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The resistance of the cowpea cv. BRS Xiquexique to infestation by cowpea weevil is related to the presence of toxic chitin-binding proteins

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ABSTRACT

The cowpea weevil (*Callosobruchus maculatus*) is the main pest that attacks cowpea (*Vigna unguiculata*) seeds during storage, causing nutritional and economic losses in the cowpea crop. Thus, studies aiming to identify resistant cowpea cultivars have been developed. Chitin-binding proteins (CBP), such vicilins and chitinases, have been detected in seeds and related with the toxicity to insects. In this work, we investigated the presence of chitin-binding proteins in the partially resistant cowpea cv. BRS Xiquexique and evaluated their toxicity towards cowpea weevil. The CBP fraction was isolated by chitin affinity chromatography. CBP fraction showed, through 15% SDS PAGE, protein bands with varying molecular masses, mainly below 55 kDa. Proteins present in CBP fraction were identified by *Western blotting* and mass spectrometry analysis, as vicilins and chitinases. CBP fraction, at 5%, was able to interfere with the development of cowpea weevil, decreasing larval mass and length. A CBV (chitin-binding vicilin) fraction isolated from CBP fraction was toxic, at 2.0%, to *C. maculatus*, decreasing larval mass and length in 64.3% and 33.23%, respectively. These results suggest that chitin binding proteins, such vicilins and chitinases, may be related to the resistance of cowpea cv. BRS Xiquexique to the infestation by *C. maculatus*.

1. Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is one of the important food legumes in the semi-arid tropics covering Asia, Africa, Central and South America. Cowpea seeds are grown mainly in Nigeria, Niger, and Brazil (Boukar et al., 2018). These grains represent relevant protein sources to populations in developing countries (Keneni et al., 2011). The cowpea weevil infestation is an important trouble during cowpea seed storage (Keneni et al., 2011). The attack by this bruchid causes nutritional and economic losses for the cowpea crop (Rees, 2007) because *C. maculatus* larvae develop inside the cotyledons, feeding on the nutritional reserves of these seeds (Beck and Blumer, 2011). Currently, available control methods against these storage insect pests are insecticides that present several disadvantages such as environmental contamination (Satya et al., 2016) and selection of resistant insects (Munawar et al., 2020). Therefore, development of new cowpea cultivars and discovery of resistant ones is a strategy to decrease pesticides use on stored grains (Togola et al., 2017).

Although cowpea is an important crop, only a few countries have planned improvement programs, including Brazil, India, Niger, Nigeria, Senegal and the USA, and among the improved characteristics, the lower susceptibility of seeds to insect infestation is one of the desired properties. Several studies have reported cowpea resistant cultivars to cowpea weevil infestation, such as TVu 2027, TVu 11,952, TVu 11,953, IT81D-1032 and IT81D-1045 (Singh et al., 1985; Singh, 1999; Appleby and Credland, 2003; Cruz et al., 2016; Kpoviessi et al., 2019). Resistant cultivars can have some of the following effects on insect development: decrease in larval hatching, larval mass and adult emergence. Delays in larval hatching and adult emergence are also desirable characteristics (Jackai and Asante, 2003). Cruz et al. (2016) showed that cowpea cv. BRS Xiquexique, developed by EMBRAPA (Brazilian Agricultural Research Corporation), negatively interfered with the cowpea weevil development, reporting that 70% of the insect larvae did not survive inside the seeds. The surviving larvae at 20 days after oviposition (DAO)

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had suffered a decrease of 50% in their body mass and the number of adults emerged was ten times lower when compared to the susceptible cultivar.

Although many cowpea cultivars have been identified as having some degree of resistance to infestation by *C. maculatus*, few studies have identified the chemical factors related to this resistance. Vicilins (7S storage globulins) with chitin affinity properties have been related with cowpea resistance to infestation by the cowpea weevil (Macedo et al., 1993; Sales et al., 2001; Uchôa et al., 2009; Miranda et al., 2020). The chitin-binding vicilins isolated from cowpea-resistant IT81D-1045 Nigerian line were toxic and able to bind to chitin present in peritrophic matrices that line the midguts of insect larvae from several species, such as *C. maculatus* (Sales et al., 2001), *Diatraea saccharalis* (Mota et al., 2003) and *Tenebrio molitor* (Paes et al., 2008). Vicilins from other legume species which are non-host seeds to *C. maculatus*, such as *Phaseolus vulgaris*, *Phaseolus lunatus*, *Canavalia ensiformis* and *Glycine* max also showed chitin-binding affinity and had strong detrimental effects on *C. maculatus* development (Yunes et al., 1998).

Chitinous structures have been studied as a target for the control of insects pests, therefore proteins capable of binding to chitin may be an important tool in the success of this approach (Tetreau and Wang, 2019). The use of chitinases as a bio control agent is one attractive and environmentally safe strategy (Singh et al., 2014). Chitinases (EC 3.2.1.14) are hydrolases that cleave glycosidic bonds (β-1 → 4-linkages) in chitin, an abundant carbohydrate component of the fungal cell walls, the exoskeleton and gut lining (peritrophic matrix) of insects, and the shells of crustaceans. Chitinases are present in a wide range of organisms including bacteria, fungi, plants, insects and other animals (Oyeleye and Normi, 2018). In plants, chitinases have been investigated as defense proteins related to protection against insects and pathogens (Singh et al., 2014; Uzma-Jalil et al., 2015). The toxicity of chitinases to *C. maculatus* has been previously reported. The seed coat chitinase isolated from soybeans reduced larval survival and weight about 77% and 60%, respectively. The insect fed with the FITC-labeled chitinase showed fluorescence in the gut and feces (Silva et al., 2018). A chitinase isolated from cowpea seeds was also toxic to *C. maculatus,* significantly affecting larval mass (Gomes et al., 1996).

The present work investigated the presence of chitin-binding proteins in the cowpea cv. BRS Xiquexique and their toxicity to the cowpea weevil.

2. Materials and methods

2.1. Insects

Cowpea weevil insects were obtained from a colony, maintained at 28 °C and 60% relative humidity, in the Laboratório de Química e Função de Proteínas e Peptídeos/LQFPP, Centro de Biociências e Biotecnologia, UENF, Campos dos Goytacazes, RJ, Brazil.

