Vicilin Storage Proteins from *Vigna unguiculata* (Legume) Seeds Inhibit Fungal Growth

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Vicilin storage proteins (7S globulins) isolated from *Vigna unguiculata* (cowpea) seeds were shown to interfere with the germination of spores or conidia of the fungi *Fusarium solani*, *Fusarium oxysporum*, *Colletotrichum musae*, *Phytophtora capsici*, *Neurospora crassa*, and *Ustilago maydis* sporidia. Cowpea vicilins have been shown to bind to fungal structures, possibly chitin-containing structures of the cell wall, and can be desorbed by strong acid. The results presented in this paper are in agreement with data previously obtained on the chitin-binding properties of cowpea vicilins and the effect they exert on the development and survival of the storage pest insect *Callosobruchus maculatus* (Coleoptera: Bruchidae).

Keywords: *7S storage globulins; cowpea seeds; Vigna unguiculata; chitin-binding proteins; fungi; spore germination*

INTRODUCTION

Chitin is a homopolysaccharide of *N*-acetyl-D-glucosamine (Muzzarelli, 1985) that is present in the cell walls of fungi and green algae, in the exoskeleton of many crustacean and insects (BeMiller, 1965), and in the midgut of some insects as a constituent of the peritrophic membrane (Richards and Richards, 1977; Peters, 1992). Chitin and chitin oligosaccharides bind to many proteins including specific lectins such as wheat germ agglutinin (WGA) and defense-related proteins such as hevein and class I chitinases (Raikhel *et al.*, 1993). Most of these proteins bind to chitin through a conserved amino acid sequence known as the chitin-binding domain (Chrispeels and Raikhel, 1991).

Vicilins are oligomeric seed storage proteins (7S globulins) of high molecular mass (ca. 150 kDa) that are utilized during germination (Casey *et al.*, 1986). We have recently shown that the vicilins from cowpea (*Vigna unguiculata*) and other legume seeds strongly associate with chitin, chitosan, and fully acetylated chitin (Sales *et al.*, 1996). These proteins could be desorbed from the polysaccharide by high concentrations of *N*-acetylglucosamine, by high DP (110−280) soluble chitosan, and by acetic or hydrochloric acids. We have also shown that vicilins associate with chitin-containing structures present in the midgut of insects (Firmino *et al.*, 1996) and that this association could possibly explain the effect of variant vicilins, found in some cowpea varieties, on the development and survival of *Callosobruchus maculatus*, an insect pest of stored cowpeas (Macedo *et al.*, 1995; Sales *et al.*, 1996).

In this paper we report some results of our investigations on the effects of vicilins, isolated from cowpea seeds, on spore/conidia germination and on the development of some phytopathogenic fungi.

MATERIALS AND METHODS

Seeds. Cowpea (*V. unguiculata)* seeds of the line IT81D-1045 (R), which are resistant to the bruchid insect *C. maculatus* (Xavier-Filho *et al.*, 1989), were obtained from IITA, Ibadan, Nigeria, through the Centro Nacional de Pesquisa Arroz-Feijão (CNPF/EMBRAPA), Goiânia, Goiás, Brazil. Cowpea seeds of the local cultivar CE-31 (S), which are susceptible to the same insect, were supplied by the Centro de Ciências Agrárias, Universidade Federal do Ceará, Fortaleza, Brazil, where they were developed.

Preparation of Albumins, Globulins, and Vicilins (7S Storage Proteins). Finely ground cowpea seed meal (1:10 meal to buffer ratio) was extracted with 50 mM borate buffer, pH 8.0, for 30 min at room temperature. After centrifugation for 30 min at 8000*g* and 4 °C, the proteins in the supernatant were fractionated into albumins (water soluble proteins) and globulins (salt soluble proteins), which were obtained after dialysis against water and freeze-drying. Vicilin type proteins from *V. unguiculata* were prepared following the protocol developed by Sammour *et al.* (1984) with some modifications (Macedo *et al.*, 1993, 1995). The borate buffer (pH 8.0) supernatant obtained as above was fractionated by ammonium sulfate precipitation, and the 70−90% fraction was dialyzed against water and freeze-dried. The vicilins from cowpea seeds were further purified by chromatography on a Sephacryl S-200 column (3 × 40 cm) equilibrated and eluted with the same buffer used for extraction. The vicilin-rich fractions were recovered and submitted to an ion-exchange chromatography step on a DEAE-Sepharose column (2 × 12 cm) equilibrated with 50 mM Tris-HCl, pH 8.0, and eluted with an NaCl gradient (0−1 M) in the same buffer. Vicilin-rich fractions were recovered and submitted to chromatography on a Sephacryl S-400 column (2.5 × 60 cm) in 100 mM Tris-HCl/0.25 M NaCl, pH 8.0. Vicilins were recovered by dialysis and freeze-drying. The degree of purification of the vicilins obtained according to the above procedure was ascertained during the purification step itself since only the most quantitatively significant and symmetrical protein peaks were collected. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) of the purified vicilins showed patterns similar to those obtained

