Postharvest Biology and Technology 56 (2010) 181–187

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Biocontrol of postharvest blue mold (*Penicillium italicum* Wehmer) on Mexican lime by marine and citrus *Debaryomyces hansenii* isolates

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ARTICLE INFO

Article history:
Received 14 May 2008
Accepted 21 December 2009

Keywords:
Debaryomyces hansenii
Penicillium italicum
Blue mold
Citrus aurantifolia
Mexican lime

ABSTRACT

Mexican lime produced on the Pacific coast of Mexico is frequently spoiled by blue mold during postharvest handling. Methods to control it include chemical fungicide applications and cold storage. Nevertheless, the increasing incidence of this disease necessitates the search for industrially compatible alternatives, such as the use of yeasts as biocontrol agents that are convenient for their easy handling and high osmotolerance, and suitable for the stressful conditions found at the surface of citrus fruits. In this work we tested the performance of twelve native isolates of *Debaryomyces hansenii* obtained from the marine environment and the pericarp of Mexican lime (*Citrus aurantifolia* Christm. Swingle). Native pericarp isolates were more effective both *in vitro* and in simulated industrial packinghouse conditions for the postharvest control of blue mold on Mexican lime. The performance of the yeast was partially linked to a rapid consumption of available sugars in the medium, and *D. hansenii* isolates DhhBCS06, LL1 and LL2 were able to reduce incidence of the disease by up to 80% after two weeks of storage.

1. Introduction

The fungus *Penicillium italicum* Wehmer (causal agent of blue mold) is responsible for important economic losses in citrus production worldwide (Filtenborg et al., 1996). Application of chemical treatments is the primary means of controlling this disease postharvest. However, the development of fungicide-resistant pathogens and public demand to reduce pesticide use have increased the search for alternative control strategies (Wilson et al., 1993; Janisiewicz and Korsten, 2002). Postharvest biocontrol activity exhibited by some yeasts and bacteria over phytopathogenic fungi has been extensively studied and there are several examples of successful disease control on stored fruits, as those obtained with the commercial bioproducts Aspire (Ecogen, Langhorn, PA, USA), YieldPlus (Anchor Yeast, Cape Town, South Africa) and BIOSAVE-110 and -111 (EcoScience, Orlando, FL, USA) (Janisiewicz and Korsten, 2002; Bar-Shimon et al., 2004). Several studies have evaluated the potential of *Debaryomyces hansenii* to control postharvest diseases (Droby et al., 1989; Chalutz and Wilson, 1990), and its mode of action was reported to be related to the production of cell-wall lytic enzymes (El-Ghaouth et al., 1998) and the induction of host resistance (Droby et al., 2002). Nonetheless, competition (for nutrients and space) seems to be the most important mechanism (Droby et al., 1989). It is important to note that the chemical composition of fruit flavedo (peel) determines its susceptibility to fungal attack, and the available substrates (mainly essential oils and carbohydrates) influence the speed and timing of fungal attack (Caccioni et al., 1998; Digrak and Ozcelik, 2001). *D. hansenii* is capable of rapidly consuming the available sources of carbon in the broken flavedo, thus retarding fungal growth by depletion of limiting nutrients (Pelser and Eckert, 1977). Such capability must therefore be present in yeasts that are native to highly competitive, nutrient deprived environments such as unbroken citrus fruits (Janisiewicz and Korsten, 2002), and deep seawater.

The objectives of the present study were: (i) to compare *D. hansenii* isolates from deep seawater and Mexican lime pericarp for *in vitro* performance in preventing spore germination and radial growth of *P. italicum* in flavedo and albedo based media; (ii) to assess the *in vivo* biocontrol performance of the selected yeasts under simulated packinghouse conditions for Mexican lime, and finally, (iii) to select the *D. hansenii* isolate(s) with the highest biocontrol capabilities.

