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ARTICLE

Purification of Legumin-Like Proteins from *Coffea arabica* and *Coffea racemosa* Seeds and Their Insecticidal Properties toward Cowpea Weevil (*Callosobruchus maculatus*) (Coleoptera: Bruchidae)

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Legumin-like proteins from seeds of *Coffea arabica* (CaL-1 and CaL-2) and *Coffea racemosa* (CrL-1 and CrL-2) were characterized and isolated by gel filtration and reverse-phase chromatography. The insecticidal properties of the purified proteins were tested against *Callosobruchus maculatus* using artificial diets. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analyses indicated that CaL-1 is composed of two subunits of 33 and 24 kDa, while CaL-2, CrL-1, and CrL-2 were monomeric with a single band of 14 kDa. The LD_{50} values were 0.5% (w/w) for CaL-1 and 0.3% (w/w) for CaL-2, CrL-1, and CrL-2. ED_{50} at 0.3% was assessed for all protein concentrations. The legumin-like proteins were not digested by midgut homogenates of *C. maculatus* until 8 h of incubation. CaL-1 and CaL-2 (*C. arabica*) and CrL-1 and CrL-2 (*C. racemosa*) are chitin-binding proteins, and their insecticidal properties toward *C. maculatus* larvae might be related to their capacity to bind chitin present in the larval gut and their associated low digestibility.

KEYWORDS: Coffee; endosperm; globulin; legumin-like; seed

INTRODUCTION

Coffee (*Coffea* sp.) is one of the most important tropical crops in the world, generating important revenues for producer countries as well as the whole coffee chain (*1*). Coffee beans need to be roasted and ground to produce the beverage. Usually, temperatures of around 220 °C are used to roast coffee beans, producing enormous biochemical alterations and making the coffee beverage a complex mixture of compounds. In this process, proteins and amino acids are very important precursors of many volatile compounds, because they participate in the Maillard reaction with sugars (*2*).

Although the importance of nitrogen compounds to beverage quality is well-known (*2*, *3*), little is known about the proteins in coffee seeds. Most of the studies have focused on globulin proteins. Globulins are the major storage proteins in legume seeds, and with some few exceptions, legume seeds contain two major types of globulins, named vicilin (7S) and legumin (11S) (*4*). An 11S legumin-like protein represents almost 45% of the total coffee bean protein content (*5*, *6*). Rogers et al. (*6*) reported a detailed investigation of proteins in *Coffea arabica* seeds using 2D electrophoretic analysis coupled to mass spectrometry (MS) characterization and found that the most abundant spots were subunits of the 11S globulin. Amino acid sequencing showed that these belong to the 11S family of plant storage proteins. The 11S legumin-like protein is a 55 kDa polypeptide, which under denaturating conditions is cleaved into two polypeptides of 22 kDa (β arm) and 33 kDa (α arm). However, dependent upon the coffee species, there might be a large variation of the 11S coffee seed legumin-like protein. While no differences were observed among varieties of *C. arabica*, other species showed variations in terms of protein mass and electrophoretic profile (*7*). *Coffea racemosa* had a unique profile, presenting a main protein band of about 14 kDa. Further molecular studies on the 11S protein from *C. arabica* showed the existence of a multigene family, and the exclusive expression in coffee endosperm is driven by a tissue-specific promoter (*8*). Previous reports mainly focused on the total protein content of the bean, and little attention was given to the 11S legumin-like coffee protein (see ref *8*). During seed germination, the 11S legumin-like protein level in coffee beans decreases, indicative of a storage role for this protein (*9*). Recently, it was shown that this protein is also formed in the coffee seed embryo during fruit development (*10*).

