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MICROBIOLOGY

Biocontrol mechanisms of the Antarctic yeast *Debaryomyces hansenii* UFT8244 against post-harvest phytopathogenic fungi of strawberries

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Abstract: The use of yeasts has been explored as an efficient alternative to fungicide application in the treatment and prevention of post-harvest fruit deterioration. Here, we evaluated the biocontrol abilities of the Antarctic yeast strain *Debaryomyces hansenii* UFT8244 against the post-harvest phytopathogenic fungi *Botrytis cinerea* and *Rhizopus stolonifer* for the protection and preservation of strawberry fruit. The strongest inhibition of germination of *B. cinerea* (57%) was observed at 0 °C, followed by 40% at 25 °C. In addition, germ tubes and hyphae of *B. cinerea* were strongly surrounded and colonized by *D. hansenii*. Production of the enzymes β-1,3-glucanase, chitinase and protease by *D. hansenii* was detected in the presence of phytopathogenic fungus cell walls. The activity of β-1,3-glucanase was highest on day 12 of incubation and remained high until day 15. Chitinase and protease activities reached their highest levels on the day 15 of incubation. *D. hansenii* additionally demonstrated the ability to resist oxidative stress. Our data demonstrated that the main biocontrol mechanisms displayed by *D. hansenii* were the control of phytopathogenic fungal spore germination, production of antifungal enzymes and resistance to oxidative stress. We conclude that isolate *D. hansenii* UFT8422 should be further investigated for use at commercial scales at low temperatures.

Key words: Antagonistic yeasts, biological control, phytopathogen, post-harvest disease.

INTRODUCTION

Strawberry is a valuable commercial fruit highly susceptible to post-harvest fungal diseases, with rotting caused by a wide range of phytopathogens. Soft rot caused by *Rhizopus stolonifer*, and gray mold caused by *Botrytis cinerea*, are two primary causative agents of post-harvest diseases of strawberry and can cause considerable economic loss (Feliziani & Romanazzi 2016, Zhao et al. 2022, Ferreira et al. 2023). Conventionally, synthetic fungicides are applied for post-harvest disease control as a standard business practice worldwide (Ons et al. 2020). However, with growing global concerns about the negative effects of fungicide residues, and the development of phytopathogen resistance to these products, there is increasing pressure to explore alternative approaches to control these fungal diseases and maintain fruit quality through the commercial chain (Ons et al. 2020, Hernandez-Montiel et al. 2021, Ferreira et al. 2023).

Biological control is now being explored as a potentially efficient alternative to the use of synthetic fungicides for the treatment and prevention of post-harvest fruit deterioration. Several yeast species have been reported to show positive effects in the control of the most diverse rots (Freimoser et al. 2019,

Hernandez-Montiel et al. 2021). However, the application of biological control to fruit that are widely stored under refrigeration, such as strawberries, remains limited, and the use of cold-adapted yeast strains is largely unexplored. The limited literature available pertaining to the use of psychrotolerant yeasts isolated from cold environments in biocontrol indicates that they can be effective for fruit stored under low temperature conditions, as illustrated in studies of apple (Vero et al. 2013, Arrarte et al. 2017), cherry tomato (Hu et al. 2017) and pear (Lutz et al. 2020) storage.

The mechanisms of action expressed by yeasts in biological control are key to the inhibition of phytopathogens (Pimenta et al. 2009, Janisiewicz et al. 2011, Droby et al. 2016). These include antibiosis, predation, mycoparasitism, lytic enzyme production, induced resistance, competition for space and nutrients, and tolerance to oxidative stress (Spadaro & Droby 2016). Improved knowledge of the mechanisms expressed by yeasts is essential for the identification and development of biological control agents and can provide a powerful tool for understanding the complex pathogen-host-antagonist interactions (Spadaro & Droby 2016).

