JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Larvicidal Effects of a Chitin-Binding Vicilin from *Erythrina velutina* Seeds on the Mediterranean Fruit Fly *Ceratitis capitata*

Leonardo L. P. Macedo,[†] Ticiana M. L. Amorim,[†] Adriana Ferreira Uchôa,[‡] Adeliana S. Oliveira,[†] Jannison K. C. Ribeiro,[†] Francisco P. de Macedo,[‡] Elizeu A. Santos,[†] and Maurício P. de Sales*,[†]

Departamento de Bioquímica and Departamento de Biologia Celular e Genética, Centro de Biociências, Universidade Federal do Rio Grande do Norte, Natal, RN, 59072-970, Brazil

Chitin-binding vicilin from *Erythrina velutina* seeds was purified by ammonium sulfate followed by affinity chromatography on a chitin column and gel filtration on Superose-6-10-300-GL. The *Erythrina velutina* vicilin, called EvV, is a tetrameric glycoprotein composed of 1.85% carbohydrates and M_r of 216.6 kDa, consisting of two subunits of M_r of 54.8 and two subunits of M_r of 50.8 kDa. The EvV homogeneity was confirmed in native PAGE where it was observed to be a unique acid–protein band with slow mobility in this gel. Effect of EvV on *C. capitata* larvae was examined by bioassay and its mechanism of action was determined by immunodetection techniques and fluorescence localization in chitin structures that are present in *C. capitata* digestory system. EvV when added to diet caused strong effect on mortality (ED_{50} of 0.14%) and larval mass (WD_{50} of 0.12%). These deleterious effects were associated to the binding to chitin structures present in peritrophic membrane and to gut epithelial cells, and its low digestibility in *C. capitata* digestive tract. These results are the first demonstration of a proteinaceous bioinsecticide from plant origin effective against *C. capitata* larvae. EvV may be part of the pest management programs or an alternative in plant improvement program.

KEYWORDS: *Ceratitis capitata*; peritrophic membrane; *Erythrina velutina*; vicilin; bioinsecticide; chitin-binding protein

1. INTRODUCTION

Fruit flies of the family Tephritidae are among the most destructive pests of fruits and vegetables around the world (*1*). One of the most notorious of these species is the mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), which is found worldwide attacking a wide host range that includes more than 350 species of plants (*2*). Females oviposit in ripening and ripe fruits, which are destroyed by larval feeding (*3*). The ever-increasing demands on yield and the intensification of farming practices have increased the problem of pest and pathogen damage and hence control. The preferred pest management practices against many fruit flies are based on chemical methods. Bait-sprays, using insecticides mixed with an attractant, were found to be effective for suppressing fruit fly pest populations of *C. capitata* (*4*). Besides the negative environmental aspects and potential risk for human health, nowadays there are populations of insects which are resistant to organochlorine, organophosphorus and carbamate insecticides (*5*). To overcome this resistance it would be necessary to seek for new environment-friendly pesticides with different mechanisms of action (*6*).

Many plant defense proteins with insecticidal activity, such as lectins and chitinases, bind to suitably glycosylated targets in the insect gut. The major action sites for these proteins are the peritrophic membrane (PM) (*7*) and the membrane glycans in the brush border epithelium (*8*). PM exists in most phytophagous insects and is composed primarily of chitin (containing *N*-acetylglucosamine residues) and proteins (*9*). The peritrophic membrane forms a barrier to protect the midgut epithelium from abrasive food particles, microorganism attack, and facilitates recycling of digestive enzymes among other functions (*10*). Changes in the permeability of PM (*11*), morphological damage or changes in the gut epithelial membrane (*8*) could cause interference with absorption of various nutrients and subsequents deleterious effects on insects.

Vicilins are legume seed storage proteins of globulin nature and classified as 7S globulins according to their sedimentation coefficients. These protein are oligomeric molecules with variable degree of glycosylation (*12*). Chemical and im-

* To whom correspondence should be addressed: Laboratório de Química e Função de Proteínas Bioativas, Departamento de Bioquímica, Centro de Biociências, Universidade Federal do Rio Grande do Norte, Natal, RN, 59072-970, Brazil. Fax: 155842119208. E-mail: msales@cb.ufrn.br.

[†] Departamento de Bioquímica.

[‡] Departamento de Biologia Celular e Genética.

10.1021/jf072746n CCC: $40.75
Published on Web 01/15/2008

munological investigations have shown that variant vicilins from the *Vigna unguiculata* genotype IT81D-1045, which is resistant to the attack of cowpea weevil, bind to the chitin-containing structure of insect midgut (*13*). These vicilins also showed deleterious effects on the sugar cane stalk borer *Diatraea saccharalis* larvae (*14*). These results demonstrate that non-adapted insects can also be affected by of vicilin ingestion and that the mechanism of action of this protein may be similar to that attributed to lectins, antibodies and other chitin-binding proteins, such as chitinases and hevein (*7*).

In this paper, we describe the purification and characterization of a chitin binding vicilin from *Erythrina velutina* seeds and report its effects on larval development and survival of *C. capitata* larvae. Also, we investigated the mechanisms involved in the larvacidal effect of *E. velutina* vicilin (EvV). This is the first report of a proteinaceous bioinsecticide from plant origin effective against *C. capitata*.

2. MATERIAL AND METHODS

2.1. Chemicals. All substrates, peroxidase-conjugated goat anti-Rabbit IgG and native protein deglycosylation kit were purchased from Sigma Chemical Co. (St. Louis, MO). Electrophoresis chambers and reagents were obtained from Bio-Rad Laboratories (Richmond, CA).

2.2. Insects. The colony of *C. capitata* used in this work was maintained with insects obtained from Departamento de Biologia Celular e Genética of the Universidade Federal do Rio Grande do Norte, Brazil. *C. capitata* larvae were reared on artificial diets which were prepared using 10.4% finely ground sugar cane fibers, 6.5% wheat flour, 15% crystal sugar, 9.9% brewers yeast, 0.3% sodium benzoate (VETEC), 0.9% HCl, and 57% H_2O. Insect colonies were maintained at 28 ± 2 °C, 70–80% relative humidity, and a photoperiod of 12 h.

