

Improved smallest peptides based on positive charge increase of the γ -core motif from PvD_1 and their mechanism of action against *Candida* species

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Background: Plant defensins have a hallmark γ -core motif (GXCX₃₋₉C) that is related to their antimicrobial properties. The aim of this work was to design synthetic peptides based on the region corresponding to the PvD_1 defensin γ -core that are the smallest amino acid sequences that bear the strongest biological activity.

Methods: We made rational substitutions of negatively charged amino acid residues with positively charged ones, and the reduction in length in the selected PvD_1 γ -core sequence to verify whether the increased net positive charges and shortened length are related to the increase in antifungal activity. Herein, we opted to evaluate the action mechanism of $\gamma_{33-41}PvD_1^{++}$ peptide due to its significant inhibitory effect on tested yeasts. In addition, it is the smallest construct comprising only nine amino acid residues, giving it a better possibility to be a prototype for designing a new antifungal drug, with lower costs to the pharmaceutical industry while still maintaining the strongest antimicrobial properties.

Results: The $\gamma_{33-41}PvD_1^{++}$ peptide caused the most toxic effects in the yeast *Candida buinensis*, leading to membrane permeabilization, viability loss, endogenous reactive oxygen species increase, the activation of metacaspase, and the loss of mitochondrial functionality, suggesting that this peptide triggers cell death via apoptosis.

Conclusion: We observed that the antifungal activity of PvD_1 is not strictly localized in the structural domain, which comprises the γ -core region and that the increase in the net positive charge is directly related to the increase in antifungal activity.

Keywords: defensin, antimicrobial peptide, cationic, mechanism of action

Introduction

Plant defensins are peptides identified in different plant tissues and organs, and several studies demonstrated the important role they played in the innate immune system of plants.^{1,2} The participation of plant defensins in the defense responses is supported by reports of their strong antimicrobial activity in vitro, not only against plant pathogenic fungi, but also against human ones;^{3,4} the gene expression pattern in response to pathogen infection,⁵ along with the demonstration that transformed plants engineering to constitutively express defensin genes, is resistant to fungal diseases.⁶

Plant defensins present a diversity of primary structures coded in 45–54 amino acid residues. The amino acid composition of plant defensins make them highly basic, giving the molecules a net positive charge at neutral pH. Several amino acid residues are conserved among the plant defensin primary structures, such as the strictly conserved eight cysteine residues.^{7,8} The eight cysteine residues bond to each other in specific pairs, forming four disulfide bridges, which are responsible for the stabilization of

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their three-dimensional structure,^{7,8} allowing this family of plant peptides to resist harsh environmental conditions, such as protease degradation and pH and temperature extremes.⁹ Despite the variation in the primary structure, a completely opposite effect is observed at the tertiary structure level, which is well defined and conserved, generally consisting of one α -helix and three antiparallel β sheets.¹⁰ The diversity of plant defensins at their primary structure level is responsible for their different biological activities in vitro.^{7,11,12} Among the activities already described, the best characterized is their growth inhibition of a large variety of filamentous fungi and yeasts, including those pathogenic to humans.^{3,7,13–17}

The mechanism of action involved in the fungal growth inhibition by plant defensins has been investigated by several authors, and the evidence demonstrates several steps involving an extracellular mechanism acting on the cell wall and/or plasma membrane, further acting on intracellular targets.^{7,9,15,18} In addition to this whole membrane permeabilization process involving plant defensins, some defensins have been shown to induce apoptosis or programmed cell death in susceptible yeast and fungal species.^{16,17}

In regard to the plant defensin structures, studies have demonstrated that two disulfide bridges, those formed between the Cys₂₁ and Cys₂₅ located in the α -helix and the Cys₄₅ and Cys₄₇ located in the last β -sheet, form a structural arrangement named the cysteine-stabilized $\alpha\beta$ motif (CS $\alpha\beta$ motif), characteristic of peptides with antimicrobial activity.^{7,19,20} In addition to the CS $\alpha\beta$ motif, plant defensins also have another conserved framework region characterized, called the γ -core, which has the conserved sequence (GXCX_{3,9}C, where X may be any amino acid residue).^{1,21} Some studies have shown that the region responsible for the biological activity of plant defensins resides in the γ -core region. De Samblanx et al²² showed that the substitution or removal of the amino acid residues located in this region, which encompasses β 2 and β 3 sheets, reduced the biological activity of RsAFP₂ (a defensin from *Raphanus sativus*). Sagaram et al²³ demonstrated that the main determinants of the antifungal activity of MsDef₁ and MtDef₄ (defensins from *Medicago sativa* and *Medicago truncatula*, respectively) reside in their γ -core motifs. In another study, it was shown that the interaction of Psd₁ defensin from *Pisum sativum* with the membrane is mediated, in part, by the amino acid residues that compose the γ -core region.¹

Knowing that the region comprising the plant defensin γ -core is related to the antimicrobial properties of these peptides, the principal aim of this work was to design synthetic peptides based on the region corresponding to the PvD₁ defensin γ -core that are the smallest amino acid sequences that bear the strongest biological activity. PvD₁ is an isolated

defensin from *Phaseolus vulgaris* seeds with antifungal properties,^{14,15,18} anti-*Leishmania* activity,²⁴ and anticancer activity.²⁵ Additionally, knowing that the charge, length, and hydrophobicity influence the activity of antimicrobial peptides,²⁶ we made rational substitutions of negatively charged amino acid residues with positively charged ones, as well as the reduction in length in the selected PvD₁ defensin γ -core sequence to verify whether the increased net positive charge and the shortened length are related to the increase in antifungal activity. In addition, some tests were carried out to better understand the mechanism of action of the peptides derived from the γ -core region of PvD₁ defensin.

Materials and methods

Microorganisms

The yeast species *Candida albicans* (CE022) and *Candida buinensis* (3982) were maintained in Sabouraud agar (1% peptone, 2% glucose, and 1.7% agar; Merck). The yeast cells were provided by Laboratório de Fisiologia e Bioquímica de Microrganismos, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, Rio de Janeiro, Brazil.

Analysis of amino acid sequence of PvD₁ defensin

The amino acid sequence of the PvD₁ defensin from *P. vulgaris* was obtained as previously described by Games et al.¹⁴ In brief, the first 21 amino acid residues of PvD₁ were obtained by Edman degradation of the purified peptide. Based on this 21 amino acid sequence, a degenerated primer was designed and used in an RT-PCR together with an oligo-dT primer. The amplicon was cloned into the pTZ57R/T vector (InsTAclone™ PCR Cloning Kit, Fermentas) and then submitted to sequencing by Big Dye1 Terminator v3.1 Kit (Applied Biosystems) in an ABI 3110 Genetic Analyzer (Applied Biosystems).

