



Responses of *Sporothrix globosa* to the cell wall perturbing agents Congo Red and Calcofluor White

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Abstract It is well documented that disturbance of cell surface by some agents triggers compensatory responses aimed to maintain the cell wall integrity in fungi and other organisms. Here, the thermomorphogenic fungus *Sporothrix globosa*, a member of the pathogenic clade of the *Sporothrix* complex, was propagated in yeast–peptone–dextrose medium under conditions to obtain the mycelium (pH 4.5, 27–28 °C) or the yeast (pH 7.8, 32–34 °C) morphotypes in the absence and presence of the wall-interacting dyes Congo Red (CR) and Calcofluor White (CFW) either alone or in combination. After different periods of time, growth, cell morphology and activity of glucosamine-6-phosphate synthase (GlcN-6-P synthase), an ubiquitous enzyme that plays a crucial role in cell wall biogenesis, were determined. CR and to a lower extent CFW affected growth and morphology of both fungal morphotypes and significantly increased enzyme activity. Notoriously, CR or CR in combination with CFW induced the transient conversion of yeasts into conidia-forming filamentous cells even under culture conditions adjusted for yeast development, most likely as a strategy to evade the

noxious effect of the dye. After sometime, hypha returned to yeast cells. An hypothetical model to explain the effect of CR on morphology and enzyme activity based on the possible role of membrane-spanning proteins known as mechanosensors is proposed. Results are discussed in terms of the fungal responses to cell wall damage.

Keywords *S. globosa* · Dimorphism · Congo Red · Calcofluor White · Cell wall · GlcN-6-P synthase

Introduction

Sporothrix globosa is one of the four pathogenic species associated to the pathogenic clade *Sporothrix schenckii* complex, altogether with *S. schenckii* sensu stricto, *S. brasiliensis* and *S. luriei* that cause sporotrichosis, a human mycosis of low fatality of humans and some animals (Romeo and Criseo 2013; Zhang et al. 2015; Huang et al. 2016; Lopes-Bezerra et al. 2018). These species exhibit differences in terms of virulence, clinical manifestation of the disease, source of infection, transmission and geographic distribution (Lopes-Bezerra et al. 2018) and are dimorphic organisms whose morphology is controlled by temperature, pH and other factors. Accordingly, at 25–28 °C and an acidic pH they grow as mycelium or saprophytic phase whereas at 32–35 °C and at a basic pH they grow as

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yeast cells, which is the morphotype isolated from infected tissues (Rodríguez-del-Valle et al. 1983; Resto and Rodríguez-del-Valle 1988; López-Romero et al. 2011; Lopes-Bezerra et al. 2018).

The fungal cell wall (CW) is a dynamic structure that adapts to changes in growth conditions and can be remodeled during morphogenetic processes such as mating, sporulation and morphological transition (Orlean 1997; Smits et al. 1999; Molina et al. 2000). The adapting behavior of the cell to environmental stress largely depends on compensatory mechanisms that result in changes in the chemical composition and/or structure of the CW (Kapteyn et al. 1997; Popolo et al. 2001; Klis et al. 2002). Most of our knowledge of the molecular basis of these responses comes from studies carried out in five mutants of *Saccharomyces cerevisiae* affected in the construction of the CW. Results demonstrated that these responses depend on complex regulatory systems. Accordingly, about 10% (33 out of 290) of the differentially expressed genes in the mutants were involved in the remodeling of the CW and some were related to chitin synthesis such as *GFA1*, *AGM1* and *CHS3* (Levin and Jung 1999; Lagorce et al. 2002, 2003). In the same line, treatment of *S. cerevisiae* with CR and Zymolyase triggered the regulation of numerous genes implicated in CW synthesis and degradation (García et al. 2004). Further studies demonstrated that some of these genes are under the control of a phosphorylation cascade involving protein kinase C (Arellano et al. 1999; Heinisch et al. 1999; Katayama et al. 1999; Calonge et al. 2000; Valdivia and Schekman 2003; Ichinomiya et al. 2007).

The fluorescent brightener CFW and the dye CR are commonly used to experimentally disturb the cell surface as they interact with various polysaccharides of the CW (Wood 1980), preferentially with chitin and cellulose (Haigler et al. 1980; Herth 1980). This results in a severe alteration of the CW and cell morphology (Bartnicki-Garcia et al. 1994; Bermejo et al. 2010). Furthermore, it has been shown that these agents inhibit β -glucan and chitin synthases in several fungi (Selitrennikoff 1984; Roncero and Durán 1985; Vermeulen and Wessels 1986) whereas chitin synthase from *Neurospora crassa* is not affected by CR (Selitrennikoff 1984). In vivo, CR and CFW stimulate chitin and glucan formation in *Saprolegnia monoica* (Fèvre et al. 1990; Nodet et al. 1990a, b) and *Geotrichum lactis* (Roncero and Durán 1985). In other

studies, CFW has been used to investigate septum formation by fluorescence microscopy (Ribas and Cortés 2016) and, in combination with other fungal inhibitors, in the treatment of mycoses produced by *Trichophyton rubrum* and *Candida albicans* (Kingsbury et al. 2012). Also, the chitin-binding ability of CR has been useful for the classification of ascomycete yeasts (Linder 2018) and for live-cell imaging of filamentous fungi (Lichius and Zeilinger 2019).

Recently, we demonstrated that CR affects growth, morphology and activity of GlcN-6-P synthase of *S. schenckii* (Sánchez-López et al. 2019). Accordingly, under conditions of yeast development, 15 μ M CR drastically inhibited conidia (CN) germination; notoriously, when CR was added to yeast cells pre-grown for 6–8 days in its absence, these rapidly converted into filamentous cells. In contrast, under conditions adjusted to obtain the mycelial form, CR up to 150 μ M did not affect CN germination but produced filamentous cells underwent several structural changes including the lost of polarity and the presence of internal CR-pigmented structures as observed by optical and transmission electron microscopies. Moreover, when added to a 16 h-old mycelium culture, CR induced a significant and transient increase in the activity of GlcN-6-P synthase, an ubiquitous enzyme whose activity has been detected in a vast diversity of organisms including bacteria and fungi (Milewski 2002; González-Ibarra et al. 2010) where it plays a crucial role in the biosynthesis of a plethora of biomacromolecules including chitin and other polysaccharides. The enzyme has been purified and characterized from *S. schenckii* both in its native (González-Ibarra et al. 2010) and recombinant (Sánchez-López et al. 2015) forms.

On this background, we considered it important to analyze these responses in more detail and extend our previous studies to *S. globosa*, a pathogenic member of the *S. schenckii* complex that exhibits a number of differences with the other three species, by examining the effect of CR and CFW, either alone or in combination, on essentially the same parameters investigated earlier.

Materials and methods

Organism and culture conditions

The EH230 strain of *Sporothrix globosa*, code number *BMFM-UNAM WDCM834*, was used in this study. It was kindly provided by Prof. Conchita Toriello, Facultad de Medicina, Universidad Nacional Autónoma de México (UNAM). The effect of CR and CFW was investigated on both the mycelium morphotype (MM) and the yeast (YM) morphotype which were propagated using conidia (CN) or vegetative yeast cells as inocula, respectively. In the first case, Petri dishes containing solid YPD medium (yeast extract, 1%; peptone, 2%; glucose, 2%; agar, 2%; pH 4.5) were inoculated with the organism and incubated at 27–28 °C. After 6 days, CN were harvested with sterile deionized water (SDW), washed by low speed centrifugation, resuspended in sterile deionized water (SDW) and counted. Fresh CN were used to inoculate Erlenmeyer flasks containing liquid YPD, pH 4.5, at a final cell density of 1×10^6 cells/ml and incubated at 27–28 °C with shaking. To propagate the YM, yeast vegetative cells were prepared by growing yeast cells at 32–34 °C for 4 days and adjusting the OD_{600nm} to 0.5. Prior to inoculation, the yeast suspension was filtered in a Millipore unit and mycelium-free cells were inoculated in flasks containing YPD, pH 7.8, at the same cell density (1×10^6 cells/ml) and incubated at 32–34 °C with shaking. In both cases, the volume of medium and time of incubation varied depending on the experimental needs.

