytical standard.

A previously described methodology was used to determine the antitryptic activity based on the following steps: a) bovine trypsin assay with seed total crude extract; b) bovine trypsin assay with partial purified seed trypsin inhibitor; c) insect digestive enzyme preparations assay with seed total crude extract; d) insect digestive enzyme preparations assay with partial purified seed trypsin inhibitor. A summary of the methodology is described below (Kakade et al., 1969).

In a microtube, 1.5 ml was performed following reaction: 5 µl of bovine trypsin (1 µg/µl) or 5 µl of insect digestive enzyme preparations (1 µg/µl), 20 µl of seed total crude extract or partial purified seed trypsin inhibitor (5 µg), 125 µl of 50 mM Tris-HCl buffer pH 8.5. The reaction was pre-incubated at 37°C for 20 min and 200 µl azocasein (1.5%, m/v) was added and again incubated at the same temperature and period. The reaction was discontinued with 300 µl of 20% trichloroacetic acid. After 5 min at room temperature, 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 (Femto, model 700S) at 440 nm. All assays were performed with three repetitions. Reagents from Sigma Aldrich (USA) were used in this assay.

Intestinal extract assays

Twenty third-instar larvae of *A. diaperinus*, *T. molitor*, *T. castaneum* and *Spodoptera frugiperda* (Lepidoptera: Noctuidae) were fed on artificial diet and then dissected for collecting the guts. *S. frugiperda* was included because it is an important crop pest and is susceptible to protease inhibitors of soybean (*Glycine max* L.) and bean (*Vigna radiata* L. Wilczek) seeds (Brioschi et al., 2007; Paulillo et al., 2000).

Dissected tissues were immediately immersed in 50 µl of 50 mM sodium phosphate buffer pH 7.5 and maintained at 4°C. After macerated, the homogenates were centrifuged at 12.000 x g for 20 min at 4°C. The supernatants were collected for further use in enzymatic assays for antitryptic activity assays with the seed total crude extract (Terra et al., 1977).

Hatching bioassay with *A. diaperinus*

Bioassays were carried out in order to estimate the hatching rate of

Table 2. Inhibitory activity of the seed total crude extract of peanut genotypes with bovine trypsin.

Genotype	Inhibition rate (%)
175 AM	94.2[a]
Florunner	92.6[a]
176 AM	92.2[a]
BRS Havana	90.3[ab]
186 AM	89.7[b]
173 AM	89.7[b]
IAC Caiapó	88.2[bc]
L7 Bege	79.3[bc]
BRS Pérola Branca	78.5[c]
LGoPE-06	78.0[c]
BRS 151 L7	70.7[d]

Means followed by the same letter do not differ significantly by the Tukey test (p≤0.05). Variance analysis to Inhibition rate: Mean square of treatment: 292.31, Standard error: 0.32, Freedom degree: 10, F test: 929.24**, Average: 85.60, Coefficient of variation: 0.66. **significant by the Friedman test (p≤0.01).

A. diaperinus fed on peanuts seeds (50 g). The seeds of each genotype were infested with 20 adults sexed and placed in plastic pots (8.0 cm of height x 11.0 cm in diameter). The pots were stored at room temperature for 90 days. Corn bran was used in control treatment. The bioassays were completely randomized with four replications.The number of larvae was registered at 53 and 90 days and hatching rates were estimated (Azevedo et al., 2010). The data were analyzed by the Friedman test (p ≤ 0.05) and the means were compared by Student Newman Keuls test (p ≤ 0.05).

