

# Ultrastructural Changes in *Candida albicans* Induced by Benzyl Isothiocyanate (BITC) †

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**Abstract:** The search for new antifungal substances is increasingly relevant due to growing antifungal resistance. *Candida albicans* is the most common pathogen yeast in humans, primarily in immuno-compromised individuals. Isothiocyanates, derived from glucosinolates, are compounds with an antimicrobial effect at low concentrations. The purpose of this study was to analyse ultrastructural changes in three *C. albicans* isolates after exposure to benzyl isothiocyanate (BITC) at different lengths of exposure time (2.5-, 5- and 24 h). Before exposure to BITC, cells presented a regular round or oval shape, with a uniform cell wall. After exposure to BITC, cell wall damage and loss occurred in the three strains. The cells developed extensive indentations, and a band of electron-dense material was formed in the cortical cytoplasm. Although for one isolate no intact cells were detected, at the highest exposure time, two of the isolates showed relevant response, regaining almost normal cell shape with nearly complete cell wall recovery. Cell lysis led to the deposition of a melted and unmixed mass with two apparently distinct fractions, the cell wall fraction and the cytoplasmic fraction. The present work demonstrates that, through targeting the *C. albicans* cell wall, BITC may prove to be a promising antifungal compound.

**Keywords:** *Candida albicans*; ultrastructure; cell wall; cell damage; BITC

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## 1. Introduction

Most of the antifungals, such as polyenes, azoles and allylamine/thiocarbamates target ergosterol, the major lipid component of yeast membrane [1]. In fighting fungal infections, some antifungal substances have three main disadvantages: limited range of action, self-medication, which may interact negatively with different types of antifungal agents, and the resistance of microorganisms [2,3]. Furthermore, despite improvement of antifungal therapies over the last 30 years, antifungal resistance is still of major concern in clinical practice [4]. The structure and biosynthesis of a fungal cell wall is unique and is therefore an excellent target for the development of new antifungal drugs. ITCs are an important class of compounds derived from glucosinolates, secondary plant compounds present mainly in the Brassicaceae family. They are volatile substances with an inhibitory effect on a variety of microorganisms at low concentrations and appear to be potential antimicrobial agents [5]. In the current study, the ultrastructure of three *C. albicans* isolates was analyzed before and after incubation in BITC and a comparative morphological study was

performed in order to identify the progressive morphological changes induced throughout over time.

