Curr Microbiol (2011) 62:1209–1217
DOI 10.1007/s00284-010-9847-3

Antifungal Activity of *Pv*D1 Defensin Involves Plasma Membrane Permeabilization, Inhibition of Medium Acidification, and Induction of ROS in Fungi Cells

Erica O. Mello · Suzanna F. F. Ribeiro · André O. Carvalho · Izabela S. Santos · Maura Da Cunha · Claudete Santa-Catarina · Valdirene M. Gomes

Received: 21 June 2010 / Accepted: 2 December 2010 / Published online: 19 December 2010
© Springer Science+Business Media, LLC 2010

Abstract In recent years, studies have demonstrated the function of many antimicrobial peptides against an extensive number of microorganisms that have been isolated from different plant species and that have been used as models for the study of various cellular processes linked to these peptides' activities. Recently, a new defensin from *Phaseolus vulgaris* (L.) seeds, named *Pv*D_1, was isolated and characterized. *Pv*D_1 was purified through anion exchange and phase-reverse chromatography. *Pv*D_1's antifungal activity was tested. A SYTOX Green uptake assay revealed that the defensin *Pv*D_1 is capable of causing membrane permeabilization in the filamentous fungi *Fusarium oxysporum*, *Fusarium solani,* and *Fusarium laterithium* and in yeast strains *Candida parapsilosis*, *Pichia membranifaciens*, *Candida tropicalis*, *Candida albicans*, *Kluyveromyces marxiannus,* and *Saccharomyces cerevisiae* at a concentration of 100 μg/ml. Ultrastructural analysis of *C. albicans* and *C. guilliermondii* cells treated with this defensin revealed disorganization of both cytoplasmic content and the plasma membrane. *Pv*D_1 is also able to inhibit glucose-stimulated acidification of the medium by yeast cells and filamentous fungi, as well as to induce the production of reactive oxygen species and nitric oxide in *C. albicans* and *F. oxysporum* cells.

E. O. Mello · S. F. F. Ribeiro · A. O. Carvalho · I. S. Santos · V. M. Gomes (✉)
Laboratório de Fisiologia e Bioquímica de Microrganismos, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ 28013-602, Brazil
e-mail: valmg@uenf.br

M. Da Cunha · C. Santa-Catarina
Laboratório de Biologia Celular e Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ 28013-602, Brazil

Introduction

Plant defensins are small, basic peptides of 45–54 amino acids comprised in a three-dimensional structure formed by three anti-parallel β-strands and one α-helix. This structure is stabilized by four disulfide bounds which form a cysteine-stabilized α-helix β-strands motif common to these peptides [4]. Plant defensins, such as insect and mammal defensins, possess antimicrobial activity. The discovery of the antimicrobial activity of plant defensins was reported in the early 1990s by Terras et al. [20]. The antimicrobial activity of plant defensins is principally observed against fungi. However, some bacteria, especially Gram-positive species, are also inhibited, though the activity is less pronounced in comparison with the activity against fungi. Growth of several fungal species, including several filamentous fungi and yeast cells, was inhibited when incubated with these peptides [3, 5, 7, 11–14, 19, 21, 22, 24, 28–32]. More recently, it was shown that *Rs*-AFP_2 (*Raphanus sativus* antifungal peptide 2) induces reactive oxygen species (ROS) production in *Candida albicans* in a dose-dependent manner but not at all in an *Rs*-AFP_2-resistant Δ*gcs C. albicans* mutant that lacks the *Rs*-AFP_2-binding site in its membranes. These findings indicate that upstream binding of *Rs*-AFP_2 to GlcCer is needed for ROS production, which leads to yeast cell death. Moreover, the antioxidant ascorbic acid blocks *Rs*-AFP_2-induced ROS generation and *Rs*-AFP_2 antifungal activity [1].

PvD_1 defensin was purified from *Phaseolus vulgaris* (cv. Perola) seeds. *Pv*D_1 inhibited the growth of the different yeast cells and filamentous fungi [8]. In this study,

the antimicrobial activity of *Pv*D_1 was investigated by studying its permeabilization of fungal membranes, inhibition of glucose-stimulated acidification of the medium, and also ultrastructural analysis, and production of ROS and nitric oxide (NO) in the different filamentous fungi cells and yeast cells.

Materials and Methods

Plant Material

Phaseolus vulgaris L. (cv. Pérola) seeds were provided by the Laboratório de Melhoramento Genético Vegetal, Centro de Ciências e Tecnologias Agropecuárias, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, Rio de Janeiro, Brazil.

Fungi

The yeasts *Candida parapsilosis* (CE002), *Candida guilliermondii* (CE013), *Candida tropicalis* (CE017), *Candida albicans* (CE022), *Kluyveromyces marxiannus* (CE025), *Pichia membranifaciens* (CE015), and *Saccharomyces cerevisiae* (1038) were obtained from the Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil. The phytopathogenic fungi *Fusarium oxysporum*, *Fusarium solani,* and *Fusarium laterithium* were obtained from the Laboratório de Fisiologia e Bioquímica de Microrganismos, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, Rio de Janeiro, Brazil. Yeasts and fungi were maintained on Sabouraud agar (1% peptone, 2% glucose and 1.7% agar-agar).

Purification of the *Phaseolus vulgaris* Defensin *Pv*D1

Purification of the defensin from *P. vulgaris* (cv. Pérola) seeds was performed largely as described by [8].

Gel Electrophoresis

*Pv*D1 defensin purification was monitored by SDS-Tricine-gel electrophoresis performed according to the method of Schägger and Von Jagow [18].