Assay of susceptibility of MM and YM to CR and CFW

To test the susceptibility of the mycelium to the dyes, CN were obtained as described above, resuspended in SDW and OD_{600nm} was determined. The CN suspension was diluted with SDW to adjust the cell density to an OD_{600nm} of 0.5. Ten-ml aliquots of this suspension were placed in 50-ml Falcon tubes and were mixed with increasing concentrations of CR (0, 75, 150 and 300 μ M), CFW (0, 0.5, 1 and 2%) or a mixture of CFW + CR: 0.5% + 75 μ M, 1% + 150 μ M, 2% + 300 μ M. Flasks were incubated at 27 °C with shaking. After 4 h, OD_{600nm} was determined, one ml-aliquots of samples were diluted with SDW to obtain an OD_{600nm} of 0.5, centrifuged at $10,000 \times g$ for 5 min

to eliminate the dyes and the cell pellet was resuspended in one ml of SDW. Using 96-well ELISA plates (Thermo Fisher Scientific), 10^0 – 10^{-5} ten-fold exponential dilutions were prepared by sequentially mixing 20 ml of sample and mock cells with 180 ml of SDW. Drops (5 ml) of each dilution were then inoculated in Petri dishes containing solid YPD medium and incubated at 27 °C. Growth was inspected after 24 h. Essentially the same protocol was used in the case of the YM, except that growth conditions were set to obtain this morphology and concentrations of CR and CW were varied as follows: CR (0 to 30 μ M), CFW (0 to 2%) and CFW + CR (0.5% + 5 μ M, 1% + 15 μ M and 2.5% + 30 μ M).

Effect of CR and CFW on mycelium and yeast morphology

The effect of perturbing agents on both morphotypes was observed by brightfield and epifluorescence microscopies. Accordingly, for the MM, 125-ml Erlenmeyer flasks containing 40 ml of YPD medium, pH 4.5, were inoculated with fresh CN at a final cell density of 1×10^6 cells/ml in the absence or presence of 150 μ M CR, 1% CFW or a combination of 150 μ M CR and 1% CFW. Flasks were shaken at 27 °C and after different intervals for a total experimental time of 24 h, aliquots were taken and observed by brightfield (control and CR-treated cells) and epifluorescence (CFW and CFW + CR-treated cells; Leica DMLS coupled to the AxioCam ICc 1 ZEISS camera) microscopies. Essentially the same protocol was used for the YM except that growth conditions were adjusted to obtain this morphology and CR and CFW concentrations were 15 μ M and 1%, respectively. Fungal morphology was inspected every 24 h during 8 days as described above for the MM.

Assay of GlcN-6-P synthase activity

One-liter Erlenmeyer flasks containing 300 ml of YPD medium were inoculated with either CN or vegetative yeast cells and grown in the conditions to obtain mycelium and yeast cells, respectively, in the absence or presence of 150 μ M CR or 1% CFW for mycelium and 15 μ M CR or 1% CFW for yeast cells. After different times of incubation for a total experimental period of 24 h (mycelium) or 6 days (yeast), cells were collected by vacuum filtration in a Millipore

unit (mycelium) or by centrifugation for 5 min at $2800\times g$ (yeasts). Cells were washed thrice with 25 mM potassium phosphate, pH 7.0, containing 1 mM EDTA, 1 mM DTT and 1 mM PMSF (buffer A) and resuspended in 25 ml of the same buffer. Cells were disrupted mechanically with glass beads (0.45–0.50 mm diameter) in a MSK cell homogenizer (Braun Melsungen, Germany) cooled by a stream of liquid CO_2 by alternate two 3-min periods of breakage and one-min cooling to complete 6 min of breakage. Cell disruption (over 90%) was assessed by phase contrast microscopy. The homogenate was centrifuged at $3000\times g$ for 15 min, the pellet was resuspended in buffer B (same as buffer A but without PMSF), centrifuged again in the same conditions and washed sequentially with Milli-Q water and buffer B until a clear supernatant was obtained. The pellet, containing CW's, unbroken cells and debris, was discarded and the supernatant was carefully decanted and further centrifuged at $100,000\times g$ for 1 h. The pellet was discarded and the supernatant, labeled as $100,000\text{ g}$ or S_{100} fraction, was carefully aspirated with a Pasteur pipette and used freshly to assay GlcN-6-P synthase activity by the modified Morgan-Elson method as previously described (González-Ibarra et al. 2010). The concentration of GlcN-6-P was calculated from a standard curve prepared with pure GlcN-6-P. One unit of the enzyme was defined as the amount that catalyzed the formation of one nmol of GlcN-6-P in one min of incubation at $30\text{ }^\circ\text{C}$ and at pH 7.0. Specific activity was expressed as units per mg of protein.

Statistics

Data of enzyme activity and cell counting were obtained from three and two independent experiments with mycelium and yeast, respectively and assays of activity and protein were carried out in triplicate. Data were processed to obtain the area under the curve (AUC) using the MATLAB version 8.4 (R14) software. Then, One-Way ANOVA followed by a Dunnett post hoc with a 0.05 alpha was carried out.

Results

Susceptibility of the mycelium and yeast morphotypes to CFW and CR

To determine the most appropriate concentrations of CFW and CR to be used in this study, their effect on viability of both morphotypes was investigated as described in the Materials and methods. Cell viability of mycelium cells was not affected by 0.5% CFW whereas at 1% and 2% discrete and low inhibitions, respectively, were observed (Fig. 1a). On the other hand, the mycelium of *S. globosa* was resistant to $75\text{ }\mu\text{M}$ CR whereas at $150\text{ }\mu\text{M}$ viability was affected though it was not lethal (Fig. 1b). Exposure of cells to a mixture of 1% CFW and $150\text{ }\mu\text{M}$ CR did not affect cell viability in contrast with the other CFW and CR combinations: 0.5% + $75\text{ }\mu\text{M}$ and 2% + $300\text{ }\mu\text{M}$ (Fig. 1c). Viability of yeast cells in the presence of 0.5, 1 and 2% CFW was either not affected, slightly reduced or strongly inhibited, respectively (Fig. 2a). At $5\text{ }\mu\text{M}$, CR did not alter viability whereas at 15 and $30\text{ }\mu\text{M}$ it reduced growth and was lethal, respectively (Fig. 2b). Exposure of yeast cells to a mixture containing 1% CFW and $15\text{ }\mu\text{M}$ CR reduced viability without being lethal as observed with higher concentrations (Fig. 2c). Based on these results, 1% CFW and $150\text{ }\mu\text{M}$ CR, either alone or in combination, were used in experiments carried out with the MM. Corresponding concentrations were 1% and $15\text{ }\mu\text{M}$ for the YM.

Effect of CFW and CR on growth of the mycelium and yeast morphotypes

Growth rate of the MM in liquid medium in the absence and presence of CW and CR is depicted in Fig. 3. In the presence of 1% CFW, growth of mycelia, as measured as mg dry weight, exhibited an apparently similar kinetics to that of the mock culture. However, both CR and the mixture of CR and CFW notoriously reduced growth after 4 and 8 h of incubation. Thereafter, growth was partially recovered under both conditions but without reaching that of the control (Fig. 3a). Statistics analysis of data carried out by comparing the AUC values calculated for all four conditions revealed that inhibition of growth by CFW was, however, slightly significant as compared with the control culture whereas that by CR and the mixture was highly significant (Fig. 3b).