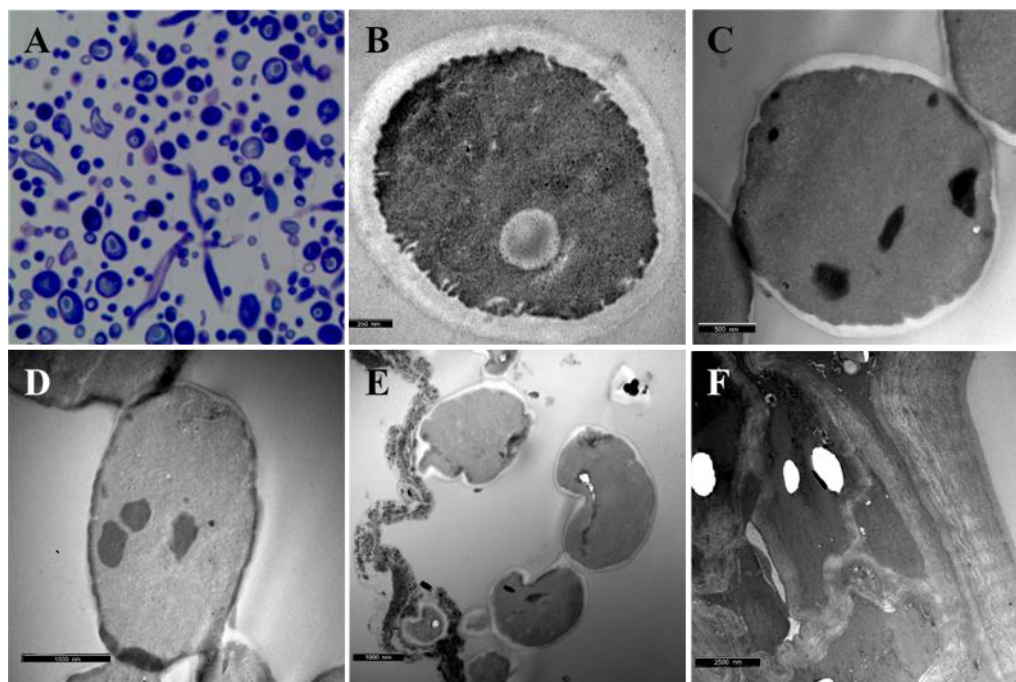
## 2. Materials and Methods

Three, *C. albicans* isolates (two oral isolates, O33 and O5, and the collection culture strain *C. albicans* ATCC 90028, used for quality control purposes when chemical compounds are tested against yeast, were used in this study, based on their susceptibilities to BITC (range 1.43–143.0 µg/mL) determined by the disk diffusion method (DD) [6]. All the isolates were susceptible for fluconazole (FLU) (breakpoint ≥ 19 mm) and, at 4.3 µg of BITC/mL, had inhibition zones diameters below FLU breakpoint) [6]. The isolates were maintained at –80 °C until needed. Cultures of *C. albicans* grew on Mueller-Hinton (MH) agar, and after 24 h a loopfull of each was collected for Transmission Electron Microscope (TEM) methodologies. For the TEM samples exposed to BITC, the same cultures were inoculated in 50 mL of MH broth, supplemented with glucose (0.9% *w/v*). After incubation (3 h) in an orbital shaker (Certomat S, B. Braun Biotech International) at 150 rpm in 35 °C, the cultures were inoculated in 250 mL of MH supplemented with BITC to a final concentration of 0.004 M. Several samples were collected at different times of incubation (2.5, 5 and 24 h) for cell fixation. The samples were washed with a solution of polyphosphate buffer saline (PBS) pH 7.3 and centrifuged at 16.000 rpm for 4 min.

The isolates samples were subjected to double fixation at 4 °C, with OsO<sub>4</sub>, dehydrated, infiltrated and embedded in Epon. Ultrathin sections were and examination and capture of images were performed on a TEM at Electron Microscopy Unit (UME) UTAD.

## 3. Results

*C. albicans* cells presented a round or oval shape and were covered by a uniform cell wall firmly attached to the plasma membrane. It was evident the presence of two to three cell wall sublayers with smooth appearance. Cells possessed a well-developed intracytoplasmic cistern membrane system closely associated with the plasmatic membrane (Figure 1A,B). After exposure to BITC for 2.5 h, most of *C. albicans* cells maintained their round shape, despite the identification of bevels or indentations in the cell surface. Cells revealed a heterogenous wall, with notorious thickness decrease. The cell wall sublayers were not identifiable, as before BITC exposure. The plasmatic membrane presented less invaginations, without connections to intracellular canaliculi, when compared to unexposed cells (Figure 1C). *C. albicans* cells exposed to BITC for 5 h revealed a very irregular contour with cell surface damage, forming pronounced bevels and indentations. Cells accumulated dense vesicles and vacuoles, and in some portions along cell surface, there was complete cell wall loss. In these situations, cortical cytoplasm, devoid of cell wall, accumulated high electron dense material that was irregular in shape and size (Figure 1D). After 24 h-exposure to BITC, most of cells recovered a round shape, tending to a regular outline. Dense intracytoplasmic inclusions remained visible, but no accumulation of dense material in the cell periphery was observed. The most remarkable change was the almost total recovery of the cell wall structural integrity (Figure 1E). There was also a decrease on the volume of the accumulated extracellular material, although it seemed denser, more heterogeneous and granular in appearance. In one isolate only vestigial cells were found in the semithin sections and no cells are found in ultrathin sections. We found an extraordinary amount of cell melted and unmixed debris, being compatible with the cell wall semblance, while the other fraction had a cytoplasmic appearance (Figure 1F).



**Figure 1.** (A) Semithin section of *C. albicans* before exposure to BITC, stained with toluidine blue 1%. (B) TEM image of *C. albicans* before exposure to BITC. (C) TEM image of *C. albicans* after an exposure of 2.5 h to BITC. (D) TEM image of *C. albicans* after an exposure of 5 h to BITC. (E) TEM image of *C. albicans* after an exposure of 24 h to BITC. (F) TEM image of debris obtained after *C. albicans* exposure of 24 h to BITC.

#### 4. Discussion

The present work revealed drastic changes in *C. albicans* cellular morphology and in cell wall removal caused by BITC. Because the cell wall structure of yeast is unique, is an important target to pharmaceutical research. Although we do not know the action mechanism of BITC at the level of the cell wall, the present work presents images that undoubtedly demonstrate the effect of this agent at the level of cell wall, constituting, alone or combined, a promising antifungal.

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