Synthetic peptide design

By comparison analysis of the primary structure of PvD₁ defensin with other defensin primary structures⁷ and by superimposing onto these sequences the sequence of the γ -core, as defined by Yount and Yeaman,²⁷ we identified and selected the region corresponding to the PvD₁ γ -core. This region encompasses the γ -core itself and amino acid residues that are part of the flanking β 2 and β 3 sheets. Based on these comparative analyses, two peptides with 15 amino acid residues and two peptides with 9 amino acid residues each were then chemically synthesized by the GenOne Soluções em Biotecnologia (Rio de Janeiro, Brazil). The purity level of the synthetic peptides was >95% as determined by

high-performance liquid chromatography (4.6×250 mm PLRP-S 100A), using a solution A (0.1% trifluoroacetic acid in 100% acetonitrile) and a solution B (0.1% trifluoroacetic acid in 100% water) with the following gradient: 0%–15% of solution A from 0 to 25 minutes; 40%–100% of solution A from 25 to 30 minutes. The Cys (C) residues of these peptides were replaced by Ala (A) residues. Additionally, two Asp (D) residues were substituted by two Arg (R) residues in two peptides. All these peptides were resuspended in ultrapure water. Their molecular masses and isoelectric points were determined by the ExPASy Compute pI/Mw tool.^{28–30}

Effect of synthetic peptides on yeast growth

Yeast cell inocula from *C. albicans* and *C. buinensis* were removed from stock tubes containing Sabouraud agar and transferred to Petri dishes containing Sabouraud agar. Cells were grown at 30°C for 2 days. After this period, each cell aliquot was added to 10 mL sterile culture medium (Sabouraud broth, 1% peptone, 2% glucose, Merck). Yeast cells were quantified in a Neubauer chamber (LaborOptik). Initially, the *C. albicans* and *C. buinensis* yeast cells (1×10^4 cells mL⁻¹) were incubated in 100 µL of Sabouraud broth containing the selected synthetic peptides at increasing concentrations from 18.35 to 293.6 µM.

The assay was performed on cell culture microplates (96 wells; Nunc) at 30°C for 24 hours. Optical readings at 620 nm (EZ Read 400, Biochrom) were collected 24 hours after the start of assay. Control cells were grown in the absence of synthetic peptides. The assay was done three times according to a methodology adapted from Broekaert et al.³¹ The yeast growth inhibition data were evaluated by the one-way ANOVA, and the differences of the mean at $P < 0.05$ were considered significant. All statistical analyses were performed using the GraphPad Prism software (version 6.0 for Windows).

Cell viability

To verify whether the growth inhibition of *C. buinensis* cells was caused by the fungicidal or fungistatic effect of the smallest active peptide, the control cells (without the peptide) were washed and diluted 1,000-fold. A 100 µL aliquot of the dilution was spread with a Drigalski spatula on the surface of a Petri dish containing Sabouraud agar and grown at 30°C for 48 hours. At the end of this period, the colony forming units were determined, and Petri dishes were photographed.³² The same procedure was repeated with the yeasts treated with 36.7 and 73.4 µM of the smallest and the strongest active peptide for 24 hours. The experiments were performed in triplicate, and the results are shown assuming that the control represents 100%

cell viability. These data were evaluated by one-way ANOVA, and the differences of the mean at $P < 0.05$ were considered significant. All statistical analyses were performed using the GraphPad Prism software (version 6.0 for Windows).

Effect of a synthetic peptide on plasma membrane permeabilization

The plasma membrane permeabilization of *C. buinensis* cells treated with 25 µM of the smallest and the strongest active peptide was evaluated using Sytox green fluorescent dye (Invitrogen), according to the methodology described by Thevissen et al.³³ Sytox green is a dye that has high affinity for nucleic acids and penetrates the cell only when its membrane is compromised, emitting a strong green fluorescent.

After the growth inhibition assay, *C. buinensis* yeast cells grown in the absence (control) and in the presence of the smallest and the strongest active peptide were incubated with Sytox green at a final concentration of 0.2 µM (dissolved in dimethyl sulfoxide), according to instructions provided by the manufacturer. A positive control with 300 mM ethanol was done. After 15 minutes incubation at 25°C with constant agitation at 500 rpm, the cells were observed under an optical microscope (Axioplan.A2, Zeiss) coupled to an AxioCAM MRc5 (Zeiss) camera and the images were analyzed by the Axiovision software version 4.0 (Zeiss). The microscope was equipped with a set of fluorescent filters for fluorescein detection (excitation wavelength between 450 and 490 nm and emission of 500 nm). The results represent triplicate experiments.

Determining the induction of intracellular oxygen reactive species

To evaluate whether the mechanism of action of the smallest and the strongest active peptide involves the induction of oxidative stress, the fluorescent probe 2',7'-dichloro-fluorescein diacetate (H₂DCFDA) was used according to the methodology described by Mello et al.¹⁵ This dye enters the cell passively, is deacetylated by intracellular esterases and after being oxidized by reactive oxygen species (ROS), becomes fluorescent. After 24 hours incubation with 25 µM of the smallest and the strongest active peptide, 50 µL of *C. buinensis* cells grown in the absence (control) and in the presence of the peptide were incubated with 20 µM of the H₂DCFDA probe (Calbiochem), for 2 hours at 25°C with constant agitation at 500 rpm. A positive control was done with 300 mM hydrogen peroxide. After this period, the cells were observed under an optical microscope (Axioplan. A2, Zeiss) coupled to an AxioCAM MRc5 (Zeiss) camera, and the images were analyzed by the Axiovision software version 4.0 (Zeiss). The microscope was equipped with a

set of fluorescent filters for fluorescein detection (excitation wavelength between 450 and 490 nm and emission of 500 nm). The results represent triplicate experiments.

Analysis of mitochondrial functionality

Mitochondrial functionality was assessed by fluorescent dye Rhodamine 123 (Sigma). Rhodamine 123 is a cationic fluorescent dye that has a high affinity to the electrical potential of membranes; thus, it marks active mitochondria in living cells resulting in a bright red fluorescence. In contrast, the treated samples present a weak or absent fluorescent signal. This test was done as described in the section “Effect of synthetic peptides on yeast growth” with the following differences: after incubation with 25 μM of the smallest and the strongest active peptide for 12 and 24 hours, the *C. buinensis* yeast cells were resuspended, incubated with 10 $\mu\text{g mL}^{-1}$ of Rhodamine 123 with constant agitation at 500 rpm for 2 hours and protected from light and then analyzed by differential interference contrast (DIC) under an optical microscope equipped with a fluorescence filter (with an excitation wavelength of 506 nm and emission wavelength of 530 nm). Control cells (in the absence of the peptide) had the same treatment as cells treated with the peptide and a positive control with cells treated with 300 mM ethanol for 30 minutes before the dye addition.

Detection of caspase activity induced by a synthetic active peptide

The detection of caspase activity was performed using the CaspACE FITC-VAD-FMK marker (Promega), as described

by the manufacturer. The FITC-VAD-FMK marker is an analog of the Z-VAD-FMK caspase inhibitor (carbobenzoxycarbonyl-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone) that enters the cell and irreversibly binds to activated caspases. This test was done as described in the section “Effect of synthetic peptides on yeast growth” with the following differences: after incubation with 25 μM of the smallest and the strongest active peptide for 24 hours, the *C. buinensis* cells were resuspended, washed once in 500 μL PBS (10 mM NaH_2PO_4 , 0.15 M NaCl) with pH 7.4 and resuspended in 50 μL of the staining solution (supplied by the kit) containing 50 μM FITC-VAD-FMK marker. After incubation for 20 minutes at 30°C with constant agitation at 500 rpm, the cells were again washed in 500 μL PBS and resuspended in 20 μL PBS. Negative control (in the absence of the peptide) and positive control cells (incubated with 300 mM acetic acid) had the same treatment as cells treated with the peptide. The cells were analyzed by DIC on the optical microscope equipped with a fluorescence filter for fluorescein detection (excitation wavelength 450–490 nm and emission wavelength 500 nm).