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doi:10.1016/j.postharvbio.2009.12.010

2. Materials and methods

2.1. Fungal inoculum

Decayed Mexican limes (*Citrus aurantifolia* Christm. Swingle) that exhibited symptoms of blue mold were collected in orchards from the State of Colima (Mexico) and sampled to obtain a pure culture of *P. italicum* that was subsequently morphologically and molecularly characterized (Hernández-Montiel and Ochoa, 2007). The fungus was multiplied by inoculation of conidia in 90 mm Petri dishes of potato-dextrose agar (PDA, Difco, Becton Dickinson & Co., USA) and incubation at 25 °C for 7–10 d. Conidia were collected from the mycelium surface and re-suspended in a sterile saline solution (0.9% NaCl) and conidial concentration was diluted to a final value of 1×10^7 conidia L^{-1} which was then immediately used as inoculum.

2.2. Antagonistic yeasts

Nine marine isolates of *D. hansenii*, originally obtained from sea water samples collected at a depth of 100 m at the Mar de Cortés (Baja California, Mexico) and belonging to the Yeast Collection of the CIBNOR (Centro de Investigaciones Biológicas del Noroeste, La Paz, B.C.S., Mexico) (labelled as: DhhBCS01, DhhBCS02, DhhBCS03, DhhBCS04, DhhBCS05, DhhBCS06, DhhBCS07, DhfBCS01 and DhfBCS02), plus three isolates (labelled as: LL1, LL2 and LL3) obtained from the pericarp of harvested Mexican limes (*Citrus aurantifolia* Christm. Swingle) from the State of Colima (Mexico) were used in this study. An isolate of baker's yeast (*Saccharomyces cerevisiae*) was used as a control strain. All the yeasts were grown in YPD broth (Yeast Extract-Dextrose-Peptone medium, Difco, Becton Dickinson & Co., USA) on a rotary shaker at 1.66 Hz for 20 h at 28 °C. Yeast cells were harvested by centrifugation at $6000 \times g$ for 10 min, washed twice with sterile distilled water and cell pellets were re-suspended in sterile distilled water, and diluted to an initial concentration of 1×10^9 cells L^{-1}.

2.3. Biocontrol assessment in vitro

Three different media were used: PDA, homemade flavedo agar (PA) and albedo agar (AA), which were modified as described in Eayre et al. (2003). The last two of these media were prepared using 28 g of flavedo or albedo peelings from mature and healthy Mexican limes (*Citrus aurantifolia* Christm. Swingle) and 8 g of agar dissolved in 0.4 L of distilled water. The media were autoclaved at 121 °C for 15 min, and then only the liquid phase was poured on Petri dishes. Aliquots of 30 μL of each yeast suspension were evenly distributed on plates using a glass rod and incubated for 24 h at 25 °C. An agar plug of 5 mm diameter, obtained from the edge of a 7 d colony of *P. italicum* growth on PDA, was placed in the center of the yeast's plates and incubated at 25 °C. The colony diameter was recorded periodically for 7 d. Each treatment was replicated three times and the test was repeated twice.

2.4. Population dynamics of the yeasts

Growth kinetics of the yeasts was determined in lime-based media. The yeasts were inoculated in 0.1 L of flavedo or albedo broth at an initial concentration of 1×10^9 cells L^{-1} and incubated at 25 °C with orbital shaking at 1.66 Hz. Aliquots were withdrawn every 4 h for 2 d and absorbance at 580 nm measured using a spectrophotometer (DU-640 UV-Vis Beckman Coulter, D.F., Mexico). An increase of 0.1 in absorbance under these conditions is equivalent to 3×10^9 cells L^{-1} according to the standard curve. Three different flasks were used for each isolate. The entire experiment was repeated twice (i.e. results from 6 flasks were available for each isolate) and data of both experiments was combined for statistical analysis.

2.5. Sugar consumption

Assessment of carbohydrate consumption rate for each individual yeast and the fungus was performed by inoculating 100 μL of the tested yeast suspension and/or 100 μL of the *P. italicum* spore suspension into 0.05 L of flavedo or albedo broth in 0.25 L Erlenmeyer flasks. The experiments were incubated at 25 °C and 1.66 Hz for 2 d and were sampled every 12 h for fructose, glucose and sucrose. Each treatment was comprised of three replicates and the entire experiment was repeated twice.

