

Capsicum annuum L. trypsin inhibitor as a template scaffold for new drug development against pathogenic yeast

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Abstract A 6,000 Da peptide, named *CaTI*, was isolated from *Capsicum annuum* L. seeds and showed potent inhibitory activity against trypsin and chymotrypsin. The aim of this study was to determine the effect of *CaTI* on *Saccharomyces cerevisiae*, *Candida albicans*, *Candida tropicalis* and *Kluyveromyces marxianus* cells. We observed that *CaTI* inhibited the growth of *S. cerevisiae*, *K. marxianus* as well as *C. albicans* and induced cellular agglomeration and the release of cytoplasmic content. No effect on growth was observed in *C. tropicalis* but morphological changes were noted. In the spot assay, different degrees of sensitivity were shown among the strains and concentrations tested. Scanning electron microscopy showed that *S. cerevisiae*, *K. marxianus* and *C. albicans*, in the presence of *CaTI*, exhibited morphological alterations, such as the formation of pseudohyphae, cellular aggregates and elongated

forms. We also show that *CaTI* induces the generation of nitric oxide and interferes in a dose-dependent manner with glucose-stimulated acidification of the medium mediated by H^+ -ATPase of *S. cerevisiae* cells.

Keywords Trypsin inhibitor · Antimicrobial peptides · *Capsicum annuum* · Yeast · *CaTI*

Introduction

Antimicrobial peptides (AMPs) are important components of the first line defense of innate immune systems in various species, including mammals, amphibians, insects and plants (Broekaert et al. 1995; Thomma et al. 2002; Castro and Fontes 2005; Senyürek et al. 2009; Padovan et al. 2010). AMPs show broad-spectrum antimicrobial activity against a wide range of pathogens (Zasloff 2002; Senyürek et al. 2009), and their mode of action is not completely understood (Brodgen 2005). It is believed that most AMPs inhibit or kill microorganisms in membrane interactions, which can be accomplished by membrane penetration or membrane disintegration or even by pore formation (Yeaman and Yount 2003; Brodgen 2005; Senyürek et al. 2009). During recent years, many plant proteins and peptides that can inhibit fungal development have been isolated and characterized. Examples of these compounds include 2S

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albumins (Agizzio et al. 2003, 2006; Ribeiro et al. 2011), thionins (García-Olmedo et al. 1998; Stec 2006; Berrocal-Lobo et al. 2009), defensins (Thevisen et al. 2003; Games et al. 2008; Mello et al. 2011; Rogozhin et al. 2011), lipid transfer proteins (LTPs) (Diz et al. 2006; Carvalho and Gomes 2007; Zottich et al. 2011) and proteinase inhibitors (PIs) (Terras et al. 1993; Macedo et al. 2007; Ribeiro et al. 2007; Lopes et al. 2009).

Various classes of AMPs have been implicated in the resistance mechanism of plants against pathogens including PIs (Sels et al. 2008). PIs have been isolated and identified in different species of organisms, including plants. In plants, PIs are generally either found as constitutive components in storage tissues, such as seeds and tubers, or are expressed in response to pest and pathogen attacks (Ryan 1990; Prasad et al. 2010). PIs can inhibit proteolytic activity of enzymes from different sources by competing with substrates for access to enzyme active sites (Clementea and Domoney 2006). In seeds, PIs maintain several physiological processes, such as regulating endogenous proteinase levels (Chye et al. 2006), acting as storage proteins (Mandal et al. 2002), regulating programmed cell death (Solomon et al. 1999), and protecting plant tissues from pests (Haq et al. 2004) and invading microorganisms (Mosolov and Valueva 2006; Sels et al. 2008).

During the last 20 years, the incidence of invasive fungal infections has increased considerably; *Candida* bloodstream infections have steadily increased since the 1980s and account for 8–15% of all bloodstream infections (Hobson 2003; Garbino 2004; Thevisen et al. 2007). A number of surveys have revealed that, in the 1980s, more than 75% of *Candida* infections were caused by *Candida albicans* (Pfaller et al. 2006). The cell wall of microorganisms such as yeast and filamentous fungi determines the cell shape and integrity of the organism during growth and cell division. Because the cell wall is a structure that is both essential for the fungus and absent from the mammalian host, the cell wall represents an attractive target for new antifungal agents that are needed to treat a growing number of human infections (Georgopapadakou and Tkacz 1995). In this work, we will demonstrate the potential of *CaTI*, which is a proteinase inhibitor from chili pepper seeds, as a new template scaffold for the development of new drugs, especially against pathogenic yeast.

Materials and methods

Yeast strains

The yeast *Candida albicans* (CE022), *Candida tropicalis* (CE017), *Kluyveromyces marxianus* (CE025) and *Saccharomyces cerevisiae* (1038) were obtained from the Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil. Yeast were maintained on Sabouraud agar (1% peptone, 4% glucose and 1.7% agar–agar).

Antimicrobial activity assay

CaTI was purified from seeds of *C. annuum* L., as previously described by Ribeiro et al. (2007). For the preparation of yeast cell cultures, an inoculum from stock was transferred to Petri dishes containing Sabouraud agar and allowed to grow at 30°C for 2 days; after this period, the cells were transferred to sterile culture medium (10 ml). Yeast cells were quantified in a Neubauer chamber for further calculation of appropriate dilutions. A quantitative assay for yeast growth inhibition was performed following the protocol developed previously by Broekaert et al. (1990) with some modifications. Antifungal activity of *CaTI* was assayed based on microspectrophotometry of liquid cultures grown in 96-well microtiter plates, at 30°C, in the presence of 16, 32 and 64 $\mu\text{g ml}^{-1}$ *CaTI* and with 1×10^4 cells ml^{-1} in 1 ml of Sabouraud agar. Negative (without *CaTI*) and positive (with 10 $\mu\text{g ml}^{-1}$ fluconazole added) controls were also run. The optical densities were plotted as a function of *CaTI* concentration.

For the spot assay a suspension of 1×10^4 cells ml^{-1} of each yeast culture at the end of the antimicrobial activity assay were directly diluted 20-fold. This first dilution was serially diluted five times each dilution at 1:5. Aliquots (3 μl) of each serially dilution were spotted onto Petri dishes containing fresh Sabouraud agar (with no *CaTI*) and incubated at 30°C for 24 h, following the protocol developed previously by Koo et al. (2004) with some modifications.

Optical microscopy analysis

After 24 h of the yeast growth inhibition assay, yeast cells were separated from the growth medium using centrifugation, washed in Sabouraud broth and

visualized using an optical microscope at 1000× magnification (Axiovert 135). The yeast cells grown in the absence of *CaTI* were also determined.