The yeast isolate *Debaryomyces hansenii* UFT8244 was originally obtained from biofilm samples in Antarctic meltwater streams (Ferreira et al. 2019). The yeast has been confirmed as an antagonist with potential as a biocontrol agent due to its strong inhibitory activity against the fungus *Botrytis cinerea* (Ferreira et al. 2023). The present study aimed to more thoroughly characterize this yeast strain in terms of its potential mechanisms of activity against the fungi *B. cinerea* and *Rhizopus stolonifer* in the post-harvest protection of strawberry fruit.

MATERIALS AND METHODS

Target pathogens

The fungus *Botrytis cinerea* was obtained from the culture collection of the Biotechnology Laboratory of the Department of Biosciences, School of Chemistry, University of the Republic of Uruguay. It was reactivated on potato dextrose agar medium (PDA, Difco) at 25 °C for 5 days and then maintained at 0 °C. *Rhizopus stolonifer* was previously isolated from strawberry fruit that presented symptoms of soft rot disease and was characterized morphologically and using molecular approaches. It was grown on PDA at 25 °C for 2 days.

Yeast

The isolate *Debaryomyces hansenii* UFT8244 was originally obtained from Antarctic biofilm samples in a meltwater stream and identified by sequencing of the D1/D2 region of the 26S ribosomal gene (Ferreira et al. 2019). It was cultivated in Sabouraud Dextrose Agar (Kasvi, Brazil), at 25 °C for 2 days.

In vitro studies

Effect of diffusible and volatile compounds on phytopathogen growth

The ability to inhibit phytopathogen growth was tested based on the evaluation of the production of diffusible substances in Sabouraud Dextrose Agar (Kasvi, Brazil) culture medium, as described by Vero et al. (2013). The strain *D. hansenii* UFT8244 was inoculated from a 2-day-old culture in the form of a line on one side of a Petri dish. After 2 d incubation at 25 °C, a 5-mm diameter disk containing mycelia of the fungi *B. cinerea* or *R. stolonifer* was inoculated approximately 30 mm from the antagonist, on the opposite side of the plate. Plates were then incubated at 0 °C for 7 d before evaluation of *B. cinerea* growth, or at 25 °C for 2 days for evaluation of *R.*

stolonifer growth. As a control, a 5mm diameter disk of mycelia of each of the phytopathogens was placed in the center of individual Petri dishes containing Sabouraud Dextrose Agar, in the absence of the yeast. The radial growth of the pathogen in both experimental and control conditions was compared using the formula: Mycelial Growth (%) = [(C- T)/C] × 100, where: C = control and T = treatment. The tests were performed in triplicate.

To determine the volatile antifungal compounds, 50 µL of a suspension with 1×10^7 cells/mL from 2-day-old culture of the strain *D. hansenii* UFT8244 previously determined by Neubauer chamber, were spread evenly using a sterile Drigalski loop in Petri plates containing Sabouraud Dextrose Agar. At the same time, disks (5 mm diameter) of 5-day-old *B. cinerea* culture or 1-day-old *R. stolonifer* culture (both without spores) were inoculated onto other plates containing Sabouraud Dextrose Agar. The lids of all Petri dishes (pathogen and inoculated yeast) were removed. The sets of dishes containing the pathogen and yeast were joined and sealed using two layers of Parafilm® to create a closed chamber (Arrarte et al. 2017). Negative controls consisted of pathogen cultures alone in a closed chamber. The plates were incubated at 0 °C for 7 d for the evaluation of *B. cinerea* growth, and at 25 °C for 2 days to evaluate growth of *R. stolonifer*. The percentage of pathogen growth inhibition achieved by the volatile compounds produced by the yeast was calculated based on the difference in growth of the test colonies compared to that of the control fungal colonies as described above. Treatments were carried out in triplicate. The data obtained were submitted to variance analysis and compared using the Scott-knott test (5% significance level).

