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**Abstract**

Seed coat is a specialized maternal tissue that interfaces the embryo and the external environment during embryogenesis, dormancy and germination. In addition, it is the first defensive barrier against penetration by pathogens and herbivores. Here we show that *Albizia lebbeck* seed coat dramatically compromises the oviposition, eclosion and development of the bruchid *Callosobruchus maculatus*. Dietary supplementation of bruchid larvae with *A. lebbeck* seed coat flour causes severe weight loss and reduces survival. By means of protein purification, mass spectrometry and bioinformatic analyses, we show that chitin-binding vicilins are the main source of *A. lebbeck* tegumental toxicity to *C. maculatus*. At concentrations as low as 0.1%, *A. lebbeck* vicilins reduce larval mass from 8.1 ± 1.7 (mass of control larvae) to 1.8 ± 0.5 mg, which corresponds to a decrease of 78%. Seed coat toxicity constitutes an efficient defense mechanism, hindering insect predation and preventing embryo damage. We hypothesize that *A. lebbeck* vicilins are good candidates for the genetic transformation of crop legumes to enhance resistance to bruchid predation.

**Key words:** Chitin-binding proteins; Seed coat; *Callosobruchus maculatus*; Insect bruchid

**Introduction**

During fruit development, seeds from several plants accumulate proteins, forming a critical nutritional source that is metabolized during germination and seedling growth (1). In eudicot seeds, 7S vicilins (globulins) are the main reserve proteins (1,2). In addition to their major role as energy source, alternative functions for vicilins have been proposed in sucrose binding (3), desiccation (4) and defense against insects and fungi (5-8). Several cotyledonary and tegumental vicilins can confer protection against *Callosobruchus maculatus*. Dietary supplementation of bruchid larvae with *A. lebbeck* seed coat flour causes severe weight loss and reduces survival. By means of protein purification, mass spectrometry and bioinformatic analyses, we show that chitin-binding vicilins are the main source of *A. lebbeck* tegumental toxicity to *C. maculatus*. At concentrations as low as 0.1%, *A. lebbeck* vicilins reduce larval mass from 8.1 ± 1.7 (mass of control larvae) to 1.8 ± 0.5 mg, which corresponds to a decrease of 78%. Seed coat toxicity constitutes an efficient defense mechanism, hindering insect predation and preventing embryo damage. We hypothesize that *A. lebbeck* vicilins are good candidates for the genetic transformation of crop legumes to enhance resistance to bruchid predation.

Chitin-binding proteins; Seed coat; *Callosobruchus maculatus*; Insect bruchid
Material and Methods

Seeds and insects
A. lebbeck seeds were collected at Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ, Brazil. Commercial cowpea (V. unguiculata cv. fradinho) seeds were purchased from local markets at Campos dos Goytacazes, RJ, Brazil. C. maculatus (Coleoptera: Bruchidae) adults were maintained in an in-house colony dos Goytacazes, RJ, Brazil. A. lebbeck seeds were placed in glass flasks and exposed to ten 3-day-old C. maculatus females for 24 h. The experiment was conducted in a BOD incubator chamber at 28°C and 70% relative humidity. Females were removed and larval development was monitored using a stereoscopic microscope coupled to a digital CCD video camera, from oviposition to complete seed coat perforation or larval death. Control experiments using cowpea as host seeds were performed in parallel under the same conditions.

Seed infestation
Sixty A. lebbeck seeds were placed in glass flasks and exposed to ten 3-day-old C. maculatus females for 24 h. The experiment was conducted in a BOD incubator chamber at 28°C and 70% relative humidity. Females were removed and larval development was monitored using a stereoscopic microscope coupled to a digital CCD video camera, from oviposition to complete seed coat perforation or larval death. Control experiments using cowpea as host seeds were performed in parallel under the same conditions.

Extraction and purification of seed coat proteins
Seed coats were separated from cotyledons and ground with a pestle and mortar. Proteins were extracted (1:10 flour to buffer ratio) with 100 mM phosphate buffer with 500 mM NaCl, pH 7.6, for 3 h at 4°C and centrifuged at 10,000 g for 30 min. The supernatant was treated with 90% saturation with ammonium sulfate for 24 h at 4°C and centrifuged at 10,000 g for 30 min at 4°C. The precipitated fraction was dialyzed against water for 24 h at 4°C using 7-kDa cut-off membranes and freeze-dried.