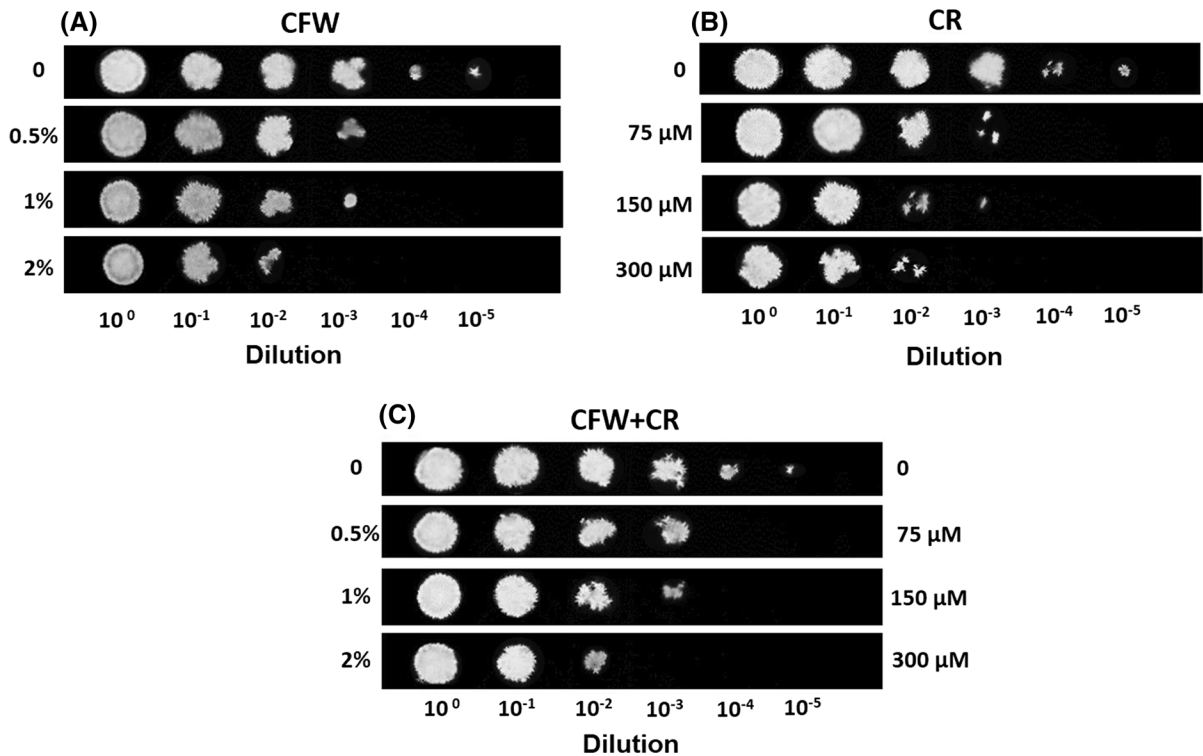


Fig. 1 Susceptibility of the MM to CFW and CR. The assay was carried out at the indicated concentrations of the dyes and fungal growth was measured by the dilution-drop method in ELISA plates as described in the text

The effect of dyes on growth of the YM is illustrated in Fig. 4a. It was observed that CFW reduced yeast growth to a significant level as compared with the control culture after one h of incubation and thereafter; in contrast, CR and particularly the mixture were clearly more inhibitory reducing the number of cells to about 50% and 92% after one day of incubation, respectively. Observation of yeast cultures incubated with CR and the mixture revealed the presence of numerous CN resulting from conidiogenic mycelial cells. Therefore, we considered it important to evaluate fungal growth by counting the number of CN. Results are illustrated in Fig. 4b. As expected, growth curves obtained after the different conditions were closely the inverse of those shown in Fig. 4a. In other words, control and CFW-containing cultures failed to form CN up to 3 h of incubation; thereafter, the amount of CN started to increase slowly up to the end of the experimental period. On the contrary, cultures incubated with CR or the mixture gave rise to high amounts of CN derived from mycelia formed even under conditions for yeast development. It is

worth noting that after 4 h of incubation in the presence of CR but not of the mixture, the amount of CN started to decline reaching values close to those of control and CFW-containing cultures. Statistics analyses using the AUC values are given in Fig. 4c, d for data presented in Fig. 4a, b, respectively. Growth of the YM was significantly affected by the combined and individual dyes in an increasing order of effectiveness of CFW > CR > CFW + CR with respect to control (Fig. 4c). Inversely, formation of mycelia and thereby CN counts, followed an increasing order of magnitude of CFW > CR > CFW + CR with respect to control (Fig. 4d).

Morphological changes induced by CFW and CR

The effect of dyes on the morphology of the MM and YM was analyzed by brightfield and epifluorescence microscopies as described in the text. In the first case, the most notorious effects were observed after 4 days of incubation (Fig. 5). Accordingly, regular septate hyphae were observed in control culture whereas in

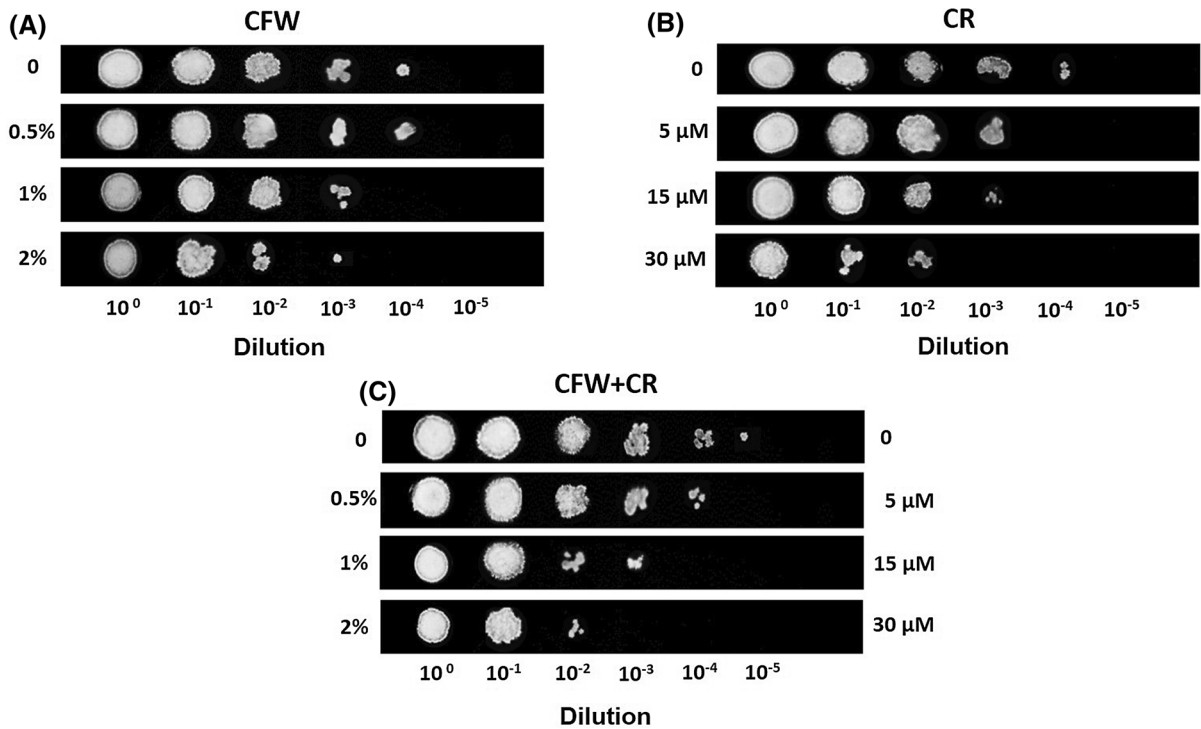


Fig. 2 Susceptibility of the YM to CFW and CR. The assay was carried out at the indicated concentrations of the dyes and fungal growth was measured by the dilution-drop method in ELISA plates as described in the text