Effect on spore germination of phytopathogens

The effect of *D. hansenii* on the germination of *B. cinerea* and *R. stolonifer* spores was evaluated in sterile strawberry juice, adapting the approach described by Vero et al. (2013). Aliquots of 10 µL of spore suspension containing 1×10^6 spores/mL of each pathogen were prepared in sterile distilled water and transferred to 96-well microtiter plates (Abano Terme, Padua, Italy) containing 100 µL of sterile strawberry juice. Ten microlitres of a yeast cell suspension containing 1×10^7 cells/mL were added to each well of the plates, except for the control wells, where only the pathogen spores were inoculated. The assays were performed in triplicate at 0 and 25 °C for *B. cinerea* and at 25 °C for *R. stolonifer*. After 12 h of incubation, the spores were microscopically observed, at 40× magnification, and germination percentage was assessed.

Determination of siderophore production

Siderophore production was evaluated by assay in Petri dishes containing Chrome Azurol Sulfonate (CAS) Agar (Schwyn & Neilands 1987), using Grimm Allen medium as a base (Baakza et al. 2004). *Debaryomyces hansenii* was inoculated from a 2-day-old culture grown at 25 °C onto a section of the plate and incubated at 0 °C for 5 d. After incubation, the presence of siderophores was determined by a color change in the medium from blue to orange around the growing culture. The siderophore-producing yeast *Rhodotorula mucilaginosa* was used as a positive control (Vero et al. 2013).

Determination of biofilm formation capacity

The ability to form biofilm was tested using a 2-day-old yeast culture grown in Sabouraud Dextrose Agar (Kasvi) at 25 °C. The cells were suspended in sterile water to reach a turbidity of McFarland scale value 4, corresponding to a concentration of 1×10^7 cells/mL. Then, 20 µL of the yeast suspension was inoculated into a

96-well microtiter plate (Abano Terme, Padua, Italy) containing 180 µL of sterile strawberry juice, and the plates were incubated for 10 d at 0 °C. Negative controls were included, containing only sterile strawberry juice. After incubation, the wells were emptied, rinsed with sterile water, and dried at room temperature. The adherent biofilm layer was stained using a 1% (w/v) aqueous crystal violet solution for 20 min, and then the wells were again rinsed with sterile water and air dried at room temperature. The bound dye was diluted in each well with 200 µL of a mixture of ethanol (80%) and acetone (20%). The absorbance of each well was then measured at 620 nm. The treatment was repeated three times for each plate. Biofilm formation was considered positive in the wells where the absorbance was greater than the mean of the negative control plus three times the standard deviation (Ruzicka et al. 2007). The averages of three replicates obtained were examined using ANOVA followed, where significant, by Tukey's pairwise *post hoc* tests.

Determination of antifungal enzymes

The production of antifungal enzymes by *D. hansenii* was confirmed by growing the yeast in glass flasks with 5 mL of YNB minimal medium (containing: 0.67% Yeast-Nitrogen Base) (Sigma-Aldrich), supplemented with 1 mg of fungal wall/mL of medium, as described by Hernández-Montiel et al. (2010). These flasks were incubated at 25 °C for 15 d. After 2, 6, 9, 12 and 15 days, a 200 µL aliquot of medium was taken, which was centrifuged at 100 rpm for 5 min. The contents of β-1,3 glucanase, chitinase and protease were then quantified using the supernatant.

β-1,3 glucanase activity

The activity of β-1,3 glucanase was determined by the amount of reducing sugars released from laminarin, using glucose as standard (Vero et al. 2013). Laminarin (1%) (*Laminaria digitata*, Sigma-Aldrich) was used as a substrate. Three microlitres of supernatant were mixed with the same amount of the substrate, then the mixture was incubated at 37 °C for 1 h. After that, 125 µL of 3,5 dinitrosalicylic acid (DNS) was added to each sample and heated in a dry bath for 5 min at 100 °C. Absorbance was then measured using a UV-vis spectrophotometer (DU-640 UV-Vis Beckman Coulter, D.F., Mexico) at 540 nm. One unit (U) of β-1,3 glucanase was defined as 1 mg of reducing sugars released per mg of protein per min. The total protein content was quantified using the method described by Abeles & Forrence (1970). Three replicate analyses were performed.