Plasma membrane permeabilization assay

Plasma membrane permeabilization was measured by SYTOX Green (Molecular Probes Invitrogen, EUA) uptake, as described previously by Thevissen et al. (1999) with some modifications. Cells of *S. cerevisiae* and *C. albicans* (1×10^4 cells ml⁻¹) were incubated in the presence of *CaTI*, at the concentration of 32 µg ml⁻¹ for 24 h. One hundred-microlitre aliquots of the suspension of yeast cells were incubated with 0.2 µM SYTOX Green in 1.5 ml microcentrifuge tubes for 30 min at 25°C with periodic agitation. The cells were observed in an optical microscope (DIC, Axiophoto Zeiss) equipped with a fluorescence filter set for fluorescein detection (excitation wavelengths, 450–490 nm; emission wavelength, 500 nm).

Scanning electron microscopy analysis

For scanning electron microscopy, the yeast cells after the yeast growth inhibition assay in the absence or presence of *CaTI* (32 µg ml⁻¹) were fixed for 30 min at room temperature in a solution containing 2.5% glutaraldehyde and 4.0% formaldehyde in 0.05 M cacodylate buffer at pH 7.0. Subsequently, cells were rinsed three times in 0.05 M cacodylate buffer at pH 7.0, post-fixed for 30 min at room temperature with 2.0% osmium tetroxide diluted in the same buffer, and rinsed again with the same buffer. After this procedure, the yeast cells were dehydrated in alcohol, critical point dried in CO₂, covered with 20 nm gold and observed with a DSEM 962 Zeiss scanning electron microscope. The yeast cells grown in the absence of *CaTI* were also evaluated.

Transmission electron microscopy analysis

For transmission electron microscopy (TEM), the yeast cells grown for 24 h in Sabouraud broth in the presence or absence of *CaTI* (32 µg ml⁻¹) were then fixed for 30 min at room temperature in a solution containing 2.5% glutaraldehyde (vol/vol) and 4.0% paraformaldehyde (vol/vol) in 50 mM cacodylate buffer (pH 7.2). After fixation, the materials were washed and post-fixed in 1.0% (wt/vol) osmium

tetroxide in the corresponding buffer for 1 h at room temperature. The samples were dehydrated in a graded acetone series [30, 50, 70, 90 and 100% (vol/vol)] and embedded in Epon resin (Polybeded). Ultrathin sections (0.1 µm) were laid on copper grids, stained with uranyl acetate for 10 min followed by lead citrate for 5 min and were then observed with a ZEISS 900 transmission electron microscope (Zeiss company, Germany) operating at 80 kV.

Nitric oxide induction determination assay

To evaluate whether the action mechanism of *CaTI* involves the induction of oxidative stress, we have used dyes that indicate the presence of reactive oxygen species. After the growth inhibition assay in the absence and presence of 32 µg ml⁻¹ of *CaTI*, the induction of endogenous production of nitric oxide in yeast cells was evaluated using the fluorescent probe, 3,4-diaminomethyl-2',7'-difluorescein diacetate (DAF-2 DA), following methods previously described by Mello et al. (2011), with some modifications. Fifty-microliter aliquots of the yeast cell suspension were incubated with 200 µM of the fluorescent probe for 2 h at room temperature with periodic agitation. After this period, the cells were transferred to slides, covered with cover slips and analyzed with a fluorescence microscope (Axiophoto Zeiss) equipped with a fluorescence filter set for fluorescein detection (excitation wavelengths 450–490 nm and emission wavelengths 500 nm). The results are representative of one triplicate experiment.

Inhibition of the glucose-stimulated acidification of the medium by *S. cerevisiae*

The Sabouraud broth (200 ml) containing the *S. cerevisiae* culture was shaken for 16 h at 30°C with good aeration. The culture ($A_{600} = 0.2$) was pelleted using centrifugation at 3000×g (5 min, 4°C) followed by three water-washing steps. The effect of *CaTI* was determined by incubation of *S. cerevisiae* cells (1×10^7 cells) with 0.8 ml of 10 mM Tris–HCl, at pH 6.0. *CaTI* was added to the final concentrations of 16, 32 and 64 µg ml⁻¹. After the pre-incubation time (30 min), 0.2 ml of 0.5 M glucose solution was added to a final concentration of 100 mM. Then, the pH was measured at each subsequent minute for the next 30 min. Control experiments (Tris–HCl buffer was

added instead of *CaTI*) were performed to evaluate the influence of *CaTI* on H^+ extrusion by yeast cells (Gomes et al. 1998). The concentration of H^+ extrusion was calculated as the difference between initial ($T = 0$) and final ($T = 60$ min) pH (ΔpH), the value of which was obtained from the equation, $pH = -\log[H^+]$. The graphs were drawn based on the assumption that the values obtained using the control corresponded to 100% acidification. The results of inhibition of the glucose-stimulated acidification are shown by calculating the average values and standard deviations of triplicate measurements for each experiment.

Results and discussion

Antimicrobial activity of *CaTI* against yeast

After the purification processes (Ribeiro et al. 2007), we tested the effects of *CaTI* as an inhibitor of the growth of various pathogenic and non-pathogenic yeast strains. We noted a complete growth inhibition of *S. cerevisiae* in the presence of $64 \mu g ml^{-1}$ *CaTI* (Fig. 1a). No growth inhibition of *C. tropicalis* was observed in the presence of all *CaTI* concentrations (Fig. 1b). An inhibition of 71, 76 and 88% in the growth of *C. albicans* was observed in the presence of 16, 32 and $64 \mu g ml^{-1}$, respectively (Fig. 1c). For *K. marxianus*, an inhibitory effect of 53, 51 and 68% was observed in the presence of 16, 32 and $64 \mu g ml^{-1}$ of *CaTI* (Fig. 1d). Fluconazole at $10 \mu g ml^{-1}$ presented antifungal activity for all strains tested, especially for *C. albicans* and *K. marxianus* that had their growth inhibited at 100%. In addition to *CaTI*, the antimicrobial action of other PIs has also been described. For example, Kim et al. (2005) demonstrated that PT-1, a 5.6 kDa trypsin-chymotrypsin protease inhibitor isolated from the tubers of potato (*Solanum tuberosum* L cv. Gogu), has antimicrobial activity against the human pathogenic fungus *Candida albicans*, the plant pathogenic fungus *Rhizoctonia solani* and the pathogenic bacterium *Clavibacter michiganense* subsp. *michiganinse*. The antifungal activity of PIs was also observed by Lopes et al. (2009) in experiments with three PIs isoforms isolated from *Acacia plumosa* seeds, ApTIA, ApTIB and ApTIC. The ApTI isoforms inhibited normal development of *Aspergillus niger*, *Colletotrichum* sp. P10 and *Fusarium moniliforme* hyphae by