2.5.1. Sugar quantification

Fructose was quantified using the method of Taylor (1995): A mixture of 30 μL of sample, 20 μL of tryptamide–HCl and 600 μL of concentrated HCl was prepared and heated at 60 °C for 15 min followed by cooling at room temperature for 40 min, after which the absorbance at 518 nm was measured and the result interpolated from a fructose calibration curve. Quantification of glucose was made according to the GOD-PAP method of Barham and Trinder (1972), using the Randox kit (Randox México S.A. de C.V.). In this method, 20 μL of sample is mixed with 200 μL of the reactive solution provided by the kit and allowed to react for 30 min, after which absorbance at 490 nm was measured on a plate reader (Bio-Rad 550, Tokyo, Japan). Sucrose was quantified with the modified DNS method of Bruner (1964), whereby a mixture of 200 μL of sample and 10 μL of 3 M HCl is prepared and heated for 15 min in a boiling water bath, followed by neutralization with 10 μL of 3N KOH and mixing with 600 μL of DNS (3,5-dinitrosalicylic acid) reagent. The mixture was then heated for 10 min in a boiling water bath, allowed to cool to room temperature, after which the absorbance at 570 nm was measured and the result interpolated from a calibration curve for fructose.

2.5.2. Inhibition of spore germination

The effect of the yeasts on spore germination was tested in the inoculated flavedo and albedo broths described previously. The germination rate of *P. italicum* was determined microscopically by measuring the percentage of germination every 12 h for 2 d, in samples of approximately 200 spores. Spores were considered germinated when the germ tube length was equal to or longer than the spore diameter (Yao et al., 2004). Each treatment was replicated three times and the entire experiment was repeated twice.

2.6. Biocontrol test on Mexican lime

The efficacy of the yeast in controlling the development of blue mold on Mexican limes was tested by using lightly wounded fruit, simulating small common injuries found in the packinghouse facilities. The fruit were immersed in the yeast suspension being tested followed by inoculation with fresh spores of *P. italicum*. For this purpose, the limes were disinfected with 2% (v/v) sodium hypochlorite for two minutes, allowed to dry at room temperature for 1 h, as described in Lima et al. (1999), then submerged for 10 min in the yeast suspension (at 1×10^9 cells L^{-1}) to promote yeast attachment to the fruit, and then allowed to dry at room temperature for 30 min. An equatorial wound (2 mm wide and 1 mm deep) was punctured on each fruit using a disinfected needle, followed by inoculation with 20 μL *P. italicum* spore suspension. Control treatment solutions were distilled sterile water, imazalil spray (IUPAC: (RS)-1-(β-allyloxy-2,4-dichlorophenethyl)imidazole) at 500 mg L^{-1} (Sanazil 75 PS, VELSIMEX®, Mexico), and submerging the limes in a suspension of *S. cerevisiae*. Experiments were performed at 13 °C and 90% RH by placing the fruit in 3 L plastic containers in an environmental chamber (KBF 720 Binder GmbH,

Postfach, Germany), thereby simulating industrially used storage conditions for Mexican lime. Disease incidence (percentage of infected wounds) and severity (lesion diameter) were measured over a two-week period. Each treatment contained ten replicates (limes) and the entire experiment was repeated twice.

2.7. Statistical analysis

Values of percentage of disease incidence were transformed with the arcsine of the square root of the proportion of infected fruits, and analyzed by using STATISTICA program (StatSoft, OK, USA). The effect of the treatments was analyzed using one-factor general linear model procedure (ANOVA), and the least significant difference (LSD) test was used ($P < 0.05$) for means separation.

3. Results

3.1. Biocontrol efficacy of D. hansenii isolates in vitro

Isolates of *D. hansenii* varied in their degree of inhibition of *P. italicum*, on PDA, flavedo and albedo agars. Isolates LL1 and LL2 were the most effective *in vitro* in reducing growth of *P. italicum* (Table 1), although marine isolates DhhBCS05 and DhhBCS06 also performed well. The *S. cerevisiae* control strain did not stop the rapid growth of the pathogen on PDA, a culture medium that supports rapid growth of this yeast, confirming the antagonistic nature of the *D. hansenii* isolates used (Fig. 1).