Chitinase activity

Chitinase activity was determined using a mixture of 90 µL of p-nitrophenyl N-acetyl-β-D-glucosamine with 10 µL of the sample supernatant, incubated at 40 °C for 30 min. After that, the reaction was interrupted using 10 µl of 1M NaOH. The absorbance of the sample was measured using a spectrophotometer (DU-640 UV-Vis Beckman Coulter, D.F., México) at 405 nm. One unit (U) of chitinase was defined as the capacity of the enzyme to release one micromol of p-nitrophenol per mg of protein. Three replicate analyses were performed (Vero et al. 2013).

Protease activity

Protease activity was determined by mixing 100 µL of the sample supernatant with 100 µL of azocaine (1%) and acetate buffer at pH 5, followed by incubation at 40 °C for 30 min. Then, 400 µL of tricycloacetic acid (10%) were added and the samples were centrifuged at 12000 rpm for 15 minutes. From the supernatant, 500 µL were removed, to which 500 µL of NaOH (525 mM) were added. The samples were evaluated using a spectrophotometer (DU-640 UV-Vis

Beckman Coulter, D.F., Mexico) at 442 nm. Ten units (U) of protease activity were defined as the amount that produces an absorbance of 0.1 under the reaction conditions (Gacto et al. 2000, Benitez et al. 2001). Three replicate analyses were performed.

Resistance to oxidative stress

To test oxidative stress tolerance, *D. hansenii* was grown from a 2-day culture on Sabouraud Dextrose Agar. One millilitre of a 1×10^7 cells/mL suspension was prepared in sterile distilled water using a Neubauer chamber. To this suspension H_2O_2 was added to a final concentration of 20 or 40 mM, and stirred at 25 °C at 450 rpm for 1 h. The culture was then centrifuged at 100 rpm, the supernatant discarded, and washed three times with sterile distilled water to remove any residual H_2O_2. The number of viable cells was determined using serial decimal dilutions and 100 µl of each dilution was inoculated onto Sabourand agar plates. The plates were incubated at 25 °C for 2 d and colony forming units (CFU) were counted (Hu et al. 2017). Each treatment consisted of three replicates. The data obtained were subjected to ANOVA followed, where significant by Tukey's *post hoc* pairwise tests.

RESULTS

Effect of diffusible and volatile compounds on phytopathogen growth

No inhibition of phytopathogen growth was observed in the test based on the evaluation of the production of diffusible or volatile compounds against *R. stolonifera*. With the phytopathogen *B. cinerea*, in the test based on the evaluation of the production of volatile substances, the yeast was able to inhibit mycelial growth by 16%, although the result showed a statistically significant inhibition. There was no evidence of inhibition by diffusible compounds.

Effect on spore germination of phytopathogens

Spore germination of both phytopathogens was reduced in the presence of the yeast. Spore germination in *B. cinerea* was reduced by 57% at 0 °C and by 40% at 25 °C. *R. stolonifera* spore germination reduced by 12% at 25 °C. The spores, germ tubes and hyphae of *B. cinerea* were densely surrounded by the yeast cells (Fig. 1).

Determination of siderophore production

No siderophores were produced by *D. hansenii* UFT8244, with no halo apparent around the colony in CAS medium. In contrast, orange halos were observed around the *R. mucilaginosa* colonies that were used as a positive control (Supplementary Material - Fig. S1).

Determination of biofilm formation capacity

The ability to form biofilm was evaluated through the absorbance of wells containing yeast suspension plus sterile strawberry juice or just sterile strawberry juice as a negative control. Values of absorbance and standard deviation of 0.178 ± 0.012 (negative control) and 0.151 ± 0.006 (*D. hansenii* UFT8244) were not significantly different ($p<0.05$) (Tukey's test), indicating no formation of biofilm by *D. hansenii* UFT8244.

Determination of antifungal enzymes

Production of β-1,3-glucanase, chitinase and protease by the yeast was detected in the presence of phytopathogenic fungus cell walls. Fig. 2 shows the levels of each enzyme assayed over the incubation period. The activity of β-1,3-glucanase (Fig. 2a) was greatest on day 12 of incubation and remained high until day 15. Chitinase (Fig. 2b) and protease (Fig. 2c) activities were greatest on day 15 of incubation.