2.3. Preparation of Larval and Insect Gut Homogenates. A total of 50 larvae from the third instar of development were dissected in 0.15 M NaCl and the guts homogenized in 50 mM Tris-HCl buffer at pH 8.0 in a glass homogenizer. The homogenate was centrifuged at 10000*g* for 30 min at 4 °C, and the clear supernatant was used in enzyme assays. All homogenates were freshly prepared (*15*).

2.4. Purification of EvV and Antibody Production. *E. velutina* seeds were obtained from Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) seed bank at Natal/RN-Brazil. Finely ground seed meal was extracted with 0.05 M Borax buffer at 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 0–70 and 70–90% concentration. The 70–90% fraction (F70–90) corresponding to globulin proteins in legumes, was then dialyzed and freezer-dried. Globulin (10 mg mL^{-1}) was dissolved in borax buffer and applied to an affinity chromatography on a chitin column (5 mL), equilibrated with 0.05 M borax buffer at pH 7.5. The matrix was washed with the equilibrium buffer and adsorbed proteins were eluted with 100 mM glycine-HCl buffer at pH 2.0. Protein peaks were collected and recovered after dialysis against water, freeze-drying and sample (1 mg/mL) in borax buffer subjected to gel filtration on Superose-6-10-300-GL using an AKTA Purifier System (GE Healthcare Bio-Sciences Corp.), previously calibrated with protein markers: thyroglobulin (669 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), and carbonic anhydrase (29 kDa). All chromatographies were monitored at 280 nm. The peak obtained, denominated EvV, was pooled, and freezer-dried and subjected to further analysis.

Polyclonal antibodies against deglycosylated EvV was prepared by immunization of rabbits according with Thorpe (*16*). Deglycosylation of EvV was performed according with technical instructions obtained from native protein deglycosylation kit, product code N-DEGLY (Sigma Co.). IgG was obtained by affinity chromatography of the crude immune sera using a protein A column (protein A bound to Sepharose CL-4B). Preimmune sera were collected before immunization and used as control.

2.5. Protein and Carbohydrate Determination. Protein content was measured by Bradford (*17*) procedure with bovine serum albumin (BSA) as protein standard. Carbohydrate content was measured according to the Dubois et al. procedure (*18*), with glucose as the carbohydrate standard. All assays were made in triplicate.

2.6. Detection of Proteinase Inhibitory Activities in EvV. Globulin inhibitory assays against proteinase extracts from *C. capitata* larvae were measured using 1% azocasein solution at pH 8.0 (serine proteinase activities). A 50 μL aliquot of EvV (2 mg mL^{-1}) was incubated with 50 μL of midgut homogenates and 400 μL of appropriate buffer (50 mM Tris-HCl and 20 mM $CaCl_2$ at pH 8.0) at 37 °C for 15 min before adding the 500 μL substrate (1% azocasein, 50 mM Tris-HCl, and 20 mM $CaCl_2$ at pH 8.0). After 30 min of incubation, the reaction was stopped by adding 150 μL of 20% TCA solution. Results were monitored at 440 nm. Samples were centrifuged at 10000*g* for 10 min and supernatants were alkalinized with 0.2 N NaOH solution. The soluble peptides were measured by absorbance at 440 nm. Appropriate controls without EvV were prepared in the same conditions as the tests, without the addition of substrate, which was added after the addition of 20% TCA solution. One inhibitory unit (IU) was defined as the amount of inhibitor activity that decreased absorbance by 0.01 at 440 nm. All of the assays were performed in triplicate.

2.7. Detection of Hemeagglutinating Activities in EvV. The hemeagglutinating activity was assayed in microtiter V plates (Nunc Brand products, Denmark) according to a 2-fold serial diluting procedure (*19*). One hemeagglutinating unit (HU) was defined as the amount of sample able to agglutinate and precipitate the erythrocytes in suspension after 30 min. To each well, 25 μL (2 mg mL^{-1}) of the 2-fold serially diluted EvV solution and 25 μL of 2% (v/v) human ABO erythrocyte suspension were added and allowed to incubate for 30 min at room temperature. The degree of hemeagglutinating activity was examined at naked eye. Negative and positive controls consisted of saline and 1.0 mg/mL ConA, respectively, incubated with erythrocytes.

2.8. Polyacrylamide Gel Electrophoresis and Western Blotting. The presence of subunits in EvV was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) at room temperature, as described by Laemmli (*20*), using 15% polyacrylamide separation gel and 4% polyacrylamide stacking gel. Protein 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), and β-lactoalbumin (18.4 kDa) were obtained from Fermentas Co. (Canada). EvV homogeneity was observed by native PAGE also as described by Laemmli (*20*), using 15% of polyacrylamide separation gel and 4% polyacrylamide stacking gel, without SDS. Bromophenol blue was used as the tracking dye.

Western blotting were performed using nitrocellulose membranes after SDS−PAGE according to described methods (*21*).

2.9. Insect Bioassay. To examine the effects of EvV on *C. capitata* development, neonate larvae were fed on artificial diets containing EvV at 0.1, 0.15, 0.20, and 0.25% (w/w) concentrations. Each diet sample (500 mg) was poured into a glass vial, and 15 larvae were allowed to feed on them. The bioassay was maintained at 28 ± 2 °C and 70–80% of relative humidity in a growth chamber. After 5 days, the vials were opened and the mass and number of larvae were determined. The percentage of mass decrease and mortality were calculated in relation to control. The experiments were carried out with six replicates and the mean (±SEM) was calculated. Control diets were prepared without EvV. A completely random design was used, and the comparisons of the means of the larval weight and treatments were made by Tukey's test at 5% level of probability.

