Fungicidal activity of miconazole against *Candida* spp. biofilms

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Abstract

Objectives: Although azole antifungals are considered to be fungistatic, miconazole has fungicidal activity against planktonic *Candida albicans* cells, presumably associated with the induction of reactive oxygen species (ROS) production. Only few data are available concerning the effect of miconazole against sessile *Candida albicans* cells. In the present study, the fungicidal activity of miconazole against *in vitro* grown mature *Candida* biofilms and its relationship with the induction of ROS and ROS-dependent apoptosis were examined.

Methods: The effect of miconazole on mature biofilms formed by ten *Candida albicans* strains and five strains from other *Candida* species was evaluated by plate counting and measuring the level of ROS induction. MIC tests were performed in the absence and presence of ascorbic acid, a quencher of ROS. The apoptotic population in *C. albicans* cells was determined using Annexin-Cy3.

Results: Miconazole showed a significant fungicidal effect against all mature *Candida* biofilms tested and caused elevated ROS levels, both in planktonic and sessile cells. Addition of ascorbic acid drastically reduced these levels. While ROS quenching decreased the susceptibility to miconazole of planktonic cells of most *Candida* strains, no reduced fungicidal activity of miconazole against biofilms was observed. Miconazole did not cause a significant increase in apoptosis.

Conclusions: ROS levels increased in all *Candida* biofilms upon addition of miconazole. However, ROS induction was not the only factor that underlies its fungicidal activity, as quenching of ROS did not lead to an enhanced survival of

biofilm cells. ROS-induced apoptosis was not observed in *C. albicans* cells after miconazole treatment.

Introduction

Candida species are frequently associated with nosocomial infections in immunocompromised hosts.[1] Device-related infections caused by this organism often involve biofilm formation, a process in which planktonic yeast cells adhere to a biotic or abiotic surface, ultimately resulting in the formation of a complex three-dimensional structure of yeast cells, filaments and extracellular polymeric matrix.[2,3] There are profound differences between planktonic and sessile cells, including an increased tolerance of the latter towards antifungal agents.[4]

Azole antifungals are widely used to treat infections with *Candida* spp. These compounds inhibit the 14α-demethylation of lanosterol by interacting with cytochrome P450, a crucial enzyme in the ergosterol biosynthetic pathway. The resulting decrease of ergosterol levels and the accumulation of toxic sterol intermediates in the cytoplasmic membrane lead to growth inhibition.[5] Miconazole (an imidazole) has reportedly a higher *in vitro* activity against planktonic *Candida albicans* cells than the more recently developed and presumably more active fluconazole (a triazole).[6-8] However, few data are available about the effect of miconazole against *Candida* biofilms. A fungicidal activity was observed for miconazole on *C. albicans* biofilms, but only in young (2 – 6h) biofilms.[9] Recent research has shown that the fungicidal activity of miconazole against planktonic *C. albicans* cells is related to the induction of reactive oxygen species (ROS). Although the exact mechanism of this enhanced ROS accumulation is not completely understood, combined inhibition of catalase and peroxidase, as well as changes in the actin cytoskeleton appear to be involved.[7,8] It is well known that anti-oxidants can act as a reductant for ROS. Anti-oxidative compounds are important for the prevention of peroxidation and free radical accumulation.[10] Furthermore, ROS are inducers of

apoptosis.[11] Programmed cell death was observed in *Saccharomyces cerevisiae* exposed to different types of oxidative stress.[12,13] Hyperactivation of the RAS-signaling pathway by stabilization of the actin cytoskeleton leads to an increase in cAMP, followed by the loss of the mitochondrial membrane potential and the accumulation of ROS, ultimately leading to apoptosis.[14] Apoptosis is also induced in *C. albicans* upon treatment with low doses of H_2O_2, acetic acid or amphotericin B.[15]

The aim of the present study was to investigate the activity of miconazole against *Candida* biofilms. To this end, the effect of miconazole and fluconazole against mature biofilms of ten *C. albicans* strains and five strains belonging to other *Candida* species was compared. ROS levels were determined in miconazole-treated and untreated mature *Candida* biofilms to verify whether there is a correlation with the activity of miconazole. Finally, we investigated whether increased apoptosis contributes to the antimicrobial effect of miconazole.

Materials and methods

Strains

The following strains were used: *C. albicans* SC5314 (ATCC MYA-2876) (American Type Culture Collection, Teddington, UK), *C. albicans* ATCC 10231, *C. albicans* IHEM 10284 (Institute of Hygiene and Epidemiology-Mycology Section, Brussels, Belgium), *C. albicans* IHEM 9559, *C. albicans* NCYC 1467 (National Collection of Yeast Cultures, Norwich, UK), *C. albicans* MUCL 29800 (Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium), *C. albicans* MUCL 29903, *C. albicans* MUCL 29981, *C. albicans* MUCL 29919, *C. albicans* MUCL 30112, *Candida dubliniensis* IHEM 14280, *C. glabrata* MUCL 15664, *Candida krusei* IHEM 1796, *Candida parapsilosis* IHEM 3270 and *Candida tropicalis* IHEM

4225. A stock culture of all these strains was kept in Microbank Tubes (Pro-Lab Diagnostics, Richmond Hill, ON, Canada) at -80°C. Cells were routinely transferred to Sabouraud Dextrose Agar (SDA) (Oxoid, Basingstoke, UK) plates or Sabouraud Dextrose Broth (SDB) (Oxoid) and incubated at 37°C for 24 h.

Biofilm formation on silicone disks

Candida biofilms were grown on sterile silicone disks (4 mm thickness and 13 mm in diameter) in a 24-well microtiter plate (TPP, Trasadingen, Switzerland). Silicone sheets were prepared from a medical grade silicone rubber kit (Q7-4735; Dow Corning Corp., Midland, MN, USA), according to the manufacturer's instructions. The disks were punched from the sheets, washed in 2% RBS 35 detergent (Sigma-Aldrich, St. Louis, MO, USA) and in MilliQ water (Millipore, Billerica, MA, USA) and autoclaved. Inoculum suspensions were prepared by incubating the cells in SDB for 16 h at 37°C. After removing the supernatant, the cells were washed three times with and finally resuspended in 1 mL 0.9% (w/v) NaCl (Novolab, Geraardsbergen, Belgium). This inoculum was further diluted with yeast nitrogen base 0.1x (YNB, BD, Franklin Lakes, USA) supplemented with 5 mM glucose (Sigma-Aldrich) to yield an optical density of 0.07 at a wavelength of 600 nm. One mL of a 1:100 dilution of the inoculum in YNB 0.1x was added to each well containing a silicone disk and the 24-well microtiter plates were incubated for 1 h at 37°C. Subsequently, the silicone disks were rinsed three times with 1 mL 0.9% (w/v) NaCl to remove non-adherent cells and aseptically transferred to a new well. Following the addition of 1 mL of diluted YNB (0.004x with final glucose concentration 0.2 mM) to each well, the plates were further incubated for 24 h at 37°C.

