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Effects of a Chitin-Binding Vicilin from *Enterolobium contortisiliquum* Seeds on Bean Bruchid Pests (*Callosobruchus maculatus* and *Zabrotes subfasciatus*) and Phytopathogenic Fungi (*Fusarium solani* and *Colletrichum lindemuntianum*)

FABIANO T. MOURA,[†] ADELIANA S. OLIVEIRA,[†,‡] LEONARDO L. P. MACEDO,[†] ANDRÉ L. B. R. VIANNA,[†] LUCIA B. S. ANDRADE,[‡,§] A. S. MARTINS-MIRANDA,[‡] JOSE T. A. OLIVEIRA,[‡] ELIZEU A. SANTOS,[†] AND MAURICIO P. DE SALES*[,†]

Laboratório de Química e Função de Proteínas Bioativas, Departamento de Bioquímica, Centro de Biociências, Universidade Federal do Rio Grande do Norte, Natal, RN, Brazil, Departamento de Bioquímica e Biologia Molecular, Centro de Ciências, Universidade Federal do Ceará, Fortaleza, CE, Brazil, and Universidade Estadual Vale do Acaraú, Sobral, CE, Brazil

Chitin-binding vicilin from *Enterolobium contortisiliquum* seeds was purified by ammonium sulfate followed by gel filtration on Sephacryl 300-SH and on Sephacryl 200-SH. The vicilin, called EcV, is a dimeric glycoprotein composed of 1.03% carbohydrates and a M_r of 151 kDa, consisting of two subunits of M_r of 66.2 and 63.8 kDa. The EcV homogeneity was confirmed in a PAGE where it was observed to be a unique acid protein band with slow mobility in this native gel. *E. contortisiliquum* vicilin (EcV) was tested for anti-insect activity against *C. maculatus* and *Zabrotes subfasciatus* larvae and for phytopathogenic fungi, *F. solani* and *C. lindemuntianum*. EcV was very effective against both bruchids, producing 50% mortality for *Z. subfasciatus* at an LD_{50} of 0.43% and affected 50% of the larvae mass with an ED_{50} of 0.65%. In artificial diets given to *C. maculatus*, 50% of the larvae mass was affected with an ED_{50} of 1.03%, and larva mortality was 50% at LD_{50} of 1.11%. EcV was not digested by midgut homogenates of *C. maculatus* and *Z. Subfasciatus* until 12 h of incubation, and at 24 h EcV was more resistant to *Z. subfasciatus* larval proteases. The binding to chitin present in larvae gut associated to low EcV digestibility could explain its lethal effects. EcV also exerted an inhibitory effect on the germination of *F. solani* at concentrations of 10 and 20 μg mL^{-1}. The effect of EcV on fungi is possibly due to binding to chitin-containing structures of the fungal cell wall.

KEYWORDS: Vicilin; bean bruchid pests; chitin binding; phytopathogenic fungi; anti-insect activity

INTRODUCTION

A major portion of the human diet throughout the world consists of cereals and legumes. According to FAO estimates, 70% of human food comprises cereals and legumes and the remaining 30% comes from animals that feed on these seed meals (*1*). Legume seeds represent an important food source for low income populations in subtropical and tropical areas where bean crops such as cowpeas (*Vigna unguiculata*) and common beans (*Phaseoulus vulgaris*) are rich sources of protein (20−25%), carbohydrates (50−60%), and lipids (1−2%) (*2*). However, losses in bean production caused by pests and pathogens have been estimated at more than 37% (*3*). Fungi are important pathogenic agents for these bean species since 29 species cause diseases in seeds, stems, leafs, and roots (*4*). One of the most important insect pests of the cowpea (*Vigna unguiculata*) is the bruchid weevil *Callosobruchus maculatus* (F.) (Coleoptera), which attacks seeds during storage (*5*). The Mexican bean weevil, *Zabrotes subfasciatus* (Boh.), is the major pest of legume seeds such as *Phaseolus vulgaris*, *Phaseolus lunatus*, and *V. unguiculata* (*6*). Both insects severely affect grain quality and storability.

The ever-increasing demands on yield and the intensification of farming practices have increased the problem of pest and pathogen damage and hence control. Bruchid and fungi controls are expensive and performed by treating stored seeds with fungicides, methyl bromide, carbon disulfide, and several other chemicals (*7*). These are considered environmentally undesirable and are also expensive for subsistence farmers. In order to increase the resistance of cultivated varieties, plant breeders are

* To whom correspondence should be addressed. Fax: 155842119208. E-mail: msales@cb.ufrn.br.
[†] Universidade Federal do Rio Grande do Norte.
[‡] Universidade Federal do Ceará.
[§] Universidade Estadual Vale do Acaraú.

