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,