Stability of seed trypsin inhibitors

This assay was performed in order to study the stability of seed-protease inhibitors at different temperature and pHs. The crude extracts were previously fractionated by sequential precipitation with ammonium sulfate at saturation ranges of 0 to 30, 30 to 60 and 60 to 90%. The 60 to 90% fraction showed the highest antitryptic activity (data not shown), and therefore was chosen as the peanut seed trypsin inhibitor fraction for stability assays. The procedures were based on that described by Gomes et al. (2005). To investigate thermal stability, the 60 to 90% fraction (1 ml, 1 µg/µl) was incubated for 30 min at 40, 60, 80 and 100°C, and thereafter cooled to 4°C. For characterization of the pH stability, the same volume of the 60 to 90% fraction was dialyzed for 16 h using the following buffers: 50 mM sodium phosphate, pHs of 5 to 8 and 50 mM Tris-HCl, pHs of 9 to 11. Then, the samples were incubated at 37°C and again dialyzed for 4 h in 50 mM Tris-HCl, pH 8.5. An aliquot of 5 µl of each sample was used for assessment of the remaining antitryptic activity with bovine trypsin. All assays were carried out in five replications. Data were analyzed by the Friedman test (p ≤ 0.05) and the means were compared by Tukey test (p ≤ 0.05).

RESULTS

Determination of antitryptic activity

Bovine trypsin was inhibited in all peanut samples analyzed, whose means ranged from 70 to 94% (Table 2). The genotypes 175 AM, Florunner, 176 AM and BRS Havana showed the same statistical classification with average of inhibition rate of 92%.

Based on the results in Table 2, all genotypes with inhibition rate at least 90% were selected for intestinal inhibition assays with *T. castaneum*, *T. molitor*, *S. frugiperda* and *A. diaperinus*. The genotype BRS 151 L7, with the lowest inhibition rate was also chosen considering that many have different response as to the types of protease inhibitors likely present in the seeds. As seen in Figure 1, the percentages of inhibitory activity of seed crude protein extract from different genotypes were almost uniform for *T. castaneum* (between 70 and 81%) and *S. frugiperda* (45 to 48%). However, different responses were obtained for *T. molitor* (35 and 83.6%) and *A. diaperinus* (8 to 19%).

The extract obtained from BRS Havana was very promising to inhibit *T. castaneum* and *T. molitor*, with inhibition rate up to 80%. For *S. frugiperda*, the extract of all genotypes showed average inhibition rate of 47% while for *A. diaperinus* BRS 151 L7 showed the highest inhibition rate close to 20% (Figure 1).

Feeding bioassay with *A. diaperinus*

In order to validate the information contained in Figure 1, a hatching bioassay was carried out using sexed adults of *A. diaperinus* fed on peanut seeds. This species was chosen due to high incidence in Brazilian grain storages. It was observed that larvae hatching were negatively affected by feed supplied and incubation period (Table 3). At 90 days, the number of larvae fed on peanut seeds was 33% less than at 53 days. As this average involved