Preliminary analysis in YMB agar plates (0.3% yeast extract, 0.3% malt extract, 1% glucose, 0.5% peptone and 2% agar) at different initial pH values evidenced the production of inhibitory compounds by some yeasts, which were visualized by the formation of a clear halo around a yeast plug that had been inoculated over a two-day *P. italicum* culture (data not shown). Further studies regarding this phenomenon are under way.

Based on these results, isolates DhhBCS05, DhhBCS06, LL1, LL2 and LL3 were selected for further characterization and to be tested under simulated packinghouse conditions.

3.2. Growth dynamics on albedo and flavedo broths

All the selected *D. hansenii* isolates grew rapidly on the Mexican lime-based broths, with those isolated from Mexican lime pericarp (LL1, LL2 and LL3) reaching the highest absorbance values 16 h after inoculation (Fig. 2). Nonetheless, we observed a sharp

Table 1
Maximum *Penicillium italicum* colony size on potato-dextrose agar (PDA), flavedo agar (FA) and albedo agar (AA) in the presence of *D. hansenni* and *S. cerevisiae* (control) isolates.

Strain	PDA	FA	AA
	Fungal colony diameter (mm)		
P. italicum alone	65 ± 0.05 a*	53 ± 0.11 a	51 ± 0.05 a
S. cerevisiae	64 ± 0.10 a	52 ± 0.05 a	48 ± 0.05 a
DhhBCS01	40 ± 0.10 b	23 ± 0.05 d	20 ± 0.05 c
DhhBCS02	19 ± 0.05 e	13 ± 0.05 f	15 ± 0.17 e
DhhBCS03	7 ± 0.10 g	20 ± 0.05 e	15 ± 0.05 e
DhhBCS04	16 ± 0.10 f	27 ± 0.20 b	17 ± 0.05 d
DhhBCS05	2 ± 0.20 i	10 ± 0.15 g	10 ± 0.05 f
DhhBCS06	1 ± 0.15 i	9 ± 0.05 g	10 ± 0.05 f
DhhBCS07	37 ± 0.11 c	24 ± 0.05 cd	20 ± 0.05 c
DhfBCS01	26 ± 0.05 d	26 ± 0.20 bc	20 ± 0.07 c
DhfBCS02	5 ± 0.10 h	25 ± 0.05 c	23 ± 0.05 b
LL1	1 ± 0.11 i	5 ± 0.05 h	8 ± 0.10 g
LL2	2 ± 0.2 i	5 ± 0.05 h	6 ± 0.05 h
LL3	2 ± 0.2 i	6 ± 0.15 h	6 ± 0.05 h

Data were measured at 7 d of incubation at 25 °C. Values in each column followed by the same letter are not significantly different according to least significant difference (LSD) test ($P < 0.05$). *Standard deviation of the mean.