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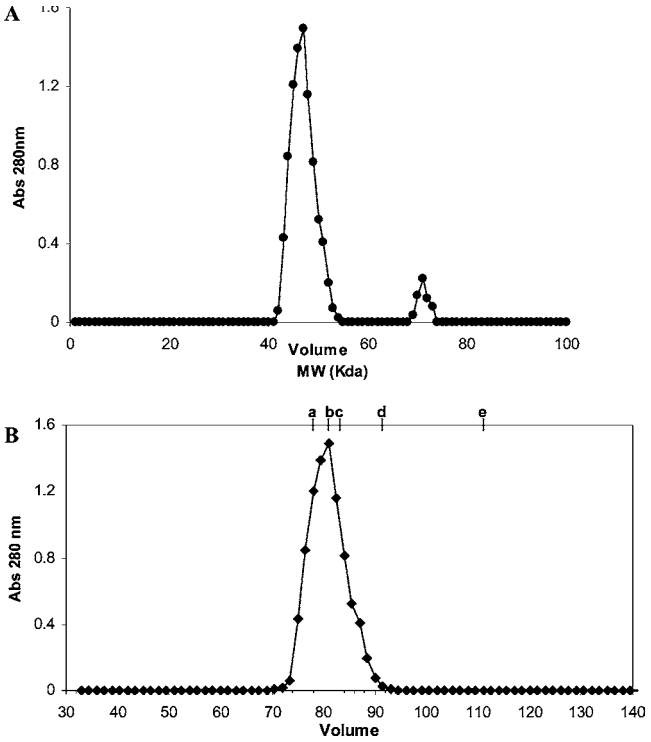

Figure 1. (**A**) Elution profile on Sephacryl 300-SH of F_{70}–90, from *E. contortisiliquum* seeds. Approximately 22 mg of protein was applied to the column equilibrated with 0.05 M Borax buffer, pH 7.5, and fractions were eluted and monitored at 280 nm. (**B**) Elution profile on a Sephacryl 200-SH calibrated column of PI (10 mg). Protein markers: (a) β-Amylase (200 kDa); (b) EcV (151 kDa); (c) alcohol dehydrogenase (150 kDa); (d) bovine serum albumin (66 kDa); (e) SBTI (20kDa).

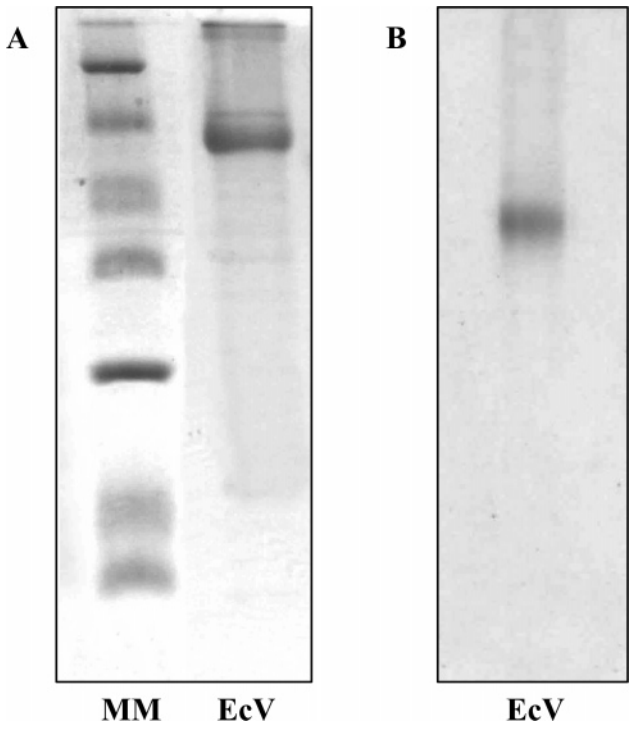

Figure 2. (**A**) SDS–PAGE (15%) analysis of purified EcV stained with Coomassie Blue. (M) β-galactosidase (116.0 kDa); bovine serum albumin (66.2 kDa); ovalbumin (45.0 kDa); lactate dehydrogenase (35.0 kDa); restriction enzyme Bsp98 (25.0 kDa); β-lactoalbumin (18.4 kDa); lysozyme (14.4 kDa). (EcV) *E. contortisiliquum* vicilin (10 μg). (**B**) PAGE (15%) analysis of purified EcV stained with Coomassie Blue. (EcV) *E. contortisiliquum* vicilin (10 μg).

interested in understanding resistance mechanisms involving defense proteins and other metabolites that operate in wild varieties.

Storage proteins of the 7S vicilin type from cultivar IT81D-1045 of *Vigna unguiculata* and others from leguminous seeds have been included as some of the most recent defensive protein plant groups (*8*−*13*). The involvement of vicilins in the resistance of cowpea seeds to *C. maculatus* was first suggested during investigations into the possible role of trypsin inhibitors in this process. Analysis of protein fractions from the seeds indicated that the detrimental effects observed were associated with the globulin fraction (*8*). Purification of vicilins from both *C. maculatus*-resistant and -susceptible cowpea seeds and their incorporation into artificial seeds showed that the purified 7S globulins were responsible for at least some of the detrimental effects caused by resistant seeds (*8*, *14*). The involvement of IT81D-1045 vicilins and others from legume seeds in the germination and development of fungi and yeast was studied by Gomes et al. (*15*, *16*). Results showed that these proteins inhibited the growth of these pathogens.

In this paper, we describe the purification and characterization of a chitin-binding vicilin from *Enterolobium contortisiliquum* plant seeds and report on its action against two phytopathogenic species of fungi (*Fusarium solani* and *Colletrichum lindemuntianum*) and two species of bean bruchid pests (*Callosobruchus maculatus* and *Zabrotes subfasciatus*).