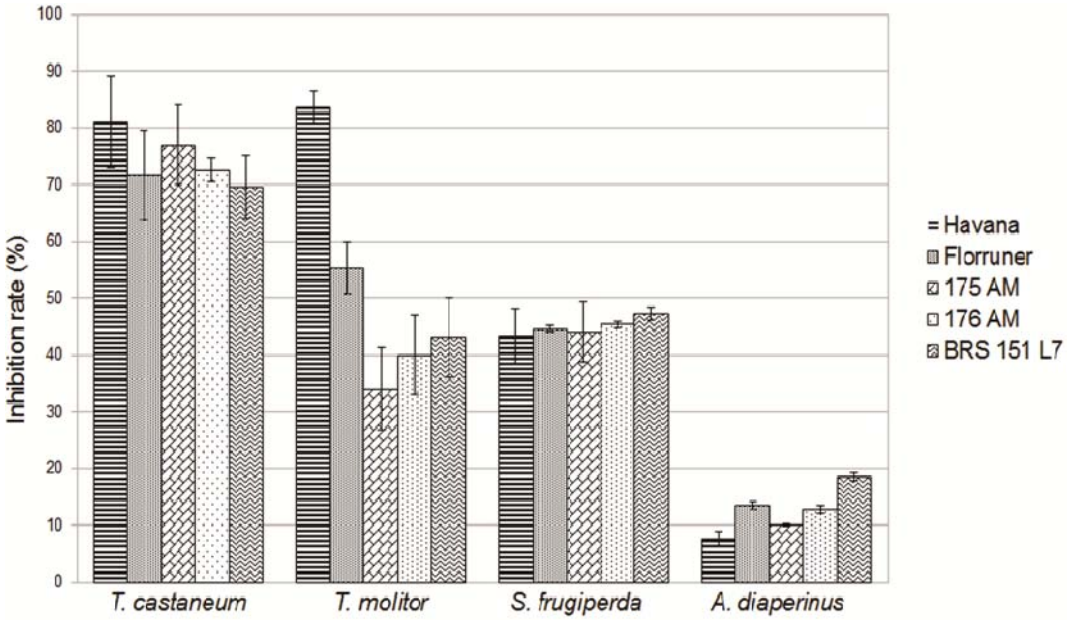

Figure 1. Inhibitory activity of seed total crude protein extracts from the peanut genotypes with insect digestive enzyme preparations of *T. castaneum*, *T. molitor, S. frugiperda* and *A. diaperinus.* Data are the mean values ± SD of three biological replicates.

Table 3. Number of hatched larvae of *A. diaperinus* fed on peanut seeds and corn bran.

Treatment	Number of larvae	DRC (%)
Period of incubation (days)		
53	9.00[a]	-
90	6.08[b]	33
Diet		
BRS 151 L7	5.52[bc]	51
Florunner	5.89[b]	47
175 AM	6.34[b]	43
176 AM	6.51[b]	42
BRS Havana	9.39[ab]	16
Corn bran (control)	11.17[a]	-

DRC- difference in hatching rate. Means with the same letter do not differ significantly by the Tukey test ($p \leq 0.05$). Means were transformed into $\sqrt{x}$ for statistical analysis. Variance analysis to period of incubation: Standard error: 91.16, Freedom degree: 1, F test: 24.41**, Average: 7.54; Variance analysis to Diet - Standard error: 40.91, Freedom degree: 5, F test: 10.77**; Average: 7.47. Coefficient of variation: 22.47%. ** significant by the Friedman test ($p \leq 0.01$).

most treatments with peanut seeds, it was suggested that this reduction may be associated with the greater period of insect feeding. This can be evident by the number of hatched larvae in different treatments. It was also verified that insects fed for 90 days on seeds from BRS 151 L7, Florunner, 175 and 176 AM had hatching rate reduced around 46% compared to control (corn bran). Among these genotypes, however, BRS 151 L7 revealed greater inhibitory rate (51%), which was in agreement with data recorded in Figure 1.

Stability assay of peanut seed trypsin inhibitors

The partial purified seed trypsin inhibitors from peanut genotypes showed thermostability in an interval of 40 to 100°C, with inhibition rate up to 60%, highlighting 175 AM who kept the inhibitory rate above 85% at all temperatures (Figure 2). An exception was verified to Florunner that had inhibitory activity substantially decreased at 100°C. As to pH stability of protease inhibitors, it was verified that the inhibitors from most

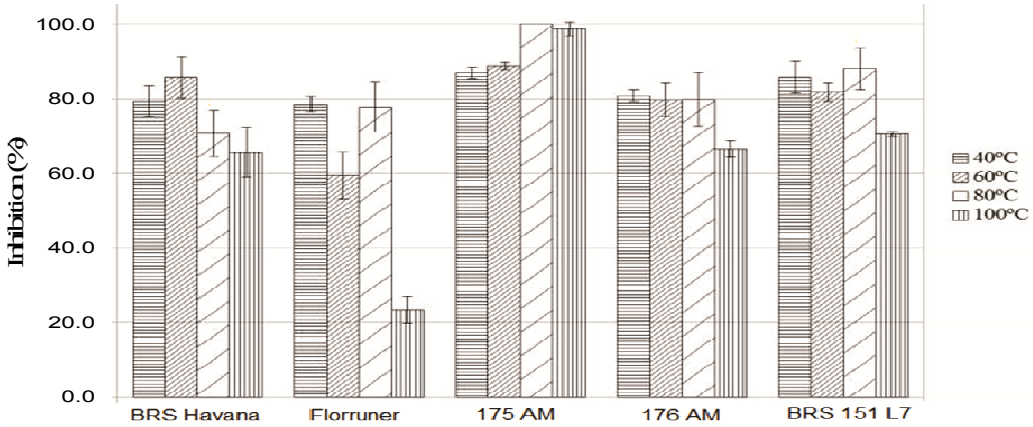

Figure 2. Thermal stability of peanut seed trypsin inhibitors from 60 to 90% fraction pre-incubated at different temperatures and tested with bovine trypsin. Data are the mean values ± SD of three biological replicates.

