DOI:10.1021/jf903254b

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

# A ConA-like Lectin from *Dioclea guianensis* Benth. Has Antifungal Activity against *Colletotrichum gloeosporioides*, unlike Its Homologues, ConM and ConA

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This study reports on the antifungal activity of Dgui, a ConA-like lectin from *Dioclea guianensis* seeds. Dgui inhibited conidial germination but not mycelial growth of *Colletotrichum gloeosporioides*. The lectins ConA and ConM from *Canavalia ensiformis* and *Canavalia maritima*, respectively, share high levels of amino acid sequence similarity (>84%) with Dgui and have the same specificity toward glucose/mannose but had no effect on the fungus. Fluorescence microscopy showed that both Dgui and ConM bind to *C. gloeosporioides* ungerminated conidia. However, Dgui did not bind to *C. gloeosporioides* germinated conidia and germ tubes and was not inhibitory to mycelial growth. Because only Dgui inhibited germination of the fungus, *C. gloeosporioides* conidia might have surface-specific germination targets recognized by Dgui but not by its homologues, ConM and ConA. Therefore, Dgui is a candidate for biotechnological approaches for improving the resistance of various nutritionally and commercially important crops that are affected by *C. gloeosporioides*.

**KEYWORDS:** ***Dioclea guianensis*; Leguminosae; plant defense; lectin; antifungal protein; *Colletotrichum gloeosporioides***

## INTRODUCTION

Plants are excellent food sources for heterotrophic organisms. As such, they are constantly challenged by viruses, bacteria, fungi, and phytophagous nematodes and insects that eat plants directly as a source of macro- and micronutrients.

During domestication by humans, most cultivated plants have lost the ability to resist pathogen and pest attack. This side effect of domestication has led to the development and excessive use of biocides to protect plant crops. Unfortunately, biocides contaminate the atmosphere, soil, surface and groundwater, drinking water, plant-eating organisms, and nontarget animals. Therefore, to sustain or even improve plant productivity, there is an urgent need to discover new, environmentally friendly, bioactive defense molecules in wild plant genetic resources that could be expressed in cultivated plants.

Lectins are one type of potential candidate molecule for disease and pest controls. These proteins recognize and bind reversibly to either specific free carbohydrates or cell-wall polysaccharides and cell-membrane glycoconjugates (*1*, *2*). Binding of lectins to accessible carbohydrate residues of cell-wall targets or cell membranes triggers a variety of biological responses. Various lectins exhibit insecticidal and antifungal properties, and the insertion of some lectin genes into transgenic plants through genetic engineering has given pest and pathogen resistance to some economically important crops (*1*, *3*).

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The seeds of leguminous plants are rich sources of bioactive lectins that could serve as defensive proteins. Dgui is a well-characterized ConA-like lectin, extracted from the seeds of the leguminous plant *Dioclea guianensis* (*4*). It is a tetramer composed of four identical polypeptide chain of 237 amino acid residues that has been characterized by sequence analysis (**Figure 1**; NCBI accession number 1H9W_A), mass spectrometry, analytical ultracentrifugation equilibrium sedimentation, and X-ray crystallography (*5*). However, its fungicidal activity against phytopathogenic fungi has not been reported.

The aim of this study was to assess whether Dgui can act as a plant chemical defense weapon against two important phytopathogenic fungi, *Colletotrichum gloeosporioides* and *Fusarium solani*. It was compared with two other glucose/mannose-specific Diocleinae lectins, ConM and ConA, from the seeds of *Canavalia maritima* Thou. and *C. ensiformis* L., which have a high degree of primary structure similarity (**Figure 1**; Dgui/ConM, 84%; Dgui/ConA, 86%; ConM/ConA, 98%) (*5*, *6*).

## MATERIALS AND METHODS

**Biological Material and Reagents.** Seeds of *D. guianensis*, *C. ensiformis*, and *C. maritima* were collected from trees growing at Ceará or Rio Grande do Norte States, Brazil. Cowpea seeds (*Vigna unguiculata* cv. BR 3 Tracuateua) were from Empresa Brasileira de Pesquisa Agropecuária - Meio Norte, Piauí State, Brazil. Erythrocytes for hemagglutination assays were collected from New Zealand male albino rabbits reared in a bioterium with a standard diet at the Zootecny Department, Universidade Federal do Ceará, Brazil. The filamentous phytopathogen fungi *Colletrotrichum gloeosporioides* (URM 3114) and *Fusarium solani*

 Published on Web 03/04/2010 

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ConA            ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQNGKVGTAHIIYNSVDKR 60
ConM            ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQNGKVGTAHIIYNSVGKR 60
Dgui            ADTIVAVELDSYPNTDIGDPSYPHIGIDIKSIRSKSTARWNMQTGKVGTAHISYNSVAKR 60
                **********:********************:***.**:****.******** **** **

ConA            LSAVVSYPNADSATVSYDVDLDNVLPEWVRVGLSASTGLYKETNTILSWSFTSKLKSNST 120
ConM            LSAVVSYPNGDSATVSYDVDLDNVLPEWVRVGLSASTGLYKETNTILSWSFTSKLKSNST 120
Dgui            LSAVVSYTGSSSTTVSYDVDLNNVLPEWVRVGLSATTGLYKETNTILSWSFTSKLKTNSI 120
                *******....*:********:**************:********************:**

ConA            HETNALHFMFNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGRALFYAPVH 180
ConM            HETNALHFVFNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGRALFYAPVH 180
Dgui            ADANSLHFSFNQFSQNPKDLILQGDATTDSDGNLELTKVSSSGDPQGSSVGRALFYAPVH 180
                 ::*:*** *****:: ***********.:*******:***.*.****************

ConA            IWESSAVVASFEATFTFLIKSPDSHPADGIAFFISNIDSSIPSGSTGRLLGLFPDAN 237
ConM            IWESSAVVASFDATFTFLIKSSDSHPADGIAFFISNIDSSIPSGSTGRLLGLFPDAN 237
Dgui            IWEKSAVVASFDATFTFLIKSPDRDPADGITFFIANTDTSIPSGSGGRLLGLFPDAN 237
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**Figure 1.** Amino acid sequence alignment of Diocleinae lectins from *C. ensiformis* (ConA), *C. maritima* (ConM), and *D. guianensis* (Dgui). The following symbols denote the degree of conservation observed in each column: "∗" residues or nucleotides in the columns are identical in all sequences; ":" conserved substitutions; "." semiconserved substitutions. Accession numbers of ConA, ConM and Dgui are, respectively, NCBI ID ABW87339, NCBI ID AAB20834; and NCBI ID 1H9W_A.

(URM 3708) were from the Mycology Department of the Universidade Rural de Pernambuco, Recife, Brazil. Fungus structures were visualized with an Olympus system microscope BX 60. Photomicrographs were taken with an Olympus photomicrographics system PM 20 or with a digital camera (Sony, MCV-CD350 model, 3.2 megapixels).