Inhibition of Glucose-Stimulated Acidification of the Medium by Yeasts

In order to evaluate the influence of *Pv*D1 interference with fungal metabolism, the authors have analyzed the H^+ extrusion by yeast. It is known that this acidification is due to the activity of the plasma membrane H^+-ATPase, and it is stimulated by glucose [9]. The effect of *Pv*D1 was determined by incubation of yeasts cells (1×10^7) or filamentous fungi cells (1×10^7) with 0.8 ml of 10 mM Tris–HCl, pH 6.0. *Pv*D_1 was added to make up to final concentrations of 100 and/or 200 μg/ml. After the pre-incubation time (1, 2, and 4 h for yeasts and 30 min and 1 h for filamentous fungi), 0.2 ml of 0.5 M glucose solution was added. Measurements of pH were taken at each subsequent minute for the next 30 min. Controls (Tris–HCl buffer was added instead of *Pv*D_1) were run to evaluate the influence of peptides on H^+ extrusion by yeast cells [22]. The concentration of H^+ extrusion was calculated as the difference (ΔpH) between initial ($T = 0$) and final ($T = 30$ min) pH, and the obtained values were applied to the equation $\mathrm{pH} = -\log [H^+]$. The results of inhibition of the glucose-stimulated acidification are shown by average values and standard deviations of triplicate for each experiment. Graphs were drawn based on the assumption that the values obtained for controls corresponded to 100% acidification and the significant test were done according to the Student's *t*-test, calculated with the Statistica software, among the pre-incubation time and their respective controls.

SYTOX Green Uptake Assay

Fungal plasma membrane permeabilization was measured by SYTOX Green uptake as described previously by Thevissen et al. [26] with some modifications. SYTOX Green is a dye that only penetrates cells when the plasma membrane is structurally compromised. Once inside the fungal cytoplasm, it binds to nucleic acids, resulting in a fluorescent complex. Therefore, this dye could be used for visualization of the permeabilization of the fungal plasma membrane. The different fungal species (1×10^4 cells/ml) were incubated in the presence of *Pv*D_1 at the concentration of 100 μg/ml for 36 and 60 h growth for yeast and filamentous fungi, respectively. One hundred-microliter aliquots of the fungal cell suspension were incubated with 0.2 μM SYTOX Green in 1.5 ml microcentrifuge tubes for 2 h at 25°C with periodic agitation. The cells were observed with a DIC microscope (Axiophoto Zeiss) equipped with a fluorescence filter set for fluorescein detection (excitation wavelengths, 450–490 nm; emission wavelength, 500 nm). The results of membrane permeabilization are representative of one triplicate experiment.

Transmission Electron Microscopy

In order to evaluate the influence of *Pv*D_1 on fungal cells morphology, the authors performed analyses of transmission electron microscopy. *C. albicans* and *C. guillermondii* cells, grown for 36 h in Sabouraud broth in the presence (100 μg/ml) or absence of *Pv*D_1, were fixed for 30 min at

room temperature in a solution containing 2.5% glutaraldehyde (vol/vol) and 4% paraformaldehyde (vol/vol) in 50 mM cacodylate buffer (pH 7.2). After fixation, the materials were washed, post-fixed in 1% (wt/vol) osmium tetroxide in the same 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 (TEM) (Zeiss company, Germany) operating at 80 kV. The results of electron microscopy are representative of one triplicate experiment.

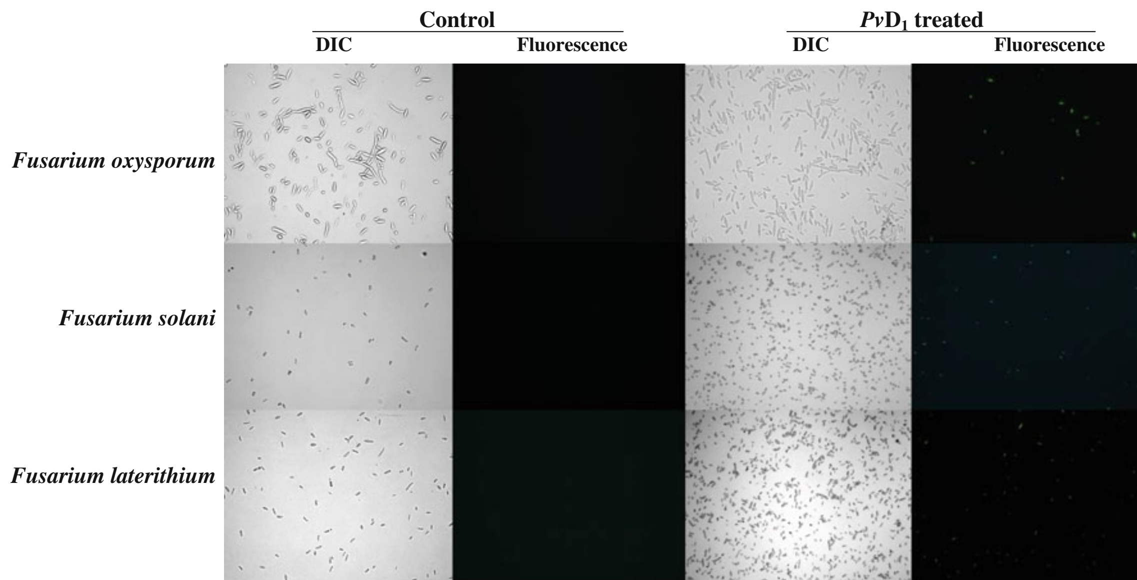

Fig. 1 Membrane permeabilization assay of different filamentous fungal cells treated with *Pv*D_1 (100 μg/ml). 200×

