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Evaluation of seed coagulant *Moringa oleifera* lectin (cMoL) as a bioinsecticidal tool with potential for the control of insects

Caio Fernando Ramalho de Oliveira[a,c], Luciana Andrade Luz[b], Patrícia Maria Guedes Paiva[b], Luana Cassandra Breitenbach Barroso Coelho[b], Sergio Marangoni[a], Maria Lígia Rodrigues Macedo[c,*]

[a] *Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas, Campinas 13083-970, SP, Brazil*
[b] *Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Pernambuco, Recife 50670-901, PE, Brazil*
[c] *Departamento de Tecnologia de Alimentos e Saúde Pública, Centro de Ciências Biológicas e da Saúde, Universidade Federal de Mato Grosso do Sul, Cidade Universitária s/n, Caixa Postal 549, Campo Grande 79070-900, MS, Brazil*

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ABSTRACT

Lectins have demonstrated significant levels of protection against different pests when expressed in transgenic plants. The effects of the coagulant *Moringa oleifera* lectin (cMoL) on moth flour (*Anagasta kuehniella*) were tested by incorporating the protein in an artificial diet at levels of 0.5%, 1% and 2% (w/w), respectively. cMoL showed a dose-dependent effect on average larval weight and a series of nutritional disturbances. A significant increase in total development time of 15 days was observed in the group fed with cMoL at 1%, increasing the rate of pupal mortality by 27.6%. The *A. kuehniella* midgut proteases were unable to digest cMoL for up to 12 h of incubation. The lectin presented a tight binding to a chitin column, suggesting that the insecticidal activity of cMoL involves carbohydrate–lectin interactions on the surface of the digestive tract, with glycoproteins and others glycolsylated structures in the midgut and resistance to enzymatic digestion.

1. Introduction

The steady increase in worldwide agricultural production and the creation of large crop areas has facilitated the spreading of insect pests that increase production costs, in addition to reducing farming productivity and causing losses in stored products. Problems associated with widespread insecticide usage, together with the development of insect resistance to *Bacillus thuringiensis* (Bt) toxins in genetically engineered crops, have resulted in a greater interest of scientists in exploiting the potential of using plant defensive proteins, such as lectins, to help in combating crop damage [1].

Plant seeds contain several biologically active proteins that play various specialized functions. The most representative molecules are hydrolytic enzymes, inhibitors, lectins and the ribosome inactivating proteins [2,3]. Plant lectins are defined as proteins possessing at least one non-catalytic domain, which binds reversibly to specific mono or oligosaccharides [4]. Indeed, many highly-abundant plant lectins have been found to combine a role in storage with a role in plant defense whenever the plant is under attack by predators [5]. Several lectins have shown effects during different life stages of many insect orders such as Coleoptera [6–8], Diptera [9], Hemiptera [10], Homoptera [11], Hymenoptera [12], Isoptera [13] and Lepidoptera [14–16]. This feature demonstrates the potential of using plant lectins as naturally occurring insecticide agents against the pests that diminish crop production [16]. Generally, *in vitro* bioassays are undertaken to judge this biological characteristic, which consists of inclusion of the studied lectin into artificial diets offered to the target insect during a given period of time [8].

Lectins are naturally occurring proteins/glycoproteins with substantial structural diversity [17]. Transgenic crops expressing genes that confer resistance against insect herbivory have been produced for most of the world's economically important crops [18] and several studies have shown the efficiency of lectin expression in transformed crops, such as the snowdrop lectin (*Galanthus nivalis* agglutinin, GNA), which is one of the most studied lectins. Genes that encode GNA have been incorporated into a range of crops, including potato [19,20], rice [21–23], maize [24], tobacco [25], wheat [26], tomato [27] and sugarcane [28–30]. In addition to GNA (mannose specifies), ConA (mannose/glucose specifies), PNA (galactose specifies), morniga-G (galactose/N-acetylgalactosamine specifies) and WGA (N-acetylgalactosamine specifies) are examples of other lectins that have also proven insecticidal activity and are widely studied. These observations support the search for new

* Corresponding author. Tel.: +55 67 33457612; fax: +55 67 33457400.
E-mail address: bioplant@terra.com.br (M.L.R. Macedo).

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lectins with insecticidal activity of different carbohydrate specificities, to act against the most distinct pest species.

cMoL (coagulant lectin from *Moringa oleifera*), belongs to a group of lectins isolated from *Moringa oleifera* seeds (Moringaceae family) [31,32]. This lectin is constituted of a monomeric protein with an approximate weight of 26.5 kDa, as revealed by SDS-PAGE and showed coagulant activity, similar to aluminium sulphate, the coagulant most widely used in water treatment [31]. Another lectin from *M. oleifera*, WSMoL (water-soluble *M. oleifera* lectin), presented larvicidal activity to *Aedes aegypti*, the vector of dengue [33]. The aim of this study was to evaluate the insecticidal activity of cMoL, as judged by its effect on the survival and growth of the Mediterranean flour moth, *Anagasta kuehniella* (Zeller) (Lepidoptera: Pyralidae). This is a polyphagous pest that feeds on a wide variety of stored products, particularly in stored grains, such as dried cocoa beans, dried grains, nuts, tobacco, coconut and dried fruits [34]. *A. kuehniella* is of major economic importance as a flour and grain feeder; it is often a severe pest in flour mills [16,35].

2. Materials and methods

2.1. cMoL purification

Seeds of *M. oleifera* were collected in Recife city, northeastern Brazil and purified according to Santos et al. [31]. Seed flours of *M. oleifera* were extracted with 0.15 M NaCl for 6 h at room temperature. The proteins in this extract were precipitated using 0–60% ammonium sulphate fractionation for 4 h at room temperature. The 0-60F was dialyzed with distilled water (two changes) and 0.15 M NaCl, overnight. The 0-60F was chromatographed (10 mg of protein) on a guar gel column (10.0 cm × 1.0 cm), previously equilibrated with 0.15 M NaCl (20 ml/h flow rate). cMoL was eluted with 1.0 M NaCl.

2.2. Insects and effects of cMoL feeding on insect growth, survival and development

The eggs of flour moths (*A. kuehniella* (Zeller); Phycitinae, Pyralidae, Lepidoptera) were supplied by the Laboratório de Purificação de Proteínas e suas Funções Biológicas (LPPFB), Universidade Federal de Mato Grosso do Sul, Campo Grande, MS, Brazil. The colony was housed in standard conditions of 28 ± 1 °C, 65–75% relative humidity and a 16:8 (light:dark) photoperiod and routinely maintained on an artificial diet prepared by mixing whole wheat flour, whole wheat husks, whole wheat, and yeast (8:2:1.9:0.1, w/w), according to Macedo et al. [16].