Results

Synthetic peptide design

After the analysis of the primary structure of PvD_1 , previously obtained by Games et al,¹⁴ and other defensin peptides,⁷ the region corresponding to the PvD_1 γ -core was selected and is highlighted in blue (Figure 1A). Based on the PvD_1 γ -core sequence, together with parts of the $\beta 2$ and $\beta 3$ sheets (from Arg₃₁ to Lys₄₅), an amino acid stretch giving rise to

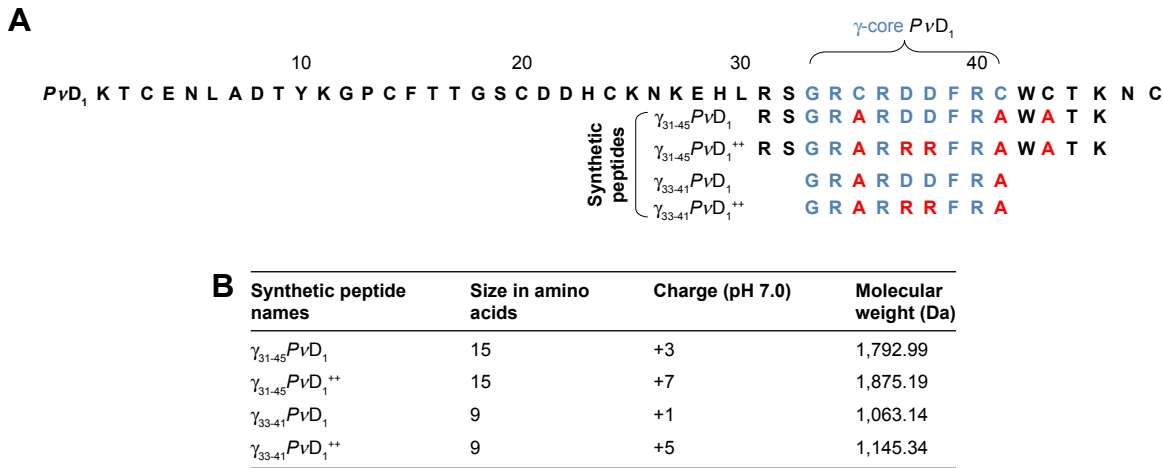


Figure 1 Design, alignment and biochemical characteristics of the synthetic peptides. **Notes:** (A) Alignment of the primary structures of PvD_1 and the four synthetic peptides in amino acid one-letter code. Numbers above the PvD_1 sequence indicate the peptide size in amino acids. The amino acid residues in blue represent the PvD_1 γ -core region. The amino acid residues in red represent the replaced residues in the original PvD_1 sequence as follows: C residues were replaced by A, and D residues were replaced by R. Amino acid residues in black are not part of the γ -core region and were not changed. Numbers in the synthetic peptide names stand for the amino acid position in the original PvD_1 . Double-plus (++) indicates the double replacement from the original D to R amino acid residues to increase the synthetic peptide positive net charge. (B) Biochemical characteristics of the synthetic peptides.

the peptide of 15 residues with the following amino acid sequence was chosen: RSGRARDDFRAWATK, which was called γ_{31-45} PvD₁ (Figure 1A). Another peptide, based on the γ_{31-45} PvD₁ design, had its net positive charge increased with the replacement of the two Asp residues, at positions 37 and 38, by two Arg residues, giving rise to the following amino acid sequence: RSGRARRRRFRAWATK, which was called γ_{31-45} PvD₁⁺⁺ (Figure 1A). Two other peptides of nine residues each, smaller than γ_{31-45} PvD₁ and γ_{31-45} PvD₁⁺⁺, and had the sequences comprising Gly₃₃ to Cys₄₁ (PvD₁ γ -core itself) with the amino acid sequences GRARDDFRA and GRARRRRFRA, which were called γ_{33-41} PvD₁ and γ_{33-41} PvD₁⁺⁺, respectively, were also synthesized (Figure 1A). All the synthesized peptides had their Cys residues replaced by Ala residues

(Figure 1A). These substitutions were made to prevent the formation of disulfide bridges. Similarly, Schaaper et al³⁴ replaced the Cys residues by α -aminobutyric acid, to avoid the formation of such undesired bridges. The biochemical characteristics of synthesized peptides are shown in Figure 1B.

Growth inhibition assay

To analyze the effect of the synthetic peptides on the growth of yeasts, *C. albicans* and *C. buinensis*, γ_{31-45} PvD₁, γ_{31-45} PvD₁⁺⁺, γ_{33-41} PvD₁, and γ_{33-41} PvD₁⁺⁺ peptides were used at increasing concentrations from 18.35 to 293.6 μ M (Figures 2 and 3).

With the γ_{31-45} PvD₁ peptide, we observed that all used concentrations inhibited the growth of yeast *C. albicans*, reaching

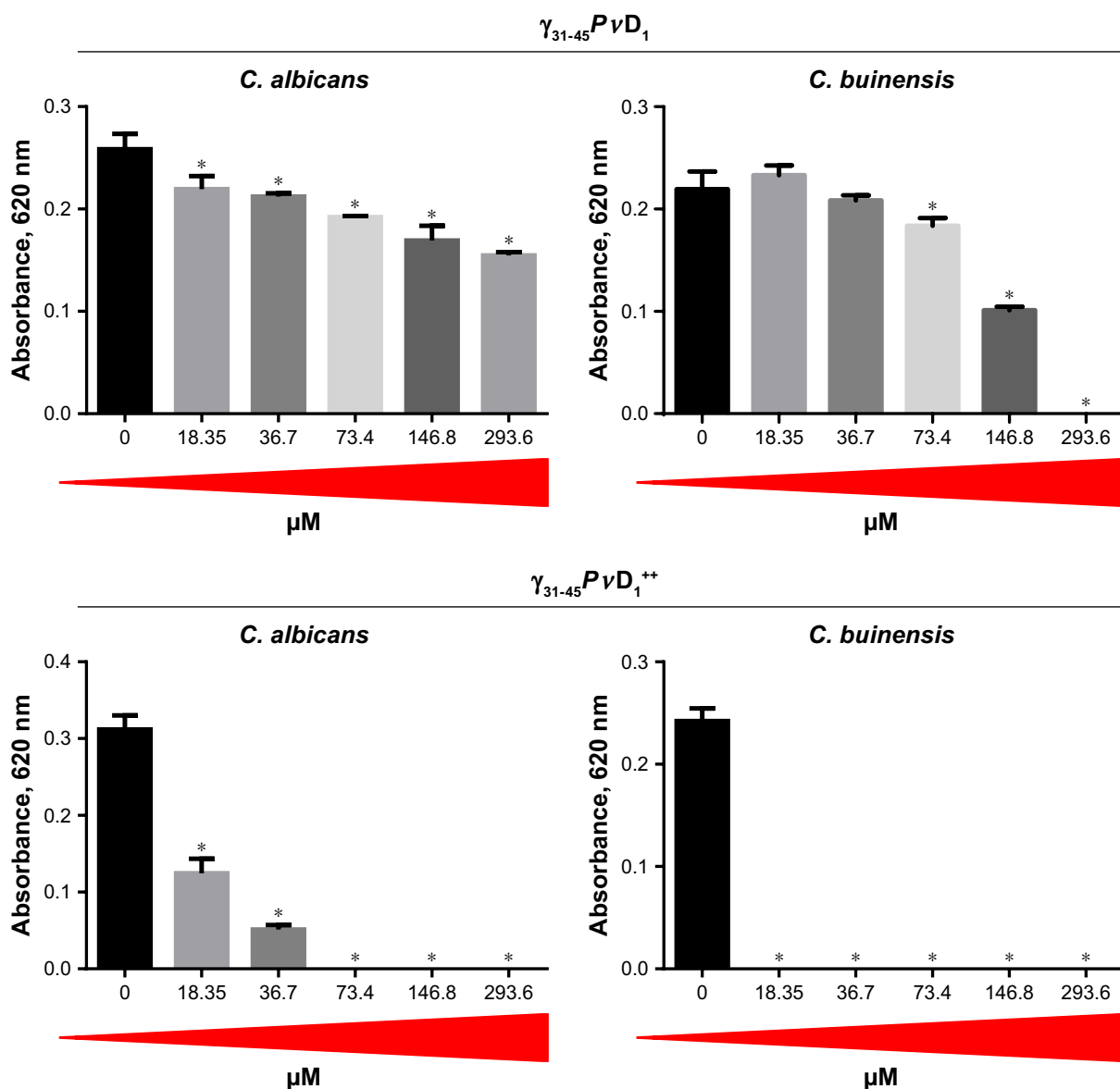


Figure 2 Antifungal effects of γ_{31-45} PvD₁ and γ_{31-45} PvD₁⁺⁺ incubated for 24 hours at different concentrations on *Candida albicans* and *Candida buinensis*. *Indicates significance by the one-way analysis of variance (ANOVA) ($P < 0.05$).