2.10. Mechanism of Actions of EvV. *2.10.1. Detection of Chitin in the Gut of C. capitata Larvae.* The gut of third instar larva was separated from windpipes and Malpighian tubes under stereomicroscopy in 0.15 M cold NaCl and aided by a pair of tweezers. Its luminal content was aspirated and kept for analysis, whereas the gut lumen washed thoroughly with NaCl to remove any residual diet. The presence of chitin in larval guts was ascertained by the von Wisselingh color test (*22*). This qualitative test detects chitosan produced after treatment of the chitin-containing materials with saturated KOH for 15 min at 160

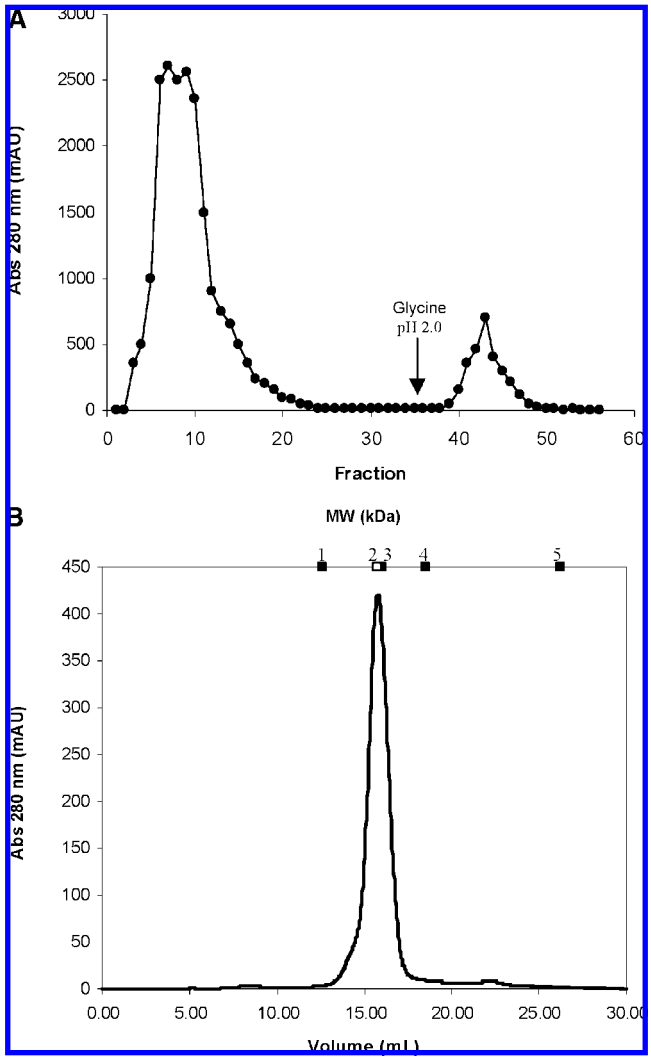

Figure 1. (**A**) Elution profile of F70–90 from *E. velutina* seeds on chitin column. The column (5 mL bed volume) was equilibrated with 50 mM Borax buffer (pH 7.5) and eluted with glycine buffer 100 mM (pH 2.0). (**B**) Elution profile on Superose-6-10-300-GL calibrated column of the EvV peak eluted from chitin column. Protein markers: (1) thyroglobulin (669 kDa), (2) EvV (216 kDa), (3) β-amylase (200 kDa), (4) alcohol dehydrogenase (150 kDa), and (5) carbonic anhydrase (29 kDa).

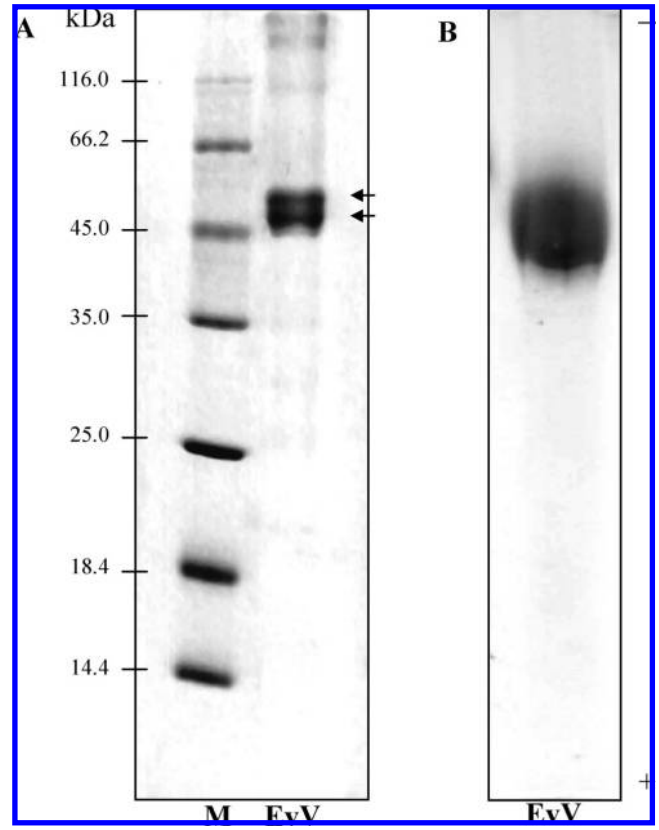

Figure 2. (**A**) SDS−PAGE (15%) analysis of purified EvV stained with Coomassie Blue. (M) Molecular mass markers: β-galactosidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), restriction enzyme Bsp98 (25.0 kDa), β-lactoalbumin (18.4 kDa), and lysozyme (14.4 kDa). (EvV) *E. velutina* vicilin (10 μg). Arrows indicate the major polypeptides chains from EvV. (**B**) Native PAGE (15%) analysis of purified EvV stained with Coomassie Blue. (EvV) *E. velutina* vicilin (10 μg).

°C. After reaction, the presence of chitin was observed with a KI/iodine solution. Controls employing cellulose (−) and lobster chitin (+) were used.

2.10.2. In Vivo Digestibility and Binding of EvV to Peritrophic Membrane. Third instar larvae were fed on artificial diet containing 2.0% starch insoluble, 20% sucrose, and 0.9% NaCl, for 3 h, at room temperature. After this time, they were transferred to a suspension containing 0.125% (w/v) of EvV for 3 h and retransferred for more 3 h to other suspension without EvV. Larvae were then removed, dissected, peritrophic membranes were excised and excreta collected. Excreta suspension was centrifuged at 10000*g* for 10 min and supernatant collected dialyzed against 0.1 M PBS at pH 7.4 and freeze-drying. Membrane peritrophic excised were washed with PBS until removal of nonadsorbed proteins and adsorbed proteins were eluted with 0.1 M glycine-HCl buffer at pH 2.0. Excreta and eluted proteins from peritrophic membranes were subjected to SDS−PAGE and Western blotting analysis. Control treatment with BSA was carried out in the same described conditions.