The freeze-dried material was diluted (500 mg/46 mL 0.1 M sodium acetate buffer, pH 5.0) and fractionated by CM-Sepharose ion exchange chromatography. The sample was applied to a 2.0 x 21.0 cm column equilibrated with 0.1 M sodium acetate buffer, pH 5.0. The non-retained fraction (CI) was eluted and adsorbed proteins were sequentially desorbed using 0.25 and 0.5 M NaCl solutions (CII and CIII fractions, respectively).

Gel filtration chromatography was used to separate a 50 mg/mL (50 mM potassium phosphate buffer, pH 7.8) sample of CI on a Sephacryl S-100 column (2.0 x 70 cm). The fractions (SI, SII, and SIII) were eluted using the sample dissolution buffer. The SI fraction was used for protein identification and characterization and toxicity to insects.

Protein identification and characterization
The protein fraction obtained by Sephacryl S-100 gel filtration chromatography (SI fraction) was analyzed by 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (19), conducted at 15 mA. Gels were stained with 0.05% Coomassie blue and destained in 10% acetic acid.

In-gel tryptic digestion of SI fraction proteins was performed as described (20). For mass spectrometry analysis the peptides were co-crystallized with 0.3 μL 10 mg/mL α-cyano-4-hydroxycinnamic acid solution in 0.1% (w/v) trifluoroacetic acid, 50% (v/v) acetonitrile directly onto a MALDI target plate. Raw data for protein identification were obtained with a 4700 Proteomics Analyzer (Applied Biosystems, USA). Both mass spectrometry (MS) and MS/MS data were acquired in the positive and reflectron modes using a neodymium-doped yttrium aluminum garnet (Nd:YAG) laser with a 200-Hz repetition rate. Typically, 1600 shots were accumulated for spectra in the MS mode while 3000 shots were accumulated for spectra in the MS/MS mode. Up to 10 of the most intense ion signals with a signal-to-noise ratio above 20 were selected as precursors for MS/MS acquisition. Peptides from trypsin autolysis peaks and matrix ion signals were discarded. External calibration in the MS mode was performed using a mixture of four peptides: des-Arg1-bradykinin (m/z = 904.47), angiotensin I (m/z = 1296.69), Glu1-fibrinopeptide B (m/z = 1570.68), and ACTH (18-39) (m/z = 2465.20). MS/MS spectra were externally calibrated using fragments of known ion masses observed in the MS/MS spectrum of angiotensin I. MS/MS results were searched against the NCBI nr databases using the Mascot software (www.matrixscience.com). The peptide sequences were analyzed using the Scaffold™ software to determine peptide identification probability. Bit scores were computed by aligning the MS peptides using BLAST. Search parameters were as follows: two tryptic missed cleavages allowed, non-fixed modifications of methionine, tryptophan, histidine (oxidation), cysteine (carbamidomethylation and propionamide). Thepps and ppw files were generated from the raw (or native) MS data according to the following parameters using the Data Explorer Software (Applied Biosystems, USA). For MS1: mass range: 900-4000; peak density: 15 peaks per 200 Da; signal-to-noise ratio: 20; minimum area: 100, and maximum peaks per spot: 60. For MS2: mass range: 60 and precursor 20; peak density: 55 peaks per 200 Da; signal-to-noise ratio: 2; minimum area: 10, and maximum peaks per precursor: 200.

