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Internalization of a sunflower mannose-binding lectin into phytopathogenic fungal cells induces cytotoxicity

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ABSTRACT

Lectins are carbohydrate-affinity proteins with the ability to recognize and reversibly bind specific glycoconjugates. We have previously isolated a bioactive sunflower mannose-binding lectin belonging to the jacalin-related family called Helja. Despite of the significant number of plant lectins described in the literature, only a small group exhibits antifungal activity and the mechanism by which they kill fungi is still not understood. The aim of this work was to explore Helja activity on plant pathogenic fungi, and provide insights into its mechanism of action. Through cellular and biochemical experimental approaches, here we show that Helja exerts an antifungal effect on *Sclerotinia sclerotiorum,* a sunflower pathogen. The lectin interacts with the fungal spore surface, permeabilizes its plasma membrane, can be internalized into the cell and induces oxidative stress, finally leading to the cell death. On the other hand, Helja is inactive towards *Fusarium solani,* a non-pathogen of sunflower, showing the selective action of the lectin. The mechanistic basis for the antifungal activity of an extracellular jacalin lectin is presented, suggesting its initial interaction with fungal cell wall carbohydrates and further internalization. The implication of our findings for plant defense is discussed.

1. Introduction

Plants have naturally developed different mechanisms to counteract the attack of phytopathogenic fungi, including antifungal proteins and peptides. These have been isolated from diverse plant species, and can be classified according to their structure and/or function into different groups including chitinases, glucanases, thaumatin-like proteins, defensins, lipid transfer proteins, lectins, peroxidases, protease inhibitors, among others (Wong et al., 2010; Yan et al., 2015). The lectins have the unique ability to recognize and reversibly bind specific carbohydrate ligands without any chemical modification; this feature distinguishes lectins from other carbohydrate binding proteins and enzymes (Peumans and Van Damme, 1995). Plants were the first discovered source of lectins, and although these proteins are ubiquitously distributed in nature, plants remain their most frequent source due to both ease of extraction and the relatively high yields that can be obtained (Lam and Ng, 2010; Dang and Van Damme, 2015). Although currently still under study, it is generally accepted that plant lectins can play a defense role recognizing the pathogens and initiating the stress response through protein-carbohydrate interactions (De Schutter and Van Damme, 2015). Related to their binding-ability to glycoconjugates, lectins exhibit a diversity of activities including antibacterial, anti-insect, antitumor, immunomodulatory, HIV-1 reverse transcriptase inhibitory activities, which make them valuable tools in different practical applications (Lam and Ng, 2010; Dang and Van Damme, 2015; Lagarda-Diaz et al., 2017; Poiroux et al., 2017). Despite the large numbers of lectins that have been purified and characterized, only a few of them displayed antifungal activity (Silva et al., 2014; Wu et al., 2016; Neto et al., 2017). Carbohydrates present on the fungal cell wall represent the main structures engaged in primary interaction with host cells and constitute ideal targets for antifungal protective intervention. Thus, the lectins are particularly attractive since they can affect the plant-pathogen interaction at the first point of contact. It is generally accepted that plant lectins are unable to bind glycoconjugates present on fungal membranes or penetrate into the fungal cytoplasm owing to the cell wall barrier (Wong et al., 2010; Dang and Van Damme, 2015). Hence, it is unlikely that lectins directly interfere with fungal growth by altering the structure and/or permeability of their membranes. However, there may be indirect effects produced by the binding to the carbohydrates on the surface of the fungal cell wall (Wong et al., 2010).

We have previously isolated a sunflower mannose-binding lectin belonging to the jacalin-related lectins (JRL) family, which was called Helja (*Helianthus annuus* jacalin) (Pinedo et al., 2012). Helja was initially detected through a proteomic study of sunflower seedlings apoplast and its putative identification as mannose binding jacalin was taken as an advantage to purify it by D-mannose affinity

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chromatography. Biochemical and cellular approaches demonstrated the extracellular localization of Helja in sunflower seeds and seedlings (Pinedo et al., 2012). The characterization of the biological activity revealed that the protein displayed agglutination capacity on *Saccharomyces cereviceae* cells and antifungal activity on human pathogens of *Candida* genus (Regente et al., 2014). Although the physiological role of jacalin-related lectins is still not understood, in general, it has been shown to be involved in resistance to abiotic and biotic stress (Xiang et al., 2011; Song et al., 2014; Esch and Schaffrath, 2017). For instance, the rice protein OsJRL is up-regulated in response to salt, drought, cold, and heat stress (He et al., 2017). The jacalin-related lectin RTM1 restricts the long distance movement of tobacco etch virus in *Arabidopsis thaliana* (Chisholm et al., 2000). The apoplastic localization of Helja and its inhibitory activity on a human pathogenic fungus model suggest that the lectin could be a component of the plant defense system towards the fungal attack. The aim of this paper was to explore the activity of Helja on plant pathogenic fungi, and provide mechanistic insights on its mode of action. The research design was based on the evaluation of Helja biological activity on *Sclerotinia sclerotiorum*, the causal agent of sunflower head rot. In addition, the antifungal activity of Helja on *Fusarium solani,* a non-pathogen of sunflower, was also evaluated. The comparison of the results obtained after Helja treatments on the two fungus species, both from the point of view of its mechanism of action as well as from its role in the interaction between microbial and host plant cell will be discussed.

2. Materials and methods

2.1. Biological material

Sunflower seeds (*Helianthus annuus* L., line 10347) were provided by Advanta Semillas SAIC, Venado Tuerto, Argentina. *Sclerotinia sclerotiorum* (Lib.) de Bary ascospores from a local virulent isolate were a gift of M. E. Bazzalo (Advanta Semillas SAIC, Centro Biotecnológico Balcarce, Argentina) and were collected from Petri dishes containing imprints of apothecia. *Fusarium solani* f. sp. *eumartii*, isolate 3122, (INTA Collection, Balcarce, Argentina) was kindly provided by Dr. C. Casalongué. *F. solani* was grown at 25 °C on potato dextrose agar (PDA) plates and spores were collected from cultures by suspension in sterile water.

2.2. Helja purification

Sunflower seeds were imbibed for 16 h and subjected to the extraction of the extracellular fluids (EF) by a standard infiltration-centrifugation procedure (Regente et al., 2008). Briefly, seeds were immersed in 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% 2-mercaptoethanol and subjected to three vacuum pulses of 10 s, separated by 30 s intervals. The infiltrated seeds were recovered, dried on filter paper, placed in filters and centrifuged for 20 min at 400*g* at 4 °C. The EF was recovered in the filtrate and subjected to Helja purification according to Pinedo et al. (2012), with some modifications. The EF was loaded on a 1 ml D-mannose-agarose resin (Sigma M6400) equilibrated with 50 mM HCl-Tris pH 7.5, 100 mM NaCl (buffer A). Non-bounded proteins were washed with buffer A before the elution of retained proteins with 0.2 M mannose in the same buffer. The eluted fraction was exhaustively dialyzed against distilled water to allow the release of mannose from the protein fraction.