2.10.3. Visualization of Peritrophic Membranes and Localization of FITC−EvV Binding. Fluorescein-5-isothiocyanate (FITC) was covalently coupled to EvV by the method of Johnston et al. (*23*) with modifications. FITC solution (20 mg in 1 mL anhydrous DMSO) was immediately added in 0.75 M bicarbonate buffer at pH 9.5 before use, followed by the addition of EvV (1.0−0.1 mg of FITC). Mixture was incubated in the dark glass tube and rotated for 1 h at room temperature. The FITC-labeled EvV was recovered by ammonium precipitation, dialyzed against distilled water, and freeze-dried.

Third instar larvae was fed on artificial diet containing 0.125% (w/w) of FITC−EvV for 3 h at room temperature and then fed on artificial diet without EvV for more 3 h. These larvae were removed and dissected, and peritrophic membrane and gut epithelial cells obtained were washed in PBS. The tissue pieces were examined in a fluorescence microscope (Nikon Eclipse E200). Control diet containing 2.5% (w/w) *N*-acetyl glycosamine were carried out in the same described conditions.

3. RESULTS

3.1. Isolation, Purification, and Characterization of *E. velutina* Vicilin (EvV). Crude soluble protein extract obtained from the mature seeds of the *E. velutina* tree was initially precipitated with ammonium sulfate at 0–70 and 70–90% saturation. The F70–90 protein fraction (globulins) obtained was chromatographed on affinity chitin column, one peak was obtained (**Figure 1A**). The peak adsorbed on the chitin matrix was assayed to detect contaminants such as gut proteinase inhibitors, and lectin activities. This fraction showed no inhibitory and lectin activities (data not shown). After analysis, this retained peak was loaded on Superose-6-10-300-GL gel filtration previously calibrated using an AKTA purifier system. The EvV peak had a molecular mass of 216.57 kDa (**Figure 1B**). EvV is a glycoprotein, composed of 1.85% carbohydrates and SDS−PAGE,

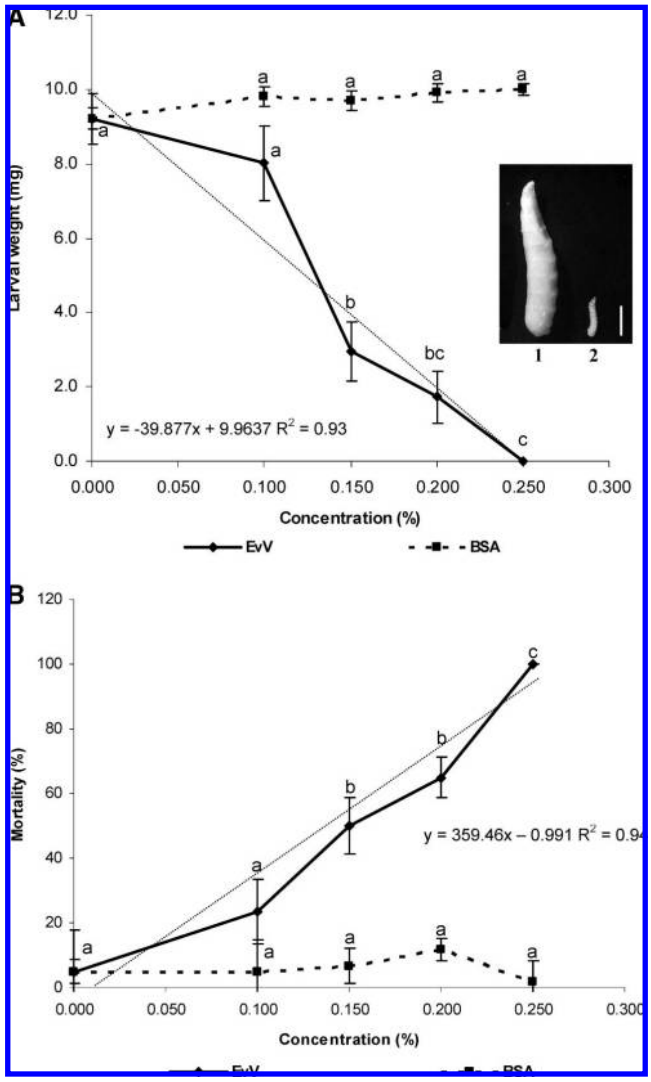

Figure 3. (**A**) Effects of EvV (%, m/m) in the larval mass and in the (**B**) mortality. *y* intercept in A is mortality, and B is larvae mass in control diet. (Inset) (1) Larva fed the control diet and (2) larvae fed on 0.20% EvV. Each data point is the mean of four replicates. Mean values followed by the same letter were not statistically different ($p < 0.05$) by Tukey's test. The error bars represent the standard deviation.

proved to be a tetrameric protein with two major relative molecular mass subunits of 54.8 and 50.8 kDa (**Figure 2A**). EvV homogeneity was confirmed by native PAGE as a unique band with low mobility, characteristic of acid protein, was observed (**Figure 2B**).

3.2. Insect Bioassays. The effect of EvV on larval development of *C. capitata* was assessed by determining the number and mass of surviving larvae fed a diet containing increasing amounts of EvV. The curve dose response of the effect of EvV on the mass and mortality of the larvae is represented in **Figure 3**. The chitin-binding vicilin added to artificial diet was very effective, causing a decrease of 50% of the larva mass with an ED_{50} (effective dose) of 0.12% and 50% mortality at a level of 0.14% (LD_{50}, lethal dose). Regression analysis showed that for every 0.1% increase in EvV dose, there was a 66.7% decrease in *C. capitata* larval mass. For each 0.1% increase in EvV fed instead applied to *C. capitata*, there was a 35.0% increase in mortality. Unrelated proteins, such as bovine serum albumin, had no effect on larval growth over the same concentration range as used for EvV.