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previously by us and by others (Sammour *et al.*, 1984; Casey *et al.*, 1986; Macedo *et al.*, 1995).

Fungi. Fungal isolates utilized were as follows: *Ustilago maydis* (strain FB_2 genotype a_2 b_2), obtained from Dr. Flora Benuett, University of California, San Francisco, CA); *Phytophtora capsici* (strain P1319), obtained from Dr. M. D. Coffey, University of California, Riverside, CA); *Fusarium solani* (URM 8071) and *Colletotrichum musae* (URM 3330), obtained from the Departmento de Micologia, Universidade Federal de Pernambuco, Recife, Brazil; *Fusarium oxysporum*, kindly supplied by CNPF/EMBRAPA, Goiania, Goiás, Brazil; and *Neurospora crassa* (wild type strain), supplied by Dr. Patricia Ponce, IIBE, Guanajuato, Mexico. The fungi were maintained on agar Sabouraud (*F. solani*, *F. oxysporum*, and *C. musae*), potato dextrose agar (*U. maydis* and *N. crassa*), or V-8 juice agar (*P. capsici*) and cultured in Sabouraud broth (*F. solani*, *F. oxysporum*, and *C. musae*), potato dextrose broth (PDB) (*U. maydis* and *N. crassa*), or V-8 juice liquid medium (*P. capsici*). For the preparation of conidia of *F. solani*, *F. oxysporum*, and *C. musae* or zoospores (*P. capsici*), fungal cultures were transferred to Petri dishes containing agar Sabouraud or V-8 juice agar, respectively, for 12 days for the deuteromycetes and 4 days for the oomycete (Joseph and Coffey, 1984); after this period, sterile distilled water (10 mL) was added to the dishes, and these were gently agitated for 1 min for spore liberation with the help of a Drigalski loop. Spores were quantified in a Neubauer chamber for appropriate dilutions. Sporidia from *U. maydis* were obtained from cultures in PDB medium and quantified in a Neubauer chamber (Bannuet and Herskowitz, 1988).

Effect of Different Protein Fractions from Cowpea on the Growth of Fungi. The effect of different protein fractions isolated from cowpea seeds on the growth of *U. maydis* and *P. capsici* was observed in liquid media (PDB or V-8 juice liquid medium) using 1.5 mL Eppendorf tubes at room temperature (28 °C) with constant shaking. The several protein fractions (albumins, globulins, and vicilins) were used at the final concentration of 2.4 mg mL^{-1}, and spores were added to give 20 000 mL^{-1} in the appropriate medium. Growth was followed for 72 h by taking measurements every 4 h of the absorbance at 660 nm (*U. maydis*) or of the protein content by the Lowry (Lowry *et al.*, 1951) method (for *P. capsici*). Readings were taken against a blank containing the corresponding medium. Each experiment was repeated three times, each one in duplicate with a control without protein addition. The averages, standard errors, and coefficients of variation were calculated. The same type of experiment was also done with *N. crassa*, but results are not reported here.

Effect of Cowpea Vicilins on the Growth of Fungi. For observation of the growth of *F. solani*, *F. oxysporum*, and *C. musae* in the presence of vicilins, we employed essentially the same protocol as described above. Spores (to give 20 000 mL^{-1}) were incubated in 1 mL spectrophotometer plastic cuvettes followed by the addition of proteins (0.8 mg mL^{-1} final concentration). The cuvettes were then maintained at ambient temperature (25 °C). Optical readings at 660 nm were taken at zero time and every 6 h for the following 92 h. A control containing bovine serum albumin, at the same concentration, was also run. A general control without addition of protein was also utilized. Readings were taken against a blank containing only the culture medium. After a 92 h growth period, mycelia were separated from the growth medium by centrifugation, washed in 0.1 M Tris-HCl, pH 8.0, and plated for observation in an optical microscope at 400× magnification. Each experiment was repeated three times, each one in triplicate, and the averages, standard errors, and coefficients of variation were calculated.