$\beta$-Mercaptoethanol, Coomassie Brillant Blue R-250, and *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine (TEMED) were from Sigma Chemical Co. (St Louis, MO), sodium dodecyl sulfate (SDS) was obtained from Merck (Darmstadt, Germany), acrylamide was from Serva Feinbiochemica GmbH & Co., Sephadex G-50 was from Pharmacia Fine Chemical, Inc. (Uppsala, Sweden), and YPD medium was from Invitrogen (Carlsbad, CA). All other chemicals were of analytical grade.

**Purification of Dgui, ConA and ConM.** Dgui, ConA, and ConM were purified from mature seeds as previously described for similar lectins (*4*, *7*), with minor modifications. Dehulled seeds were ground in a coffee grinder, and 100 g of flour was extracted with 1 L of 0.15 M NaCl containing $5.0 \times 10^{-3}$ M $CaCl_2$ and $5.0 \times 10^{-3}$ M $MnCl_2$ (extraction solution). The suspension was stirred at 25 °C for 4 h, filtered through cheesecloth, and centrifuged at 10 000*g* at 5 °C for 20 min (Sorvall RC-5B). The resulting supernatant was precipitated with 80% solid ammonium sulfate for 4 h at 5 °C and centrifuged as described above. The precipitate was resuspended and dialyzed exhaustively against the extraction solution, and the mixture was centrifuged to remove undissolved protein. The clear supernatant was submitted to affinity chromatography on Sephadex G-50 (2 cm × 40 cm) equilibrated with the extraction solution, and the unbound proteins were eluted until absorbance at 280 nm was below 0.005. Elution of the bound lectin was achieved with 0.2 M D-glucose in extraction solution. The recovered lectin was dialyzed against 0.1 M AcOH overnight to remove reversibly bound D-glucose, followed by Milli-Q grade $H_2O$ for 24 h, at 10 volumes of purified lectin. After dialysis, the lectin was freeze-dried and stored at −20 °C. Lectin concentration was determined according to Bradford's (*8*) procedure. Hemagglutination was assayed using rabbit erythrocytes as described (*7*). One unit of hemagglutinating activity (HA) was defined as the reciprocal of the highest dilution of protein sample exhibiting cell hemaggluatination. The potency of D-glucose to fully inhibit hemagglutination was determined in 96-well V-shaped microtiter plates by a 2-fold serial dilution as described previously (*4*). The purity of the lectin preparations was assessed by SDS (15%)−polyacrylamide gel electrophoresis (PAGE) with $\beta$-mercaptoethanol (*9*). To assay for chitinase and $\beta$-1,3-glucanase activities, which are characteristic of genuine antifungal proteins, lectins were assayed using colloidal chitin and laminarin as substrates (*10*), respectively.

**Antifungal Assays.** *C. gloeosporioides* and *F. solani* were grown for 7 days in Petri dishes on potato dextrose agar in a development chamber (BOD) at 25 °C with a 12 h photoperiod. Fresh conidia were obtaining by gently rinsing the surface of 7-day-old sporulated cultures with sterile $H_2O$, using a triangular Drigalsky rod, and filtering through cheesecloth in a laminar flux chamber under sterile conditions. Antifungal assays were performed to verify the ability of the lectins to inhibit conidium germination and to check for their vegetative growth inhibition properties. Inhibition of conidial germination was conducted in reticulated plates with 10 $\mu$L of ConA, ConM, or Dgui (25, 50, 100, and 200 $\mu$g/mL in 0.05 M NaOAc buffer, pH 6.0, containing 0.15 M NaCl, $5.0 \times 10^{-3}$ M $CaCl_2$, and $5.0 \times 10^{-3}$ M $MnCl_2$), incubated with 10 $\mu$L of conidium suspension ($2 \times 10^5$/mL). In negative, noninhibitory controls, conidia were incubated with only Na-acetate buffer or sterile Milli-Q grade $H_2O$. Positive controls used $H_2O_2$ (0.5 M). Plates were placed into a plastic box maintained at close to 100% relative humidity at 30 °C in the dark for 24 h. Subsequently, 50 conidia were selected randomly from the treatment groups and evaluated for germination with an Olympus System microscope BX 60. Photomicrographs were taken with an Olympus Photomicrographics System PM 20 with 400 Asa Kodak color film or with a digital camera (Sony, MCV-CD350 model, 3.2 megapixels). A conidium having one hyphal strand at least twice the length of an ungerminated conidium was considered successfully germinated. Experiments were done in triplicate, and statistical analysis was done (*11*) to check for differences between the means of treatments (Tukey, $P \leq 0.05$).

To verify whether the effects of the studied lectins on fungal germination were related to fungal structure binding, the lectins were pretreated with 0.2 M D-glucose for 30 min in 0.05 M Na-acetate buffer, pH 6.0, containing 0.15 M NaCl, $5.0 \times 10^{-3}$ M $CaCl_2$, and $5.0 \times 10^{-3}$ M $MnCl_2$, and incubated with the fungi as described above. As reported earlier, D-glucose fully inhibits the hemagglutination activity of red blood cells induced by the studied lectins (*4*, *7*).

To check whether the antifungal effect of Dgui on conidial germination could be observed in a natural environment (*in situ*), *C. gloeosporioides* conidia were inoculated in the presence of Dgui on fully expanded primary leaves of a susceptible cowpea (cv. BR 3 Tracuateua) (*12*) as follows. Cowpea seeds were surface-sterilized with 1% (v/v) active NaOCl for 3 min, washed exhaustively, and imbibed (10 min) in distilled $H_2O$. Seeds were germinated (five seeds per pot) and grown in plastic pots (1 L capacity) containing river sand washed exhaustively with tap water and autoclaved. Seedlings were maintained under greenhouse conditions, 27−35 °C, 40−60% relative humidity (RH), exposed to natural light, irrigated daily with autoclaved distilled $H_2O$ for 3 days after emergence and subsequently at intervals of 3 days, with 10-, 5-, and 2-fold-diluted Hoagland and Arnon nutritive solution as modified by Silveira et al. (*13*). Thereafter, undiluted nutritive solution was used until the end of the experimental period. Twelve days after planting, when the primary leaves were expanded completely, healthy seedlings were transferred to a growth chamber at 25 °C dark, 30 °C light, 65% ± 5% RH, with a 12 h photoperiod and a light intensity of 200 $\mu$mol/(m·s). Two 25 $\mu$L drops

of *C. gloeosporioides* conidium suspension ($2 \times 10^5$/mL) were placed on the adaxial surface of primary leaves, on either side of the central vein, and 25 $\mu$L of Dgui (25 $\mu$g/mL and 100 $\mu$g/mL of the extraction solution) was added to each of the four conidium drops. Controls were the extraction solution and distilled $H_2O$. After inoculation, cowpea seedlings were maintained at 80% ± 5% RH to avoid evaporation. At 12 h intervals, beginning 60 h after inoculation, the primary leaves were excised, depigmented with $Cl_3CO_2H$ (1.5 g in 750 mL ethanol and 250 mL chloroform), and treated with lactophenol aniline blue (*14*, *15*) to stain the fungal structures. Fungal germination and development on cowpea leaves in the presence of Dgui were observed and photographed microscopically as described above. To test the feasibility of Dgui transgenic plants developed to express Dgui in the leaf apoplastic space, a 1-mL needleless plastic disposable syringe was used to pressure-infiltrate the intercellular space of fully expanded detached cowpea primary leaves of a susceptible cultivar (BR 3 Tracuateua) with Dgui solution (200 $\mu$g/mL prepared in the extraction solution), followed immediately by *C. gloeosporioides* conidia ($2 \times 10^5$/mL) deposition on the leaves at the diffusely infiltrated areas. Leaves were kept in Petri dishes over a filter-paper wetted with sterile $H_2O$ to provide 100% RH, in the dark, at 37 °C. Conidial development was examined microscopically, and micrographs were taken as described above after leaf depigmentation with $Cl_3CO_2H$ and treatment with lactophenol aniline blue (*14*, *15*).