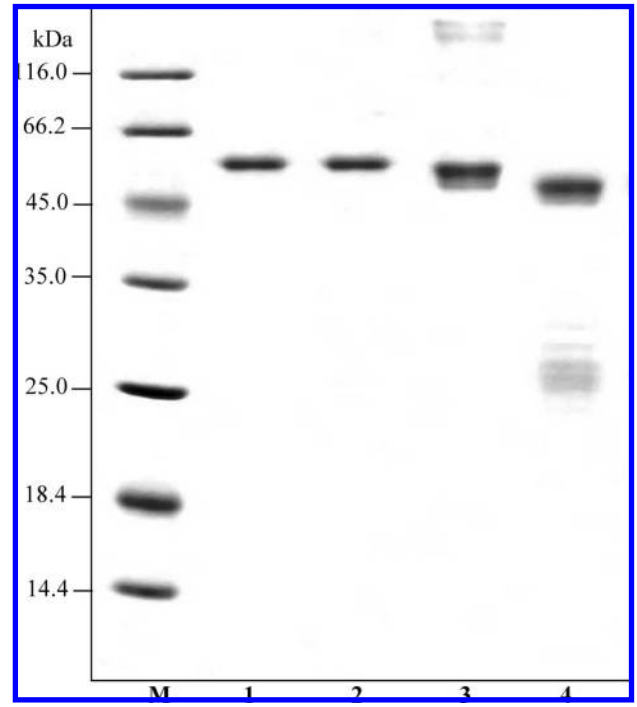

Figure 4. *In vivo* digestibility of EvV analyzed by SDS−PAGE. (M) Molecular-mass markers. (1) BSA control, (2) BSA excreta, (3) EvV control, and (4) EvV excreta.

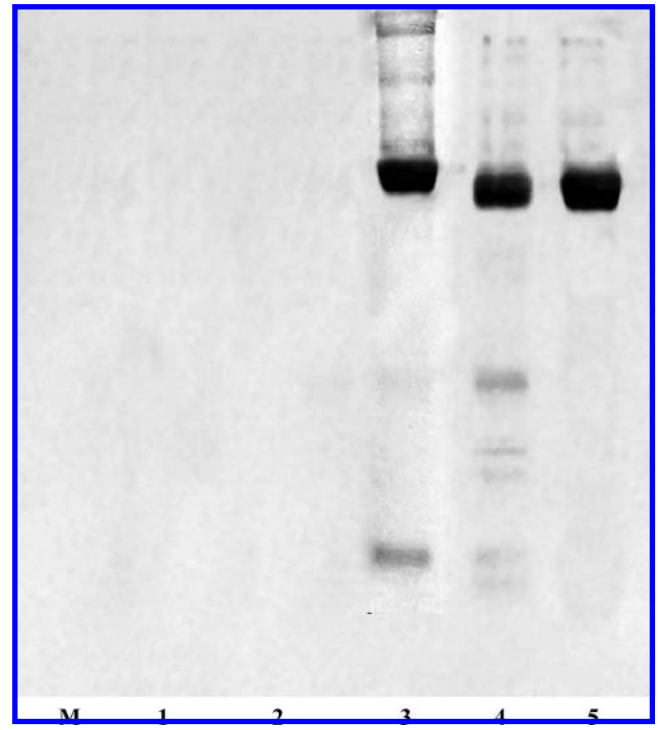

Figure 5. *In vivo* digestibility and binding of EvV to peritrophic membrane analyzed by immunoblotting. (M) Molecular-mass markers. (1) BSA control, (2) BSA excreta, (3) EvV control, (4) EvV excreta, and (5) eluted EvV from peritrophic membrane.

3.3. Mechanism of Actions of EvV. *3.3.1. Detection of Chitin in the Fruit Fly Larvae Gut.* The presence of chitin in the midgut of *C. capitata* larvae was revealed by the Wisselingh color test for the qualitative determination of chitin. KOH-treated larvae midguts were placed in a KI/iodine solution and the appearance of a brown color was indicative