2.2. Seeds

Cowpea cv. Fradinho seeds were obtained commercially in local markets at Campos dos Goytacazes, RJ, Brazil. Cowpea cv. BRS Xiquexique was developed by the Cowpea Breeding Program from the Embrapa Meio-Norte, situated in Teresina, Piauí, Brazil.

2.3. Isolation of chitin-binding proteins

Seeds of cowpea from cv. BRS Xiquexique and cv. Fradinho were decoated and the cotyledons were ground to obtain a flour. The proteins from cotyledon flour were extracted with 100 mM sodium acetate buffer, pH 6.0 (1:10 *w/v* ratio), under stirring for 1 h, at 4 °C. The extract was centrifuged at 10.000 x*g* during 10 min at 4 °C and the obtained supernatant was used to isolate the chitin-binding proteins.

Preparation of chitin (Sigma) was performed according to Uchôa et al. (2009). A chitin suspension (30 mL) in 100 mM sodium acetate, pH 6.0, was mixed with 18 mL of seed extract supernatant, from cv. BRS Xiquexique or cv. Fradinho and incubated, under stirring, during 30 min at room temperature. The mixture was packed in a glass column at a flow of 1.3 mL/min. Non-retained fraction was eluted with 100 mM sodium acetate pH 6.0 and the chitin-binding proteins (CBP) retained fraction was eluted with 100 mM hydrochloric acid. Fractions of 4 mL were collected and the absorbances were read at 280 nm. The fractions were dialyzed against water for 48 h, at 4 °C and freeze dried.

2.4. Isolation of chitin-binding vicilins

To isolate the chitin-binding vicilins (CBV) fraction, the CBP fraction from cv. BRS Xiquexique was diluted in water (1 mg/mL), reserved at 4 °C for 1 h and centrifuged at 10.000 x*g* during 20 min at 4 °C. The precipitate was named chitin-binding vicilins (CBV) fraction.

2.5. Determination of chitinase activity

The presence of chitinase in CBP and CBV fractions from cv. BRS Xiquexique was analyzed through the detection of chitinase activity, according to Silva et al. (2018). Chitinase activity was detected using chitin azure (chitin covalently linked to Remazol Brilliant Violet 5R dye; Sigma-Aldrich) as substrate. The fractions were diluted with 100 mM sodium acetate, pH 5.0 (3 μg/μL). A volume of 100 μL of the suspensions was incubated with 4 mg of chitin azure, at 37 ˚C, for 1 h. The reaction was stopped with the addition of 50 μL of 1 M HCl, and the reactions were centrifuged at 2000 x*g* for 1 min. The absorbance of the supernatants was measured at 540 nm. A pure chitinase (1.95 mU/mg) from *Streptomyces griseus* (Sigma-Aldrich) was used as a standard to determine the activity present in the fractions.

2.6. SDS-PAGE and Western blotting

The protein profile from CBP and CBV fractions from cv. BRS Xiquexique were visualized by 15% SDS-PAGE according to Laemmli (1970). The gels were stained with 0.05% Coomassie blue and destained with 10% acetic acid.

After electrophoresis, an unstained gel containing the separated proteins from CBP fraction was blotted onto a nitrocellulose membrane and subjected to *Western blotting*, according to Towbin et al. (1979). An anti-*V. unguiculata* vicilin antibody, produced in rabbits, diluted 1:1000, was used to detect vicilins and an anti-*Adenanthera pavonina* chitinase antibody, produced in rabbits, diluted 1:1000, was used to detected chitinases. A peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) antibody, at 1:1000, was used as secondary antibody. Peroxidase activity was revealed by using 5 mg of diaminobenzidine (DAB), 100 μL of 2 M Tris–HCl, pH 8.0, 300 μL of 0.1 M imidazole, 10 μL of 30% H_2O_2 and 4.9 mL of distilled water.

2.7. Mass spectrometry analysis

CBP fraction from cv. BRS Xiquexique was separated by 15% SDS-PAGE, stained with 0.05% Coomassie blue and destained with 10% acetic acid. Four bands with molecular masses between 20 and 40 kDa were excised from the gel and digested with pure trypsin, as Shevchenko et al. (1996). C18 Zip-Tip micropipette tips were used to desalt the peptides. Reversed-phase nanochromatography coupled with nano-electrospray high-resolution mass spectrometry was performed for tryptic digest identification. For each sample, 4 μL of desalted tryptic peptide digest were initially applied to a 2 cm long trap column (Michrom Bioresources, USA), followed by separation on a 12 cm long separation column (New Objective, USA). Chromatography was carried out on an EASY-nLC II instrument (Thermo Scientific, USA). The eluted peptides were introduced to an LTQ XL/Orbi/Trap MS (Thermo, USA) for analysis. The full ion trap value and the MSn AGC target value were

30,000 and 10,000, respectively. The FTMS full AGC target value was set to 500,000. The MS1 spectra were acquired on the Orbitrap analyser. For each spectrum, the 10 most intense ions were submitted to CID fragmentation, followed by MS2 acquisition on the linear trap analyser. The dynamic exclusion option was enabled. The search parameters for monoisotopic peptide masses tolerance 10 ppm and fragment tolerance 0.02 Da allowed two missed enzymatic cleavage and accepted the carbamidomethylation of the cysteine residues and the oxidation of methionine as modifications fixed and variable, respectively. The peptide mass profiles were analyzed using Peaks Studio X (Bioinformatics Solutions Inc.) and the alignment of the peptides was done using BLASTp.

2.8. Cowpea weevil feeding trials

The CBP fraction isolated from cv. BRS Xiquexique and cv. Fradinho was incorporated in artificial seeds, according to Macedo et al. (1993). A concentration of 5.0% (*w*/w) of CBP fraction was mixed with cowpea cv. Fradinho cotyledonary flour in order to make up 400 mg. The mixture was placed into a cylindrical brass mold and pressed using hand press. Control artificial seeds were performed using only 400 mg of cowpea cv. Fradinho cotyledon flour. The artificial seeds were infested with 2 days-old females for 24 h, and after this time, the laid eggs were counted, and excess eggs were removed to leave three eggs per artificial seed. Larval neonate development and hatching on artificial seeds were monitored at 2, 3, 6 and 11 days after oviposition (DAO). At 20 DAO, the seeds were opened and surviving larvae were weighed, photographed, and measured using the Image J software.