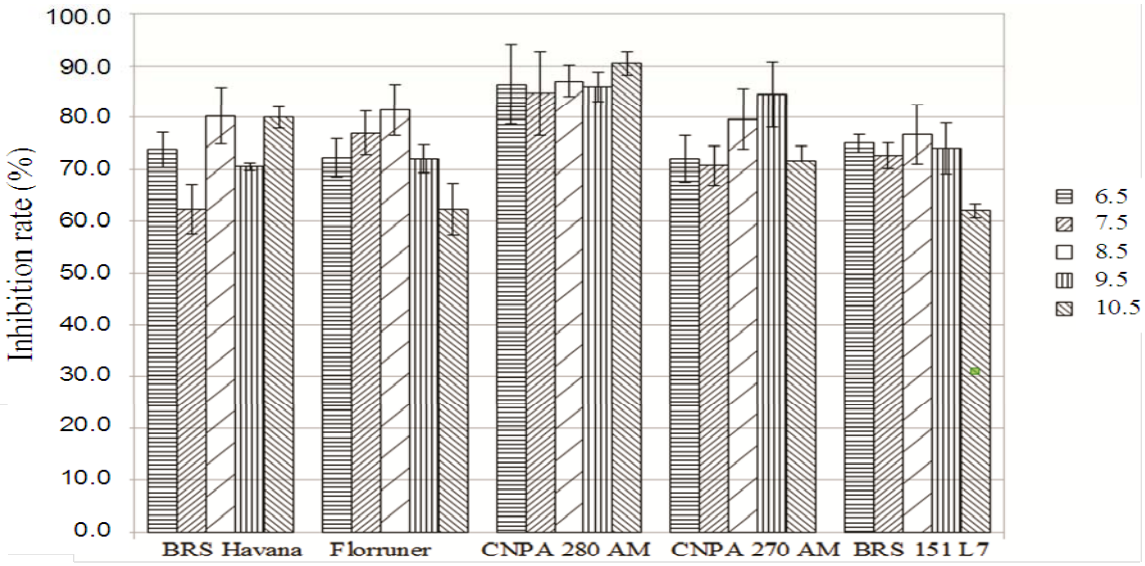

Figure 3. pH stability of peanut seed trypsin inhibitors from 60 to 90% fraction pre-incubated at different pHs and tested with bovine trypsin. Data are the mean values ± SD of three biological replicates.

genotypes maintained inhibition rate up to 70% at pH ranging between 6.5 and 9.5. Above this value, a small reduction in the rate was seen in extracts from Florunner and BRS 151 L7. Even so, it surpassed 60% which is still a reasonable rate of inhibition (Figure 3). The top line 175 AM showed large pH stability keeping the inhibition rate up to 80% at all pH range evaluated.

DISCUSSION

Based on high inhibition rates obtained in antitryptic activity assays using different peanut genotypes, we concluded that all materials showed great potential for further use in breeding programs aiming to tolerance to stored grain pests. Considering the rates obtained (70 to 94%, Table 2), it is suggested that proteases inhibitors are abundant in the seeds and this trait must be quantitative with high heritability. Such inference is based on studies developed by Dam and Baldwin (2003) and Kollipara et al. (1996), involving genetic inheritance of genes encoding to high expression of IPs in *Glycine tomentella* and *Nicotiana attenuata*. According to these authors, the inheritance is dominant and highly heritable. These findings are relevant when considering the perspective of transferring IP genes by conventional

hybridization.

Despite relevance of these data, *in vitro*-inhibition assays are not enough to define promising genotypes for a breeding program aiming to tolerance to stored grain pests. The bioassays with insects are essential to complement the information, mainly because a high inhibition rate to a given insect cannot be the same to another, due to particular differences in the families of intestinal proteases as well as to variations in pHs of insect guts. These are what really influence on binding and expressiveness of IPs to target proteins associated to insect digestion (Linser et al., 2009; Vinokurov et al., 2006; Dow, 1992).