MATERIALS AND METHODS

Isolation of *E. contortisiliquum* Globulin. *E. contortisiliquum* plant seeds were obtained from the IBAMA (Brazilian Environmental Institute) seed bank of Natal/RN-Brazil. The globulin fraction was isolated by the procedure developed by Macedo et al. (*17*) with modifications. Finely ground seed meal was extracted with 0.05 M Borax buffer, pH 7.5, for 30 min at room temperature. After centrifugation for 30 min at 8000*g* at 4 °C, the supernatant (crude extract) was precipitated with ammonium sulfate at a concentration of 70−90%. This fraction, corresponding to the globulin fraction in legume seeds, was then dialyzed and freezer dried.

Preparation of the Larvae Midgut Homogenates. Bean bruchids, *C. maculatus*, and *Z. subfasciatus* were supplied from Laboratório de Química e Função de Proteínas, Departamento de Bioquímica, UFRN, Natal, Brazil. Permanent insect colonies were established on cowpea and common bean seeds and reared at 28−30 °C and 55−60% relative humidity. Homogenate enzymatic extracts were obtained after dissection and extraction of the midguts. For this, 200 guts were surgically removed from the each insect larvae and placed into 1000 μL of an iso-osmotic saline (0.15 M NaCl) solution. Midgut tissue was stirred and centrifuged for 10 min at 10000*g* at 4 °C. The supernatants were then removed and used for *in vitro* assays.

Detection of Proteinase Inhibitory Activities in *E. contortisiliquum* Globulin. Globulin inhibitory assays against proteinase extracts from both larvae were measured using 1% azocasein solution at pH 7.5 (serine proteinase activities), 1% azocasein solution at pH 5.6 (cysteine proteinase activities), and 1% hemoglobin solution at pH 3.5 (acid proteinase activities) as substrates (*18*).

A 50 μL aliquot of globulin (2 mg mL^{-1}) was incubated with 40 μL of midgut homogenates and 450 μL of appropriate buffer (50 mM Tris-HCl, 20 mM $CaCl_2$, pH 7.5; 50 mM sodium acetate, 3 mM cysteine, EDTA 2 mM buffer pH 5.6) at 37 °C for 15 min before adding the 200 μL of substrate (1% azocasein, 50 mM Tris-HCl, 20 mM $CaCl_2$, pH 7.5; 50 mM sodium acetate, 3 mM cysteine, EDTA 2 mM buffer pH 5.6). After 30 min incubation, the reaction was stopped by adding 150 μL of 20% TCA solution. Results were monitored at 410 nm. Samples were centrifuged at 10000*g* for 10 min and supernatants alkalinized with 0.2 N NaOH solution. The soluble peptides were measured by absorbance at 440 nm. Appropriate controls without

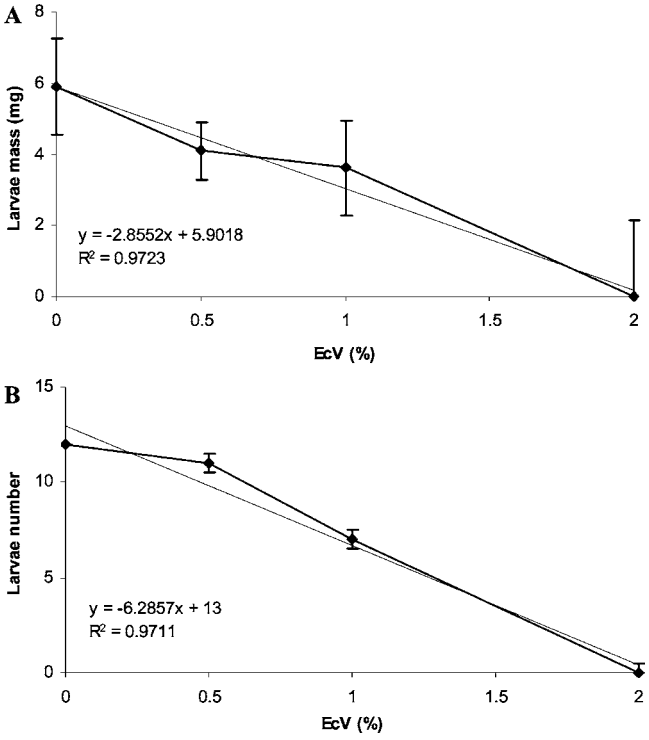

Figure 3. Effects of EcV (%, w/w) on *C. maculatus* larvae development in an artificial diet system. (**A**) Mass of *C. maculatus* larvae and (**B**) number of *C. maculatus* larvae. The *Y*-intercept in parts **A** and **B** is the mass of larvae and the number of larvae, respectively, in control seeds. Each mean represents four replicates (±SE).