The CBV fraction, isolated from cv. BRS Xiquexique, was also incorporated in artificial seeds at the concentration of 2.0% (w/w). The processes for artificial seed experiments, infestation with the insect and analyzes of the results, were performed as described above.

Experiments were done in triplicate with 3 seeds per experiment (total of nine seeds and 27 eggs). Statistical significance was assessed using Student's *t*-test ($P < 0.05$).

2.9. Cysteine protease assay

Cysteine protease activity was previously described as the major midgut proteolytic activity of *C. maculatus* larvae (Lemos et al., 1990). In order to analyze any possible inhibitory effect over larval protein digestion of this CBP fraction, we measured the levels of cysteine protease activity in larvae developed in natural seeds of *Vigna unguiculata,* cv. Fradinho. (14, 15, 16, 17, 18, 19, 20 and 21 DAO) and in larvae, at 20 DAO, developed in artificial seeds containing 0 or 5% of CBP fraction. The larvae were macerated in citrate phosphate buffer (15 μL/mg larvae) and 40 μL of larval extract was incubated with 80 μL of azocasein solution during 1 h at 37 °C. Then, 300 μL of 10% of trichloroacetic acid (TCA) was added to the samples which were further centrifuged at 10.000 x*g* for 5 min. A volume of 300 μL of 1 M NaOH was added to 300 μL of the supernatant obtained from each sample and absorbance readings were taken at 450 nm. The assay was performed according to Michaud et al. (1994), using azocasein (azocasein 1% in citrate phosphate buffer pH 5.6, 100 mM sodium citrate, 100 mM sodium phosphate, 0.1% triton X-100 and 1.5 mM DTT) as substrate. Cysteine protease activity was determined in reference to a standard curve of pure papain (Sigma-Aldrich).

2.10. Statistical analysis

Assays were performed in independent biological triplicates. Averages, standard deviation, and statistical treatments were performed using software GraphPrism 7.0. Tukey tests (Test T; $p < 0,05$), according to Bridge and Sawilowsky (1999), were performed for artificial seed assays.

3. Results

By chitin affinity chromatography, a chitin binding protein (CBP) fraction from cowpea cv. BRS Xiquexique (Fig. 1A) was isolated. In this CBP fraction, protein bands with varying molecular masses, mainly below 55 kDa, were revealed by 15% SDS-PAGE (Figure 1B1). Bands of about 100 and 40 kDa reacted with an anti-vicilin antibody (Figure 1B2) and less intense bands above 70 kDa and close to 35 and 30 kDa reacted with an anti-chitinase antibody (Figure 1B3).

Bands with molecular masses below 55 kDa, named b1, b2, b3 and b4 (Fig. 2A) were identified by mass spectrometry analysis. Peptide sequences obtained from b1, b2, b3 and b4 bands were similar to the cowpea vicilin (UniProt accession number: A8YQH5_VIGUN) (Fig. 2B). Peptide sequences from b4 band showed similarity with a chitinase from *Canavalia ensiformis* (UniProt accession number: O81934_CANEN) (Fig. 2C), indicating that the b4 band cut out from the gel was not pure, and contained, in addition to vicilin, chitinase.

CBP fraction isolated from cv. BRS Xiquexique and cv. Fradinho was incorporated at 0% (control seeds) and 5% level in the insect diet and the monitoring of larval neonate development and hatching showed that, at 2 DAO, the egg contents were clear and transparent on both seeds (control and experimental seeds); at 3 DAO, the larvae begin to differentiate and at 6 DAO, the larvae were apparently fully formed inside the egg (Fig. 3). At 11 DAO, the eggs had a whitish appearance due to the presence of flour inside them. At this time, the larvae had ecloded and penetrated the artificial seed. In artificial seeds containing 5% of CBP fraction from cv. Fradinho (data not shown) and from cv. BRS Xiquexique, the neonate larvae development and hatching were similar to those of control larvae (Fig. 3). The CBP fraction from cv. BRS Xiquexique was able to interfere with the post hatch development of the larvae. At 20 DAO, these larvae showed decreases in their body mass (Fig. 4A and B) and length (Fig. 4C) of about 79.6% and 51.23%, respectively when compared to the larvae developed in the control seeds (containing only cv. Fradinho flour). Larvae developed in seeds containing 5% of cv. Fradinho CBP fraction had no decreases in masses and sizes in relation to the control larvae (Fig. 4B and C). No difference in larval survival was observed at 20 DAO (data not shown).

The cysteine protease activity was decreased about 45% in larvae fed with 5% of cv. BRS Xiquexique CBP fraction, when compared with control larvae at 20 DAO (Fig. 5A). The cysteine protease activity of the 20 DAO larvae developed in the seeds containing CBP fraction was also lower than the activity detected in the larvae developed in natural seeds of *V. unguiculata* (cv. Fradinho) between 14 and 21 DAO (Fig. 5B).

Based on the water insolubility of vicilins, proteins which were not solubilized in water were considered chitin-binding vicilins (CBV). The two main *Vigna* vicilin bands were observed in the cv. BRS Xiquexique CBV fraction, with molecular masses between 55 and 40 kDa, by SDS-PAGE (Fig. 6A). The CBV fraction did not show considerable chitinase activity, when compared to the CBP fraction (Fig. 6B), indicating an effective separation between vicilins and chitinases.

CBV fraction was incorporated, at 0% (control) and 2%, in insect artificial diet and the results showed that there was no difference in the larval hatching in artificial seeds (control) or containing CBV fraction (data not shown). At 20 DAO, the larvae showed decreases in body mass (Fig. 7A and B) and length (Fig. 7C) of about 64.3% and 33.23%, respectively. No difference in larval survival was observed at 20 DAO (data not shown).

