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A Sunflower Lectin with Antifungal Properties and Putative Medical Mycology Applications

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Abstract Lectins are carbohydrate-binding proteins with a high specificity for a variety of glycoconjugate sugar motifs. The jacalin-related lectins (JRL) are considered to be a small sub-family composed of galactose- and mannose-specific members. Using a proteomics approach, we have detected a 16 kDa protein (Helja) in sunflower seedlings that were further purified by mannose-agarose affinity chromatography. The aim of this work was to characterize the biological activity of Helja and to explore potential applications for the antifungal activity of this plant lectin against medically important yeasts. To initially assess the agglutination properties of the lectin, *Saccharomyces cerevisiae* cells were incubated with increasing concentrations of the purified lectin. At a concentration of 120 μg/ml, Helja clearly agglutinated these cells. The ability of different sugars to inhibit *S. cerevisiae* cell agglutination determined its carbohydrate-specificity. Among the monosaccharides tested, D-mannose had the greatest inhibitory effect, with a minimal concentration of 1.5 mM required to prevent cell agglutination. The antifungal activity was evaluated using pathogenic fungi belonging to the *Candida* and *Pichia* genera. We demonstrate that 200 μg/ml of Helja inhibited the growth of all yeasts, and it induced morphological changes, particularly through pseudohyphae formation on *Candida tropicalis*. Helja alters the membrane permeability of the tested fungi and is also able to induce the production of reactive oxygen species in *C. tropicalis* cells. We concluded that Helja is a mannose-binding JRL with cell agglutination capabilities and antifungal activity against yeasts. The biological properties of Helja may have practical applications in the control of human pathogens.

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Introduction

Lectins are proteins that interact with various cellular structures through their carbohydrates moieties, and in particular, with cell walls and cell membranes. They can trigger several biological effects through their interaction with cell walls and membranes, including agglutination of erythrocytes [20, 31]. Due to their carbohydrate-binding properties, lectins are able to recognize specific glycoconjugate sugar structures that occur on cell surfaces or in solution. This characteristic supports cell agglutination and antitumoral, immunomodulatory, antiviral, antibacterial, antifungal, and insecticidal activities that are associated with this family of proteins, and could contribute to the development of important practical applications [5, 11, 13]. Lectins are ubiquitously distributed in nature, and especially abundant in the *Plantae* kingdom. They have been documented as one of the bioactive compounds from plants that can be used against pathogens of diverse origins

[31, 32]. Recent studies have revealed important information about the inhibitory activity of lectins, specifically against various fungi [5, 8, 11, 22]. It was suggested that the antifungal activity of lectins occurs through their interaction with the fungal cell wall, which is composed mainly of glucans, chitin, and other sugar polymers [3].

Among the lectin groups, the jacalin-related lectins (JRLs) are a sub-group that is composed of galactose and mannose-specific members [31] able to display relevant antipathogenic activities. This family comprises proteins that share sequence similarity with the agglutinin from jack fruit (*Artocarpus integrifolia*) which, in turn, is characterized by its high specificity for the T-antigen [26]. Using a proteomic approach, we have detected a 16 kDa protein predicted to be a mannose-specific JRL in the extracellular fluids of *Helianthus annuus* (sunflower) seedlings. Hereafter, we will refer to this protein as *H. annuus*-like jacalin (Helja). Further, mannose-agarose affinity chromatography allowed us to obtain a purified protein whose identity was confirmed to be Helja by MALDI-TOF spectrometric assays [21]. In this work, we have assessed potential applications of Helja isolated from seeds and characterized its biological activity against a group of selected yeasts that are of major importance in medical mycology.

Materials and Methods

Biological Material

Candida albicans (CE0022), *Candida parapsilosis* (CE0020), *Pichia membranifaciens* (CE0015), *Candida tropicalis* (CE0017), and *Saccharomyces cerevisiae* (1038) were maintained in the Laboratório de Fisiologia e Bioquímica de Microrganismos, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, Rio de Janeiro, Brazil. The yeasts were maintained on Sabouraud agar (1 % peptone, 2 % glucose, and 1.7 % agar–agar).

Sunflower (*Helianthus annuus* L) seeds, line 10347, were kindly supplied by Advanta Semillas SAIC, Argentina.