Biofilm formation in 96-well microtiter plates

Candida biofilms were grown in round-bottomed 96-well microtiter plates (TPP), as described previously,[16] with an adhesion phase of 1 h followed by a growth phase of 24 h (both at 37°C in SDB).

Treatment of biofilms with antifungal agents

The silicone disks containing the mature biofilms were transferred to a new 24-well microtiter plate. One mL of a solution of fluconazole (Diflucan, Pfizer, Brussels, Belgium) or miconazole nitrate (Certa, Braine–l'Alleud, Belgium) (final concentration 5 mM, corresponding to 1531 mg/L for fluconazole and 2081 mg/L for miconazole) in PBS (phosphate buffered saline) with 2% DMSO (Sigma-Aldrich) was added to the biofilms. Appropriate controls were also included. The plates were incubated at 37°C for 24 h. The silicone disks were subsequently washed three times with 1 mL 0.9% (w/v) NaCl. The number of colony forming units (cfu) on each silicone disk was determined by pour plating. To this end, the silicone disks with biofilms were transferred to 10 mL of SDB and biofilm cells were removed from the silicone by three cycles of 30 s of sonication and 30 s of vortex mixing. With this procedure, all sessile cells were removed from the silicone disks and clumps of cells were broken apart.[17] Serial tenfold dilutions of the resulting cell suspension were made and 1 mL of each dilution was plated and SDA was added, resulting in a lower limit of detection of 10 cfu per disk. Plates were incubated for 24 h at 37°C, after which the number of cfu per disk was calculated by counting colonies on the plates. For each strain and treatment, biofilms formed on at least three silicone disks in at least three independent experiments ($n \geq 9$) were included.

Detection of ROS

Mature biofilms formed in round bottomed 96-well microtiter plates were rinsed with 100 μL 0.9% (w/v) NaCl and treated for 24 h at 37°C with 100 μL of a miconazole suspension (5 mM in PBS with 2% DMSO). Appropriate controls were included and incubated under identical conditions. ROS accumulation was measured in a fluorometric assay using 2',7'-dichlorofluorescein diacetate (DCFHDA) (Invitrogen, Carlsbad, CA, USA).[6] To this end, biofilms were incubated with 10 μM DCFHDA, simultaneously with the antifungal treatment. Fluorescence was measured after 24 h incubation using a Wallac Victor Multilabel Counter (Perkin Elmer, Wellesley, MA, USA) (λ_{EX} = 485 nm; λ_{EM} = 535 nm). Values obtained were corrected for background fluorescence (measured in the absence of cells) and compared to those obtained with untreated biofilms. ROS levels were quantified in duplicate on at least three biofilms ($n \geq 6$) for each strain. To determine whether fluorescence is generated in the extracellular environment or intracellularly, the entire content of the well (supernatant and biofilm) was removed and cells were separated from the supernatant by centrifugation. The fluorescence of the supernatant and of the resuspended sessile cells in PBS was measured separately, as described above.

Influence of anti-oxidants on miconazole-treated biofilms

A set of anti-oxidative compounds was used to investigate their possible protective effect against miconazole activity, including cysteine (0.025% w/v and 0.25% w/v) (Sigma-Aldrich), mannitol (10 mM and 100 mM) (Merck, Darmstadt, Germany), glutathione (1.5 mM and 15 mM) (Sigma-Aldrich), ascorbic acid (10 mM and 100 mM) (Merck) and pyrrolidinedithiocarbamate (PDTC) (10 μM and 1 mM) (Sigma-Aldrich). The level of ROS was determined in miconazole-treated biofilms using

DCFHDA (10 μM) in the presence and absence of the anti-oxidants. The number of cfu on silicone disks of *C. albicans* SC5314 treated with miconazole in combination with the selected anti-oxidative compounds was determined by plating in at least three independent experiments on at least three silicone disks for each condition ($n \geq 9$).

Antifungal susceptibility assay

The minimal inhibitory concentration (MIC) of miconazole in the presence and absence of ascorbic acid (10 mM) was determined according to the protocol of the European Committee on Antimicrobial Susceptibility Testing.[18] The medium used for these experiments was RPMI-1640 2x with L-glutamine and without sodium bicarbonate (Sigma-Aldrich), supplemented with 2% w/v glucose (Sigma-Aldrich) and buffered to pH 7.0 with MOPS (Sigma-Aldrich). Flat-bottomed 96-well microtiter plates were inoculated with *Candida* to obtain $5x10^5$ cells per mL in each well. After 24 h incubation at 35°C, the absorbance was measured at a wavelength of 590 nm using a Wallac Victor microtiter plate reader (Perkin Elmer). DCFHDA (10 μM) was also added to each well and the absorbance measurement was immediately followed by a measurement of fluorescence (λ_{EX} = 485 nm; λ_{EM} = 535 nm).

Detection of apoptosis

To investigate a possible apoptosis inducing effect of miconazole on *C. albicans* SC5314 biofilms the Apoptosis Detection Kit Annexin V-CY3 (Sigma-Aldrich) was used. This test allows to differentiate between living (green fluorescence), necrotic (red fluorescence) and apoptotic cells (green and red fluorescence). Biofilms were grown in 96-well microtiter plates and treated with miconazole, as described above. Sessile cells were removed and diluted 1:10 in PBS, after which 50 μL was spotted on

a microscope slide and left at room temperature, allowing the cells to be adsorbed to the slide. The adsorbed cells were carefully washed three times with binding buffer (supplied with the apoptosis kit) and treated with a mixture of 6-carboxyfluorescein diacetate (500 μM) and annexin V-Cy3 conjugate (1 mg/L) for 10 min. Excess labelling agent was removed by washing the cells three times with binding buffer. *C. albicans* biofilms treated with acetic acid (60 mM and 300 mM) and hydrogen peroxide (5 mM and 25 mM) were included as positive controls in this assay. Apoptosis in planktonic *C. albicans* SC5314 cells was also tested. Therefore overnight cultures were treated with miconazole (10 x MIC) for 24h and compared to untreated planktonic cultures. The staining procedure was performed as described above for sessile cells. Results were observed using a fluorescence microscope (Olympus BX40, Olympus, Tokyo, Japan). For each condition, between 241 and 1174 cells were photographed and co-localisation of the fluorescein and Cy3 fluorescence signal was quantified on a cell per cell basis with an in house developed image processing program based on Matlab to differentiate between living, necrotic and apoptotic cells.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software. The non-parametric Mann-Whitney test was used to compare the results.