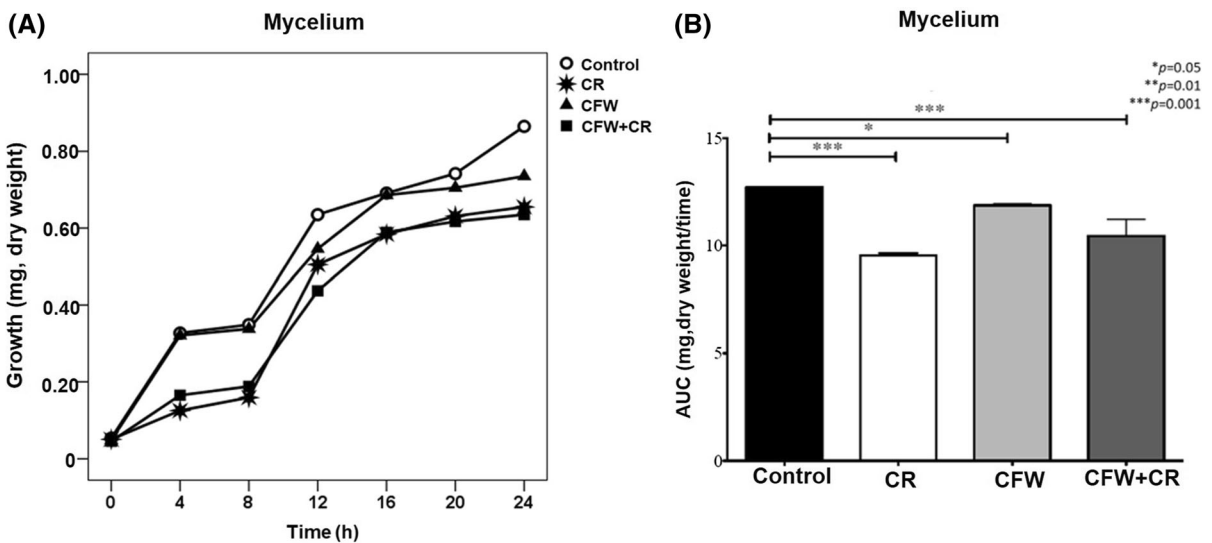


Fig. 3 Influence of CFW and CR on growth of the MM of *S. globosa*. Fungal growth was measured after the indicated times of incubation of CN in the medium and in the conditions to obtain mycelium cells in the absence and presence of the dyes either alone or combined at the optima concentrations

determined in the experiment described in Fig. 1. The statistics analysis of the mycelial growth using the values obtained from the area under the curve (AUC) for each growth condition is shown in (b)

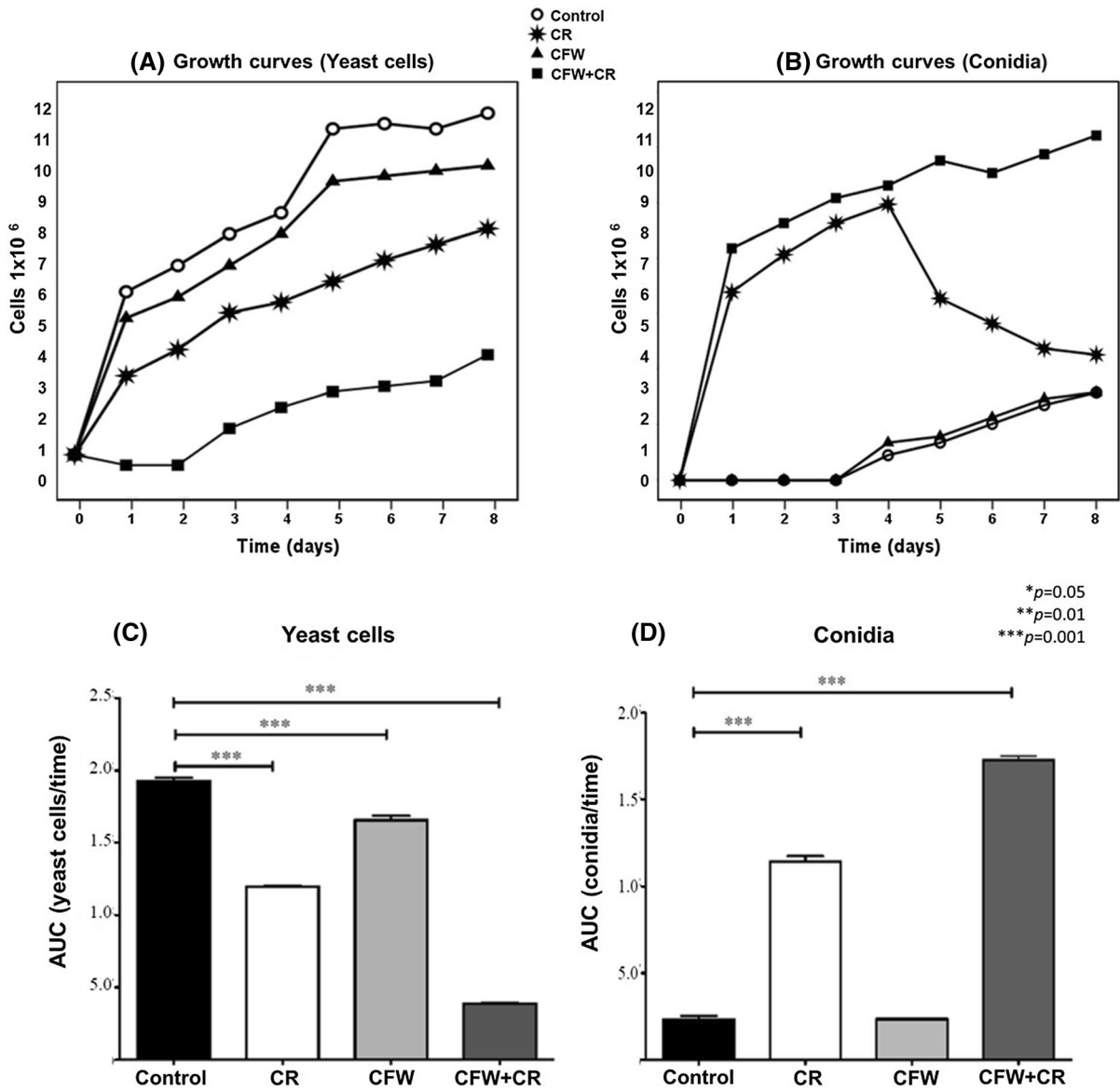


Fig. 4 Influence of CFW and CR on growth of the YM of *S. globosa*. Fungal growth was measured by counting the number of yeast cells (a) or CN (b) after the indicated times of propagation of vegetative yeast cells in the medium and in the conditions to obtain yeast cells in the absence and presence of

the dyes either alone or combined at the optima concentrations determined as described in Fig. 2. The statistics analyses of growth data shown in (a, b) were carried out by calculating the area under the curve (AUC) for each condition and are presented in (c, d), respectively

those incubated with CR, T-shaped cells and globose structures appeared close to red-pigmented deposits (arrows) presumptively corresponding to recruited and segregated CR. On its part, CFW accumulated mainly on the hyphal apex and a similar deposition was observed when it was used in combination with CR. After 8 h, T-shaped cells and minor amounts of red-pigmented deposits still appeared in the presence of

CR and septa partitions were occasionally seen. CFW, either alone or combined with CR, remained positioned mainly in the hyphal, globose region.

The effect of dyes on the YM within a 6-day period of incubation is illustrated in Fig. 6. Accordingly, after the first day of incubation, CR induced a noticeable transition of yeasts to conidiogenic hypha decorated with bunches of sympodial- or daisy-shaped CN.