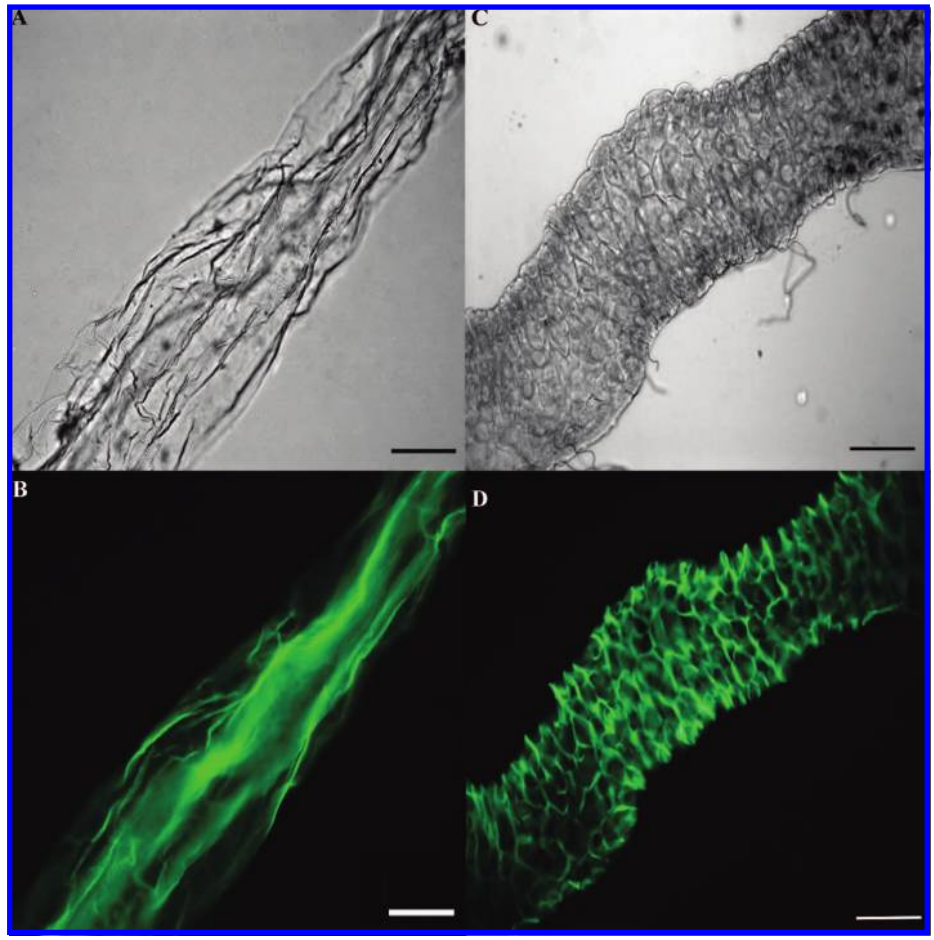

Figure 6. Fluorescence micrographs showing FITC−EvV binding in *C. capitata* gut larvae. (**A**) Light microscopy and (**B**) fluorescence microscopy of the peritrophic membrane from larvae fed on artificial diet containing FITC−EvV. (C) Light microscopy and (D) fluorescence microscopy of gut epithelial cells from larvae fed on artificial diet containing FITC−EvV. Scales in A and B are 50 μm. Scales in C and D are 200 μm.

of the presence of chitin in larvae midguts. A positive test was confirmed by the addition of 0.1% H_2SO_4 when the color turned violet.

3.3.2. In Vivo Digestibility and Binding of EvV to Peritrophic Membrane of C. capitata Larvae. The susceptibility of EvV to gut digestive enzymes of *C. capitata* larvae was demonstrated *in vivo*. Analysis of the larvae excreta by SDS−PAGE showed that EvV was resistant to action of gut digestive enzymes (**Figure 4**), where immunoreactive EvV subunities refractory bands to hydrolysis were detected in larval excreta (**Figure 5**).

In vivo binding of vicilins to peritrophic membrane from *C. capitata* larvae was evaluated by Western blotting. The presence of the immunoreactive vicilin liberated from peritrophic membrane was confirmed and showed in line 5 of **Figure 5**.

3.3.4. Fluorescent Localization of Bound EvV−FITC. Binding of EvV−FITC to the peritrophic membrane and gut epithelial cells was confirmed as shown in **Figure 6**. These interactions could have occurred directly with chitin components and/or with specific membrane-associated glycoproteins. The lack of fluorescence in control samples demonstrated the specificity of EvV binding.

DISCUSSION

Toxicological studies based on acute and chronic effects upon exposure revealed that many classical insecticides are highly toxic not only to nontargeted insect species, but also to mammals and humans (*24, 25*). Consequently, a search for safer alternatives for pest control is necessary. Biological and physiological studies resulted in the development of insecticides with a better environmental profile, with different mechanisms of actions and with reduced risks for living systems. In this context, proteinaceous compounds such as plant proteinase inhibitors, α-amylase inhibitors, lectins and chitin binding proteins may affect development by interfering in the digestive system of a wide range of potentially damaging insects. These effects on insects can be observed when digestive enzymes are inhibited or when defense proteins bind to the gut structures of larvae and adult insects, such as chitin and or others binder molecules (*26*). Among the defense proteins, Vicilins or 7S globulins consist of multisubunit combinations with molecular masses between 20.1 and 94 kDa (*27*), which pass by extensive post-translational process resulting in a high degree of polymorphism of 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 vicilin from *E. velutina* seeds, denoted of EvV, was purified through a chitin matrix, and its effect was tested against *C. capitata* larvae. EvV is a glycoprotein with molecular mass of 216 kDa with two subunities of 54.8 and 50.8 kDa, in agreement with similar data previously reported by several authors for other legume vicilins (*30–33*). Many seed defense proteins are able to bind on chitin matrix as chitinases (*34–36*), lectins (*37, 38*), proteinase inhibitors (*39, 40*), and hevein (*41*). The first study that showed that chitin binding vicilins could be involved in plant defense was related by Sales et al. (*42*). These authors demonstrated that variant vicilins, isolated from resistant *Cal-*

losobruchus maculatus nigerian cowpea seeds (*V. unguiculata*), strongly bound to chitin matrix and it had high detrimental effect to the Bruchid (*43*). Posterior study showed that vicilins isolated from others legume seeds of distantly related species such as jack beans (*Canavalia ensiformis*), soybeans (*Glycine max*), and lima bean (*Phaseolus lunatus*) showed a high detrimental effect on the larval development of *C. maculatus* (*33*). Through bioassays, it was observed that EvV, similar to other chitin binding vicilins, had lethal and or detrimental effects on organisms that contain chitin in the peritrophic membrane. This matrix forms a barrier to protect the gut epithelium from abrasive food particles (*44*). EvV was very lethal to *C. capitata* with LD_{50} (0.14%) and ED_{50} (0.13%) lower than those observed in nigerian resistant *V. unguiculata* vicilin (ED_{50} of 1.07%) (*43*) and among other legume vicilins, such as *G. max* vicilin (ED_{50} of 1.66%), *P. lunatus* vicilin (ED_{50} of 1.74%), *C. ensiformis* vicilin (ED_{50} of 2.15%) to *C. maculatus*. The lethal EvV dose was comparable to the concentrations (0.1–1.0%) of WGA, rice lectins, nettle lectins, TEL (*Talisia esculenta* lectin) and *Enterolobium contortisiliquum* vicilin (EcV) tested for *C. maculatus* (*40, 45, 46*), which are not target hosts to this bruchid. Possibly the strong effect of EvV to *C. capitata* could be related with the distance evolutive between dipteran pest and the legume species *E. veluntina*. The EvV action mechanism was due to the indigestibility of EvV associated to the binding with chitin present in the peritrophic membrane and also on gut epithelium cells of this dipteran, as proved here by imunoblotting and fluorescent binding of EvV−FITC. These effects on the gut of *C. capitata* suggests that the binding of vicilins to both structures of larvae gut caused interference with nutrient absorption (*47*) either preventing or enhancing movement between the endo and exoperitrophic space or preventing the formation of the membrane itself (*48*) and binding to epithelium cells could affect various functions of the cell membranes (*11*).