Helja Purification and Characterization

The purification of Helja was performed according to Pinedo et al. [21], with some modifications. Ten ml of extracellular fluid was obtained from seeds and loaded onto a 1 ml D-mannose-agarose resin (Sigma M6400) that was equilibrated with buffer A (50 mM HCl-Tris pH 7.5, 100 mM NaCl). The resin was washed exhaustively to remove the non-bound proteins before the retained proteins were eluted with 4 ml of 0.2 M D-mannose in buffer A. The eluted fraction was loaded into a centrifugal filter device, in a centricon YM 3 (Millipore), to allow the separation of the protein fractions from mannose. Electrophoretic separation was performed on 12 % SDS-PAGE gels using standard methods [12].

For MALDI-TOF spectrometric analysis, gels were stained with Sypro reagent, and the bands were automatically excised from the gel by using an Investigator ProPic robotic workstation (Genomic Solutions). The gel pieces were digested with trypsin, according to standard protocols, in a ProGest station (Genomic Solutions). MS and MS/MS analyses of peptides from each sample were performed in a 4700 Proteomics Station (Applied Biosystems, USA), as previously described by Pérez-Reinado et al. [19]. Protein identification was completed by comparing the obtained peptide mass fingerprint with the non-redundant plant database, using the Mascot 1.9 search engine (Matrixscience, UK).

Agglutination Assay

The agglutination assay was performed using yeast (*S. cerevisiae*) cells, because of their easy growth and cultivation as well as the occurrence of D-mannose on the surface of their cell walls [14]. The assay was carried out on micro slides that contained increasing concentrations of Helja and 5 µl of yeast cells diluted 1/100 (Undiluted cell cultures displayed OD 600 nm = 0.25). After 15 min of incubation, the samples were evaluated using an optical microscope (Leica Galen III). The controls were performed by replacing the Helja sample with the same volume of water. The inhibition of cell agglutination was evaluated through the addition of different concentrations of D-mannose, D-galactose, D-glucose, or D-fructose (all from Sigma) to the incubation mix.

Yeast Growth Inhibition Assay

For the preparation of *C. tropicalis*, *C. parapsilosis*, *P. membranifaciens*, and *C. albicans*, an inoculum of each yeast was transferred to Petri dishes containing agar Sabouraud and allowed to grow at 28 °C for 2 days. After this period, the cells were transferred to sterile Sabouraud broth (1 ml). The yeast cells were quantified in a Neubauer chamber for further calculation of appropriate dilutions. To monitor the effect of Helja on the growth of the yeasts, cells (10^4 in 1 ml Sabouraud broth) were incubated in the presence of 100 or 200 µg/ml of Helja at 28 °C in 200 µl in 96-well microplates (Nunc). The spectrophotometer was blanked with culture medium alone, then optical readings were taken at the zero time point and every 6 h for the following 24 h at 670 nm [4]. At the end of the assay, yeast cells were separated from the growth medium using centrifugation, washed in Sabouraud broth and plated for observation by optical microscopy (AxioImager.A2, Zeiss).

Plasma Membrane Permeabilization Assay

The permeabilization of the yeast plasma membrane was measured by SYTOX Green uptake, as described previously by Thevissen et al. [29]. SYTOX Green is a dye that only penetrates cells with a structurally compromised plasma membrane. Once inside the cell cytoplasm, SYTOX Green binds to nucleic acids, and the complex becomes fluorescent. Therefore, this dye was used for visualization of the permeabilization of the fungal plasma membrane. At the end of the growth inhibition assay, 100 µl aliquots of the yeast cell suspensions 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 (AxioImager.A2, Zeiss) equipped with a fluorescence filter set for fluorescein detection (excitation wavelengths, 450–490 nm; emission wavelength, 500 nm). The membrane permeabilization results shown are representative of three experiments.