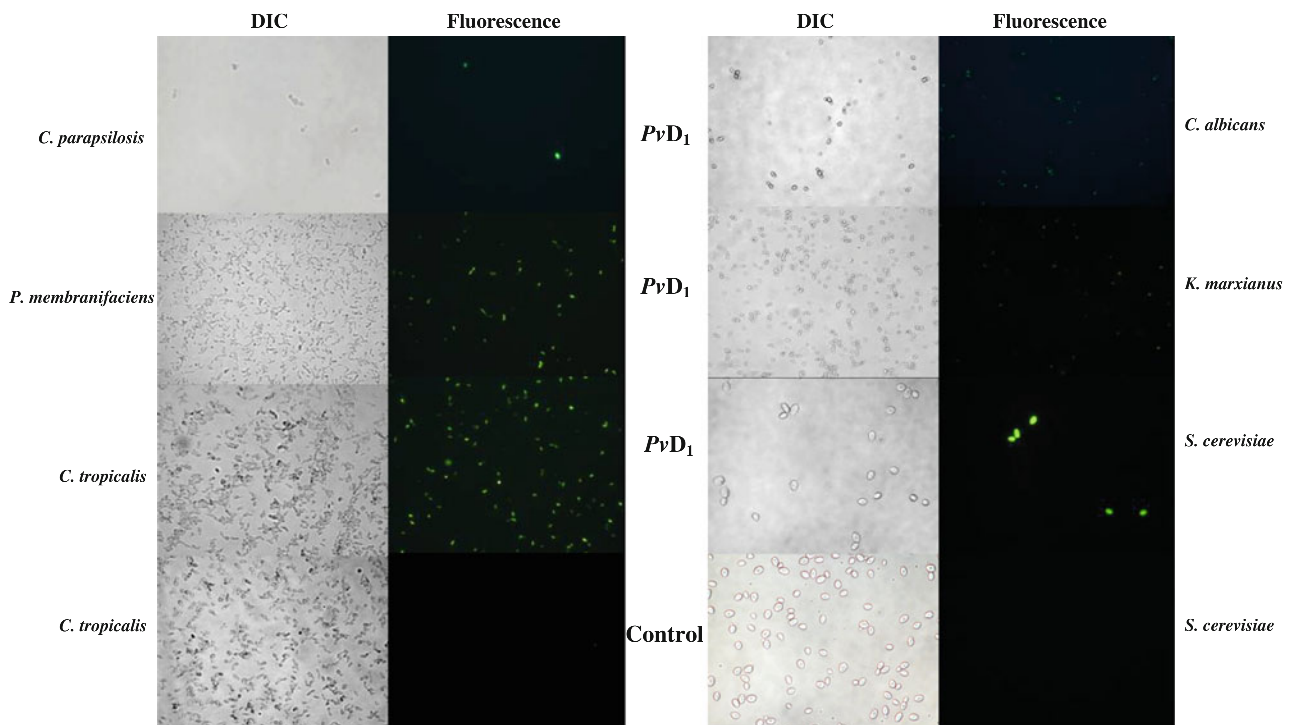

Fig. 2 Membrane permeabilization assay of different yeast cells treated with *Pv*D_1 (100 μg/ml). 400×

ROS and NO Induction Assay

It has been demonstrated that some plant defensins could induce oxidative stress in fungal cell exposed to them [1]. In order to evaluate whether the action mechanism of PvD_1 involve the induction of oxidative stress the authors have used two dyes that indicates the presence of reactive species. Induction of endogenous production of ROS in *C. albicans* and *F. oxysporum* fungi and NO in *C. albicans* yeast cells, treated with 100 µg/ml of the defensin PvD_1 after growth inhibition assay, was evaluated using the fluorescent dye 2′,7′ dichlorofluorescein diacetate (Calbiochem - EMD) and 3-amino, 4-aminomethyl-2′,7′-difluorofluorescein diacetate (Calbiochem - EMD), respectively, using methods described by [1] with some modifications. Incubations were performed as described in the SYTOX Green uptake assay section. The incubation time for this analysis was 24 h of growth in the presence or absence of PvD_1. An aliquot was incubated with constant agitation for 2 h with fluorescent dye to a final concentration of 20 µM, according to instructions provided by the manufacturers. After this period, these cells were transferred to slides, covered with coverslips, and analyzed with a fluorescence microscope (Axiophoto Zeiss) equipped with a fluorescence filter set for fluorescein detection (excitation wavelengths, 450–490 nm; emission wavelength, 500 nm). The results of oxidative stress are representative of one triplicate experiment.