2.3. Microscopic antifungal activity assays

Fresh spores from *S. sclerotiorum* and *F. solani* were collected in sterile water and subjected to counting in a Neubauer chamber for further calculation of appropriate dilutions. The antifungal activity was evaluated on micro slides in a final volume of 20 µl containing the protein sample (0.01; 0.05; 0.1 and 0.2 µg µl^{-1}), 1 × 10^5 spores and 4% sucrose (Regente and de la Canal, 2000). Controls were performed replacing Helja solution with the same volume of water. After 16 h of incubation at 25 °C and 100% relative humidity the slides were microscopically evaluated for inhibition of spore germination or hyphae growth reduction. The MIC of Helja was defined as the lowest concentration that led to a microscopically visible reduction of hyphal length compared to controls. Three independent biological replicates of antifungal test were performed.

2.4. Viability assays on PDA plates

A *S. sclerotiorum* spore suspension (10^4 cells ml^{-1}) was incubated with protein sample (0.2 µg µl^{-1}) or water as control during 18 h at 4 °C. Subsequently, 10 µl of each treatment were plated in the center of a PDA Petri dish. Antifungal activity was evaluated by observation of mycelial growth and comparing them to controls. The plates were prepared in triplicate and incubated at 25 °C for 7 days.

2.5. Quantitative antifungal activity assays

A quantitative test was performed in 96-well flat microplates containing the protein sample (0.2 µg µl^{-1}) and 1 × 10^4 spores in Sabouraud broth in a final volume of 100 µl (Regente et al., 2014). The fungal growth was monitored by 595 nm optical reading at the indicated times during 3 days of incubation at 25 °C. The percentage of growth inhibition in the presence of Helja was calculated relative to the control in the absence of the lectin, which was considered as 100% growth. At the end of the assay, *S. sclerotiorm* cells were collected from the growth medium and washed in sterile water for further evaluation of morphology and viability. Quantitative tests were performed by triplicate and repeated at least twice. Growth curves were compared using a regression analysis (Infostat/L). The Tukey's 2-tailed *t*-test, assuming unequal variance, was used to determine whether there was a significant difference between two sets of data. *P*-values of 0.05 were considered significant.

2.6. Evans blue and propidium iodide staining

The fungal viability and permeabilization of the plasma membrane were evaluated by Evans Blue and propidium iodide uptake. After evaluation of the microscopic antifungal activity in microslides containing the protein sample (0.05 and 0.2 µg µl^{-1}), Evans Blue dye (Levine et al., 1994) was added to a final concentration of 0.05% and fungal cells were observed by optical microscopy. Following the quantitative antifungal activity test containing the protein sample (0.2 µg µl^{-1}), iodide propidium was added to a final concentration of 50 µg ml^{-1} and the assay was observed under fluorescence microscopy using a Eclipse E200 microscope (Nikon) equipped with an epi-fluorescence unit and a G-2E/C filter set containing an excitation filter at 540/25 nm, a suppressor filter at 630/60 nm and a dichroic mirror at 565 nm (Mansilla et al., 2015).

2.7. Detection of hydrogen peroxide

Detection of hydrogen peroxide in fungal spores was performed by a staining procedure using 3,3′-diaminobenzidine (DAB) (Giudici et al., 2004). Briefly, the fungal spores (1 × 10^5) were incubated on microslides with water (control) or the protein sample (0.05 and 0.2 µg µl^{-1}) in the presence of 0.5 mg ml^{-1} DAB. 2 mg ml^{-1} DAB solution was prepared and one-quarter of the final volume was added to the assay. The slides were microscopically evaluated for production of endogenous H_2O_2 as a brown pellet.

2.8. Covalent conjugation of FITC to Helja and binding to fungal spores

Fluorescein isothiocyanate (FITC) was covalently coupled to Helja

and tested for binding to fungal cells according to the methodology described by Taveira et al. (2016) with some modifications. FITC (50 mg in 1 ml of 99.9% anhydrous DMSO) was immediately diluted in 0.75 M bicarbonate buffer (pH 9.5) before use. Following the addition of FITC to give a ratio of 1 mg FITC per mg of lectin, the tube was wrapped in foil and incubated in an orbital shaker at room temperature for 4 h. Non-coupled FITC was removed by dialysis against distilled water, and the resulting solution was stored at −20 °C until use. The fungal spores were treated with 0.05 μg μl^{-1} of Helja-FITC for 30 min. After the treatments, the materials were mounted on glass slides and visualized by confocal laser scanning microscopy. FITC was excited at 450–490 nm and detected at 500 nm. To evaluate the effect of sugars on the binding of Helja-FITC to fungal cells, 500 mM D-mannose was added in the assay. When indicated incubations were supplemented with 10 mM sodium azide, an inhibitor of endocytosis in fungal cells (Fischer-Parton et al., 2000; Higuchi et al., 2006). In order to monitor endocytic pathways, spores of *S. sclerotiorum* were stained with N-(3-triethylammoniumpropyl)-4-(6-(4-diethylaminophenylhexatrienyl) pyridinium dibromide (FM4-64). FM4-64 was excited at 488 nm and detected at 650–750 nm. When indicated, a simultaneous staining with 40 μM FM4-64 and Helja-FITC (0.05 μg μl^{-1}) was performed (Fischer-Parton et al., 2000). Microscopic analysis was performed using a Nikon C1 confocal laser scanning microscope (Nikon Instruments Inc., Melville, NY, USA). The screening along the Z axis of fungal cells was performed taking optical sections of 1 μm thickness. All images were acquired with Super Fluor 40.0x/1.30/0.22 oil-immersion lens. The post-processing of images was performed with the aid of EZ-C1 Free-Viewer version 3.2 software.

