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ARTICLE

Soybean Toxin (SBTX), a Protein from Soybeans That Inhibits the Life Cycle of Plant and Human Pathogenic Fungi

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Soybean toxin (SBTX) is a 44 kDa glycoprotein that is lethal to mice (LD_{50} = 5.6 mg/kg). This study reports the toxicity of SBTX on pathogenic fungi and yeasts and the mechanism of its action. SBTX inhibited spore germination of *Aspergillus niger* and *Penicillium herguei* and was toxic to *Candida albicans*, *Candida parapsilosis*, *Kluyveromyces marxiannus*, *Pichia membranifaciens*, and *Saccharomyces cerevisiae*. In addition, SBTX hampered the growth of *C. albicans* and *K. marxiannus* and inhibited the glucose-stimulated acidification of the incubation medium by *S. cerevisiae*, suggesting that SBTX interferes with intracellular proton transport to the external medium. Moreover, SBTX caused cell-wall disruption, condensation/shrinkage of cytosol, pseudohyphae formation, and *P. membranifaciens* and *C. parapsilosis* cell death. SBTX is toxic to fungi at concentrations far below the dose lethal to mice and has potential in the design of new antifungal drugs or in the development of transgenic crops resistant to pathogens.

KEYWORDS: ***Glycine max*****; soybean; soybean toxin; antifungal protein; plant toxin**

INTRODUCTION

Heterotrophic organisms depend directly or indirectly on plants that provide proteins, oils, carbohydrates, vitamins, and minerals for their growth. Consequently, plants are continuously threatened by these organisms, including predators and phytopathogens. To counteract this biotic pressure and survive, plants have developed complex defense mechanisms (*1*, *2*). In fungal infection, plant resistance is achieved by both constitutive and inducible defense mechanisms. These involve physical barriers and chemical weapons such as proteins and other organic molecules that are synthesized prior to infection or during pathogen attack (*3*, *4*).

In plants, a large number of antifungal proteins have been described and characterized (*5*−*7*). They usually act by inhibiting the synthesis of the fungal cell wall or disrupting cell wall structure and/or function (*8*). Others interact with the plasma membrane, leading to pore formation, fungal cell lysis, disruption of the efflux and uptake of cellular components, and changes in the membrane potential (*9*, *10*). Moreover, they eventually cross the fungal cell wall and membrane and interact with potential fungal intracellular targets, resulting in cell death (*11*).

Antifungal proteins have been shown to be strong candidates for plant improvement programs through biotechnological approaches to prevent plant fungal diseases and hence massive economic losses (*12*). In addition, new antifungals could potentially treat fungal diseases that are resistant to commercial drugs (*13*). To fully explore the potential use of these proteins as chemical weapons against fungi, it is essential to thoroughly understand their mechanisms of action.

Thus, our research group has systematically investigated the role of plant proteins in defense against pathogens. Recently, we have shown that soybean toxin (SBTX), a lethal protein to mice (LD_{50} = 5.6 mg/kg by intraperitoneal (ip) injection), inhibited the mycelial growth of *Cercospora sojina* (*14*). SBTX is a 44 kDa basic glycoprotein composed of two polypeptide chains (27 and 17 kDa) linked by a disulfide bond. In agreement with the proposed defensive role of SBTX, the NH_2-terminal amino acid sequences of the 27 and 17 kDa subunits share high similarities to SC24, an abundant 24 kDa protein from soybean seed hull, and to cyclophilins, respectively, to which protective function was attributed (*15*−*17*).

To test the hypothesis that SBTX is an antifungal protein, its inhibitory activity on the conidial and mycelial growth of the phytopathogenic fungi *Aspergillus niger*, *Fusarium oxysporum*, *Fusarium solani*, and *Penicillium herguei* was evaluated. Moreover, to assess the action of SBTX toward yeasts, some of which are pathogenic to humans, its ability to inhibit the cell growth of *Candida albicans*, *Candida parapsilosis*, *Kluyveromyces marxiannus*, and *Pichia membranifaciens* was tested. In addition, to gain better insights into the mechanisms by which this toxin acts on these organisms, its effects on the glucose-stimulated acidification of the external medium was assessed in *Saccharomyces cerevisiae*. Finally, plasma membrane permeabilization and ultrastructural alterations were analyzed in *C. parapsilosis* and *P. membranifaciens*.

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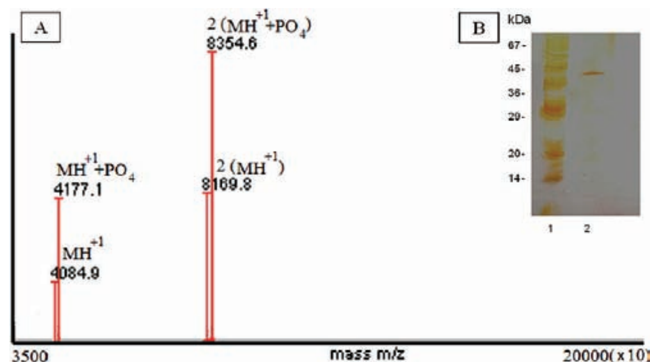

Figure 1. (**A**) Mass spectrometry analysis of SBTX. (**B**) Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) of soybean toxin (SBTX). Lanes: 1, molecular mass standards are shown (in kDa) on the left; 2, SBTX (5 μg). Molecular mass markers were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), egg white albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.2 kDa).