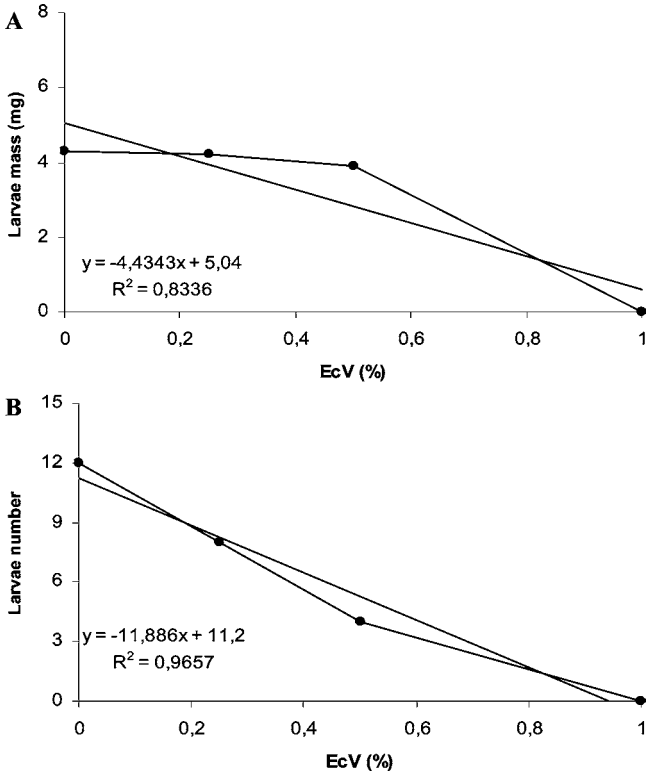

Figure 4. Effects of EcV (%, w/w) on *Z. subfasciatus* larvae development in an artificial diet system. (**A**) Mass of *Z. subfasciatus* larvae and (**B**) number of *Z. subfasciatus* larvae. The *Y*-intercept in parts **A** and **B** is the mass of larvae and the number of larvae, respectively, in control seeds. Each mean represents four replicates (±SE).

globulin were included, and inhibitory activity was determined by measuring the remaining enzymatic activity at pH 5.6 after preincubation with samples. One inhibitory unit (IU) was defined as the amount of inhibitor activity that decreased absorbance by 0.01 at 440 nm. All the assays were performed in triplicate.

A 50 μL aliquot of globulin (2 mg mL^{-1}) was preincubated with 50 μL of midgut homogenates and 50 μL of appropriate buffer (0.2 M sodium acetate buffer pH 3.5) at 37 °C for 15 min. After this time, 500 μL of substrate solution (1.0% hemoglobin in 0.2 M sodium acetate buffer pH 3.5) was added to preincubated solution at 37 °C for more 60 min. The reaction was stopped by the addition of 100 μL of 40% TCA solution. Samples were centrifuged at 10000*g* for 10 min and supernatants alkalinized with 2.0 N NaOH solution. The supernatants were submitted to Folin's method, modified by Lowry et al. (*19*), and soluble peptides were measured by absorbance at 750 nm. Appropriate controls without globulin were included, and inhibitory activity was determined by measuring the remaining enzymatic activity at pH 3.5 after preincubation with samples. One inhibitory unit (IU) was defined as the amount of inhibitor activity that decreased absorbance by 0.01 at 740 nm. All the assays were performed in triplicate.

Detection of Amylase Inhibitory Activities in *E. contortisiliquum* Globulin. Globulin inhibitory assays against midgut amylase from both larvae were measured using 1% starch solution at pH 5.5) by the Bernfeld method (*20*) as modified by Baker (1983). A 40 μL aliquot of midgut homogenates was preincubated with 50 μL of globulin (2.0 mg mL^{-1}) at 30 °C for 15 min, prior to the addition of 2 mL of substrate solution (1% soluble starch solution in 0.1 M acetate buffer, pH 5.5 containing 20 mM NaCl and 0.1 mM $CaCl_2$). After 60 min at 30 °C, the reaction was interrupted. Aliquots of 100 μL were added to 10 mL of a solution containing 1 mM iodine and 24 mM potassium iodide solution. Absorbencies were read at 656 nm. Appropriate controls without globulin were included, and inhibitory activity was determined by measuring the remaining enzymatic activity at pH 5.5 after preincubation with samples. One inhibitory unit (IU) was defined as the amount of inhibitor activity that decreased absorbance by 0.01 at 656 nm. All assays were performed in triplicate.

Detection of Hemeagglutinating/Hemolytic Activities in *E. contortisiliquum* Globulin. The hemeagglutinating/hemolytic activity was assayed in microtiter V plates (Nunc Brand products, Denmark) according to a twofold serial diluting procedure (*21*). One hemeagglutinating unit (HU) was defined as the amount of sample able to agglutinate and hence precipitate the erythrocytes in suspension after 30 min. To each well was added 25 μL (2 mg mL^{-1}) of the twofold serially diluted vicilin solutions and 25 μL of human ABO erythrocytes (2% v/v suspension) and allowed to incubate for 30 min at room temperature. The degree of hemeagglutinating/hemolytic activity was examined. The controls were set up with saline and erythrocytes and 1 mg mL^{-1} of Con A solution and erythrocytes.

Purification of *E. contortisiliquum* Vicilin. Globulin (10 mg mL^{-1}) (enzyme inhibitor and lectin free) was applied to a size exclusion column Sephacryl 300-SH (84 cm × 2.5 cm column), equilibrated with 0.05 M Borax buffer, pH 7.5. Fractions of 1.5 mL were collected using a flow rate of 30 mL h^{-1}. The first peak PI (5 mg mL^{-1}) was then applied to another gel filtration Sephacryl 200-SH column (72 cm × 2.5 cm) calibrated with protein markers: cytochrome *c* (12 kDa), SBTI (20 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa). Fractions of 1.5 mL were collected using a flow rate of 30 mL h^{-1}. All chromatography was monitored at 280 nm. The peak obtained, denominated EcV, was pooled, freezer dried, and subjected to further analysis.

Protein and Carbohydrate Determination. Protein content was measured by Bradford's (*22*) procedure with bovine serum albumin (BSA) as protein standard. Carbohydrate content was measured by Dubois et al.'s procedure (*23*) with glucose as carbohydrate standard. All assays were made in triplicate.