3. Results

3.1. Helja inhibits S. sclerotiorum hyphal growth and does not affect F. solani

In order to explore the biological activity of Helja on phytopathogenic fungi, we have tested its effect on *Sclerotinia sclerotiorum* and *Fusarium solani*. Fungal spores were incubated with increasing doses of Helja (from 0.01 to 0.2 μg μl^{-1}) to perform an optical microscopy evaluation. Helja antifungal activity on *S. sclerotiorum was* observed as a reduction of mycelial growth (Fig. 1), nevertheless we could not detect the complete inhibition of spore germination even at the highest dose tested. While the hyphae length reduction was barely perceptible at lower doses, the effect was clearly visible at 0.2 μg μl^{-1} (Fig. 1). Therefore, it was assumed that the minimum inhibitory concentration (MIC) of Helja on *S. sclerotiorum* was 0.2 μg μl^{-1}. Interestingly, Helja showed no apparent effect on *F. solani* at the same doses, since the fungus treated with the lectin displayed similar hyphal length and morphology compared to controls (Fig. 1). Cell viability was further analysed using Evans Blue staining taking advantage of its penetration into dead cells. Fungal spores were treated with Helja at the dose indicated and after 16 h of incubation *S. sclerotiorum* hyphae appeared blue stained, clearly revealing non-viable cells (Fig. 2). By contrast, *F. solani* hyphae excluded the dye and its appearance was similar to the control demonstrating that its viability was not affected (Fig. 2).

3.2. Helja modifies plasma membrane permeability and hydrogen peroxide production in S. sclerotiorum

To further characterize the antifungal effect of Helja on the fungus *S. sclerotiorum*, a first experimental approach to assess fungal survival was performed. The spores were incubated in the absence or in the presence of Helja during 16 h, and then the samples were plated on solid rich medium Petri dishes. At 6 days of growth, the control culture showed a dense mass of white mycelium while in Helja treated culture a barely detectable growth was visualized (Fig. 3A). In order to quantify this effect, spores were incubated in liquid medium with the protein and the optical density of the cultures was monitored for 3 days. As visualized in Fig. 3B, a 30% reduction of fungal growth was even observed in rich medium in the presence of Helja. To determine whether this reduction was a consequence of a cytotoxic effect, aliquots of the culture were evaluated for membrane permeability through the propidium iodide method. Viable cells exclude the fluorophore, however, if the membrane integrity is compromised the probe diffuses and appear red-labeled upon binding to DNA. Fig. 4 shows red dyed hyphae after the treatment indicating that Helja modifies the plasma membrane permeability of fungal cells. This result is consistent with that shown in Fig. 2 and taken together highlight the cytotoxic action of the lectin, which was observed both in a limiting medium and in a nutrient-rich medium.

In order to asses if Helja ability to inhibit fungal growth involves the induction of reactive oxygen species (ROS), we used the 3, 3-diaminobenzidine (DAB). The oxidation of DAB in the presence of peroxides

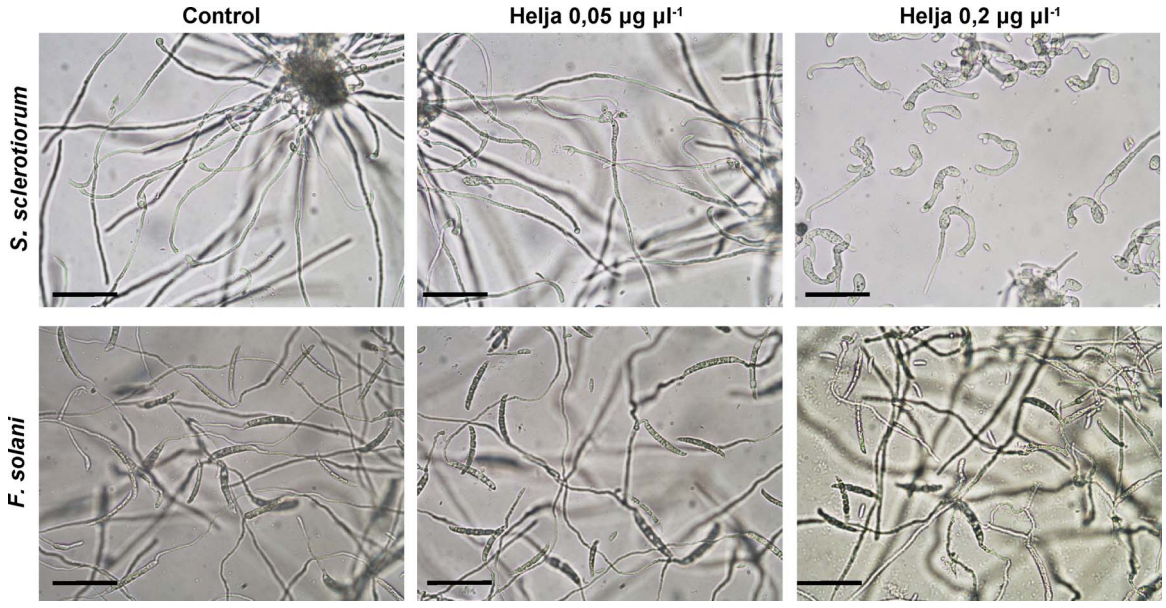

Fig. 1. Helja selectively affects *S. sclerotiorum* and *F. solani* phytopathogenic fungi. Fungal spores were incubated in the presence of water (control) or Helja (0.05 and 0.2 μg μl^{-1}) during 16 h. The images are representative of 3 biological replicates. Scale bar: 60 μm.

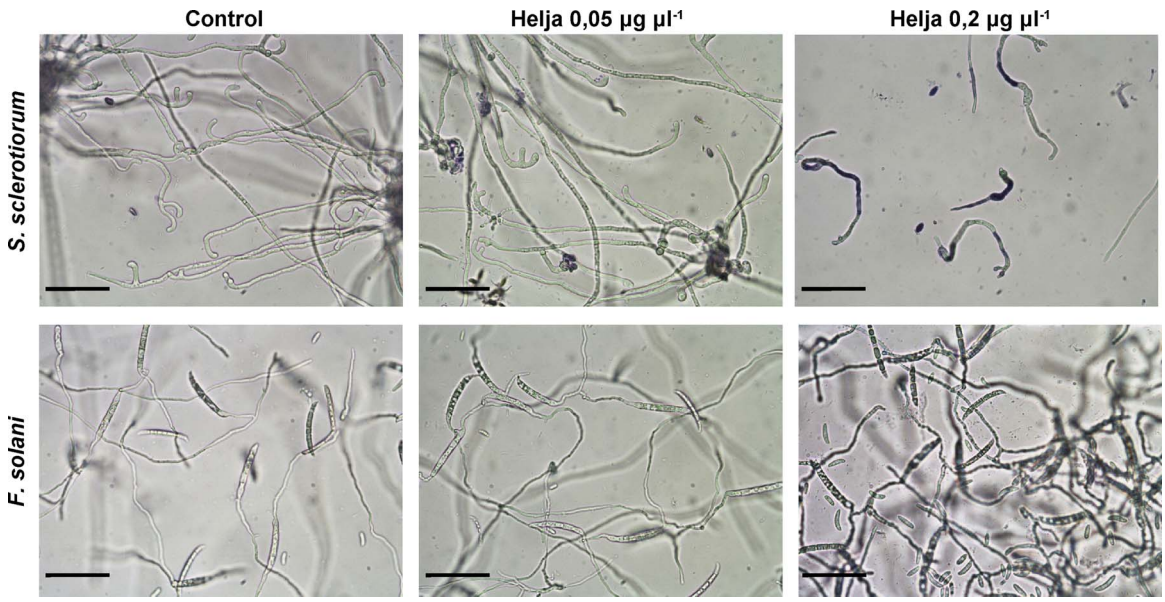

Fig. 2. Helja selectively affects the viability of *S. sclerotiorum* and *F. solani*. Spores were incubated in the presence of water (control) or Helja (0.05 and 0.2 µg µl^{-1}) during 16 h, and then stained with 0.05% Evans Blue to detect non-viable cells. The images are representative of 3 biological replicates. Scale bar: 60 µm.