MATERIALS AND METHODS

Biological Material and Reagents. Mature seeds of soybean [*Glycine max* (L.) Merr.] (genotype IAC-24) were supplied by Instituto Agronômico de Campinas (IAC, São Paulo, Brazil). The filamentous fungi *A. niger*, *F. oxysporum*, *F. solani*, and *P. herguei* were provided by the Departamento de Micologia of the Universidade Rural de Pernambuco, Recife, Brazil. The yeasts *C. albicans*, *C. parapsilosis*, *K. marxiannus*, *P. membranifaciens*, and *S. cerevisiae* were obtained from the Departamento de Biologia, Universidade Federal do Ceará (UFC), Fortaleza, Brazil. All chemicals used were of analytical grade.

Protein Determination. Protein concentration was measured according to the method of Bradford (*18*), using bovine serum albumin (BSA) as a standard. Absorbance at 280 nm was also used to determine the protein content of column eluates.

Toxicity Assay. The toxicity assay was reviewed, approved by the Animal Ethics Committee (CEPA) of UFC, Brazil, and realized according to the methodology described by Vasconcelos et al. (*14*, *19*). Toxic activity was defined as mortality observed in Swiss mice within 24 h after ip injections of SBTX at varied concentrations (mg of protein/kg of mouse body weight) to allow calculation of LD_{50}. LD_{50} was taken as the amount of protein producing convulsions and death of 50% of tested animals (six doses; six animals).

SBTX Preparation. SBTX preparations were obtained according to the method of Vasconcelos et al. (*14*). Briefly, mature seeds were ground in a coffee grinder and passed through a 1 mm mesh screen, and the resulting fine powder was treated with petroleum ether (1:10, m/v). This defatted powder was extracted with 0.025 M Tris-HCl/0.005 M dithiothreitol (DTT), pH 7.5, in a proportion of 1 g of meal to 5 mL of buffer for 3 h at 4 °C under constant stirring and filtered through cheesecloth. The press cake was re-extracted for 2 h under the same conditions. After centrifugation at 21000*g* and 4 °C for 30 min, the supernatant, now denoted the crude extract, was fractioned by saturation to 20−55% with solid ammonium sulfate. This protein fraction was dissolved in and dialyzed against the extracting buffer and applied to a DEAE-cellulose column equilibrated with the same buffer. After elution of the unbound proteins from the column with the equilibrating buffer, proteins were concentrated by precipitation with 90% ammonium sulfate, exhaustively dialyzed against 0.025 M Tris-HCl/0.005 M DTT, pH 7.5, and applied to a CM-Sepharose column equilibrated with the buffer. The toxic fraction was eluted with the equilibrating buffer containing 0.2 M NaCl, concentrated with 90% ammonium sulfate, dialyzed against 0.025 M Tris-HCl, pH 7.5, and applied on a Superdex 200 HR 10/30 column equilibrated with 0.025 M Tris-HCl containing 0.5 M NaCl, pH 7.5. The fractions displaying toxicity were pooled, concentrated with 90% ammonium sulfate, and dialyzed against 0.025 M Tris-HCl, pH 7.5. Finally, DTT was added to a final concentration of 0.005 M. Purity of SBTX was checked by denaturing gel electrophoresis (*20*) and by mass spectrometry analysis (Voyager-DE STR mass spectrometer, MALDI-ToF). The identity of SBTX was confirmed by N-terminal amino acid sequence analysis by Edman degradation (Shimadzu PPSQ-10 automated protein sequencer).

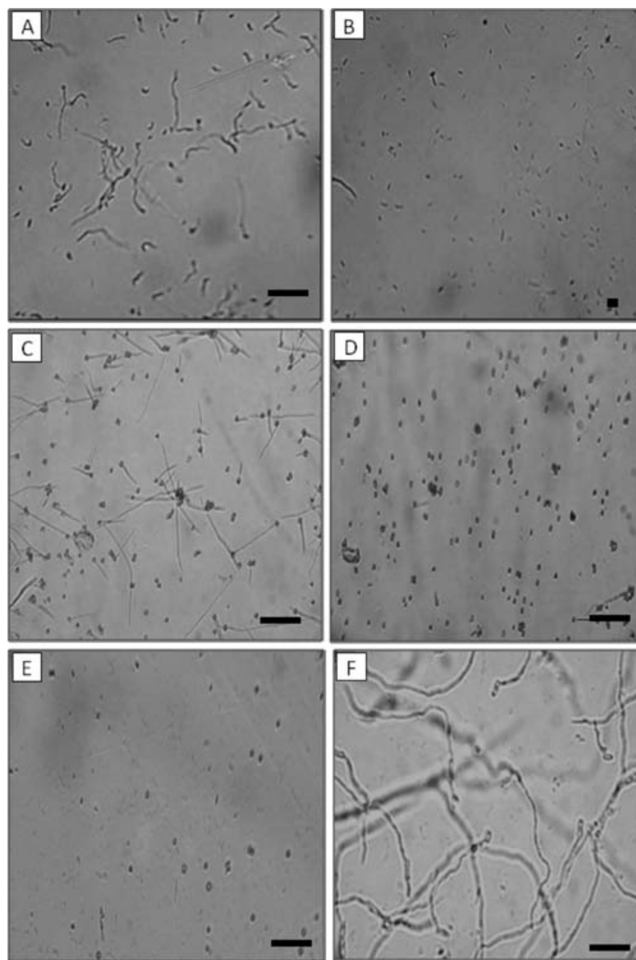

Figure 2. Light micrographs of *Aspergillus niger* (**A**, **B**, **E**), *Penicillium herguei* (**C**, **D**), and *Fusarium solani* (**F**) spores after 16 h of germination in culture medium (**A**, **C**) and in the presence of soybean toxin (SBTX, 70 μg/mL) (**B**, **D**, **F**). Negative control: *A. niger* in the presence of 100 mM hydrogen peroxide (**E**). Bars (**A**−**F**): 2.5 μm.