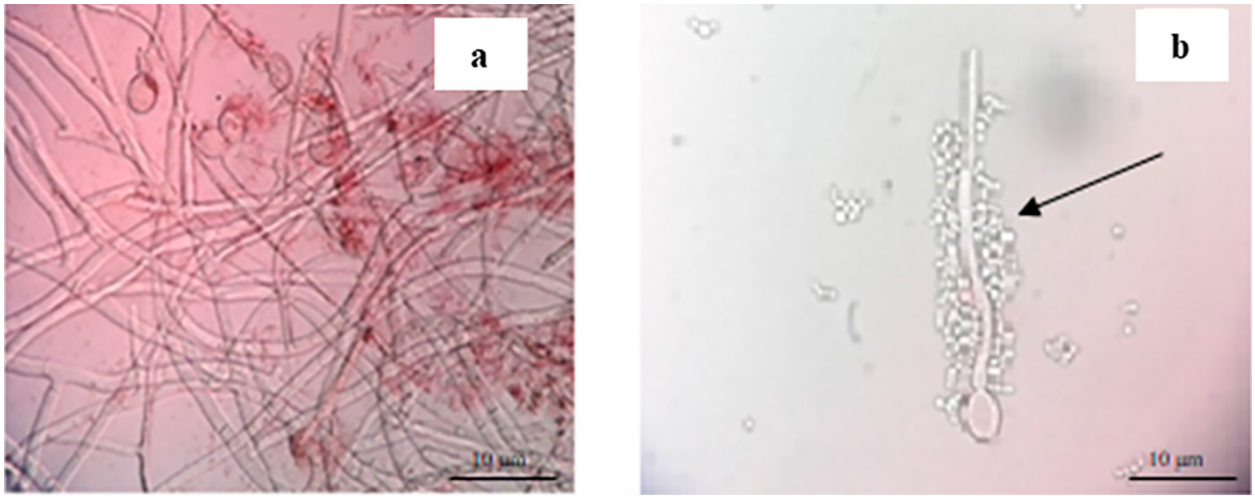

Figure 1. Photomicrography showing (a) Hyphae of *Botrytis cinerea* and (b) interaction between *Debaryomyces hansenii* UFT8244 and germ tube of *B. cinerea*.

Resistance to oxidative stress

The viability of *D. hansenni* cells was lower after treatment with H_2O_2, although the treatments did not cause lethal stress to the yeast, and there was no significant difference in cell viability between the two concentrations of H_2O_2 trialled. At 20 and 40 mM the yeast displayed 2.4×10^5 and 1.4×10^5 UFC/mL, respectively, in comparison with the control (not exposed to H_2O_2) that displayed 1×10^8 UFC/mL This indicates that *D. hansenii* UFT8244 has some ability to resist oxidative stress.

DISCUSSION

The development of resistance to several classes of fungicide has been frequently reported worldwide, emphasizing the need for alternative control strategies, such as through the use of antagonistic yeasts (Feliziani & Romanazzi 2016, Zhang et al. 2017, Ferreira et al. 2019). *Debaryomyces hansenii* is a yeast that is commonly present on the surface of different fruits and in other habitats. It has been described as an antagonistic agent effective against different phytopathogens affecting grapefruit (Droby et al. 1989), lemon (Hernández-Montiel et al. 2010), tangerine (Asrey et al. 2012), peach (Singh & Sharma 2009), papaya (Hernandez-Montiel et al 2018) and blueberry (Ramos-Bell et al. 2022) amongst other fruit. It has the status of qualified presumption of safety (QPS) from the European Food Safety Authority (EFSA) and is therefore considered suitable to be considered in trials as a biological control yeast (Koutsoumanis et al. 2020).