is observed as a dark-brown signal indicative of the local formation of an insoluble precipitate at the reaction site (Thordal-Christensen et al., 1997). The ascospores of *S. sclerotiorum* were incubated with the lectin for 4 h in the presence of 0.5 mg ml^{-1} DAB and observed by optical microscopy. The cells treated with 0.05 µg µl^{-1} Helja show light brown coloration compared to controls that appeared unstained (Fig. 5). In addition, spores treated with 0.2 µg µl^{-1} of the lectin developed a strong brown pellet, clearly revealing that the production of endogenous H_2O_2 is a dose dependent reaction (Fig. 5).

3.3. Helja binds to the surface and penetrates into S. sclerotiorum cells but not to F. solani cells

The selectivity of the antifungal activity of Helja was further analysed. Helja lectin might contact ligands of glycosidic nature at the surface of the cell discriminating different fungi. In order to explore the interaction between the lectin and the spores of *S. sclerotiorum* and *F. solani*, fluorescent Helja was prepared by fluorescein isothiocyanate (FITC) conjugation. Interaction of Helja-FITC with fungal cells was monitored by confocal laser scanning microscopy. As observed in Fig. 6 the spores of *S. sclerotiorum* were completely labeled with green fluorescence of Helja-FITC while *F. solani* spores remained unlabeled, clearly revealing that Helja was able to bind to *S. sclerotiorum* cells but not to *F. solani* cells. To assess whether the binding of Helja-FITC to *S. sclerotiorum* was mediated by its ability to recognize putative carbohydrate ligands of fungal cell surface, 500 mM D-mannose was added in the assay. As shown in Fig. 6, the fluorescent signal was abolished in the presence of sugar, demonstrating Helja-FITC binding to fungal carbohydrate. To elucidate whether Helja remained attached to the spore surface or if it could be incorporated inside the cells, we have performed a screening along the z axis of the cells by confocal laser scanning microscopy. The images clearly show that Helja was localized within fungal cells and not restricted to the spore surface (Fig. 7A). To explore the effect of sugars on the uptake of Helja, the fungal spores were treated simultaneously with 0.05 µg µl^{-1} of Helja-FITC and 500 mM D-mannose for 30 min, and visualized as described above (Fig. 7B). The absence of the fluorescent signal within the cells treated with mannose indicates that the sugar inhibited the internalization of the lectin. Endocytosis is a widespread process by which eukaryotic cells internalize proteins and other molecules that cannot pass through the hydrophobic plasma membrane by engulfing them in vesicles that fuse with the endosomal system (Peñalva, 2010). The endocytic uptake of lectins by animal cells has already been documented (Yau et al., 2015). Thus, most cytotoxic lectins for tumor cells exhibit their effect through the binding to cell surface carbohydrates and subsequent internalization into the cell, finally leading to cell death (Gabor et al., 2001; Yau et al., 2015). In order to determine if the internalizationof Helja takes place by this active transport process, we first performed a pharmacological approach. The spores were incubated with Helja in the presence of sodium azide, which inhibits endocytosis in fungal cells (Fischer-Parton et al., 2000; Higuchi et al., 2006). The confocal microscopy approach allowed us to visualize that Helja was incorporated into *S. sclerotiorum* cells in the presence of 10 mM sodium azide (Fig. 7C). In a second approach, Helja-FITC uptake was compared to the incorporation of the red fluorescent dye FM4-64. This is an amphipathic fluorophore unable to cross membranes thus constituting a valuable

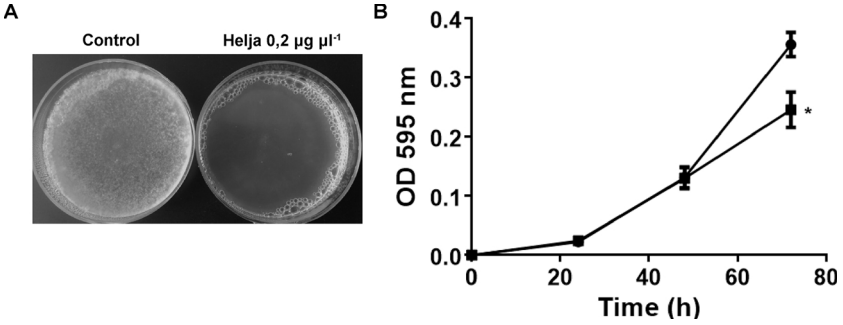

Fig. 3. Helja inhibits mycelial growth of *S. sclerotiorum*. A: Fungal spores were incubated for 16 h in the presence of water (control) or Helja (0,2 µg µl^{-1}), then plated in the center of PDA Petri dishes and the mycelial growth was observed after 6 days. B: Spores of *S. sclerotiorum* were incubated in the absence (●) or presence of 0.2 µg µl^{-1} of Helja (■) in Sabouraud broth. Fungal growth was monitored by OD595 nm for 3 days. The experiments were performed in triplicate, and the values are shown as the average. The means of the slopes of both growth curves were significantly different (*) from each other, as derived from regression analyses, $P < 0.05$.

