Emirates Journal of Food and Agriculture. 2017. 29(2): 114-122
doi: 10.9755/ejfa.2016-09-1273
http://www.ejfa.me/

REGULAR ARTICLE

Use of a marine yeast as a biocontrol agent of the novel pathogen *Penicillium citrinum* on Persian lime

Ramsés Ramón González Estrada[1], Felipe de Jesus Ascencio Valle[2], Juan Arturo Ragazzo Sánchez[1], Montserrat Calderón Santoyo[1]*

[1]Laboratorio Integral de Investigación en Alimentos, Instituto Tecnológico de Tepic. Av. Tecnológico No. 2595, Tepic, Nayarit, México, C.P. 63175, [2]Centro de Investigaciones Biológicas del Noroeste, S.C., Mar Bermejo No. 195, Col. Playa Palo de Santa Rita, La Paz, BCS, Mexico. C.P. 23090

ABSTRACT

The effectiveness of the yeast *Debaryomyces hansenii* against *Penicillium citrinum* in Persian lime was evaluated. Postharvest decay is a serious problem in the storage of many fresh fruits and vegetables. *P. citrinum* was isolated from fruits of a packinghouse in San Pedro Lagunillas, Nayarit, Mexico and turned out to be an endemic pathogen against Persian lime. This is the first report that involves this strain as a pathogen for citrus. The control of Persian lime decay caused by *P. citrinum* in postharvest and yeast population dynamics were evaluated. In order to enhance yeast establishment on the fruit carposphere Dextrose and Tween 80 were added as adjuvants to the *D. hansenii* suspension. The use dextrose and Tween 80 promoted a better establishment of the yeast ($3.2x10^8$ UFC/fruit - 25 days storage), however the infection by *P. citrinum* on Persian lime was higher (20 to 60% of infected fruits). The application of yeast suspension without adjuvants strongly inhibited fungal decay (100%) even though yeast population counts were minimal ($3.3x10^5$ CFU/fruit) at the end of the storage period (25th day) at 25°C and 60-75% of relative humidity. Application of *D. hansenii* can be an alternative of postharvest disease management.

Keywords: Biological control; *Debaryomyces hansenii*; *Penicillium citrinum;* Persian lime; Postharvest

INTRODUCTION

Citriculture in Mexico is an activity of economic and social importance: it is carried out in just over half a million hectares in regions with tropical and subtropical climate in 23 states (SIAP, 2012). Citrus postharvest diseases caused by fungi cause economic losses throughout the world (Eckert and Eaks, 1989). In Mexico losses are as high as 40% of the total production (Ochoa et al., 2007). Harvesting, packaging and transport can damage fruit allowing fungal infections through surface wounds (Sharma et al., 2009). Important pathogens of the genus *Penicillium* found in citrus are: *P. digitatum* [(Pers.) Sacc.] (Green mold) which is ubiquitous to the whole citrus growing regions. Decayed areas appears as a soft watery spots with softened rind, the entire fruit is soon encompassed by a mass of olive green spores. *P. italicum* Wehmer (Blue mold) whose initial symptoms are similar to those of green mold encompassed by a definite band of water-soaked rind, the infected fruit can be covered entirely by a mass of blue spores (Brown and Eckert, 1988). A study of fungus in Persian lime (*Citrus latifolia* Tanaka) in San Pedro Lagunillas, Nayarit, Mexico revealed a high frequency of *P. citrinum* Thom on fruits in local packinghouses (Data not published). However, there is not information in the literature about *P. citrinum* pathogenicity, then it could be an emerging pathogen or an endemic one. Emerging infectious diseases are caused by pathogens with increased incidence. They have changed pathogenesis, are newly evolved; or have been discovered or newly recognized. In this way plant diseases impact negatively on human wellbeing through agricultural and economic losses (Anderson et al., 2004). Traditionally, control postharvest decay is mainly based on the use of synthetic fungicides, however the demand from consumers for products free of chemical residues and the appearance of fungi resistant to these compounds has hampered the control of pathogens (Liu et al., 2013). Thus, an alternative to chemical methods could be the biocontrol of pathogenic fungi by antagonistic microorganisms. The establishment of the antagonist at the carposphere of the