The strong negative effects of EvV on *C. capitata* larvae, observed in this study, suggest that EvV might be able to provide a viable alternative in designing transgenic crops to control insect pests or be used as a pesticide when tested against adult *C. capitata*.

ACKNOWLEDGMENT

This study was supported by the following Brazilian Agencies: FINEP, CAPES, CNPq, and BNB-FUNDECI.

LITERATURE CITED

(1) White, I. M.; Elson-Harris, M. M. *Fruit Flies of Economic Significance: Their Identification and Bionomics*; CAB International: Wallingford, U.K., 1992; p 600.

(2) Liquido, N. J.; Shinoda, L. A.; Cunningham, R. T. Host plants of Mediterranean fruit fly: An annotated world review. *Misc. Publ. Entomol. Soc. Am.* **1991**, *77*, 1–52.

(3) Duarte, A. L.; Malavasi, A. Tratamentos quarentenários. In *Moscas-das-frutas de importancia econômica no Brasil. Conhecimento Básico e aplicado*; Malavasi, A., Zucchi, R. A., Eds.; Holos: Ribeirão Preto, 2000.

(4) Bateman, M. A.; Morton, T. C. The importance of ammonia in proteinaceous attractants for fruit flies (family, Tephritidae). *Aust. J. Agric. Res.* **1981**, *32* (6), 883–903.

(5) Hemingway, J.; Ranson, H. Insecticide resistance in insect vectors of human disease. *Annu. Rev. Entomol.* **2000**, *45*, 371–391.

(6) Grapov, A. F. New insecticides and acaricides. *Uspekhi Khimii* **1999**, *68* (8), 773–784.

(7) Wang, P.; Granados, R. R. Molecular structure of the peritrophic membrane (PM): Identification of potential PM target sites for insect control. *Arch. Insect Biochem. Physiol.* **2001**, *47* (2), 110–118.

(8) Fitches, E.; Woodhouse, S. D.; Edwards, J. P.; Gatehouse, J. A. In vitro and in vivo binding of snowdrop (*Galanthus nivalis* agglutinin; GNA) and jackbean (*Canavalia ensiformis*; Con A) lectins within tomato moth (*Lacanobia oleracea*) larvae; mechanisms of insecticidal action. *J. Insect Physiol.* **2001**, *47* (7), 777–787.

(9) Peters, W. *Peritrophic Membranes*; Springer-Verlag: New York, 1992.

(10) Lehane, M. Peritrophic matrix structure and function. *Annu. Rev. Entomol.* **1997**, *42*, 525–550.

(11) Eisemann, C. H.; Donaldson, R. A.; Pearson, R. D.; Cadogan, L. C.; Vuocolo, T.; Tellam, R. L. Larvicidal activity of lectins on *Lucilia cuprina*—Mechanism of action. *Entomol. Exp. Appl.* **1994**, *72* (1), 1–10.

(12) Shutov, A. D.; Kakhovskaya, I. A.; Braun, H.; Baumlein, H.; Muntz, K. Legumin-like and vicilin-like seed storage proteins: Evidence for a common single-domain ancestral gene. *J. Mol. Evol.* **1995**, *41* (6), 1057–1069.

(13) Sales, M. P.; Pimenta, P. P.; Paes, N. S.; Grossi-de-Sa, M. F.; Xavier, J. Vicilins (7S storage globulins) of cowpea (*Vigna unguiculata*) seeds bind to chitinous structures of the midgut of *Callosobruchus maculatus* (Coleoptera: Bruchidae) larvae. *Braz. J. Med. Biol. Res.* **2001**, *34* (1), 27–34.

(14) Mota, A. C.; DaMatta, R. A.; Lima, M.; Silva, C. P.; Xavier, J. Cowpea (*Vigna unguiculata*) vicilins bind to the peritrophic membrane of larval sugarcane stalk borer (*Diatraea saccharalis*). *J. Insect Physiol.* **2003**, *49* (9), 873–880.

(15) Silva, F. C. B. L.; Alcazar, A.; Macedo, L. L. P.; Oliveira, A. S.; Macedo, F. P.; Abreu, L. R. D.; Santos, E. A.; Sales, M. P. Digestive enzymes during development of *Ceratitis capitata* (Diptera: Tephritidae) and effects of SBTI on its digestive serine proteinase targets. *Insect Biochem. Mol. Biol.* **2006**, *36* (7), 561–569.

(16) Thorpe, R. Producing antibodies. In *Immunochemistry—Labfax*, 1st ed.; Kerr, M. A., Thorpe, R., Eds. BIOS Scientific Publishers Limited: Oxford, U.K., 1994; pp 63–81.

(17) Bradford, M. M. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein−dye binding. *Anal. Biochem.* **1976**, *72* (1–2), 248–254.

(18) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28* (3), 350–356.

(19) Debray, H.; Decout, D.; Strecker, G.; Spik, G.; Montreuil, J. Specificity of 12 lectins towards oligosaccharides and glycopeptides related to *N*-glycosylproteins. *Eur. J. Biochem.* **1981**, *117* (1), 41–55.

(20) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227* (5259), 680–685.

(21) Towbin, H.; Staehelin, T.; Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76* (9), 4350–4354.

(22) Rogers, H. J.; Perkins, H. R. *Cell Walls and Membranes*; Spon Ltd.: London, U.K., 1968.

(23) Johnston, L. A.; Pearson, R. D.; Leatch, G. Evaluation of an indirect fluorescent antibody test for detecting babesia argentina infection in cattle. *Aust. Vet. J.* **1973**, *49* (8), 373–377.

(24) Burton, A. Pesticides may promote Parkinson's disease. *Front. Ecol. Environ.* **2006**, *4* (6), 285–285.

(25) Wolkers, H.; van Bavel, B.; Ericson, I.; Skoglund, E.; Kovacs, K. M.; Lydersen, C. Congener-specific accumulation and patterns of chlorinated and brominated contaminants in adult male walruses from Svalbard, Norway: Indications for individual-specific prey selection. *Sci. Total Environ.* **2006**, *370* (1), 70–79.

(26) Xavier-Filho, J. Sementes e suas defesas contra insetos. In *Projeto Multinacional de Biotecnologia e Alimentos*; Organizacão dos Estados Americanos (OEA): Washington, D.C., 1993; pp 1−31.