To examine the effects of cMoL on *A. kuehniella* development, neonate first instar larvae were selected and fed an artificial diet containing 0.5%, 1% and 2% of cMoL (w/w). Controls were fed with untreated diet. Each treatment was set up in 250-mg clear plastic, airtight containers and five larvae were transferred to each plastic container ($n = 60$). After the larvae reached the 4th instar at standard conditions, the relationship between protein content and the weight and number of larvae were determined. Larval consumption and faecal production were analyzed on a dry mass basis. The protein content and tryptic activity of the faecal and midgut samples were also determined.

Linear regression analysis was used to evaluate the response of *A. kuehniella* to the concentrations of cMoL. The treatment that provided the effective dose for a 50% response (ED_{50}), defined as the concentration of cMoL that reduced the larval mass by 50% compared to the control larvae, was utilized for further assays. The fresh weight of the resulting pupae and adults was determined on the first day following pupation and upon adult eclosion, respectively. The number of adults that emerged was counted in order to determine the percentage survival to adult emergence (S). The time at which the adults emerged was also recorded to allow estimation of the mean time of development (T). Howe's index [36] was calculated by dividing the log10 of the percentage survival to adult emergence ($\%S$) by the mean time of development (T).

2.3. Measurement of nutritional parameters

Several nutritional parameters were used to compare fourth instars fed on the control diet with those fed on a diet containing 1% cMoL. The larvae, faeces and remaining uneaten food were separated, dried and weighed. The indices of consumption, digestion and food utilization were calculated as described by Scriber and Slansky [37]:

The efficiency of the conversion of ingested food (ECI) estimates the percentage of ingested food that is converted to biomass, and was calculated as: [biomass gained (mg fresh mass)/food ingested (mg dry mass)] × 100.

The efficiency of the conversion of digested food (ECD) estimates the efficiency with which digested food is converted to biomass, and was calculated as: biomass gained (mg fresh mass)/[food ingested (mg dry mass) – feces (mg dry mass)] × 100. Approximate digestibility (AD) estimates the amount of ingested food that is digested, and was calculated as: [food ingested (mg dry mass) – feces (mg dry mass)]/food ingested (mg dry mass) × 100.

Metabolic cost (MC) was calculated as: 100 – ECD.

2.4. Midgut preparation

Homogenates of the larval guts were prepared according to Macedo et al. [38]. Fourth instar larvae were cold-immobilized and dissected in cold 150 mM NaCl. The midguts were surgically removed from the larvae using tweezers. The gut portion taken was posterior to the proventriculus and anterior to the malpighian tubules. After removing all extraneous tissue and freeing the lumen of its contents by rinsing in 150 mM NaCl, the midgut tissues were homogenized in cold 150 mM NaCl in a hand-held Potter–Elvehjem homogenizer immersed in ice. Midgut homogenates were centrifuged at 14,000 × g for 20 min at 4 °C and the supernatants were collected in a known volume of phosphate buffer and used immediately as enzymes sources for enzymatic assays or stored at −20 °C.

2.5. Faecal pellet preparations

Faeces were prepared according to Ramos et al. [34]. Faeces were collected and homogenized in cold 150 mM NaCl in a hand-held Potter–Elvehjem homogenizer. The homogenates were centrifuged at 14,000 × g for 20 min at 4 °C and the supernatants were collected in a known volume of phosphate buffer and used as a source of enzymes for enzymatic assays or stored at −20 °C.

2.6. Protein quantification

Protein concentrations were determined by the dye-binding method of Bradford [39], with bovine serum albumin (1 mg/mL) as the standard.

2.7. Enzymatic assays

Trypsin-like enzymes from gut extracts and faecal samples from *A. kuehniella* larvae were determined using the chromogenic substrate, N-benzoyl-DL-arginine-p-nitroanilide (BAPNA), prepared in 1% (v/v) DMSO (dimethyl sulfoxide) and 100 mM Tris–HCl, pH 8.0 buffer. Samples of 10 μL of trypsin-like enzymes were added to 60 μL of assay buffer, and then 200 μL of BAPNA was added a final concentration of 1 mM. Six replicates were made for each assay. The reaction rate was determined by monitoring the absorbance change at 410 nm for 30 min in a VersaMax Microplate Reader (Molecular Devices, US).

2.8. Digestion of cMoL

The digestion of lectin was carried out according to Macedo et al. [16]. cMoL (2 mg/mL) was incubated with midgut homogenate in Tris–HCl 100 mM pH 8.0 buffer. The cMoL/midgut protein ratio was 1:10 (w/w). Digestion was performed for 1, 3, 6, 12, 24 and 48 h at 37 °C and was stopped by immersing the tubes in boiling water for 2 min. The degradation of bovine serum albumin was used as a positive control for protease activity. The proteins were subsequently separated by SDS-PAGE on 12.5% as described by Laemmli [40]. The proteins were detected by silver staining.

2.9. Protease activity of midgut and faecal extracts in polyacrylamide gels containing 0.1% gelatin

Proteins extracted from the midguts and faecal extracts of *A. kuehniella* larvae fed on control diet and diet containing 1% cMoL were run on 12.5% SDS-PAGE containing 0.1% gelatin [41]. The samples were incubated without prior boiling for 30 min at 37 °C. The samples had also been incubated with TLCK, a synthetic trypsin inhibitor (N-p-tosyl-lysine chloroketone, 1 mM) for 30 min at 37 °C to inhibit proteolytic activity prior to SDS-PAGE. Following electrophoresis at 4 °C, the gels were washed with 2.5% Triton X-100 solution for 2 h with shaking to remove the SDS, after which the gels were incubated with 100 mM Tris–HCl buffer, pH 8.0, for 2 h. The gels were subsequently stained with Coomassie brilliant blue R-250. Bands of proteolytic activity appeared as clear (white) zones against a blue background.

2.10. Chitin-column chromatography

To examine the possible interaction of cMoL with chitin, cMoL was chromatographed on a chitin column (2 ml) and equilibrated with 150 mM NaCl. After adsorption of the protein (2 mg), the column was washed with 150 mM and 1 M NaCl, respectively. The bound cMoL was then eluted with 1 M acetic acid. The bound cMoL was then eluted with 0.1 M acetic acid. Fractions (2 ml) were collected, dialyzed against water and used in assays of hemagglutinating activity and protein quantification.

2.11. Hemagglutinating activity assays

HA was performed in microtiter plates, according to Correia and Coelho [42]. The peaks from chromatography (50 μL) were serially two-fold diluted in 150 mM NaCl before addition of 50 μL 2.5% (v/v) suspension of rabbit glutaraldehyde treated erythrocytes. The titer was expressed as the highest dilution exhibiting hemagglutination.