Results

Effect of azoles on Candida *biofilms*

The effect of fluconazole and miconazole on *Candida* biofilms formed on silicone disks was determined for 15 strains (Figure 1). Untreated biofilms contained 10^5-10^6

cfu/disk depending on the strain tested. Treatment with fluconazole did not result in a significant reduction in cfu. In contrast, treatment with miconazole resulted in a substantial reduction (ranging from 89.3% to 99.1% ; $p < 0.05$) in the number of cfu recovered from the disks for all strains investigated. The lowest reductions were observed for *C. parapsilosis* IHEM 3270 (89.3%) and *C. tropicalis* IHEM 4225 (90.3%) and the highest reductions were observed for *C. albicans* MUCL 30112 (99.1%) and *C. albicans* IHEM 10284 (99.1%).

Our results showed only a fungistatic effect for fluconazole, whereas miconazole showed fungicidal activity against *Candida* biofilms.

ROS accumulation in miconazole-treated biofilms

Accumulation of ROS following treatment with miconazole was measured using DCFHDA. As the conversion of this dye depends on the number of metabolically active cells in the biofilm, results were normalized to the number of cfu/disk. Treatment with miconazole resulted in a significant increase of ROS accumulation for all strains investigated ($p < 0.005$) (Figure 2). No ROS accumulation could be observed for untreated *C. dubliniensis* IHEM 14280, *C. glabrata* MUCL 15664 and *C. parapsilosis* IHEM 3270 biofilms. In contrast, all untreated *C. albicans* biofilms tested showed a basal ROS accumulation. A remarkable high fluorescence could be observed for *C. albicans* MUCL 30112. The highest increases (more than 100-fold) in ROS accumulation after miconazole treatment could be observed for *C. albicans* MUCL 29919, *C. albicans* IHEM 9559 and *C. albicans* MUCL 29800. The lowest impact of miconazole treatment was detected for *C. tropicalis* IHEM 3270 (2-fold increase) and *C. albicans* 1467 (7-fold increase). Measurements were also carried out separately in sessile *C. albicans* SC5314 cells and in the supernatant. The ROS-

induced increase in fluorescence was only observed for the cells and not for the supernatant (data not shown), indicating an intracellular origin.

In conclusion, miconazole caused a significant intracellular increase of ROS accumulation in all *Candida* strains investigated.

Effect of anti-oxidants on miconazole-treated biofilms

The effect of the addition of five compounds with anti-oxidative properties on miconazole-treated *C. albicans* SC5314 biofilms was investigated using DCFHDA ($n \geq 9$ for each treatment). Only cysteine (0.25% w/v), glutathione (15 mM), PDTC (10 μM) and ascorbic acid (10 mM and 100 mM) significantly reduced ($p < 0.05$) miconazole-induced ROS accumulation (Figure 3). However, addition of anti-oxidants to miconazole-treated biofilms did not result in a statistically significant increase in survival (Figure 3). Ascorbic acid (10 mM) was selected to further examine the effect of ROS quenchers on other miconazole-treated *Candida* biofilms. The addition of ascorbic acid did not significantly increase the number of cfu on the silicone disks, but resulted in a reduction of ROS accumulation following treatment with miconazole for all strains tested (Table 1). This reduction was statistically significant ($p < 0.05$) when compared to biofilms treated with miconazole alone ($n \geq 9$ for each strain), except for *C. albicans* MUCL 30112, MUCL 29919 and *C. glabrata* MUCL 15664.

Ascorbic acid reduced the miconazole-induced ROS accumulation in sessile cells, but did not cause an enhanced survival.

Effect of ascorbic acid on the susceptibility of planktonic cells

Miconazole also induced ROS accumulation in planktonic *Candida* cultures. Addition of ascorbic acid to planktonic cultures, incubated with miconazole reduced ROS production (47.8% to 89.9% reduction) for all strains tested (data not shown). The MIC of miconazole increased 2 to 64 fold for most strains following the addition of ascorbic acid. This increase was not observed for strains with an intermediate (0.125 mg/L) MIC (*C. albicans* IHEM 10284 and MUCL 29919) and for most strains with a high (1.0 – 4.0 mg/L) MIC (*C. glabrata* MUCL 15664, *C. krusei* IHEM 1796 and *C. parapsilosis* IHEM 3270) (Table 2).

For planktonic cells of most strains the addition of ascorbic acid reduced the ROS accumulation and the susceptibility to miconazole.

Apoptosis in miconazole-treated planktonic and sessile C. albicans *cells*

To determine whether the effect of miconazole was due to increased apoptosis, we quantified the number of apoptotic cells in treated and untreated biofilms and planktonic cultures (see supplementary data). Untreated planktonic *C. albicans* SC5314 cultures contained 14.0% $\pm$ 7.0% apoptotic cells, which was not significantly different from miconazole-treated cells (14.6% $\pm$ 4.5%). In contrast, a slight but not significant increase in the number of necrotic cells could be observed when planktonic cells were treated with miconazole (13.2% $\pm$ 3.6%) compared to untreated cells (7.5% $\pm$ 2.3%). Untreated *C. albicans* SC5314 biofilms contained 9.7% $\pm$ 4.0% apoptotic cells. A slight increase was observed following treatment with miconazole alone (14.4% $\pm$ 11.5%) or in combination with ascorbic acid (18.5% $\pm$ 6.5%). However, these increases were not significant. In contrast, the fraction of necrotic cells increased significantly ($p < 0.05$) compared to the untreated biofilms (8.9% $\pm$ 3.7%) following treatment with miconazole alone (25.1% $\pm$ 1.9%) or in combination with

ascorbic acid (32.7% ± 8.6%). Low concentrations of acetic acid (60 mM) and hydrogen peroxide (5 mM) resulted in a significant increase ($p < 0.05$) in the fraction of apoptotic cells (34.6% ± 5.1% and 20.6% ± 5.2%, respectively). The hydrogen peroxide-treated biofilms also showed a significant increase in the amount of necrotic cells (26.1% ± 3.5%).

Miconazole did not result in increased apoptosis in planktonic or in sessile *Candida* cells.

Discussion

In the present study the antifungal activity of miconazole against *Candida* biofilms was investigated.

Results from our study showed that miconazole, unlike fluconazole, has a pronounced anti-biofilm effect against *C. albicans* and other *Candida* spp. It should be noted that the antifungal concentration used in our *in vitro* experiments (5 mM) was higher than the common therapeutic *in vivo* concentrations. However, this high concentration is achievable during antifungal lock therapy,[19,20] and allowed us to investigate the mechanism of action of miconazole.