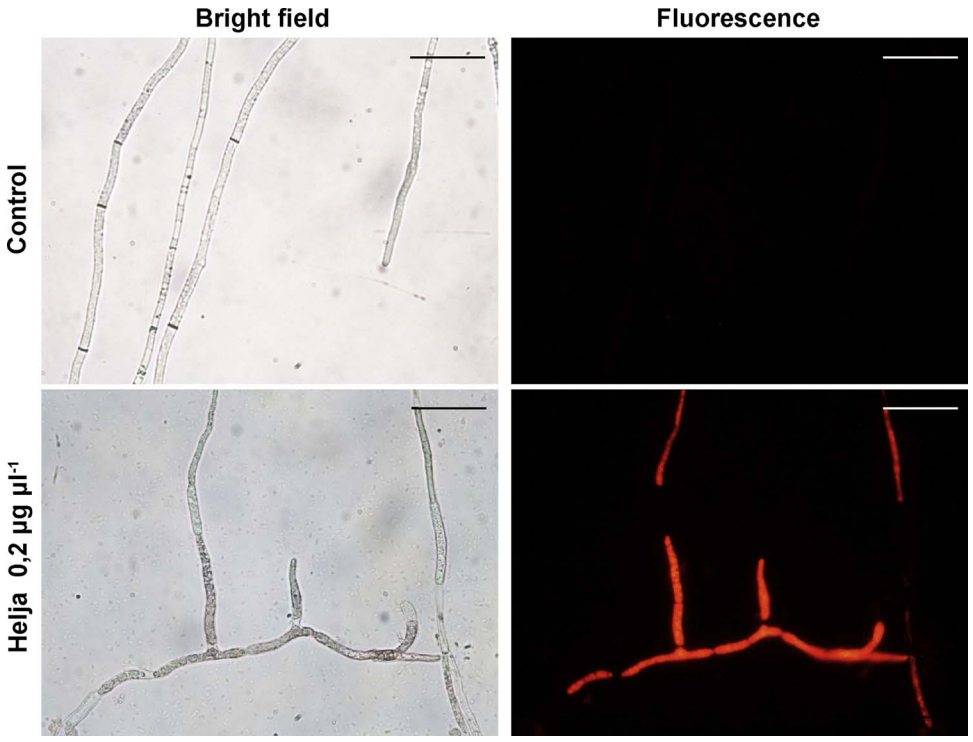

Fig. 4. Helja permeabilizes *S. sclerotiorum* plasma membrane. The mycelium from control or 0.2 µg µl^{-1} Helja treatments showed in Fig. 3B was stained with iodide propidium and observed under bright field and fluorescence microscopy. The images are representative of 3 biological replicates. Scale bar: 60 µm.

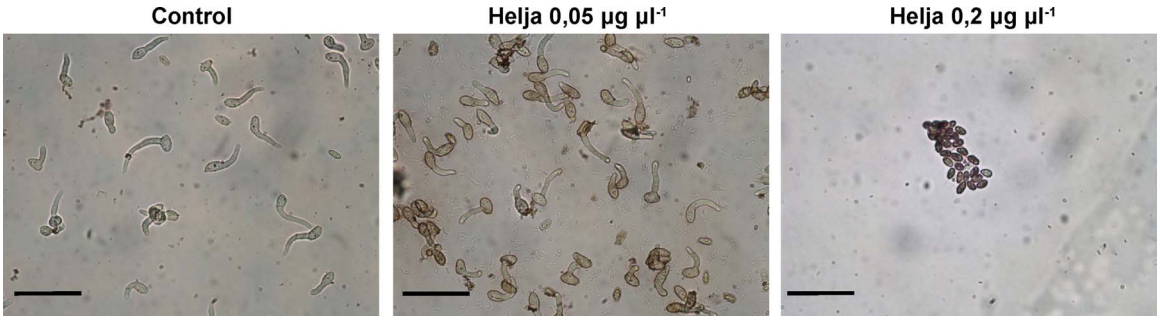

Fig. 5. Helja induces oxidative stress in spores of *S. sclerotiorum*. Spores were incubated in the presence of water (control) or Helja (0.05 and 0.2 µg µl^{-1}) for 4 h. The incubation was performed in the presence of 0.5 mg ml^{-1} DAB to detect hydrogen peroxide. The images are representative of 3 biological replicates. Scale bar: 60 µm.

tool since it can only enter living cells through endocytic events (Bolte et al., 2004). Fig. 8 shows that at 10 min of treatment, Helja-FITC appears uniformly associated to the cell surface of *S. sclerotiorum*. At 30 min of incubation, the fluorescence was clearly detected inside the cells, displaying increased intensity at 60 min of treatment. Interestingly, in the same spores incubated for 10 min with FM4-64, the red fluorescence already appears inside the cells. Unlike the entry of Helja, during the internalization of FM464 apparent endocytic vesicles stained with the dye are evident (Fig. 8, arrows). These results allow us to establish clear differences between the internalization of the lectin and FM4-64. Taken together the results of Figs. 7 and 8 indicate that Helja penetrates into fungal cells through an endocytosis-independent mechanism.

4. Discussion

Our study was focused towards the exploration and characterization of Helja bioactivity on plant pathogenic fungi to deepen the basis of its antifungal action. Two phytopathogenic fungi which show different impact on sunflower were selected; *Sclerotinia sclerotiorum,* the fungus that produces serious damages on this crop, and *Fusarium solani,* that is not pathogenic (Boland and Hall, 1994; Mestries et al., 1998). We demonstrate that Helja presents the ability to reduce the hyphal growth and viability of *Sclerotinia sclerotiorum* but does not show any visible effect on *Fusarium solani* at equivalent doses. Interestingly, the results obtained in this work showed that the antifungal activity of Helja appeared to be selective since the two microorganisms showed differential susceptibilities to the lectin, thus emerging as an experimental system attractive to provide mechanistic insights of the lectin bioactivity. It is accepted that lectins may exhibit antifungal activity through their binding to carbohydrates of the fungal cell wall (Wong et al., 2010). In this sense, the interaction analysis of Helja-FITC with the spores of the two microorganisms offers key information on lectin selectivity. The differential antifungal activity of Helja appears to be determined by its specific binding ability to fungal cells, suggesting differences in ligand recognition between *S. sclerotiorum* and *F. solani*. In fact, Smith et al. (2008) used ConA, a mannose-binding lectin, conjugated to FITC as a tool to detect mannose exposed to the outer surface of fungal cell walls. Thus, our results suggest that *S. sclerotiorum* could present mannose structures exposed towards the outer surface of the cell wall, evidenced by the binding of the lectin to the spores and the inhibition in the presence of mannose, whereas *F. solani* would not present exposed mannose. In this sense, the composition and/or spatial distribution of carbohydrates in the wall would be the cause of the Helja differential action on both species. Interestingly, Araújo-Filho et al. (2010) have reported the antifungal activity of a mannose binding lectin (Dgui) from