For hemagglutinating activity inhibition, carbohydrate or glycoprotein in solution (50 μL) were added to microtiter plates. Samples of peaks (50 μL) were serially two-fold diluted in monosaccharide solution and incubation (45 min, 27 °C) was performed before the addition of a 2.5% (v/v) suspension of rabbit erythrocytes (50 μL) and HA was observed after 45 min. Inhibition of HA was performed using a D-fructose solution (200, 100, 50 and 25 mM) and azocasein (0.5 mg/mL).

2.12. Statistical analysis

For each treatment and control, data regarding the reduction in weight gain of the larvae, pupae and adults are expressed as means ± SEM. To detect significant differences between treatments, data were analyzed by one-way analysis of variance (ANOVA) and then means ± SEM were separated using a post-hoc Tukey test. A p value ≤ 0.05 was considered to be significant.

3. Results and discussion

The effect of cMoL incorporated in artificial diet on growth, nutritional physiology and survival of larvae was accompanied until fourth instar, pupae and adults (Fig. 1). All nutritional parameters, as well as the total development time (TDT) and growth index (GI), were evaluated together with survival and weight gain to better understand the action of cMoL on this insect. Fig. 1(A and B) shows the effect of cMoL on weight and survival of the larvae of the fourth instar, respectively. According to the graph, it may be clearly seen that the reduction in larval weight gain is dose-dependent. At 1%, cMoL caused a reduction in the average weight of ca. 45%, approaching the ED_{50}. Regression analysis demonstrated that the addition of cMoL to the artificial diet, at 0.1%, resulted in a 0.25 mg decrease in mass; however, no concentration of cMoL tested caused any significant reduction in larval survival. Similar results have been reported for other lectins. Coelho et al. [43] incorporated ACLEC (*Anona coriacea* lectin) in an artificial diet at a level of 1% for *A. kuehniella* larvae and observed a reduction in larval mass of ca. 50%. A lectin from *Koelreuteria paniculata* seeds (Kplec), added to the artificial diet at a concentration of 1%, was toxic to *A. kuehniella,* reducing the larval weight by 84% [44]. Recently, Macedo et al. [16] tested a *Bauhinia monandra* leaf lectin (BmoLL) for anti-insect activity against *A. kuehniella*, *Callosobruchus maculatus* and *Zabrotes subfasciatus*. BmoLL in the artificial diet at 1% produced a decrease in larval weight of 40% for *A. kuehniella* while at 0.5% and 0.4% it produced 20% and 50% decreases in the mass of *Z. subfasciatus* and *C. maculatus*, respectively.

With regard to mortality rate, the same lectin may present different lethality for different insects species. For example, transgenic rice plants expressing GNA show resistance to both *Nephotettix virescens* and *Nilaparvata lugens* [45,46]. In both studies, the survival of nymphs on GNA-expressing plants was significantly reduced (up to 50%) [47]. Nagadhara et al. [48] tested resistance against *Sogatella furcifera* by expressing GNA specifically in the phloem of transgenic rice plants. Survival of nymphs and fecundity of adults both dropped by 90%, feeding decreased, and surviving nymphs suffered delayed development [47]. However, Gatehouse et al. [49] reported the expression of GNA in transgenic potatoes for the control of the tomato moth, *Lacanobia oleracea*, with a significant effect on larval size and adverse affect on the developmental rate, whilst effects on survival were not so marked. This difference in larvicidal activity of GNA towards different insect species illustrates the peculiar specificity of lectins as insect pest management molecules. Therefore, we can infer that other factors intrinsic to each species of insect study can result in different effects on larval mortality; this does not rule out an insecticide role for cMoL, but indicates that significant rates