To check for the presence of contaminating antifungal proteins, the SBTX preparation was assayed for chitinase and β-1,3-glucanase activities according to the protocol of Xue et al. (*21*). Chitinase activity was measured colorimetrically on the basis of the release of *N*-acetyl-D-glucosamine produced by the hydrolytic action of chitinases upon colloidal chitin used as substrate (*22*). Colloidal chitin was prepared following the protocol described by Molano et al. (*23*), except that nonradioactive acetic anhydride was used to acetylate the crab shell chitosan obtained from Sigma Chemical Co.

Effect of SBTX on Spore Germination and Mycelial Growth of Filamentous Fungi. The filamentous fungi *A. niger*, *F. oxysporum*, *F. solani*, and *P. herguei* were grown in Petri dishes containing Saboraud dextrose agar for 7 days at room temperature (25 °C). Fresh conidia were obtained by gently rinsing the surface of 7-day-old sporulated cultures with sterile water, spreading with a triangular Drigalsky rod, and filtering through cheesecloth in a laminar flux chamber under sterile conditions. Conidia were quantified using a Neubauer chamber under an optical microscope. Antifungal assays were performed to verify the ability of SBTX to inhibit conidium germination and to check for its vegetative growth inhibition properties. Inhibition of conidium germination was conducted in reticulated plates where 10 μL of the conidia suspension (2 × 10^5/mL) was incubated with 10 μL of SBTX (50, 70, 100, 125, and 250 μg/mL in 0.025 M Tris-HCl, pH 7.5). In negative, noninhibitory controls, conidia were incubated in the culture medium alone, Tris buffer or sterile Milli-Q grade water, all in the absence of SBTX. As a positive inhibitory control, 0.5 M H_2O_2 was used. The reticulated plates were placed in a

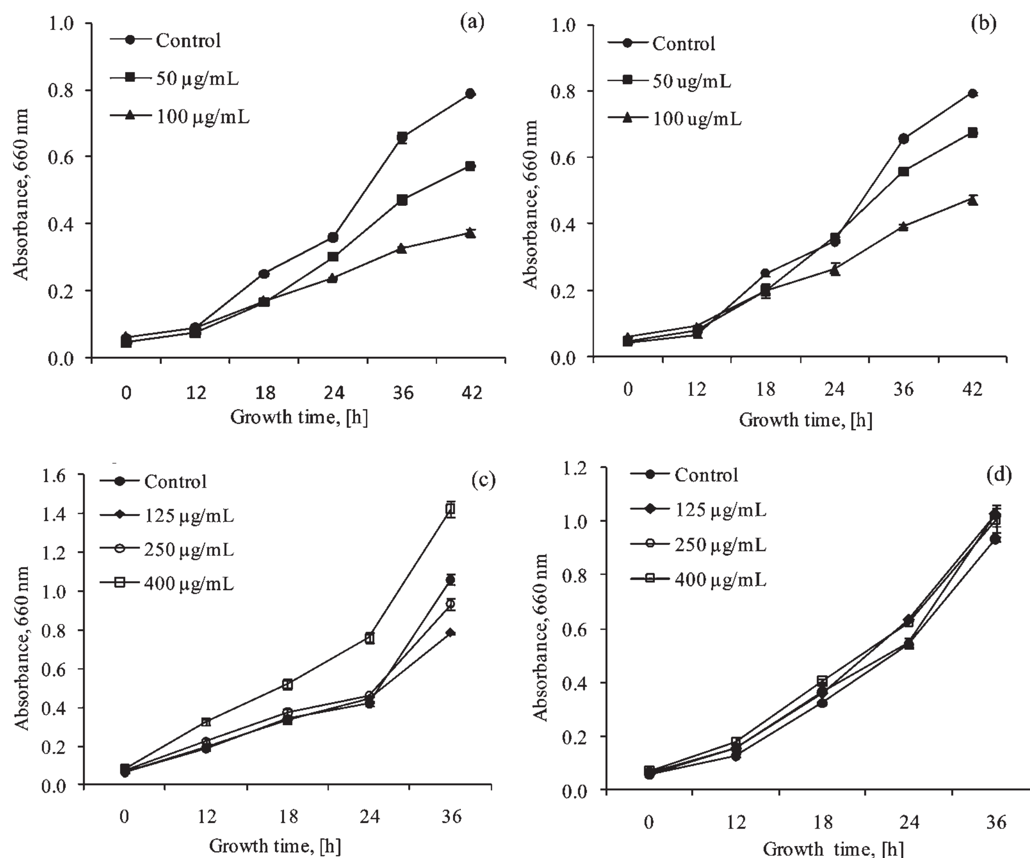

Figure 3. Growth curves of *Candida albicans* (**A**), *Kluyveromyces marxiannus* (**B**), *Pichia membranifaciens* (**C**), and *Candida parapsilosis* (**D**) cells cultured in the absence and presence of soybean toxin (SBTX). Results are means ± SD (n = 3).

plastic box maintained near 100% relative humidity at 22 °C in the dark for 16 h. After this time, 50 conidia were randomly selected from the treatments in the presence or absence of SBTX and evaluated for germination under an optical microscope. Pictures were taken with a digital camera (Sony, MCV-CD350 model, 3.2 megapixels). A conidium having germinating hyphae at least twice the length of the ungerminated conidium was considered to have successfully germinated. Assay for fungal growth inhibition was performed following the protocol developed by Broekaert et al. (*24*). Briefly, 100 μL of the conidia suspension (2 × 10^4/mL) was incubated in 96-well flat microplates (Nunc) with the same volume of SBTX (50 or 100 μg/mL final concentration) prepared in 1% peptone dextrose broth, pH 6.0, and sterilized using a 0.22 μm membrane (Millipore). Cell growth without the addition of SBTX was also determined. The fungal growth was monitored from 0 to 72 h by turbidimetry at 630 nm using an automated microplate reader (Bio-Tek Elx800). All experiments were run in triplicate. Statistical analysis to check whether the rates of fungal growth in the presence of SBTX depart significantly from the growth rate of controls was performed according to the method described by Johnston (*25*).