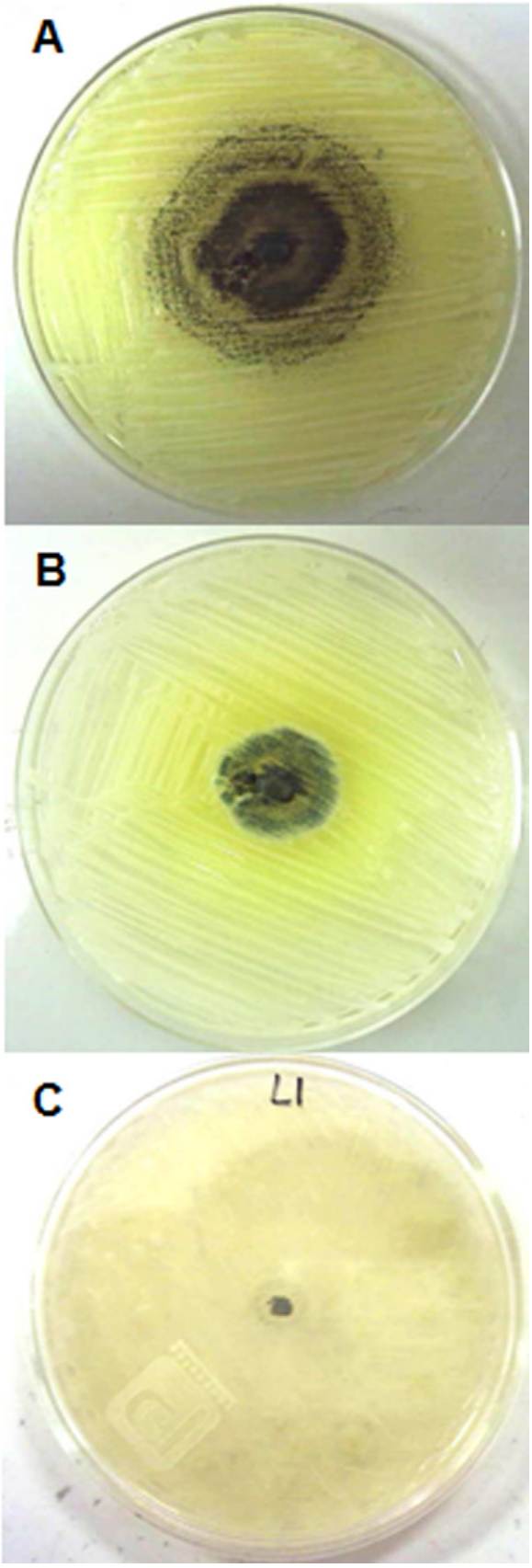

Fig. 1. Typical colony diameter of *P. italicum* on flavedo agar (FA) plates in the presence of (A) *Saccharomyces cerevisiae*, and *D. hansenii* isolates: (B) DhhBCS05, and (C) LL1.

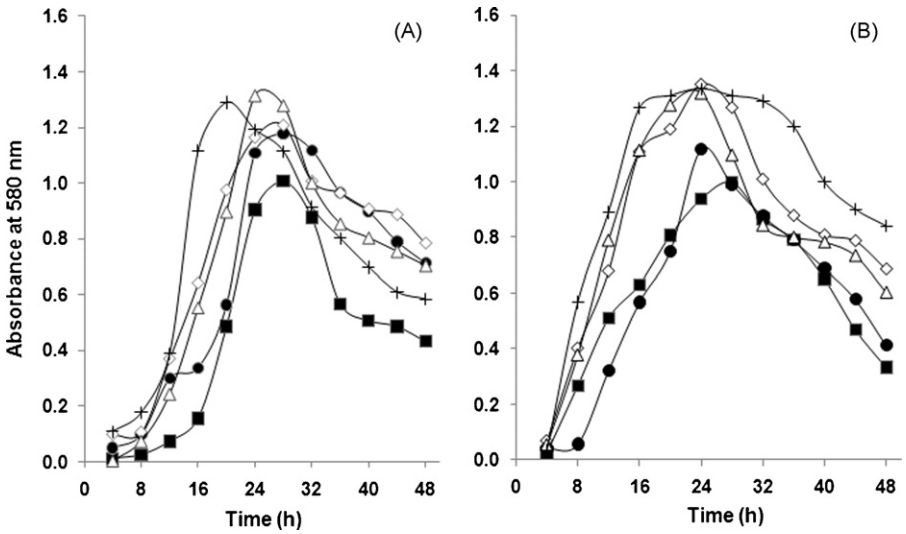

Fig. 2. Growth curves of *D. hansenii* isolates DhhBCS05 (■), DhhBCS06 (●), LL1 (◊), LL2 (△) and LL3 (+), in (A) flavedo and (B) albedo broths. Standard deviation of the mean was always less than 8% for all data.