Characterization of EcV by Polyacrylamide Gel Electrophoresis. The presence of subunits in EcV was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at room temperature, as described by Laemmli (*24*) using 15% of polyacrylamide separation gel and 4% polyacrylamide stacking gel. Protein

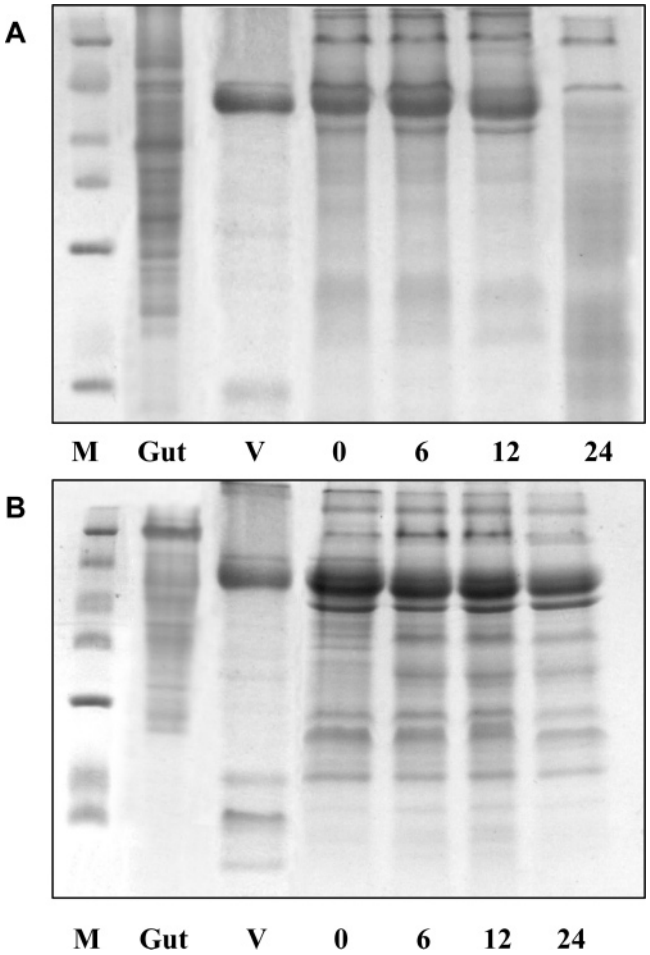

Figure 5. SDS-PAGE (15%) patterns of EcV (0.8 μg μL^{-1}) digested by midgut homogenate of (**A**) *C. maculatus* and (**B**) *Z. subfasciatus* stained with Coomassie Blue. (M) Molecular mass markers. (M): β-galactosidase (116.0 kDa); bovine serum albumin (66.2 kDa); ovalbumin (45.0 kDa); lactate dehydrogenase (35.0 kDa); restriction enzyme Bsp98 (25.0 kDa); β-lactoalbumin (18.4 kDa); (Gut): midgut homogenate; incubation time (h): 0, 6, 12, and 24 h. Assays were performed at pH 6.0.

molecular weight markers β-galactosidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), restriction enzyme Bsp98 (25.0 kDa), β-lactoalbumin (18.4 kDa), and lysozyme (14.4 kDa) were obtained from Fermentas Co. EcV homogeneity was observed by native polyacrylamide gel electrophoresis (PAGE) also as described by Laemmli (*24*), using 15% of polyacrylamide separation gel and 4% polyacrylamide stacking gel, without SDS. Bromophenol blue was used as the tracking dye.

Binding of EcV to Chitin Matrix. To examine the possibility of EcV interaction with chitin matrix, the vicilin was chromatographed on a chitin column (5 mL bed volume) equilibrated with 50 mM borax, pH 7.5. After protein adsorption (10 mg mL^{-1}), the column was washed with the same buffer until the absorbance at 280 nm returned to zero, after which the adsorbed protein was eluted with 0.1 M HCl. Fractions (2 mL) were collected. Controls were chromatographed on the same chitin column and under the same conditions described above. Positive protein controls were IT81D-1045 *Vigna unguiculata* vicilin, *Canavalia ensiformis* vicilin, *Phaseolus lunatus* vicilin, chitin-binding lectins (WGA, wheat germ agglutinin), *Vigna unguiculata* chitinase, and negative protein controls: chymotrypsin, SBTI (soybean trypsin inhibitor), BSA (bovine serum albumin), ovalbumin, PHA (*Phaseolus* phytohemeaglutinin) lectin, and rabbit gamma globulin.

Insect Bioassay. The developmental performance of *C. maculatus* and *Z. subfasciatus* in artificial seeds system was assessed following the previously described method by Macedo et al. (*8*). Artificial seeds (400 mg) were prepared with finely ground cowpea (*C. maculatus*) and common bean (*Z. subfasciatus*) seed meal containing EcV, at 0.06, 0.125, 0.25, 0.5, 1.0, and 2.0% (w/w) final concentrations. The seeds were made by pressing the meal into a cylindrical brass mold with the help of a press. After a 48 h adjustment period in the growth chamber, the seeds were presented to three fertilized females (2−3 day old) per seed in a glass vial containing four seeds, for 24 h at 28 °C, 60% RH. The excess eggs laid were removed from the seeds, leaving three eggs per seed. After 20 days, infested seeds were opened and the mass and number of the larvae was calculated. The experiments were run in four replicates and the mean (±SEM) was determined. Control artificial seeds were made with cowpea and common bean seed meal.