To assess the ability of the lectins to inhibit fungal vegetative growth, 10 $\mu$L of *C. gloeosporioides* and *F. solani* conidium suspensions ($2 \times 10^5$/mL) were placed in flat-bottomed 96-well microplates (Nunc, Denmark) containing 100 $\mu$L of liquid YPD medium and allowed to germinate for 12 h, at 30 °C, in the dark. Next, 90 $\mu$L of Dgui, ConA, or ConM (25, 50, 100, and 200 $\mu$g/mL in 0.05 M NaOAc buffer, pH 6.0, containing 0.15 M NaCl, $5.0 \times 10^{-3}$ M $CaCl_2$ and $5.0 \times 10^{-3}$ M $MnCl_2$) was added to each well and after incubation for 12, 24, 36, 48, and 72 h, mycelial growth was monitored by turbidimetry at 630 nm using an automated microplate reader (model Elx800, Bio-Tek Instruments, Inc., Winooski, VT). The assay was also done with lectins incubated previously for 30 min with 0.2 M D-glucose and with lectins denatured by boiling at 98 °C, for 30 min. In parallel, conidia were incubated with either NaOAc buffer or sterile $H_2O$ as negative controls or $H_2O_2$ (0.25 and 0.5 M) as positive control. Experiments were done in triplicate, and statistical analysis (ANOVA) was performed (*11*).

**Conjugation of Lectins with Fluorescein Isothiocyanate (FITC).** Fluorescein-labeled Dgui (FITC-Dgui) and ConM (FITC-ConM) were prepared as described (*16*) with modifications. Purified lectin (10 mg) was dissolved in 1 mL of conjugation buffer (0.5 M carbonate/bicarbonate buffer, pH 9.5), and 3.0 $\mu$g of FITC (10% dispersed in Celite) was added under gentle stirring. The mixture was incubated in the dark at 25 °C for 1.5 h and centrifuged at 14 000*g* for 10 min at 4 °C (Eppendorf MSE Micro Centaur). The supernatant (FITC-lectin + free FITC) was loaded onto a Sephadex G-25 column (HiTrap Desalting, 5 mL, Amersham Pharmacia Biotech) previously equilibrated and eluted with 0.15 M NaCl containing 0.2 M D-glucose, to avoid lectin association with the column matrix. The faster-eluting FITC-lectin complex was dialyzed exhaustively against 0.5 M AcOH to remove noncovalently bound D-glucose and then against Milli-Q grade $H_2O$ followed by centrifugation at 14 000*g* for 10 min at 4 °C. The efficiency of FITC-lectin coupling was evaluated by the ratio between the absorbance at 495 nm and that at 280 nm, for FITC and the lectin, respectively (*16*).

**Interaction of FITC-Dgui and FITC-ConM with Conidia.** FITC-conjugated lectins were used to determine whether the inhibitory effect of Dgui on the conidium germination of *C. gloeosporioides* was associated with binding to specific conidial sites. FITC-Dgui, FITC-ConM, and their nonfluorescent forms, dissolved in 0.05 M Na-acetate buffer, pH 6.0, containing 0.15 M NaCl, $5.0 \times 10^{-3}$ M $CaCl_2$, and $5.0 \times 10^{-3}$ M $MnCl_2$, were incubated with conidia ($2 \times 10^5$/mL) for 1 h in reticulated plates, in triplicate, according to the following protocols: (A) FITC-ConM (100 $\mu$g/mL) + conidia; (B) FITC-Dgui (100 $\mu$g/mL) + conidia; (C) unlabeled ConM (100 $\mu$g/mL) preincubated with conidia followed by FITC-Dgui (100 $\mu$g/mL); (D) unlabeled Dgui (100 $\mu$g/mL) preincubated with conidia followed by FITC-Dgui (100 $\mu$g/mL); (E) unlabeled Dgui (50 $\mu$g/mL) + unlabeled ConM (50 $\mu$g/mL) preincubated with conidia followed by FITC-Dgui (100 $\mu$g/mL); (F) unlabeled Dgui (100 $\mu$g/mL) preincubated with conidia followed by FITC-ConM (100 $\mu$g/mL); and (G) control of conidia without lectins. The above protocols were also followed with conidia that had germinated for 12 h before lectin incubation. After treatment for 0, 12, and 24 h, conidia in the same field were observed by transmission light microscopy and epifluorescence microscopy (Olympus System microscope BX 60) with the barrier filter set for green. Photomicrographs were taken as described above.

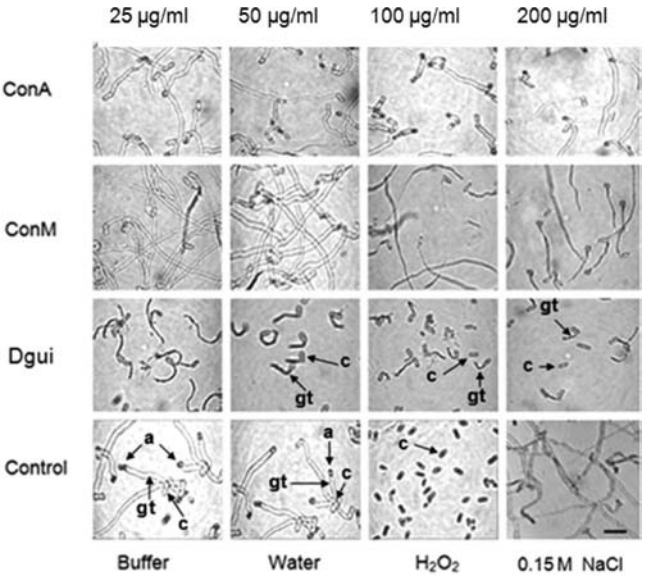


**Figure 2.** In culture effects of Dgui, ConA, and ConM on the conidium germination of *C. gloeosporioides*, after incubation for 24 h. Negative controls: sterile $H_2O$, acetate buffer, and 0.15 M NaCl. Positive control: 0.50 M $H_2O_2$. Scale bar = 20 $\mu$m (light microscopy, magnification 400×). Arrows indicate (a) appressorium, (c) conidium, and (gt) germ tube.