Effect of Vicilin Preparations on the Hyphal Growth of Fungi. Antifungal activity of the vicilin preparations was observed under sterile conditions using the disk-plate diffusion assay as described by Roberts and Selitrenikoff (1988). Fungi (*F. solani*, *F. oxysporum*, and *C. musae*) were inoculated at the center of Petri dishes (8.5 cm diameter) containing Sabouraud dextrose agar. After incubation for 48 h at 25 °C to allow for spore germination and vegetative growth, sterile filter paper disks (Whatman 3MM) were radially distributed on the agar surface, close to the expanding mycelial mat, and 50 μL of the protein solutions (CE-31 and IT81D-1045 vicilins at 1% and 2% in 0.1 M Tris-HCl buffer, pH 8.0) were applied to the disks. The Petri dishes were then incubated for 24 h at 25 °C. A negative control was run by adding buffer instead of the protein solutions. In this manner a crescent-shaped zone of inhibition of fungal growth could be observed around the paper disk if the solution being tested showed any antifungal property.

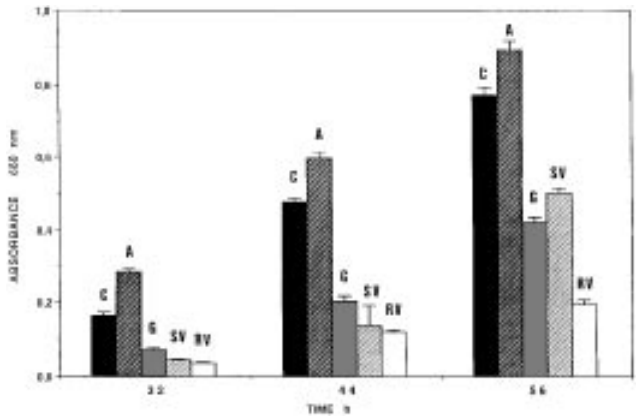

Figure 1. Effect of cowpea proteins on the growth of *U. maydis*. Bar heights correspond to the absorbance at 660 nm, taken as a measure of fungal growth. Measurements were taken every 4 h but graphed at only 32, 44, and 56 h for the sake of simplicity. (C) Control medium; (A) albumins; (G) globulins; (SV) cowpea vicilins from CE-31 seeds; (RV) cowpea vicilins from IT81D-1045 seeds. Experiments were run in triplicate, and the standard errors are indicated.

Extraction of Proteins Associated with Fungal Cell Walls. After 92 h, when growth experiments with *F. solani*, *F. oxysporum*, and *C. musae* were terminated, the material in suspension and corresponding to each treatment was centrifuged at 12 000 rpm in an Eppendorf microcentrifuge for 2 min. The supernatant was discarded, and the precipitate was washed with 0.1 M Tris-HCl, pH 8.0, until no more protein could be measured by using the Bradford (1976) method. The final precipitate was treated with 0.1 M HCl, and the resulting supernatant was concentrated in a SpeedVac apparatus. The dried samples were taken in 0.1 mL of 0.1 M Tris-HCl, pH 8.0, and utilized for SDS−PAGE and Western blotting.

Gel Electrophoresis. SDS−PAGE was carried out according to the denaturing method of Laemmli (1970). Protein markers employed were bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), and lysozyme (14 kDa).

Western Blotting. Antisera against purified vicilins (from CE-31 seeds) were prepared by immunization of white rabbits (Macedo *et al.*, 1995). Purified antisera were obtained by affinity chromatography of the crude immune sera in a column of protein A covalently bound to Sepharose CL-4B. Preimmune sera were collected before immunization. Western blotting was done to nitrocellulose membranes after SDS−PAGE according to described methods (Towbin *et al.*, 1979).

RESULTS

Effect of Different Protein Fractions from Cowpea Seeds on Growth of *U. maydis* and *P. capsici*. The growth of *U. maydis* was measured by estimating the absorbance at 660 nm of the culture suspensions, while the Lowry (1951) method was utilized to measure the growth of *P. capsici*. Figures 1 and 2 show bar graphs of the values taken at three representative times (at the log phase and maximal growth) for each fungus. Growth of both *U. maydis* and *P. capsici* seems to be stimulated by the presence of cowpea albumins during all stages of the experimental period (Figures 1 and 2).