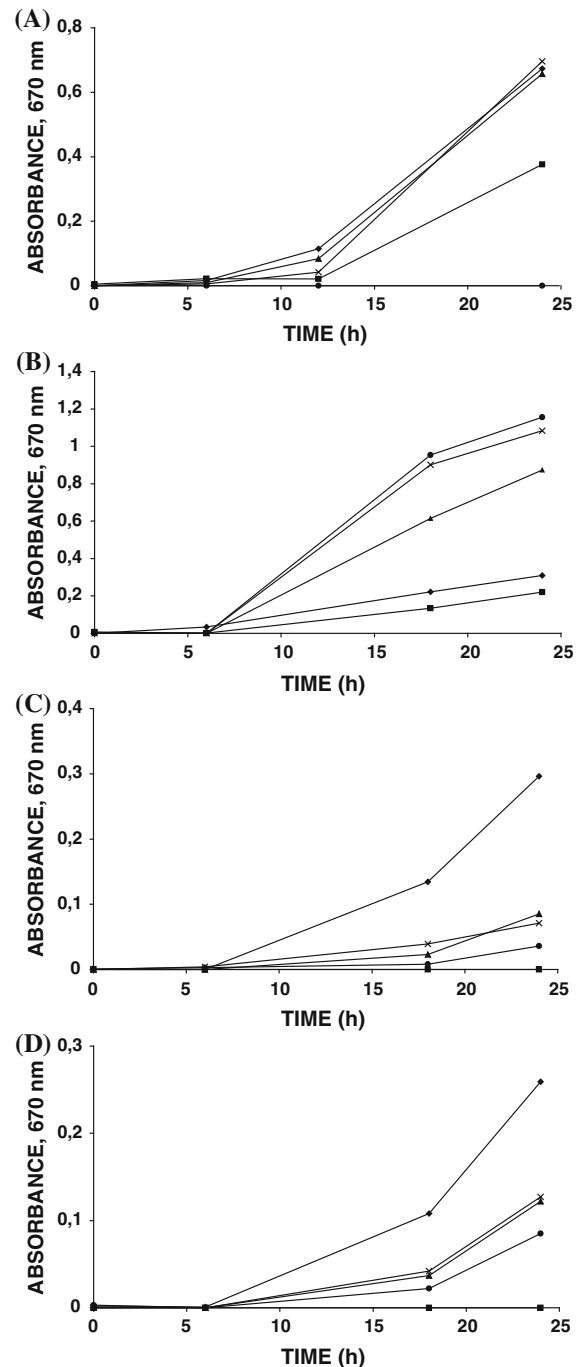


Fig. 1 Antimicrobial activity of *CaTI* against different yeast. **a** *S. cerevisiae*, **b** *C. tropicalis*, **c** *C. albicans* and **d** *K. marxianus*. Filled diamond Control; filled square Fluconazole $10 \mu g ml^{-1}$; filled triangle *CaTI* $16 \mu g ml^{-1}$; times *CaTI* $32 \mu g ml^{-1}$ and filled circle *CaTI* $64 \mu g ml^{-1}$. Experiments were performed in triplicate and the standard errors (coefficients of variations were $<20\%$) were omitted for clarity

generating an inhibition halo around the disk containing the higher concentrations of the fungus spores. In several studies, it has been shown that proteases secreted by fungi could be important pathogenic factors because the inhibition of these factors significantly reduces fungal infection (Soares-Costa et al. 2002; Satheesh and Murigan 2011).

The effects of *CaTI* on *S. cerevisiae*, *C. tropicalis*, *C. albicans* and *K. marxiannus* growth was also observed in spot assays (Fig. 2). Different degrees of sensitivity were observed among the strains and concentrations tested. *CaTI* was most toxic to *S. cerevisiae*, whereby a marked inhibition was detected on incubating medium containing $64 \mu\text{g ml}^{-1}$ *CaTI* but not on media containing lower concentrations (Fig. 2a). Based on this assay, it is also clear that there is a toxic effect of *CaTI* on *S. cerevisiae*; at this concentration and incubation time, *CaTI* functions as a fungicide. *C. tropicalis* was the least sensitive strain; no marked growth inhibition was detected compared to control cells regardless of the *CaTI* concentration (Fig. 2b). For *C. albicans* (Fig. 2c) and *K. marxiannus* (Fig. 2d), different degrees of sensitivity to *CaTI* were observed and, at the higher concentration, a reduction in the number of colonies also indicated a fungicide effect on some cells. When we analyzed the growth curves of *C. albicans* and *K. marxiannus* (Fig. 1c and d, respectively), at least a 50% growth inhibition (IC_{50}) was observed for 16 and $32 \mu\text{g ml}^{-1}$ *CaTI*. This range of IC_{50} is similar to the range of values observed in the *C. albicans* (Fig. 2c) and *K. marxiannus* (Fig. 2d) spot assays. Another plant peptide, *PvDI* defensin, which is purified from *Phaseolus vulgaris* seeds, presented significant antifungal activity by inhibiting the growth of *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii*, *K. marxiannus* and *S. cerevisiae* and displaying inhibitory activity against the growth of various species of phytopathogenic fungus at concentrations that ranged from 25 to $100 \mu\text{g ml}^{-1}$ (Games et al. 2008). A 14.3 kDa proteinase inhibitor that was isolated and purified from the leaves of *Coccinia grandis* strongly inhibited various pathogenic and non-pathogenic microorganisms, including *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Candida albicans*, *Penicillium notatum* and *Aspergillus flavus*. This PI induced a complete inhibition of the germination of spores from *K. pneumoniae* and *A. flavus* at a concentration of 0.5 mg ml^{-1} , whereas a reduction in the growth of hyphae was observed at a concentration of 0.01 mg ml^{-1} . The growth of *S. aureus*,