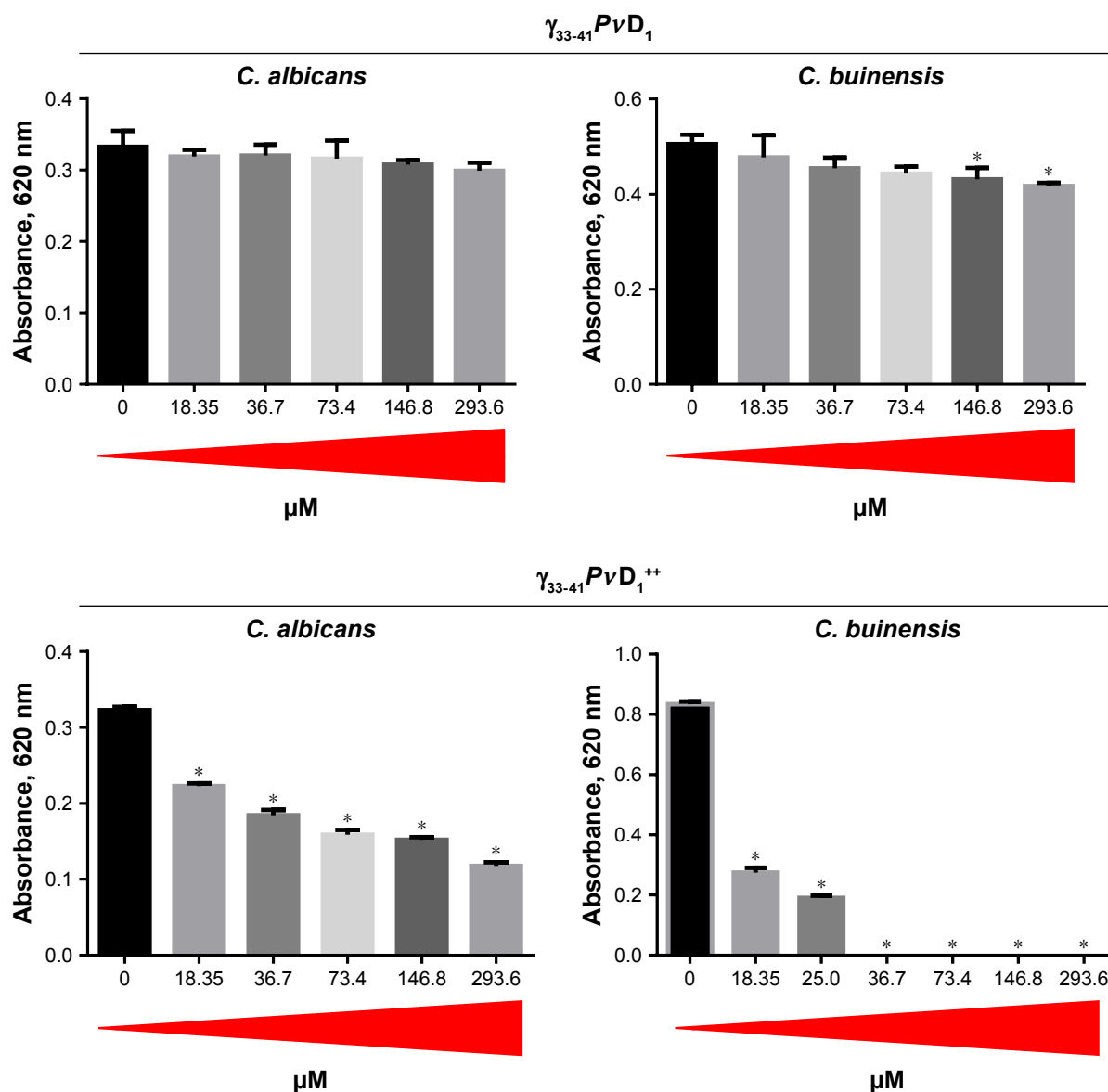


Figure 3 Antifungal effect of $\gamma_{33-41}PvD_1$ and $\gamma_{33-41}PvD_1^{++}$ incubated for 24 hours at different concentrations on *Candida albicans* and *Candida buinensis*. *Indicates significance by the one-way analysis of variance (ANOVA) ($P < 0.05$).

40% inhibition at the concentration of 293.6 μM . The yeast *C. buinensis* had its growth inhibited starting from the 73.4 μM concentration, and it was possible to observe the 100% inhibition, when the peptide concentration was 293.6 μM (Figure 2). When the positive net charge of this peptide was increased by the synthesis of $\gamma_{31-45}PvD_1^{++}$, the peptide concentration of 18.35 μM inhibited 60% growth of the yeast *C. albicans*, and starting from the concentration of 73.4 μM , the yeast growth was completely inhibited (Figure 2). For yeast *C. buinensis*, the lowest used peptide concentration of 18.35 μM completely inhibited yeast growth (Figure 2).

When we used the smallest peptides, we observed that $\gamma_{33-41}PvD_1$ was not able to inhibit the growth of *C. albicans*

at any concentrations tested (Figure 3). For *C. buinensis*, this peptide inhibits growth by 15% and 17% at the two highest used concentrations, respectively (Figure 3). However, the peptide $\gamma_{33-41}PvD_1^{++}$ that had its positive net charge increased showed a greater inhibition potential, being able to inhibit the growth of *C. albicans* at all concentrations tested and reaching 63% inhibition at the concentration of 293.6 μM (Figure 3). For *C. buinensis*, this peptide had an even greater inhibitory potential, being able to inhibit 100% of its growth starting from the concentration of 36.7 μM (Figure 3).

Based on the results from Figures 2 and 3, the designed smallest peptide that had the strongest antimicrobial activity

was γ_{33-41} PvD₁⁺⁺ when incubated with *C. buinensis*. Because of this outcome, this peptide and this yeast species were chosen for the next experiments.

Cell viability

Based on the results obtained in the growth inhibition assay, we found that the minimal inhibitory concentration (MIC) of γ_{33-41} PvD₁⁺⁺ peptide was 36.7 μ M for *C. buinensis* cells (Figure 4A). The γ_{33-41} PvD₁⁺⁺ peptide at 36.7 and 73.4 μ M induced the viability loss in *C. buinensis* cells. The cells treated with 36.7 μ M of this peptide had an 84% loss of viability and the cells treated with 73.4 μ M had a 100% loss of viability, showing that the effect of this peptide is fungicidal to *C. buinensis* (Figure 4B and C). Starting from these experiments, all following assays were performed with

yeast *C. buinensis* with the γ_{33-41} PvD₁⁺⁺ at 25 μ M, because at this concentration, below the determined MIC, we were able to obtain reasonable amounts of cells for visualization by optical microscopy.