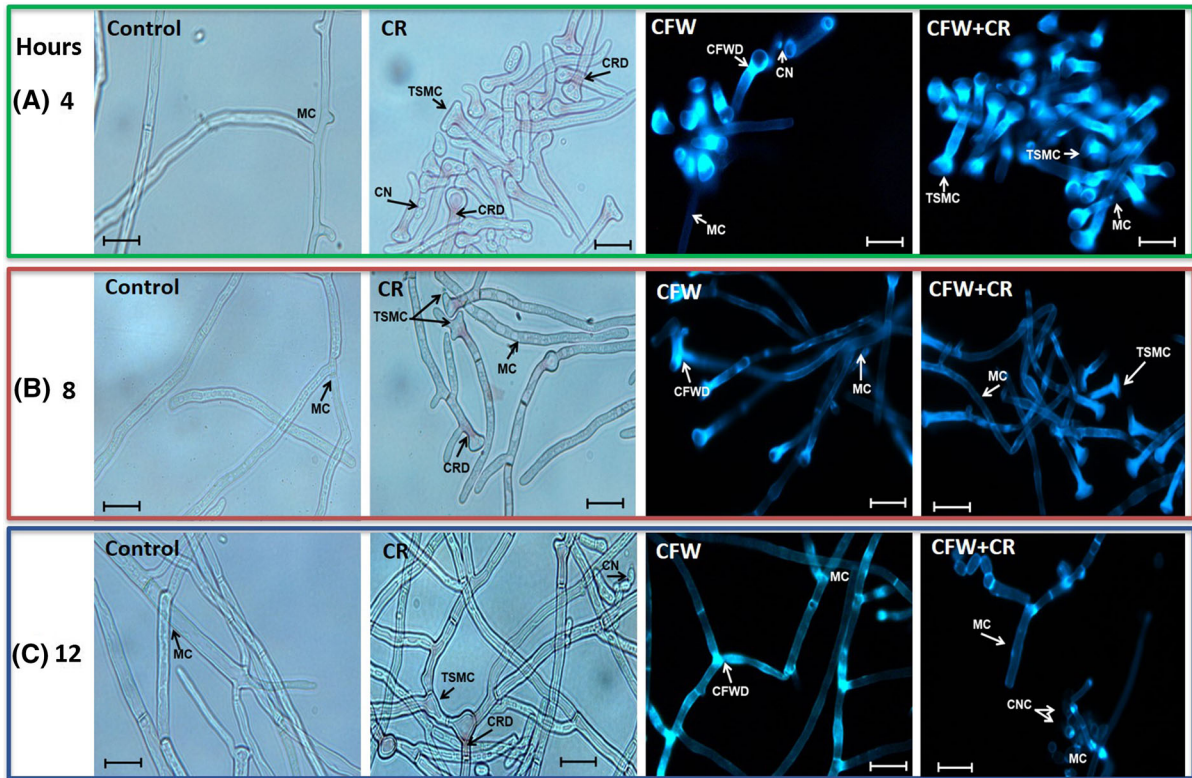


Fig. 5 Effect of CFW and CR on the morphology of the MM of *S. globosa*. Fresh CN were incubated in the medium and in the conditions of pH and temperature to obtain mycelium cells in the absence and presence of the dyes either alone or in combination. After the indicated times, a sample of the cultures

was observed by brightfield and epifluorescence microscopies. *CN* Conidia; *MC* Mycelium cell; *TSMC* T-shaped mycelium cell; *CRD* CR deposit; *CFWD* CFW deposit; *CNC* CN clusters. Bar, 20 μm

Secondary or sessile conidia were occasionally seen. By itself, CFW remained slightly and homogeneously distributed on the yeast cell surface without inducing the morphological change. With the mixture, the morphological transition was confirmed and some slightly fluorescent CN were also seen.

After 4 days, the presence of CR gave rise to a complex, heterogeneous population of yeast and filamentous cells in what it seemed to be a regressive phase of mycelia to yeasts. In concordance with this view, a decrease in the number of CN was also noticed. Though CFW failed to induce the morphological transition, some fluorescent structures that may correspond to transition cells were observed in its presence. On the other hand, in response to the combination of dyes, mycelium and yeast cells as well as some CN heterogeneously labeled with CFW were observed.

After 6 days in the presence of CR, cells transitioned back to the YM as inferred from an increase in the

amount of yeast cells and a decrease in the amount of mycelia, presumably after degradation or otherwise conversion of CR into an innocuous product. The image obtained in the presence of CFW confirmed the observation made with CR in terms of the amount of yeasts.

Activity of GlcN-6-P synthase in response to cell surface stress

An important goal of this study was to investigate how the CW stress affected the activity of GlcN-6-P synthase. To this purpose, both mycelium and yeast cells were grown in the absence and presence of the dyes for total periods of 24 h and 6 days, respectively, and at different intervals enzyme activity was measured as described in the text. Results for the MM are depicted in Fig. 7. As shown in Fig. 7a, control cells expressed two clearly separated peaks of activity after

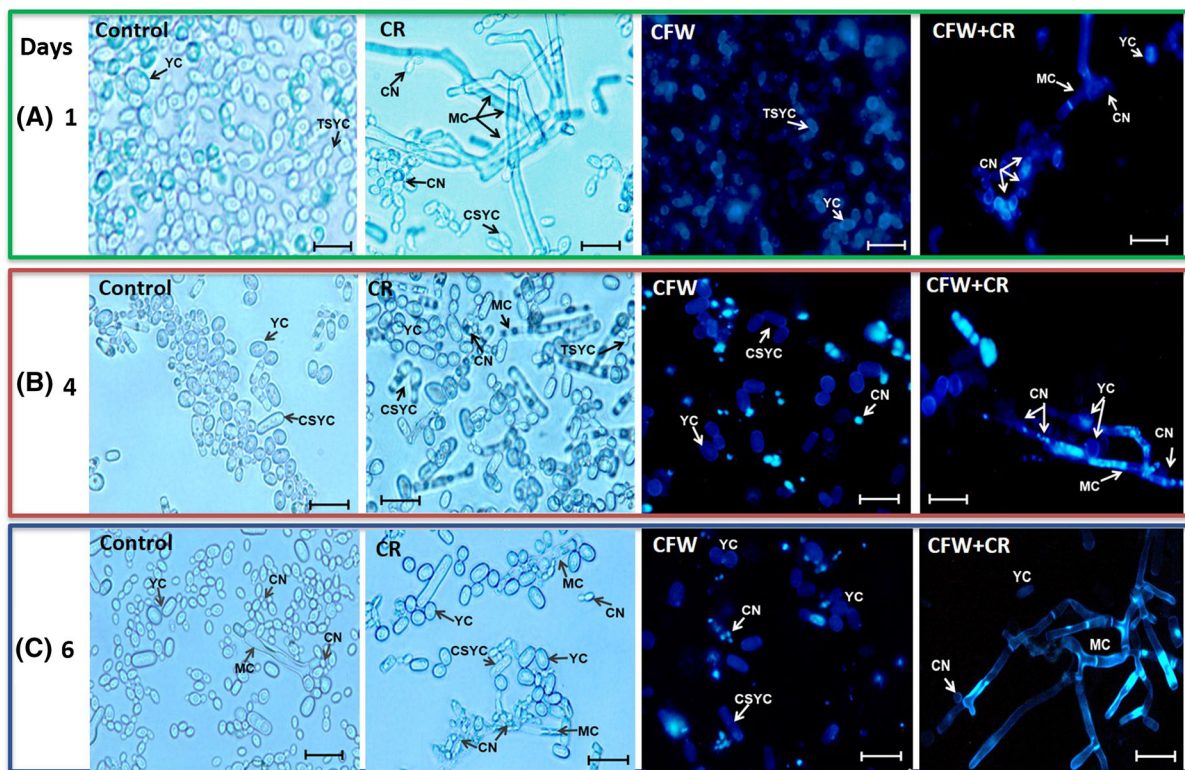


Fig. 6 Effect of CFW and CR on the morphology of the YM of *S. globosa*. Freshly obtained yeast vegetative cells were propagated in the medium and in the conditions of pH and temperature to obtain yeast cells in the absence and presence of the dyes either alone or in combination. After the indicated

times, a sample of the cultures was observed by brightfield and epifluorescence microscopies. CN, Conidia; YC, Yeast cell; MC, Mycelium cell; TSYC, Tear-shaped yeast cell; CSYC, Cigar-shaped yeast cell; Bar, 20 μ m

4 and 16 h of incubation with essentially the same level of specific activity: 5.4 and 5.5 units/mg. In the presence of CR (Fig. 7b), two peaks were also observed but they appeared earlier (2 and 12 h) and exhibited specific activities of 120 and 36 units/mg, i.e., 22- and 6.6-fold higher with respect to non-exposed cells. In the presence of CFW (Fig. 7c), two peaks were also observed after 4 and 16 h of incubation and specific activity values of 6.9 and 6.3 were comparable to those shown in Fig. 7a. In the presence of the mixture (Fig. 7d), the first peak was significantly delayed with respect to the other conditions, appearing after 8 h and with a specific activity of 23 units/mg, that is, much lower than that induced by CR alone. In contrast, instead of the second peak, a plateau within 12 and 16 h followed by a slow decline was observed. Anyway, under these conditions, peak and plateau levels of activity were either fourfold

higher or slightly higher than those shown by peaks in Fig. 7a, respectively.