We observed that the accumulation of ROS was strongly increased in sessile *Candida* cells treated with miconazole, indicating that ROS may be responsible for the fungicidal effect. ROS are generally described as important inducers of apoptosis in yeasts,[10] but ROS induced by treatment with miconazole did not cause an increase in programmed cell death in sessile *C. albicans* cells. The majority of cells killed by miconazole were necrotic.

The addition of ascorbic acid to miconazole-treated *Candida* biofilms considerably reduced ROS accumulation for all strains. Surprisingly, this did not lead to a reduction

of the fungicidal activity of miconazole. Sessile *Candida* cells are reportedly more tolerant to oxidative stress than their planktonic counterparts.[21] This inherent tolerance may explain why several anti-oxidative compounds did not result in an additional protection. Furthermore, previous studies have shown that prior to the induction of ROS miconazole affects the organization of the actin cytoskeleton in yeasts.[8] The coupling of mitochondria to the actin cytoskeleton might lead to an association of actin with channels in the mitochondrial membranes. The opening of these channels is followed by reduction of the membrane potential and finally the release of ROS into the cytoplasm.[22] The targeting of the actin cytoskeleton by miconazole may have other effects, which are not counteracted by ascorbic acid. Alternatively, a yet unknown mechanism may contribute to the fungicidal activity of miconazole against sessile *Candida* cells.

An increase in ROS accumulation caused by miconazole treatment was also observed in all planktonic *Candida* cultures tested, confirming earlier observations in *C. albicans*.[6,7] The addition of ascorbic acid to miconazole-treated planktonic *Candida* cells reduced ROS induction for all strains. Furthermore, a simultaneous decrease in susceptibility to miconazole was observed for most strains, which confirmed earlier findings concerning the protective effect of anti-oxidants during miconazole treatment.[6] However, we demonstrated that the protective effect of ascorbic acid was limited in case of strains with intermediate to high MICs for miconazole.

Our data suggest that miconazole may be useful for the treatment of biofilm-related *Candida* infections. We have also shown that ROS induction is probably not directly responsible for the reduction of the number of cfu. So far, the basis for the fungicidal activity of miconazole remains unclear and further investigations are needed.

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Transparancy declaration

None to declare.

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Figure legends

Figure 1. Number of colony forming units (logarithmic) per silicone disk of *Candida* biofilms in the presence and absence of antifungals. Black bars, untreated mature biofilms; grey bars, mature biofilms treated with 5 mM fluconazole; white bars, mature biofilms treated with 5 mM miconazole. Data presented are the mean and SEM of at least three independent experiments on at least three biofilms ($n \geq 9$). Statistical analysis with Mann-Whitney Test indicated significant differences in biofilm biomass between miconazole-treated and untreated biofilms for all strains tested ($p < 0.05$), but not between fluconazole-treated and untreated biofilms.

Figure 2. Accumulation of ROS expressed as fluorescence per 1000 cells in mature *Candida* biofilms in the presence and absence of miconazole. Black bars, untreated mature biofilms; grey bars, mature biofilms treated with 5 mM miconazole. Data presented are the mean and SEM of two independent experiments on at least three biofilms ($n \geq 6$). Statistical analysis with Mann-Whitney Test indicated a significant difference ($p < 0.05$) in fluorescence between miconazole-treated and untreated biofilms. * Fluorescence below background level

Figure 3. Number of cfu of *C. albicans* SC5314 biofilms (left Y-axis) and ROS accumulation (right Y-axis) following treatment with miconazole in the absence or presence of anti-oxidants. Black bars, log cfu per silicone disk; grey bars, fluorescence. Data presented are the mean and SEM of at least three independent

experiments on at least three biofilms ($n \geq 9$). All treated biofilms showed a significant increase ($p < 0.05$) in cfu compared to the control. Significant reductions ($p < 0.05$) in ROS accumulation compared to the control are marked with an asterisk.

Table 1. Number of cfu of *Candida* biofilms and ROS accumulation after miconazole treatment and the addition of ascorbic acid (10 mM). Plate counts correspond to the mean ± SEM of at least three independent experiments on at least three silicone disks ($n \geq 9$). There was no significant increase in cfu after the addition of ascorbic acid compared to miconazole-treated biofilms without ascorbic acid. Fluorescence results correspond to the mean ± SEM of two independent experiments on three biofilms ($n = 6$). Statistically significant reductions ($p < 0.05$) in ROS accumulation are marked with an asterisk.

	PLATING (log cfu / silicone disk)	FLUORESCENCE (% compared to miconazole treatment without ascorbic acid)
C. albicans SC5314	4.13 ± 0.41	10.4 ± 2.3*
C. albicans MUCL 29800	2.00 ± 0.02	32.2 ± 7.7*
C. albicans MUCL 29981	2.99 ± 0.20	30.7 ± 4.2*
C. albicans MUCL 29903	3.47 ± 0.04	60.3 ± 14.1*
C. albicans MUCL 30112	2.53 ± 0.31	17.9 ± 5.6*
C. albicans NCYC 1467	4.16 ± 0.21	25.0 ± 5.9*
C. albicans IHEM 10284	3.19 ± 0.27	47.6 ± 5.5*
C. albicans ATCC 10231	2.89 ± 0.32	33.4 ± 2.5*
C. albicans IHEM 9559	3.28 ± 0.15	11.5 ± 3.9*
C. albicans MUCL 29919	3.80 ± 0.19	81.7 ± 16.9
C. dubliniensis IHEM 14280	3.90 ± 0.21	23.3 ± 2.3*
C. glabrata MUCL 15664	3.21 ± 0.19	64.4 ± 13.3
C. krusei IHEM 1796	2.73 ± 0.16	20.3 ± 4.5*
C. parapsilosis IHEM 3270	3.36 ± 0.22	53.2 ± 8.5*
C. tropicalis IHEM 4225	4.05 ± 0.22	26.5 ± 3.8*

461 **Table 2.** Minimal inhibitory concentrations (MIC) (mg/L) of miconazole in the absence and presence of ascorbic acid.
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463

	MIC miconazole (mg/L)	MIC miconazole (mg/L) in the presence of 10 mM ascorbic acid	Fold change in MIC
C. albicans SC5314	0.063	0.250	4
C. albicans MUCL 29800	0.031	0.125	4
C. albicans MUCL 29981	0.031	2.000	64
C. albicans MUCL 29903	0.063	0.250	4
C. albicans MUCL 30112	0.063	0.500	8
C. albicans NCYC 1467	0.063	0.500	8
C. albicans IHEM 10284	0.125	0.125	1
C. albicans ATCC 10231	0.125	0.250	2
C. albicans IHEM 9559	0.125	0.250	2
C. albicans MUCL 29919	0.125	0.125	1
C. dubliniensis IHEM 14280	0.063	0.125	2
C. glabrata MUCL 15664	1.000	1.000	1
C. krusei IHEM 1796	4.000	4.000	1
C. parapsilosis IHEM 3270	1.000	1.000	1
C. tropicalis IHEM 4225	1.000	4.000	4