Bioinformatic analyses
We used a computational strategy based on BLAST searches (command line version) (21) to identify the proteins (or close homologs from other species) from which the MS peptides were derived. Since the sequenced peptides are short, we used non-default BLAST parameters (word size 2, no filters or compositional based statistics and PAM30 as score matrix). The full-length amino acid sequences of the aligned hits were submitted to a second BLAST search, which allowed us to obtain a more comprehensive list of homologs, not biased by the short alignments from MS peptides. Sequences aligning with an E-value <0.001, 40% similarity and 50% query and hit coverage were recovered, clustered using blastclust (21), and aligned using MUSCLE (22). BLAST results were parsed using BioPerl (23) and custom Perl scripts. Taxonomic information was obtained from the Taxonomy database (NCBI) (24) using custom scripts.
Feeding trials
To test the effects of the seed coat flours and chromatographic fractions on larval development, we used an artificial seed system (9). Samples were added to a cowpea-based meal at concentrations of 0.1, 0.25, 0.5, and 1.0%. Artificial seeds were exposed to 3-day-old fertilized females for 24 h (28°C, 70% relative humidity). Females were removed and laid eggs were left at a concentration of 3 eggs per seed. After 20 days, infested seeds were opened and larvae were counted and weighed. Control experiments were performed under the same conditions using V. unguiculata (cv. fradinho) cotyledons. Experiments consisted of 3 seeds per assay and were run in triplicate (a total of 9 seeds and 27 eggs per tested dose). Statistical significance was assessed using the Student t-test (P value <0.05) (25).

ELISA
Vicilin levels were quantified by enzyme-linked immunosorbent assay (ELISA) (26), using an anti-V. unguiculata (cv. EPACE-10) vicilin antibody produced in rabbits (primary antibody) diluted 1:1000 and a peroxidase-conjugated anti-rabbit IgG antibody (secondary antibody) diluted 1:2000. Cotyledonary EPACE-10 vicilin samples (5 to 0.0024 µg/100 µL) were used as reference. Peroxidase activity was developed using ortho-phenylenediamine.

Chitin-binding assay
Chitin from lobster shells was obtained from Sigma-Aldrich (USA) and treated with a previously established protocol (27). The finely ground chitin was used to prepare an affinity column, subsequently used to purify chitin-binding proteins from the CI (162 mg/2 mL) and SI (18 mg/mL) fractions (dissolved in 80 mM sodium acetate buffer, pH 5.0). The non-retained fraction was eluted with the dilution buffer and adsorbed proteins were desorbed using 0.1 M HCl. Both fractions were dialyzed against water for 24 h at 4°C and freeze-dried.

Results
Toxicity of A. lebbeck seed coat to C. maculatus
By comparing the survival and development of C. maculatus on A. lebbeck and V. unguiculata cv. fradinho (host seed) we found that oviposition, larval eclosion and adult emergence were drastically compromised in the former. The reduction of oviposition was striking (96%), whereas seed coat penetration, larval eclosion and adult emergency were not observed before 30 days after oviposition. The amount of adults that emerged from the V. unguiculata seeds after this time was about 86% (data not shown).

Additional experiments using flour prepared from A. lebbeck seed coats showed a clear dose-dependent toxicity to C. maculatus larvae, with strong weight loss and mortality by 20 days (Figure 1). At concentrations of 0.25, 0.5, and 1%, we observed 40, 83.8, and 87.1% larval weight loss, respectively. In addition, after 20 days of incubation, only 14.1% of the larvae survived on a diet with 1% of A. lebbeck seed coat flour (P < 0.05, t-test).

Purification and characterization of seed coat proteins and toxicity to C. maculatus
A. lebbeck seed coat proteins were initially precipitated using 0-90% ammonium sulfate and submitted to ion exchange chromatography on a CM-Sepharose column, resulting in a non-retained fraction (CI) and two adsorbed fractions that were desorbed with 0.25 and 0.5 M NaCl (CII and CIII, respectively). Higher NaCl concentrations (0.75 and 1.0 M NaCl) did not desorb additional protein fractions (Figure 2). The three chromatographic fractions...
Chitin-binding vicilins are toxic to the insect *C. maculatus* were incorporated into the larval diet and CI was the one with highest toxicity, with 67 and 100% larval mortality at concentrations of 0.1 and 0.25%, respectively (Figure 3A).