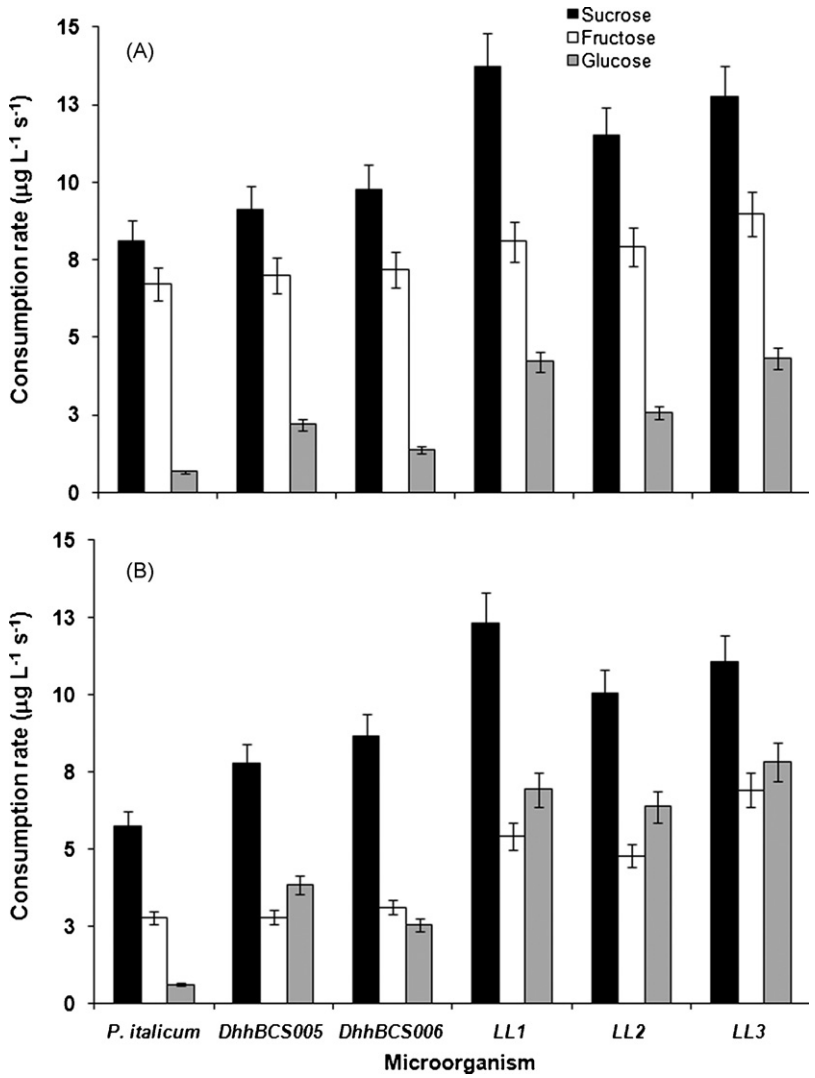

Fig. 3. Carbohydrate consumption rates, (A) flavedo broth, and (B) albedo broth. Bars represent the standard deviation of the mean.

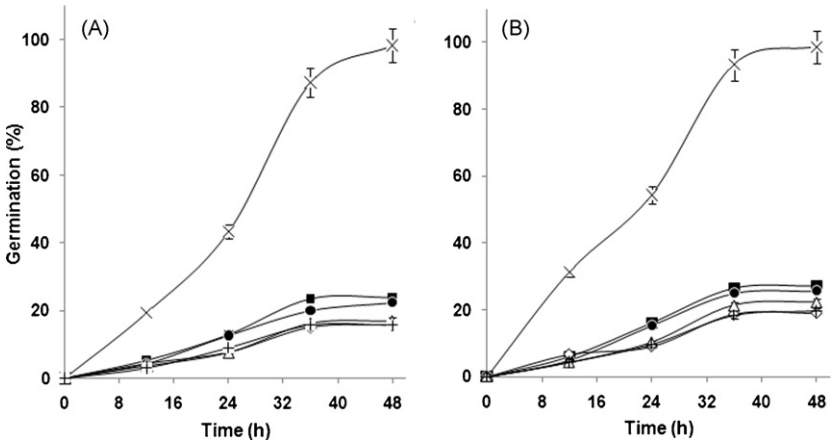

Fig. 4. Percentage of spore germination of *P. italicum* alone (×) and in the presence of *D. hansenii* isolate DhhBCS05 (■), DhhBCS06 (●), LL1 (◊), LL2 (△) and LL3 (+) inoculated in: (A) flavedo broth, and (B) albedo broth. Bars represent the standard deviation of the mean.

decline in absorbance for all cultures 32 h after inoculation which was expected due to the lack of available nutrients in all the broths, and evidencing yeast lysis.