***Corresponding author:**
Montserrat Calderón Santoyo, Laboratorio Integral de Investigación en Alimentos, Instituto Tecnológico de Tepic. Av. Tecnológico No. 2595, Tepic, Nayarit, México, C.P. 63175. Tel: 00 52 1 3112119400. E-mail: montserratcalder@gmail.com

Received: 29 September 2016; **Revised:** 17 January 2017; **Accepted:** 23 January 2017; **Published Online:** 29 January 2017

fruit is an important trial to facilitate their colonization and efficacy (Ippolito and Nigro, 2000). In addition, the level of protection of antagonistic yeasts against pathogens can be influence by the concentration of yeast inoculum, as previously reported (Hernández-Montiel et al., 2012). In the wounds, yeast growths rapidly, but on intact fruit surfaces the antagonist populations usually diminish to the level of natural epiphytic microflora (Droby et al., 2009; Liu et al., 2013). In these sense, the use of adjuvants like Tween 80 (as surfactant) or dextrose (as nutrient) could be an alternative to a better distribution and maintaining of antagonistic yeast population on carposphere at concentrations suitable for biological control, as previously reported (De Cal et al., 2012; Spadaro and Droby, 2016). Several studies have evaluated the potential of *D. hansenii* [(Zopf) Lodder & Kreger] in controlling postharvest fungal pathogens in citrus fruits, in lemon [*Citrus limon* (L.) Burm.f.] against green and blue mold (*P. digitatum* and *P. italicum*); in grapefruit (*Citrus paradisi* Macfad.) against *P. digitatum;* in sweet orange (*Citrus sinensis* Osbeck) against *P. digitatum* (Chalutz and Wilson, 1990; Droby et al., 1999; Taqarort et al., 2008). In a recent study marine *D. hansenii* was capable to inhibit the mycelial growth of pathogenic fungi of maize (*Zea mays* L.) (Medina-Córdova et al., 2016). The mechanisms of action reported to *D. hansenii* are related to competition for nutrients and space, parasitism, production of cell-wall lytic enzymes, induction of host resistance and recently the production of extracellular soluble and volatile compounds (Droby et al., 2002; Hernández-Montiel et al., 2010a; Medina-Córdova et al., 2016). Then the aim of this study was to evaluate the potential of *D. hansenii* with and without adjuvants in the control of *P. citrinum* in Persian lime.

MATERIALS AND METHODS

Fungi isolation and purification

Persian limes were taken without apparent damage, same size, in the same area, from a specific point in the packinghouse process, and placed in a sterile sampling bag (BioPro Sample Bag, International BioProducts, Inc. USA). Individually fruits were washed by immersion in sterile solution, 100 µL of peptone solution 1% (Bioxon, USA) was added and manually homogenized 2 min and then a serial dilution was performed. Then inoculated in Petri dishes on potato dextrose agar (PDA) (Bioxon, USA) with 50 µg/mL of ampicillin (Hormone, México) and rose bengal dye 0.5% w/v as inhibitors (Fernández, 2000). Finally, they were incubated at 25ºC during 5 days until mycelial development. Fungal mycelium was transferred in PDA Petri dishes with ampicillin until purification. Purified strains were stored at 4ºC in sabouraud agar (Bioxon, USA). The pre-identification of isolated fungi was performed using traditional morphological methods. Macroscopic (colony type, color and growth type) as well as microscopic (mycelium type and spore morphology) characteristics were considered for identification.

Molecular analysis: Identification of genus and species of strains

DNA extraction was performed first with an alkaline lysis and then by enzymatic digestion (Chitinase and Proteinase K). Due to the nature of some samples, ZR Fungal/Bacterial DNA Kit™ (Zymo Research, USA) was used. Once the mold DNA was obtained, we proceeded to amplification by end-point PCR method (Polymerase chain reaction) of the intergenic region ITS (ITS1-5, 8s-ITS-2) of ribosomal DNA using universal primers ITS1 (5'- CAACTCCCAAACCCCTGTGA-3') and ITS4 (5'- GCGACGATTACCAGTAACGA-3') for molds. These oligos were obtained from the region ITS (Internal Transcriber Spacer) found in DNA ribosomal (White et al., 1990). PCR products obtained were sent to GENEWIZ Inc. (USA), for sequencing and identification of species. The results were analyzed using the BLAST (The Basic Local Alignment Search Tool) of NCBI (National Center of Biotechnological Information) with the support of CodonCode Aligner 2.0 editing program sequences.