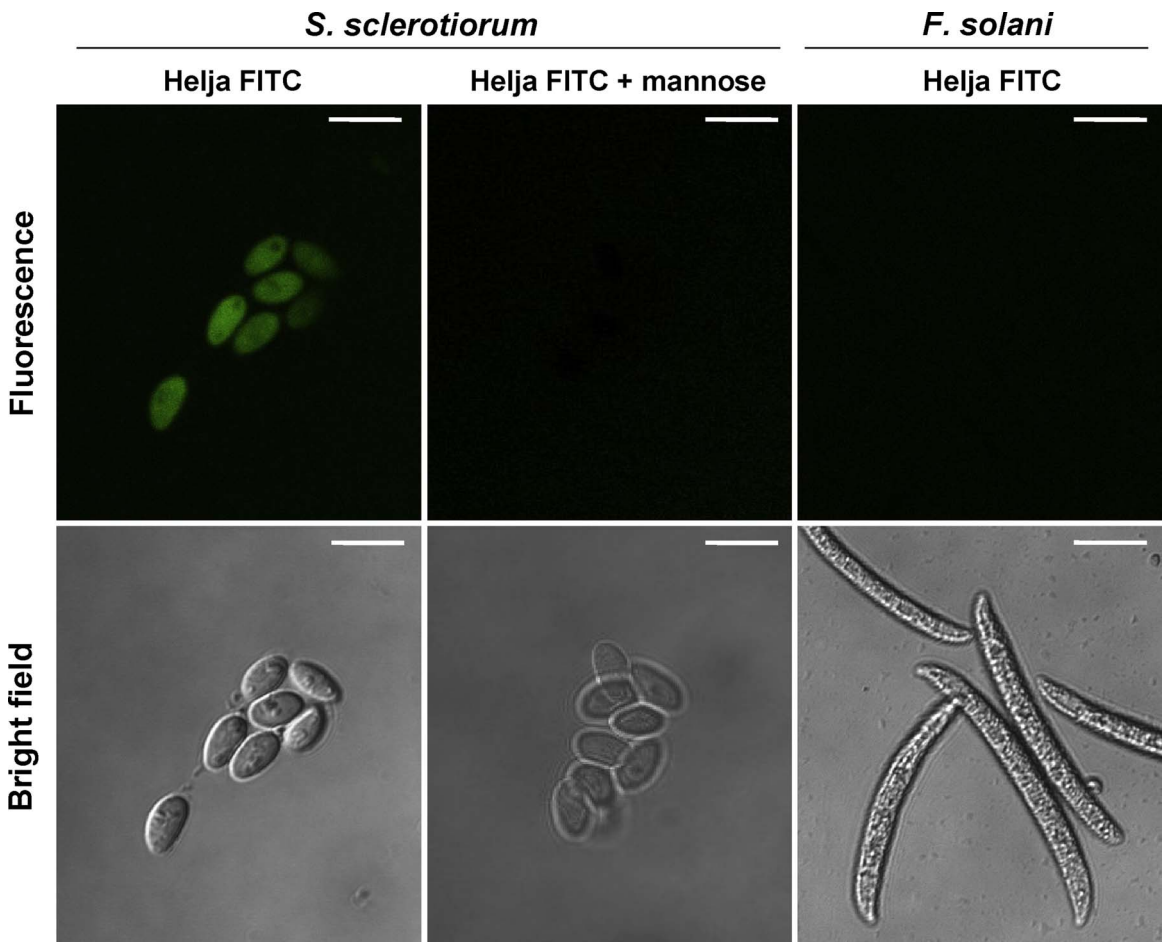

Fig. 6. Binding of Helja to *S. sclerotiorum* and *F. solani* spores. Fungal spores were incubated with 0,05 μg μl^{-1} Helja-FITC or 0,05 μg μl^{-1} Helja-FITC and 500 mM mannose for 30 min and observed under bright field or laser scanning confocal fluorescence microscopy. The images are representative of 3 biological replicates. Scale bar: 10 μm.

Dioclea guianensis. Dgui binds to the conidia and inhibits the germination of *Colletotrichum gloeosporioides* but not of *Fusarium solani*. The authors suggest that the ability of Dgui to specifically inhibit *C. gloeosporioides* might be due to recognition of fungal surface-specific target carbohydrates. Even though the presence of specific targets on *S. sclerotiorum* surface recognized by Helja could be the basis of the fungal selectivity, additional experiments are required to identify the putative specific ligand/s.

The binding selectivity and antifungal action of the lectin Helja on *S. sclerotiorum*, added to its extracellular location, gain particular interest in the context of the interaction between this pathogen and sunflower. The carbohydrates present on the fungal cell wall represent the initial point of contact in primary interaction with host cells. Thus, Helja could counteract the fungal attack in the first line of defense of the plant cells through the binding to carbohydrate ligands. On the other hand, this interaction could contribute to the perception of the pathogen and its translation into plant defense response. In this sense, Esch and Schaffrath (2017) have recently discussed the function of JRL domains as a decoy for help to alert plants of the presence of attacking pathogens. This hypothesis was based on demonstration that a jacalin-like lectin domain of a chimeric protein was responsible for relocating the protein towards the site of fungal pathogen attack, most likely by binding to oligosaccharide signatures typical for the infection process (Weidenbach et al., 2016).

Despite the large number of lectins that have been characterized, only a few of them have been associated with antifungal properties (Reviewed by Lam and Ng, 2010; Silva et al., 2014; Wu et al., 2016; Neto et al., 2017). The results of this work and our previous report (Regente et al., 2014) allow us to include the lectin Helja within this small group. For instance, the maize lectin-like protein ZmCORp exhibited inhibitory activity on conidia from *A. flavus* exposed to 18 mM of the protein (Baker et al., 2009). *Dendrobium findleyanum* agglutinin showed antifungal activity against *Alternaria alternata* and *Collectotrichum* sp. at concentration of 0.1 μg μl^{-1} (Sattayasai et al., 2009). A galactose-binding lectin purified from seeds of *Bauhinia ungulata* L. showed antifungal activity against *Fusarium* spp. at dose of 0.16 μg μl^{-1} (Silva et al., 2014). A novel lectin, designated Lunatin, was isolated from edible seeds of *Phaseolus lunatus billb* exerted antifungal activity toward a variety of fungal species, including *Sclerotium rolfsii, Physalospora piricola, Fusarium oxysporum,* and *Botrytis cinerea* at concentration between 0.05–0.1 μg μl^{-1} (Wu et al., 2016). Mo-CBP2, a chitin-binding protein recently purified from *Moringa oleifera*, possesses *in vitro* antifungal activity against *Candida* spp. with MIC50 and MIC90 values ranging between 9.45–37.90 mM and 155.84–260.29 mM, respectively (Neto et al., 2017). Based on the literature data concerning the active doses of lectins towards fungi, Helja exhibits similar potency to the antifungal lectins described, showing a MIC on *S. sclerotiorum* of 0.2 μg μl^{-1}.

