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Biocontrol activity of the marine yeast *Debaryomyces hansenii* against phytopathogenic fungi and its ability to inhibit mycotoxins production in maize grain (*Zea mays* L.)

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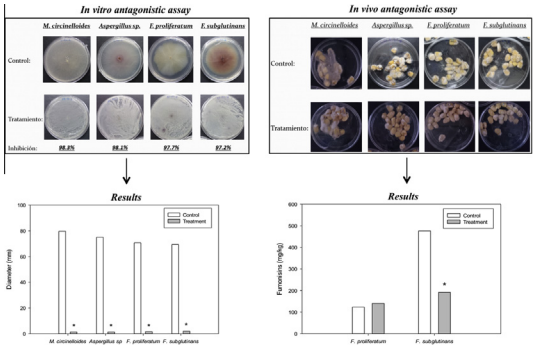
HIGHLIGHTS

- *In vitro* tests *D. hansenii* significant decreases the mycelial growth of four maize postharvest pathogens.
- Presence of *D. hansenii* reduced the production of fumonisins of *F. subglutinans* in 59.8% *in vivo*.
- First time that marine yeast is used to the mold control in maize grain.

GRAPHICAL ABSTRACT



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ABSTRACT

Debaryomyces hansenii is an antagonistic yeast that has been shown high effectiveness against different phytopathogenic fungi in diverse habitats. Both, the antagonistic activity of *D. hansenii* BCS003 and its mechanism against four pathogenic fungi (*Mucor circinelloides*, *Aspergillus* sp., *Fusarium proliferatum* and *Fusarium subglutinans*) in maize grains (*Zea mays* L.) were evaluated. Results indicated an almost entirely decrease (97.2–98.3%) of mycelial growth in a radial inhibition assay against four strains of pathogenic fungi by the *D. hansenii* action. In diffusible and volatile compounds as well cell-free supernatants *in vitro* assays *D. hansenii* showed significant inhibitory activity ($p < 0.05$) against *Aspergillus* sp., *F. proliferatum* and *F. subglutinans*. The four isolated fungi were used to infect maize grains and results showed a partial inhibition of the mycelial growth and a 24 h delay in the disease appearance when compared to control (not *D. hansenii* treated maize grain). Moreover, the application of *D. hansenii* reduced the production of *F. subglutinans* fumonisins by 59.8% after seven days of co-incubation without affecting the chemical and mineral composition of maize grain. According to these results, *D. hansenii* BCS003 could be considered a potential biocontrol agent against toxigenic molds in maize grains, probably due to the synergistic effects of the factors such as the competition for nutrients and space, as well as the production of extracellular soluble and volatile compounds. The potential of marine *D. hansenii* as biocontrol agent merits further research to improve its efficacy.

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1. Introduction

Cereal grains are exposed to several phytopathogen contaminants and improper handling could favor conditions for the growth of phytopathogens, such as fungi (Mylona et al., 2014). Mycotoxins secreted by fungi not only affect the nutritional and organoleptic properties of the grains, but also represents a worldwide problem for human and animal health (Magan and Aldred, 2007; Nicolaisen et al., 2008; Battilani et al., 2008). Particularly, maize grain is one of the most important agricultural crops for animal feed as well human diet in many regions of Asia, Africa and America (Thompson et al., 2013). Both maize grain production and storage are seriously affected by phytopathogenic fungal contamination (Ruiz de Galarreta et al., 2015; Giorni et al., 2015).

The use of synthetic fungicides is the primary effective strategy to control post-harvest fungal diseases in several fruits and grains (Magan and Aldred, 2007; Lu et al., 2014; Zhou et al., 2014a,b). However, an increase in the public concern regarding the negative human health and environmental effects associated with the use of pesticide in agriculture had made a compulsory search of alternative control methods (Bautista-Rosales et al., 2014; Lahlali et al., 2014; Oro et al., 2014). Thus, the biocontrol of toxigenic fungi in food by antagonistic microorganisms could be an alternative to those chemical methods. Currently, the biological control of postharvest fungal diseases of fruits and vegetables by antagonistic yeasts helps on replacing or reducing the use of synthetic fungicides (Spadaro et al., 2013; Bautista-Rosales et al., 2013; Liu et al., 2013a,b; Nally et al., 2013). In this sense, yeasts could be used as biocontrol agents since they posses important characteristics: i) a faster growth compared to molds, ii) simple nutritional requirements and iii) the ability to colonize dry surfaces of several niches to compete for nutrients and space (Nally et al., 2012; Guo et al., 2014). The biological control process of yeast against fungi involves several biochemical mechanisms and their functional roles are not fully understood (Armando et al., 2013). These mechanisms can include the yeast's ability to adhere itself on specific sites of both host and pathogen cells, to secrete specific enzymes and antimicrobial substances, to induce host resistance and the capacity to form biofilms on the inner surface of wounds (Lu et al., 2013; Lutz et al., 2013). Additionally, several yeasts reduce the production of mycotoxins as described Abrunhosa et al., 2010 and Gil-Serna et al., 2011. Therefore, the knowledge about mechanisms of potential antagonistic yeasts as biocontrol agents are useful to improve their performance against toxigenic fungi.