In order to confirm the genus and identify the specie for the fungus, identification based on the polymerase chain reaction was applied. DNA extraction was performed according a previous protocol proposed (Sambrook and Russell, 2001). In a sterile eppendorf tubes 400-600 µL of lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM pH 8.0 HCl Tris and 1 mM EDTA) were added, depending on the amount of biomass 50-100 mg of fungal was added in the tube. The sample containing the mix and glass beads (0.4 mm) was mixed for 2 min at high speed using a vortex. Then, 5 µL of chitinase (25 U) and 4 µL of proteinase K (20 mg/mL) were added. The mixture was mixed and incubated overnight at ambient temperature (25ºC). After the incubation 100 µL of NaCl (5M) was added and gently mixed. Then, 400-600 µL of phenol chloroform (1:1 v/v) was added, depending on the volume of lysis buffer added. The mixture was turned gently and centrifuged for 8 min at 13000 rpm, then the watery portion was removed and 10 µL of ammonium acetate 5M were added and mixed gently. 700 µL of isopropanol (depending on the amount of biomass) were added and mixed gently, if different densities were observed at the beginning or is not evident, then, is recommended to place the sample at -80 ° C for about 30 min, to aid DNA precipitation. The mixture was centrifuged for 10 min at 14000 rpm. The supernatant was discarded and DNA was washed with 70% ethanol (500 µL) and mixed gently. Tubes were dried in a laminar flow chamber at 25ºC for 10 min. DNA

pellet was dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA; 50-100 µL) and incubated at 60ºC 10 min to dissolve the pellet. Finally 4 µL of RNAsa (1 mg/ml) was added and incubated at 37°C for 30 min. PCR was performed using the primers ITS1 and ITS4 (White et al., 1990). For amplification 1 µL of isolated DNA was used in 25 µL of PCR mixture. The reaction was performed in H_2O MQ (17.3 µL), Buffer 10X (2.5 µL), 50 mM $MgCl_2$ (1 µL), each primer (1 µL), 2.5 mM dNTP's (2 µL) and Taq DNA polymerase (0.2 µL). Amplifications were carried out in Technethermocycler (iCycler Biorad, USA) with thermal cycling parameters: initial denaturation at 95ºC for 2 min, followed by 30 cycles of heat denaturation at 90ºC for 1 min, annealing at 50ºC for 30 s, extension at 72ºC for 10 min and final extension at 72ºC for 10 min. PCR products from these amplifications were separated by electrophoresis on 1% (w/v) agarose gel (100 mL), stained in ethidium bromide (0.6 µL), the sample is mixed with 0.1 mL of buffer bromophenol blue as a contrast buffer. On the banks of a marker molecule weight of 100 bp ladder is placed. Electrophoresis was performed under the following conditions: 30 min, 90 volts and 400 mA. PCR products were visualized using a UV transilluminator (UVP BioDoc-IT Imaging System, USA).

Fruits

Persian limes were harvested in the municipality of San Pedro Lagunillas, Nayarit. Fruits were washed with water and disinfected with 2% sodium hypochlorite solution for 2 min, rinsed with sterile distilled water and allowed to dry in a biosafety hood for 10 min.

Fungal inoculum

P. citrinum spore´s suspension was prepared using one week old fungal cultures in a PDA Petri dish. Ten ml of a sterile distilled water containing 0.1% (v/v) Tween 80 and NaCl 0.9% (w/v), were added to the cultures and Petri dishes were scraped using a sterile loop. The liquid was filtered in sterile gauze and recovered in a dilution flask. Spore concentration was determined by microscopic counting in a hemocytometer.

Antagonistic yeast

D. hansenii DhhBCS03 originally was obtained from seawater samples collected at a depth of 100 m at the Cortés Sea (Baja California, Mexico) and belonging to the Yeast Collection of the CIBNOR A.C. The yeast strain was conserved at -80ºC in YE broth with 40% glycerol as cryoprotector (Guyot et al., 2000).