Effect of γ_{33-41} PvD₁⁺⁺ on plasma membrane permeabilization

The ability of the γ_{33-41} PvD₁⁺⁺ to permeabilize the *C. buinensis* plasma membrane was analyzed using Sytox green. The analysis by fluorescence microscopy revealed that *C. buinensis* cells were labeled by the dye, when treated with γ_{33-41} PvD₁⁺⁺ at 25 μ M; thus, these data suggest that γ_{33-41} PvD₁⁺⁺ acts on the plasma membrane of *C. buinensis*, structurally compromising it and allowing its permeabilization for the labeling dye. In the control (in the absence of peptide),

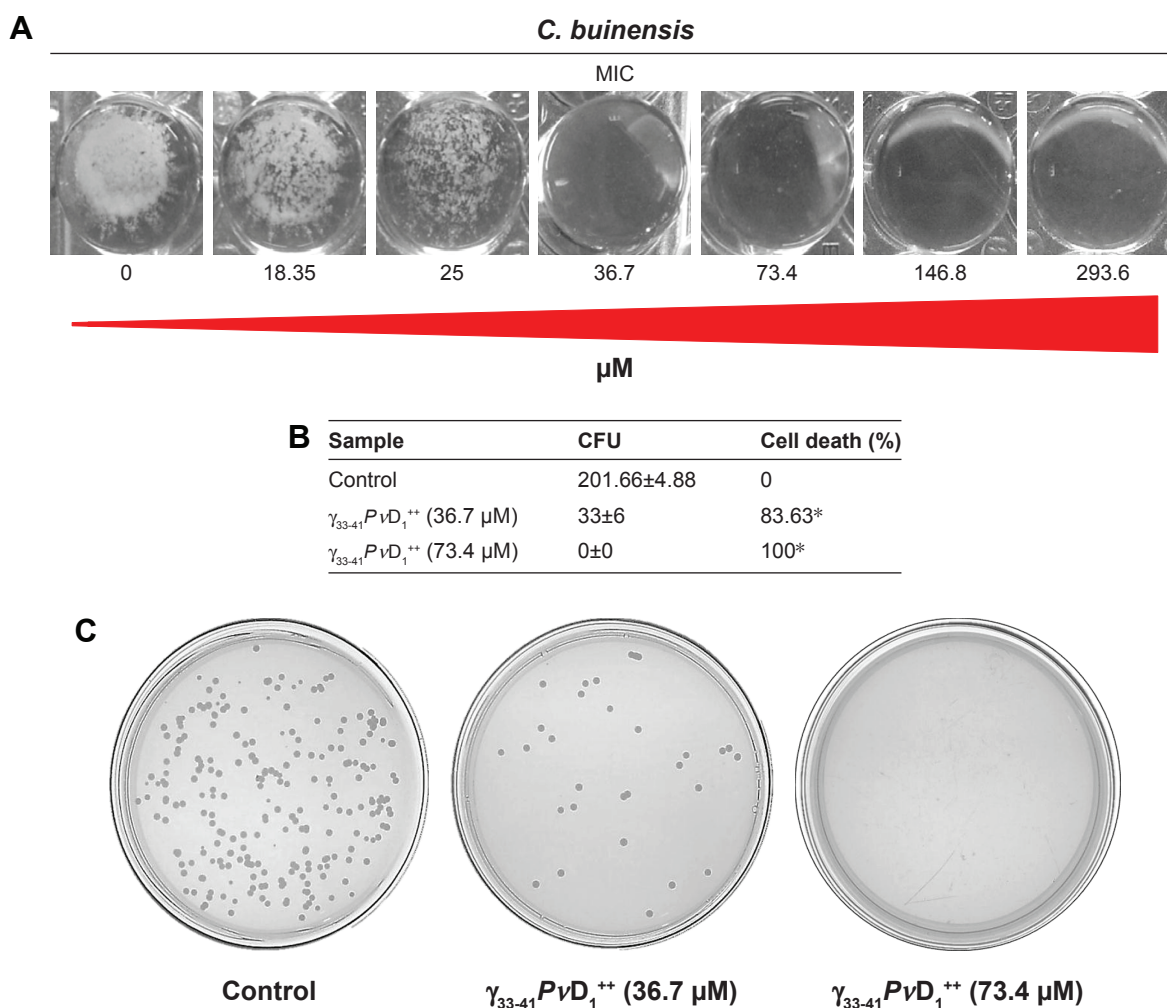


Figure 4 MIC determination and cell viability loss.

Notes: (A) Images of the plate wells at the end of the growth inhibition assay (at 24 hours) showing the growth pattern of *Candida buinensis* cells at the bottom of the wells in the absence (control) and in the presence of different γ_{33-41} PvD₁⁺⁺ concentrations. (B) Table showing the number of CFU and the percentage of viability loss of *C. buinensis* cells after treatment with 36.7 and 73.4 μ M of γ_{33-41} PvD₁⁺⁺ for 24 hours, respectively. *Indicates significance by the one-way analysis of variance (ANOVA) ($P < 0.05$). (C) Images of Petri dishes showing the CFU as described in (B). The experiments were carried out in triplicate.

Abbreviations: CFU, colony forming units; MIC, minimal inhibitory concentration.

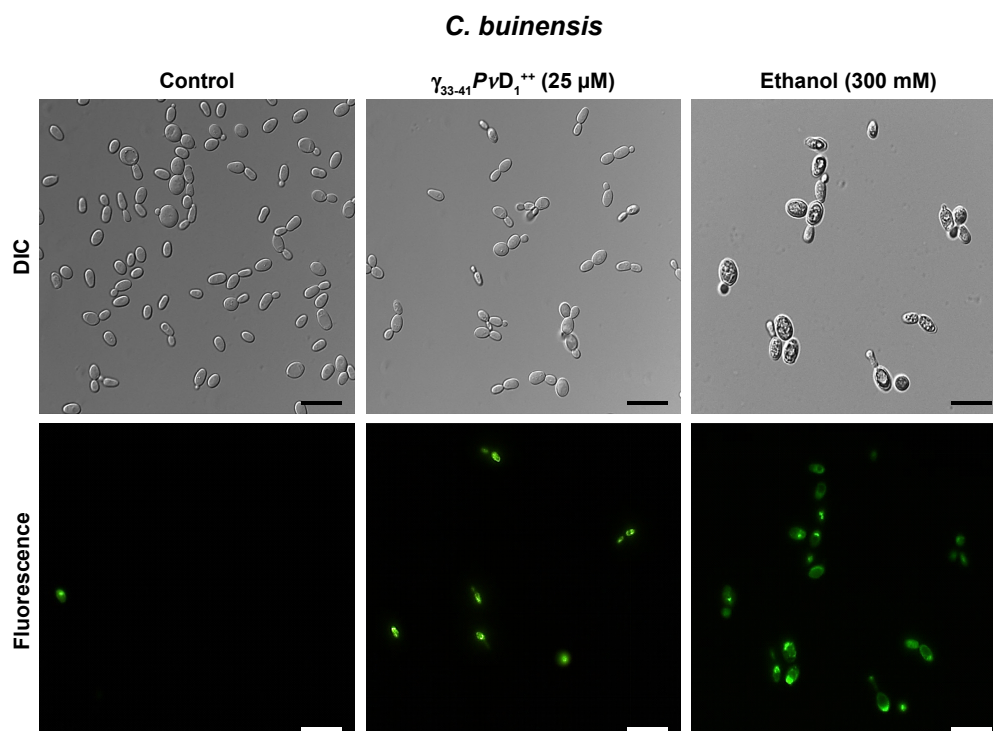


Figure 5 Images of membrane permeabilization assay of *Candida buinensis* cells after treatment with $\gamma_{33-41}PvD_1^{++}$ (25 μ M) for 24 hours. Control cells were treated only with the Sytox green probe and positive control cells were treated with 300 mM ethanol. Bars =20 μ m.