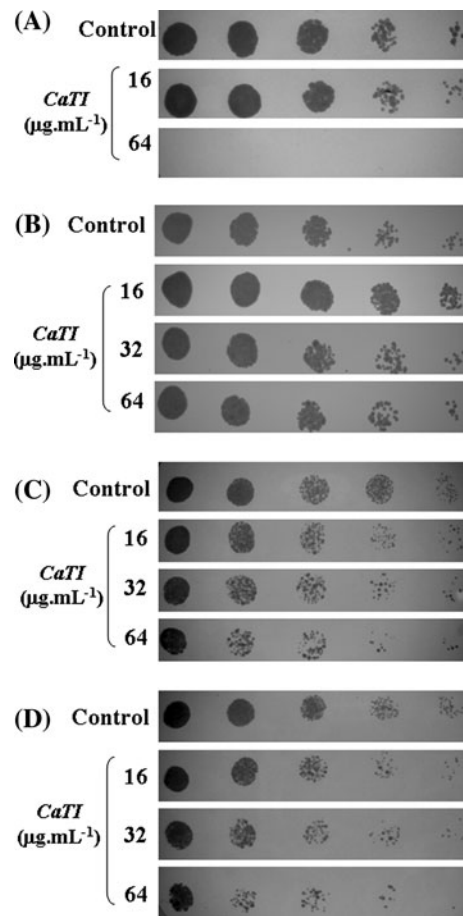


Fig. 2 Sensitivity of yeast cell growth to *CaTI* (spot assay). A suspension of 1×10^4 cells ml^{-1} , at the end of the antimicrobial activity assay, of **a** *S. cerevisiae*, **b** *C. tropicalis*, **c** *C. albicans* and **d** *K. marxiannus* were diluted 20-fold and subsequently serially diluted five times at 1:5. Aliquots (3 μl) of each serially diluted were spotted onto Petri dishes containing fresh Sabouraud agar (with no *CaTI*) and incubated at 30°C for 24 h

B. subtilis, *C. albicans* and *C. neoformans* was also inhibited, but these strains were less sensitive compared to *K. pneumoniae* and *A. flavus*. Morphologically, the fungi treated with this PI showed significantly fewer hyphal tips. These effects from PIs suggest that PIs can exert their activity by inhibiting serine proteases involved in the growth and differentiation of microbial cells (Satheesh and Murigan 2011).

To get an understanding of the mode of action of *CaTI*, we examined the yeast cells using optical (Figs. 3, 4), scanning electron (Fig. 5) and TEM (Fig. 6) to assess the details of the morphological changes observed at the end of the growth inhibition

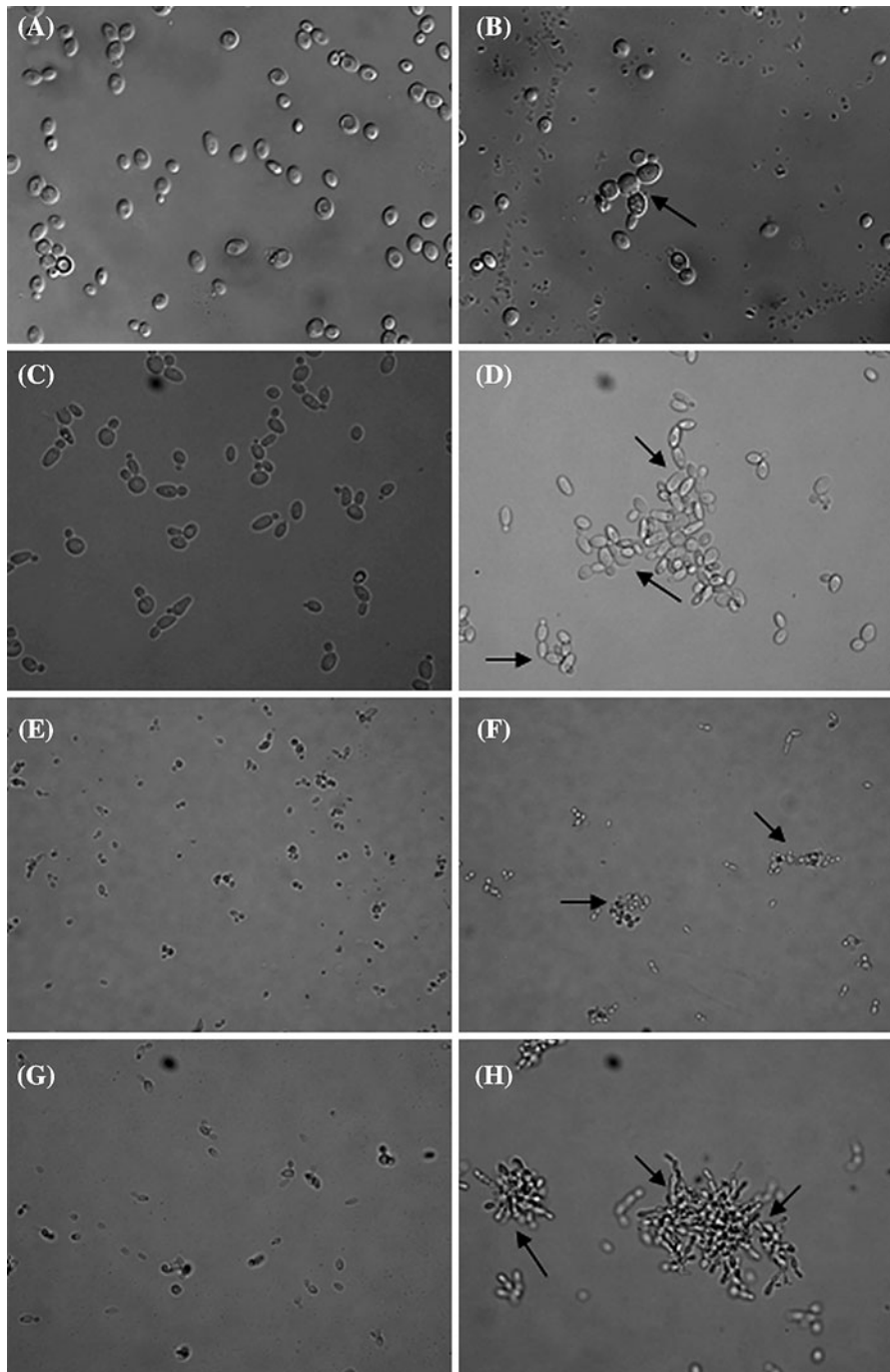


Fig. 3 Optical microscopy of yeast cells in the presence of *CaTI*. **a** Control cells of *S. cerevisiae*; **b** cells of *S. cerevisiae* treated with *CaTI* ($32 \mu\text{g ml}^{-1}$); **c** control cells of *C. tropicalis*; **d** cells of *C. tropicalis* treated with *CaTI* ($32 \mu\text{g ml}^{-1}$); **e** control

cells of *C. albicans*; **f** cells of *C. albicans* treated with *CaTI* ($32 \mu\text{g ml}^{-1}$); **g** control cells of *K. marxianus*; **h** cells of *K. marxianus* treated with *CaTI* ($32 \mu\text{g ml}^{-1}$). Arrows shows cellular agglomeration

assay. Normal growth and development were observed for all control cells. For the samples incubated with *CaTI*, a cellular agglomeration was observed for all

yeast strains (Fig. 3b, d, f, h); for Fig. 3h, there were noticeable elongated cells, which possibly indicate the transition from yeast to hyphal growth.