Pathogenicity test

In order to discard saprophytic fungi from phytopathogens, a pathogenicity test based on Koch´s Postulates was applied. A wound was performed using a sterile needle to artificially infect five limes with 10 µl of spore's suspension (10^5 spores/mL) of the fungus in study. The fruits were placed in plastic chambers (25 fruits per chamber) with elevated relative humidity (60-75%), at 25°C. For fungus re-isolation, cuts (1x1 cm) 50% affected surface and 50% healthy surface were made in the fruits flavedo with severe symptoms of disease. Tissue sections were superficially disinfected for 1 min (2% sodium hypochlorite), then rinsed with sterile distilled water and placed in the middle of PDA agar plates and incubated at 28°C for 8 days. Control fruits were inoculated with sterile distilled water.

In vitro antifungal assay

In order to evaluate the *in vitro* interactions between the antagonist and the fungus, inhibition for *P. citrinum* was tested in flavedo agar (FA) and albedo agar (AA). These media were prepared using 28 g of flavedo or albedo peelings from mature and healthy Persian limes and 8 g of agar dissolved in 400 mL of distilled water. Yeasts were re-suspended into sterile distilled water containing 10 mL of 0.1% Tween 80 (Hycel, México) (v/v) and 0.9% NaCl (w/v) and adjusted to concentration of $1x10^8$ cells/mL. Thirty µL of yeast suspension were added and incubated 24 h at 28ºC in flavedo and albedo agar, subsequently plugs (7 mm diameter) were cut from 7-days-old PDA cultures of *P. citrinum* and then were replaced in the medium and incubated at 28ºC. Diameter growth was registered during 7 days. The effect of *D. hansenii* on spore germination was tested in 5 ml of potato dextrose broth. 100 µL of yeast suspension (10^8 cells/mL) and at the same time 100 µL of fungal spores (10^5 spores/mL) were added and incubated on a rotary shaker at 110 rpm at 28°C. The germination rate of *P. citrinum* was determined microscopically by measuring the percentage of germinated spores every 2 h for 14 h in samples of approximately 200 spores (Spadaro, 2003). Spores were considered germinated when the germ tube length was equal to or longer than the spore diameter (Yao et al., 2004).

Biocontrol assessment *in vivo*

Five fruits with no apparent flavedo injury, similar size and randomly selected, were used per treatment. The surfactant Tween 80 at 2.5 ml/L and dextrose (Jalmek, Mexico) as additive at 2% (w/v) were added to the yeast suspension (Alvindia and Natsuaki, 2007; Guijarro et al., 2007). Persian lime were sprayed with approximately 2 mL per fruit with the antagonistic suspension (10^8 cells/mL) of the yeast plus the following treatments: containing Tween 80 + Dextrose, Dextrose alone, Tween 80 alone, and only yeast cells. An untreated control (UTC) was sprayed with 2 mL of sterile distilled water. Twenty µL of a suspension of 10^5 spores/mL was added to a fruit center wound made using a sterile needle. Fruits were stored at room temperature and high

relative humidity for 25 days in a plastic chamber. Plastic chamber used was 89 × 42.5 × 15.6 cm, 25 fruits were placed in the chamber. High relative humidity (60-75%) was maintained introducing 8 flasks containing 50 ml of distilled water. Disease incidence and damaged surface were measured on days 20, 22 and 25. Damaged surface was evaluated on a visual scale as a percentage, where 0% no symptoms showed; 25% quarter of the fruit surface showing symptoms; 50% half of the fruit; 75% three quarters of the fruit; and 100% entire fruit decayed surface.

Population dynamics of biological control agent

In order to determine the population dynamics, the same treatments applied at *in vivo* test but without pathogen addition were used. Fruits were individually immersed into a beaker with 100 mL of distilled water supplemented with Tween 80 (0.05%), shaken using a rotary shaker at 110 rpm (28°C) for 30 min. After that, an aliquot of 100 µL was inoculated into YPD culture medium and incubated at 28°C for 24 h. The counts were made during 25 days. Fruits sprayed with sterile distilled water were used as controls (UTC). Fruits were stored at room temperature and high relative humidity in plastic chambers.