Reactive Oxygen Species and Nitric Oxide Induction Assays

To evaluate whether Helja's mechanism of action involved the induction of oxidative stress, we used two dyes that indicated the presence of oxygen (ROS) and nitric oxide (NO) reactive species. At the end of the growth inhibition assay, 100 µl aliquots of each yeast cell suspensions were separately incubated with fluorescent dyes: 2′,7′-dichlorofluorescein diacetate (Calbiochem—EMD) for ROS detection and 3-amino, 4-aminomethyl-2′,7′-difluorofluorescein diacetate (Calbiochem—EMD) for NO detection. We used a previously described method by Mello et al. [16] with some modifications. The incubations were performed as described for the membrane permeabilization assay using the fluorescent dyes at a final concentration of 20 µM, according to the instructions provided by the manufacturers, with constant agitation for 2 h. After this period, these cells were transferred to slides, covered with coverslips and analyzed with a fluorescence microscope (AxioImager.A2, Zeiss) that was equipped with a fluorescence filter set for fluorescein detection (excitation wavelengths, 450–490 nm; emission wavelength, 500 nm). The results shown are representative of three experiments.

Results and Discussion

Affinity purification of mannose-binding proteins from seed extracellular fluids of sunflower was performed reproducing the procedure previously described for the isolation of Helja from seedlings. Elution of agarose-mannose columns with 0.2 M D-mannose yielded a unique protein peak representing seed extracellular mannose-binding proteins (Supplementary Fig. S1). As shown in Fig. 1, SDS-PAGE separation of this fraction retained in the D-mannose matrix revealed a protein of 16 kDa, in agreement with the molecular mass expected for Helja and its in silico assigned binding properties [21]. This 16 kDa mannose-binding protein isolated from seeds was further identified as Helja by MALDI-TOF spectrometry (Supplementary Table S1).

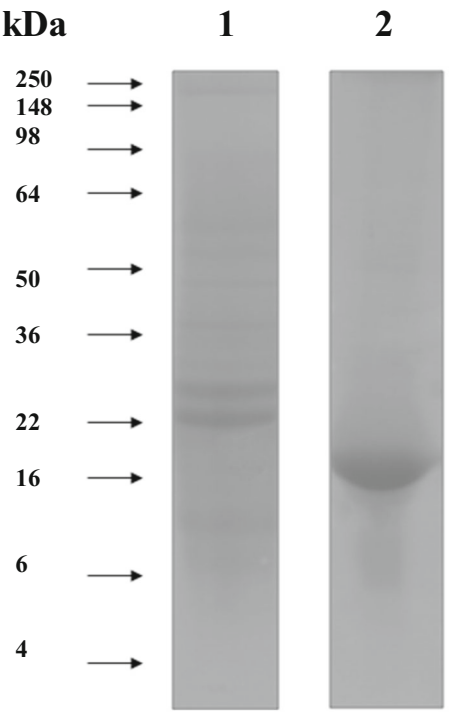

Fig. 1 Electrophoretic visualization of purified Helja after fractionation of the apoplastic fluid from sunflower seeds by D-mannose affinity chromatography. 12 % SDS-PAGE gel was stained with colloidal coomassie blue. 1: Non-retained fraction. 2: D-mannose-eluted fraction. Molecular mass *markers* are indicated on the *left*

To characterize the biological activity of Helja, we analyzed its ability to agglutinate *S. cerevisiae* cells, which are known to be enriched with mannan oligosaccharides in their cell wall [14]. Helja exhibited agglutinating effect at 120 µg/ml, and 1.5 mM D-mannose was found to inhibit this action (Fig. 2). Helja specificity and binding affinity for carbohydrates were evaluated by adding different concentrations of particular sugars to the agglutination mix, and the minimum inhibitory concentration (MIC) was estimated. Table 1 shows the MIC observed for some monosaccharides. Mannose showed the most potent inhibitory capacity (MIC 1.5 mM), and other sugars, such as galactose, glucose, and fructose, were 2–10 times less inhibitory than mannose (Table 1). Hence, it was concluded that Helja preferentially bounds mannose residues. These MIC values