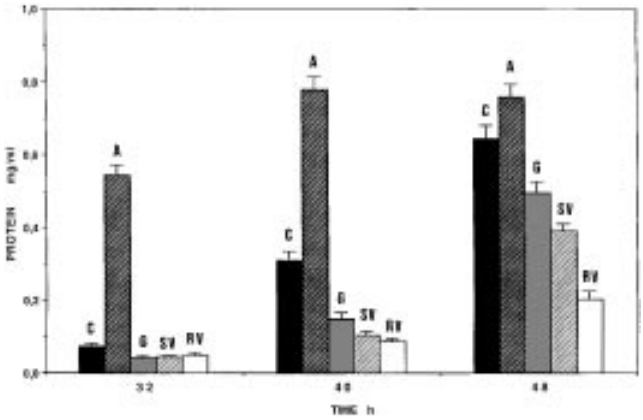

Figure 2. Effect of cowpea proteins on the growth of *P. capsici*. Bar heights correspond to the protein concentrations by the Lowry method, which are taken as a measure of fungal growth. Measurements were taken every 4 h but graphed at only 32, 40, and 48 h for the sake of simplicity. (C) Control medium; (A) albumins; (G) globulins; (SV) cowpea vicilins from CE-31 seeds; (RV) cowpea vicilins from IT81D-1045 seeds. Experiments were run in triplicate, and the standard errors are indicated.

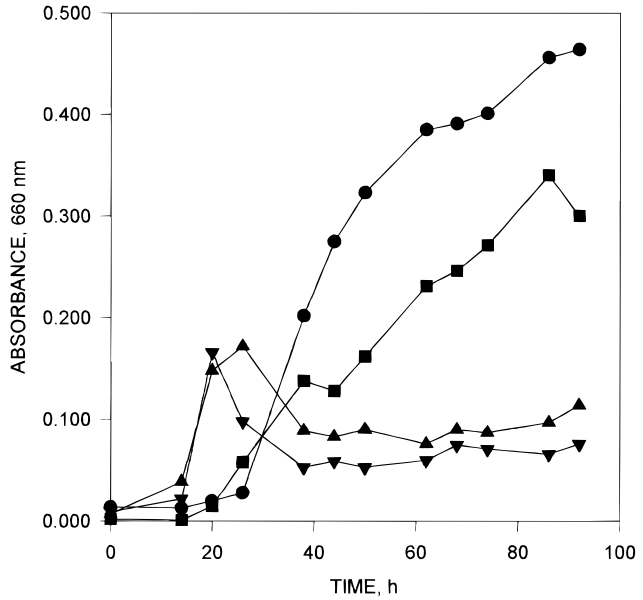

Figure 3. Effect of cowpea vicilins on the growth of *F. solani.* The absorbance was measured at 660 nm, taken as a measure of fungal growth. (●) Control; (■) BSA; (▲) CE-31 (S) vicilins; (▼) IT81D-145 (R) vicilins. Experiments were run in triplicate, and the standard errors (coefficient of variation not more than 20%) are omitted for clarity.

Cowpea globulins, on the other hand, show strong inhibitory effects (up to 57%) on the growth of these same fungi (Figures 1 and 2). When we examine the effects of vicilins from seeds of both cultivars utilized, we find a very strong inhibition (up to 70%) of the growth of the two fungi above (Figures 1 and 2).

Effect of Vicilins from Cowpea Seeds on Growth of *F. solani*, *F. oxysporum*, and *C. musae*. Figures 3−5 show the patterns of growth for *F. solani*, *F. oxysporum*, and *C. musae* in the presence of bovine serum albumin (BSA) and vicilin preparations isolated from seeds of both CE-31 (S) and IT81D-1045 (R) cowpea cultivars and in control medium. As shown in the figures, BSA, used as a control, exerts a small inhibitory effect on the growth of all three above fungi as compared with the control medium. On the other hand, cowpea vicilins are more inhibitory than BSA. Vicilins from