Scanning electron microscopy

Wounds inoculated with both microorganisms were used to observe the arrangement of the hyphae, fungal conidia and yeast cells. Flavedo tissue samples using for treatments to evaluate the population dynamics were used to observe the presence of surface yeast. Samples were mixed by immersion in 2.5% glutaraldehyde in phosphate buffer at pH 7 for 24 h, rinsed with phosphate buffer and dehydrated through ethanol (30, 50, 70 and 100%), 2 h in each stage and two changes in 100% ethanol at room temperature. Then, a drying with a liquid CO_2 using its critical point for replacing ethanol and evaporation at high temperature was performed. Finally, a palladium plated surfaced was carried out. The specimens were observed under a scanning electron microscope (Hitachi S-3000N, San Jose, CA, USA) (Usall et al., 2001).

Data analysis

All treatments were replicated three times, 5 fruits were used per replicate for infected fruits and damaged surface, 3 fruits were used per replicate for population dynamics. The experiments were repeated twice. The data were analyzed by analysis of variance (ANOVA) with significance level set at $p<0.05$ with the statistical software SAS v. 9.0. Significant differences among treatments were established with the LSD test ($p<0.05$). In order to improve the homogeneity of variances, population dynamics data of *D. hansenii* (CFU/fruit) were transformed to logarithms (logCFU/fruit).

RESULTS AND DISCUSSION

Fungal isolation and identification

The pre-identification of fungi showed the following morphological characteristics: colony radially furrowed, velvety, dense; with mycelium pale yellow to grayish yellow; abundant conidiogenesis, grayish green to dull green; yellowish white to light yellow exudate. The identification of *P. citrinum* performed by PCR using the primers ITS1 and ITS4 gave a 519 bp fragment (Fig. 1). Sequences reported at BLAST v2.3.0 (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/) at National Center for the Biotecnology Information, NCBI, USA, were used to compare the obtained sequence. Then, the pathogen tested was identified with a homology of 100% (accession number KF986420.1) as *P. citrinum*.

Pathogenicity test

During the storage of Persian lime in high relative humidity conditions, disease symptoms appeared twelve days after incubation. Fruits inoculated with *P. citrinum* showed diffuse green spots on the flavedo (12th day) with the subsequent emergence of a spot located (yellow pale) in the area of the lesion with softening. The lesion changed of pale yellow to dark brown and extended through the lesion periphery within half of flavedo surface and absence of mycelium (19th day) (Fig. 2). Control fruits did not show damage on the flavedo surface. *P. citrinum* isolated from the fruit lesions presented macroscopic characteristics similar to the first inoculated fungus. The observed characteristics for *P. citrinum* on PDA culture were green fungus with white halo, velvety appearance and yellowish on the reverse of the plate. Microscopic characteristics (100×)

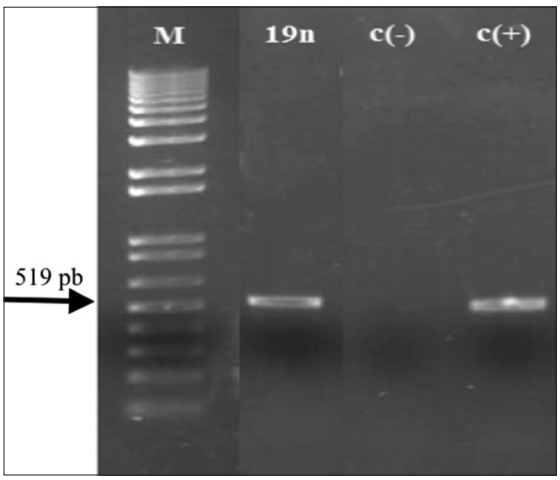

Fig 1. Gel electrophoresis of PCR products amplified from DNA extracted from *Penicillium citrinum*. The PCR products (519 pb) were amplified by the primers ITS1 and ITS4. M: Molecular weight marker, 1KB plus DNA ladder (Invitrogen); 19N: *Penicillium citrinum*; C-: Negative control; C+: Positive control *Aspergillus niger*.

for *P. citrinum* are readily recognized by its penicilli, which consist of 3-5 divergent and usually vesiculate metulae, bearing long well defined columns of conidia (Pitt and Hocking, 2009). These characteristics were observed in the re-isolated fungus. Thus, *P. citrinum* showed pathogenicity on Persian lime fruits. *P. citrinum* is usually reported as a pathogen fungus for cereals such as rice (*Oryza sativa*), wheat (*Triticum* L.), barley (*Hordeum vulgare* L.), corn grits and flour but not for citrus (Pitt and Hocking, 2009). The results of this study provide important information on the pathogenicity of this fungus on Persian lime from Nayarit. Worldwide rots caused by *Penicillium* spp. are important by the level of damage on citrus.