The ability of *CaTI* to permeabilize the plasma membrane of yeast cells was examined in this study. Membrane permeabilization was assessed after 24 h of growth in the presence of *CaTI* and 30 min after the addition of SYTOX Green. When observed with a fluorescence microscope, the cells of *S. cerevisiae* (Fig. 4d) and *C. albicans* (Fig. 4h) showed SYTOX Green fluorescence in the presence of *CaTI*, as compared to controls where cells were grown in the absence of *CaTI* (Fig. 4b, f). Different plant proteins/peptides have also been found to permeabilize membranes and to modulate ion flux across membranes and have since become popular models for understanding how ion channel proteins function (Abad et al. 1996; Regente et al. 2005; Agizzio et al. 2006).

When the samples were analyzed using scanning electron microscopy, fine details could be observed. *S. cerevisiae* cell growth in the presence of *CaTI* (Fig. 5b) exhibited morphological alterations, especially in the cell morphology and in the cell wall. In addition, agglutinated material aggregates on the surface of the cells were also observed. The presence of these aggregates may have been caused by the leakage of cytoplasmic materials. Cells exposed to *CaTI* also showed an irregular wrinkled cell wall surface, which was not observed in untreated cells (Fig. 5a). For *C. tropicalis*, a significant variation in cell size was observed; in addition, this strain showed cellular aggregates and elongated forms but did not show pseudohyphae (Fig. 5d). For *C. albicans*, although it is a dimorphic yeast that can grow alternately between a yeast phase and a hyphal phase depending on environmental conditions, compared to control cells (Fig. 5e), treatment with an inhibitory concentration of *CaTI* ($32 \mu\text{g ml}^{-1}$) (Fig. 5f) did not cause the cells to lose their yeast phase morphology; no significant variation in cell size was noted. The presence of a large amount of aggregate on the cell surface and a complete agglomeration of the cells was also observed (Fig. 5f). A significant inhibition in the growth of the *K. marxianus* strain in the presence of *CaTI* occurred, and morphological changes were observed in these cells; therefore, growth inhibition can be detected in this yeast species. Pseudohyphae formation was observed during the development of treated cells (Fig. 5h), which confirms the observations made under a light microscope. Moreover, significant variations in cell size were noticed, and

aggregates on the cell surface were observed (Fig. 5h). Other plant peptides, which have also been isolated from seeds, have been found to induce changes in yeast morphology, and these changes may be associated with the presence of these peptides in the medium. A purified 9 kDa peptide, *Cc-LTP1*, with homology to LTPs was isolated from *Coffea canephora* seeds and promoted morphological changes in yeast. These changes included the formation of pseudohyphae by *C. tropicalis* cells; in addition, *Cc-LTP1* exhibited antifungal activity against *C. albicans* (Zottich et al. 2011). Koo et al. (2004) showed that *Pn-AMP1*, a plant defense protein, induces various morphogenic alterations, such as cell bursts or wrinkling, in the cells of *S. cerevisiae* and *C. albicans*. Several lines of evidence indicate that the main action of *Pn-AMP1* is actin depolarization, and cell bursts or wrinkling may be a downstream phenotype of actin depolarization, depending on the mode of growth and the activity of actin regulatory proteins. We conclude that the ability of a plant defense protein to induce actin depolarization is important for its antifungal activity. *C. tropicalis* cells observed with TEM showed normal ultrastructure development in the control cells (Fig. 6a, b); however, cells treated with *CaTI* exhibited disorganization of the plasma membrane (Fig. 6c) as well as disorganization of the cytoplasm (Fig. 6d) and cell wall disassembly (Fig. 6e, f). Not all of these features were observed in every cell, but they occurred frequently. PR-5 proteins, similar to many AMPs, can cause membrane leakage in target microorganisms and artificial membranes (Anzlovar et al. 1998; García-Olmedo et al. 1998). It has been demonstrated that osmotin induces rapid cell death in yeast. In addition, there is specificity between the antifungal activity of a specific PR-5 family protein and the plasma membrane of its target cell (Abad et al. 1996; Yun et al. 1998; Anzlovar et al. 1998). Agizzio et al. (2006) demonstrated that a 2S albumin-homologous protein from passion fruit seeds induces several morphological alterations in cell shape, cell surface, cell wall and bud formation, as well as in the organization of intracellular organelles in *S. cerevisiae* cells. Another peptide, isolated from seeds, *PvDI*, was also shown to induce morphological alterations of yeast cells, including plasma membrane blebbing, disappearing and shrinking cytosol and disorganization of the nucleus and other organelles (Mello et al. 2011).