Several reports have shown globulins acting as plant defenses against insects and other herbivores. A toxic vicilin (EcV) isolated from *Enterolobium contortisiliquum* seeds was highly toxic to *Callosobruchus maculatus* (*11*), as were globulins and albumins isolated from *Luetzelburgia auriculata* seeds (*12*). The glycoprotein zeatoxin (globulin) isolated from *Zea mays* seeds was toxic to

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C. maculatus when supplied in an artificial diet (*13*). In coffee, there are no reports until now on the insecticidal properties of the seed proteins.

In this study, we purified legumin-like proteins from *C. arabica* and *C. racemosa* seeds, characterized their physicochemical properties, and for the first time, tested their effects on the development of *C. maculatus* larvae, a bruchid used as a model in studies to test insecticidal activity of isolated proteins. We chose these coffee species to study because the former is the most cultivated species and the latter has a unique electrophoretic protein profile (*7*). The isolated proteins were also evaluated for their susceptibility to digestion by proteolytic enzymes present in the midgut of this weevil species. To our knowledge, this is the first report on the insecticidal activity of coffee proteins.

MATERIALS AND METHODS

Seeds. Seeds of *C. arabica* and *C. racemosa* were kindly supplied by Dr. Luiz Gonzaga Esteves Vieira from the Instituto Agronômico do Paraná, Londrina, PR, Brazil. Fruits were collected from the trees in the field, and only those at the cherry stage were selected and dry-processed (*14*).

Purification of Legumin-Like Proteins. Seeds with 10% water content were used to extract the legumin-like proteins according to the protocol described by Baú et al. (*7*). Seeds were frozen in liquid N_2 and quickly and finely ground in a knife mill, prechilled with liquid N_2. The resulting powder was defatted in Soxhlet and hexane for 12 h. Proteins were extracted from 0.5 g of defatted powder in a mortar with pestle using 10 mL of 100 mM sodium borate ($NaBO_3$) at pH 8.0, 50 mM sodium diethyldithiocarbamate (DIECA), 50 mM ethylenediaminetetraacetic acid (EDTA), 300 mM NaCl, 114 mM ascorbate, and 0.5 g of polyvinylpolypyrrolidone (PVPP) at 4 °C for 20 min. The supernatant was recovered by centrifugation (39200*g* for 20 min), and the pellet was extracted again (10 mL of buffer). Proteins in the combined supernatants were precipitated with 80% $(NH_4)_2SO_4$, and the pellet recovered by centrifugation was dissolved in a small volume of distilled H_2O and dialyzed against distilled H_2O for 24 h at 4 °C. Proteins in the dialyzed extract were lyophilized, divided in batches, and stored at −20 °C until use. Lyophilized proteins were dissolved in 50 mM Tris-HCl at pH 8.0 containing 50 mM KCl and applied to a Sephacryl S-200 column (3.0 × 50 cm) equilibrated with the same buffer. Collected fractions were dialyzed against distilled water, lyophilized, and then purified by high-performance liquid chromatography (HPLC) using a C-18 reversed phase column (Spherisob ODS2, 3.9 × 30 cm) using 100% solvent A [0.1% trifluoroacetic acid (TFA) in water] for 10 min and a linear gradient (0−100%) of solvent B (0.08% TFA in 80% acetonitrile) over 30 min at a flow rate of 1.0 mL/min. Protein elution was monitored with a UV detector at 230 nm.

Protein concentrations were determined by the dye-binding method (*15*) using a ready-to-use reagent from BioRad, and bovine serum albumin was used as a standard.

Neutral Sugar Content. The carbohydrate content of the legumin-like proteins was determined using the phenol−sulphuric acid method (*16*), and D-glucose was used as a standard.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE of legumins was carried out according to Laemmli (*17*) using 5% stacking and 17% separating gels under reducing and non-reducing conditions. Proteins were stained with Coomassie Brilliant Blue R-250, and molecular mass was determined using molecular-mass references of phosphorylase B (94 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin (20.1 kDa), and α-lactalbumin (14.4 kDa).