Effect of SBTX on Yeast Growth. Yeast cells (*C. albicans*, *C. tropicalis*, *P. membranifaciens*, *C. parapsilosis*, *K. marxiannus*, and *S. cerevisiae*) were obtained from inocula of each stock culture transferred to Petri dishes containing Sabouraud agar and allowed to grow at 28 °C for 2 days. Next, cells were transferred to sterile 0.15 M NaCl (10 mL) and counted in a Neubauer chamber to allow calculation of appropriate dilutions. Yeast growth inhibition was performed following the protocol developed by Broekaert et al. (*24*), with some modifications. Cells (10000 cells/mL of 0.15 M NaCl) were incubated at 28 °C in 200 μL microplates in the presence of different concentrations of SBTX (50−400 μg/mL). Cell growth was also determined without the addition of SBTX. Optical readings at 660 nm were taken at time zero and every 6 h for 36 or 42 h. At the end of this period, control and SBTX-treated cells were pelleted by centrifugation, washed in 0.1 M Tris-HCl, pH 8.0, and observed in an optical microscope. All experiments were carried out in triplicate.

SYTOX Green Uptake Assay. Plasma membrane permeabilization was measured by SYTOX Green uptake, as described previously by Thevissen et al. (*26*), with some modifications. *P. membranifaciens* and *C. parapsilosis* cells were cultured in the presence of SBTX (400 μg/mL), as described previously. Cell suspensions (100 μL) were incubated with 0.2 μM SYTOX Green in 96-well microplates for 30 min at 25 °C under occasional agitation. Next, harvested cells were observed in a DIC microscope (Axiophoto Zeiss) set for fluorescein detection (excitation wavelengths, 450−490 nm; emission wavelength, 500 nm). Negative (no SBTX incubation) and positive (thermal treatment at 100 °C for 10 min) controls were also run to evaluate cell membrane permeabilization.

Inhibition of the Glucose-Stimulated Acidification of the Medium by Yeast Cells. This assay was carried out to assess whether SBTX had any effect on the ability of the cells *S. cerevisiae* to transport intracellular protons to the external medium. The assay was performed by measuring the pH of the external culture medium as previously described by Monk and Perlin (*27*). Briefly, *S. cerevisiae* cells were grown in Sabouraud broth for 16 h at 30 °C on a gyratory shaker at 160 rpm. The cells were harvested by centrifugation at 3000*g* and 4 °C for 5 min and washed three times with 0.005 M Tris-HCl, pH 6.0; the pellet was resuspended in the Tris buffer at a cell density of 1.0 × 10^7 cells/mL. SBTX (4 mL at 400 μg/mL) was added to 50 μL of this cell suspension and incubated in an ice bath for 30 min with gentle stirring. After incubation, a 0.5 M glucose solution was added to a final concentration of 0.15 M, and the pH of the external medium was monitored for 60 min. Control experiments (BSA at 800 μg/mL instead of SBTX or 0.005 M Tris-HCl, pH 6.0) were conducted in parallel to measure the extent of acidification of the external medium in the absence of SBTX. The variation in the proton (H^+) concentration was calculated on the basis of the difference of pH at 0 and 60 min.

Scanning Electron Microscopy (SEM). For SEM, yeast cells were grown for 36 h in Sabouraud broth in the presence (400 μg/mL) or absence of SBTX. Cells were harvested and fixed with 2.5% (v/v) glutaraldehyde and 2% (v/v) formaldehyde in 0.1 M phosphate buffer, pH 7.3, for 30 min at room temperature (25 °C). Subsequently, the samples were rinsed three times with the above buffer, postfixed with 1.0% (m/v) osmium tetroxide diluted in the same buffer for 30 min at room temperature, and rinsed with distilled water. After this procedure, the fungi were dehydrated in acetone,