In addition, we noticed that larval death can happen either before or after hatching (Figure 3B, Panels a-d). When the larval development on natural seeds of *A. lebbeck* and *V. unguiculata* (*C. maculatus*-host seeds) was monitored, during the initial 6 days after oviposition no morphological differences in larval development were observed between the two seeds (Figure 3B, Panels e-j). Egg content was clear and larvae were virtually formed by the fifth day, when the larvae started to hatch and perforate the seed coat (Figure 3B, Panels e,f). Although they stayed alive for up to 16 days when feeding on *A. lebbeck* (Figure 3B, Panel g), *C. maculatus* larvae were unable to reach the cotyledonary tissue (Figure 3B, Panel h). On the other hand, *C. maculatus* larvae were able to reach and intensively feed on the *V. unguiculata* cotyledons by the 6th day after oviposition (Figure 3B, Panels i,j).

Although CI administration resulted in a clear reduction of larval counts, the body weight of surviving individuals was apparently unaffected (Figure 3C). CII and CIII were also toxic at 1%, both showing a 67% mortality rate (Figure 3A) and CIII also reduced larval body weight by 77.2% (Figure 3C). Therefore, these results suggest that *A. lebbeck* seed coat has physicochemically distinct sets of toxic proteins. Due to its higher toxic potential against *C. maculatus*, CI was elected for additional characterization experiments.

CI was submitted to Sephacryl S-100 gel filtration chromatography, resulting in three fractions (SI, SII, and SIII; Figure 4A). Due to its high abundance, we chose SI for further experiments. When the SI protein profile was examined by SDS-PAGE, three major bands with molecular masses between 50 and 40 kDa (Figure 4B) were demonstrable. These three major bands were cut out, subjected to trypsin digestion and MS analysis. The results showed that two peptides originated from bands 1 and 8 were derived from band 3 (Table 1). The sequences obtained were analyzed by BLAST in order to identify the protein. The peptides were identified as components of vicilin from *V. unguiculata* (ID GenBank emb|CAP19902.1|) and *V. radiata* 8S globulin (ID GenBank ABG02260). Collectively, the peptides account for approximately 24.7% of the *V. radiata* 8S protein and 25.9% of *V. unguiculata* vicilin sequences. No peptide was obtained from band 2 (45-kDa

![Figure 3. Toxicity of flour and of the CI fraction prepared from Albizia lebbeck seed coat to Callosobruchus maculatus larvae. A and B, Larval mortality. B, a-d: larval mortality in artificial seeds containing the CI fraction (arrows). a: the larva died inside the egg; b: perforation of artificial seed; c and d: dead larvae found inside the artificial seed. e-j: development of larvae on natural A. lebbeck and Vigna unguiculata seeds. e: larva inside the egg on the 5th day after oviposition; f: inferior surface of the egg on the 5th day after oviposition; g: larva inside the egg on the 16th day after oviposition; h: A. lebbeck seed coat partially perforated on the 16th day after oviposition; i: hole across the host seed coat from V. unguiculata on the 6th day; j: hole on the V. unguiculata cotyledon surface on the 6th day after oviposition. The bar inside Panel a indicates magnification for Panels a-d. The bar inside Panel e indicates magnification for Panels e-d. The bar inside Panels h,i indicates magnification for Panels h-j. C, Larval mass at 20 days after oviposition. The seed coat fraction was incorporated into artificial seeds at different concentrations (0.1, 0.25, 0.5, and 1.0%). Experiments were done in triplicate and the data shown are the means ± SD of these results. *P < 0.05 compared to control (t-test).
The peptide sequences REQQQQQQQEESWEVQRY and RLHEITPEKNPQLRD obtained from protein band 3 were analyzed using the Scaffold 3™ software and the results showed a peptide identification probability of 95% with V. unguiculata vicilin (ID GenBank 160332746).