Lectin Activity Assay. The hemagglutinating activities of coffee legumin-like proteins were tested against rat and human erythrocytes. Serial dilutions (2-fold) of legumins (50 μL) in saline solution (50 μL) were prepared in microtiter plates, and 50 μL of a 2% erythrocyte suspension was then added to each well. Agglutination was recorded after 1 h at room temperature, and hemagglutinating activity was expressed as the reciprocal of the highest dilution, producing positive agglutination. Five replicates were made for each analysis.

Total Proteinase Activity. The total proteolytic activity was determined using *N*-benzoyl-D,L-arginyl-*p*-nitroanilide (BAPNA) as the substrate. Protein (50 μg) was incubated in 100 mM Tris-HCl at pH 8.0 in a final volume of 0.1 mL for 10 min before the addition of 1 mL of 1 mM substrate. The reaction proceeded at 37 °C for 20 min and was then stopped by adding 0.2 mL of 30% acetic acid, and absorbance was then determined at 410 nm (*18*). Incubations were performed for at least three periods to calculate the initial rates of hydrolysis. The assays were performed under conditions in which enzyme activity was proportional to the protein concentration and the time of incubation. One enzyme unit was defined as the amount of protein that catalyzes the cleavage of 1 μmol of substrate per minute. Five replicates were made for each analysis.

Affinity Chromatography on a Chitin-Binding Column. A chitin column (2 × 6 cm; 20 mL bed volume) (*19*) was equilibrated with 50 mM phosphate buffer at pH 7.6. Protein (2 mg of protein in 1 mL of phosphate buffer) was loaded into the column, and the column was washed with the same buffer until the absorbance at 280 nm was zero. Then, the adsorbed proteins were further eluted with 100 mM HCl, and 2 mL fractions were collected. The protein concentration was estimated on the basis of the absorbance at 280 nm. Three replicates were made for each sample.

Insect Bioassays. The colony of *C. maculatus* was reared at 28 ± 1 °C, with a relative humidity of 65−75%, and the insects were fed with seeds of a susceptible cowpea cultivar (Epace-10). To examine the effects of the purified proteins on *C. maculatus* development, an artificial seed system was used (*20*). Artificial seeds (ca. 400 mg each) were made from finely ground cowpea seeds (Epace 10 cultivar) using a cylindrical brass mold and a hand press. Artificial seeds containing coffee legumin-like proteins at concentrations of 0.1, 0.3, and 0.5% (w/w) were obtained by thoroughly mixing the proteins with cowpea meal and pressing, as described above. Each treatment had five artificial seeds and was replicated 3 times for each of the above concentrations. After a 48 h period of insect acclimation in the growth chamber, the seeds were offered to nine 2−3-day-old fertilized females. After allowing 24 h for oviposition, the number of eggs per seed was reduced to five ($n = 75$). After incubation for 20 days at 28 °C and 70−75% relative humidity, the seeds were opened and the weight and number of larvae were determined. Control artificial seeds were made with Epace-10 cultivar meal, which did not contain coffee protein. The effective dose for 50% response (ED_{50}) is the protein concentration that decreases the mass of the larvae to 50% of the mass of the control. The lethal dose for 50% mortality (LD_{50}) is the protein concentration that decreases the number of larvae to 50% of the number found in control seeds.

Midgut Preparation and Digestion of Legumin-Like Protein. Homogenates of the insect larval guts were prepared using fourth instar larvae (*20*). The larvae were cold-immobilized and dissected in cold 250 mM NaCl, and 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 all extraneous tissue was removed and the lumen was freed of its contents by rinsing in 250 mM NaCl, 200 midgut tissues were homogenized with 1 mL of 0.1 M acetate buffer at pH 5.6, 1 mM cysteine, and 3 mM EDTA in a hand-held Potter−Elvehjem homogenizer immersed in ice. Midgut homogenates were centrifuged at 17000*g* for 20 min at 4 °C, and the supernatants were collected and stored at −20 °C until use. The protein concentration in the extracts was determined according to Bradford (*15*).