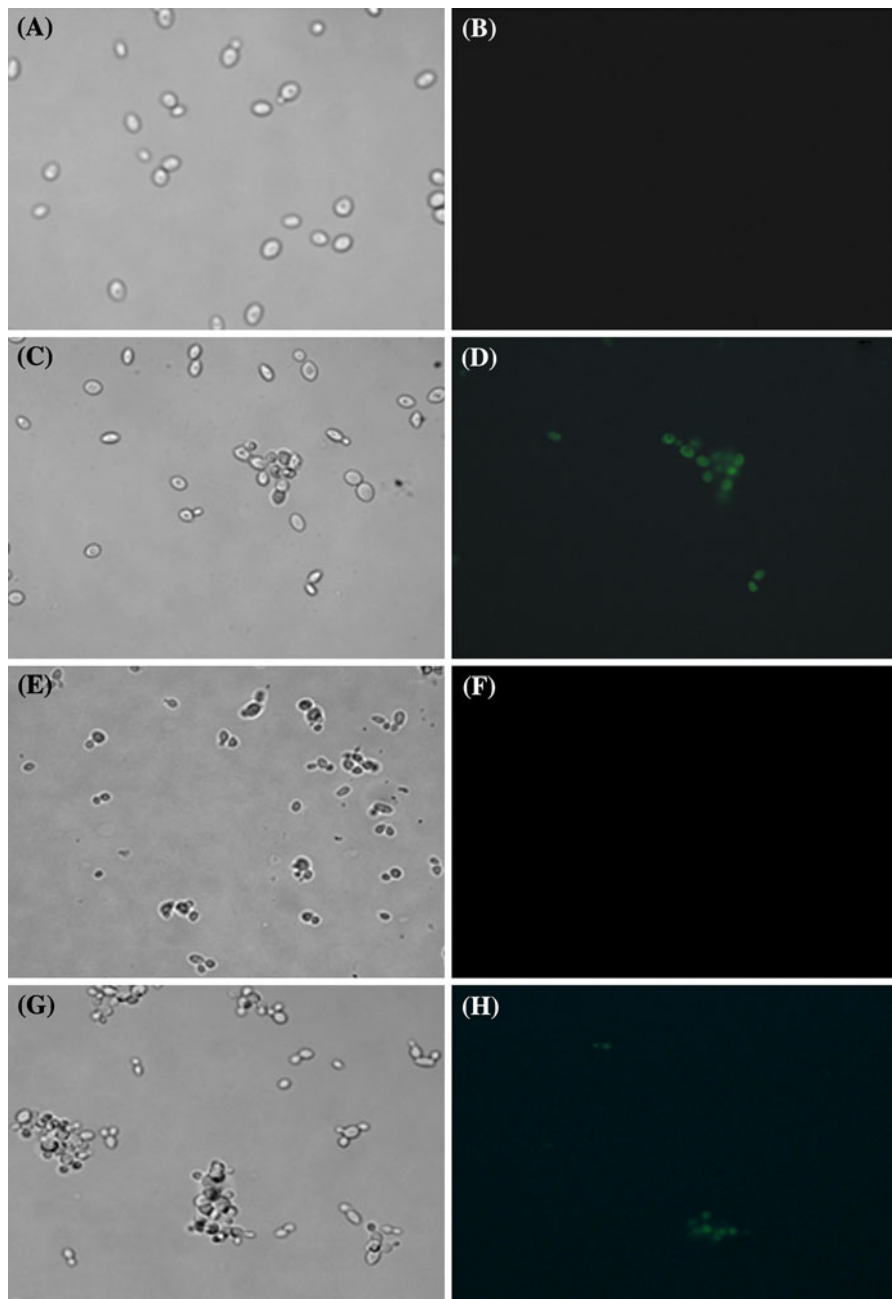


Fig. 4 Membrane permeabilization assay performed with fluorescence microscopy of different yeast cells treated with SYTOX Green. **a, b** Control cells of *S. cerevisiae*; **c, d** cells of *S. cerevisiae* treated with *CaTI* ($32 \mu\text{g ml}^{-1}$); **e, f** control cells

of *C. albicans*; **g, h** cells of *C. albicans* treated with *CaTI* ($32 \mu\text{g ml}^{-1}$). **a, c, e, g** Cells viewed by DIC; **b, d, f, h** cells viewed by fluorescence

Nitric oxide induction determination assay

Some authors have reported that some plant defensins may be involved in the induction of oxidative stress in fungal cells (Aerts et al. 2007). Most of the oxygen

free radicals can be generated in elevated quantities during the reduction of oxygen and lead to cell injury; however, under normal physiological conditions, minimal amounts of oxygen free radicals are produced (Maiese et al. 2010). Using a fluorescence microscope,

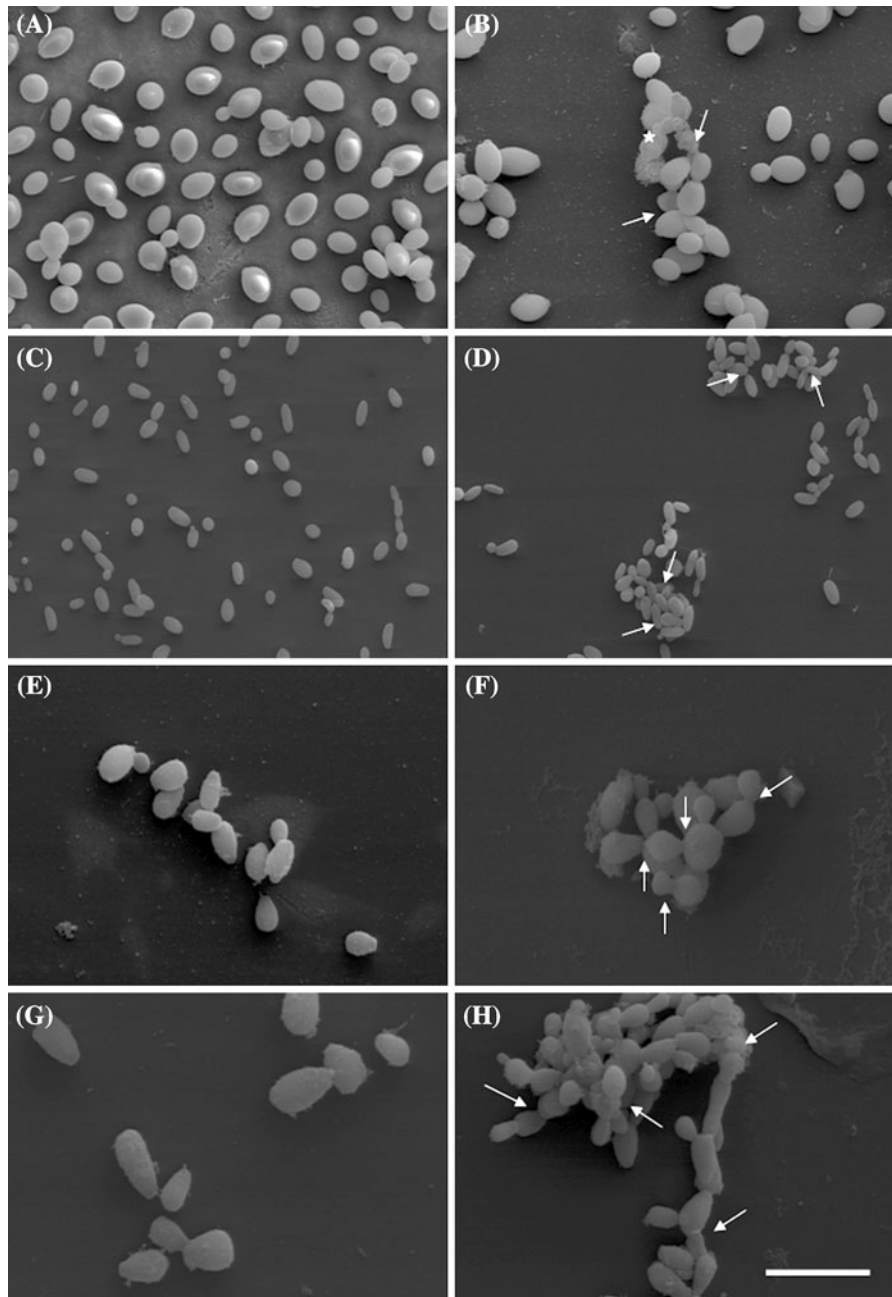


Fig. 5 Scanning electron microscopy of yeast cells in the presence of *CaTI*. **a** Control cells of *S. cerevisiae*; **b** cells of *S. cerevisiae* treated with *CaTI* ($32 \mu\text{g ml}^{-1}$); **c** control cells of *C. tropicalis*; **d** cells of *C. tropicalis* treated with *CaTI* ($32 \mu\text{g ml}^{-1}$); **e** control cells of *C. albicans*; **f** cells of