**Treatment of Conidia with Proteases.** To identify the possible glycoprotein nature of *C. gloeosporioides* conidial surface binding sites, conidia were subjected to enzymatic treatment with pepsin, trypsin, and chymotrypsin. Pepsin was dissolved in 0.1 M HCl, pH 1.5, and trypsin and chymotrypsin were dissolved in 0.1 M Tris−HCl, pH 8.1, each at a final concentration of 0.2 $\mu$g/mL. Fungal cultivation and inoculum preparation were performed as described above. *C. gloeosporioides* conidia ($2 \times 10^5$/mL) were incubated in triplicate for 3 h at 37 °C with each one of the proteases. Controls were untreated. After treatment, suspensions were washed exhaustively by centrifugation (15 000*g* for 20 min) to fully remove the proteases. Protease-treated conidia ($2 \times 10^5$/mL) were incubated with lectins for 1 h according to the following protocols: (A) FITC-ConM (100 $\mu$g/mL) + protease-treated conidia; (B) FITC-Dgui (100 $\mu$g/mL) + protease-treated conidia; (C) ConM (100 $\mu$g/mL) + protease-treated conidia followed by incubation with FITC-Dgui (100 $\mu$g/mL); and (D) controls of protease-pretreated conidia without lectins. After treatment for 0, 12, and 24 h, conidia were observed as described above.

## RESULTS AND DISCUSSION

**Lectin Purification.** The SDS-PAGE patterns of the seed lectins Dgui, ConA, and ConM have subunit structures characteristic of lectins from subtribe Diocleinae seeds (data not shown) (*4*, *7*). They migrated as three protein bands consisting of the full-length intact polypeptide chain (α-chain) and two fragments, $\beta$ and $\gamma$, that result from post-translational modification of the 237-residue polypeptide protein (*17*). They were purified to homogeneity and did not show either chitinase or $\beta$-1,3-glucanase activity, indicating that they were not contaminated with these antifungal proteins. Moreover, Dgui, ConA, and ConM showed hemagglutinating activity (228, 819, and 579 HA/mg protein, respectively) against rabbit erythrocytes and were fully inhibited by 0.2 M D-glucose.

**Effects of Dgui, ConA, and ConM on in Culture Germination of *C. gloeosporioides* Conidia.** Interestingly, although Dgui, ConA,

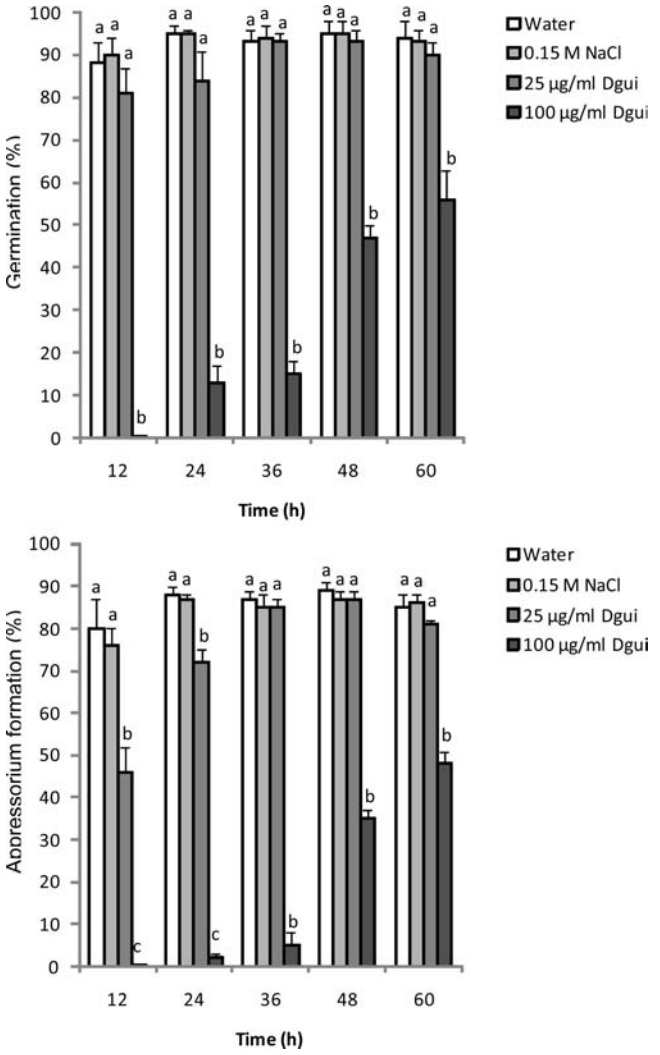


**Figure 3.** In culture germination and appressorium formation by *C. gloeosporioides* conidia in the presence of Dgui. Sterile $H_2O$ and the extraction solution were used as negative controls. Different letters at each time point denote statistical differences by Tukey test ($P \leq 0.05$) among treatments. Bars indicate the standard deviation ($n = 150$).

and ConM are all mannose/glucose-specific lectins, and ConM (NCBI ID AAB20834) and Dgui (NCBI ID 1H9W_A) have 90% and 86% protein sequence similarity (**Figure 1**) with ConA (NCBI ID ABW87339), respectively (*5*, *18*), only Dgui effectively inhibited *C. gloeosporioides* conidium germination at concentrations >50 $\mu$g/mL (**Figure 2**). The inhibitory effect was abolished either in the presence of 0.2 M D-glucose or when Dgui was denatured by boiling (data not shown). In a time-course experiment, Dgui caused a delay in both *C. gloeosporioides* conidium germination and appressorium formation (**Figure 3**), from 12 to 60 h, after incubation with 100 $\mu$g/mL of Dgui. Such a delay of germination/growth may allow the host to trigger other defense mechanisms to avoid pathogen infection. Plant defense mechanisms such as hypersensitive response (HR) and systemic acquired resistance (SAR) are induced after recognition of specific pathogen-derived molecules, called avirulence (Avr) proteins, to restrict pathogen proliferation (*19*).