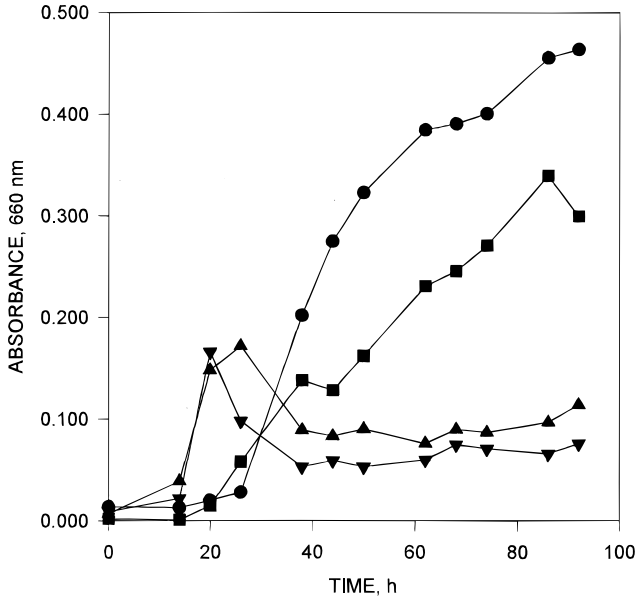

Figure 4. Effect of cowpea vicilins on the growth of *F. oxysporum*. The absorbance was measured at 660 nm, taken as a measure of fungal growth. (●) Control; (■) BSA; (▲) CE-31 (S) vicilins; (▼) IT81D-145 (R) vicilins. Experiments were run in triplicate, and the standard errors (coefficient of variation not more than 20%) are omitted for clarity.

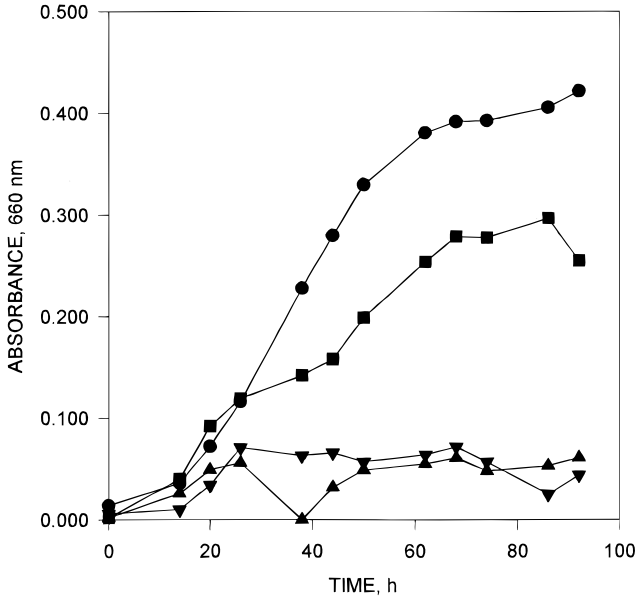

Figure 5. Effect of cowpea vicilins on the growth of *C. musae.* The absorbance was measured at 660 nm, taken as a measure of fungal growth. (●) Control; (■) BSA; (▲) CE-31 (S) vicilins; (▼) IT81D-145 (R) vicilins. Experiments were run in triplicate, and the standard errors (coefficient of variation not more than 20%) are omitted for clarity.

IT81D-1045 (R) seeds seem to be more effective in the inhibition of the growth of *F. solani* and *C. musae* than vicilins from CE-31 (S) seeds, while for the growth of *F. oxysporum* there is no difference when both vicilins are compared (Figure 4). For *F. solani* and *C. musae*, we notice an apparent stimulation of growth at the beginning of the experiment for both vicilins employed (Figures 3 and 5). Photomicrographs of the mycelia of the above fungi were taken after the 92 h growth period, but only those for *F. oxysporum* are shown (Figure 6) since other fungi had similar appearances. Normal hyphal development (Figure 6, plates A and B) was observed in control and BSA treatments, but in both