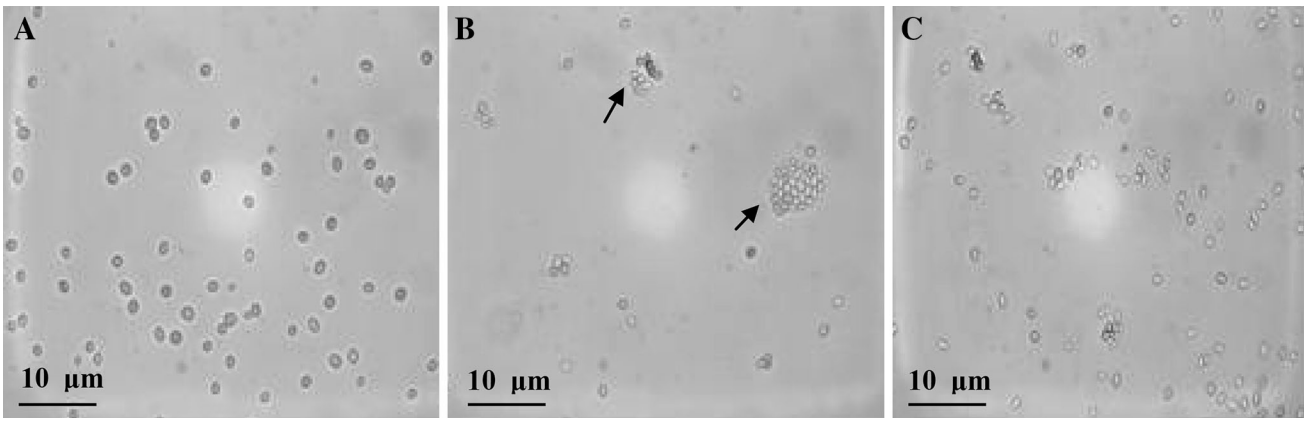

Fig. 2 Agglutinant effects of Helja on *Saccharomyces cerevisiae* cells observed by microscopy (*arrows*). **a** In the absence of Helja; **b** in the presence of 120 µg/ml Helja; **c** in the presence of 120 µg/ml Helja and 1.5 mM of D-mannose

Table 1 Sugar specificity of Helja on *Saccharomyces cerevisiae* agglutination

Sugar	MIC (mM)
Mannose	1.5
Galactose	5.0
Glucose	10
Fructose	10

Inhibition of *Saccharomyces cerevisiae* agglutination was determined by adding increasing concentrations of the indicated sugars in the presence of 120 µg/ml of Helja. The lowest concentration of sugar that inhibited agglutination was defined as MIC (minimum inhibitory concentration)

observed for Helja are similar to those previously reported for other plant mannose-binding lectins [9].

Together, mass spectrometry results and D-mannose affinity confirmed that Helja isolated from seeds is a JRL, since D-mannose binding is a typical feature of members of this family [21]. Lectin preparations from other organisms have also been shown to agglutinate *S. cerevisiae*, and this agglutination was inhibited by solutions of D-mannose [17].

Further characterization of this JRL showed that Helja affects important pathogenic yeasts such as *C. tropicalis*, *C. parapsilosis*, *C. albicans*, and *P. membranifaciens*, and these findings support potential therapeutic applications for Helja. In fact, 200 µg/ml Helja produced growth inhibition of all of these yeasts. The growth of *C. tropicalis*, *P. membranifaciens*, and *C. albicans* was inhibited by 50, 40, and 45 %, respectively (Fig. 3a, b, d) and, more interestingly, the growth of *C. parapsilosis* was significantly blocked by Helja. This yeast exhibited a 98 % inhibition of growth in the presence of 200 µg/ml Helja (Fig. 3c). Purified Helja was also tested at concentrations of 100 µg/ml, and its inhibitory activity diminishes resulting in approximately 60 and 18 % for *C. parapsilosis* and *C. albicans*, respectively (Fig. 3e, f). Even if several articles report that lectins have antimicrobial activity, only some members of the family have been associated to fungal inhibitory properties [10, 15, 27, 30]. Thus, a lectin isolated from the Egyptian *Pisum sativum* seeds inhibited the growth of the fungi *Aspergillus flavus, Trichoderma viride*, and *Fusarium oxysporum* [28]. Another lectin obtained from *Talisia esculenta* seeds inhibited the growth of *Fusarium oxysporum*, *Colletotrichum lindemuthianum, S. cerevisiae*, and *Microsporum canis* [7, 22]. A lectin from *Eugenia uniflora* L. seeds inhibited the growth of *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Klebsiella* sp. [18]. More recently, a protein of the chitin-binding lectin group isolated from *Schinus terebinthifolius* leaves was shown to be active against *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, P. aeruginosa, Salmonella enteritidis*, and *S. aureus* [8]. Regarding the JRLs, two members denoted jackin (from jackfruit) and frutackin (from breadfruit) are known to impair *Fusarium moniliforme* and *Saccharomyces cerevisiae* growth [30]. Interestingly, Klafke et al. [11] showed the inhibitory potential of six plant lectins, which were tested in vitro against several yeasts of medical importance: *C. albicans, C. tropicalis, C..parapsilosis, Cryptococcu.* However, to our knowledge, Helja is the first member of the subgroup of JRL able to display antifungal activity against human pathogenic yeasts.