None of the three lectins tested showed any antifungal effect on *F. solani*. The ability of Dgui, but not ConA or ConM, to specifically inhibit *C. gloeosporioides* germination might be because of differential specificities of these related lectins toward complex carbohydrates and glycoproteins (*20*). Interestingly, the carbohydrate-binding sites of these lectins are exactly the same, with the amino acids $Tyr^{12}$, $Asn^{14}$, $Thr^{15}$, $Asp^{16}$, $Leu^{99}$, $Tyr^{100}$, $Asp^{208}$, and $Arg^{228}$ (**Figure 1**) participating in the establishment of hydrogen bonds, and $Tyr^{12}$, $Pro^{13}$, $Asn^{14}$, $Thr^{15}$, $Asp^{16}$, $Gly^{98}$, $Leu^{99}$, $Tyr^{100}$, $Ala^{207}$, $Asp^{208}$, $Gly^{227}$, and $Arg^{228}$ involved in van der Waals interactions with carbohydrates (*6*, *20*, *21*). However, despite these common biochemical and structural features, subtle differences are present at the carbohydrate-recognition region of the molecules regarding atomic distances involved in the hydrogen bond and van der Waals interactions between lectins and the carbohydrates (*6*). For example, crystallographic studies showed that the relative distances between $Tyr^{100}$ and $Arg^{228}$ are 13.00 and 13.70 Å, and between $Tyr^{12}$ and $Gly^{227}$ are 9.58 and 8.97 Å, in ConA and ConM, respectively (*6*). The authors concluded that although only five amino acid residues differ between ConA and ConM, the most relevant was the substitution of $Pro^{202}$ to $Ser^{202}$ in the ConM structure, which led to three-dimensional alterations in the carbohydrate-binding sites, with this residue promoting the approximation of $Tyr^{12}$ to the carbohydrate-binding site. This was supported by crystal structure studies of ConM complexed with trehalose and maltose, which revealed significant differences in the position of H-bonds (*22*). While the O-6′ of the second glucose ring in maltose interacts with $Tyr^{12}$, in trehalose the interaction is established by O-2′ and $Tyr^{12}$, explaining the higher affinity of ConM for disaccharides over monosaccharides. Dam et al. (*21*) reported significant differences in the inhibitory potencies of various monosaccharides, disaccharides, and the trimannoside 3,6-di-*O*-(α-D-mannopyranosyl)-D-mannose, which is present in the core of all asparagine-linked carbohydrates (*23*), and its deoxy analogues, on hemagglutination of rabbit erythrocytes by Dgui, ConA, ConM, and other Diocleinae lectins. Moreover, differential ability of Leguminosae lectins from members of the Diocleinae subtribe to stimulate histamine release from rat peritoneal mast cells, correlated with differential specificities against a biantennary complex carbohydrate with terminal GlcNAc residues. Ramos et al. (*20*) used surface plasmon resonance technology to show differences in fine glycan specificity for Diocleinae lectins toward various glycoproteins (*Phaseolus vulgaris* lectin E, soybean agglutinin, arcelin-1, hen ovalbumin, orosomucoid or acid α-glycoprotein, ovomucoid, bovine lactotransferrin, and human serotransferrin). They concluded that ConBr (*Canavalia brasiliensis* agglutinin) is the most reactive lectin, while ConA or ConM often react more weakly with the above glycoproteins. Furthermore, Ramos et al. (*20*) speculate that in addition to the residues that constitute the lectin carbohydrate-binding site, neighboring residues at the surface of the lectins form an extended carbohydrate-binding site. According to Young and Oomen (*24*) the replacement of a few of these residues could lead to differences in glycan-binding specificities and differences in the biological properties induced by these closely related plant lectins.

Recently, a comparative crystallographic study of ConM (PDB code 2CWM) and CGL (a lectin from *Canavalia gladiata*; PDB code 1WUV) complexed with the disaccharides Man(α1−2)Man(α1−*O*)Me, Man(α1−3)Man(α1−*O*)Me and Man(α1−4)Man(α1−*O*)Me under different conditions revealed that the binding modes of these two lectins with the dimannosides present different interaction patterns. This may account for a structural explanation of the distinct biological properties observed in the lectins of Diocleinae subtribe (*25*). Based on the above data, different spacings between the carbohydrate-binding sites of closely related Diocleinae lectin tetramers combined with different distances between equivalent epitopes on multivalent ligands are hypothesized to modulate their ability to cross-link and aggregate specific glycoprotein and glycolipid receptors on the same or different cell types. This is proposed to lead to distinct capabilities to trigger cellular responses (*6*).

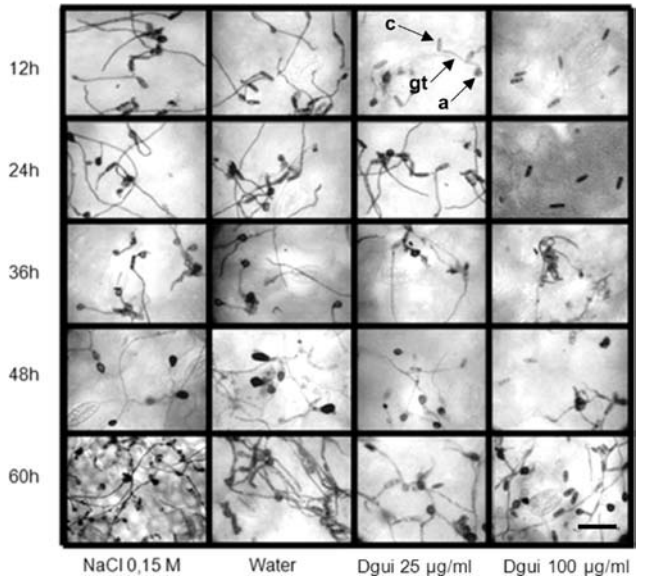


**Figure 4.** *In situ* germination and development of *C. gloeosporioides* on cowpea (cv. BR3 Tracuateua) leaves incubated with Dgui. Negative controls: sterile $H_2O$ and 0.15 M NaCl. Scale bar = 20 $\mu$m (light microscopy magnification, 400×). Arrows indicate (a) appressorium, (c) conidium, and (gt) germ tube.

Besides the fine variations in the tridimensional structures of the Diocleinae lectins, the specificity of Dgui to inhibit *C. gloeosporioides* germination but not *F. solani* might depend on the absence of proper Dgui glycoconjugate targets in the fungal cell wall or cellular membrane of this latter fungus, inaccessibility of the lectin to the proper fungus target, or even subtle structural differences in the surface glycoconjugates of these fungi. Slight modifications in the sequences of these lectins or subtle differences in the distances of the atoms involved in hydrogen bond and van der Waals interactions between the lectins and the carbohydrates may also confer distinct biological properties to these molecules (*26*).

**Effects of Dgui on Germination of *C. gloeosporioides* Conidia on Cowpea Leaves.** Dgui was the only lectin tested, since it was the only one of the three evaluated that exhibited antifungal properties against *C. gloeosporioides in vitro*. The extent of fungal germination inhibition by Dgui *in situ* was essentially the same as that observed *in vitro*. At a concentration of 25 $\mu$g/mL of Dgui, conidium germination and apressorium formation on cowpea leaves were impaired slightly, whereas at 100 $\mu$g/mL, strong inhibition of both parameters occurred compared with controls (**Figure 4**). A similar antifungal property was observed for wheat-germ agglutinin (WGA). A conidial suspension pretreated with WGA lost its infection potential on freshly cut potato tuber disks compared with untreated conidia, which caused profound necrosis of potato tuber tissue (*27*). Thus, seed lectins may serve as fungistatic agents in the plant kingdom, particularly during seed imbibition, germination, and early growth of seedlings, when they are most vulnerable to pathogen attacks. For instance, it was demonstrated that *Luetzelburgia auriculata* lectin, which is inhibitory to the mycelial mass development of *Colletotrichum lindemuthianum*, *F. solani*, and *Aspergillus niger*, is released from seeds upon imbibition (*28*).