Statistics analysis of data by calculating the area under the curve (AUC) for each growth condition revealed a highly significant difference ($p = 0.001$) when values obtained with CR and the mixture of CR and CFW were compared with control cells. This difference was less significant ($p = 0.01$) when CFW was used (Fig. 8a). The time (hours) required by the enzyme to reach maximum activity was also calculated for each culture condition. Results revealed that in the presence of CR, the enzyme reached an overpeak after 2 h whereas it required 8 h to do so in control cells, i.e., a highly significant difference ($p = 0.001$). The corresponding time in the case of CW and the mixture was 12 h (Fig. 8b).

The analysis of enzyme activity in yeast cells in response to the dyes unveiled significant differences with respect to mycelium cells. Results are depicted in

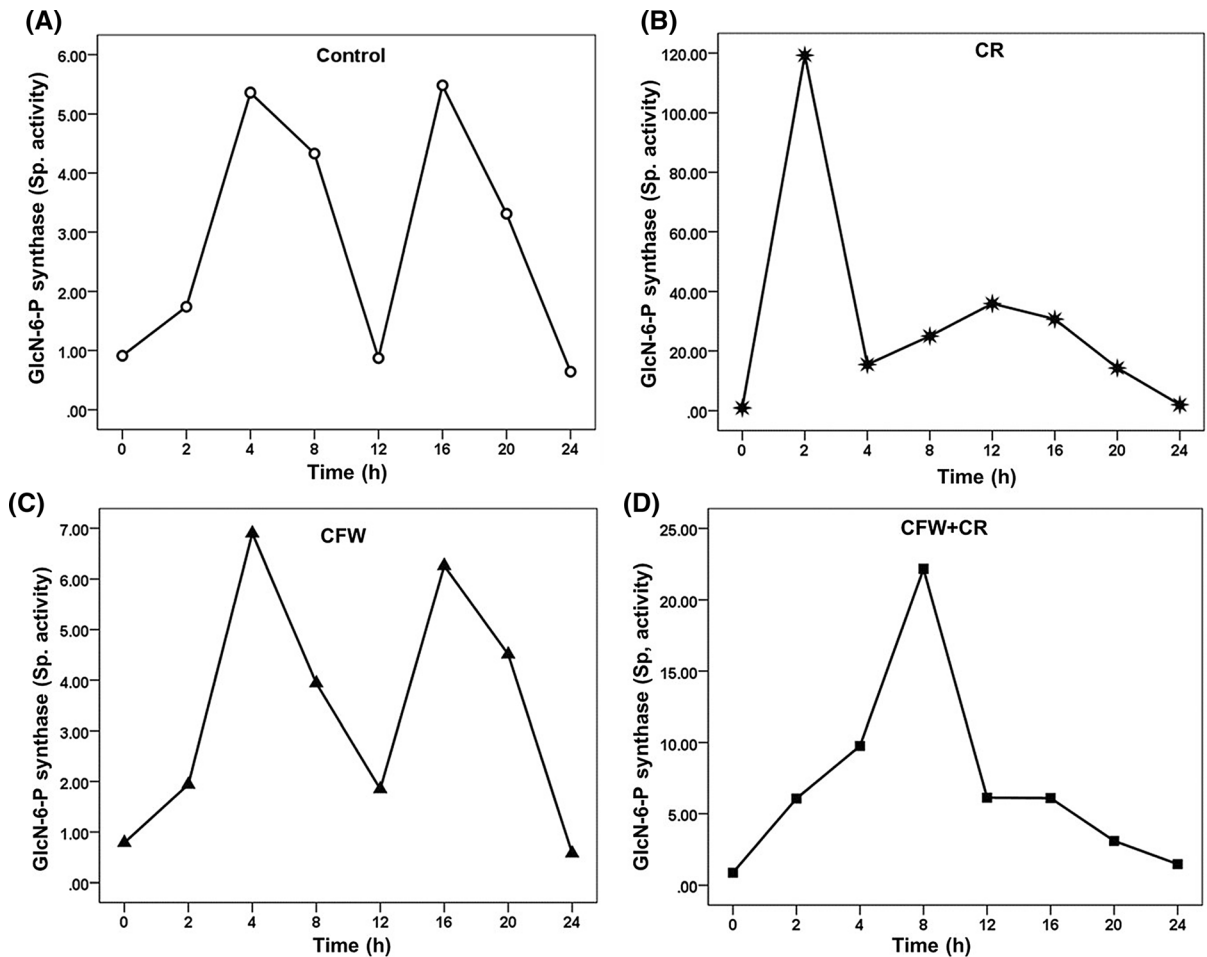


Fig. 7 Influence of CW-perturbing agents on activity of GlcN-6-P synthase from the MM of *S. globosa*. Fresh CN were incubated in the medium and in the conditions of pH and temperature to obtain mycelium cells in the absence and

presence of the dyes either alone or in combination. After the indicated times, cells were harvested, disrupted mechanically with glass beads and processed to measure enzyme activity as described in the text

Fig. 9. As with mycelium cells, control yeast cells also expressed two peaks of activity after 1 and 4 days of incubation with similar values of 2.3 and 2.5 units/mg, respectively, i.e., much lower than control mycelium cells (Fig. 9a). In the presence of CR (Fig. 9b), two peaks were again observed, namely, one after 1 day and a smaller one after 3 days with corresponding activity values of 5.0 and 3.2 units/mg, respectively (Fig. 9b). In response to CFW, cells expressed a peak between days 2 and 3 with a specific activity of 2.5, i.e., very similar to mock cells (Fig. 9c). When used in combination, CR and CFW gave rise to a peak after 2 days with a maximum activity of 4.0. Thereafter, activity slowly decreased reaching a value of 3.5 at the end of the experimental period (Fig. 9d).

Statistics analysis of data by calculating the AUC for each growth condition revealed a highly significant difference ($p = 0.001$) when values obtained with CFW, CR and the mixture of CR and CFW were compared with mock cells (Fig. 10a). The time (hours) required by the enzyme to reach maximum activity was also calculated for each culture condition. Results revealed that in control cells and in those exposed to CR, CW and the mixture, the enzyme reached an overpeak after 3.5, 1, 2.8 and 2.3 days, respectively (Fig. 10b).