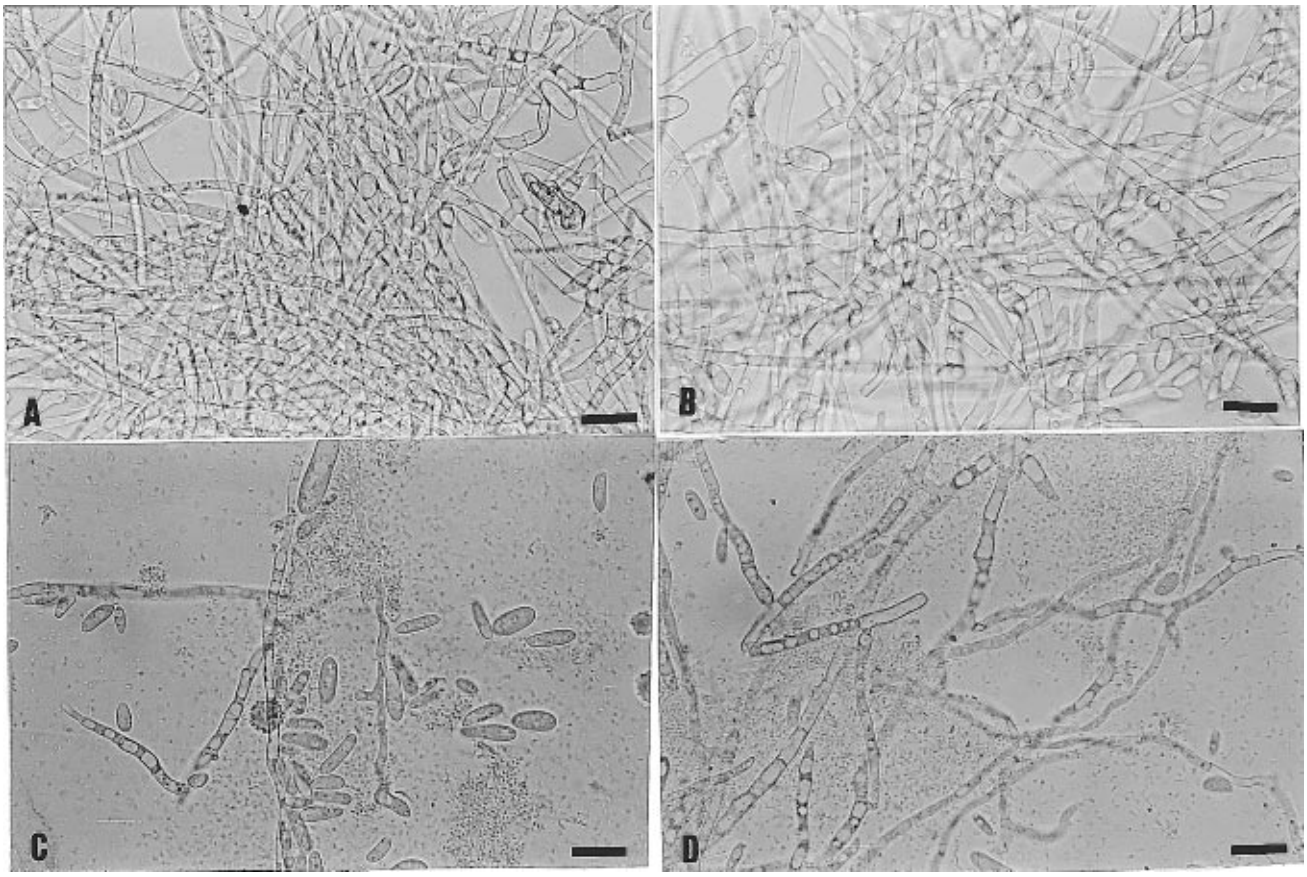

Figure 6. Light micrographs of *F. oxysporum* mycelia after 92 h of growth of the fungus in the presence of (A) control medium, (B) bovine serum albumin, (C) CE-31 (S) vicilins, and (D) IT81D-1045 (R) vicilins. Magnification bars = 15.6 μm.

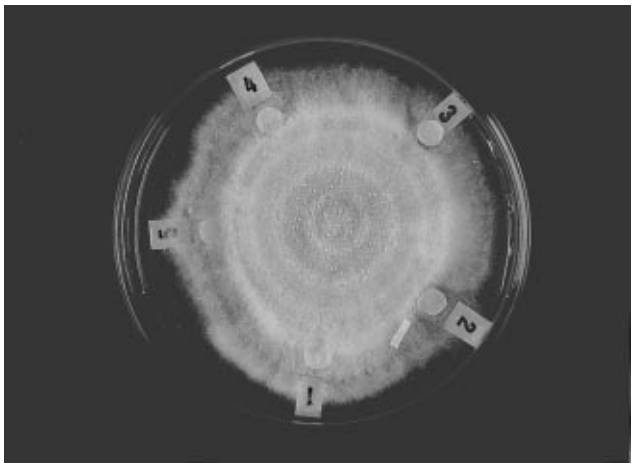

Figure 7. Disk-plate diffusion assay on the effects of vicilins on the mycelial growth of *F. oxysporum*. (1) 0.1 M Tris-HCl buffer control; (2) CE-31 [S] vicilins [10 mg mL^{-1}]; (3) IT81D-1045 (R) vicilins [10 mg mL^{-1}]; (4) CE-31 [S] vicilins [20 mg mL^{-1}]; (5) IT81D-1045 (R) vicilins [20 mg mL^{-1}].

vicilins from IT81D-1045 (R) and CE-31 (S) cowpea seeds there is a noticeable inhibition of conidial germination (Figure 6, plates C and D).