C. albicans treated with *CaTI* ($32 \mu\text{g ml}^{-1}$); **g** control cells of *K. marxianus*; **h** cells of *K. marxianus* treated with *CaTI* ($32 \mu\text{g ml}^{-1}$). The *arrows* show cellular agglomeration and presence of pseudohyphae and the *star* indicates cell wall wrinkling. *Bars*: **a, b** $4 \mu\text{m}$; **c, d** $9 \mu\text{m}$; **e–g** $2 \mu\text{m}$; **h** $3 \mu\text{m}$

we demonstrated NO induction in *S. cerevisiae* (Fig. 7d), *C. tropicalis* (Fig. 7h), *C. albicans* (Fig. 7l) and *K. marxianus* (Fig. 7p) cells after pre-incubation with $32 \mu\text{g ml}^{-1}$ of *CaTI* for 24 h, compared to

control cells (Fig. 7b, f, j, n, respectively). Reactive oxygen species (ROS) include superoxide free radicals, hydrogen peroxide, single oxygen, nitric oxide and peroxynitrite, and most of these species are

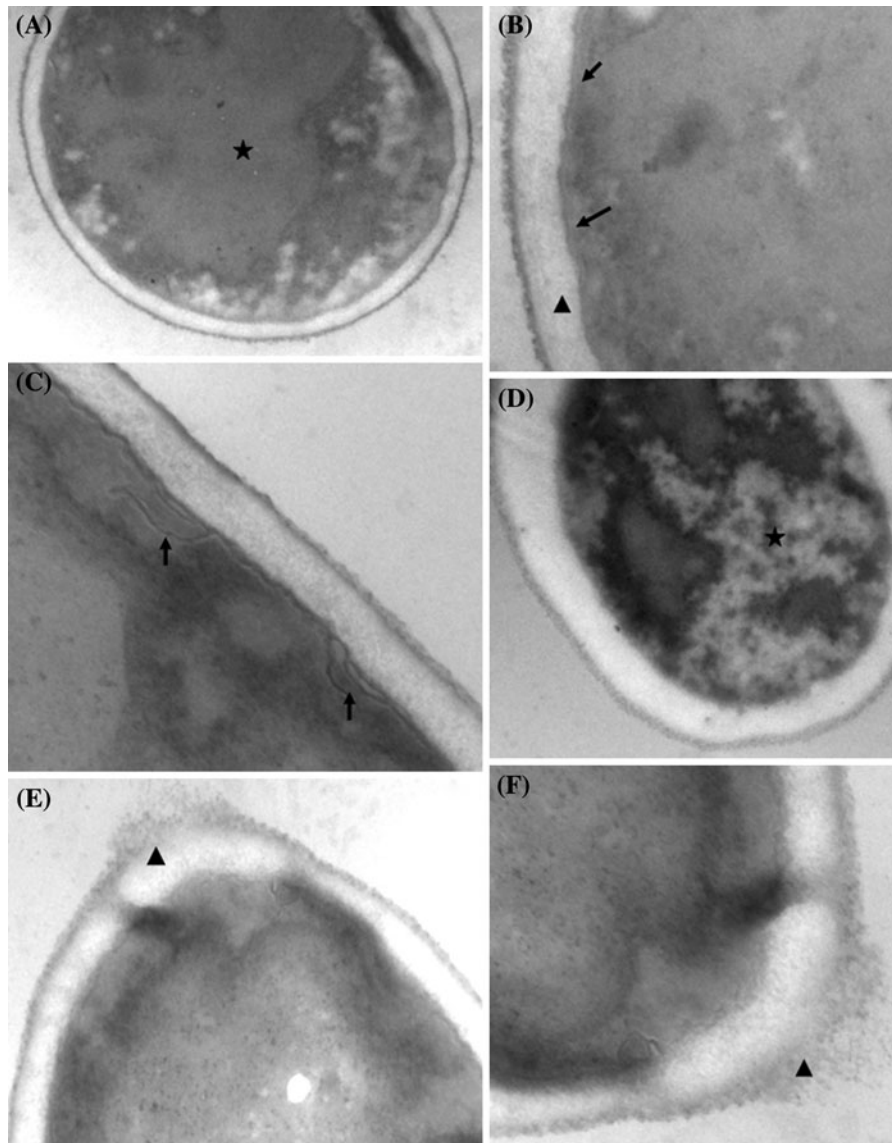


Fig. 6 Transmission electron microscopy micrographs in transverse sections of *C. tropicalis* in the presence of *CaTI*. **a**, **b** Control (absence of *CaTI*). The arrows indicate the integrity of

the plasma membrane. **c**, **f** Presence of *CaTI*. **c**, **d** Arrows indicate disorganization of the plasma membrane and of the cytoplasm. **e**, **f** Cell wall disassembly. Star nucleus; arrow head cell wall

produced at basal levels at normal physiological conditions, which explains the low fluorescence that was observed in the control cells (Fig. 7b, f, j, n). *CaTI* may induce higher levels of NO, which may be involved in different metabolic mechanisms of yeast growth arrest or death. Additional studies have demonstrated that increased metabolic rates could be detrimental to animals in an elevated oxygen environment (Muller et al. 2007). Current studies show that oxygen free radicals are associated with cellular

injury and accumulated toxicity in an organism (Yui and Matsuura 2006), and that this oxidative stress leads to the destruction of multiple cell types through apoptotic pathways (Lin and Maiese 2006; Chong et al. 2006; De Felice et al. 2007). Aerts et al. (2007) also utilized the dye, DAF-2 DA, to demonstrate the generation of ROS by *C. albicans* treated with *Rs*-AFP₂. This finding provided a direct link between ROS generation and the antifungal effects of this peptide. Mello et al. (2011) have