(27) Pedalino, M.; Durzo, M. P.; Delledonne, G.; Grillo, S.; Rao, R. The structure of cowpea (*Vigna unguiculata* L. Walp) seed storage proteins. *Seed Sci. Technol.* **1992**, *20* (2), 223–231.

(28) Higgins, T. J. V. Synthesis and regulation of major proteins in seeds. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1984**, *35*, 191–221.
(29) Shewry, P. R.; Napier, J. A.; Tatham, A. S. Seed storage proteins—Structures and Biosynthesis. *Plant Cell* **1995**, *7* (7), 945–956.
(30) Casey, R.; Domoney, C.; Ellis, N. *Legume Storage Proteins and Their Genes*; Oxford University Press: Oxford, U.K., 1986; Vol. 3, p 95.
(31) Khan, R. I.; Gatehouse, J. A.; Boulter, D. The seed proteins of cowpea (*Vigna unguiculata* L. Walp). *J. Exp. Bot.* **1980**, *31* (125), 1599–1611.
(32) Macedo, M. L. R.; Fernandes, K. V. S.; Sales, M. P.; Xavier, 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**, *28* (2), 183–190.
(33) Yunes, A. N. A.; de Andrade, M. T.; Sales, M. P.; Morais, R. A.; Fernandes, K. V. S.; Gomes, V. M.; Xavier-Filho, J. Legume seed vicilins (7S storage proteins) interfere with the development of the cowpea weevil (*Callosobruchus maculatus* (F)). *J. Sci. Food Agric.* **1998**, *76* (1), 111–116.
(34) Krishnaveni, S.; Liang, G. H.; Muthukrishnan, S.; Manickam, A. Purification and partial characterization of chitinases from sorghum seeds. *Plant Sci.* **1999**, *144* (1), 1–7.
(35) Santos, I. S.; Da Cunha, M.; Machado, O. L. T.; Gomes, V. M. A Chitinase from *Adenanthera pavonina* L. seeds: Purification, characterisation and immunolocalisation. *Plant Sci.* **2004**, *167* (6), 1203–1210.
(36) Taira, T.; Yamagami, T.; Aso, Y.; Ishiguro, M.; Ishihara, M. Localization, accumulation, and antifungal activity of chitinases in rye (*Secale cereale*) seed. *Biosci., Biotechnol., Biochem.* **2001**, *65* (12), 2710–2718.
(37) Santi-Gadelha, T.; Gadelha, C. A. D. A.; Aragao, K. S.; de Oliveira, C. C.; Mota, M. R. L.; Gomes, R. C.; Pires, A. D. F.; Toyama, M. H.; Toyama, D. D. O.; de Alencar, N. M. N.; Criddle, D. N.; Assreuy, A. M. S.; Cavada, B. S. Purification and biological effects of *Araucaria angustifolia* (Araucariaceae) seed lectin. *Biochem. Biophys. Res. Commun.* **2006**, *350* (4), 1050–1055.
(38) Harper, M. S.; Hopkins, T. L.; Czapla, T. H. Effect of wheat germ agglutinin on formation and structure of the peritrophic membrane in European corn borer (*Ostrinia nubilalis*) larvae. *Tissue Cell* **1998**, *30* (2), 166–176.
(39) Macedo, M. L. R.; Freire, M. D. M.; Cabrini, E. C.; Toyama, M. H.; Novello, J. C.; 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* (2), 170–182.
(40) Macedo, M. L. R.; Mello, G. C.; Freire, M. D. G. M.; Novello, J. C.; Marangoni, S.; de Matos, D. G. G. Effect of a trypsin inhibitor from *Dimorphandra mollis* seeds on the development of *Callosobruchus maculatus*. *Plant Physiol. Biochem.* **2002**, *40* (10), 891–898.
(41) Vanparijs, J.; Broekaert, W. F.; Goldstein, I. J.; Peumans, W. J. Hevein—An antifungal protein from rubber tree (*Hevea brasiliensis*) latex. *Planta* **1991**, *183* (2), 258–264.
(42) Sales, M. P.; Gomes, V. M.; Fernandes, K. V. S.; Xavier, J. Chitin-binding proteins from cowpea (*Vigna unguiculata*) seeds. *Braz. J. Med. Biol. Res.* **1996**, *29* (3), 319–326.
(43) Macedo, M. L. R.; Andrade, L. B. D.; Moraes, R. A.; Xavierfilho, J. Vicilin variants and the resistance of cowpea (*Vigna unguiculata*) seeds to the cowpea weevil (*Callosobruchus maculatus*). *Comp. Biochem. and Physiol., Part C: Pharmacol., Toxicol., Endocrinol.* **1993**, *105* (1), 89–94.
(44) Richards, A. G.; Richards, P. A. Peritrophic membranes of insects. *Annu. Rev. Entomol.* **1977**, *22*, 219–240.
(45) Huesing, J. E.; Murdock, L. L.; Shade, R. E. Rice and stinging nettle lectins—Insecticidal activity similar to wheat germ agglutinin. *Phytochemistry* **1991**, *30* (11), 3565–3568.
(46) Moura, F. T.; Oliveira, A. S.; Macedo, L. L. P.; Vianna, A. L. B. R.; Andrade, L. B. S.; Martins-Miranda, A. S.; Oliveira, J. T. A.; Santos, E. A.; de Sales, M. P. 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*). *J. Agric. Food Chem.* **2007**, *55* (2), 260–266.
(47) Higgins, T. J. V.; Chrispeels, M. J.; de Sa, M. F. G. Genetic engineering with α-amylase inhibitors makes seeds resistant to bruchids. *Seed Sci. Res.* **1998**, *8* (2), 257–263.
(48) Czapla, T. H.; Lang, B. A. Effect of plant lectins on the larval development of European corn borer (Lepidoptera, Pyralidae) and southern corn rootworm (Coleoptera, Chrysomelidae). *J. Econ. Entomol.* **1990**, *83* (6), 2480–2485.

Received for review September 14, 2007. Revised manuscript received November 24, 2007. Accepted November 30, 2007.

JF072746N