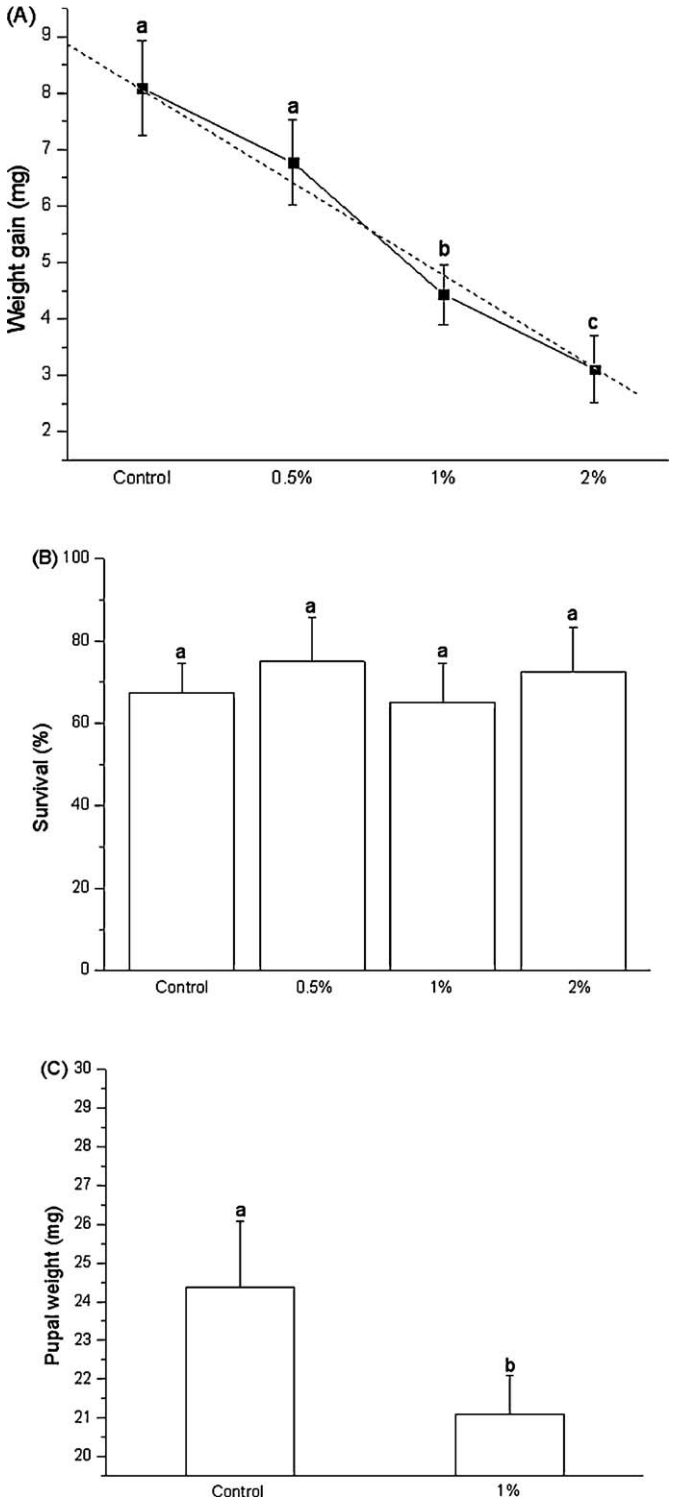

Fig. 1. Effect of dietary cMoL on the (A) mass and (B) survival of *A. kuehniella* larvae. Inset: variation in the size of fourth-instar larvae fed on the control diet (1) or a diet containing 0.5% (2), 1.0% (3) or 2.0% (4) cMoL. (c) Effect of cMoL against pupa of the *A. kuehniella,* resulting from larvae fed on diet containing 1% cMoL. Inset: variation in the size of control pupa (1) or that of larvae fed on 1% cMoL (2). Each point is the mean ± S.E. of ≥60 larvae. The different letters indicate significant differences ($p < 0.05$; Tukey test). Bar = 1 cm.

of larval mortality were not observed for *A. Kuehniella* larvae. The cMoL concentration in the artificial diet ranged from 0.5% to 2%, as these values constitute the range of lectin concentrations found in transgenic crops and were similar to concentrations used in other studies with purified plant lectins [16,20,50,51].

Table 1
Nutritional parameters of *A. kuehniella* fourth-instar larvae fed on 1% cMoL-treated and control diets.

Treatment (%)	ECI (%)	ECD (%)	AD (%)	CM (%)
Nutritional parameters (mean ± SE)				
Control	26.58 ± 1.1a	31.13 ± 1.5a	80.67 ± 4a	68.87 ± 1.5a
1	18.28 ± 1.9b	19.42 ± 1.8b	94.06 ± 2.3b	80.23 ± 1.6b

ECI: efficiency of the conversion of ingested food; ECD: efficiency of the conversion of digested food; AD: approximate digestibility; CM: 100 – ECD.
Means within a column followed by a different letters are significantly different, $p < 0.05$; based on Tukey's test.

Table 2
Effect of cMoL (1% in the diet) on the development of the *A. kuehniella* larvae.

Treatment (%)	%S[a]	TDT[b]	GI[c]
Control	90.1 ± 1	46	0.0424
1	62.5 ± 1.9	61	0.0294

Values are the mean ± S.E. $p < 0.05$ compared to corresponding control (Student's *t*-test).
[a] Survival during the adult stage.
[b] TDT = total developmental time (mean developmental time per insect in days).
[c] GI = growth index [(in % adult emergence)/TDT].

Food intake and faecal production of larvae of *A. kuehniella* treated on a diet containing 1% cMoL decreased by 18.7% and 66.5%, respectively. These results were followed by profound nutritional changes (Table 1). The diet containing 1% cMoL decreased (by approximately 14%) the efficiency by which ingested food was assimilated (AD) by the larvae of *A. kuehniella,* when compared to the control group. The efficiency by which *A. kuehniella* larvae converted food into body mass as shown by the amount of food consumed (ECI), or the amount of food absorbed in the intestine (ECD) decreased by 31.2% and 37.6%, respectively, while the metabolic cost (MC) increased by 16.5%, compared to respective controls. The decrease in dietary utilization suggested that the reduced growth and decrease in faecal production resulted from behavioral and physiological (post-ingestive) effects [43,52,53], possibly caused by the longer retention of food in the gut to maximize AD. A greater AD would help to meet the increased demand for nutrients [54,55] and compensate for the deficiency in foodstuff conversion (reduction in ECI and ECD), perhaps by diverting energy from biomass production into detoxification [43,56]. ECD also decreases as the proportion of digested food metabolised for energy increases [57]. The low values of ECI and ECD observed suggest that chronic toxicity and the reduction in ECD likely resulted from an increase in the proportion of assimilated energy diverted from growth to cover MC associated with detoxification and excretion of cMoL. Boleti et al. [15] described an increase in MC in *A. kuehniella* larvae that was similar to that seen in our study. Coelho et al. [43] observed similar results in their study, where *A. kuehniella* and *C. cephalonica* larvae feed on ACLEC at 2% presented lower food consumption (33% and 28%) and faecal production with decreases of 25% and 40%, respectively, accompanying similar nutritional changes.

The vast majority of individuals fed on cMoL at 1% developed to the pupal stage. Interestingly, a significant delay in larval development time of 15 days was found, compared to the control group. In addition to the significant increase in TDT, treatment with cMoL at 1% caused an increase in pupal mortality of 27.6%, where the surviving pupa were adversely affected and presented a smaller size and a reduction in the average weight of 13.5%, compared to the control group (Fig. 1C). Neither type of deformation was observed in the pupae in both groups. Li and Romeis [30] reported that the presence of GNA in food of green lacewing *Chrysoperla carnea* significantly prolonged the larval development time by 3.7 days on average. Machuka et al. [50] incorporated different lectins from African yam beans (*Sphenostylis stenocarpa*) for *C. maculatus* larvae. The Enugu 95-3 lectin caused an average delay in developmental time of 7.5, 7.8 and 11.3 days at 0.2%, 2.0% and 5.0% dietary levels, respectively, compared to susceptible control seeds. Murdock et al. [55] showed that WGA, lectin, which has N-acetylglucosamine carbohydrate binding specificity, delays *C. maculatus* development by 6.5 and 22.8 days at 0.2% and 1.0%, respectively. The growth index (GI) measures the effects of the food substrate, such as dietary lectin, on both survival and developmental time [36]. The principle behind this parameter is that adverse conditions, such as an unsuitable food substrate, may prolong the developmental period, while fewer individuals survive, resulting in a low GI value. Conversely, a suitable food substrate should result in a high GI value [50,58,59]. At 1%, cMoL caused a significant drop in GI value of more than 30% (Table 2). Similar effects of plant lectins on the development of insects are well documented [60]. The low mortality observed for *A. Kuehniella* larvae fed with cMoL was offset by low rates of adult emergence. Another important result was the delayed development. The extension of the life cycle of a pest is very desirable, since any delay in its development is advantageous because it reduces the emergence ratio of new generations and, thus, reduces the damage caused by the species in the target crop.