All of the yeast strains that were incubated in the presence of Helja became agglutinated and/or formed pseudohyphae, especially *C. tropicalis*. These alterations were not observed in untreated control cells (Fig. 4). A significant variation in the number of cells was also observed for all yeasts treated with Helja. Previous studies have also described yeast/fungus structural changes caused by seed proteins displaying a wide range of functions. Agizzio et al. [2] demonstrated that a 2S albumin-like protein from passion fruit seeds induced several morphological alterations, including changes to the

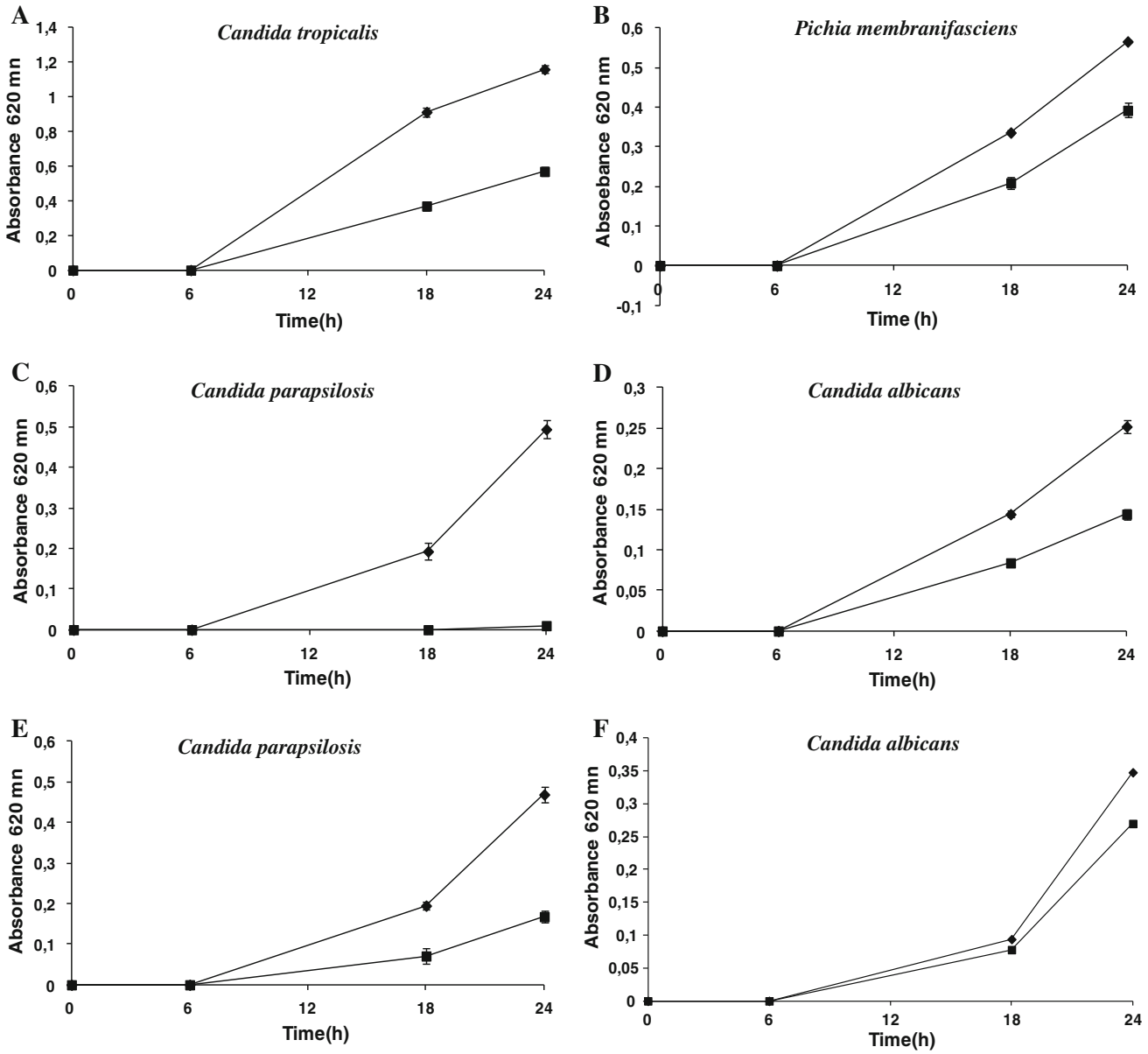

Fig. 3 The effect of Helja on the growth of the yeast strains *Candida tropicalis*, *Pichia membranifaciens*, *Candida parapsilosis*, and *Candida albicans*. (*Diamond*) control; (*square*) 200 μg/ml (**a**–**d**) or 100 μg/ml (**e**, **f**). The experiments were performed in triplicate, and the values are shown as the average

cell shape, cell surface, cell wall, and bud formation. In another work, Ribeiro et al. [25] showed that a trypsin inhibitor isolated from *Capsicum annuum* seeds caused morphological alterations, such as the formation of pseudohyphae, cellular aggregates, and elongated forms of yeasts that were incubated with it.

The ability of Helja to permeabilize the plasma membrane of yeast cells was examined in this study. Membrane permeabilization was assessed after 24 h of growth in the presence of Helja and 2 h after the addition of SYTOX Green. As measured by fluorescence microscopy, *C. tropicalis*, *P. membranifasciens*, and *C. albicans* cells fluoresced with SYTOX Green in the presence of Helja, unlike controls, indicating that the protein altered the membrane permeability (Fig. 4). Some plant proteins and peptides have also been found to permeabilize membranes [2, 6, 24].

Another part of our work investigated the ability of Helja to induce the production of ROS and NO in yeasts cells. Other authors have reported that some plant peptides may be involved in the induction of oxidative stress in fungal cells [1, 16]. Using a microscopic assay, we were able to demonstrate that ROS induction occurred in *C. tropicalis* cells that were incubated with 200 μg/ml Helja for 24 h. In comparison, control cells that were not treated