The isolated yeast *D. hansenii* yeast UFT8244 proved to be an efective biocontrol antagonist against *B. cinerea*, reducing the incidence of gray mold (Ferreira et al. 2023). However, our data demonstrated that *D. hansenii* UFT8244 did not significantly inhibit the *in vitro* growth of the two tested phytopathogens through the production of diffusible or volatile antagonistic substances. Hernandez-Montiel et al (2018) considered that volatile production may be a mechanism by which yeasts can act against phytopathogens. The production of volatile compounds at low temperatures by some Antarctic yeasts has been documented, for instance by Arrarte et al. (2017) who investigated two isolates of *Candida sake* (strains 41E and F36A) obtained from soils on King George Island, maritime Antarctica. These isolates were able to produce volatile

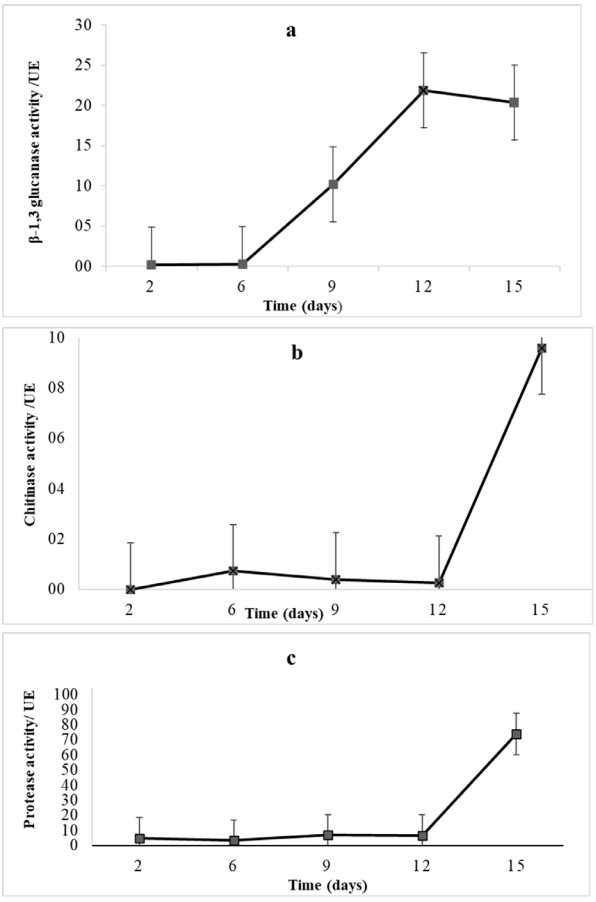

Figure 2. Enzymatic activity of the yeast *Debaryomyces hansenii* UFT8244 grown in YNB medium, (a) β-1,3 glucanase activity, (b) chitinase activity, (c) protease activity.

compounds that inhibited the growth of *B. cinerea* at 0 °C.

Spore germination was significantly inhibited for *B. cinerea* at 0 and 25 °C. The colonization of hyphae by yeast cells is also an important action mechanism in the control of phytopathogens (Zhang et al. 2011) and has been noted in studies of other antagonistic yeasts, such as *Pichia guilliermondii* in the control of *B. cinerea* (Zhang et al. 2011), *Candida oleophila* strain I-182 under the hyphae walls of *B. cinerea* (Saligkarias et al. 2002), and *Pichia membranefaciens* and *Cryptococcus albidus*, which can bind to the hyphae walls of *Monilinia fructicola*, *Penicillium expansum* and *R. stolonifera* (Chan & Tian 2005). This mechanism was also observed with *D. hansenii* colonizing mycelia of *P. italicum* on lemons (Hernández-Montiel et al. 2012) and inhibiting *B. cinerea* on blueberries (Ramos-Bell et al. 2022).