The assay in which Dgui was infiltrated on the epidermal surface of fully expanded detached cowpea primary leaves of a susceptible cultivar (BR 3 Tracuateua), followed by inoculation of *C. gloeosporioides* ungerminated conidia, showed no detrimental effect on fungal development, compared with controls

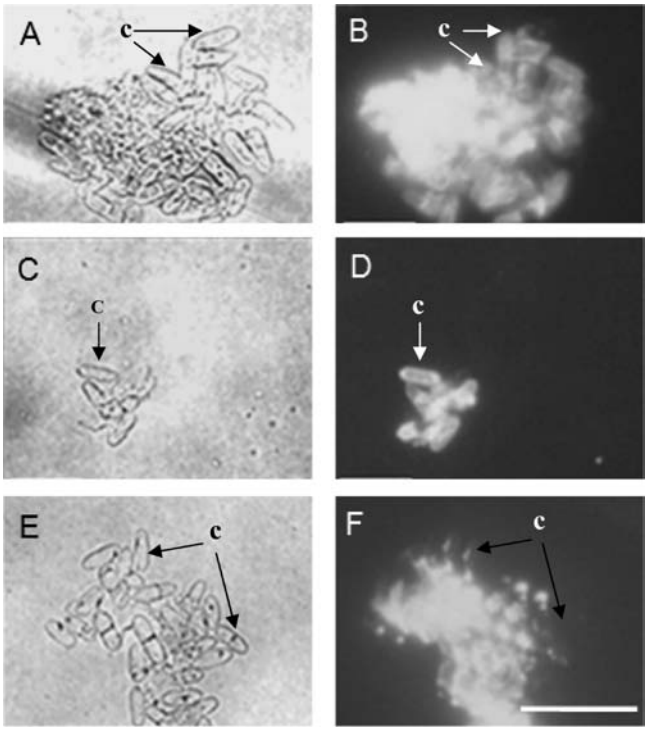


**Figure 5.** Light (**A**, **C**, **E**) and fluorescence (**B**, **D**, **F**) microscopy of *C. gloeosporioides* ungerminated conidia incubated for 1 h with 100 $\mu$g/mL FITC-ConM (**A**, **B**), 100 $\mu$g/mL FITC-Dgui (**C**, **D**), and 100 $\mu$g/mL unlabeled ConM followed by 100 $\mu$g/mL FITC-Dgui (**E**, **F**) for an additional 1 h. Paired pictures from the same treatment were taken from the same field of view. Scale bar = 20 $\mu$m (light microscopy, magnification 400×). Arrows indicate (c) conidium.

(data not shown). Indeed, conidia adhered to the leaf cuticle and germinated to produce germ tubes that differentiated a melanized appressorium from which narrow penetration pegs emerged, characteristic of normal vegetative growth. At least four factors, alone or in combination, may have hampered the inhibitory action of leaf-infiltrated Dgui on fungal germination: the small amount of Dgui in the infiltrated zones resulting from its diffusion into the surrounding areas; degradation of Dgui by leaf enzymes; Dgui inhibition by free haptenic sugars (glucose/mannose); binding of Dgui to polysaccharides or glycoconjugates bearing glucose/mannose in the leaf structure.

**Effects of Dgui, ConA, and ConM on Vegetative Growth of *C. gloeosporioides* in Culture.** No inhibitory effect on the vegetative (mycelial) growth of *C. gloeosporioides* or *F. solani* was observed when the germinated conidia were incubated with Dgui, ConA, or ConM. Since Dgui inhibited *C. gloeosporioides* conidia germination, the lack of inhibitory activity on mycelial growth strongly suggests modification of the fungal cell wall or cellular membrane composition in the course of fungal development that makes Dgui-target glycoconjugates absent or inaccessible. These findings highlight the importance of the different fine specificities of the above-mentioned lectins, which possess the same specificity toward simple sugars.

**Interaction of Fluorescein-Labeled Dgui (FITC-Dgui) and ConM (FITC-ConM) with *C. gloeosporioides*.** To study the mechanism of action of Dgui during inhibition of fungal germination, *C. gloeosporioides* conidia were incubated with 100 $\mu$g/mL FITC-Dgui or FITC-ConM. Interestingly, fluorescence was observed on the ungerminated conidia incubated with FITC-ConM or FITC-Dgui (**Figure 5**), indicating that they both bind to the fungal ungerminated conidium structure. The same results were

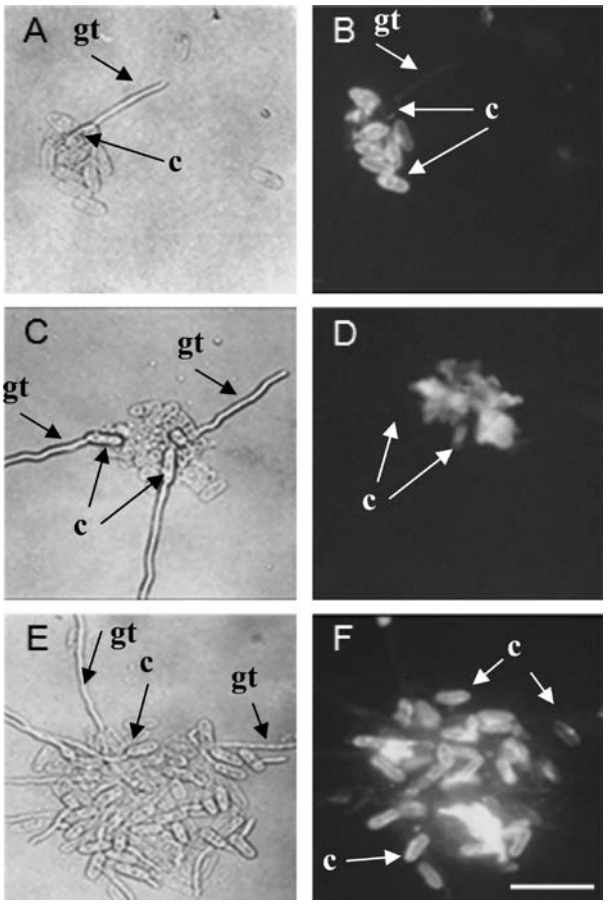


**Figure 6.** Light (**A**, **C**, **E**) and fluorescence (**B**, **D**, **F**) microscopy of 12 h germinated conidia of *C. gloeosporioides* incubated for 1 h with 100 $\mu$g/mL FITC-ConM (**A**), 100 $\mu$g/mL FITC-Dgui (**C**), and 100 $\mu$g/mL unlabeled ConM followed by 100 $\mu$g/mL FITC-Dgui (**E**) for an additional 1 h. Paired pictures of the same treatment were taken from the same field of view. Scale bar = 20 $\mu$m (light microscopy, magnification 400×). Arrows indicate (c) conidium and (gt) germ tube.

obtained with 200 $\mu$g/mL lectin (data not shown). However, because only Dgui was inhibitory to conidium germination, Dgui may attach to specific target(s) on the fungus that are not recognized by ConM, leading to inhibition of conidium germination. These results are consistent with the hypothesis that the glycoconjugates on the conidium structure to which ConM binds are not involved in *C. gloeosporioides* germination. Remarkably, fluorescence was observed when ungerminated conidia were first incubated with unlabeled ConM followed by incubation with FITC-Dgui (**Figure 5**), demonstrating that FITC-Dgui was able to recognize and bind to specific targets on the conidia that were not recognized by ConM. In contrast, no fluorescence was observed when ungerminated conidia of *C. gloeosporioides* were first incubated with unlabeled Dgui followed by incubation with FITC-ConM (data not shown), suggesting that Dgui binds receptors recognized by ConM in addition to Dgui-specific ones or provokes steric hindrance that renders the receptors inaccessible to ConM. Thus, the binding of Dgui to specific targets of ungerminated conidia of *C. gloeosporioides* may lead to inhibition of conidium germination. Because ConM does not recognize Dgui targets, it does not inhibit fungal germination. Not surprisingly, no fluorescence was observed when ungerminated conidia were first incubated with unlabeled ConM followed by incubation with FITC-ConM (data not shown).