Detection of Chitin in the Midgut of Bruchid Larvae. Larvae were dissected under magnification in cold 0.15 M NaCl with the help of tweezers, and midguts were separated from the windpipes and Malpighian tubes. Midguts were perforated, and luminal contents were aspirated and reserved. Midguts were then thoroughly washed to remove remaining luminal contents. The presence of chitin in both larval midguts was ascertained by the von Wisselingh color test (*25*). This qualitative test detects chitosan produced after treatment of the chitin-containing materials with saturated KOH for 15 min at 160 °C. After reaction, the presence of chitin was observed with a KI/iodine solution. Controls employing cellulose (−) and lobster chitin (+) were used.

***In Vitro* Digestibility of EcV to Midgut Homogenate of *C. maculatus* and *Z. subfasciatus* Larvae.** Vicilin was dissolved in 0.01 M phosphate buffer, pH 6.0, at 0.8 μg μL^{-1} concentration. Vicilin (aliquot of 240 μL) was incubated with 240 μL of midgut homogenate from both larvae (0.2 μg midgut μL^{-1}) at 37 °C for periods of 0, 6, 12, and 24 h. The ratio of vicilin to midgut was 4:1. Adding a 10% SDS solution stopped the digestion. Protein hydrolysis was observed by SDS-PAGE (*24*), using 15% of polyacrylamide separation gel and 4% polyacrylamide stacking gel.

Effect of EcV on Fungal Germination. The effect of EcV on fungi (*F. solani* and *C. lindemuthianum*) germination was assessed following the previously described method by Ji and Kuc (*26*). Accordingly, 200 μL of spores (20 000 spores mL^{-1} of Sabouraud broth) was placed in multiwell plates followed by the addition of 5 and 10 μL of vicilin in 100 mM Tris-HCl, pH 8.0 (2 μg μL^{-1}). Controls were 100 mM Tris-HCl, pH 8.0, without the addition of protein, distilled H_2O, and 100 mM H_2O_2 solution. Plates were placed in the humidity chamber (100% RH) for 24 h at 30 °C. Results were observed by light microscopy (Olympus BX60 at 400× magnification and photographed (Olympus Photomicrographics System PM 20). Assays were performed in triplicate.

Statistical Analysis. The data were examined using one-way analysis of variance (ANOVA). The Student *t*-test was used to identify the means that differed if ANOVA indicated significance.

RESULTS

Isolation, Purification, and Characterization of *E. contortisiliquum* Vicilin (EcV). The crude soluble protein extract obtained from the mature seeds of the *E. contortisiliquum* tree was initially precipitated at 0−70 and 70−90% saturation with ammonium sulfate. The F70−90 protein fraction (globulins) obtained was assayed to detect contaminants such as midgut proteinase inhibitors, midgut amylase inhibitors, and hememaglutunating (lectin)/hemolytic activities. This fraction showed no inhibitory, hemolytic, and hememaglutinating activities (data not showed). The F70−90 was applied to Sephacryl 300-SH gel filtration, and the peak PI was then applied to previously calibrated Sephacryl 200-SH gel filtration. The EcV peak had a molecular mass of 151 kDa (**Figure 1**). EcV is a glycoprotein, composed of 1.03% carbohydrates, and when applied to a SDS-PAGE, proved to be a multimeric protein with 2 relative molecular mass subunits of 66.2 and 63.8 kDa (**Figure 2A**). EcV homogeneity was confirmed in a PAGE where it is a unique protein band with slow mobility in this native gel, similar to acid protein (**Figure 2B**).