4. Discussion

The resistance of cowpea cultivars to *C. maculatus* infestation has been studied as an alternative to reduce the amount of insecticides used to protect these seeds, especially during storage (Cruz et al., 2016). Over the years, some *V. unguiculata* genotypes have been identified as resistant to *C. maculatus* infestation. The International Institute for Tropical Agriculture (IITA) identified resistant genotypes, such TVu 2027, TVu

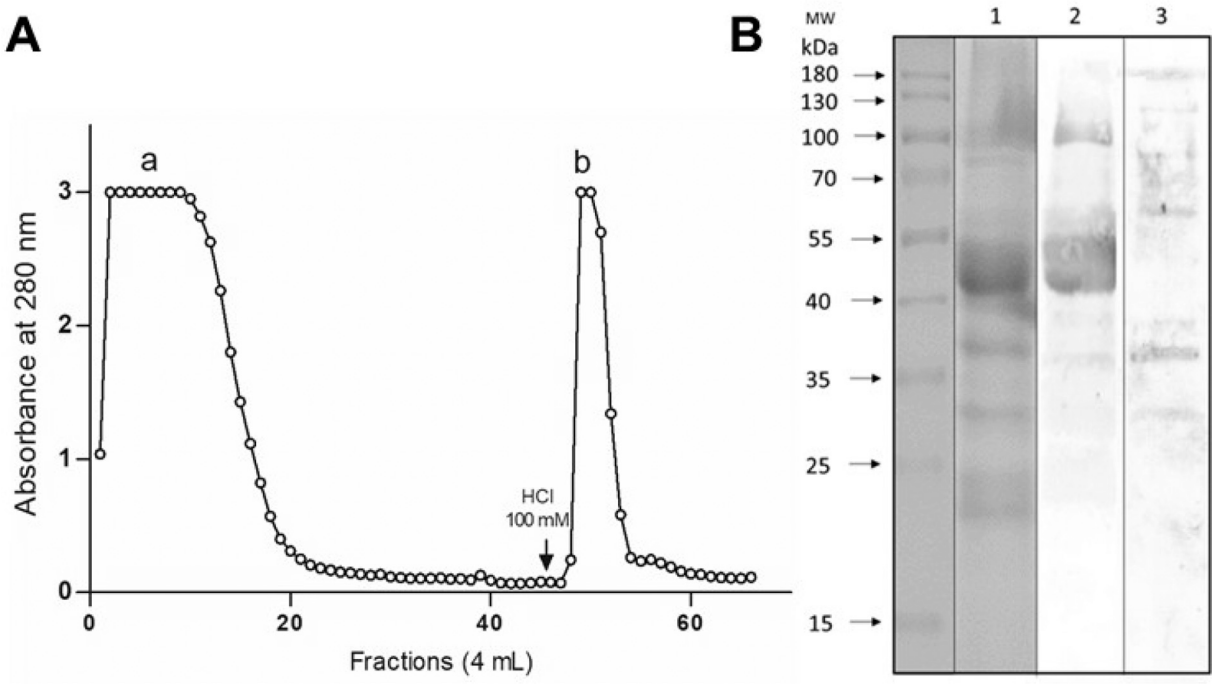


Fig. 1. (A) Chitin affinity chromatography of BRS Xiquexique cultivar seed proteins. Non-retained proteins (a) were washed with sodium acetate, and adsorbed proteins (b) were desorbed with 100 mM HCl. (B) SDS-PAGE and *Western blotting* of the BRS Xiquexique CBP fraction. MW- molecular mass markers; 1- CBP fraction; 2-Vicilin detection. 3- Chitinase detection.

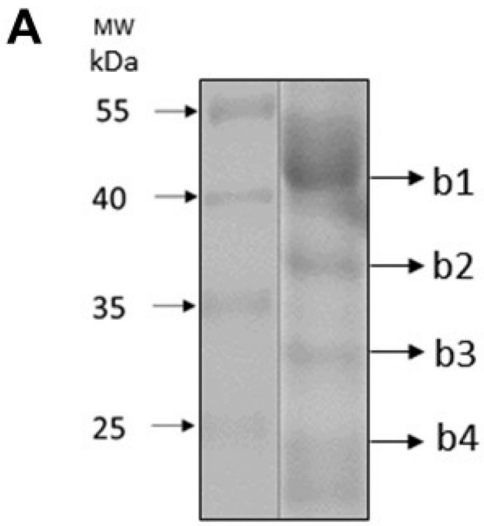
11,952, and TVu 11,953 (Singh et al., 1985), that later originated cultivars with important degrees of resistance to infestation, such as IT81D-1032 and IT81D-1045 (Singh, 1999).

Cruz et al. (2016) studied the susceptibility and resistance of nine different cowpea cultivars to infestation and damage by *C. maculatus* and showed that some cultivars interfered in female oviposition, increased the time necessary for the larvae to perforate the seed coat, decreased larval survival and the activity of insect digestive enzymes. In cowpea cv. BRS Xiquexique, only 30% of the larvae survived at 20 days after oviposition. The weight of surviving larvae decreased about 50%. The larvae fed with cowpea cv. BRS Xiquexique showed decreases in intestinal activities of cysteine protease, α-glucosidase, and α-amylase. Although the toxic effects had been quite evident, the compounds related to the insect toxicity of this cultivar remained unclear.

Previous studies have related the presence of chitin-binding proteins, such as vicilins and chitinases, with the seed toxicity towards insects (Macedo et al., 1993; Mota et al., 2003; Paes et al., 2008;Silva et al., 2018; Miranda et al., 2020). In this work, we showed that chitin-binding proteins, as vicilins and chitinases, are present in cotyledons of cowpea cv. BRS Xiquexique and are toxic to *C. maculatus* larvae. Both chitin binding protein (CBP) and chitin binding vicilin (CBV) fractions were able to interfere with the development of cowpea weevil, decreasing larval mass and length. The CBP fraction was seen to contain vicilins and chitinases and, when present at a level of 5% in the insect diet, led to decreases of 79.6% and 51.2% in the larval mass and length, respectively. CBP fraction isolated from cowpea weevil-susceptible seeds (cv. Fradinho) had no harmful effect on the insect larvae, which showed development parameters, such as weight and size, similar to the control larvae. The cv. BRS Xiquexique CBV fraction, at 2.0%, led to decreases of 64.3% to the larval mass and of 33.23% to the larval length. These results suggest that chitin binding proteins, such as vicilins and chitinases, may be related to the resistance of cowpea cv. BRS Xiquexique to the infestation by *C. maculatus*.