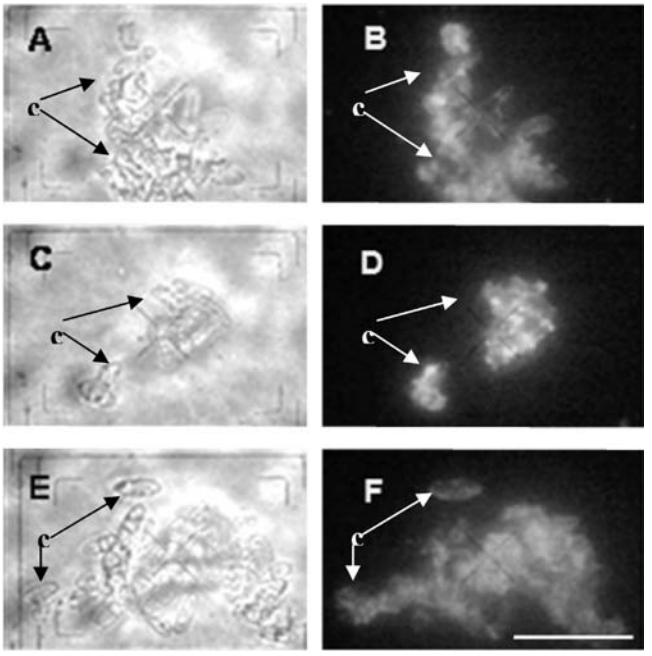


**Figure 7.** Light (**A**, **C**, and **E**) and fluorescence (**B**, **D**, and **F**) microscopy of ungerminated conidia of *C. gloeosporioides* after treatment with pepsin followed by incubation for 1 h with 100 $\mu$g/mL FITC-ConM (**A** and **B**), 100 $\mu$g/mL FITC-Dgui (**C** and **D**), and 100 $\mu$g/mL of unlabeled ConM followed by 100 $\mu$g/mL FITC-Dgui (**E** and **F**) for an additional 1 h. Paired pictures of the same treatment were taken from the same field of view. Scale bar = 20 $\mu$m (light microscopy, magnification 400×). Arrows indicate (c) conidium.

The lack of fluorescence on the germinating conidia and corresponding germ tubes of *C. gloeosporioides* at 12 h (**Figure 6**) and 24 h (figure not shown) suggests the absence or inaccessibility of receptors to Dgui and ConM on these fungal structure. This explains why neither Dgui nor ConM inhibited the vegetative (mycelial) growth of *C. gloeosporioides* (data not shown).

**Fungus Treatment with Proteases.** To assess the chemical nature of the lectin receptors on ungerminated conidia of *C. gloeosporioides*, conidia were treated with pepsin, trypsin, or chymotrypsin, followed by incubation with FITC-Dgui and FITC-ConM. Fluorescence was observed after incubation of the protease-treated conidia with both FITC-labeled lectins, regardless of protease (**Figure 7** shows representative pepsin). Dgui and ConM were still able to bind fungal receptors after enzyme treatment, suggesting that the lectin receptors are not glycoprotein in nature, or else they are refractory to the action of pepsin, trypsin, and chymotrypsin. Dgui target receptors may be polysaccharides or glycolipids, which are not substrates for the proteases used in the assay.

In conclusion, Dgui is unlike ConA and ConM with regard to its antifungal activity toward *C. gloeosporioides*. Although these lectins share similar specificity for simple sugars (mannose/glucose) and have high levels of amino acid sequence similarity (> 84%), only Dgui is able to inhibit the germination of *C. gloeosporioides* conidia. The Diocleinae lectins, despite their phylogenetic proximity and conserved primary amino acid sequences, possess different biological activities, such as lymphocyte proliferation and interferon-$\gamma$ production (*29*), and stimulation of peritoneal macrophages and the inflammatory reaction (*30*). These differences in biological activity are probably because Diocleinae lectins have conserved binding sites for the core trimannoside of asparagine-linked oligosaccharides (*21*), but differential specificities for complex carbohydrates (*24*). Experimental evidence from this work shows that specific targets of the *C. gloeosporioides* surface are recognized and bound by Dgui, but

not by ConM and ConA. Thus, in addition to the possibility of using Dgui in biotechnological approaches to improve the resistance of crops to *C. gloeosporioides*, it might be employed as a tool for purifying the target surface receptor(s) of its conidia, to shed light on the mechanisms of fungal germination and development. In the case of development of Dgui transgenic plants, studies will have to include an estimate of the effectiveness of the lectin expression toward avoiding infection by *C. gloeosporioides* and the safety of its use as food, because some legume lectins are antinutritional factors, even taking into consideration that most of the legume grains are cooked before consumption.

## LITERATURE CITED


(1) De Hoff, P. L.; Brill, L. M.; Hirsch, A. M. Plant lectins: The ties that bind in root symbiosis and plant defense. *Mol. Genet. Genomics* **2009**, *282*, 1–15.

(2) Sharon, N. Lectins: Carbohydrate-specific reagents and biological recognition molecules. *J. Biol. Chem.* **2007**, *282*, 2753–2764.

(3) Vasconcelos, I. M.; Oliveira, J. T. A. Antinutritional properties of plant lectins. *Toxicon* **2004**, *44*, 385–403.

(4) Vasconcelos, I. M.; Cavada, B. S.; Moreira, R. A.; Oliveira, J. T. A. Purification and partial characterization of a lectin from the seeds of *Dioclea guianensis*. *J. Food Biochem.* **1991**, *15*, 137–154.

(5) Calvete, J. J.; Thole, H. H.; Raida, M.; Urbanke, C.; Romero, A.; Grangeiro, T. B.; Ramos, M. V.; Almeida-da-Rocha, I. M.; Guimarães, F. N.; Cavada, B. S. Molecular characterization and crystallization of Diocleinae lectins. *Biochim. Biophys. Acta* **1999**, *1430*, 367–375.

(6) Gadelha, C. A. A.; Moreno, F. B. M. B.; Santi-Gadelha, T.; Cajazeiras, J. B.; Rocha, B. A. M.; Assreuy, A. M. S.; Mota, M. R. L.; Pinto, N. V.; Meireles, A. V. P.; Borges, J. C.; Freitas, B. T.; Canduri, F.; Souza, E. P.; Delatorre, P.; Criddle, D. N.; Azevedo, W. F., Jr.; Cavada, B. S. Native crystal structure of a nitric oxide-releasing lectin from the seeds of *Canavalia maritime*. *J. Struct. Biol.* **2005**, *152*, 185–194.