The results shown in Tables 2 and 3 demonstrated the assertive in inclusion of cv. BRS 151 L7 in additional assays planned in this work. Although, this cultivar showed the lowest inhibition rate *in vitro* assay (70%, Table 2), it revealed expressive reduction in hatching rate (51%, Table 3), by using *A. diaperinus. T. castaneum* and *T. molitor*, the cv. BRS Havana was the most promising genotype due to its high inhibition rate (about 80%) in bioassays with intestinal homogenate (Figure 1). This is an expressive result comparing inhibition rate of others leguminous species. With *Crotalaria pallida*, Gomes et al. (2005) found 74% inhibition in intestinal homogenate of *Callosobruchus maculatus*. In further works, it would be interesting to test the extract of BRS Havana also with this coleoptera.

The inhibition rates obtained to *S. frugiperda* were reasonable but should be taken with caution in a breeding program since larvae are able to overcome the deleterious effects of IPs, possibly due to wide ability to activating new trypsin-like enzymes, which are less sensitive to inhibitors produced by plants (Paulillo et al., 2000; Xavier et al., 2005). This is a natural defense process also verified in several classes of insects.

In relation to thermal and pH stabilities of peanut seed trypsin inhibitors, these traits are quite important for further selection of genotypes because all seeds can be facing thermal variations in post-harvest processes. The instability of the trypsin inhibitors may be a negative factor for selection in a breeding program, even though genotype shows satisfactory results in inhibition assays. In this work, the results of pH and thermal stabilities of the partial purified trypsin inhibitors of the peanut genotypes were very expressive. The inhibitory potential of the seed extracts remained between pH 6.5 to 9.5 (Figure 3) indicating that peanut seed-IPs can affect a range of insect pests, especially Coleoptera and Lepidoptera, which intestinal lumen-pH varies from neutral to alkaline, acid to neutral or acid to alkaline, depending on the species (Linser et al., 2009; Vinokurov et al., 2006; Dow, 1992). The thermostability of the proteins were also satisfactory, since all extracts kept the inhibition above 60°C, excepting to Florunner that did not tolerate temperatures at 100°C. Plants rich in IPs and that hold the stability of inhibition in this range of pH and temperature are excellent sources genetic resources for plant defense by conventional hybridization or by transgenesis.

For food crops, such as peanuts, this information becomes more relevant due to several commercial products that are processed from the grains. Since many cereals are consumed boiled, losses in the activity of this compound may lead to the inactivation of its function. Serquiz (2012) tested the thermal and pH stabilities of trypsin inhibitor (TI) from peanut candy and verified that peanut-TI was fairly resistant, keeping the total inhibitory activity over trypsin when heated to 80°C and reducing only 8% when tested at 100°C. Gomes et al. (2005) evaluated the effect of trypsin inhibitor from *Crotalaria pallida* seeds on *Callosobruchus maculates*, *Ceratitis capitata* and obtained good results of inhibition from fresh seeds. However, when the extract was heated at 100°C for 30 min, the inhibition activity was reduced to 50%.

The results presented in this work can contribute greatly to the planning of a peanut breeding program aiming to tolerance to stored grain pests. The both earliness BRS Havana and BRS 151 L7, developed by Embrapa were promising candidates for this proposal. Additionally, the segregating arising from this crossing would also offer a range of variability for grain and oil, based on the characters presented in Table 1. The top line 175 AM was also a promising material based on the results obtained in inhibition assays against *T. castaneum* (Figure 1) and feeding assay with *A. diaperinus* (Table 3). Furthermore, the thermal and pH stabilities also contribute to explain its selection. The combination of this genotype, that is a Florunner-descendant (Gomes et al., 2007), with BRS Havana and BRS 151 L7 would generate large variability populations due to their broad genetic base which involves ancestors of fastigiata, hypogaea and vulgaris subspecies (Duarte et al., 2013; Gomes et al., 2007). The perspective in obtaining rich-IPs lines would naturally increase considering the dominant inheritance that controls the trait and also the percentage of inhibition shown in Table 2. Before beginning the breeding procedures, however, it is recommended to validate the information in natural storage conditions.

Conflict of Interests

The authors have not declared any conflict of interests.

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