The disk-plate diffusion assay employing vicilins from both cowpea cultivars shows that the proteins do not interfere with hyphal development of the fungi *C. musae*, *F. solani*, and *F. oxysporum*. Results are shown only for *F. oxysporum* (Figure 7).

Proteins Associated with Hyphae after Fungi Growth in the Presence of Vicilins. After the 92 h growth period in the presence of BSA, vicilins from IT81D-1045 (R) and CE-31 (S) cowpea seeds and control medium fungal hyphae were obtained by centrifugation, washed with buffer, and extracted by acid. Extracted proteins were analyzed by SDS−PAGE (Figure 8a). The corresponding immunoblot using an antiserum raised against cowpea vicilins (Figure 8b) demonstrates that vicilins were extracted from these fungal pellets.

DISCUSSION

Albumins isolated from cowpea seeds stimulate the development of the fungi *U. maydis* and *P. capsici* (Figures 1 and 2) throughout the experimental growth period, while globulins extracted from the same seeds show an inhibitory effect for the duration of the observations. In experiments not shown, albumins led to an early stimulation of growth for *N. crassa*, which was followed by inhibition. This finding could be tentatively explained by an increased amino acid availability due to the action of proteolytic enzymes of both fungi and seed origin on the many different soluble proteins found in the albumin fraction (Bewley and Black, 1994). The globulin fraction in legume seeds contains the reserve proteins known as legumins and vicilins, which are also referred to as the 11S and 7S storage proteins, respectively (Casey *et al.*, 1986). In the case of cowpea seeds, the globulins are mainly composed of vicilins (Macedo *et al.*, 1995). Our experiments show that vicilins are highly inhibitory to the above fungi, indicating that the effect observed with the globulin fraction is due to these reserve proteins. When we repeated these growth experiments now utilizing the phytopathogenic fungi *F. solani*, *F. oxysporum*, and *C. musae*, we observed the same strong inhibitory effect of vicilins (Figures 3−5). BSA, utilized as a control protein, did not show such an effect. An apparent stimulation of germination by vicilins at the beginning of the experiment (Figures 3

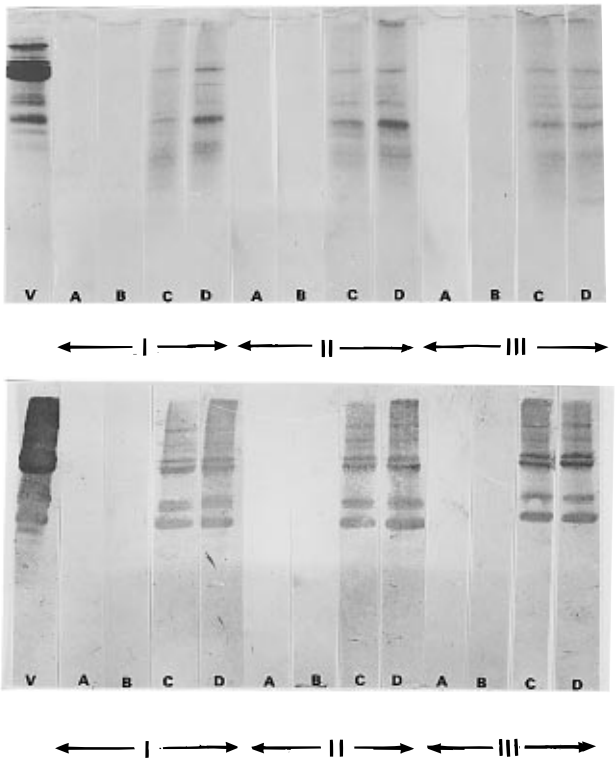

Figure 8. (a, top) SDS−PAGE of the proteins extracted from the mycelial pellet after a 92 h growth period. (V) Vicilin standard: (panel I) *C. musae*; (panel II) *F. oxysporum*; (panel III) *F. solani*. (A) Control medium; (B) bovine serum albumin; (C) CE-31 (S) vicilins; (D) IT81D-1045 (R) vicilins. (b, bottom) Western blotting of the proteins extracted from the mycelial pellet after a 92 h growth period revealed by an antiserum against purified vicilins (from CE-31). (V) Vicilin standard: (panel I) *C. musae*; (panel II) *F. oxysporum*; (panel III) *F. solani*. (A) Control medium; (B) bovine serum albumin; (C) CE-31 (S) vicilins; (D) IT81D-1045 (R) vicilins.

and 5) in the case of *F. solani* and *C. musae* could be explained by the development of the small number of germinating conidia that is found in our initial spore preparations for these fungi. We notice that vicilins isolated from bruchid-resistant cowpea seeds (IT81D-1045) seem to be more inhibitory than vicilins from susceptible seeds (CE-31) with the exception of *N. crassa* (not shown) and *F. oxysporum*, in which case it seems that there is little difference between the two vicilins (Figure 4).