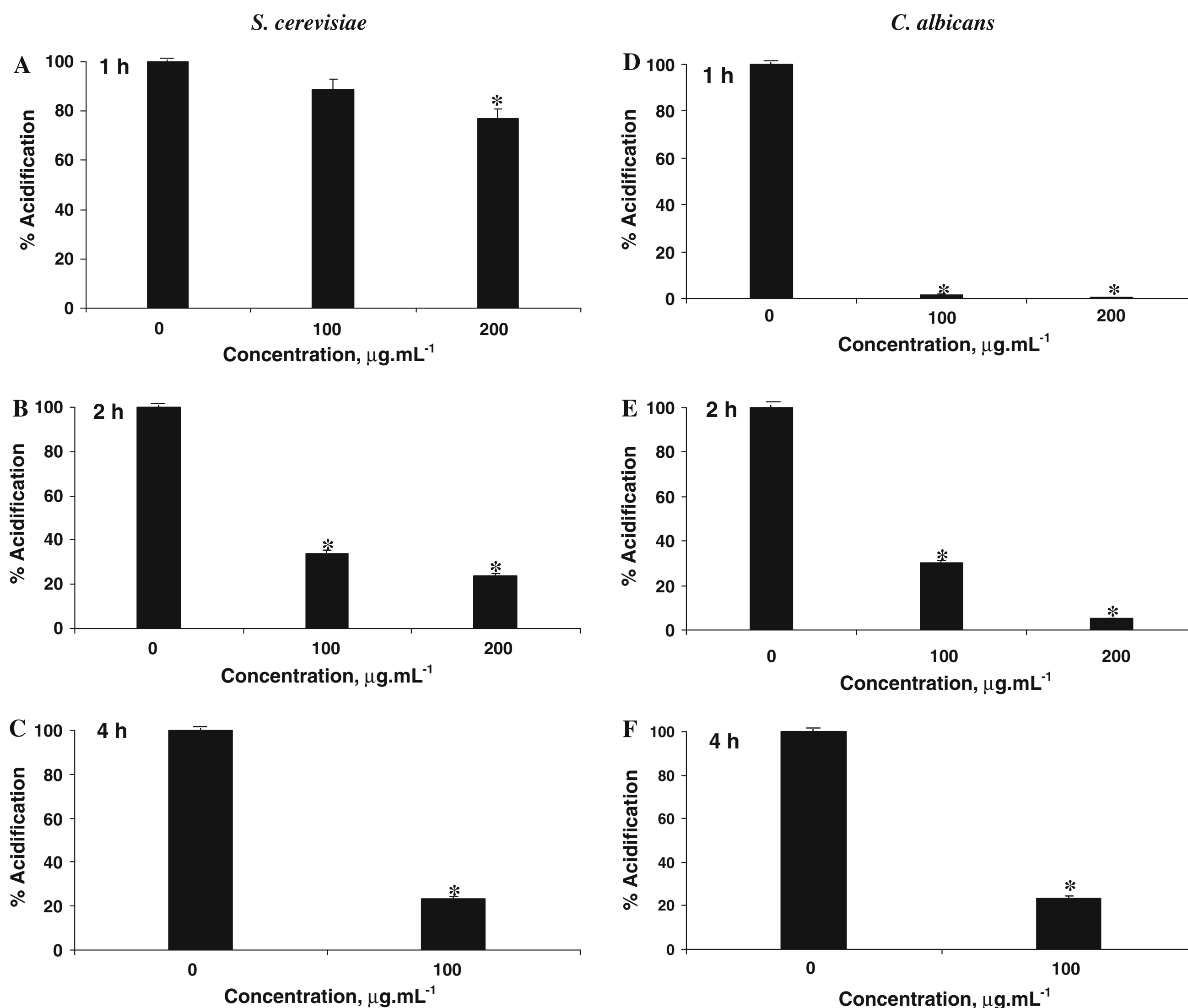

Fig. 3 The effect of PvD_1 on the glucose-dependent acidification of the medium by *S. cerevisiae* and *C. albicans* cells. PvD_1 was pre-incubated for 1, 2, and 4 h at 100 and/or 200 µg/ml before addition of glucose. Significantly different from the controls according to Student's *t*-test ($P < 0.05$)

Results and Discussion

The ability of PvD_1 to permeabilize the plasma membrane of different fungal cells, especially pathogenic yeasts, was examined. SYTOX Green membrane permeabilization was assessed after 60 and 36 h of growth for filamentous fungi and yeasts, respectively, in the presence of PvD_1 and 2 h after the addition of SYTOX Green. When observed with a fluorescence microscope, all filamentous fungal cells showed strong SYTOX Green fluorescence in the presence of PvD_1 (Fig. 1), as compared with controls, in which fungi were grown in the absence of PvD_1 (Fig. 1). All these yeast cells also showed strong SYTOX Green fluorescence in the presence of PvD_1 (Fig. 2), especially *P. membranifaciens* and *C. tropicalis*, as compared with controls, in which cells of *C. tropicalis* and *S. cerevisiae* were grown in the absence of PvD_1. The controls for the other yeasts showed the same results (data not show). Different defensins have also been found to permeabilize membranes and to modulate ion flux across membranes, and they have since become popular models for understanding how ion channel proteins function [1]. Thevissen et al. [25], for example, demonstrated that when the fungi *Neurospora crassa* and *F. culmorum* were treated with the plant defensins *Rs*-AFP_2 and *Dm*-AMP_1 (*Dhalia merckii* antimicrobial peptide 1), ion flux across the fungal plasma membrane was observed. The fungal membrane permeabilization was also shown in response to other antimicrobial peptides, such as lipid transfer proteins and 2S albumins [2, 6, 17], and membrane permeabilization is related to the inhibition of fungal growth [26].

Interference with the function of H^+-ATPase in fungi by antagonists commonly leads to cell death. In this study, the authors investigated whether PvD_1 could interfere with the fungal H^+-ATPase. For this, the authors monitored glucose-stimulated acidification of the incubation medium by *S. cerevisiae* and *C. albicans* cells in the presence of various concentrations of PvD_1 defensin, a phenomenon that is dependent on the activity of the H^+-ATPase. As shown in Fig. 3a, PvD_1, at concentrations of 100 and 200 μg/ml, was able to decrease this acidification in *S. cerevisiae* by 10 and 20%, respectively, when used for a pre-incubation time of 1 h, been the higher concentration used statistically significant. In Fig. 3b, PvD_1, at the same concentrations, was able to lower this acidification by 65 and 75%, respectively, when used for a pre-incubation time of 2 h. Finally, when held for a pre-incubation time of 4 h in the presence of PvD_1 at the concentration of 100 μg/ml, the decrease in acidification observed was 80% (Fig. 3c). These results suggest that for *S. cerevisiae* cells, the inhibition of acidification is dose and pre-incubation time-dependent. For *C. albicans*, as shown also in Fig. 3d, PvD_1, at the concentrations of 100 and 200 μg/ml, strongly inhibited acidification of the medium; the inhibitions observed were 97 and 99%, respectively, when a pre-incubation time of 1 h was used. As shown in Fig. 3e, PvD_1 was able to inhibit this acidification by 68% and 90%, respectively, when a pre-incubation time of 2 h was used. Interestingly, when a pre-incubation time of 4 h was used, the inhibition of acidification observed was 72% at the concentration 100 μg/ml, suggesting that for *C. albicans* cells (Fig. 3f), the inhibition of acidification is not dose or pre-incubation-time dependent.