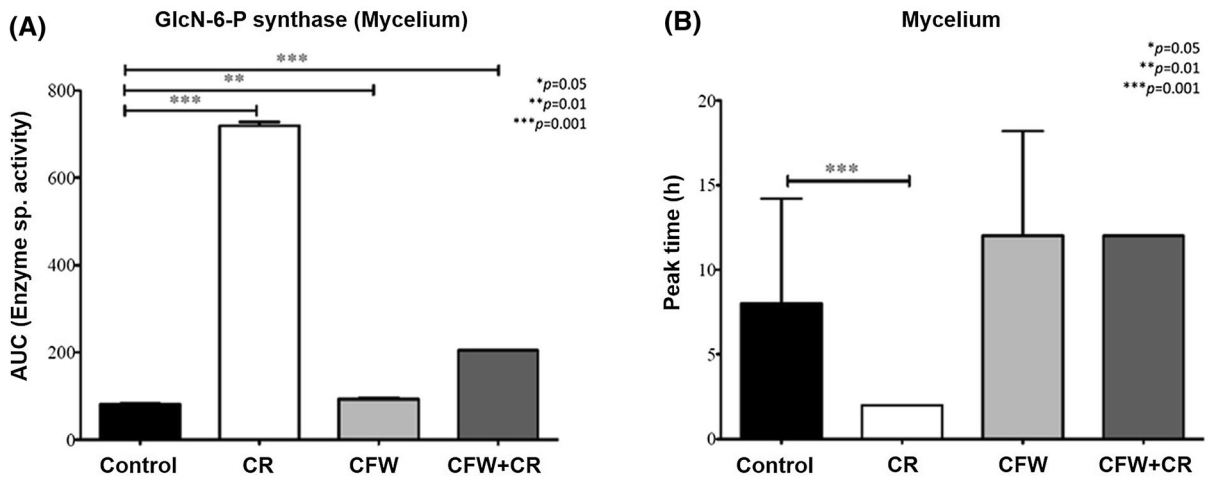


Fig. 8 Statistics analysis of data presented in Fig. 7. Data of enzyme activity for each culture condition were processed by calculating the area under the curve (AUC) (a) and the peak

time, i.e., the time (h) required for the enzyme to reach its maximum activity (b). Significance values (p) are indicated

Discussion

Here we demonstrate that perturbation of the cell surface of *S. globosa* by the CW-interacting agents CR and CFW triggers a number of responses involving growth inhibition, morphological changes and activity of GlcN-6-P synthase, a critical enzyme for CW biosynthesis. It is well documented that CR and CFW interact with β -linked structural polymers such as chitin and cellulose (Roncero and Durán 1985; Kopecká and Gabriel 1992; Kingsbury et al. 2012; Linder 2018). Here, CR and CFW were tested on both the MM and YM either alone or in combination with the idea to see whether synergistic or mutually exclusive effects occurred.

We observed that CR and the CR/CFW combination significantly reduced growth of the MM, particularly after 4 and 8 h, while CFW alone barely reduced it, suggesting that CR was the main effector. The effect of CR observed here is consistent with previous findings in *S. schenckii* whose mycelial morphology was clearly affected by this dye giving rise to the appearance of T-shaped cells, globose structures and an apparent loss of polarity (Sánchez-López et al. 2019).

The mechanism of growth inhibition by these cell stressants is not fully understood. It has been advanced that rigidity imposed to the surface by the binding of CR impairs the cell ability to properly structure the CW (Ram and Klis 2006). Recently, it was proposed

that a cell wall integrity (CWI) pathway mediates the compensatory responses of *S. cerevisiae* to cell surface stress by CR, Zymolyase and SDS. These perturbations are detected by the membrane-resident mechanosensor Mid2 that transduce the signal to a protein phosphorylation cascade and the transcription factor Rlm1, resulting in a strong inhibition of growth (Jiménez-Gutiérrez et al. 2020a, b). The presence and role of a PKC has been demonstrated in *S. schenckii* (Ichinomiya et al. 2007; Aquino-Piñero and Rodríguez-del-Valle 2002) which supports the idea that a similar mechanism may operate in *S. globosa*. Yet, a search for a Mid2 ortholog in *S. globosa* met with failure probably due to the fact that not all genes are still annotated in this organism or simply that another sensor fulfills the role of the yeast Mid2.

Exposure of yeast cells to CR triggered a rapid transition to mycelial cells most probably as a strategy to evade the toxic effect of the dye. This response occurred under culture conditions set for yeast development. Similar results were previously observed in *S. schenckii* (Sánchez-López et al. 2019). Hyphae formed by *S. globosa* under these conditions produced CN that were morphologically indistinguishable from those generated under normal growth conditions. After 4 days in the presence of CR, brightfield microscopy revealed a heterogenous population of cells including yeasts, some mycelium, CN and other unidentified cells probably associated to the process of reversion to yeast cells. After 6 days in the presence of

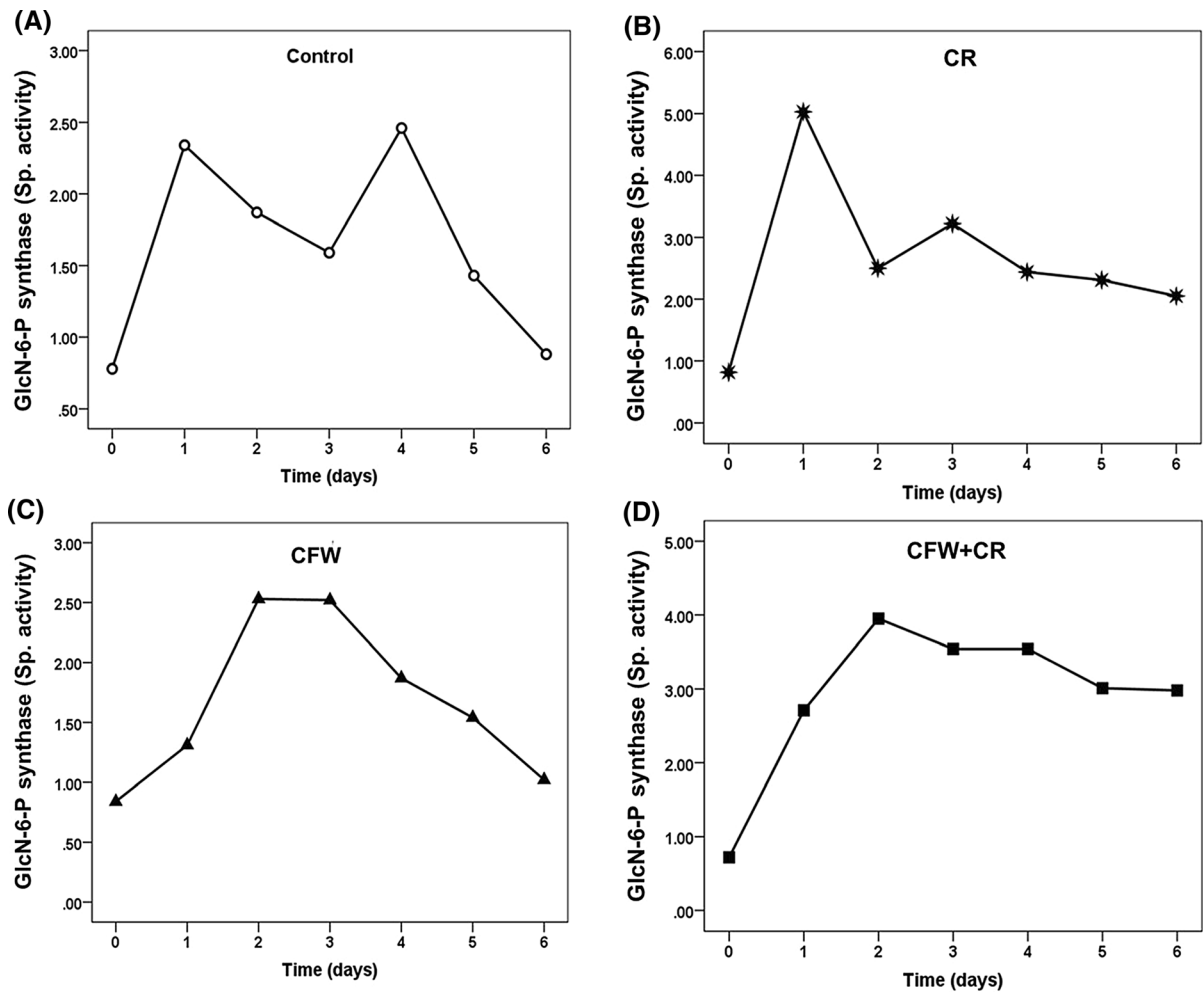


Fig. 9 Influence of CW-perturbing agents on activity of GlcN-6-P synthase from the YM of *S. globosa*. Freshly obtained yeast vegetative cells were propagated in the medium and in the conditions of pH and temperature to obtain yeast cells in the

CR, mycelium, CN and other cells were outnumbered by yeast cells. Microscopy images revealed that CFW induced the yeast-to-mycelium transition to a low extent only, and when it was used in combination with CR, it significantly blocked the conversion of mycelium back to the yeast morphology.