A prerequisite for toxicity is that the lectin should be able to survive the hostile proteolytic environment of the insect midgut [43]. The susceptibility of cMoL degradation by proteases of *A. kuehniella* was tested by incubating the lectin with these enzymes in a 1:10 ratio, followed by SDS-PAGE (Fig. 2A). In an attempt to degrade the lectin under study, the larvae of *A. kuehniella* did not show any changes in its enzymatic profile, in the total amount of enzymes or the production of different classes of proteases when analyzed by gel electrophoresis and enzymatic activity *in vitro* (Fig. 2A). cMoL was resistant to digestion during the first 12 h of incubation (Fig. 2B), in contrast to the control that was prepared with BSA, where digestion of protein occurred during the first 3 h (data not shown). Macedo et al. [60] observed that the incubation of TEL (*Talisia esculenta* lectin) with purified insect cysteine-like proteases from *C.* maculatus was unable to digest the lectin at up to 15 h. Another lectin, BmoLL was resistant to gut proteolysis by *C. maculatus* and *Z. subfasciatus* for up 48 h [16]. These results vary according to insect species. The incubation of ACLEC with gut proteases from *C. cephalonica* larvae resulted in the degradation of the lectin that started within first minutes but required several hours for completion. In contrast, ACLEC was more resistant to *A. kuehniella* gut enzymes [43]. This result confirms the effects on larval physiology, reaffirming that the lectin appears to have maintained its structure to exert its insecticidal activity.

Many proteins that bind to chitin are related to plant defense mechanisms against organisms that contain this polysaccharide as a constituent of their membranes. This group includes wheat germ agglutinin (WGA) and other lectins and proteins that are toxic to insects and affect the development of fungi [61]. The mode of action of these proteins is unknown but may interfere with the uptake of nutrients by binding to chitin in larval midgut membranes. This membrane exists in most phytophagous insects and is composed primarily of chitin (containing N-acetylglucosamine residues) and proteins [61,62]. The chromatography of cMoL on the chitin column resulted in two peaks, one of which eluted in the equilibrium buffer and the other with 1 M acetic acid. According to the results of HA assays, we can affirm that the peak eluted from the column with acetic acid is cMoL, as evidenced by the high HA and its complete inhibition by azocasein, characteristic of cMoL (data not shown).

We suggest that the binding of cMoL to chitin demonstrates the interactions of lectin with chitinous structures present in the midgut of larvae. As such, cMoL binds strongly to chitin and presents insecticidal activity, similarly to other insecticidal proteins such as the vicilins [63,64], Talisin [65] and zeatoxin [66], in addition to other lectins [16,43,44]. Because the occurrence of

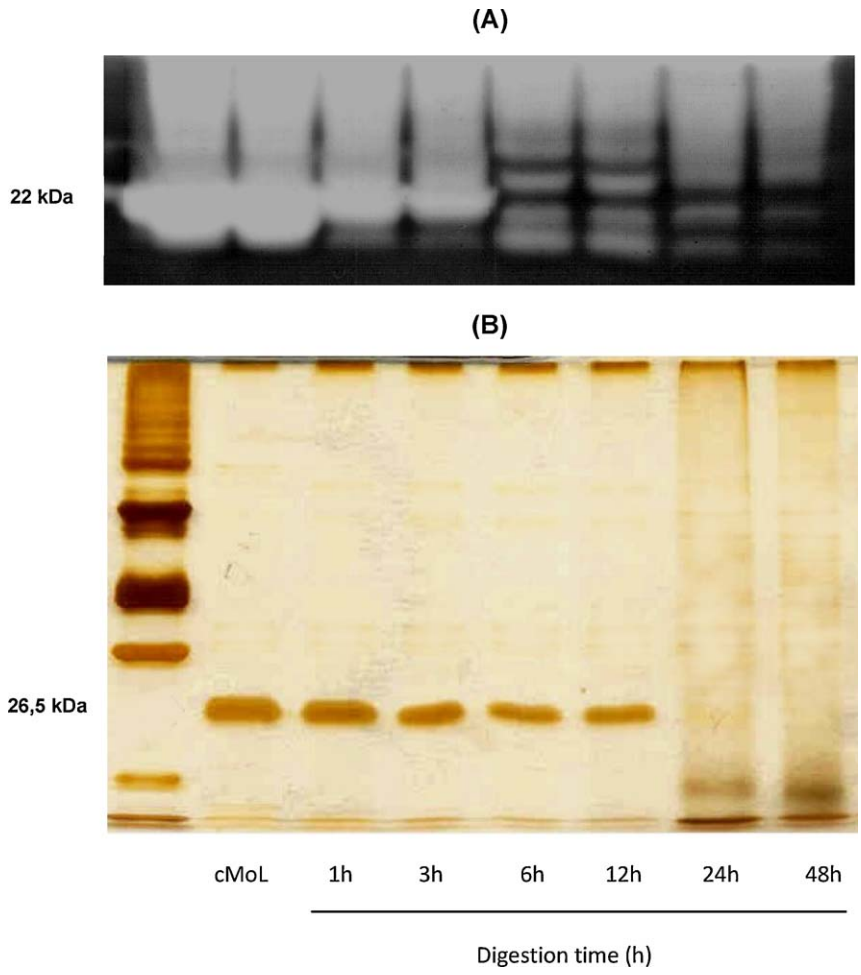

Fig. 2. (A) Proteolytic activity of *A. kuehniella* larvae fed on cMoL. Protease assay SDS-PAGE containing 0.1% gelatin: (1) midgut extract from larvae fed on the control diet, (2) midgut extract from larvae fed on 1% cMoL, (3) faecal extract from larvae fed on the control diet, (4) faecal extract from larvae fed on 1% cMoL, (5) midgut extract from larvae fed on the control diet with TLCK, (6) midgut extract from larvae fed on 1% cMoL with TLCK, (7) faecal extract from larvae fed on the control diet with TLCK, (8) faecal extract from larvae fed on 1% cMoL with TLCK. Proteolytic activity appeared as a clear zone against a dark blue background. (B) SDS-PAGE of cMoL digested by midgut extracts of *A. kuehniella*. cMoL and cMoL digested by 1 h, 3 h, 6 h, 12 h, 24 h and 48 h.

this carbohydrate as a structural component is limited to a few organs, such as the integuments of arthropods, nematodes and the gut linings of insects, chitin metabolism is an excellent target for selective pest control strategies [67]. Eisemann et al. [68] reported that ConA (a mannose/glucose ligand) bound to and increased the permeability of peritrophic membranes of the blowfly *Lucilla cuprina*. These authors concluded that the ingested lectin may restrict the bi-directional movement of nutrients and digestive enzymes across pores in these membranes, thus leading to nutritional deprivation [8,68]. Macedo et al. [16,60] suggest that the alteration in intestinal protein content may result from an interference in the digestive environment, which could increase the difficulty of the transit of molecules through the perotrophic membrane by interfering with recycling and absorption, culminating in a decrease in the protein concentration of gut contents and a greater removal of proteins in the faeces. SDS-PAGE, containing 0.1% gelatin (Fig. 2A), demonstrated that cMoL did not cause any change in the enzymatic content of the larvae, as shown by activity levels and by the similarity of the enzyme profiles obtained from the control group and that fed on cMoL. Another important observation is that, in both groups, the serine proteases are largely digestive enzymes, since the addition of a synthetic inhibitor (TLCK) upgraded its inhibition.