The relationship between vicilins (7S globulins) and resistance to *C. maculatus* infestation was first demonstrated for cowpea cv. IT81D-1045 and IT81D-1032 (Sales et al., 1992; Macedo et al., 1993). Vicilins isolated from these cultivars were more refractory to digestion by insect proteases than vicilin from susceptible cultivars (Sales et al., 1992). Vicilins from cv. IT81D-1045 at 2% were able to decrease the larval mass by 50% (Macedo et al., 1993). The toxicity mechanism of vicilins was shown to be related to their capacity to bind to the chitin present in peritrophic matrices of larval midgut, added to their lower digestibility by insect proteases (Sales et al., 1992; Sales et al., 2001). Sales et al. (2005) studied the emergence of adult insects, the total developmental period (TDP) and excretion of vicilin by *C. maculatus* reared on resistant (IT81D-1045) and susceptible (EPACE 10) cowpea seeds. The results showed that the insects raised on resistant seeds had lower emergence and longer TDP than those raised on susceptible seeds. Insects emerged from resistant seeds were seen to excrete 7 times higher levels of vicilins than insects emerged from susceptible seeds, indicating a difficulty in digesting and absorbing the vicilins from resistant cultivar. Vicilins isolated from cowpea weevil-susceptible seeds, as *V. unguiculata* (cvs. CE-31, Epace 10 and Pitiuba) and *V. angularis* are not toxic to *C. maculatus* and are more efficiently digested by the insect digestive proteases. Although some of these vicilins also have chitin-binding affinity, this interaction appears to be much weaker than the linkage between chitin and insect-resistant seed vicilins. (Sales et al., 1992; Macedo et al., 1993; Sales et al., 2001; Uchôa et al., 2009). In this work we show that although CBP are present in the cv. Fradinho, these proteins were not toxic to the insect, indicating that the binding of these proteins to chitin may be weaker than the binding of CBP from resistant cultivars, as it also happens with vicilins from other cowpea weevil-susceptible cultivars.

Chitin-binding vicilins toxic to insects have also been isolated from other species (Yunes et al., 1998). Vicilin from *Enterolobium contortisiliquum* is a dimeric glycoprotein, with two subunits of 66.2 and 63.8 kDa. This vicilin was toxic to *C. maculatus* larvae, producing 50% mortality and decreasing 50% of the larval mass, at 1.0%. The vicilin binding to larvae gut chitin, associated to its low digestibility by insect proteases, have also been reported to be related to this toxicity (Moura et al., 2007). Proteins with molecular masses between 50 and 40 kDa isolated from *Albizia lebbeck* seed coat were similar to vicilins and toxic



B

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A8YQH5  VPLLLLGVLF LASLSVSFGI VHRGHQESQE ESEPRGQNNP FYFDSDRWFH TLFRNQYGHL RVLQRFDQRS KQIQNLENYR  80
b1                          RGHQESQE ESEPRGQNNP FYFDSDRWFH TLFRNQYGHL R        S KQIQNLENYR
b2                          RGHQESQE ESEPRGQNNP FYFDSDRWFH TLFRNQYGHL R        S KQIQNLENYR
b3                                   GQNNP FYFDSDRWFH TLFRNQYGHL R            QIQNLENYR
b4                          RGHQESQE ESEPRGQNNP FYFDSDRWFH TLFRNQYGHL R        S KQIQNLENYR

A8YQH5  VVEFQSKPNT LLLPHHADAD FLLVVLNGRA ILTLVNPDGR DSYILEQGHA QKTPAGTTFF LVNHDDNENL RIVKLAVPVN 160
b1           SKPNT LLLPHHADAD FLLVVLNGRA ILTLVNPDGR DSYILEQGHA QK                     IVKLAVPVN
b2           SKPNT LLLPHHADAD FLLVVLNGRA ILTLVNPDGR DSYILEQGHA QK                     IVKLAVPVN
b3           SKPNT LLLPH               A ILTLVNPDGR DSYILEQGHA QK                     IVKLAVPVN
b4           SKPNT LLLPHHADAD FLLVVLNGRA ILTLVNPDGR DSYILEQGHA QK                     IVKLAVPVN

A8YQH5  NPHRFQDFFL SSTEAQQSYL QGFSKNILEA SFDSDFKEIN RVLFGEEEQK QQDEESQQEG VIVQLKREQI RELMKHAKST 240
b1      NPHRFQDFFL SSTEAQQSYL QGFSKNILEA SFDSDFKEIN RVLFGEEEQK QQDEESQQEG VIVQLK
b2      NPHRFQDFFL SSTEAQQSYL QGFSKNILEA SFDSDFKEIN RVLFGEEEQK QQDEESQQEG VIVQLK
b3      NPHRFQDFFL SSTEAQQSYL QGFSKNILEA SFDSDFKEIN RVLFGEEEQK QQDEESQQEG VIVQLK
b4      NPHRFQDFFL SSTEAQQSYL QGFSKNILEA SFDSDFKEIN RVLFGEEEQK QQDEESQQEG VIVQLK

A8YQH5  SKKSLSTQNE PFNLRSQKPI YSNKFGRLHE ITPEKNPQLR DLDVFLTSVD IKEGGLLMPN YNSKAIVILV VNKGEANIEL 320
b1        KSLSTQNE PFNLRSQKPI YSNKFGRLHE ITPEKNPQLR           EGGLLMPN YNSKAIVILV VNKGEANIEL
b2                 SQKPI YSNKFGRLHE ITPEKNPQLR           EGGLLMPN YNSKAIVILV VNKGEANIEL
b3                 SQKPI YSNKFGRLHE ITPEKNPQLR           EGGLLMPN YNSKAIVILV VNKGEANIEL
b4          LSTQNE PFNLRSQKPI YSNKFGRLHE ITPEKNPQLR           EGGLLMPN YNSKAIVILV VNKGEANIEL