3.3. Assimilation of carbohydrates

The low sugar content of both flavedo and albedo broths most likely promoted carbon competition. Flavedo broth sugar content was 2.37 g L^{-1} sucrose, 1.12 g L^{-1} fructose and 0.46 g L^{-1} glucose. Albedo broth sugar content was 2.05 g L^{-1} sucrose, 1.0 g L^{-1} fructose and 0.90 g L^{-1} glucose. All five *D. hansenii* isolates selected for the *in vitro* test had a higher consumption rate of glucose and sucrose than the pathogen (Fig. 3), making them effective in restricting germination of the fungal spores, as was also observed when sterile distilled water was tested for spore germination (data not shown).

The rates of assimilation of sucrose, fructose and glucose were different for each microorganism (Fig. 3A and B). Isolate *D. hansenii*

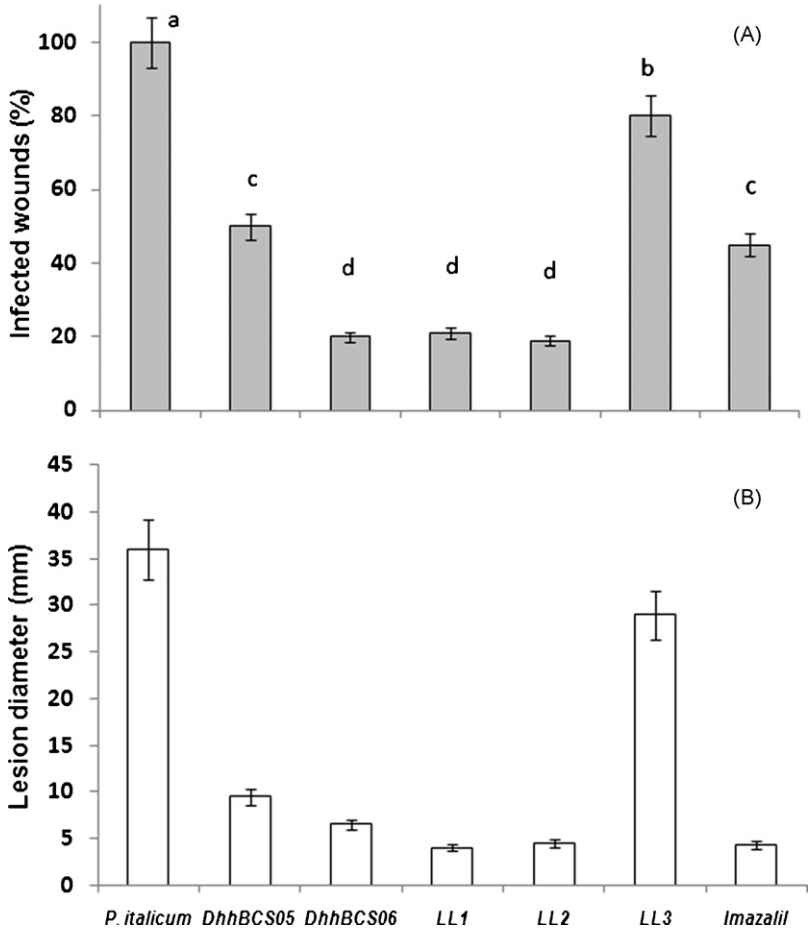

Fig. 5. Biocontrol efficacy of *D. hansenii* isolates on Mexican lime under simulated packinghouse conditions (13 °C and 90% RH) measured as: (A) incidence of blue mold, and (B) size of the lesion caused by *P. italicum*. Values of each bar with the same letter are not significantly different, according to least significant difference (LSD) test at $P < 0.05$. Lesion diameter bars represent the standard deviation of the mean.

LL1 was found to have the highest rate of sugar consumption and excellent performance as antagonist *in vivo*.

3.3.1. Inhibition of *P. italicum* spore germination

Spore germination of *P. italicum* in both flavedo and albedo broths was strongly inhibited (by at least 70%) by *D. hansenii* isolates DhhBCS05, DhhBCS06, LL1, LL2 and LL3 (Fig. 4), with the last three having best performance.

3.4. Biocontrol efficacy of *D. hansenii* isolates on Mexican limes

Fruit treated with *S. cerevisiae* (data not shown) or with sterile distilled water and inoculated *P. italicum* spores resulted in disease incidences of nearly 100%, with a mean lesion diameter of 0.037 m. In contrast, fruit co-inoculated with *D. hansenii* isolates LL1 and LL2 had the fewest infected wounds and smallest lesion sizes (Fig. 5).