Mycoparasitism could provide a further mechanism of action of the yeast, since their adhesion to the fungal structures can cause them to collapse, mediated by the secretion of hydrolytic enzymes such as glucanase and chitinase (Freimoser et al. 2019). Microorganisms are capable of producing a variety of chelating substances of low molecular mass and high affinity, capable of solubilizing iron in the environment and then transporting it into the cell (Freimoser et al. 2019). These compounds are known as siderophores and show variability in both their structure and affinity for iron (Saha et al. 2016). For instance, some yeasts are capable of producing hydroxamates, which are siderophore compounds that can influence growth under competitive conditions when nutrient availability is a limiting factor (Calvente et al 2001). However, the production of this compound by *D. hansenii* UFT8244 was not detected here. Similarly, although the ability to form biofilm is another important mechanism in the selection of biological control agents against post-harvest diseases (Droby et al 2009, Vero et al. 2013), no evidence for biofilm production by *D. hansenii* UFT8244 was obtained.

The production of hydrolytic enzymes is another mechanism that yeasts can use to inhibit phytopathogens. Our data confirm that *D. hansenii* UFT8244 showed activity of β-1, 3 glucanase and protease, both previously reported in studies of other isolates of the same species (Hernandez-Montiel et al. 2010, Hernandez-Montiel et al. 2018), and of chitinase. Distinct profile results for enzyme production have been reported for each isolate studied in a study of different isolates of *D. hansenii* as a biological

control agent against *Penicillium italicum* (Hernández-Montiel et al. 2010). This study reported higher enzyme production results after between 9- and 13-day incubations, consistent with the results obtained here. Hernandez-Montiel et al (2018) also demonstrated that the production of hydrolytic enzymes was one of the mechanisms employed by *D. hansenii* in the biological control of anthracnose in papaya fruit.

The *in vitro* verification of β-1,3-glucanase, chitinase and protease production is consistent with these enzymes being involved in the biological control process. Since β-1,3-glucanase and chitinase are able to hydrolyze the β -1,4 N-acetyl- β-D-glycosamide bonds of chitin, these enzymes are able to break down the fungal cell wall (Freimoser et al. 2019) and thereby cause the death of phytopathogens (Hernández-Montiel et al. 2010, Vero et al. 2013, Banani et al. 2015, Di Francesco et al. 2015). Proteases, in turn, are able to hydrolyze mannoproteins, which are also a component of the fungal cell wall (Hernández-Montiel et al. 2010).

Some resistance to oxidative stress was observed in *D. hansenii* UFT8244. The occurrence of oxidative stress is an initial response in plants to the presence of both pathogenic and non-pathogenic microbes and may form part of the signaling pathways of the fruit's resistance system (Zhang et al. 2017). However, the yeast may be able to tolerate oxidative stress increases at the site of an injury. Castoria et al. (2003) investigated the relationship between the efficiency of antagonistic yeasts as post-harvest biological control agents and resistance to oxidative stress, suggesting that the ability of a yeast to tolerate the high levels of ROS (reactive oxygen species) produced in apple trees in response to damage may be an essential characteristic of an effective biological control agent. Zhang et al. (2017) also investigated the mechanisms of oxidative stress tolerance of *Cryptococcus laurentii*, confirming exogenous oxidative stress has a significant effect on the efficiency of *C. laurentii* biocontrol. Hence, understanding the biocontrol mechanisms used by antagonistic yeasts is essential for the development of commercial formulations to maximize their potential to manage post-harvest diseases. Several studies have confirmed the beneficial use of antagonistic yeasts (Spadaro & Droby 2016). However, few potential biocontrol agents are ever eventually developed into commercial products, mainly due to lack of understanding of their mechanisms of action.

CONCLUSIONS

Our study demonstrated that the yeast *D. hansenii* UFT8244, originally obtained from Antarctica, possesses several features important for it potential use in biocontrol, including: (i) control of spore germination of known post-harvest phytopagenic fungi, (ii) production of enzymes with antifungal activity, and (iii) resistance to oxidative stress. However, further studies are required to elucidate the complete mechanisms possessed by the yeast, such as induction of host resistance, competition for space and nutrients, mycoparasitism, the production of antifungal secondary metabolites and *in vivo* studies, in order to further investigate the use of yeasts such as *D. hansenii* UFT8422 in commercial-scale applications at low temperatures.

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SUPPLEMENTARY MATERIAL

Figure. S1.

How to cite

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