For filamentous fungi (*F. oxysporum* and *F. solani*), as shown in Fig. 4, PvD_1, at the concentrations of 200 μg/ml, strongly inhibited acidification of the medium. The inhibition observed was 99%, for both fungi, when a pre-incubation time of 30 min was used; with pre-incubation time of 1 h, were obtained the same results (data not shown). However, the filamentous fungi are not good models for testing acidification, since even in the presence of glucose, they acidify slightly. The authors believe that this effect is due to the sum of the toxicity of the peptide and the low acidifying capacity of filamentous fungi. Different peptides have been studied, and the analysis has been performed to test the ability of some of these peptides

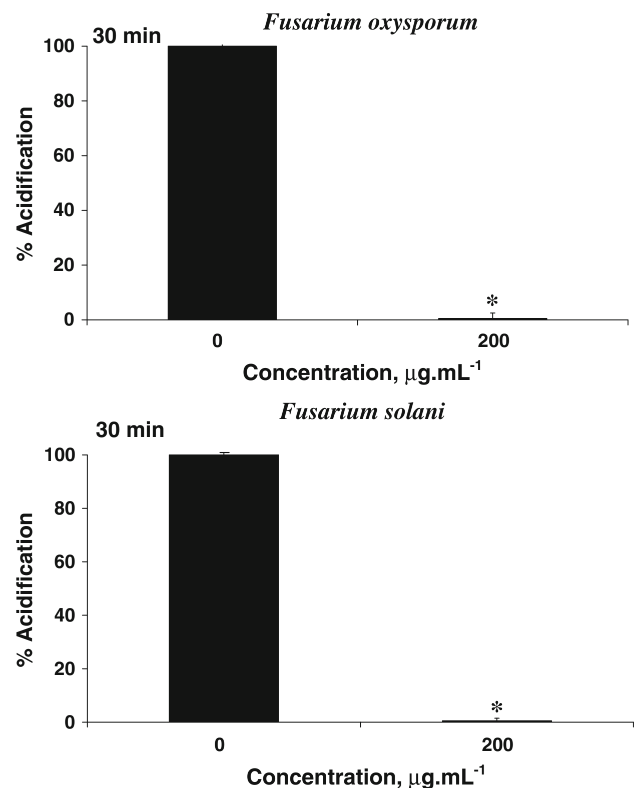

Fig. 4 The effect of PvD_1 on the glucose-dependent acidification of the medium by *F. oxysporum* and *F. solani* conidia. PvD_1 was pre-incubated for 30 min at 200 μg/ml before addition of glucose. Significantly different from the controls according to Student's *t*-test ($P < 0.05$)

to act on the plasma membrane [25–27] and consequently interfering with the function of H^+-ATPase, causing alterations in the glucose-stimulated acidification of the medium by the cells.

TEM observations of yeast cells showed normal ultrastructure development in control cells (Fig. 5a, d); however, cells treated with PvD_1 (Fig. 5b, c, e, f) exhibited plasma membrane blebbing, disappearing and shrinking cytosol, and disorganization of the nucleus and other organelles. Not all of these features were observed in every cell, but they occurred quite frequently. The percentage of cells observed with abnormal features was approximately 80% as compared with controls.

Another objective of this study was to investigate the ability of PvD_1 to induce the production of ROS and NO in yeasts cells. It was recently demonstrated that PsD_1 (*Pisum sativum* defensin 1) is internalized into the cytoplasm and targeted to the nucleus [10]. NaD_1 (*Nicotiana alata* defensin 1) has also been shown to enter the cytoplasm of *F. oxysporum f. sp. vasinfectum*, but in contrast to PsD_1, it was not observed in the nucleus, indicating that defensins may act by different mechanisms [27]. These results provide a direct link between ROS generation and the antifungal effect of the peptide. Using a microscopic assay, the authors were able to demonstrate ROS induction in *C. albicans* cells incubated with 100 μg/ml PvD_1 for 24 h (Fig. 6) compared with control cells (Fig. 6) that were not treated with PvD_1. The authors were able also to demonstrate ROS induction in *F. oxysporum* cells incubated with 100 μg/ml PvD_1 for 24 h (Fig. 6) as compared with control cells (Fig. 6) that were not treated with PvD_1. The ROS induction capacity of various antifungals has been previously reported. Azole antifungals, as well as the polyenes and other antifungals, which interact with ergosterol in the fungal membrane cause membrane permeabilization and induce ROS production in susceptible fungi [15, 23, 25, 27].