Legumin-like proteins were incubated with the midgut in 100 mM acetate buffer at pH 5.6 containing 1 mM cysteine and 3 mM EDTA. The legumin-like protein/midgut protein ratio was 1:1. Digestion was allowed for 0.5, 1, 2, 4, and 8 h at 37 °C and was stopped by immersing the tubes in boiling water for 2 min. Five replicates were made for each sample.

Statistical Analysis. All data were examined using one-way analysis of variance (ANOVA), and a p value < 0.001 was considered to be significant. Means were compared by the Tukey test. The Statistica software was used for the analysis.

RESULTS

Seed storage proteins are often difficult to purify because of their heterogeneous nature and lack of measurable functionality, such as modification of substrates. Additionally, 11S globulins, which are the major storage globulins, are oligomeric proteins that exhibit dissociation/association behavior depending upon the pH and ionic strength of the medium, which creates further complications during isolation from plant sources.

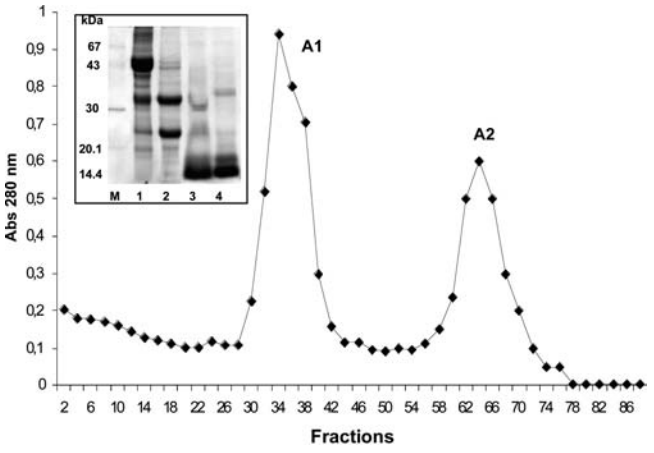

Figure 1. Sephacryl S-200 chromatography and SDS−PAGE of the crude extract of *C. arabica* seeds. (Inset) Lane M, molecular marker; lane 1, peak A1; lane 2, peak A1 in the presence of the reducing agent; lane 3, peak A2; lane 4, peak A2 in the presence of the reducing agent.

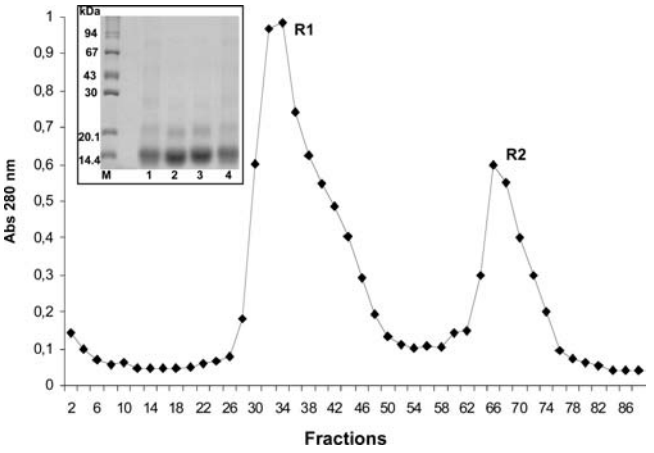

Figure 3. Sephacryl S-200 chromatography and SDS−PAGE of the crude extract of *C. racemosa* seeds. (Inset) Lane M, molecular marker; lane 1, peak R1; lane 2, peak R1 in the presence of the reducing agent; lane 3, peak R2; lane 4, peak R2 in the presence of the reducing agent.