We used a computational strategy based on BLAST (21) to identify homologs in the nr database (see Methods for details). Although the peptides sequenced from each band were totally different from each other, the hit lists from the two bands had an extremely high overlap (more than 96% of the BLAST hits were shared by the bands). Therefore, our results strongly suggest that the two bands are isoforms of the same gene (or extremely close paralogs), and hence we merged the results. The combined list is dominated by vicilins and vicilin-like proteins (e.g., gis 160332746 and 145207915), which were extracted from the nr database, clustered using blastclust and aligned [data shown at (http://dl.dropbox.com/u/7886304/supplements/BJMBR-Souza2012/BJMBR-Souza-MSA.pdf)]. We extracted taxonomic information for all the hits and found that all the detected homologs were from the Tracheophyta clade (vascular plants), encompassing homologs in the basal vascular plant Selaginella moellen-dorffii (Lycopodiophyta), the fern Matteuccia struthiopteris, confers, various monocots (e.g., Oryza sativa and Zea mays) and eudicots (e.g., Vitis vinifera, Phaseolus vulgaris and Glycine max) [data shown at (http://dl.dropbox.com/u/7886304/supplements/BJMBR-Souza2012/BJMBR-Souza-MSA.pdf)]. Although homologs could be detected in such diverse major groups, our results indicate a proliferation of vicilins in eudicots, which is coherent with their prominent nutritional roles in such these seeds.

The SI fraction showed strong toxicity to C. maculatus larval development. At a concentration of 0.1%, larval mass was reduced from 8.1 ± 1.7 (mass of control larvae) to 1.8 ± 0.5 mg, which corresponds to a decrease of 78% (data not shown).

Figure 4. A, Sephacryl S-100 gel filtration chromatography of the CI fraction of Albizia lebbeck seed coat previously obtained by CM-Sepharose chromatography resulting in three fractions (SI, SII, and SIII). The column was equilibrated and developed with 50 mM potassium phosphate buffer, pH 7.8. B, SDS-polyacrylamide gel electrophoresis of the SI fraction (106 to 116 mL).

Table 1. Mass spectrometry analysis of the peptides from the fraction of Albizia lebbeck seed coat obtained by Sephacryl S-100 gel filtration chromatography (SI fraction).

| Spot No. | Peptide sequence | Protein accession number and Blast bit score* |
|----------|------------------|---------------------------------------------|
|          |                  | Vigna radiata 8S globulin ABG02260 | Vigna unguiculata vicilin emb|CAP19902.1| |
| Band 1   | KQIQNLENYRV      | 36.3 | 40.1 |
|          | RIPAGTTFVLVNPNDDNLRI | 63.0 | 54.5 |
| Band 3   | RWFHTLRN         | 36.3 | 40.1 |
|          | KIPAGTTFVLVPNDDNLRI | 71.0 | 61.7 |
|          | RAILTLPNGDRD     | 41.4 | 43.9 |
|          | KQIQNLENYRV      | 36.3 | 40.1 |
|          | RGQNPYPFHDSDRW   | 45.6 | 52.8 |
|          | RLHITPEKNQRLD    | 42.6 | 52.8 |
|          | REQQQQQQQESWEVQRY | 53.2 | 62.2 |
|          | KLAIPVPNPHRF     | 40.1 | 40.1 |

*Bit scores were computed by aligning the MS peptides against the Vigna radiata 8S globulin protein ABG02260.1 (454 amino acids) and Vigna unguiculata vicilin protein (433 amino acids) using BLAST. Collectively, the peptides encompass approximately 24.7% of the V. radiata 8S protein and 25.9% of V. unguiculata vicilin. We used a 90% threshold for query coverage and 35 bit score (BLAST) to map the peptides on the vicilin and vicilin-like proteins.
Chitin-binding vicilins are toxic to the insect C. maculatus

The vicilin concentration in the SI fraction (1.4 µg/mg) was ~5.6-fold higher than the one found in the CI fraction that was 0.25 µg/mg (data not shown). In addition, proteins from the SI fraction also showed strong chitin-binding activity and, after desorption with 0.1 M HCl, were recognized by anti-vicilin antibodies (Figure 5).

Discussion

Before reaching the cotyledons, bruchid larvae must pass through the seed coat, which may pose not only a physical, but also a chemical toxic barrier to counter the penetration process (10,28). For instance, Acanthoscelides obtectus larvae were unable to penetrate P. lunatus seed coats (29) and only 50% of them penetrated P. vulgaris teguments (30). The seed coat of Vicia faba prevented the penetration of about 55% of the Callosobruchus chinensis and C. maculatus larvae (31,32).

In the present study, we demonstrated that C. maculatus oviposition, hatching and adult development were drastically reduced when the bruchids were grown on A. lebbeck seeds.

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