Supplementary data :

Candida albicans **sessile cells stained with the Apoptosis Detection Kit Annexin V-CY3.**

Picture A: Untreated sessile *Candida albicans* cells (green fluorescence)

Picture B: Untreated sessile *Candida albicans* cells (red fluorescence)

Picture C: Miconazole-treated sessile *Candida albicans* cells (green fluorescence)

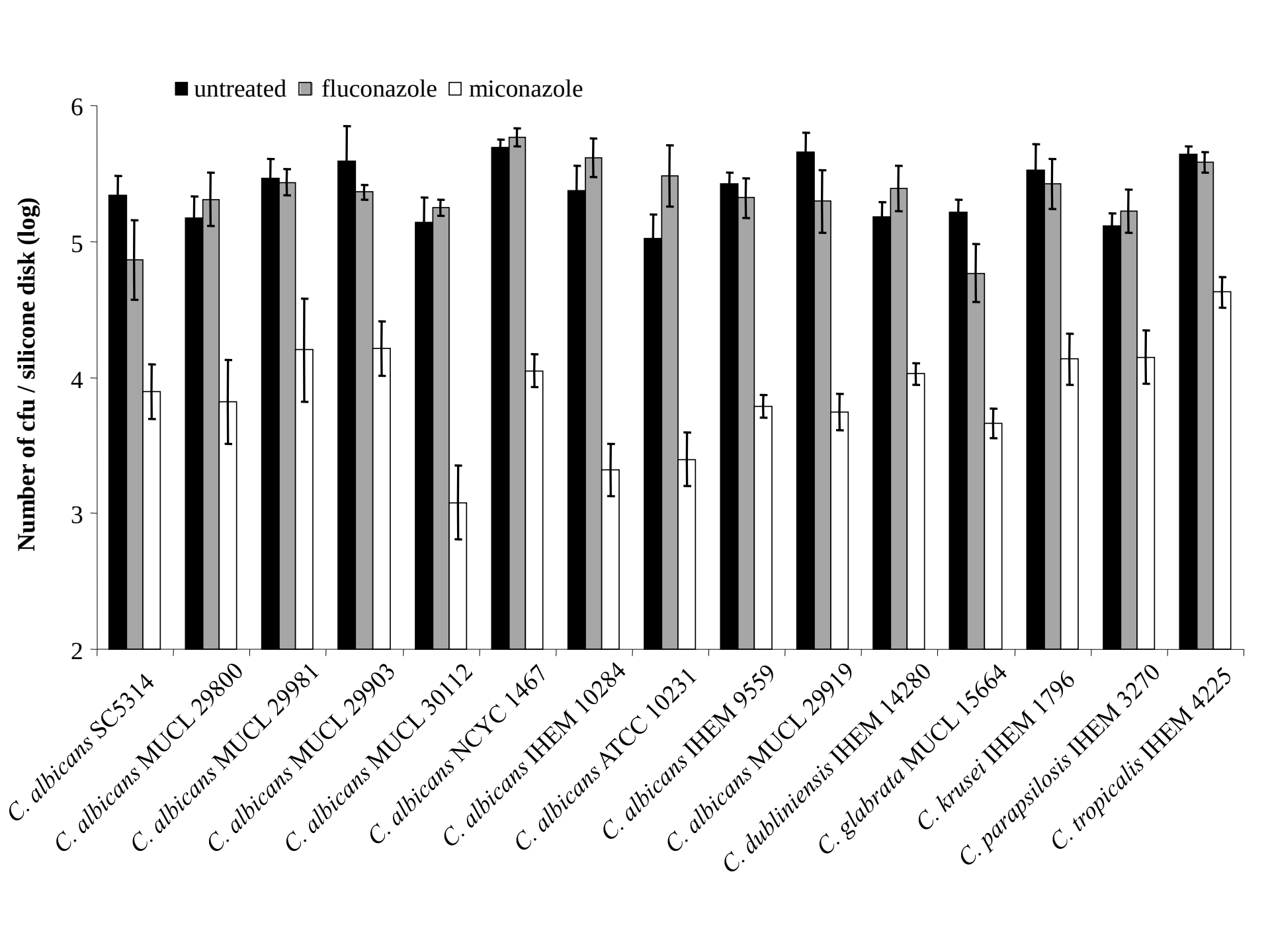
Picture D: Miconazole-treated sessile *Candida albicans* cells (red fluorescence)

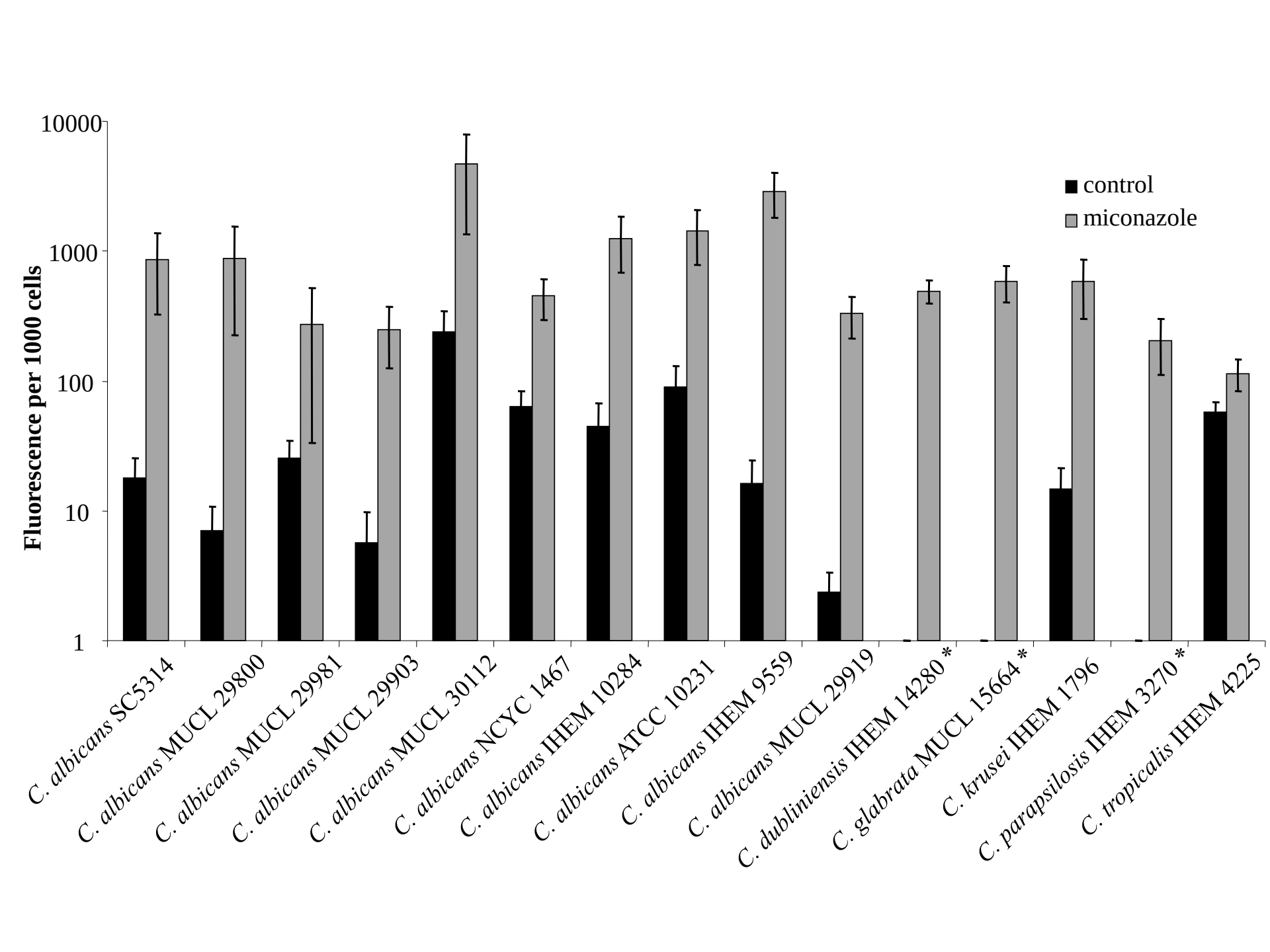
Picture E: Miconazole and ascorbic acid treated sessile *Candida albicans* cells (green fluorescence)