Abbreviation: DIC, differential interference contrast.

no fluorescence was observed and in the positive control (300 mM ethanol), fluorescent cells were observed (Figure 5).

Reactive oxygen species assay detection

After the growth inhibition assay, *C. buinensis* cells were incubated with the H_2DCFDA probe for the detection of endogenous ROS production. When incubated in the presence of 25 μ M of $\gamma_{33-41}PvD_1^{++}$, the cells showed ROS labeling (Figure 6), suggesting that a $\gamma_{33-41}PvD_1^{++}$ -induced increase in oxidative stress may underlie the growth inhibitory effect on this yeast. In the control (in the absence of peptide), no fluorescence was observed and in the positive control (300 mM hydrogen peroxide), fluorescent cells were observed (Figure 6).

Analysis of mitochondrial functionality

In *C. buinensis* cells treated with $\gamma_{33-41}PvD_1^{++}$ at 25 μ M for 12 hours is observed a diminished mitochondrial activity as indicated by the weaker red fluorescent signal of Rhodamine 123 (Figure 7) in comparison to the untreated cells, and after additional 24 hours in the presence of $\gamma_{33-41}PvD_1^{++}$ it is also observed a shrinkage and condensation of cell cytoplasm. A similar weak fluorescent signal was observed for the 300 mM ethanol-treated samples (positive control). Control cells with functional mitochondria evinced a strong signal

of Rhodamine 123 fluorescence (Figure 7). This result indicated that after treatment with $\gamma_{33-41}PvD_1^{++}$, the *C. buinensis* cells lost their electrical mitochondrial membrane potential, causing the dysfunction of their mitochondria.

Detection of caspase activity induced by $\gamma_{33-41}PvD_1^{++}$

The detection of caspase activity was performed using the CaspACE FITC-VAD-FMK in situ marker. The *C. buinensis* yeast cells grown in the absence (control) and in the presence of 25 μ M of $\gamma_{33-41}PvD_1^{++}$ were subsequently incubated with the FITC-VAD-FMK marker. It can be observed that the cell treatment with $\gamma_{33-41}PvD_1^{++}$ resulted in the activation of metacaspases, making it possible to observe a strong labeling of these cells. Positive control cells, treated with 300 mM acetic acid, a known apoptosis inducer in yeast,³⁵ also showed labeling. However, the untreated cells (negative control) did not show metacaspase activity (Figure 8). These data suggest a programmed cell death that occurs by an apoptotic pathway.

Discussion

Plant defensins are peptides whose activity and mechanisms of action are primarily described against a wide variety of filamentous fungi and yeasts; however, their mechanism of

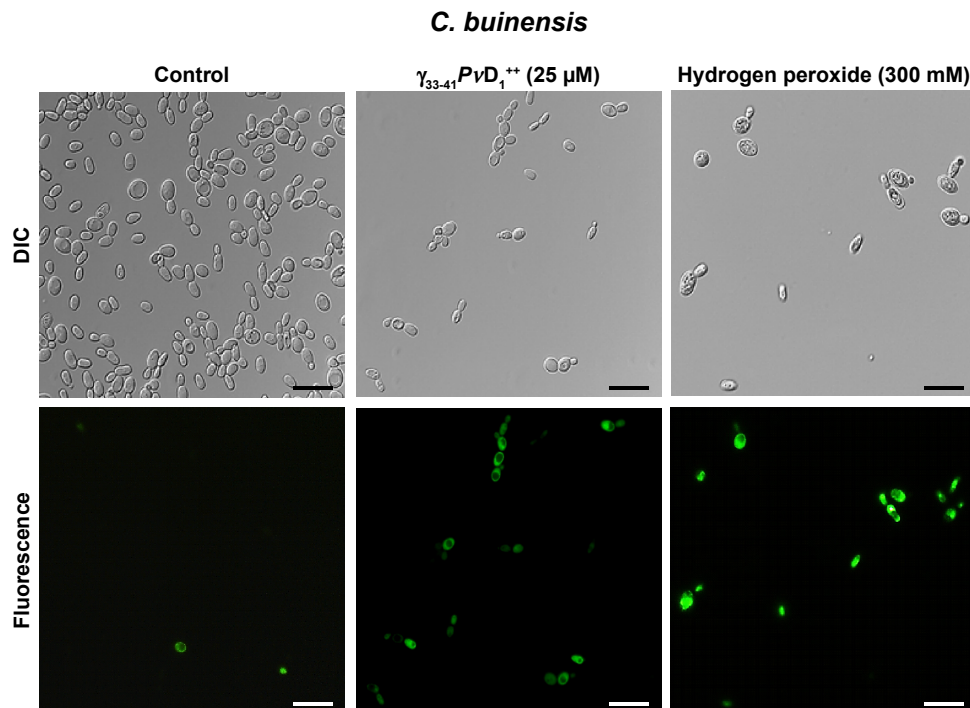


Figure 6 Images of reactive oxygen species assay detection in *Candida buinensis* cells after treatment with γ_{33-41} PvD₁⁺⁺ (25 μ M) for 24 hours. Control cells were treated only with the 2',7'-dichlorofluoresceindiacetate probe and positive control cells were treated with 300 mM hydrogen peroxide. Bars =20 μ m.

Abbreviation: DIC, differential interference contrast.

action has not yet been fully elucidated.^{3,7} The defensins have a structure composed of an α -helix and two β -sheets connected by four disulfide bridges. Yount and Yeaman²⁷ showed that the peptides stabilized by disulfide bridges possess amino

acid residues that are important for antimicrobial activity in the γ -core region that encompasses β 2 to β 3 sheets. Many studies have already shown that the activity of plant defensins is related to this structural domain.^{22,23,36}