Microphotographs of the fungi *F. solani*, *F. oxysporum*, and *C. musae* taken after a 92 h growth period in the presence of cowpea vicilins show that there is practically no hyphal development, while the presence of many spores is clearly discernible. These results, shown here only for *F. oxysporum* (Figure 6), suggest that these seed storage globulins negatively affect spore germination. Results obtained when we employed the Petri dish diffusion growth assay of Roberts and Selitrenikoff (1988) for the fungi *C. musae*, *F. solani*, and *F. oxysporum*, shown here only for the latter (Figure 7), suggest that there is no inhibition of hyphal development by vicilins. The finding that mycelial growth is not inhibited and spore or conidia germination is could be explained by a relative inaccessibility of the chitin (or *N*-acetylglucosamine-containing) component of the cell walls of growing hyphae to the high molecular size vicilins (Alexopoulos *et al.*, 1996).

We also studied the effect of several other legume vicilins on the growth of the yeasts *Candida albicans* and *Saccharomyces cerevisiae* and found the same inhibitory effect on spore germination (Gomes *et al.*, 1996).

We found that cowpea vicilins strongly bind to fungal structures when we prepared HCl-extracted proteins from fungal growth pellets exhaustively washed with Tris-HCl buffer and examined them by SDS−PAGE and Western blotting utilizing an antiserum against cowpea vicilins. The patterns show the presence of vicilin subunits (lanes C and D) and no protein bands for the control or BSA treatments (lanes A and B) in the extracted fractions corresponding to each of the fungi examined (Figure 8). These findings are an indication of the association of vicilins with the fungal cell walls, which could be due to the chitin component in these structures. It is well-known that chitin present in fungal cell walls associates with several proteins, the so-called chitin binding proteins, resulting in the inhibition of growth for these organisms (Raikhel *et al.*, 1993; Mirelman *et al.*, 1975; Callow, 1977).

We had already noted that the association of vicilins with fungi cell walls or membranes was a possibility when we first studied the association of legume vicilins in general with chitin and some of its derivatives (Sales *et al.*, 1996) and with chitin-containing membranes of the midgut of the bruchids *C. maculatus* and *Z. subfasciatus* (Firmino *et al.*, 1996). The fact that vicilins interfere with the growth of *P. capsici* (Figure 2), an oomycete pathogen that contains little or no chitin in its cell wall (Alexopoulos *et al.*, 1996), could indicate that these proteins associate with cell surface glycoconjugates rich in *N*-acetylglucosamine residues. In our previous papers on the binding of vicilins to chitin-containing structures, we observed that variant vicilins, present in cowpea seeds which are resistant to *C. maculatus*, apparently bind to chitin and chitin-containing structures in the midgut of these insects more strongly than normal vicilins, that is, those isolated from seeds that are susceptible to *C. maculatus* (Sales *et al.*, 1996). This stronger association was then postulated to be the reason these cowpea seeds are resistant to this bruchid (Xavier-Filho *et al.*, 1989; Macedo *et al.*, 1995; Sales *et al.*, 1996). The results reported here do not suggest that vicilins from bruchid-resistant cowpea seeds exert stronger inhibitory effects on fungal spore germination than those seen from susceptible seeds.

The findings reported in this paper and other results from our group (Macedo *et al.*, 1995; Sales *et al.*, 1996; Firmino *et al.*, 1996) suggest that legume seed vicilins could associate with organisms containing chitin, chitin derivatives, or *N*-acetylglucosamine-containing glycoconjugates in structures that are exposed to the external medium.

During the germination processes, when seeds imbibe water and exude many solutes, including proteins (Spaeth, 1989), exuded vicilins could get in contact with seed-borne fungal spores interfering with further fungal development or alternatively become hydrolyzed by specific proteolytic enzymes produced by them. Experiments along these lines are being developed in our laboratories.

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