The genus *Debaryomyces* Lodder et Kreger-van Rij is a genus of yeasts in the family Saccharomycetaceae which has yeasts with potential biotechnological applications. *Debaryomyces* spp. was initially isolated from seawater but can be found in many habitats with low water activity (Breuer and Harms, 2006). Among species, *Debaryomyces hansenii* has been reported as efficient antagonist yeast against pathogenic fungus in various food products, including dairy products (Liu and Tsao, 2009), dry-cured meat products (Andrade et al., 2014; Simoncini et al., 2014) and dry-fermented sausage (Núñez et al., 2015). For instance, *D. hansenii* isolated from marine environments was able to reduce in 80% the incidence of disease-caused by *Penicillium italicum* Wehmer in Mexican lime (Hernández-Montiel et al., 2010). Interesting, *D. hansenii* has been included in the list of qualified presumption of safety (QPS) for the European Food Safety Authority (BIOHAZ, 2012), supporting the industrial and commercial purposes. However, the potential of *D. hansenii* as antagonist yeast against phytopathogenic fungi in maize grain has not been explored. This study aimed to evaluate the antagonist effect of the marine yeast *D. hansenii* BCS003 against pathogenic fungi, the possible mechanisms of action, nutritional quality and mycotoxins production in maize grain (*Zea mays* L.).

2. Materials and methods

2.1. Yeast and mold strains

Yeast *D. hansenii* was taken from the collection of isolated yeast belonging to marine environments of Centro de Investigaciones Biologicas del Noroeste, S.C. (CIBNOR). The yeast was maintained in culture medium yeast extract peptone dextrose (YPD) agar at 4 °C (yeast extract 10 g, peptone 20 g, dextrose 20 g, agar 20 g, dissolved in 1 L of distilled water).

In vitro antagonism tests were conducted against four strains of fungi which are among the most common in maize grain: *Mucor circinelloides*, *Aspergillus* sp., *Fusarium proliferatum* and *Fusarium subglutinans*. Fungi were directly isolated from maize grains at agricultural field of CIBNOR, La Paz, B.C.S. Strains were grown on potato dextrose agar (39 g/L, PDA, Difco®) at 28 °C and were subsequently stored at 4 °C for identification and antagonism tests. The molecular identification of fungal strains was carried out by obtaining the region ITS1-5.8s-ITS2 of rDNA, using the primers ITS1 (5′ TCCGTAGGTGAACCCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) (White et al., 1990). DNA samples were obtained from the mycelial mass using the method described by Raeder and Broda (1985). PCR amplifications were performed with a thermocycler (Thermal Cycler 170-9701 Bio-Rad®) using the protocol described by Ochoa et al. (2007). The products obtained were sequenced by GENEWIZ, Inc., and the molecular identity was obtained by multiple alignment analysis using Blast tool in the NCBI database (http://www.ncbi.nlm.nih.gov).

2.2. Radial inhibition assay of pathogenic fungi of maize in solid media

The antagonistic potential of *D. hansenii* to inhibit fungal growth of four strains of pathogenic fungi was performed by agar plate inhibition assay as described by Núñez et al. (2015), using PDA medium (39 g/L, Difco®). One hundred microliters of 1×10^8 cells/mL suspension of *D. hansenii* were spread on the PDA plates, after drying, 10 μL of 1×10^6 spores/mL suspension of molds were inoculated in the center of each plate. Following incubation for 7 days at 28 °C, the fungal growth was calculated measuring the diameter of each colony. The radial inhibition (RI) was calculated as follows: $RI\ (\%) = [(C - T)/C] \times 100$, where C (control) was the average diameter of fungal colonies in the absence of *D. hansenii* and T (treatment) was the average diameter of fungal colonies in the cocultured plates. The experiment was performed by triplicate.

2.3. Effect of diffusible antifungal compounds of D. hansenii on fungal growth in solid media

This study was performed as previously described (Huang et al., 2012; Lutz et al., 2013; Matic et al., 2014; Núñez et al., 2015). Briefly, plates were prepared with PDA (39 g/L), added with 1 μL/mL chloramphenicol (50 mg/mL of 70% alcohol). Then 100 μL aliquot of initial culture yeast (2×10^8 cells/mL) was inoculating in 24 mL of YPD liquid medium (yeast extract 10 g, dextrose 20 g, peptone 20 g) supplemented with antibiotic (chloramphenicol 1 μL/mL), and incubated at 28 °C for 24 h at 150 rpm. Then, yeast was adjusted at 1×10^8 cells/mL concentration using a Neubauer chamber (40×). A mycelial disc of 5 mm of each fungus with five days of growth on PDA was placed at 28 °C, at 2.5 cm distance of the edge of each plate, and 3 cm on the opposite side a 10 μL aliquot of inoculum yeast was seeded (1×10^8 cells/mL). Each plate was sealed with parafilm (Parafilm® M, Sigma). Incubation was carried out for 7 days at 28 °C. The diameter of fungus was measured at day 7. A plate with each isolate was used as control without yeast. The inhibitory activity (IA) was expressed as the