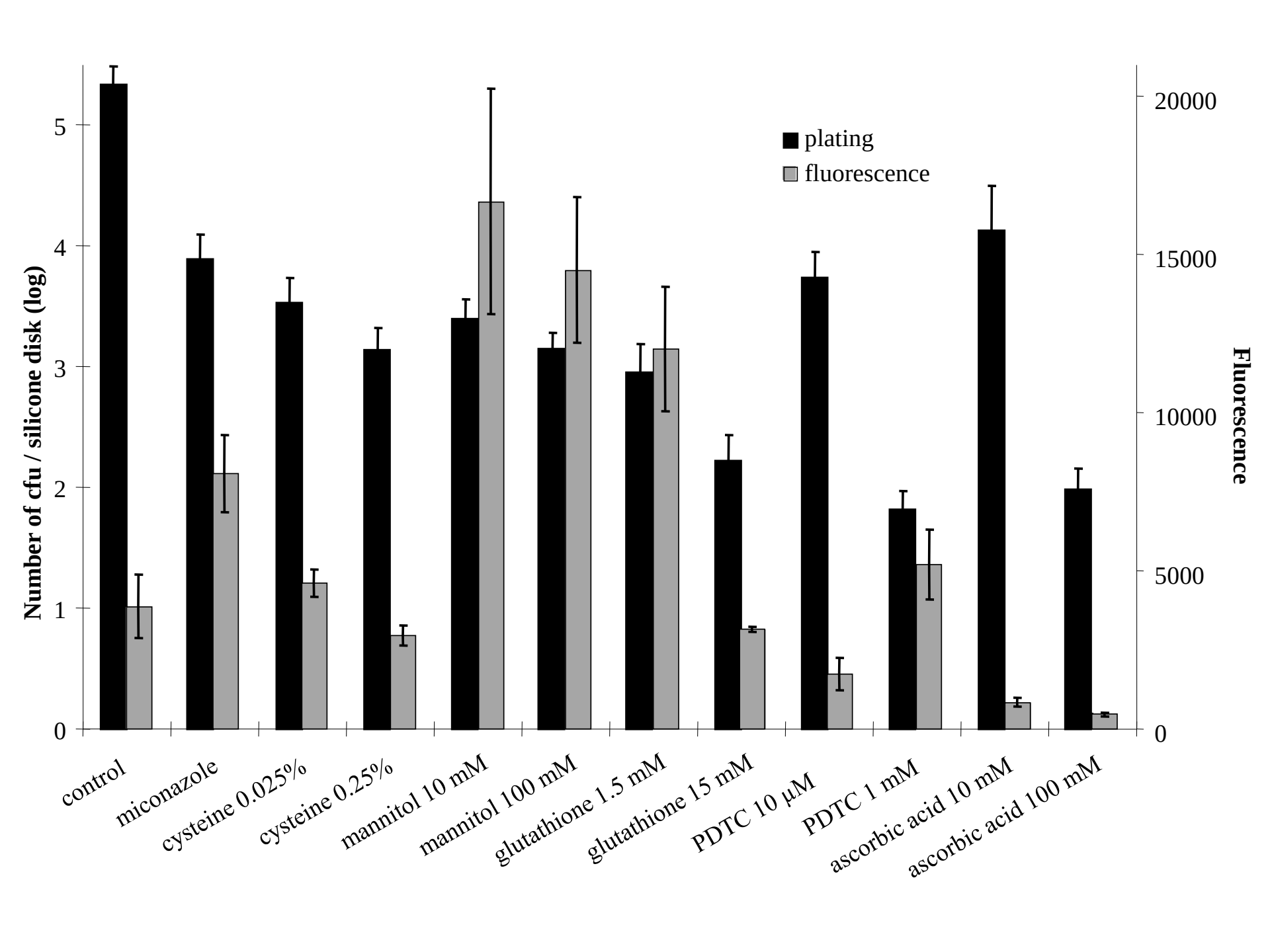
Picture F: Miconazole and ascorbic acid treated sessile *Candida albicans* cells (red fluorescence)