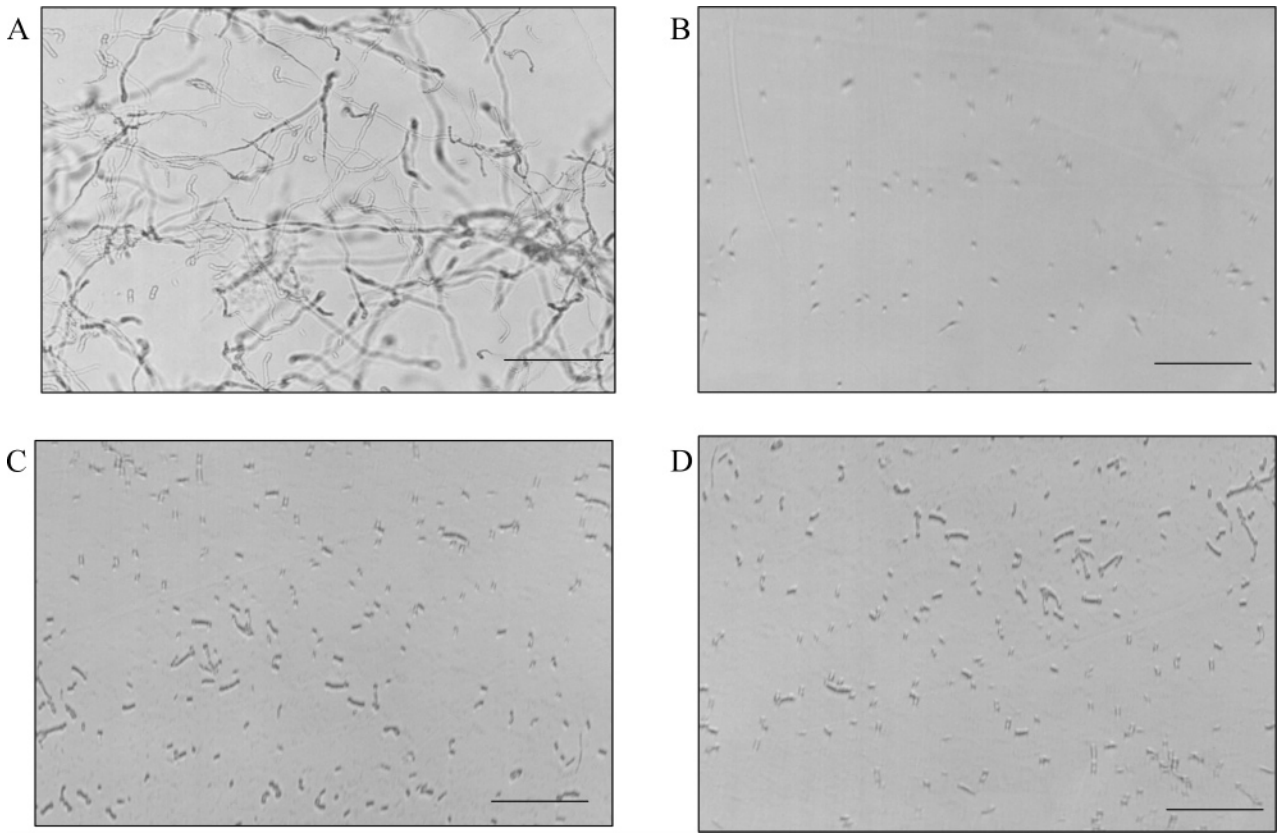

Figure 6. Light micrographs of *F. solani* mycelia after 24 h of fungal growth in the presence of (**A**) control medium, (**B**) 100 mM H_2O_2, (**C**) EcV (10 μg mL^{-1}), and (**D**) EcV (20 μg mL^{-1}). Bars = 15.0 μm.

Chitin-Binding Property of EcV. To test the affinity of EcV to chitin, this protein was applied to a chitin-matrix column. The EcV affinity was similar those chitin-binding protein, such as WGA, cowpea chitinase, and chitin-binding vicilins (IT81D-1045 *V. unguiculata* vicilin, *C. ensiformis* vicilin, and *P. lunatus* vicilin).

Insect Bioassays. Figures 3 and **4** show the influence of EcV on the weight and number of survivors of *C. maculatus* and *Z. subfasciatus* during larval development when larvae were fed a diet containing different EcV concentrations. The chitin-binding vicilin added to the diet of these pests in artificial seeds was very effective in both bruchids, causing 50% mortality in *Z. subfasciatus* at a level of 0.43% (LD_{50}, lethal dose) and affected 50% of the larvae mass with an ED_{50} (effective dose) of 0.65%. In artificial diets administered to *C. maculatus*, 50% of the larvae mass was affected with an ED_{50} (effective dose) of 1.03% and larvae mortality was 50% at a level of 1.1% (LD_{50}, lethal dose) of EcV. Regression analysis showed that for every 0.1% increase in EcV dose applied to *C. maculatus* and *Z. subfasciatus*, there was a 0.5% and 1.1% increase in mortality, respectively. For each 0.1% increase in EcV dose, there was a 0.2% and 0.3% decrease in *C. maculatus* and *Z. subfasciatus* mass, respectively.

Detection of Chitin in Midgut from Bruchid Larvae. The presence of chitin in the midgut of *C. maculatus* and *Z. subfasciatus* larvae was revealed by the Wisselingh color test for the qualitative determination of chitin. KOH-treated larvae midgets were placed in a KI/iodine solution, and the appearance of a brown color was indicative of the presence of chitin in both larvae midguts. A positive test was confirmed by the addition of 0.1% H_2SO_4 when the color turned violet.

***In Vitro* Digestibility of EcV to Midgut Homogenate of *C. maculatus* and *Z. subfasciatus* Larvae.** Larval proteases were unable to digest EcV until 12 h of digestion (**Figure 5**). EcV was completely digested by *C. maculatus* larval proteases after a 24-h assay. EcV was more resistant to *Z. subfasciatus* larval proteases, and at 24 h of digestion, the presence of protein bands of high molecular mass was still observed.

Effect of EcV on Fungal Germination. Figure 6 shows the growth of *F. solani* in the presence of EcV (at a concentration of 10 and 20 μg mL^{-1}) and in controls. EcV exerted an inhibitory effect on the germination in both EcV concentrations. Photomicrographs of the mycelia of *F. solani* fungi taken after 24 h showed normal hyphal development (**Figure 6A**) in the control culture but inhibition of hyphal development in the presence of EcV (**Figure 6B**−**D**) and 100 mM H_2O_2 solution. The fungi *C. lindemunhtianum* were not inhibited at concentrations of 10 and 20 μg mL^{-1} of EcV.