Regarding the mechanism of action of Helja, it is accepted that plant lectins can neither bind to glycoconjugates on the fungal membranes nor penetrate the cytoplasm owing to the cell wall barrier (Wong et al., 2010; Dang and Van Damme, 2015). However, the interaction of Helja with putative ligands of glycosidic nature along the cell wall could facilitate that the lectin reaches the plasma membrane. The knowledge assembled so far regarding the mode of action of lectins on fungi has led to suggest an indirect effect through the interaction with carbohydrates of the cell wall. For instance, *Urtica dioica* lectin interrupted fungal cell wall synthesis attenuating chitin synthesis and/or deposition (Van

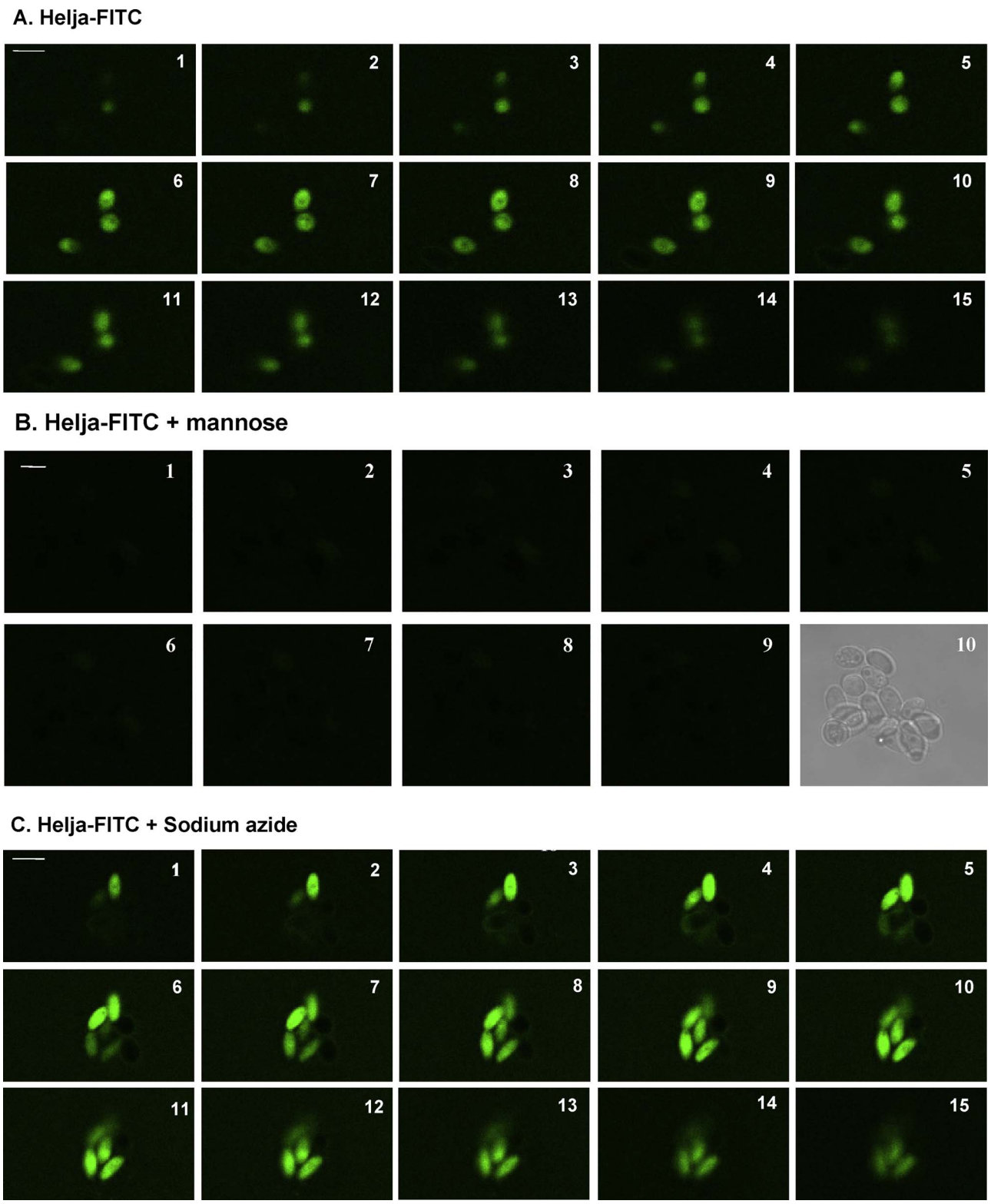

Fig. 7. Helja internalization into *S. sclerotiorum* spores. Screening along the Z axis by laser scanning confocal fluorescence microscopy of *S. sclerotiorum* spores incubated with 0.05 μg μl^{-1} Helja-FITC (A), 0.05 μg μl^{-1} Helja-FITC and 500 mM D-mannose (B), or 0.05 μg μl^{-1} Helja-FITC and 10 mM sodium azide (C) for 30 min. The figure shows consecutive optical sections (each of 1 μm thickness). The images are representative of 3 biological replicates. Scale bar: 10 μm.

Parijs et al., 1991). However, it has recently been reported the characterization of a chitin-binding protein from *Moringa oleifera*, Mo-CBP2, showing anticandidal activity by increasing cell membrane permeability and reactive oxygen species production (Neto et al., 2017). Here we show that Helja was able to permeabilize the cell membrane of *S. sclerotiorum* as it was previously demostrated for *Candida* yeasts (Regente et al., 2014). Simultaneously, we verified that fungal cells undergo oxidative stress when treated with Helja. ROS are frequently increased when intracellular oxygen metabolism is unbalanced from the equilibrium with antioxidants. When this balance is disrupted, molecules such as hydrogen peroxide, hydroxyl and superoxide radicals can be accumulated originating cellular damage (Waris and Ahsan, 2006;