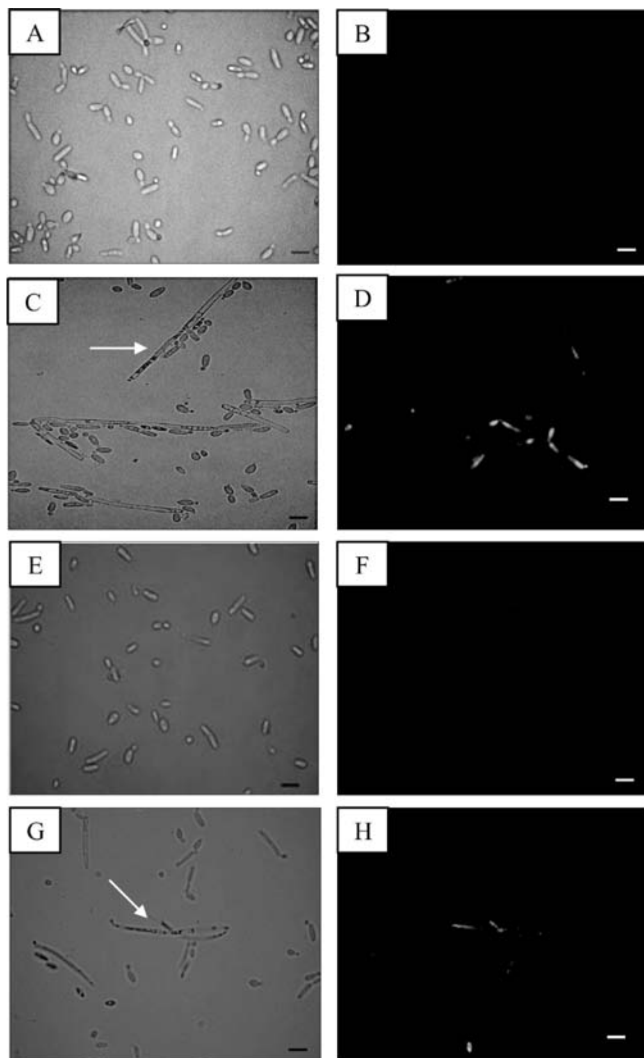

Figure 4. Fluorescence microscopy of yeast cells cultured in the absence (**A**, **B**, **E**, **F**) and presence (**C**, **D**, **G**, **H**) of soybean toxin (SBTX, 400 μg/mL) followed by SYTOX Green treatment. *Pichia membranifaciens* (**A**−**D**) and *Candida parapsilosis* (**E**−**H**) cells were viewed by DIC and fluorescence. Arrows indicate pseudohyphae. Bars (**A**−**H**): 10 μm.

critical point dried in CO_2, covered with 20 nm gold, and observed in a Zeiss 962 scanning electron microscope. Cell growth in the absence of SBTX was also evaluated (control).

Transmission Electron Microscopy (TEM). For TEM, yeast cells were grown for 36 h in Sabouraud broth in the presence (400 μg/mL) or absence of SBTX. Cells were then harvested and fixed in 2.5% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde in 0.05 M cacodylate buffer, pH 7.2, for 30 min at room temperature. After fixation, the materials were washed and postfixed in 1% (m/v) of osmium tetroxide in the above buffer for 1 h at room temperature. The samples were dehydrated in a graded acetone series (30, 50, 70, 90, and 100%, v/v) and embedded in Epon resin (Polybeded). Ultrathin sections (0.1 μm) were fixed onto copper grids and stained with uranyl acetate for 10 min and then with lead citrate for 5 min. Visualization of cells was done in a transmission electron microscope (Zeiss TEM 900) at 80 kV.

RESULTS AND DISCUSSION

SBTX Preparation. SBTX preparations used in the present study were shown to be homogeneous and free of contaminants, as shown in a representative figure (**Figure 1**). SBTX is a 44 kDa protein band with high toxicity in mice (LD_{50} = 5.6 mg/kg, ip route), confirming the findings of Vasconcelos et al. (*14*). The purity of SBTX was further demonstrated by mass spectrometry

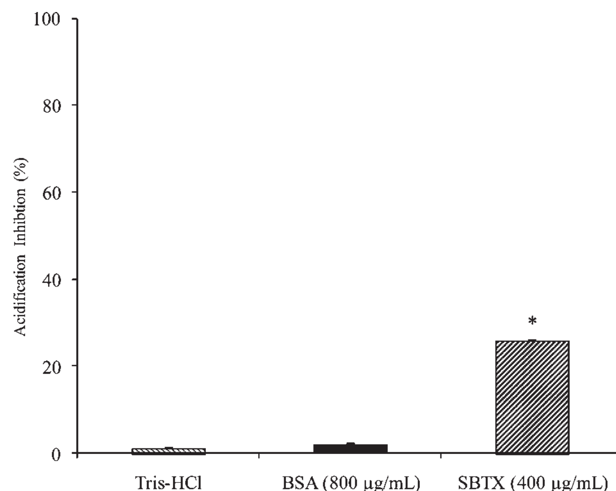

Figure 5. Influence of soybean toxin (SBTX, 400 μg/mL) on the glucose-dependent acidification of the medium by *Sacharomyces cerevisiae* cells. Glucose was added after 30 min of incubation with SBTX at a final concentration of 0.15 M. Error bars represent the SD of the mean (n = 3). ∗, difference between SBTX and the control or BSA is significant ($p < 0.05$).

and N-terminal sequencing analysis. The deconvolved mass spectrum (**Figure 1A**) indicated molecular masses around 41771 and 83546 Da for the monomer and dimer forms of SBTX, respectively. The results are in agreement with SDS-PAGE (**Figure 1B**). SBTX did not show any chitinase or β-1,3-glucanase activity at 2 mg/mL. These enzymes are well-known for their antifungal properties, and if they were present as contaminants they could interfere with the effects of SBTX on the fungi tested here.

Effect of SBTX on Spore Germination and Mycelial Growth of Filamentous Fungi. SBTX inhibited spore germination of the phytopathogenic fungi *A. niger* and *P. herguei* (**Figure 2**) at all concentrations tested (from 50 to 250 μg/mL), but it was not inhibitory to *F. oxysporum* and *F. solani* (**Figure 2**) when compared to controls (**Figure 2**). However, for mycelial (vegetative) growth, none of the fungi tested were affected by SBTX (data not shown), even at a concentration of 500 μg/mL.

Developmental stage- and species-specific inhibitory effects of other plant proteins on fungi have been previously reported. The lectin (25 μg/mL) from *Dioclea guianensis* seeds inhibited the conidium germination but not the mycelial growth of *Colletrotrichum gloeosporioides* (*28*). In contrast, the lectin (250 μg/mL) of *Luetzelburgia auriculata* halted the mycelial growth of *C. lindemunthianum*, *F. solani*, and *A. niger*, but was not inhibitory to the spore germination of these fungi (*5*). A recombinant PR10 of *Arachis hypogaea* specifically inhibited the mycelial development of *F. oxysporum* ($IC_{50\%}$ = 12.5 μg/mL) and *R. solani* ($IC_{50\%}$ = 100 μg/mL) but was ineffective on *Sclerotium rolfsii*, *A. flavus*, *A. niger*, and *Phytophthora infestans* (*29*).