In vitro antifungal assay

In vitro antagonism of yeast *D. hansenii* against *P. citrinum* was observed. The obtained inhibition was 97.4% in albedo agar (AA) and 68% in flavedo agar (FA) ($p<0.05$). No germination was observed in the fungal spores in presence of yeast. The germination percentage for the untreated control was 90% (Data not shown). Hernández-Montiel et al. (2010b) obtained a 97.8% inhibition on albedo agar against *P. italicum* with an isolate of *D. hansenii* (DhhBCS03 marine isolated) and a 68.7% inhibition on flavedo agar. Hernández-Montiel et al. (2010b) also studied the content of sugars in flavedo (2.37 g/L sucrose, 1.12 g/L fructose and 0.46 g/L glucose) and albedo (2.05 g/L sucrose, 1.0 g/L fructose and 0.90 g/L glucose). They found a higher rate of glucose and sucrose consumption by *D. hansenii* than the pathogen *P. italicum*. Probably, the low content of sugars in the flavedo and albedo is likely to promote carbon competition between *D. hansenii* and *P. citrinum*, which could explain the result of a greater inhibition on albedo agar than on flavedo agar in this study. *In vitro* antagonism of *D. hansenii* also relates *killer* toxin production, as previously reported (Hernández-Montiel et al., 2010a). The toxin interacts specifically with the cell wall of the fungus through an identification of (1-6)-β-D-glucan as receptor (Santos, 2002). Cell death is then induced as a consequence of this process. *In vitro* assays in culture media prepared from fruit tissues provide a more realistic prediction of the colonization ability of biocontrol agents in fruits wounds (Nunes et al., 2001). Spores germination process can be divided into four stages: 1) breaking of dormancy's spore, 2) isotropic swelling, 3) establishment of cell polarity, and 4) the germ tube formation and maintenance of polar growth (Barhoom and Sharon, 2004). The activation (breaking dormancy) occurs in response to favorable environmental conditions like oxygen, carbon dioxide and water are required for initiation of the activation. It also requires additional factors such as: low molecular weight nutrients (sugars and amino acids) and inorganic salts (d'Enfert, 1997). The inhibition of germination as well as germ tube elongation of *P. citrinum* could be due by the consumption of sugars by *D. hansenii* favoring their development, leading to an antagonistic effect on competition for space and nutrients and limiting access of carbon sources to *P. citrinum*.

Biocontrol assessment *in vivo*

The disease was no detected (0% of disease incidence) on fruits treated with yeast suspension without adjuvants (10^8 cells/ml) (Fig. 3 and 4). However, fruits treated with the yeast suspension with adjuvants added; disease incidence values ranged from 20% (Tween 80 + Dextrose) to 60% (Tween 80) (Fig. 4). Likewise, the surface damage percentages were 10% (Tween 80 + Dextrose), 30% (Dextrose alone) and 15% (Tween 80 alone) at the 25th evaluation day (Fig. 5). The UTC at the end of the evaluation came up to 100% disease incidence and to 55% of damaged surface. *D. hansenii* produce enzymes β-1, 3 glucanase, chitinase and protease in presence of cell walls of the fungus *P. italicum* (Hernández-Montiel et al., 2010a). Thus, these enzymes could be involved in the *in vivo* inhibition of *P. citrinum*. Hydrolytic enzymes produced by yeasts and their interaction with fungi, results in collapse and disintegration of the hyphae and affect the infective process of the pathogen (Bautista-Rosales et al., 2013; Sharma et al., 2009). Hernández-Montiel et al. (2012) obtained a 0% disease incidence and absent of lesion when using 10^8 cells/mL of *D. hansenii* DhhBCS03 as inoculum in fruit stored at 25°C and 80% RH, coinciding with the results of this research. The level of protection by *D. hansenii* against fruit decay can be explained by the ability of the yeast to assimilate carbon sources from the wounds of the fruit to settle and multiply (Hernández-Montiel et al., 2012). Furthermore, as shown in Fig. 7C, the formation of biofilm by *D. hansenii* in wounds in Persian lime favors colonization and development of antagonistic yeast. Biofilms consist primarily of viable and nonviable microorganisms embedded in polyanionic extracellular polymeric substances (EPS) anchored to