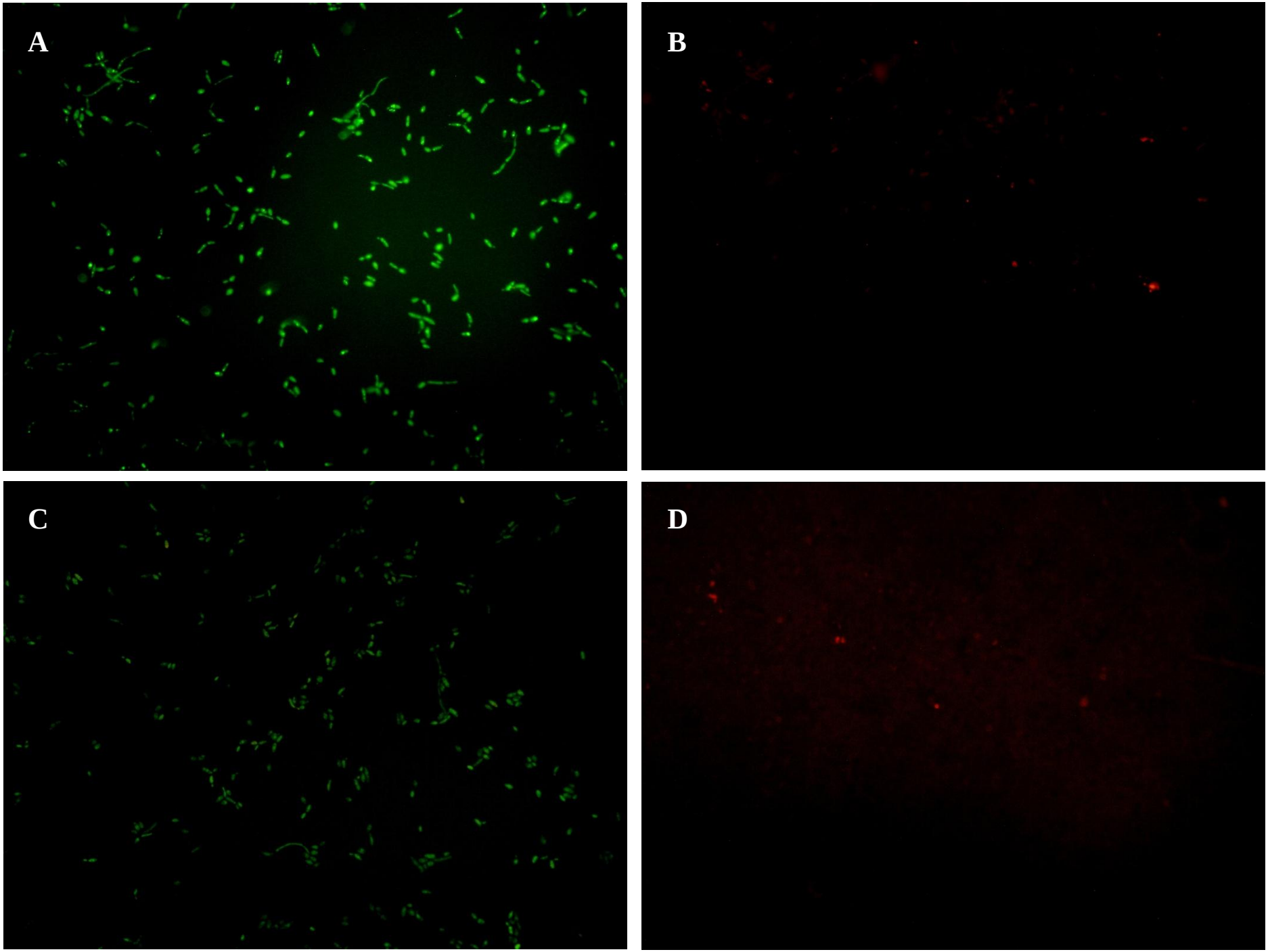
Picture G: Hydrogen peroxide treated sessile *Candida albicans* cells (green fluorescence)

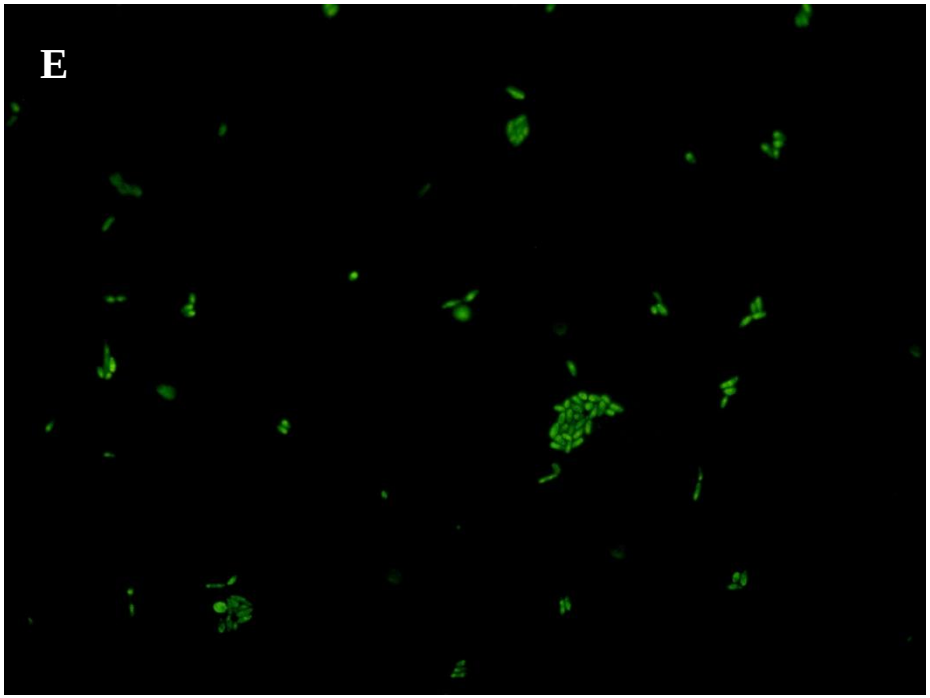
Picture H: Hydrogen peroxide treated sessile *Candida albicans* cells (red fluorescence)