During the development of fungi, several changes in cell wall composition take place that might explain the differences in the inhibitory activity of proteins against these organisms at distinct development stages. For instance, Feofilova et al. (*30*) reported that both the content and the polysaccharide composition of the chitin/glucan cell wall complex of *A. niger* change during the fungus life cycle. The highest content of the chitin/glucan complex was present in the mature mycelium, whereas the lowest content was observed in spores. Moreover, chitin predominates in spores, whereas glucan predominates in mycelia during the vegetative phase. Thus, it is possible that the inhibitory effect of SBTX on spore germination but not on the vegetative growth of *A. niger*

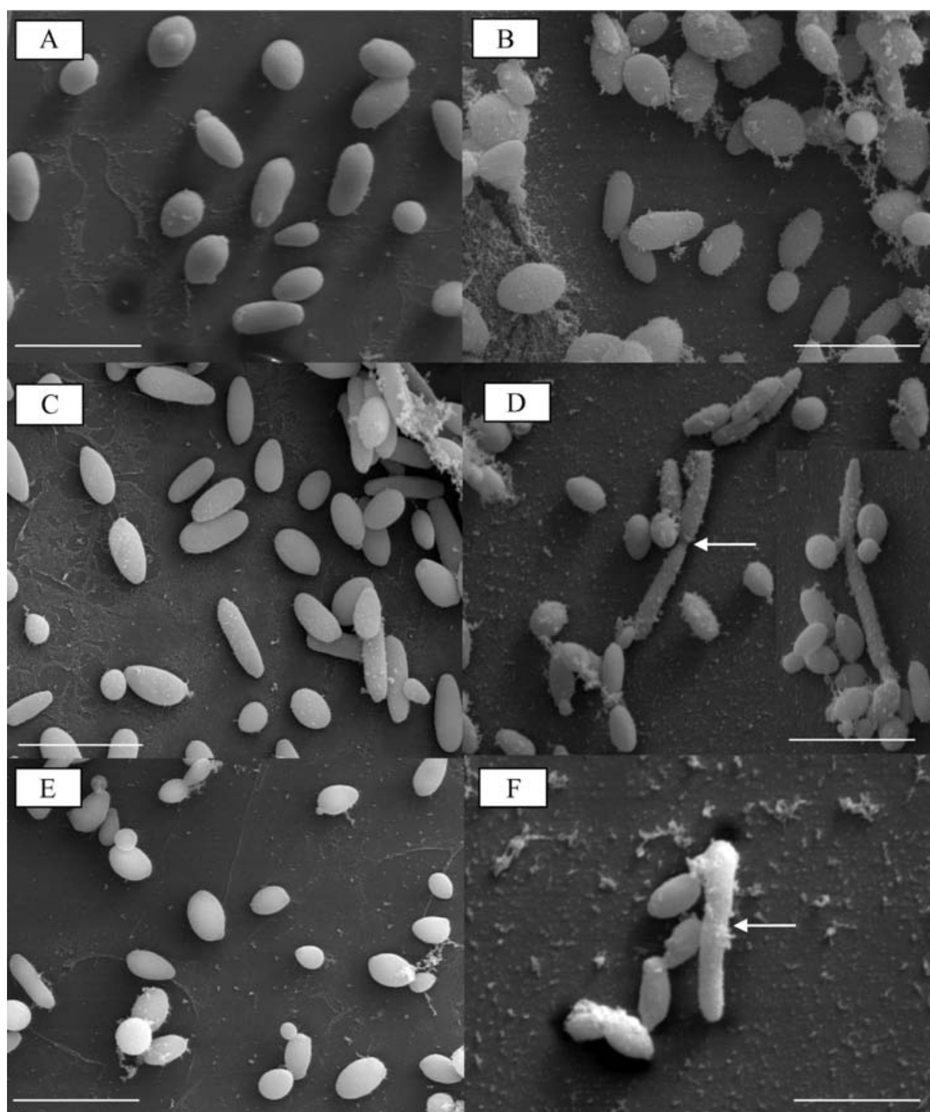

Figure 6. Scanning electron microscopy of yeast cells cultured in the absence (**A**, **C**, **E**) and presence (**B**, **D**, **F**) of soybean toxin (SBTX, 400 μg/mL): *Candida albicans* (**A**, **B**), *Pichia membranifaciens* (**C**, **D**), and *Candida parapsilosis* (**E**, **F**). Arrows point to a constriction between two cells, a characteristic of pseudohyphae. Bars (**A**−**F**): 10 μm.

and *P. herguei* might be due to differences in the cell wall chemical composition over the course of fungal development. The biological mechanism underlying the lack of inhibitory activity of SBTX toward *F. oxysporum* and *F. solani* is difficult to speculate particularly because of the scarcity of comparative studies on the structural constituents of cell walls of different fungi. However, the presence of β-1,6-glucan in addition to chitin and β-1,3-glucan, commonly found in filamentous fungi, contributing to the rigidity and integrity of the cell wall, and the presence of cell wall glycoproteins in the external layer of *F. oxysporum* (*31*) might have a bearing upon the resistance of *F. oxysporum* and *F. solani* to SBTX. Another possibility is that exposure of these fungi to SBTX might induce the cell wall integrity pathway that involves transcriptional activation of cell wall maintenance genes, as described by Jung and Levin (*32*).