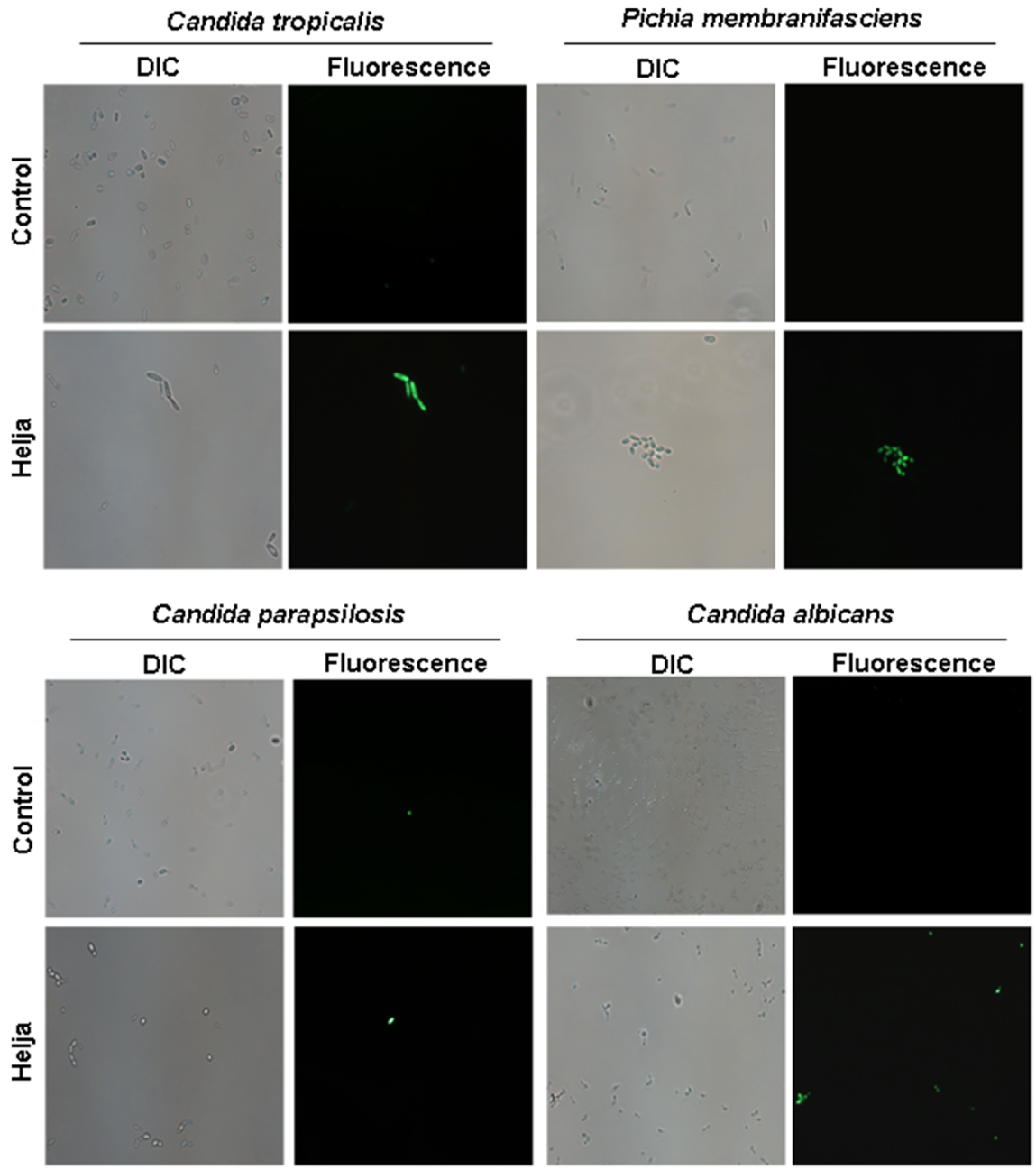

Fig. 4 Membrane permeabilization assays were performed by fluorescence microscopy of different yeast cells treated with SYTOX *Green*. Control cells (without Helja), cells treated with Helja: *Candida tropicalis*, *Pichia membranifaciens*, and *Candida albicans* (200 µg/ml) and *Candida parapsilosis* (100 µg/ml). The magnification is ×400 (Color figure online)

with Helja did not have ROS induction (Fig. 5). There was no ROS induction observed in *C. parapsilosis*, *P. membranifasciens,* or *C. albicans* after incubation with Helja (Fig. 5). In contrast, by fluorescence microscopy, we could not demonstrate NO induction in any of the yeast strains (data not show). Interestingly, it was recently shown that an antimicrobial peptide from *C. annuum* seeds, named CaTI, induced the generation of NO [25]. Reactive oxygen species (ROS) include superoxide free radicals, hydrogen peroxide, single oxygen, nitric oxide, and peroxynitrite. Most of these species are produced at basal levels under normal physiological conditions, which may explain the negative fluorescence for NO and low fluorescence for ROS assay.

The characteristic molecular function of lectins is their ability for the binding of sugar motif that occurred in different molecules. Even if a database analysis of jacalin-like lectins has established sequence-structure–function relationships,