(7) Moreira, R. A.; Cavada, B. S. Lectin from *Canavalia brasiliensis* Mart. Isolation, characterization and behavior during germination. *Biol. Plant.* **1984**, *26*, 113–120.

(8) Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

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

(10) Boller, T. Biochemical analysis of chitinases and $\beta$-1,3-glucanases. In *Molecular Plant Pathology A Pratical Approach*; Gurr, S. J., McPherson, M. J., Bowles, D. J., Eds.; IRL Press: New York, 1992; pp 23−30.

(11) Johnston, J. Further topics in the k-variable linear model. In *Economics Methods*; Johnston, J. Ed.; McGraw-Hill: Singapore, 1987; pp 204−259.

(12) Barreto, A. L. H.; Vasconcelos, I. M.; Grangeiro, T. B.; Melo, V. M. M.; Matos, T. E.; Eloy, Y. R. G.; Fernandes, C. F.; Torres, D. C.; Freire-Filho, F. R.; Freire, F. C. O.; Oliveira, J. T. A. Infection process and host defense responses in compatible and incompatible interactions between cowpea [*Vigna unguiculata*] and *Colletotrichum gloeosporioides*. *Int. J. Plant Sci.* **2007**, *168*, 193–211.

(13) Silveira, J. A. G.; Melo, A. R. B.; Viegas, R. A.; Oliveira, J. T. A. Salinity-induced effects on nitrogen assimilation related to growth in cowpea plants. *Environ. Exp. Bot.* **2001**, *46*, 171–179.

(14) Ballows, A.; Hausler, W. J.; Herrman, K. L. *Manual of Clinical Microbiology*, 5th ed.; American Society of Microbiologists: Washington, DC, 1991.

(15) Silva, M. C.; Nicole, M.; Guerra-Guimarães, L.; Rodrigues, C. J., Jr. Hypersensitive cell death and post-haustorial defence responses arrest the orange rust (*Hemileia vastatrix*) growth in resistant coffee leaves. . *Physiol. Mol. Plant Pathol.* **2002**, *60*, 169–183.

(16) Howard, G. C. *Methods in Nonradioactive Detection*, 1st ed. Simon and Schuster Business and Professional Group, Norwalk, CT, 1993.

(17) Wang, J. L.; Cunningham, B. A.; Edelman, G. M. Unusual fragments in the subunit structure of concanavalin A. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1130–1134.

(18) Perez, G.; Perez, C.; Cavada, B. S.; Moreira, R. A.; Richardson, M. Comparison of the amino acid sequences of the lectins from seeds of *Dioclea lehmanni* and *Canavalia maritima*. *Phytochemistry* **1991**, *30*, 2619–2621.

(19) van Ooijen, G.; Mayr, G.; Kasiem, M. M. A.; Albrecht, M.; Cornelissen, B. J. C.; Takken, F. L. W. Structure−function analysis of the NB-ARC domain of plant disease resistance proteins. *J. Exp. Bot.* **2008**, *59*, 1383–1397.

(20) Ramos, M. V.; Cavada, B. S.; Mazard, A. M.; Rougè, P. Interaction of Diocleinae lectins with glycoproteins based in surface plasmon resonance. *Mem. Inst. Oswaldo Cruz* **2002**, *97*, 275–279.

(21) Dam, T. K.; Cavada, B. S.; Grangeiro, T. B.; Santos, C. F.; Sousa, F. A. M.; Oscarson, S.; Brewer, F. Diocleinae lectins: A group of proteins with conserved binding sites for the core trimannoside of asparagine-linked oligosaccharide and differential specificities for complex carbohydrates. *J. Biol. Chem.* **1998**, *273*, 12082–12088.

(22) Delatorre, P.; Rocha, B. A. M.; Gadelha, C. A. A.; Santi-Gadelha, T.; Cajazeiras, J. B.; Souza, E. P.; Nascimento, K. S.; Freire, V. N.; Sampaio, A. H.; Azevedo, W. F., Jr.; Cavada, B. S. Crystal structure of a lectin from *Canavalia maritima* (ConM) in complex with trehalose and maltose reveals relevant mutation in ConA-like lectins. *J. Struct. Biol.* **2006**, *154*, 280–286.

(23) Gupta, D.; Dam, T. K.; Oscarson, S.; Brewer, F. Thermodynamics of lectin-carbohydrate interactions. *J. Biol. Chem.* **1997**, *272*, 6388–6392.

(24) Young, N. M.; Oomen, R. P. Analysis of sequence variation among legume lectins. A ring of hypervariable residues forms the perimeter of the carbohydrate-binding site. *J. Mol. Biol.* **1992**, *228*, 924–934.

(25) Bezerra, G. A.; Oliveira, T. M.; Moreno, F. B. M. B.; Souza, E. P.; Rocha, B. A. M.; Benevides, R. G.; Delatorre, P.; Azevedo, W. F., Jr.; Cavada, B. S. Structural analysis of *Canavalia maritima* and *Canavalia gladiata* lectins complexed with different dimannosides: new insights into the understanding of the structure-biological activity relationship in legume lectins. *J. Struct. Biol.* **2007**, *160*, 168–176.

(26) Sanz-Aparício, J.; Hermoso, J.; Grangeiro, T. B.; Calvete, J. J.; Cavada, B. S. The crystal structure of *Canavalia brasiliensis* lectin suggests a correlation between its quaternary conformation and its distinct biological properties from concanavalin A. *FEBS Lett.* **1997**, *405*, 114–118.

(27) Ciopraga, J.; Gozia, O.; Tudor, R.; Brezuica, L.; Doyle, R. J. *Fusarium* sp. growth inhibition by wheat germ agglutinin. *Biochim. Biophys. Acta* **1999**, *1428*, 424–432.

(28) Melo, V. M. M.; Vasconcelos, I. M.; Gomes, V. M.; Da Cunha, M.; Soares, A. A.; Oliveira, J. T. A. A cotyledonary agglutinin from *Luetzelburgia auriculata* inhibits the fungal growth of *Colletotrichum lindemuthianum*, *Fusarium solani* and *Aspergillus niger* and impairs glucose-stimulated acidification of the incubation medium by *Saccharomyces cerevisiae* cells. *Plant Sci.* **2005**, *169*, 629–639.

(29) Barral-Netto, M.; Santos, S. B.; Barral, A.; Moreira, L. I. M.; Santos, C. F.; Moreira, R. A.; Oliveira, J. T. A.; Cavada, B. S. Human lymphocyte stimulation by legume lectins of the Diocleinae subtribe. *Immunol. Invest.* **1992**, *21*, 297–303.

(30) Rodrigues, D.; Cavada, B. S.; Oliveira, J. T. A.; Moreira, R. A.; Russo, M. Differences in macrophage stimulation and leukocyte accumulation in response to intraperitoneal administration of glucose/mannose binding plant lectins. *Braz. J. Med. Biol. Res.* **1992**, *25*, 823–826.


**Received for review September 15, 2009. Revised manuscript received November 27, 2009. Accepted December 9, 2009. This work was supported by grants from the Brazilian agencies CNPq, CAPES/PROCAD, and FUNCAP. J.H.A.-F. was supported by a master's studentship from FUNCAP.**