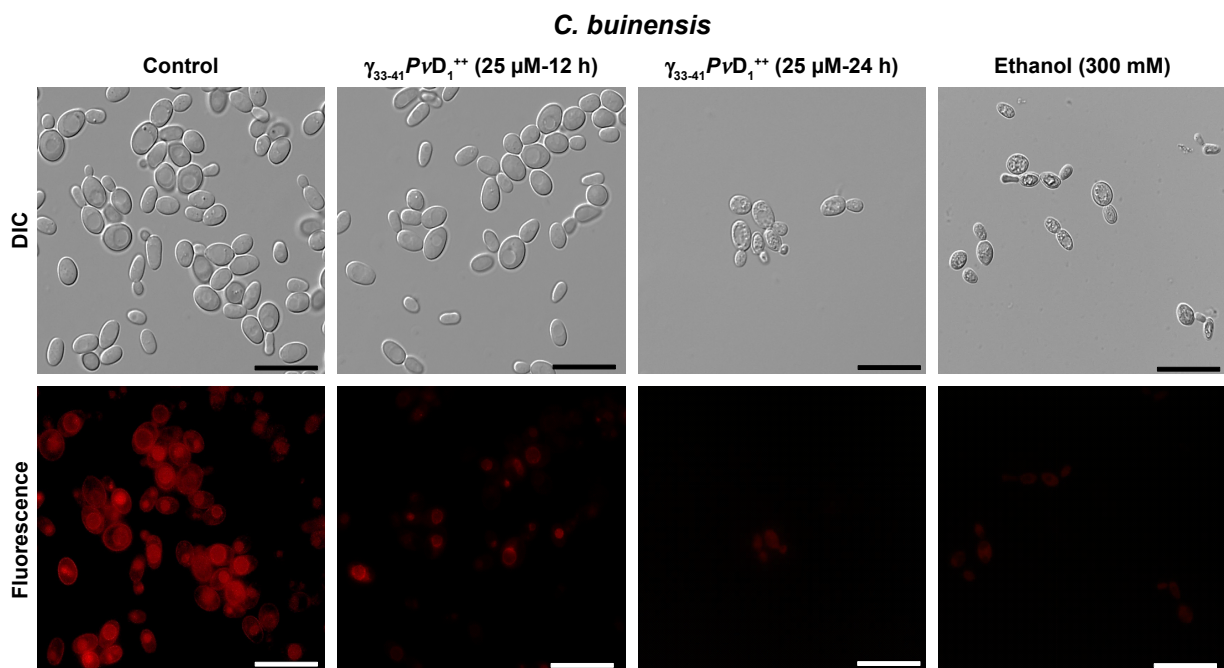


Figure 7 Images of mitochondrial functionality assay of *Candida buinensis* cells after treatment with γ_{33-41} PvD₁⁺⁺ (25 μ M) for 12 and 24 hours. Control cells were treated only with Rhodamine 123 probe and positive control cells were treated with 300 mM ethanol. Bars =20 μ m.

Abbreviation: DIC, differential interference contrast.

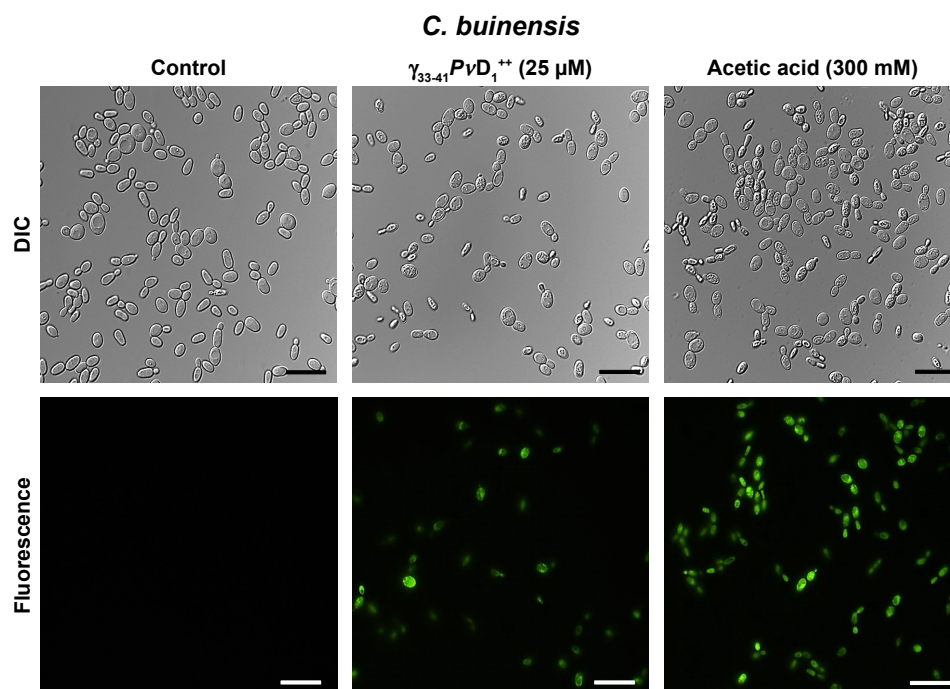


Figure 8 Images of detection of metacaspase activity assay of *Candida buinensis* cells after treatment with $\gamma_{33-41}PvD_1^{++}$ (25 μ M) for 24 hours. Control cells and cells treated with $\gamma_{33-41}PvD_1^{++}$ were incubated with CaspACE FITC-VAD-FMK probe. Positive control cells were treated with 300 mM acetic acid and analyzed by fluorescence microscopy. Bars = 20 μ m.

Abbreviation: DIC, differential interference contrast.

In the previous work performed by our group, the PvD_1 defensin isolated from *P. vulgaris* seeds inhibited growth of various yeasts.^{14,15,18} In this study, we designed synthetic peptides based on the PvD_1 γ -core. Initially, we designed two peptides of 15 residues that comprised the region from Arg₃₁ to Lys₄₅, called $\gamma_{31-45}PvD_1$ and $\gamma_{31-45}PvD_1^{++}$, the latter having its positive net charge increased (Figure 1). Some studies have identified charge, length, and hydrophobicity as the parameters that influence the activity of antimicrobial peptides.^{26,27} Regarding the length, it has already been reported that larger peptides that cover the entire $\beta 2$ and $\beta 3$ sheets, where the γ -core region is inserted, have higher activity.³⁴ Sagaram et al^{23,37} also demonstrated that synthesis of peptides extending beyond the γ -core region improves antifungal activity and that the synthetic peptide (GMA-4C) derived from the γ -core region also contained the C-terminus of $MtDef_4$ defensin with cationic and hydrophobic amino acids that were important for its antifungal activity. Regarding the charge, Lacerda et al¹⁰ demonstrated that the positively charged amino acids located in the γ -core were essential for the antifungal activity of peptides, since the substitution of neutral residues by positively charged amino acid residues in the γ -core region increased their inhibitory activity against pathogenic fungi. The mechanism behind this interconnection between positive charge increasing and antimicrobial inhibition increasing is

based mainly in the opposite charge attraction.³⁸ Fungal cells have negatively charged structures in their cell walls, such as phosphomanolipids,³⁹ that may serve as primary anchor sites for the positively charged peptides. Then the peptides became able to interact with the membrane. The interaction with the membrane is possible because of the amphipathic character of antimicrobial peptides which turning them able to interact with the negative charge of phospholipids head groups and also with the hydrophobic core of the membrane.^{11,33} This interaction explains the ability of antimicrobial peptide to cause membrane permeabilization. Similarly, the His and Arg residues present in the γ -core were important for the oligomerization and antifungal activity of the peptide derived from $MtDef_5$ defensin against the filamentous fungi *Neurospora crassa* and *Fusarium graminearum*.³⁶ Thus, we designed the peptide $\gamma_{31-45}PvD_1$, which had additional amino acids of the flanking $\beta 2$ and $\beta 3$ sheets, beside the amino acids that compose the γ -core and increased the charge of $\gamma_{31-45}PvD_1^{++}$ by replacing two Asp₃₇₋₃₈ by two Arg. When we tested the antifungal activity of $\gamma_{31-45}PvD_1$, it inhibited the growth of *C. albicans* at all concentrations tested. For *C. buinensis*, we observed inhibition only starting from the concentration of 73.4 μ M, with MIC being 293.6 μ M concentration. When we increased the net positive charge of $\gamma_{31-45}PvD_1^{++}$, it exhibited higher antifungal activity. All tested concentrations of this

peptide inhibited the *C. albicans* growth, with MIC being 73.4 μ M. For yeast *C. buinensis*, MIC was determined at the lowest concentration used, at 18.35 μ M (Figure 2).

Although some previous works have shown that the peptide length influenced its antifungal activity, if a smaller molecule obtained from the native sequence of a peptide still had biological activity, it would be more commercially interesting due to the low cost of its synthesis and could be seen as a promising molecule for the control of fungal diseases.³⁴ To find an even smaller molecule with antifungal potential that could be used as a novel drug, we designed two peptides of nine amino acid residues each that comprised the γ -core region from Gly₃₃ to Cys₄₁, called γ_{33-41} PvD₁ and γ_{33-41} PvD₁⁺⁺, where the latter also had its positive net charge increased as discussed above (Figure 1). Our choice of the smallest sequence with the strongest biological activity is based on requirements of desired characteristics for the pharmaceutical industry to alleviate problems like toxicity, drug stability, specificity, and less immunogenicity.⁴⁰ For these reasons, we chose to work with the γ_{33-41} PvD₁⁺⁺ peptide.