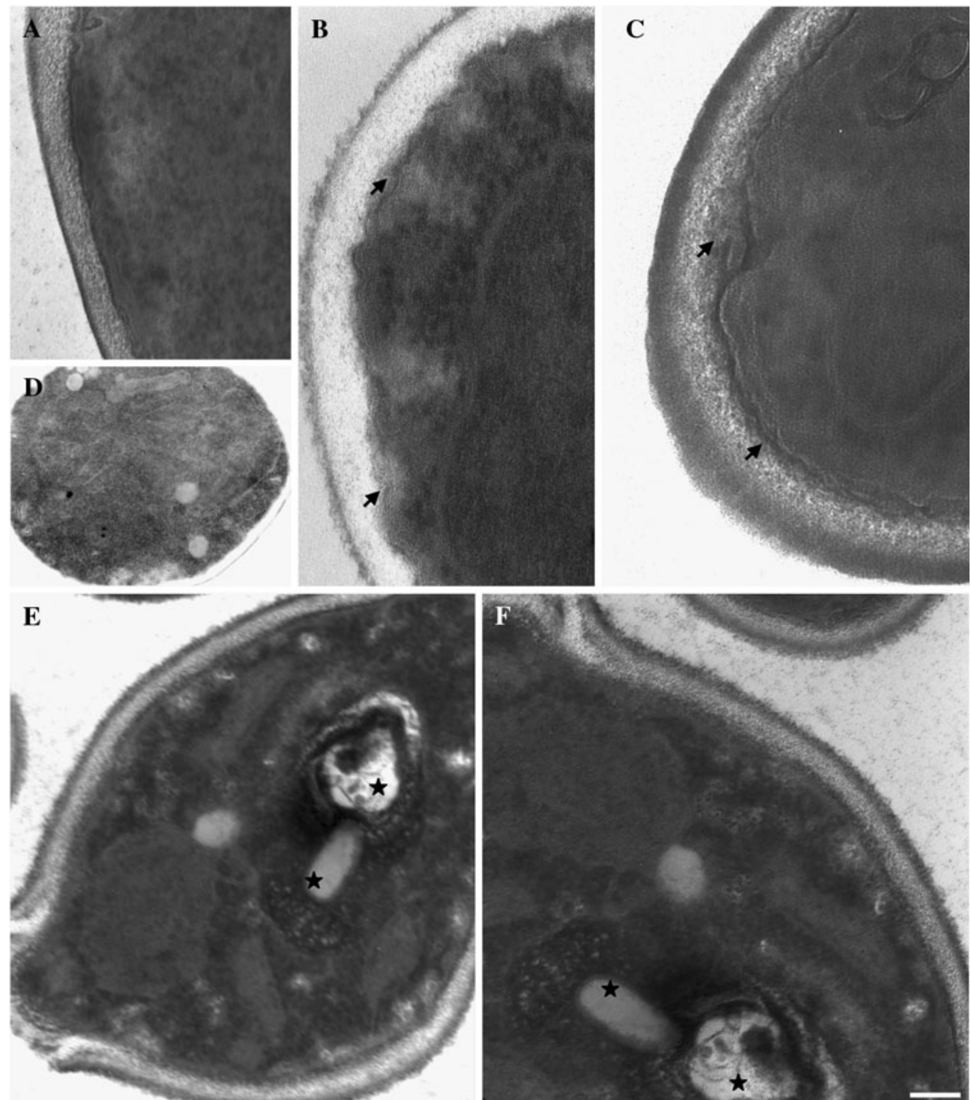

Fig. 5 Transmission electron microscopy of *C. albicans* (**a**, **b**, **d**, **e**, **f**) and *C. guilliermondii* (**c**). **a**, **d** controls; **b**, **c**, **e**, **f** cells treated with PvD_1 (100 μg/ml). A *star* indicates condensation and shrinkage of the cytosol with loss of cytosol structure and contents; *arrows* indicate cell and plasma membrane blebbing; *bars*: **a**, 0.4 μm; **b**, **c**, **e**, **f**, 0.25 μm