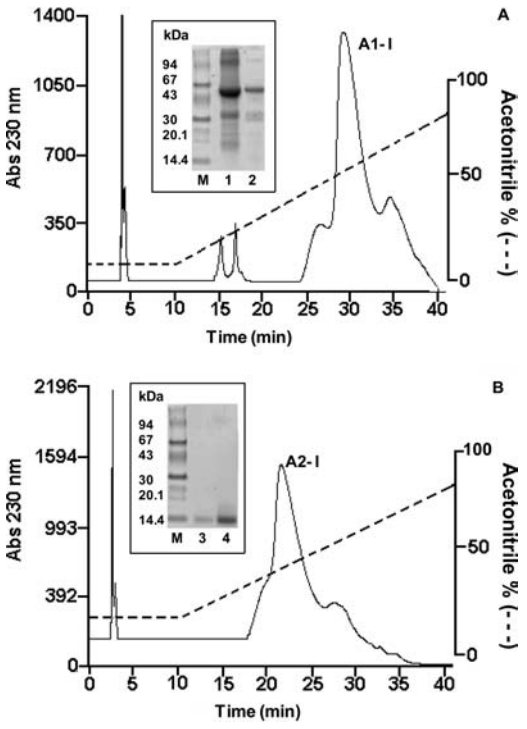

Figure 2. Reverse-phase HPLC separation of the fractions of *C. arabica* seeds: fractions corresponding to (A) peak A1 and (B) peak A2 from Sephacryl S-200. (Inset) Lane M, molecular marker; lane 1, peak CaL-1; lane 2, peak CaL-1 in the presence of the reducing agent; lane 3, peak CaL-2; lane 4, peak CaL-2 in the presence of the reducing agent.

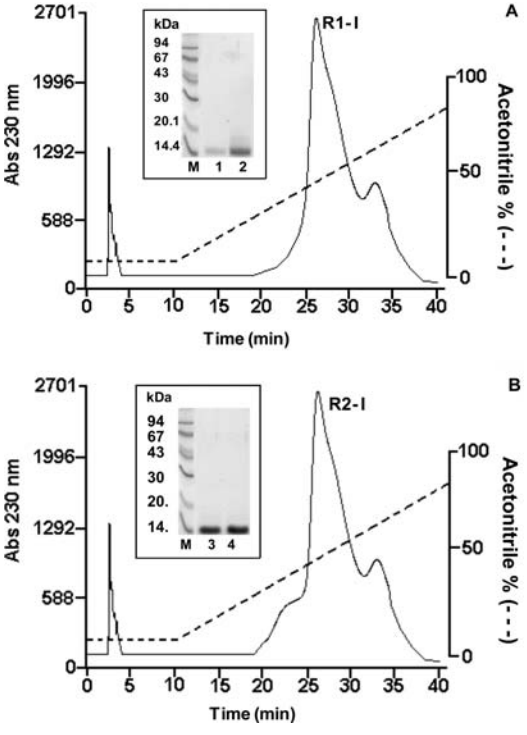

Figure 4. Reverse-phase HPLC separation of the fractions of *C. racemosa* seeds: fractions corresponding to (A) peak R1 and (B) peak R2 from Sephacryl S-200. (Inset) Lane M, molecular marker; lane 1, peak CrL-1; lane 2, peak CrL-1 in the presence of the reducing agent; lane 3, peak CrL-2; lane 4, peak CrL-2 in the presence of the reducing agent.

Pure legumin-like protein from *C. arabica* and *C. racemosa* seeds was obtained in two chromatographic steps. Crude extracts were applied to a Sephacryl S-200 column, and the elution profiles resulted in two peaks, A1 and A2 (**Figure 1**) in *C. arabica* extracts and R1 and R2 in *C. racemosa* extracts (Figure **3**). After dialysis against water, centrifugation, and lyophilization, each peak (A1, A2, R1, and R2) was chromatographed on an HPLC reverse-phase column and the elution profiles showed one major peak for each fraction of *C. arabica* (CaL-1, **Figure 2A**; CaL-2, **Figure 2B**) and *C. racemosa* (CrL-1, **Figure 4A**; CrL-2, **Figure 4B**). All isolated fractions did not show proteinase or hemagglutinating activities (data no shown).