A8YQH5  VGQREQQQQQ QEESWEVQRY RAEVSDDDVF VIPASYPVAI TATSNLNFIA FGINAENNQR NFLAGEEDNV MSEIPTEVLD 400
b1      VGQREQQQQQ QEESWEVQR                        SNLNFIA FGINAENNQR           SEIPTEVLD
b2      VGQREQQQQQ QEESWEVQR                            FIA FGINAENNQR
b3      VGQREQQQQQ QEESWEVQR                            FIA FGINAENNQR
b4      VGQREQQQQQ QEESWEVQR                            FIA FGINAENNQR

A8YQH5  VTFPASGEKV EKLINKQSDS HFTDHSSKRE ERV
b1      VTFPASGEK
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Fig. 2. (A) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the cv. BRS Xiquexique CBP fraction. The protein bands b1, b2, b3 and b4 were submitted to mass spectrometry analysis. MW- molecular mass markers. (B) Multiple sequence alignment of the peptides obtained by mass spectrometry analyses from bands b1, b2, b3 and b4 with the sequence of *V. unguiculata* vicilin (UniProt accession number A8YQH5). (C) Alignment of the peptides from band b4 with the sequence of *Canavalia ensiformis* chitinase (UniProt accession number 081934).

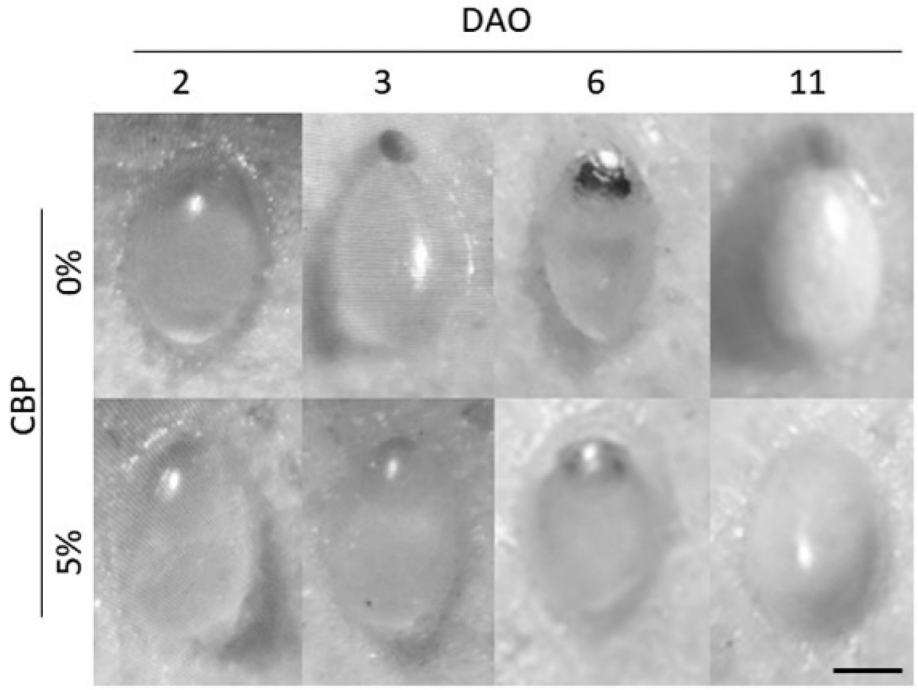

Fig. 3. Larval neonate development and hatching of *Callosobruchus maculatus* on artificial seeds containing 0 or 5% of cv. BRS Xiquexique CBP fraction. The development and hatching were monitored at 2, 3, 6 and 11 DAO (Days After Oviposition). Bar = 0.1 mm.

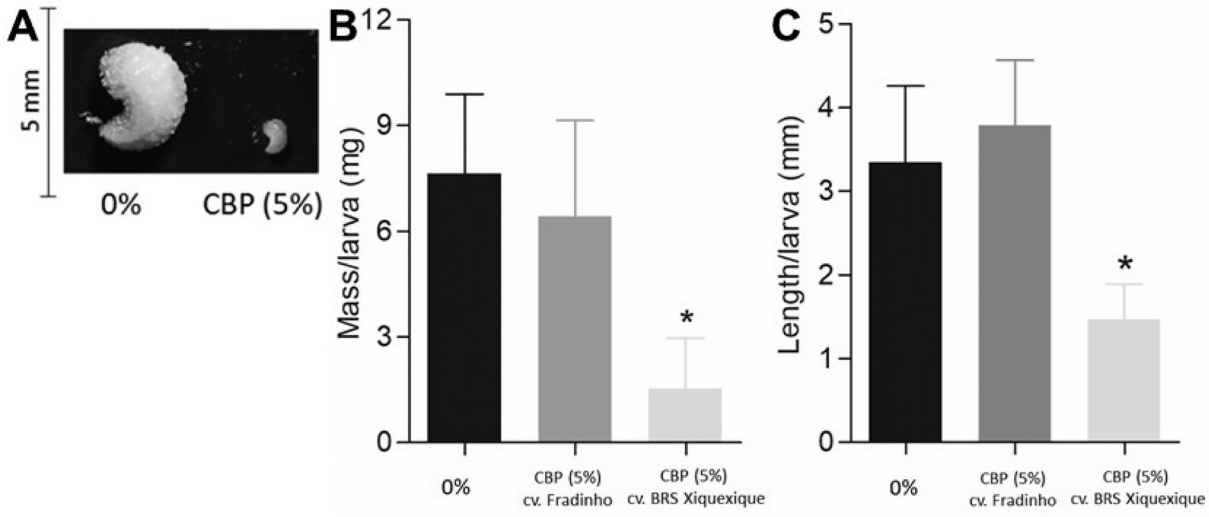

Fig. 4. Toxicity of CBP fractions from cv. Fradinho and cv. BRS Xiquexique to *Callosobruchus maculatus* larvae. (A) Photography of larvae at 20 DAO (Days After Oviposition), developed in artificial seeds containing 0 or 5% of cv. BRS Xiquexique CBP fraction. (B) Mass and (C) Length of larvae at 20 DAO, developed in artificial seeds containing 0 or 5% of CBP fractions. Experiments were run in triplicate and the data shown are the average of these results. Asterisk (*) indicates results statistically different from the control larvae ($p < 0.05$ by Student's *t*-test).

to *C. maculatus* larvae, decreasing 78% the larval mass, when provided at a 0.1% level in the insect diet. These proteins showed strong chitin-binding ability (Souza et al., 2012).