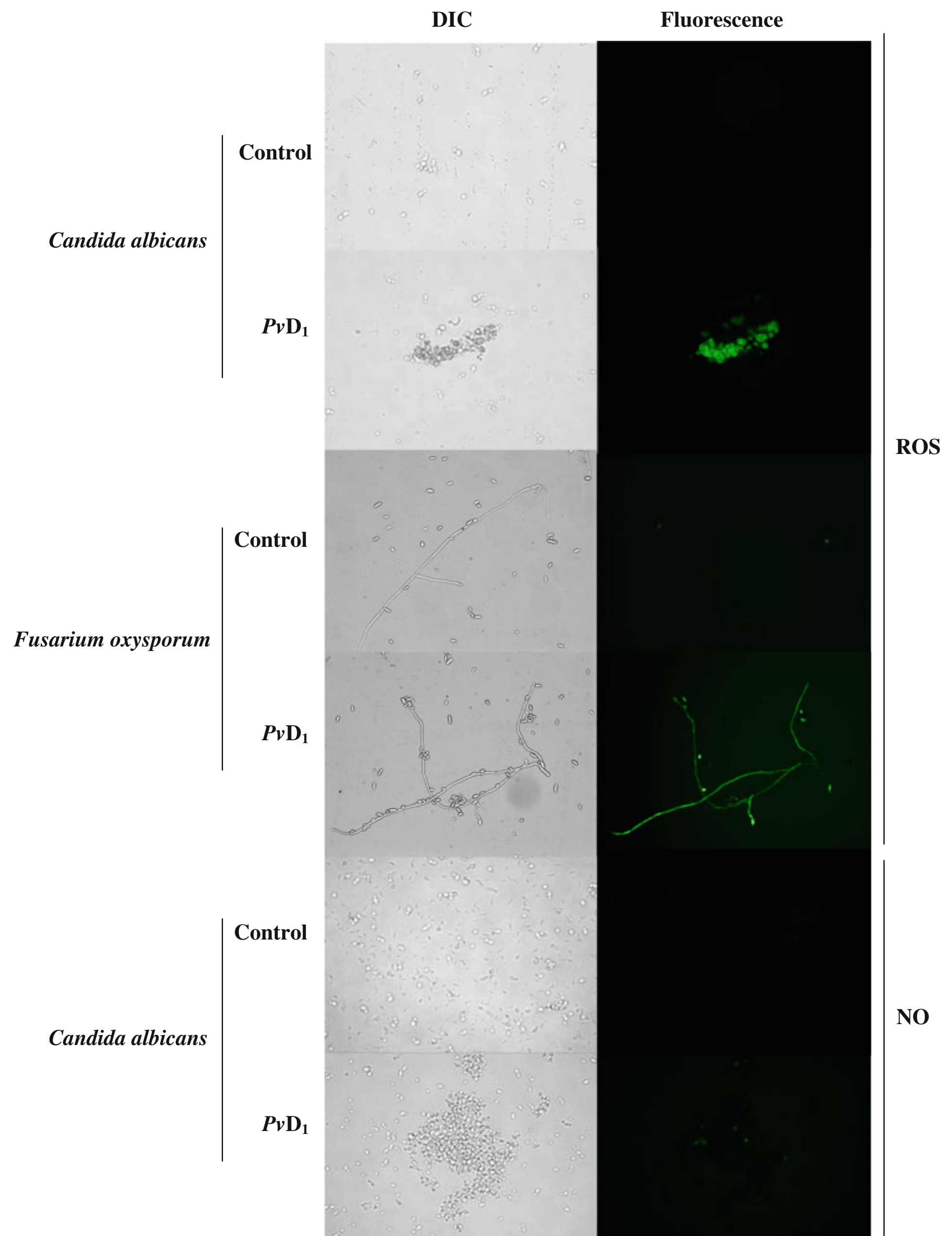

Fig. 6 Oxidative stress assay for ROS and NO of *C. albicans* and *F. oxysporum* cells previously incubated with 100 µg/ml of *Pv*D_1 for 24 h. Cells were treated with 2′,7′ dichlorofluorescein diacetate for ROS and/or 3-amino, 4-aminomethyl-2′,7′-difluorofluorescein diacetate for NO detection. 400× for yeasts and 200× for filamentous fungi

Induction of NO production in *C. albicans* cells was also demonstrated after incubation of the cells with 100 µg/ml of *Pv*D1 for 24 h (Fig. 6). Little or no fluorescence was observed in the case of control cells (Fig. 6).

The authors found in this study that the defensin isolated from *P. vulgaris*, *Pv*D_1, was able to permeabilize the membranes of filamentous fungi and yeasts. Ultrastructural analysis of yeast cells treated with this defensin revealed disorganization of both cytoplasmic content and the plasma membrane. Incubation with the *Pv*D_1 caused fungal inhibition of the glucose-stimulated acidification for the filamentous fungi and yeasts and was also able to cause induction of ROS in *C. albicans* and *F. oxysporum* and NO in *C. albicans*. The antimicrobial activity of plant defensins are initially felt at the level of the fungal plasma membrane where receptors were identified. They are for *Dm*-AMP_1 the sphingolipid manosyldiinositolphosphrylceramide [22] and for *Rs*-AFP_2 and *Ms*DEF_1 the sphingolipid glucosylceramide [16, 22]. After this initial binding on components of the fungal membranes, secondary effects induced internally in the cell have been demonstrated. Lobo et al. [10] have demonstrated that *Ps*D1 interacts with cyclin F and consequently interferes with the cell cycle [1, 27]; *Na*D_1 and *Rs*-AFP_2 induce ROS production; and that these

toxic substances are involved in fungal growth arrest. These results reinforce the idea that different plant defensins must act by different mechanisms and demonstrate a complicated and sophisticated mechanism underlying fungal growth arrest.

Acknowledgments This study forms part of the M.Sc. degree dissertation and DSc degree thesis of EOM, first author of this article, carried out at the Universidade Estadual do Norte Fluminense. The authors acknowledge the financial support of the Brazilian agencies CNPq, CAPES, and FAPERJ.