All of the legumin-like proteins isolated were identified as glycoproteins composed of approximately 33% (CaL-1), 3.26% (CaL-2), 15% (CrL-1), and 1.6% (CrL-2) carbohydrate and 10% (CaL-1), 3% (CaL-2), 5.29% (CrL-1), and 1% (CrL-2) total protein. The purified proteins of *C. arabica* (CaL-1 and CaL-2) and *C. racemosa* (CrL-1 and CrL-2) were analyzed by SDS−PAGE. The isolated legumin-like CaL-1 was composed of two bands of ∼33 and ∼24 kDa (lane 1 in the inset of **Figure 2A**), and several weakly stained bands were observed. When CaL-1 was reduced with 2-mercaptoethanol, two prominent bands of 33 and 24 kDa were observed in the gel (lane 2 in the inset of **Figure 2A**). CaL-2 (inset of **Figure 2B**) isolated from

Table 1. Effect of *C. arabica* and *C. racemosa* Legumin-Like Proteins on the Survival and Weight of *C. maculatus* Larvae

	larval survival (%)				larval weight (mg)			
protein concentratio n (%)	CaL-1	CaL-2	CrL-1	CrL-2	CaL-1	CaL-2	CrL-1	CrL-2
0.0	86 ± 2.5 a	86 ± 2.4 a	86 ± 2.4 a	86 ± 2.4 a	5.27 ± 0.1 a	5.26 ± 0.1 a	5.27 ± 0.1 a	5.27 ± 0.1 a
0.1	56 ± 1.5 b	65 ± 4.8 b	50 ± 5.0 b	50 ± 7.2 b	1.71 ± 0.1 b	3.70 ± 0.2 b	2.35 ± 0.5 b	4.75 ± 0.3 b
0.3	51 ± 0.6 b	35 ± 5.5 c	44 ± 6.2 b	33 ± 5.4 c	1.87 ± 0.1 b	2.01 ± 0.3 c	1.56 ± 0.1 d	1.20 ± 0.1 c
0.5	44 ± 1.5 b	30 ± 4.5 c	35 ± 6.1 c	35 ± 5.1 c	1.77 ± 0.1 b	2.0 ± 0.2 c	4.70 ± 0.3 c	1.44 ± 0.2 c

C. arabica and CrL-1 (inset of **Figure 4A**) and CrL-2 (inset of **Figure 4B**) isolated from *C. racemosa* seeds showed a single protein band with an apparent molecular mass of 14 kDa under non-reducing and reducing conditions.

The effects of the coffee 11S legumin-like proteins on the development of *C. maculatus* were assessed by determining the weight and number of surviving larvae (fourth instar) fed with a diet containing increasing amounts of the isolated globulins. The dose−response effect on the mortality and growth of the insect larvae is shown in **Table 1**. The survival and weight of cowpea weevil larvae feeding on control seeds were about 86% and 5.26 mg, respectively. A statistically significant reduction of about 50% (LD_{50}, lethal dose) in larval survival was observed with CaL-1 at 0.5% (w/w) and CaL-2, CrL-1, and CrL-2 at 0.3% (w/w). In addition, all protein concentrations significantly affected the larval weight relative to larvae fed on control seeds with an ED_{50} (effective dose) of 0.3%.