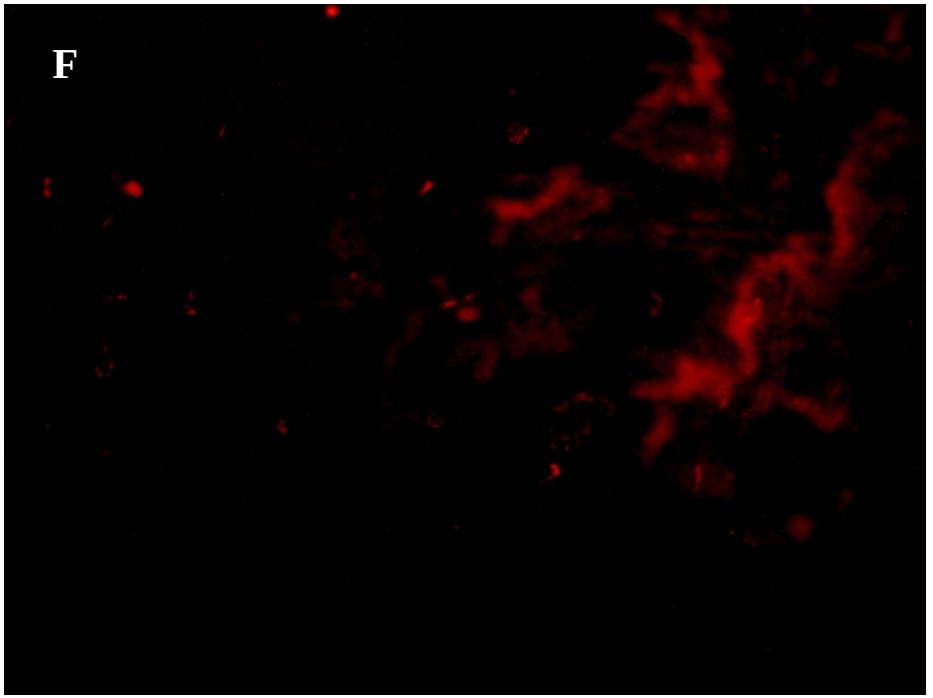

untreated
fluconazole
miconazole
Number of cfu / silicone disk (log)
6
5
4
3
2
C. albicans SC5314
C. albicans MUCL 29800
C. albicans MUCL 29981
C. albicans MUCL 29903
C. albicans MUCL 30112
C. albicans NCYC 1467
C. albicans IHEM 10284
C. albicans ATCC 10231
C. albicans IHEM 9559
C. albicans MUCL 29919
C. dubliniensis IHEM 14280
C. glabrata MUCL 15664
C. krusei IHEM 1796
C. parapsilosis IHEM 3270
C. tropicalis IHEM 4225

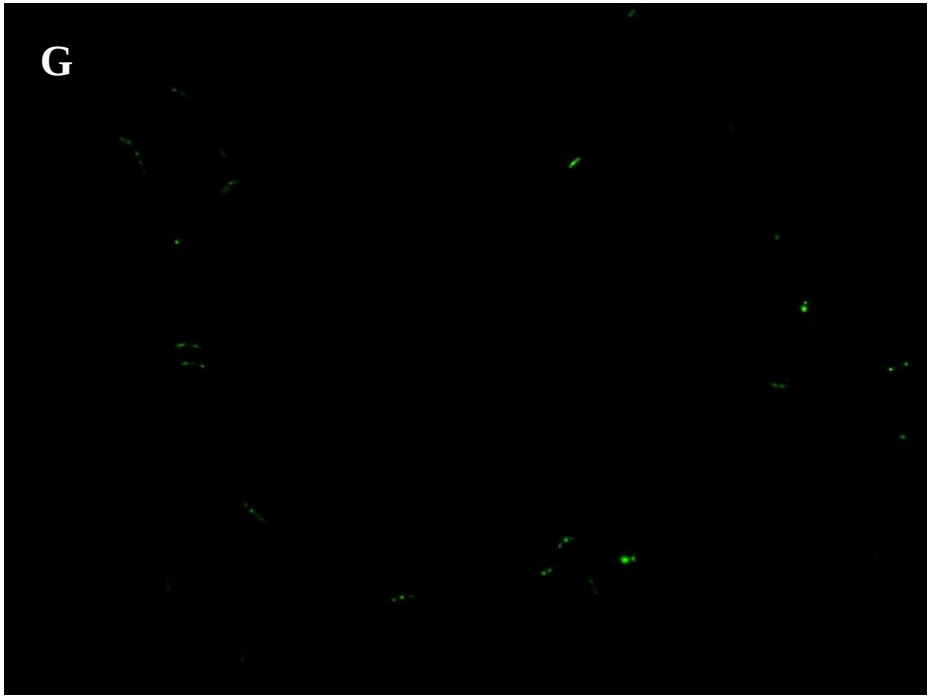

Fluorescence per 1000 cells
10000
1000
100
10
1
control
miconazole
C. albicans SC5314
C. albicans MUCL 29800
C. albicans MUCL 29981
C. albicans MUCL 29903
C. albicans MUCL 30112
C. albicans NCYC 1467
C. albicans IHEM 10284
C. albicans ATCC 10231
C. albicans IHEM 9559
C. albicans MUCL 29919
C. dubliniensis IHEM 14280 *
C. glabrata MUCL 15664 *
C. krusei IHEM 1796
C. parapsilosis IHEM 3270 *
C. tropicalis IHEM 4225

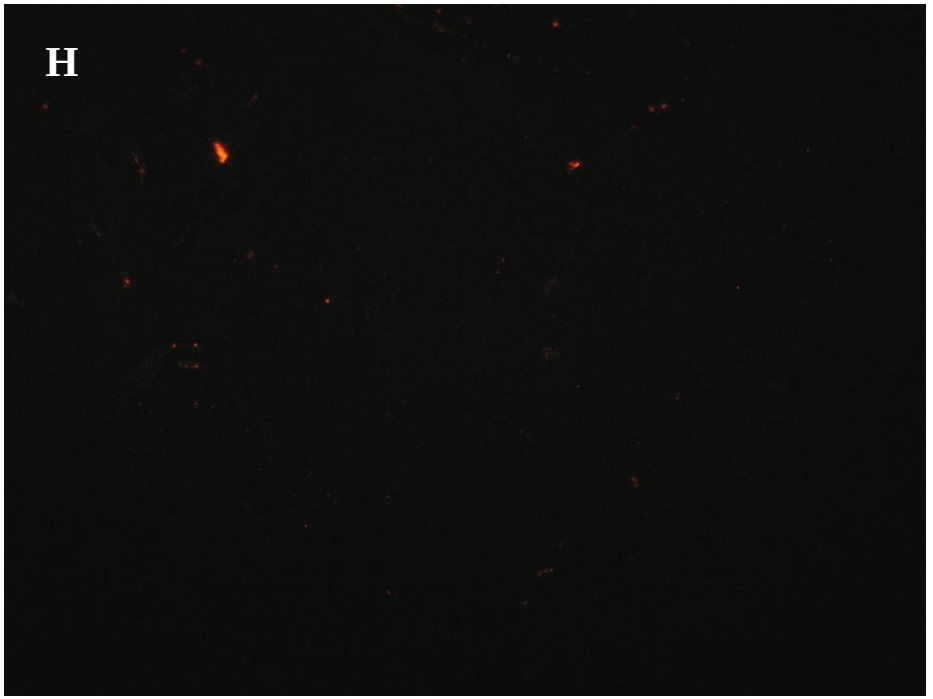

plating
fluorescence
Number of cfu / silicone disk (log)
Fluorescence
0
1
2
3
4
5
0
5000
10000
15000
20000
control
miconazole
cysteine 0.025%
cysteine 0.25%
mannitol 10 mM
mannitol 100 mM
glutathione 1.5 mM
glutathione 15 mM
PDTC 10 µM
PDTC 1 mM
ascorbic acid 10 mM
ascorbic acid 100 mM


A
B
C
D


E


F


G


H