Effect of SBTX on Yeast Growth. Many studies have focused on the antifungal activity of plant substances, but few have demonstrated their effects on the morphology and structure of fungi. Moreover, the mechanism of action is not well-understood. Thus, to assess the spectrum of action and mechanisms of inhibition of fungus development by SBTX, *C. albicans*, *C. parapsilosis*, *K. marxiannus*, *P. membranifaciens*, and *S. cerevisae* were used as model cells. Although a complete model of the cell wall of filamentous fungi is not available yet, many similarities exist in the cell walls of the above species of fungi (*11*).

SBTX at 100 μg/mL inhibited the growth of *C. albicans* (54%) and *K. marxiannus* (48%) within 42 h of incubation (**Figure 3A**, **B**). This inhibitory concentration of SBTX on the growth of both yeasts is 56 times less than that found to be lethal to mice and also lower than the amount found per gram of mature soybean seeds (300 μg/g) (*14*). In contrast, it was not inhibitory toward *P. membranifaciens* or *C. parapsilosis*, even at 400 μg/mL (**Figure 3C**,**D**). In fact, for these species, increases in absorbance were observed when compared with controls, particularly for *P. membranifaciens* (**Figure 3C**). Moreover, in both cases, SBTX promoted morphological alterations characterized as pseudohyphae formation (**Figure 4C**,**G**), which was absent in control cells (**Figure 4A**,**E**). This morphological change could explain the increase in absorbances observed (**Figure 3C**,**D**). Interestingly, SBTX (50 and 100 μg/mL) hampered the growth of *C. albicans* and *K. marxiannus* but did not promote pseudohyphae formation in these cells (data not shown). Osborn et al. (*33*) showed that plant defensins isolated from different seeds cause morphological alterations that are often very distinct in some, but not all, tested

fungi. A lipid transfer protein (LTP) from chili pepper also induced pseudohyphae formation in *C. albicans* (*34*).

A variety of conditions promote pseudohyphal growth, such as poor nitrogen, carbon sources in the medium (*35*), and possibly some stress conditions (*36*, *37*). During pseudohyphal formation, the cells become elongated, budding occurs synchronously in a unipolar fashion, and the buds do not separate. This results in chains of cells, which are called pseudohyphae (*38*). In *S. cerevisiae*, the MAP kinase and cAMP-dependent pathways are involved in the generation of pseudohyphae (*38*). Nevertheless, pseudohyphae formation seems to be a physiological reaction associated with both starvation and stress conditions. In starvation, pseudohyphae formation would allow the yeast to forage for nutrients that may be present in the vicinity; under stress condition, it would allow escape from harmful agents (*36*).

Effects of SBTX on Permeabilization and Glucose-Stimulated Acidification of the External Medium of Yeast Cells. Various antifungal proteins have the ability to disturb the cell wall and plasma membrane of fungi, leading ultimately to cell death (*8*, *11*). The ability of SBTX to permeabilize the plasma membrane of *P. membranifaciens* and *C. parapsilosis* cells was also examined. SYTOX Green permeabilization was assessed after 36 h of growth in the presence of SBTX (400 μg/mL) followed by 30 min of SYTOX Green treatment. Fluorescence was observed in both *P. membranifaciens* and *C. parapsilosis*, indicating that both yeast strains allowed penetration of SYTOX Green into the cell in the presence of SBTX (**Figure 4D**,**H**). No fluorescence was detected in control cells cultured in the absence of SBTX (**Figure 4B**,**F**). Although the yeast cells developed pseudohyphae as an attempt to defend themselves from the harmful presence of SBTX, the results reported here highlight the toxic effects of this protein to cells, as fluorescence is the result of SYTOX uptake that occurs in cells that have their membrane compromised. As SYTOX stains only dying or dead cells, the yeast cells were likely dead or dying due to the presence of SBTX (**Figure 4D**,**H**). Staining of damaged cells has been observed by treatment with other plant proteins such as 2S albumin from passion fruit (*13*), the F1 fraction from chili pepper (*34*) seeds, and juncin from *Brassica juncea* seeds (*2*).

To test whether SBTX has any effects on the ability of the yeast cells to transport intracellular protons to the external medium, the glucose-stimulated acidification of the medium was measured in *S. cerevisiae*. This phenomenon is dependent on the H^+-ATPase activity. The presence of SBTX (400 μg/mL) in the culture medium inhibited external medium acidification by 27% (**Figure 5**) when compared to controls. It is possible that such inhibition results from effects on plasma membrane permeabilization, as suggested by SYTOX uptake, and/or interference of the plasma membrane H^+-ATPase pump. The proton pump in the cell membrane of fungi is responsible for the homeostasis of intracellular pH and maintenance of the electrochemical gradient of protons necessary for nutrient uptake (*27*, *39*). Therefore, any disruption of plasma membrane can directly or indirectly alter the function of this translocating enzyme and the traffic of substances through the membrane leading to cell death (*40*). For SBTX to exert direct effects on the plasma membrane, it has to cross the cell wall. It is known that the cell wall of *S. cerevisiae* is permeable to dextrans up to 70 kDa (*41*). Thus, SBTX (44 kDa) could cross the cell wall barrier and reach the plasma membrane, where it might alter function. Alternatively, SBTX could disrupt the cell wall, facilitating its contact with the plasma membrane. Various other proteins from plants are able to inhibit the glucose-stimulated acidification of the external medium. *L. auriculata* lectin (500 μg/mL) promoted 60% inhibition of the acidification of the external medium by *S. cerevisiae* as compared with control cells (*5*).