The susceptibility of CaL-1, CaL-2, CrL-1, and CrL-2 to degradation by *C. maculatus* midgut proteolytic enzymes was evaluated by incubating the isolated proteins with a midgut protein extract followed by SDS−PAGE (**Figure 5**). Incubation of CaL-2 (**Figure 5B**), CrL-1 (**Figure 5C**), and CrL-2 (**Figure 5D**) with midgut extracts for up to 8 h showed that these legumin-like proteins were resistant to proteolysis. However, a similar incubation with CaL-1 showed enzymatic hydrolysis within the first 30 min of incubation (**Figure 5A**). BSA was hydrolyzed from the first hour onward when incubated with the midgut extract (data not shown). CaLs (1 and 2) and CrLs (1 and 2) were retained by the chitin-binding affinity chromatography column and after elution by sequential 100 mM acetic acid and 100 mM HCl washing, showing the same SDS−PAGE profile of a previous loading on the column (data not shown).

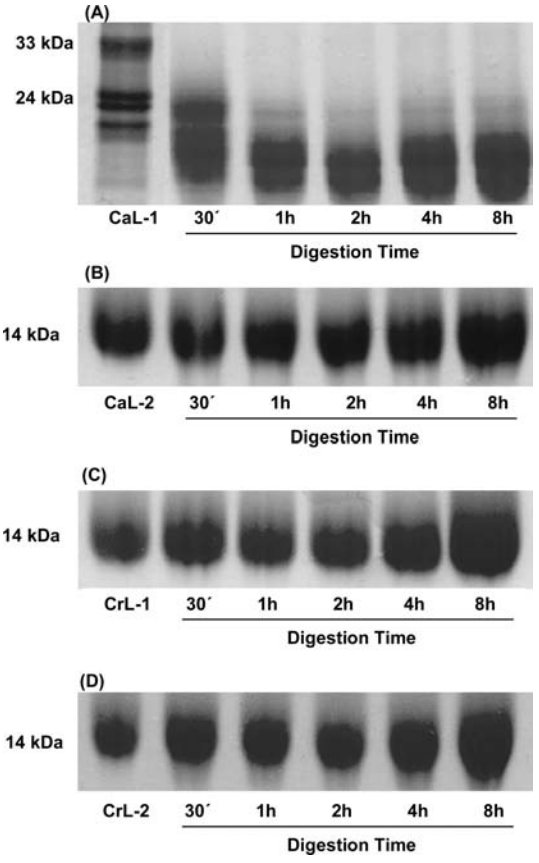

Figure 5. SDS−PAGE of legumin-like proteins from *C. arabica* and *C. racemosa* after proteolysis. Effect of proteolysis (lanes represent hours of proteolysis) on (A) CaL-1 and (B) CaL-2 isolated from *C. arabica* seeds and (C) CrL-1 and (D) CrL-2 isolated from *C. racemosa* seeds digested by midgut extracts of *C. maculatus*.

DISCUSSION

Many plants produce seeds that are rich in proteins and carbohydrates and therefore are commonly infested by pests. Because grain-producing crop plants are bred for specific seed characteristics related to human nutrition, they are generally more susceptible to insect attack, because untargeted genetic characteristics are lost. Therefore, it is expected that in some cases wild genotypes would harbor more effective insect defenses that may be of use in transgenic programs for improved pest resistance (*12*).

Investigations have shown that several seed storage proteins can be toxic to insects and interfere with their growth and survival. In these studies, growth parameters were evaluated using artificial diet bioassays, where proteins were added to the diet, and *C. maculatus* has been the preferred insect (*11*, *12*, *21*−*23*). All coffee legumin concentrations tested here significantly altered the parameters of *C. maculatus* larvae when compared to control larvae. CaL-2, CrL-1, and CrL-2 caused significant changes in survival and weight gain of *C. maculatus* larvae, with the LD_{50} corresponding to legumin concentrations of 0.3, 0.5, and 0.3% (w/w), respectively, and ED_{50} at 0.3% (w/w). Similar results have been reported for other storage proteins, such as vicilins isolated from *Vigna unguiculata*, *Vigna angulariz*, and *Canavalia ensiformis* (*24*), zeatoxin (*13*), and other storage proteins, such as lectins from *Talisia esculenta* (*25*), *Gracilaria ornata* (*26*), and *Koelreuteria paniculata* (*27*).