Other chitin-binding proteins, such as lectins and chitinases, have been reported as toxic to insects. The chitin-binding lectin from *Moringa oleifera* seeds impaired the *Anagasta kuehniella* larval weight gain by 50% and affected the activity of the digestive enzymes (De Oliveira et al., 2017). Chitin-binding defense proteins from mulberry latex, with lectin activity and hevein domain, caused abnormal swelling of the peritrophic membrane in Eri silkworm (*Samia cynthia*) gut (Konno et al., 2018). Chitinase isolated from *Bacillus subtilis* has effectively reduced the gut enzyme activity and growth of *Spodoptera litura* (Chandrasekaran et al., 2014). Chitinase from *Pseudomonas fluorescens* showed insecticidal activity against *Helopeltis theivora* (Suganthi et al., 2017). *In this work, we showed the presence of chitinases, with varied molecular masses, in the protein fraction retained in chitin (CBP); however, it was not possible to isolate and test the toxicity of chitinases separated from vicilins. Even without the test with pure chitinases, it is possible to believe that part of the toxicity of CBP fraction is related to the presence of chitinases, since previous studies have already shown that these plant chitinase are toxic to C. maculatus* (Gomes et al., 1996;Silva et al., 2018).

Chitinases *with varied molecular masses* are quite abundant in plants. A toxic chitinase previously isolated from cowpea seeds showed molecular mass of 22 kDa (Gomes et al., 1996). Class I chitinases from

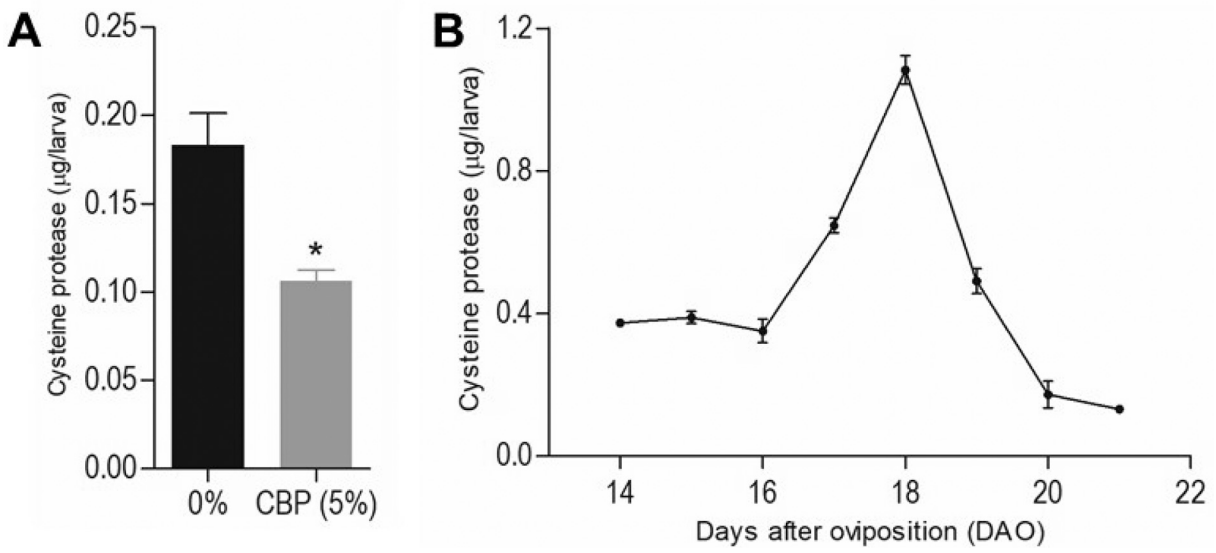

Fig. 5. (A) Cysteine protease activity of 20 DAO larvae developed in artificial seeds containing 0 or 5% of cv. BRS Xiquexique CBP fraction. (B) Cysteine protease activity of larvae developed in natural seeds of *Vigna unguiculata* (cv. Fradinho) at 14, 15, 16, 17, 18, 19, 20 and 21 DAO (Days After Oviposition). Experiments were run in triplicate and the data shown are the average of these results. Asterisk (*) in fig. A indicates results statistically different from the control larvae ($p < 0.05$ by Student's t-test).