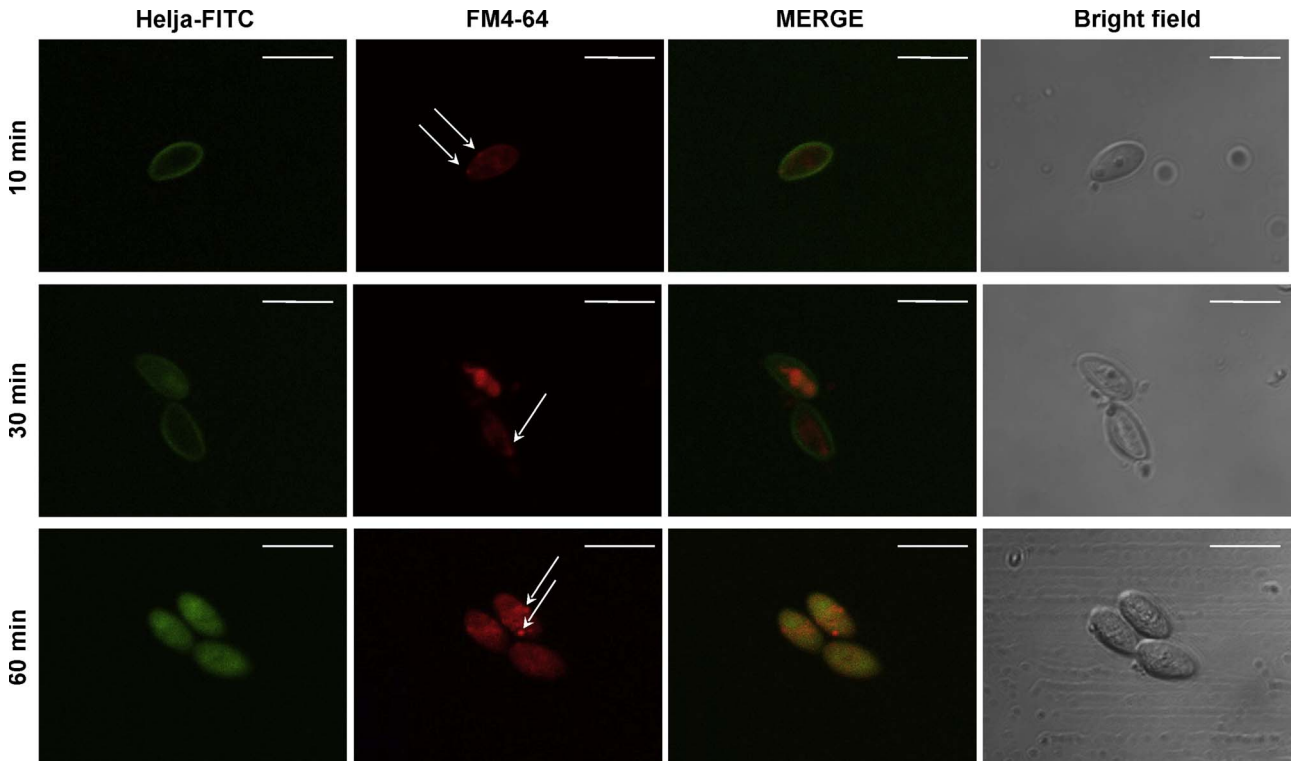

Fig. 8. Helja internalization is independent of the endocytic pathway. Laser scanning confocal fluorescence microscopy images of *S. sclerotiorum* spores simultaneously incubated with 0.05 μg μl^{-1} Helja-FITC (green) and FM4-64 (red) for 10, 30 and 60 min. Arrows (→) point to apparent endocytic vesicles. The images are representative of 3 biological replicates. Scale bar: 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Kowaltowski et al., 2009; Scandalios, 2005). The mode of action of Helja is probably linked to its ability to increase ROS levels and to disrupt the integrity of cell membrane, which in turn, could damage cell structures and ultimately lead to cell death. Interestingly, Belenky et al. (2013) demonstrated, by a system biology approach, that the antifungal drugs amphotericin, azoles and synthetic ciclopirox olamine induce the formation of lethal ROS even though they display different primary modes of action that include membrane permeabilization, accumulation of a toxic methylated sterol and DNA damage. According to the current knowledge, it could be speculated that at least the induction of ROS triggered by Helja might be responsible of killing fungal cells.

Despite showing a similar effect to that of Helja, it is unknown if Mo-CBP2 enters the cell to trigger its cytotoxic action (Neto et al., 2017). In this work we were able to determine that Helja is internalized into fungal cells, a process inhibited by 500 mM mannose, suggesting that the uptake of Helja could be mediated by its binding to ligands of a glycosidic nature. The high concentration of sugar required for inhibit the uptake of Helja could be explained by differences in the binding affinity of lectins towards monosaccharides, typically close to 10^{-3} M, or more complex glycans (like those present in the fungal cell wall) that reach values of 10^{-6} to 10^{-8} M (Duverger et al., 2003). The internalization of lectins has been documented in animal cells. For instance, lectins can be toxic to tumours via different mechanisms, which are generally initiated by interaction with specific receptors, glycosylated or not, on the membrane of the cells. After this step, the lectins can be internalized through endocytosis and addressed to different compartments leading to activation of signaling pathways related to cell death (Gabor et al., 2001; Yau et al., 2015). Another example is the plant lectin ricin from *Ricinnus communis*, ranked among the most toxic substances known, able to enter mammalian cells by endocytic mechanisms (Hartley and Lord, 2004). However, different experimental approaches indicate that the uptake of Helja into fungal cells does not occur through an endocytic process. In fact, confocal microscopy images are compatible with the uniform internalization of Helja across the cell surface, a feature that could be related to its ability to permeabilize the plasma membrane, although such a mechanism remains to be explored. Changes in membrane fluidity associated with changes in cell wall mechanical properties (Francois, 2016) or pore formation across the plasma membrane, like the reported for the human C-type lectin could not be discarded (Mukjerhee et al., 2014).

Lectins have been proposed as being part of the plant defense battery although limited experimental evidence is available. Here we show Helja exerts an antifungal effect on *S. sclerotiorum* that involves the interaction with the cell surface, the permeabilization of the plasma membrane, the internalization in the intracellular space and the production of oxidative stress, finally leading to the cell death. Even though the occurrence of multiple Helja's putative targets in the host cell might be speculated on the base of the sugar affinity of the lectin, additional experimentation is required to determine which of the above mentioned factors is the most important to kill the fungal cell. Interestingly, the antifungal action of Helja was selective on a natural sunflower pathogen, contributing to the possible function in the perception and response of the plant towards pathogens attack. Our results provided mechanistic insights of Helja, opening up novel opportunities to understand the mode of action of a reduced group of antifungal lectins.

5. Conclusion

Conclusive evidence on the Helja antifungal activity towards the sunflower phytopathogenic fungus *Sclerotinia sclerotiorum* is presented. We demonstrate its ability to interact with fungal spores, permeabilize the plasma membrane, enter into intracellular space, produce reactive oxygen species and finally kill the fungal cells. We further show the selective action of Helja, since the lectin is inactive towards *Fusarium solani,* a non-pathogen of sunflower. Our findings suggest the participation of Helja in the perception and defense of the plant against the fungal attack.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

MDR: peformed the experiments and designed the figures.
LDLC: participated in data interpretation and manuscript revision.
MP: participated in data interpretation and manuscript revision.
MR: conceived the idea, designed and supervised the experiments and wrote the manuscript.

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