The insecticidal activity of lectins makes them good candidates for the control of insect pests. However, the specific interaction of lectins with the gut epithelium of insects may have a wider potential for biotechnology than the insecticidal property of lectins alone [69]. By developing chimeric proteins that use lectins as a binding domain or subunit, they might be used, (*i*) to assist in concentrating bioactive polypeptides such as Bt toxins and insect hormones [70] at or near vulnerable target sites in the insect alimentary tract, (*ii*) to facilitate the transfer of cytotoxins across the epithelial cell membrane or (*iii*) to help prevent proteolytic digestion of other bioactive proteins to which they have been bound [69]. Other possible biotechnological applications of lectin may include the site direct mutagenesis of cMoL in order to improve its insecticide activity, similar to that performed by Zhu-Salzman et al. [69] for *Griffonia simplicifolia* lectin II (GSII). Through mutagenesis studies, the authors showed that recombinant GSII with an increase in sugar binding also increased the insecticidal activity for *Callosobruchus maculatus*.

In conclusion, this study showed that cMoL is a lectin with a significant negative impact against *A. kuehniella* and suggests that the use of this molecule may constitute an alternative method for the combat this pest. The lectin demonstrated a tight interaction with chitin and proved to be refractory to digestion by *A. kuehniella* midgut proteases, interfering in the digestive environment together with the glycosylated enzymes, other glycoproteins or even in the peritrophic membrane structure, which may hinder the transport of enzymes and their hydrolysis products, reducing the availability of amino acids for larval growth, leading to

the poor development observed. cMoL possesses a great potential as a biotechnological tool, promoting an environmentally friendly agriculture and being less harmful.