The coffee legumin-like proteins bound to the matrix of a chitin column and eluted in a similar manner using 100 mM acetic acid and 100 mM HCl, a behavior previously reported for other globulins (*22*, *28*). Therefore, these coffee storage proteins seem to be part of the family of chitin-binding proteins, such as WGA (*29*), hevein (*30*), α-amylase inhibitor/endochitinase (*31*), class I chitinase (*28*), and cowpea chitinase (*32*). Because chitin is a constituent of membrane systems, several proteins able to bind this polysaccharide have been related to defense mechanisms of plants against different organisms (*33*−*36*).

It is not known how these storage proteins become toxic to insects, but it is believed that they interfere with nutrient uptake by binding to chitin in the peritrophic membranes of the larval midgut (*29*). Also, because of the proteolytic activity of the insect midgut, these proteins have to be insensitive to proteolysis. Therefore, resistance to gut proteolysis, specificity for carbohydrate receptors, the ability to bind to different parts of the small

intestine, and functional and morphological changes caused would define the efficiency of the protein in terms of insecticidal activity (*37*). Several storage proteins with insecticidal activity are resistant to degradation by insect digestive enzymes, such as *T. esculenta* lectin (*25*), *Bauhinia monandra* leaf lectin (*38*), *Griffonia simplicifolia* seed lectin II (*39*), *V. unguiculata* vicilins (reserve protein-7S) (*20*), and maize zeatoxin (*13*). Coffee legumin-like proteins were insensitive to gut proteases from *C. maculatus* larvae, indicating that they may have a role in protecting coffee seeds from insect predators.

Therefore, according to the data obtained on the coffee legumin-like proteins from *C. arabica* and *C. racemosa*, the toxicity to *C. maculatus* larvae may involve the ability to bind chitin or equivalent components in the membranes of the insect gut, an interaction with glycoconjugates on the surface of epithelial cells along the digestive tract, an ability to bind to the sugar moiety of any of the glycosylated digestive enzymes and/or assimilatory proteins present in the insect midgut, and insensitiveness to enzymatic attack by midgut proteinases.

Hypothenemus hampei (Coleoptera: Scolytidae) is a coffee seed borer that is the main pest of coffee plantations in any producer country (*40*) and attacks only species of the genus *Coffea* (*41*). The seed endosperm is perforated, causing weight loss but mainly decreasing the quality of the resulting beverage. Although we have found here legumin-like proteins that based on the tests with *C. maculatus* might play a role in the resistance of coffee seeds to pests, both studied coffee species are attacked by *H. hampei*; however, *C. arabica* is more susceptible than *C. racemosa* (*40*). While seeds of the former lost from 22.7 to 37.7% of their weight in experiments where insects were reared with coffee seeds, seeds of the latter lost 10.4%. The only other study that tested the resistance of *C. racemosa* to *H. hampei* found that it was among the least-damaged species (*42*).

All cultivars and varieties of *C. arabica* are susceptible to *H. hampei*, and most have the same SDS−PAGE protein profile observed for CaL-1, with two main bands of about 33 and 24 kDa (*7*). On the other hand, *C. racemosa* has a unique electrophoretic profile that is similar to the profile obtained for CrL-1 and CrL-2 but also CaL-2. Therefore, a first conclusion might be that it is not possible to establish a clear relationship between *H. hampei* resistance and the protein composition of the seeds of these two coffee species. However, the amino acid composition of CaL-2, CrL-1, and CrL-2 are quite different, indicating that the legumin-like proteins of *C. racemosa* have compositional differences that might explain the resistance this species has to *H. hampei*. To test this hypothesis, we are isolating the CrL-1 and CrL-2 genes to produce heterologous protein and expressing both proteins in tobacco for insect tests. Another possibility is that other coffee seed components may have a role in resistance toward *H. hampei*.

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