Conversion of yeasts to CN-producing hyphae provided an alternative method to measure growth by counting the number of CN. This resulted in growth curves that were the inverse of those obtained when yeast cells were counted. In other words, the lowest amounts of CN were obtained in control cells and those incubated with CFW whereas the highest counts were obtained in the presence of CR and the mixture,

absence and presence of the dyes either alone or in combination. After the indicated times, cells were harvested, disrupted mechanically with glass beads and processed to measure enzyme activity as described in the text

thus confirming that CFW did not promote conidiation and when added together with CR somehow interfered with the mycelium-to-yeast reversion. Of particular interest was to observe a striking fall in the number of CN after 4 days of incubation in the presence of CR, coinciding well with the time where mycelium induced by CR started to return to yeast cells and the ensuing reduction of CN production.

In a hypothetical model, we propose two alternatives to explain the yeast-to-mycelial transition cells exposed to CR. On the one hand, sensing of the dye by an homolog of one of the five membrane-spanning proteins that function as mechanosensors in *S. cerevisiae* (known as Wsc1, Wsc2, Wsc3, Mid2 and Mtl1)

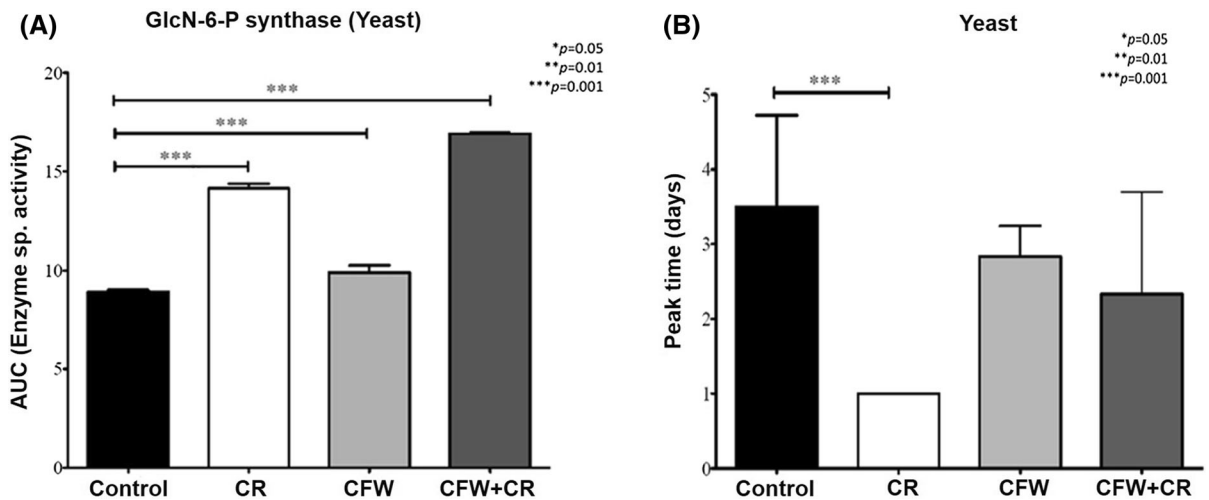


Fig. 10 Statistics analysis of data presented in Fig. 9. Data of enzyme activity for each culture condition were processed by calculating the area under the curve (AUC) (a) and the peak

time, i.e., the time (h) required for the enzyme to reach its maximum activity (b). Significance values (*p*) are indicated

activate an intracellular signalling pathway following cell surface aggression of either the CW or the plasma membrane that ultimately induces the morphological change, as has been proposed for other environmental effectors (Elhasi and Blomberg 2019). This signal transduction cascade is commonly known as the PKC1-SLT1 pathway (Gualtieri et al. 2004). In support of this notion, one of these sensors has been found in *S. schenckii* and *S. brasiliensis* (Teixeira et al. 2015) though not yet in *S. globosa*. On the other hand, the opening of mechanosensitive ion channels that underlies responses to external stimuli in many cell types (Kumamoto 2008) may also occur in *S. globosa* and allow the entrance of CR into the cell. Once inside, CR would trigger the signal for the morphological change. This alternative is sustained by the deposition of CR in the cytoplasm of filamentous cells as observed here by brightfield microscopy and earlier in *S. schenckii* by electron transmission microscopy (Sánchez-López et al. 2019). Failure in this study to observe the dye in the filamentous cells resulting from the yeast shift can be explained by the low amount of CR (15 mM) used for the YM as compared with that of the MM (150 μM).

A major goal of this study was to investigate the effect of cell surface stress on the levels of GlcN-6-P synthase, a catalytically complex enzyme with inherent variations in its functional domains (Gupta et al. 2011) involved in CW biogenesis. It has been

demonstrated that activation of chitin synthesis in CW mutants of *S. cerevisiae* correlates with the increase of GlcN-6-P synthase activity (Lagorce et al. 2002; Ram et al. 2004) and that activation of the enzyme in *Aspergillus niger* results in an augmented deposition of chitin in the CW (Ram et al. 2004). In accordance with these findings, we previously observed that exposure of mycelial cells of *S. schenckii* to 150 μM CR for as short as 3 h resulted in a transient, seven-fold increase in the enzyme activity over control cells (Sánchez-López et al. 2019).

Comparison of sensitivity to inhibition by UDP-N-GlcNAc of purified and S₁₀₀ fractions from *S. schenckii* revealed that the latter are nearly twofold more sensitive (González-Ibarra et al. 2010). Moreover, as in other organisms such as *Candida albicans* (Milewski et al. 1999) and *Blastocladiella emersonii* (Maia 1994), GlcN-6-P synthase from *S. schenckii* is modulated by phosphorylation of the enzyme protein (González-Ibarra et al. 2010). Whether modulation of activity by UDP-N-GlcNAc, phosphorylation and other factors plays a role in enzyme regulation in vivo, remains to be elucidated. It is conceivable that the increase of enzyme activity in *S. globosa* exposed to CR occurs by any of the two mechanisms proposed above to explain the yeast-to-mycelium shift.

The lower activity detected in yeast cells with respect to mycelia as well as the presence of two

enzyme peaks in control cells and some of the other conditions, particularly in the MM, are not easy to explain in simple terms. In the first case, hyphal cells may require a higher amount of UDP-N-GlcNAc to fulfill the need to form diverse structures such as long, septate and branched conidiogenic-hyphae and CN, which are not produced by yeast cells. A plausible explanation for the observed two peaks of enzyme activity is that early during incubation, fungal growth and consequently CW elaboration, depends on a pre-existing pool of aminosugars that once exhausted by the cell biosynthetic processes, it is replenished by the activity associated to the first peak. Later, after longer times of incubation (10–12 h and more), aminosugar and nutrient deprivation may function as metabolic signals that trigger the activation of *GFAI* and other genes connected with CW biogenesis resulting in the second peak.

Heretofore, this is the first report on the morphological transition of a pathogenic dimorphic fungus induced by CW damage. Experiments are in progress to determine the CW composition and structure after fungal exposure to cell surface stressful agents.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The authors confirm that not ethical approval was required as the research in this article involved micro-organisms only.

Consent for publication All authors read and consent to publish this manuscript.

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