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References

[1] Hamshou M, Van Damme EJM, Smagghe G. Entomotoxic effects of fungal lectin from *Rhizoctonia solani* towards *Spodoptera littoralis*. Mycol Res 2010;114:34–40.
[2] Duranti M, Barbiroli A, Scarafoni A, Tedeschi G, Morazzoni P. One step purification of Kunitz soybean trypsin inhibitor. Protein Expres Purif 2003;30:167–70.
[3] Richa KW, Theerapong T, Soottawat B, Saroat R. Three-phase partitioning of trypsin inhibitor from legume seeds. Process Biochem 2009;44:1307–14.
[4] Van Damme EJM, Lannoo N, Peumans WJ. Plant lectins. Adv Bot Res 2008;48:107–209.
[5] Peumans WJ, Van Damme EJM. Lectins as plant defense proteins. Plant Physiol 1995;109:347–52.
[6] Leite YFMM, Silva LMCM, Amorim RCN, Freire EA, Jorge DMM, Grangeiro TB, et al. Purification of a lectin from the marine red alga *Gracilaria ornata* and its effect on the development of the cowpea weevil *Callosobruchus maculatus* (Coleoptera: Bruchidae). Biochim Biophys Acta 2005;1724:137–45.
[7] Sadeghi A, Van Damme EJM, Peumans WJ, Smagghe G. Deterrent activity of plant lectins on cowpea weevil *Callosobruchus maculatus* (F.) oviposition. Phytochemistry 2006;67:2078–84.
[8] Macedo MLR, Freire MGM, Castro MM. Mechanisms of the insecticidal action of TEL (*Talisia esculenta* lectin) against *Callosobruchus maculatus* (Coleoptera: Bruchidae). Arch Insect Biochem Physiol 2004;56:84–96.
[9] Sá RA, Santos NDL, Silva CSB, Napoleão TH, Gomes FS, Cavada BS, et al. Larvicidal activity of lectins from *Myracrodruon urundeuva* on *Aedes aegypti*. Comp Biochem Physiol C 2009;149:300–6.
[10] Sauvion N, Nardonb G, Febvay G, Gategouse AMR, Rahbé Y. Binding of the insecticidal lectin Concanavalin A in pea aphid, *Acyrthosiphon pisum* (Harris) and induced effects on the structure of midgut epithelial cells. J Insect Physiol 2004;50:1137–50.
[11] Bandyopadhyay S, Roy A, Das S. Binding of garlic (*Allium sativum*) leaf lectin to the gut receptors of homopteran pests is correlated to its insecticidal activity. Plant Sci 2001;161:1025–33.
[12] Couty A, de la Vinã G, Clark SJ, Kaiser L, Pham-Delègue MH, Poppy GM. Direct and indirect sublethal effects of *Galanthus nivalis* agglutinin (GNA) on the development of a potato-aphid parasitoid, *Aphelinus abdominalis* (Hymenoptera: Aphelinidae). J Insect Physiol 2001;47:553–61.
[13] Silva MDC, Sá RA, Napoleão TH, Gomes FS, Santos NDL, Albuquerque AC, et al. Purified *Cladonia verticillaris* lichen lectin: insecticidal activity on *Nasutitermes corniger* (Isoptera: Termitidae). Int Biodeter Biodegr 2009;63:334–40.
[14] Coellho MB, Marangoni S, Macedo MLR. Insecticidal action of *Annona coriacea* lectin against the flour moth *Anagasta kuehniella* and the rice moth *Corcyra cephalonica* (Lepidoptera: Pyralidae). Comp Biochem Physiol C 2007;146:406–14.
[15] Boleti APA, Kubo CEG, Macedo MLR. Effect of pouterin, a protein from *Pouteria torta* (Sapotaceae) seeds, on the development of *Anagasta kuehniella* (Lepidoptera: Pyralidae). Int J Trop Insect Sci 2009;29:24–30.
[16] Macedo MLR, Freire MGM, Silva MBR, Coelho LCBB. Insecticidal action of *Bauhinia monandra* leaf lectin (BmoLL) against *Anagasta kuehniella* (Lepidoptera: Pyralidae), *Zabrotes subfasciatus* and *Callosobruchus maculatus* (Coleoptera: Bruchidae). Comp Biochem Physiol A 2007;146:486–98.
[17] Costa RMPB, Vaz AFM, Oliva MLV, Coelho LCBB, Correia MTS, Carneiro-da-Cunha MG. A new mistletoe *Phthirusa pyrifolia* leaf lectin with antimicrobial properties. Process Biochem 2010;45:526–33.
[18] Bell HA, Fitches E, Down RE, Marris GC, Edwards JP, Gatehouse JA, et al. The effect of snowdrop lectin (GNA) delivered via artificial diet and transgenic plants on *Eulophus pennicornis* (Hymenoptera: Eulophidae), a parasitoid of the tomato moth *Lacanobia oleracea* (Lepidoptera: Noctuidae). J Insect Physiol 1999;45:983–91.
[19] Gatehouse AMR, Down RE, Powell KS, Sauvion N, Rahbé Y, Newell CA, et al. Transgenic potato plants with enhanced resistance to the peach-potato aphid *Myzus persicae*. Entomol Exp Appl 1996;79:295–307.
[20] Down RE, Gatehouse AMR, Hamilton WDO, Gatehouse JA. Snowdrop lectin inhibits development and decreases fecundity of the glasshouse potato aphid (*Aulacorthum solani*) when administered in vitro and via transgenic plants both in laboratory and glasshouse trials. J Insect Physiol 1996;42:1035–45.
[21] Foissac X, Loc NT, Christou P, Gatehouse AMR, Gatehouse JA. Resistance to green leafhopper (*Nephotettix virescens*) and brown planthopper (*Nilaparvata lugens*) in transgenic rice expressing snowdrop lectin (*Galanthus nivalis* agglutinin; GNA). J Insect Physiol 2000;46:573–83.
[22] Nagadhara D, Ramesh S, Pasalu IC, Rao YK, Krishnaiah NV, Sarma NP, et al. Transgenic indica rice resistant to sap-sucking insects. Plant Biotechnol J 2003;1:231–40.
[23] Nagadhara D, Ramesh S, Pasalu IC, Kondal RY, Sarma NP, Reddy VD, et al. Transgenic rice plants expressing the snowdrop lectin gene (GNA) exhibit high-level resistance to the whitebacked planthopper (*Sogatella furcifera*). Theor Appl Genet 2004;109:1399–405.
[24] Wang Z, Zhang K, Sun X, Tang K, Zhang J. Enhancement of resistance to aphids by introducing the snowdrop lectin gene GNA into maize plants. J Biosci 2005;30:627–38.
[25] Hilder VA, Powell KS, Gatehouse AMR, Gatehouse JA, Gatehouse LN, Shi Y, et al. Expression of snowdrop lectin in transgenic tobacco plants results in added protection against aphids. Transgenic Res 1995;4:18–25.
[26] Stoger E, Williams S, Christou P, Down RE, Gatehouse JA. Expression of the insecticidal lectin from snowdrop (*Galanthus nivalis* agglutinin; GNA) in transgenic wheat plants: effects on predation by the grain aphid *Sitobion avenae*. Mol Breeding 1999;5:65–73.
[27] Wu CY, Ye ZB, Li HX, Tang KX. Genetic transformation of tomato with snowdrop lectin gene (GNA). Acta Bot Sin 2000;42:719–23.
[28] Sétamou M, Bernal JS, Legaspi JC, Mirkov TE. Effects of snowdrop lectin (*Galanthus nivalis* Agglutinin) expressed in transgenic sugarcane on fitness of *Cotesia flavipes* (Hymenoptera: Braconidae), a parasitoid of the nontarget pest *Diatraea saccharalis* (Lepidoptera: Crambidae). Ann Entomol Soc Am 2002;95:75–83.
[29] Sétamou M, Bernal JS, Mirkov TE, Legaspi JC. Effects of snowdrop lectin on Mexican rice borer (Lepidoptera: Pyralidae) life history parameters. J Econ Entomol 2003;96:950–6.
[30] Li Y, Romeis J. Impact of snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) on adults of the green lacewing, *Chrysoperla carnea*. J Insect Physiol 2009;55:136–43.
[31] Santos AFS, Luz LA, Argolo ACC, Teixeira JA, Paiva PMG, Coelho LCBB. Isolation of a seed coagulant *Moringa oleifera* lectin. Process Biochem 2009;44:504–8.
[32] Katre UV, Suresh CG, Khan MI, Gaikwad SM. Structure–activity relationship of a hemagglutinin from *Moringa oleifera* seeds. Int J Biol Macromol 2008;42:203–7.
[33] Coelho JS, Santos NDL, Napoleão TH, Gomes FS, Ferreira RS, Zingali RB, et al. Effect of *Moringa oleifera* lectin on development and mortality of *Aedes aegypti* larvae. Chemosphere 2009;77:934–8.
[34] Ramos VS, Freire MGM, Parra JRP, Macedo MLR. Regulatory effects of an inhibitor from *Plathymenia foliolosa* seeds on the larval development of *Anagasta kuehniella* (Lepidoptera). Comp Biochem Physiol A 2009;152:255–61.
[35] Van Damme EJM, Peumans WJ, Barre A, Rougé P. Plant lectins: a composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. Crit Rev Plant Sci 1998;17:575–692.
[36] Howe RW. A parameter for expressing the suitability of an environment for insect development. J Stored Prod Res 1971;7:63–5.
[37] Scriber JM, Slansky Jr F. The nutritional ecology of immature insects. Ann Rev Entomol 1982;26:183–211.
[38] Macedo MLR, Freire MGM, Cabrini EC, Toyama MH, Novello JC, Marangoni S. A trypsin inhibitor from *Peltophorum dubium* seeds active against pest proteases and its effect on the survival of *Anagasta kuehniella* (Lepidoptera: Pyralidae). Biochim Biophys Acta 2003;1621:170–81.
[39] Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[40] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[41] Michaud D, Faye L, Yalle S. Eletrophoretic analysis of plant cysteine and serine proteinases using gelatin-containing polyacrylamide gels and class-specific proteinase inhibitors. Electrophoresis 1993;14:94–9.
[42] Correia MTS, Coelho LCBB. Purification of a glucose/mannose specific lectin, isoform 1, from seeds of *Cratylia mollis* Mart. (Camaratu bean). Appl Biochem Biotechnol 1995;55:261–73.
[43] Coelho MB, Marangoni S, Macedo MLR. Insecticidal action of *Annona coriacea* lectin against the flour moth *Anagasta kuehniella* and the rice moth *Corcyra cephalonica* (Lepidoptera: Pyralidae). Comp Biochem Physiol C 2007;146:406–14.
[44] Macedo MLR, Damico DC, Freire MGM, Toyama MH, Marangoni S, Novello JC. Purification and characterization of an N-acetylglucosamine-binding lectin from *Koelreuteria paniculata* seeds and its effect on the larval development of *Callosobruchus maculatus* (Coleoptera: Bruchidae) and *Anagasta kuehniella* (Lepidoptera: Pyralidae). J Agric Food Chem 2003;51:2980–6.
[45] Foissac X, Thi Loc N, Christou P, Gatehouse AMR, Gatehouse JA. Resistance to green leafhopper (*Nephotettix virescens*) and brown planthopper (*Nilaparvata lugens*) in transgenic rice expressing snowdrop lectin (*Galanthus nivalis* agglutinin; GNA). J Insect Physiol 2000;46:573–83.
[46] Nagadhara D, Ramesh S, Pasalu IC, Rao YK, Krishnaiah NV, Sarma NP, et al. Transgenic indica rice resistant to sap-sucking insects. Plant Biotech J 2003;1:231–40.
[47] Michiels K, Van Damme EJM, Smaggle G. Plant–insect interactions: what can we learn from plant lectins? Arch Insect Biochem Physiol 2000;73:193–212.
[48] Nagadhara D, Ramesh S, Pasalu IC, Rao YK, Sarma NP, Reddy VD, et al. Transgenic rice plants expressing the snowdrop lectin gene (GNA) exhibit high-level resistance to the whitebacked planthopper (*Sogatella furcifera*). Theor Appl Genet 2004;109:1399–405.