When we tested the activity of these peptides, the γ_{33-41} PvD₁ peptide at different concentrations did not inhibit the growth of *C. albicans* cells, whereas for yeast *C. buinensis*, it showed low activity at high concentrations (Figure 3). These results are consistent with the works of Schaaper et al³⁴ and Sagaram et al³⁷ in the sense that the smallest peptides, only encompassing the γ -core, as in our study, did not bear strong biological activity, at least for *C. albicans* and *C. buinensis*. Additionally, as in another example of Sagaram et al,²³ the γ -core alone may or may not present biological activity similar to the entire defensin. These authors tested the activity of two synthetic peptides called GMA₁ and GMA₄ derived, respectively, from the γ -core of the defensins MsDef₁ and MtDef₄, against the filamentous fungus *F. graminearum*. The peptide GMA₁, even at the concentration of 96 μ M, had no antifungal activity. In contrast, the peptide GMA₄ exhibited antifungal activity at 6 μ M, and when used at 12 μ M, was able to completely inhibit the conidial germination of the tested fungus. These results make it evident that only the γ -core region of the MtDef₄ defensin is sufficient for antifungal activity; however, the γ -core of the MsDef₁ defensin by itself is not sufficient for antifungal activity, similar to the γ -core (γ_{33-41} PvD₁) of the PvD₁ defensin.

On the other hand, when we tested the antifungal activity of γ_{33-41} PvD₁⁺⁺, with the positive net charge increased by replacing two Asp₃₇₋₃₈ residues by two Arg residues, we observed that it inhibited the growth of *C. albicans* and *C. buinensis*, with the MIC for *C. buinensis* of 36.7 μ M.

Previous studies showed that higher the net positive charge, the higher will be the antifungal activity of peptides, and the positively charged amino acids, such as Arg and Lys, were very important for antifungal activity. The synthetic peptide MBGo₁ derived from RsAFP₂ defensin, which had its net positive charge increased due to the substitution of some amino acid residues by Arg residues, showed higher activity against the filamentous fungus *Fusarium culmorum*.³⁴ Sagaram et al²³ showed that MtDef₄ defensin had five basic amino acids in its γ -core, and MsDef₁ defensin had two basic amino acids and two acidic amino acids. After an exchange of the γ -core region between these two defensins, MsDef₁ had a two fold higher antifungal activity, thus showing the importance of positively charged amino acid residues for the higher antifungal activity of this peptide.

In the growth inhibition assays, γ_{33-41} PvD₁⁺⁺ peptide showed higher activity against *C. buinensis* cells, and this inhibitory effect on yeast growth was fungicidal (Figure 4). Taveira et al^{41,42} found that another cationic peptide isolated from *Capsicum annuum* fruits, belonging to thionin family, called CaThi, had a fungicidal effect against six species of *Candida* genus and *Fusarium solani*. It is worth emphasizing that the fungicidal characteristic is very important for the development of new therapeutic drugs, since substances that present fungistatic effect can contribute to the development of resistant microorganisms.^{43,44}

Considering the results obtained, we began to investigate the mechanism of action of γ_{33-41} PvD₁⁺⁺, responsible for the inhibition of *C. buinensis* growth. One characteristic of the cationic antimicrobial peptides is their ability to permeabilize the plasma membrane of microorganisms.^{15,36} Initially, we used Sytox green, a dye that has high affinity for nucleic acids and penetrates cells only when their plasma membrane is compromised. We observed that the *C. buinensis* cells, when treated with the γ_{33-41} PvD₁⁺⁺ peptide, were marked by the dye (Figure 5). Sagaram et al²³ showed that MsDef₁ γ_4 peptide was also able to permeabilize the membrane; however, the tested fungus was *F. graminearum*. In another work, Islam et al³⁶ found that the cationic amino acid residues, present in the γ -core of MtDef₅ defensin, were responsible for its antifungal activity and that at micromolar concentrations, MtDef₅ caused the membrane permeability of the filamentous fungi *F. graminearum* and *N. crassa*. These same authors also observed that there was an increase in the production of intracellular ROS in *F. graminearum* and *N. crassa* hyphae after these cells were treated with MtDef₅.

ROS have been considered as primary regulators of cell death and are linked to many crucial apoptotic pathways

in yeasts.⁴⁵ Studies have shown that an increase of ROS in the medium can be toxic to the organisms, leading to the destruction of several cell types through apoptotic pathways.^{1,17} Previously, our research group showed that PvD_1 induced ROS in *C. albicans* and *Fusarium oxysporum* cells.¹⁵ In this study, we observed that *C. buinensis* cells were labeled by the 2',7'-dichlorofluorescein diacetate probe when they were treated with $\gamma_{33-41}PvD_1^{++}$, thus indicating that it caused an increase in endogenous ROS production in these cells (Figure 6). Due to the increased ROS production in the presence of $\gamma_{33-41}PvD_1^{++}$, our next objective was to verify if the apoptotic process could be taking place when the *C. buinensis* cells were treated with $\gamma_{33-41}PvD_1^{++}$. For this process, we verified the presence of active metacaspases⁴⁶ and the dissipation of mitochondrial membrane potential in these cells. Caspases play a central role in signaling death by apoptosis. These are described as specific cysteine-containing aspartate proteases that are among the key markers of the apoptotic pathway, including yeasts.^{46,47} In these two assays, it was possible to observe that $\gamma_{33-41}PvD_1^{++}$ activated the metacaspases in *C. buinensis* cells and caused a collapse of mitochondrial membrane potential in these cells (Figures 7 and 8). Taveira et al⁴² found that *CaThi* caused an activation of metacaspase in *F. solani*, indicating that programmed cell death could be triggered by *CaThi* in this fungus. In another study, Soares et al¹⁷ showed that *ApDef₁* defensin, isolated from the seeds of *Adenanthera pavonina*, caused an increase in the ROS production and accumulation that led to permeabilization of the plasma membrane and, consequently, the death of *Saccharomyces cerevisiae* cells through a metacaspase-dependent apoptotic process. It has also been observed by Vieira et al⁴⁸ that *C. albicans* cells lost mitochondrial functionality when treated with the *Lp-Def₁* defensin, isolated from *Lecythis pisonis* seeds.

Conclusion

In conclusion, taken together, our results suggest that the antifungal activity of PvD_1 is not strictly localized in the structural domain, which comprises the γ -core region; however, we can state that the addition of amino acid residues beyond the γ -core region, which comprises parts of the $\beta 2$ and $\beta 3$ sheets, is important for antifungal activity. Additionally, we observed that the increase in the net positive charge is directly related to the increase in antifungal activity. In this work, we opted to evaluate the mechanism of action of the $\gamma_{33-41}PvD_1^{++}$ peptide due to its significant inhibitory effect on tested yeast. In addition, $\gamma_{33-41}PvD_1^{++}$ is the smallest construct comprising only nine amino acid residues, which gives it a better possibility to be the basis for a design of a new anti-

fungal drug, with lower costs to the pharmaceutical industry, but still maintaining its antimicrobial properties. In relation to the antifungal activity of $\gamma_{33-41}PvD_1^{++}$, it was fungicidal, and its effects on the growth inhibition of *C. buinensis* are related to membrane permeabilization, an endogenous increase in ROS, the loss of mitochondrial functionality and caspase activation, suggesting that $\gamma_{33-41}PvD_1^{++}$ triggers *C. buinensis* cell death via apoptosis, as demonstrated by the key markers of this pathway.

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Author contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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