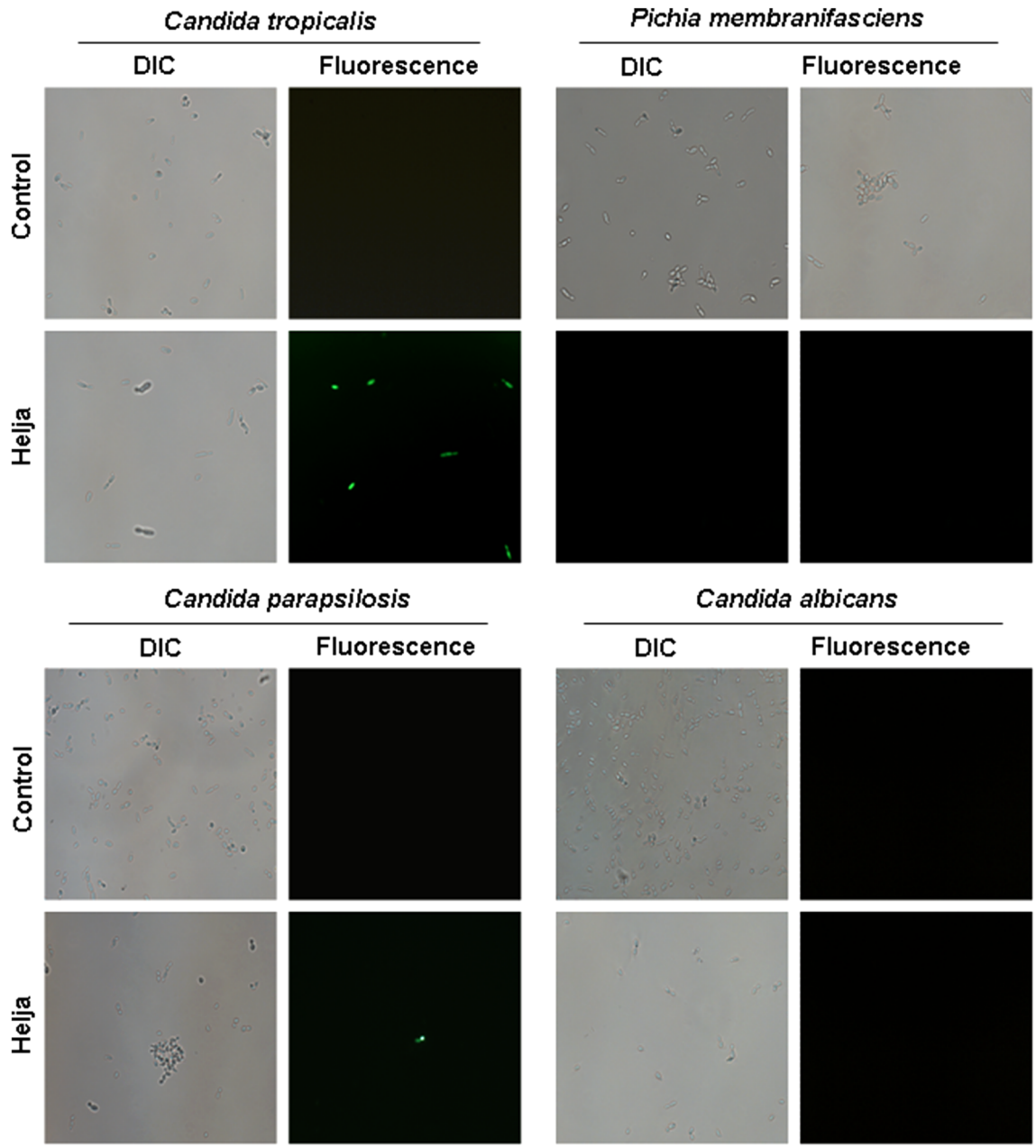

Fig. 5 Different yeast cells were treated with 2′,7′ dichlorofluorescein diacetate and an oxidative stress assay for ROS was performed with fluorescence microscopy. Control cells (without Helja), cells treated with Helja: *Candida tropicalis*, *Pichia membranifaciens*, and *Candida albicans* (200 μg/ml) and *Candida parapsilosis* (100 μg/ml). The magnification is ×400

the antimicrobial role of JRL is still unclear [23]. An interesting observation in wheat revealed that the jacalin domain of a jasmonate-induced protein appeared involved in Pseudomonas′s agglutination and increased plant defense [15]. Moreover, other JRL expressed in wheat stems and spikes named TaJRLL1 reduced the susceptibility to the facultative fungal pathogen *Fusarium graminearum* and the biotrophic fungal pathogen *Blumeria graminis* while *Arabidopsis thaliana* transformed with the gene encoding this protein increased the resistance to *F. graminearum* and *Botrytis cinerea* causing the decrease of conidial production of both fungi [33].

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