DISCUSSION

Vicilins or 7S globulins consist of multi-subunit combinations with molecular masses between 20.1 and 94 kDa (*27*). Combinations of multiple structural genes and extensive post-translational processing results in a high degree of polymorphism for these proteins (*28*). In legume seeds, vicilins exhibit a considerable amount of sequence homology and microheterogeneity and may contribute to plant defense mechanisms (*29*). In this study vicilins from *E. contortisiliquum* seeds were purified and denominated EcV. EcV is a glycoprotein with molecular mass of 151 kDa with two subunits of 66.2 and 63.8 kDa, in agreement with similar data previously reported by several authors (*13*, *17*, *30*, *31*) for other legume vicilins. EcV had neither digestive enzyme inhibitory activities nor hemeaglutinating and hemolytic activities in comparison with other

defense proteins found in *E. contortisiliquum* seeds such as thiol enzyme inhibitors (*32*−*35*) and cytolytic enterolobin (*36*−*41*).

The observation that legume vicilins bind to a chitin matrix led to the discovery that vicilins from cowpeas (*35*) and other legumes such as adzuki beans (*Vigna angularis*), jack beans (*Canavalia ensiformis*), soybeans (*Glycine max*), and lima beans (*Phaseolus lunatus*) strongly bind to chitin (*13*). Vicilins from these distantly related species showed a highly detrimental effect on the larval development of *C. maculatus* (*13*). EcV had an affinity to chitin matrix and by this way should be included to the family of the chitin-binding proteins such as WGA (*32*), Hevein (*38*), α-amylase inhibitor/endochitinase (*37*), class I chitinase (*36*) and cowpea chitinase (*16*). Through bioassays it was observed that EcV, like other chitin-binding proteins such as vicilins, lectins, and chitinases, had lethal and detrimental effects on organisms that contain chitin in cell walls (fungi) (*42*) and in the peritrophic membrane of some insects, which forms a barrier to protect the midgut epithelium from abrasive food particles (*43*). EcV was very lethal to *C. maculatus* with the LD_{50} (1.1%) and ED_{50} (1.03%) lower than those observed in IT81D-1045-resistant *V. unguiculata* vicilin (LD_{50} of 2.0% and ED_{50} of 1.07%) (*8*) and among other legume vicilins such as *G. max* vicilin (ED_{50} of 1.66%), *P. lunatus* vicilin (ED_{50} of 1.74%), and *C. ensiformis* vicilin (ED_{50} of 2.15%), which are nonhost legume seeds of *C. maculatus* (*13*). The lethal EcV dose was comparable to the concentrations (0.1−1.0%) of WGA, rice lectins, nettle lectins, and TEL (*Talisia esculenta* lectin) tested for *C. maculatus* (*44*, *45*). Differently from IT81D-1045-resistant *V. unguiculata* vicilin, which was neither lethal nor detrimental to *Z. subfasciatus* (*8*), EcV was highly lethal (LD_{50} of 0.43%) and detrimental (ED_{50} of 0.65%) to this insect. In comparison to TEL, EcV was twice as lethal as TEL (LD_{50} of 1.0%) to *Z. subfasciatus* (*45*). The EcV action mechanism is probably due to the binding with chitin or chitinous structures present in the midgut of these bruchids, as proved here by von Wisselingh's color test for chitin. This effect on the midgut of both *C. maculatus* and *Z. subfasciatus* suggests that the binding of vicilins to the peritrophic membrane of insect guts either causes interference with nutrient absorption (*46*), prevents or enhances movement between the endo- and exoperitrophic space, or prevents the formation of the membrane itself (*47*). EcV was also resistant to digestion by enzymes from the midgut of *C. maculatus* and highly resistant to those of *Z. subfasciatus*. Similar resistance was found to TEL, which was hydrolyzed with a mixture of pepsin and papain after 16 h of incubation (*45*). This fact, along with chitin binding, suggests that at least part of the effect on insects can be accounted for by these lower rates of EcV hydrolysis.

Microphotographs of the fungi *F. solani*, taken after a 24 h growth period in the presence of EcV, show that these seed vicilins negatively affect spore germination. Other lectins that also have a potent inhibitory activity on several fungi *in vitro* include hevein from the rubber tree (*Hevea brasiliensis*) latex (*48*), UDA from stinging nettle (*Urtiga dioica*) rhizomes (*49*), and GAFP-1 from *Gastrodia elata*, a parasitic plant on the fungus *Armillaria mellea* (*50*). The EcV concentration necessary to inhibit fungal growth was 10 and 20 $\mu g\ mL^{-1}$. In comparison, TEL inhibited *Fusarium oxysporum*, *Colletotrichum lindemuthianum*, and *Saccharomyces cerevisiae* at 280 $\mu g\ mL^{-1}$ (*44*), and cowpea vicilin (*16*) inhibited growth and spore germination in the fungus *F. oxysporum* and led to abnormal development of *S. cerevisae* cells at a concentration of 800 $\mu g\ mL^{-1}$. GAFP-1 also showed strong inhibitory activity against *Valsa ambiens*, *Rhizoctonia solani*, *Gibberella zeae*, *Ganoderma lucidum*, and *Botrytis cinerea in vitro* at a concentration of 360 $\mu g\ mL^{-1}$ (*50*). The negative effect of EcV on spore germination suggests that this protein could associate with chitin derivatives or *N*-acetylglucosamine-containing glycoconjugates in fungi structures that are exposed to the external medium.

The strong negative effects of EcV on bruchids and fungi observed in this study suggest that EcV might be able to provide a viable alternative in designing transgenic crops to control insect pests and fungi or be used as a fungicide and pesticide.

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