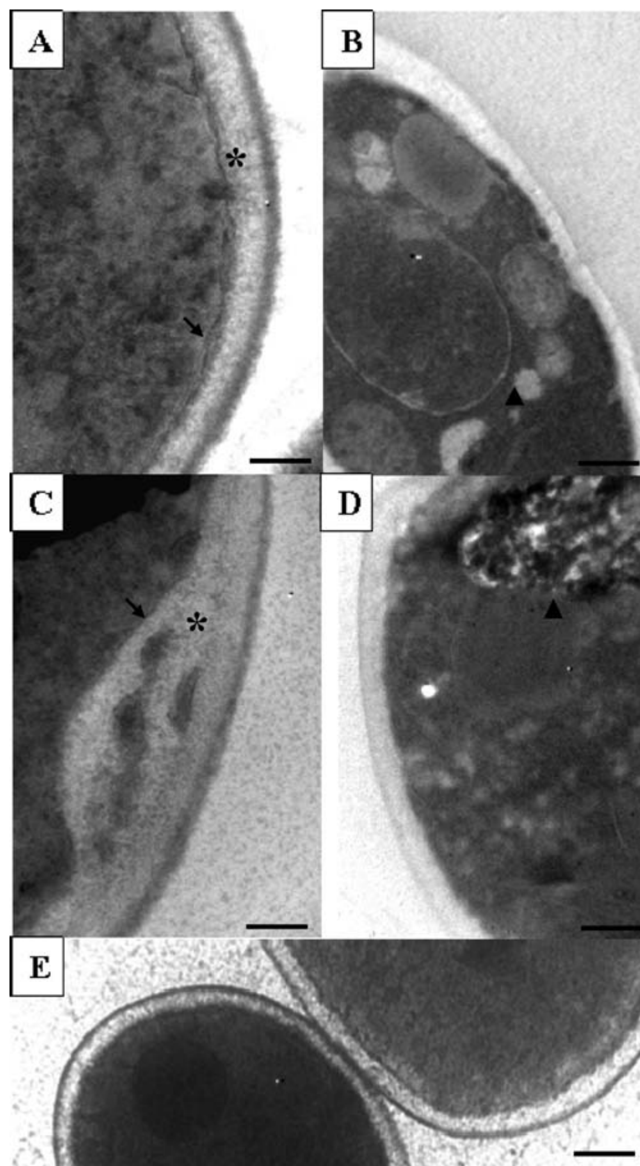

Figure 7. Transmission electron microscopy of *Pichia membranifaciens* and *Candida albicans* cells cultured in the absence (**A**, **B**) and presence (**C**–**E**) of soybean toxin (SBTX, 400 μg/mL): *P. membranifaciens* (**A**–**D**) and *C. albicans* (**E**) cells. Star indicates the disruption of cell wall; small arrows denote plasma membrane, and large arrows depict condensation and shrinkage of cytosol with increased vacuolation and loss of cytosol structure and content in *P. membranifaciens* treated with SBTX. Bars (**A**–**C**), 0.2 μm; (**B**–**D**), 0.4 μm.

The F3 fraction (2 μg/mL) and the F1 peptide (160 μg/mL) from chili pepper seeds caused 100% inhibition of glucose-stimulated acidification of the external medium by *S. cerevisiae* cells (*34*, *42*).

Ultrastructural Alterations in Yeast Cells Induced by SBTX. SEM was employed to better visualize possible morphological changes promoted by SBTX on the plasma membrane of yeast cells, as suggested by optical microscopy and fluorescence studies. Photomicrographs of *C. albicans*, *P. membranifaciens*, and *C. parapsilosis* were taken 36 h after yeast growth in the presence or absence of SBTX (400 μg/mL). Normal cell structure was observed for controls (**Figure 6A**,**C**,**E**) and for *C. albicans* incubated with SBTX (**Figure 6B**). However, morphological changes were observed for *P. membranifaciens* and *C. parapsilosis*, as aggregation of cells and pseudohyphae formation (**Figure 6D**,**F**). For instance, aggregation of

P. membranifaciens and *C. parapsilosis* cells incubated with SBTX was about 10 times higher than that of control cells.

Diz et al. (*34*) observed pseudohyphae formation in *C. albicans* cells in the presence of the F1 fraction (35 μg/mL) of chili pepper. Loss of asymmetry, deformations, clumps, and deep wrinkles in cells incubated with Pf2 (100 μg/mL), a 2S albumin-homologous protein from passion fruit seeds, were reported in *S. cerevisiae* (*13*).

TEM of *P. membranifaciens* (**Figure 7A−D**) cells indicated that there was disruption of the cell wall in SBTX-treated cells (400 μg/mL) (**Figure 7C,D**). Moreover, condensation and shrinkage of heavily granulated cytosol, increased vacuolation, and loss of normal structure and content were observed when compared with control cells (**Figure 7A,B**). Structural disarrangement of the plasma membrane caused by the presence of SBTX was verified by SYTOX treatment (**Figure 4D**). Thus, these data together suggest that the antifungal properties of SBTX are triggered by alterations in the cell surface. No morphological modifications of *C. candida* in the presence of SBTX were observed by TEM (**Figure 7E**), similar to the results from SEM (**Figure 6B**).

In conclusion, this study reinforces previous data (*14*) on the antifungal properties of SBTX and reports new information about the molecular events that take place during pathogen−protein interaction. It is noteworthy that the toxic concentration found here for SBTX, a naturally occurring toxin which is inhibitory to phytopathogenic fungi and pathogenic yeasts, is far below that found to be lethal to mice. Therefore, SBTX has potential to be used for the development of new antifungal drugs or transgenic crops with enhanced resistance to pathogens.

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