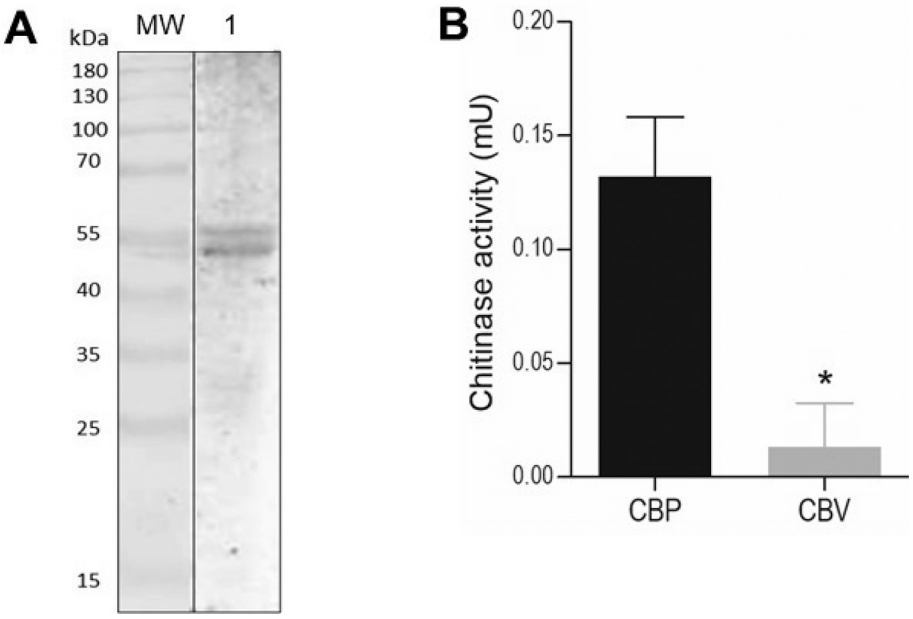

Fig. 6. (A) SDS-PAGE of the cv. BRS Xiquexique CBV fraction. MW- molecular mass markers; 1- CBV fraction profile. (B) Chitinase activity in CBP and CBV fractions. Experiments were run in triplicate and the data shown are the average of these results. Asterisk (*) indicates results statistically different from the control larvae ($p < 0.05$ by Student's t-test).

cowpea produced in *Pichia pastoris* showed molecular mass of 34 and 37 kDa (Landim et al., 2017). In the *Arabidopsis* genome appears to contain 25 chitinase or chitinase-like proteins. The length of the chitinase proteins in *Arabidopsis* varies from 211 to 430 amino, with molecular mass between 20 and 50 kDa. (Grover, 2012). The rice genome shows 49 chitinase with 178 to 479 amino acids and molecular mass between 18 and 50 kDa. (Grove, 2012).

Chitin is a polymer of β1-4 *N*-acetyl-D-glucosamine present in the cell wall of fungi, in the exoskeleton of invertebrates and in the peritrophic matrices or membranes that line insect larval midguts. The peritrophic membrane protects the intestinal epithelium from food abrasion and facilitates recycling of gut digestive enzymes (Terra, 2001; Wang and Granados, 2001; Hegedus et al., 2009). In this work, we observed that larvae fed with 5% of cowpea cv. BRS Xiquexique CBP fraction suffered a decrease in the activity of cysteine protease, the main protease of the *C. maculatus* larval midgut (Lemos et al., 1990; Nogueira et al., 2012; De Sá et al., 2018). This result is similar to that previously observed by Cruz et al. (2016). The decrease of larval enzyme activities may indicate that the binding of CBP fraction in the peritrophic membrane of larvae would prevent the gut digestive enzymes recycling. Alternatively, it could indicate an adaptative answer by the insect to the presence of toxic proteins in its diet. It is discussed in the literature, for example, the frequent cases of unsuccessful transgenic crops expressing protease inhibitors due to the rapid insect adaptation to the presence of inhibitors

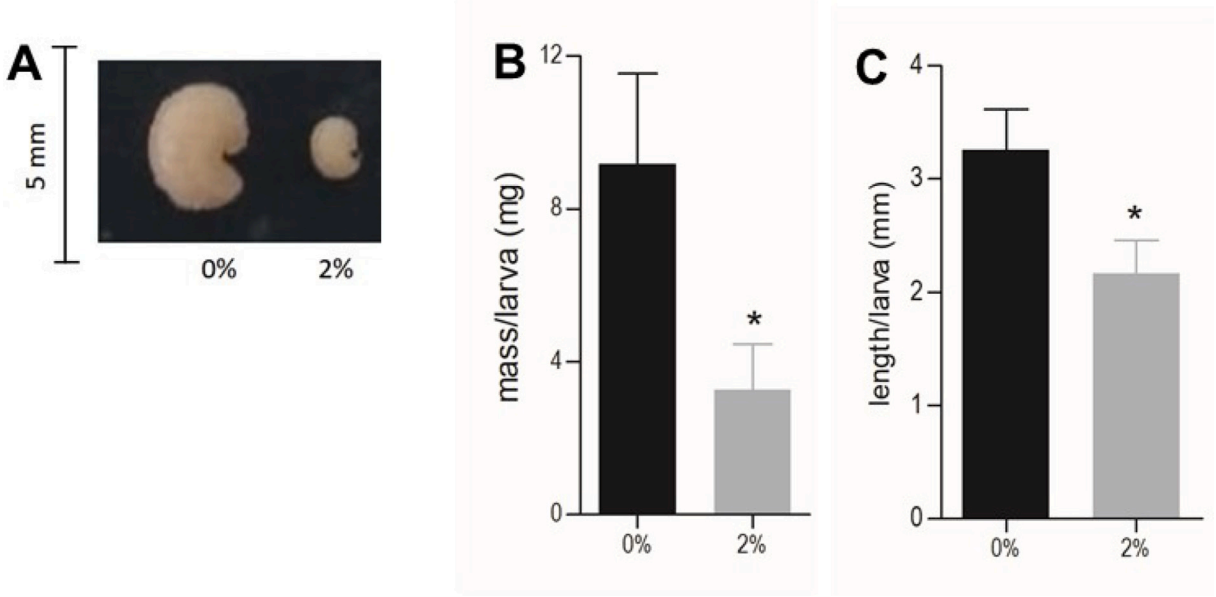

Fig. 7. Toxicity of cv. BRS Xiquexique CBV fraction to *Callosobruchus maculatus* larvae. (A) Photography of larvae at 20 DAO (Days After Oviposition), developed in artificial seeds containing 0 or 2% of CBV fraction. (B) Mass and (C) Length of larvae at 20 DAO, developed in artificial seeds containing 0 or 2% of CBV fraction. Experiments were run in triplicate and the data shown are the average of these results. Asterisk (*) indicates results statistically different from the control larvae ($p < 0.05$ by Student's t-test).

in their diet. Strategies utilized by insects include the overproduction of digestive proteases to overcome the inhibitors; an increased expression of inhibitor-insensitive protease isoforms; or activation of proteases that hydrolyse the inhibitor (Guo et al., 2012). A similar process can be carried out by insects in relation to other sort of toxic compounds in the diet, such as chitin-binding proteins.

Our data suggest that CBP fraction, including the CBV fraction, may be related to the resistance of the cowpea cv. BRS Xiquexique to the infestation by *C. maculatus* insect pest.

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