[49] Gatehouse AMR, Davison GM, Newell CA, Merryweather A, Hamilton WDO, Burgess EPJ, et al. Transgenic potato plants with enhanced resistance to the tomato moth *Lacanobia oleracea*: growth room trials. Mol Breeding 1997;3:49–63.
[50] Machuka JS, Okeola OG, Chrispeels MJ, Jackai LEN. African yam beans seed lectin affects the development of the cowpea weevil but does not affect the development of larvae of legume pod borer. Phytochemistry 2000;53:667–74.
[51] Fitches E, Gatehouse AMR, Gatehouse JA. Effects of snowdrop lectin (GNA) delivered via artificial diet and transgenic plants on the development of tomato moth (*Lacanobia oleracea*) larvae in laboratory and glasshouse trials. J Insect Physiol 1997;43:727–39.
[52] Koul O, Isman MB. Effects of azadirachtin on dietary utilization and development of variegated cutworm, *Peridroma saucia*. J Insect Physiol 1991;37:591–8.
[53] Venzon M, Rosado MC, Fadini MA, Ciociola AI, Pallini A. The potential of neem Azal for the control of coffee leaf pests. Crop Prot 2004;24:213–9.
[54] Nathan SS, Kalaivani K, Murugan K, Chung PG. Efficacy of neem limonoids on *Cnaphalocrocis medicinalis* (Guenée) (Lepidoptera: Pyralidae) the rice leafholder. Crop Prot 2005;24:760–3.
[55] Nathan SS, Chung PG, Murugan K. Effect of biopesticides applied separately or together on nutritional indices of the rice leaffolder *Cnaphalocrocis medicinalis* (Guenée) (Lepidoptera: Pyralidae). Phytoparasitica 2005;33:187–95.
[56] Nathan SS, Kalaivani K. Efficacy of nucleopolyhedrovirus (NPV) and azadirachtin on *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae). Biol Control 2005;34:93–8.
[57] Wheeler DA, Isman MB. Antifeedant and toxic activity of *Trichilia americana* extract against the larvae of *Spodoptera litura*. Entomol Exp Appl 2001;98: 9–16.
[58] Murdock LL, Huesing JE, Nielson SS, Pratt RC, Shade RE. Biological effects of plant lectins on the cowpea weevil. Phytochemistry 1990;29:85–9.
[59] Omitogun OG, Jackai LEN, Thottappilly G. Isolation of insecticidal lectin-enriched extracts from African yam bean (*Sphenostylis stenocarpa*) and other legume species. Entomol Exp Appl 1999;301:9301–11.
[60] Macedo MLR, Castro MM, Freire MGM. Mechanisms of the insecticidal action of TEL (*Talisia esculenta* lectin) against *Callosobruchus maculatus* (Coleoptera: Bruchidae). Arch Insect Biochem Physiol 2004;56:84–96.
[61] Macedo MLR, Mello GC, Freire MGM, Novello JC, Marangoni S, Matos DGG. Effect of a trypsin inhibitor from *Dimorphandra mollis* seeds on the development of *Callosobruchus maculatus*. Plant Physiol Biochem 2002;40:891–8.
[62] Richards AG, Richards PA. The peritrophic membranes of insects. Annu Rev Entomol 1977;22:219–40.
[63] Macedo MLR, Fernandes KVS, Sales MP, Xavier-Filho J. Vicilins variants and the resistance of cowpea (*Vigna unguiculata*) seeds to the cowpea weevil (*Callosobruchus maculatus*). Comp Biochem Physiol C 1993;105:89–94.
[64] Macedo MLR, Fernandes KVS, Sales MP, Xavier-Filho J. Purification and properties of storage proteins (vicilins) from cowpea (*Vigna unguiculata*) seeds which are susceptible or resistant to the bruchid beetle *Callosobruchus maculatus*. Braz J Med Biol Res 1995;26:183–90.
[65] Freire MGM, Vasconcelos IM, Oliveira MV, Souza Filho GA, Macedo MLR. Characterization of a saccharide-binding protein from *Talisia esculenta* seeds with trypsin inhibitory activity. Protein Peptide Lett 2009;16:1557–64.
[66] Macedo MLR, Coelho MB, Freire MGM, Machado OLT, Marangoni S, Novello JC. Effect of a toxic protein isolated from *Zea mays* seeds on the development and survival of the cowpea weevil, *Callosobruchus maculatus*. Protein Peptide Lett 2000;17:25–31.
[67] Kramer KJ, Muthukrishnan S. Insect chitinases: molecular biology and potential use as biopesticide. Insect Biochem Mol Biol 1997;27:887–900.
[68] Eisemann CH, Donaldson RA, Pearson RD, Cadagon LC, Vacuolo T, Tellman RL. Larvicidal activity of lectins on *Lucilia cuprina*: mechanism of action. Entomol Exp Appl 1994;72:1–11.
[69] Zhu-Salzman K, Shade RE, Koiwa H, Salzman RA, Narasimhan M, Bressan RA, et al. Carbohydrate binding and resistance to proteolysis control insecticidal activity of *Griffonia simplicifolia* lectin II. Proc Natl Acad Sci USA 1998;95:15123–8.
[70] Jeffers LA, Roe RM. The movement of proteins across the insect and tick digestive system. J Insect Physiol 2008;54:319–32.