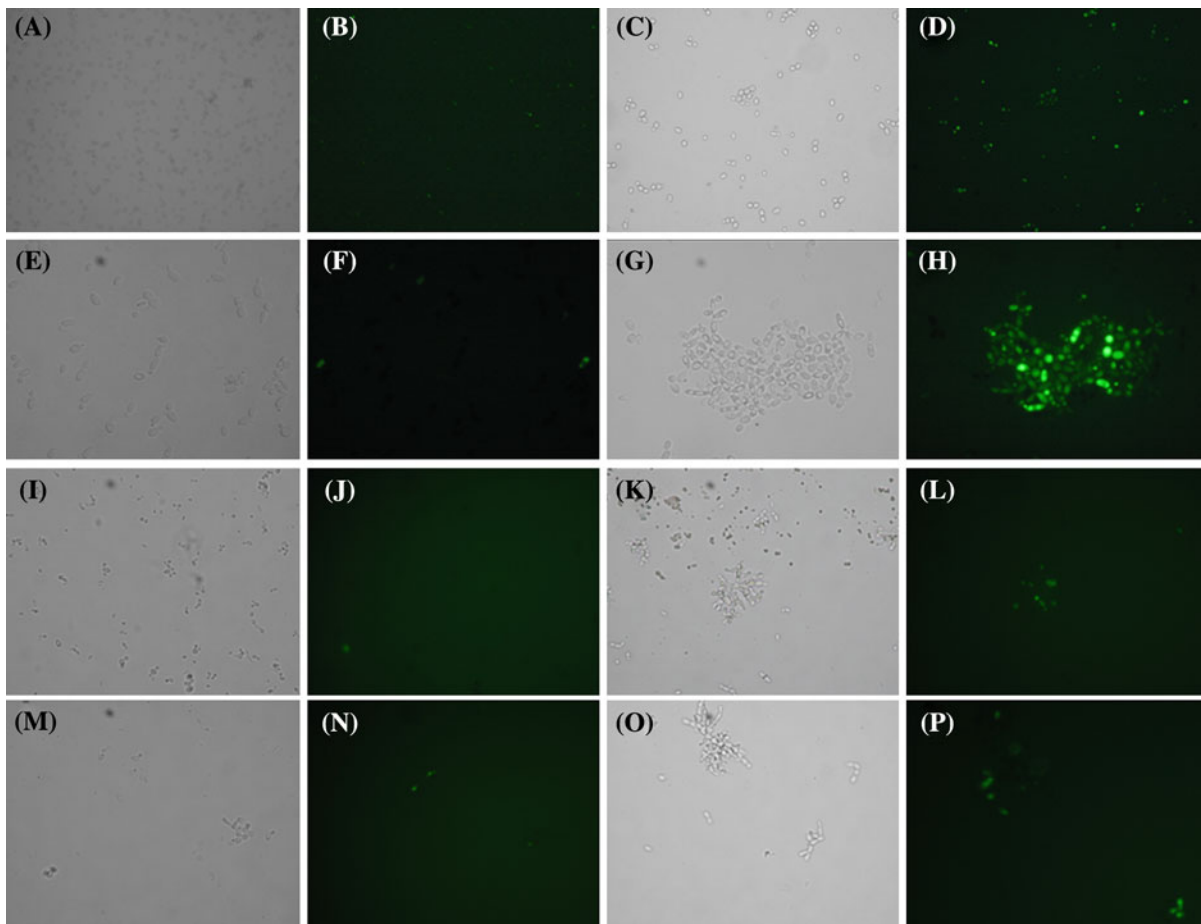


Fig. 7 Oxidative stress assay performed with fluorescence microscopy of different yeast cells for NO detection. **a, b** Control cells of *S. cerevisiae*; **c, d** cells of *S. cerevisiae* treated with *CaTI* ($32 \mu\text{g ml}^{-1}$); **e, f** control cells of *C. tropicalis*; **g, h** cells of *C. tropicalis* treated with *CaTI* ($32 \mu\text{g ml}^{-1}$); **i, j** control cells

of *C. albicans*; **k, l** cells of *C. albicans* treated with *CaTI* ($32 \mu\text{g ml}^{-1}$); **m, n** control cells of *K. marxianus*; **o, p** cells of *K. marxianus* treated with *CaTI* ($32 \mu\text{g ml}^{-1}$). **a, c, e, g, i, k, m, o** Cells viewed by DIC; **b, d, f, h, j, l, n, p** cells viewed by fluorescence

reported the induction of ROS and nitric oxide in fungal cells treated with *PvD1*. Aerts et al. (2007) have demonstrated that the growth inhibition of *C. albicans* by *Rs-AFP₂* is a consequence of apoptosis and showed that the process is independent of the metacaspase-1 pathway. Despite the intracellular signaling described above, it is not known whether *Rs-AFP₂* enters the cytoplasm or induces a signaling cascade upon interaction with the plasma membrane. However, it is known that *NaD₁* permeabilizes fungal hyphae to Sytox green and generates ROS, which indicates that these defensins may act by different mechanisms although they can both traverse the plasma membrane (van der Weerden et al. 2008).

Effects of *CaTI* on the glucose-stimulated acidification of medium by *S. cerevisiae* cells

The plasma membrane H^+ -ATPase plays an essential role in fungal cell physiology. Inhibition of H^+ -ATPase function in fungi by antagonists commonly leads to cell death. We investigated in this study whether *CaTI* could inhibit the function of yeast H^+ -ATPases. Thus, we monitored glucose-stimulated acidification, a phenomenon that is dependent on the activity of H^+ -ATPases in the incubation medium of *S. cerevisiae* cells in the presence of concentrations of 16, 32 and $64 \mu\text{g ml}^{-1}$ of *CaTI*. As shown in Fig. 8, *CaTI*, at all concentrations tested, was able to inhibit this acidification by 31, 75 and 86%, respectively, in a

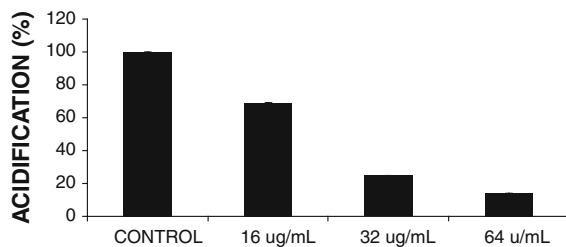


Fig. 8 Effects of *CaTI* on the glucose-dependent acidification of the medium by *S. cerevisiae* cells. *CaTI* was pre-incubated for 30 min at 16, 32 and 64 $\mu\text{g ml}^{-1}$ concentrations before the addition of glucose to a final concentration of 100 mM

dose-dependent manner and with a pre-incubation time of 30 min. Diz et al. (2006) demonstrated that the F1 fraction, isolated from *Capsicum annuum* seeds, at concentrations between 10 and 160 $\mu\text{g ml}^{-1}$, inhibited this acidification by 85–100%, suggesting that H^+ -ATPases may be a potential target for the F1 fraction. *PvD1*, in addition to stimulating ROS production, causes the inhibition of glucose-stimulated acidification of the medium, demonstrating inhibition of H^+ -ATPase pumps (Mello et al. 2011). Albumins 2S from passion fruit seeds may also inhibit glucose-dependent acidification by yeast cells and phytopathogenic fungi (Agizzio et al. 2006). It is possible that *CaTI* could interact with components of the plasma membrane, causing changes that lead to the dissipation of the H^+ gradient across the membrane, thus weakening the function of H^+ -ATPases in the plasma membrane. Thus, the identification of molecular targets and physiological responses to *CaTI* in model eukaryotes that are similar to the yeast tested may provide new insights into molecular processes that are relevant to the development of new drugs.

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