Yeasts isolates LL1, LL2 and DhhBCS06 were the best in preventing the infection by *P. italicum* on lime wounds, and a positive correlation was obtained between the *in vitro* inhibition of *P. italicum* (colony diameter) and the degree of protection provided (*in vivo* test) to Mexican limes (percentage of infected wounds) under simulated packinghouse conditions ($R^2 = 0.85$) when isolate LL3 (which tended to deviate from this trend) was not considered. The fact that yeast LL3 did not perform well in the simulated packinghouse experiments could indicate that either the fruit pericarp composition or the storage conditions (13 °C and 90% RH) were not favorable for this isolate.

4. Discussion

The twelve native isolates of *D. hansenii* tested in this work varied in their degree of inhibition of *P. italicum* on potato-dextrose, flavedo and albedo agars. The isolates LL1 and LL2 were the most effective in the 3 tested solid media for controlling the blue mold *in vitro* (Table 1) although marine isolates DhhBCS05 and DhhBCS06 also had a good performance. The use of *S. cerevisiae* as control in the test of *in vitro* inhibition of *P. italicum* had no effect on the growth of this pathogen on potato-dextrose agar, a culture medium that supports a rapid growth of this yeast, confirming the antagonistic nature of the *D. hansenii* isolates used. This result, obtained on a rich medium, suggests an additional mechanism to that of competition for nutrients and space as antagonistic isolates were able to inhibit the growth of *P. italicum* on PDA, a feature that has been observed by other authors as important for antagonist yeast to be used as control agent for postharvest pathogenic fungi (Usall et al., 2000; Duverfors et al., 2005).

Germination of *Penicillium* spp. spores usually requires a carbon source as inductor (Digrak and Ozcelik, 2001) as well as a signal for starting the infection process on the fruit (Santos et al., 1978) and the absence of a carbon source can act as a factor inducing dormancy in some fungal spores. Accordingly, isolates LL1, LL2 and LL3 had the best performance in this experiment (Fig. 3) most likely due to rapid carbon depletion, as inferred from experiments using sterile distilled water. The use of flavedo and albedo based broths also allowed a rapid selection of yeast strains for the *in vivo* test which, in theory, permits a more realistic prediction of their capabilities of colonization of the citrus wounds (Nunes et al., 2001).

Biocontrol experiments on Mexican lime (*in vivo*) showed that yeasts isolates DhhBCS05 and DhhBCS06 were most effective, and isolate LL3 had the least effective control performance among the five selected *D. hansenii* isolates. It is worth noting that the imazalil control test had a 50% disease incidence (infected wounds), suggesting that this strain of *P. italicum* has already developed some resistance to this chemical fungicide.

The use of *D. hansenii* as a postharvest biocontrol agent has been reported by Chalutz and Wilson (1990) and Lima et al. (1999) for controlling green and blue molds on citrus, and by Droby et al. (1989) against *P. digitatum* on grapefruit. However, to our knowledge, this is the first study specifically developed for Mexican lime diseased by caused *P. italicum*. We are currently working on the development of a formulation suitable for application in industrial facilities.

5. Conclusions

The use of *in vitro* selective media based on Mexican lime itself allowed us to rapidly select isolates with potential for biocontrol of blue mold. Although preliminary tests suggest that the production of an inhibiting metabolite is involved in the *in vitro* biocontrol activity observed, this phenomenon requires further study.

From a practical point of view, the *in vivo* test results obtained from this work under simulated industrial conditions suggest that selected *D. hansenii* isolates, either native to citrus pericarp or from a marine environment, can be used for biocontrol of postharvest infections caused by *P. italicum* on Mexican lime.

Acknowledgments

We thank the financial support provided to L.G. Hernández-Montiel by the CONACyT (Consejo Nacional de Ciencia y Tecnología, grant 144710, México), the grant project SAGARPA2003-C01-8 (Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación, México), and the research support from the Instituto Politécnico Nacional projects SIP2006-0913 and SIP2007-0786. We thank R. Hernández-Herrera and I.C. Rodríguez-Luna for their excellent technical assistance.

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