References

1. Aerts AM, Francois IEJA, Meert EMK, Li Q-T, Cammue BPA, Thevissen K (2007) The antifungal activity of Rs-AFP2, a plant defensin from *Raphanus sativus*, involves the induction of reactive oxygen species in *Candida albicans*. J Mol Microbiol Biotechnol 13:243–247
2. Agizzio AP, Da Cunha M, Carvalho AO, Oliveira MA, Ribeiro SFF, Gomes VM (2006) The antifungal properties of a 2S albumin-homologous protein from passion fruit seeds involve plasma membrane permeabilization and ultrastructural alterations in yeast cells. Plant Sci 171:515–522
3. Anaya-López JL, López-Meza JE, Baizabal-Aguirre VM, Cano-Camacho H, Ochoa-Zarzosa A (2006) Fungicidal and cytotoxic activity of a *Capsicum chinense* defensin expressed by endothelial cells. Biotechnol Lett 28:1101–1108
4. Carvalho AO, Gomes VM (2009) Plant defensins—prospects for the biological functions and biotechnological properties. Peptides 30:1007–1020
5. Chen G-H, Hsu M-P, Tan C-H, Sung H-Y, Kuo CG, Fan M-J, Chen H-M, Chen S, Chen C-S (2005) Cloning and characterization of a plant defensin VaD1 from azuki bean. J Agric Food Chem 53:982–988
6. Diz MSS, Carvalho AO, Rodrigues R, Neves-Ferreira AGC, Da Cunha M, ElW Alves, Okorokova-Façanha AL, Oliveira MA, Perales J, Machado OLT, Gomes VM (2006) Antimicrobial peptides from chilli pepper seeds causes yeast plasma membrane permeabilization and inhibits the acidification of the medium by yeast cells. Biochim Biophys Acta 1760:1323–1332
7. Finkina EI, Shramova EI, Tagaev AA, Ovchinnikova TV (2008) A novel defensin from the lentil *Lens culinaris* seeds. Biochem Biophys Res Commun 371:860–865
8. Games PD, Santos IS, Mello EO, Diz MSS, Carvalho AO, Souza-Filho GA, Da Cunha M, Vasconcelos IM, Ferreira BS, Gomes VM (2008) Isolation, characterization and cloning of a cDNA encoding a new antifungal defensin from *Phaseolus vulgaris* L. seeds. Peptides 29:2090–2100
9. Gomes VM, Okorokov LA, Rose TL, Fernandes KVS, Xavier-Filho J (1998) Legume vicilins (7S storage globulins) inhibit yeast growth and glucose stimulated acidification of the médium by yeast cells. Biochim Biophys Acta 1379:207–216
10. Lobo DS, Pereira IB, Fragel-Madeira L, Medeiros LN, Cabral LM, Faria J, Bellio M, Campos RC, Linden R, Kurtenbach E (2007) Antifungal *Pisum sativum* defensin 1 interacts with *Neurospora crassa* cyclin F related to the cell cycle. Biochemistry 46:987–996
11. Odintsova TI, Rogozhin EA, Baranov Y, Musolyamov AK, Nasser Y, Egorov TA, Grishin EV (2008) Seed defensins of barnyard grass *Echinochloa crusgalli* (L.). Beauv Biochimie 90:1667–1673
12. Olli S, Kirti PB (2006) Cloning, characterization and antifungal activity of defensin Tfgd1 from *Trigonella foenum-graecum* L. J Biochem Mol Biol 39:278–283
13. Osborn RW, De Samblanx GW, Thevissen K, Goderis I, Torrekens S, Van Leuven F, Attenborough S, Rees SB, Broekaert WF (1995) Isolation and characterisation of plant defensins from seeds of Asteraceae, Fabaceae, Hippocastanaceae and Saxifragaceae. FEBS Lett 368:257–262
14. Park HC, Kang YH, Chun HJ, Koo JC, Cheong YH, Kim CY, Kim MC, Chung WS, Kim JC, Yoo JH, Koo YD, Koo SC, Lim CO, Lee SY, Cho MJ (2002) Characterization of a stamen-specific cDNA encoding a novel plant defensin in Chinese cabbage. Plant Mol Biol 50:59–69
15. Phillips AJ, Sudbery I, Ramsdale M (2003) Apoptosis induced by environmental stresses and amphotericin B in *Candida albicans*. PNAS 100:14327–14332
16. Ramamoorthy V, Cahoon EB, Li J, Thokala M, Minto RE, Shah DM (2007) Glucosylceramide synthase is essential for alfalfa defensin-mediated growth inhibition but not for pathogenicity of *Fusarium graminearum*. Mol Microbiol 66(3):771–786
17. Regente MC, Giudici AM, Villalaín J, de la Canal L (2005) The cytotoxic properties of a plant lipid transfer protein involve membrane permeabilization of target cells. Lett Appl Microbiol 40:183–189
18. Schägger H, Von Jagow G (1987) Tricine-sodium dodecylsulfate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166:368–379
19. Solis J, Medrano G, Ghislain M (2007) Inhibitory effect of a defensin gene from the Andean crop maca (*Lepidium meyenii*) against *Phytophthora infestans*. J Plant Physiol 164:1071–1082
20. Terras FRG, Schoofs HME, De Bolle MFC, Van Leuven F, Rees SB, Vanderleyden J, Cammue BPA, Broekaert WF (1992) Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. J Biol Chem 267:15301–15309
21. Terras FRG, Torrekens S, Van Leuven F, Osborn RW, Vanderleyden J, Cammue BPA, Broekaert WF (1993) A new family of basic cysteine-rich plant antifungal proteins from *Brassicaceae* species. FEBS 316:233–240
22. Thevissen K, Ferket KKA, François IEJA, Cammue BPA (2003) Interactions of antifungal plant defensins with fungal membrane components. Peptides 24:1705–1712
23. Thevissen K, François IE, Winderckx J, Pannecouque C, Cammue BP (2006) Ceramide involvement in apoptosis and apoptotic diseases. Min Rev Med Chem 6:69–709
24. Thevissen K, François IEJA, Takemoto JY, Ferket KKA, Meert EMK, Cammue BPA (2003) DmAMP1, an antifungal plant defensin from dahlia (*Dahlia merckii*), interacts with sphingolipids from *Saccharomyces cerevisiae*. FEMS Microbiol Lett 226: 169–173
25. Thevissen K, Ghazi A, De Samblanx GW, Brownleei C, Osborn RW, Broekaert WF (1996) Fungal membrane responses induced by plant defensins and thionins. J Biol Chem 271:15018–15025
26. Thevissen K, Terras FRG, Broekaert WF (1999) Permeabilization of fungal membranes by plant defensins inhibits fungal growth. Appl Environ Microbiol 65:5451–5458
27. van der Weerden NL, Lay FT, Anderson MA (2008) The plant defensin, NaD_1, enters the cytoplasm of *Fusarium oxysporum* hyphae. J Biol Chem 283:14445–14452
28. Wang HX, Ng TB (2006) An antifungal peptide from baby lima bean. Appl Microbiol Biotechnol 73:576–581
29. Wisniewski ME, Bassett CL, Artlip TS, Webb RP, Janisiewicz WJ, Norelli JL, Goldway M, Droby S (2003) Characterization of a defensin in bark and fruit tissues of peach and antimicrobial activity of a recombinant defensin in the yeast, *Pichia pastoris*. Physiol Plant 119:563–572

30. Wong JH, Ng TB (2005) Sesquin, a potent defensin-like antimicrobial peptide from ground beans with inhibitory activities toward tumor cells and HIV-1 reverse transcriptase. Peptides 26:1120–1126
31. Wong JH, Ng TB (2005) Vulgarinin, a broad-spectrum antifungal peptide from haricot beans (*Phaseolus vulgaris*). IJBCB 37:1626–1632
32. Ye XY, Ng TB (2002) A new antifungal peptide from rice beans. J Peptide Res 60:81–87