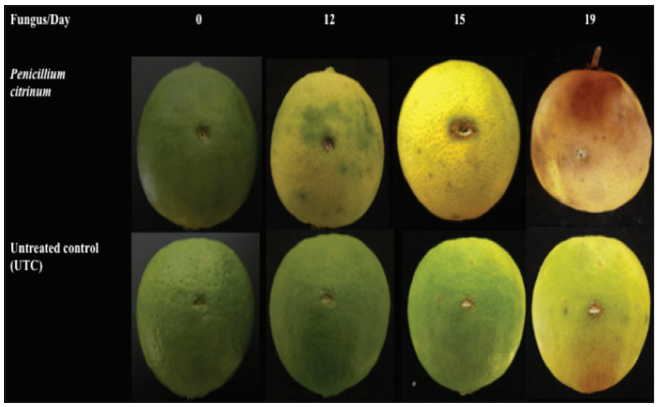

Fig 2. Evaluation of disease symptoms of fruits artificially infected with *P. citrinum*.

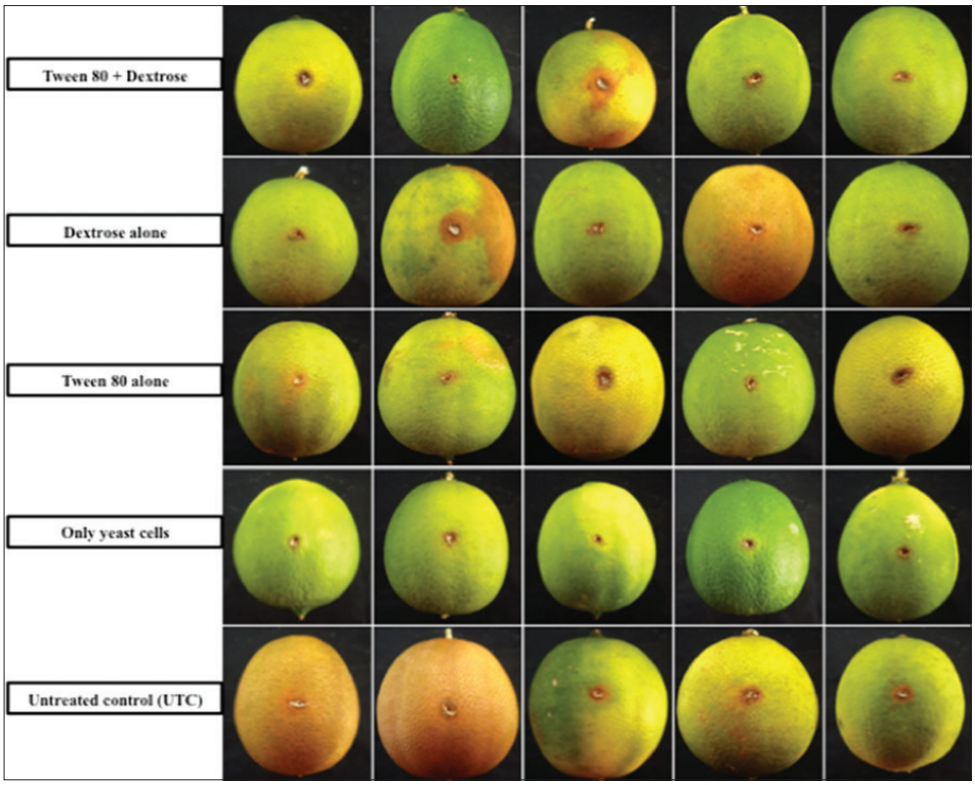

Fig 3. Fruit decay caused by *Penicillium citrinum* on Persian lime. Fruits were inoculated with *D. hansenii* (10^8 cells/mL) plus other additives. The UTC was added only with sterile distilled water.

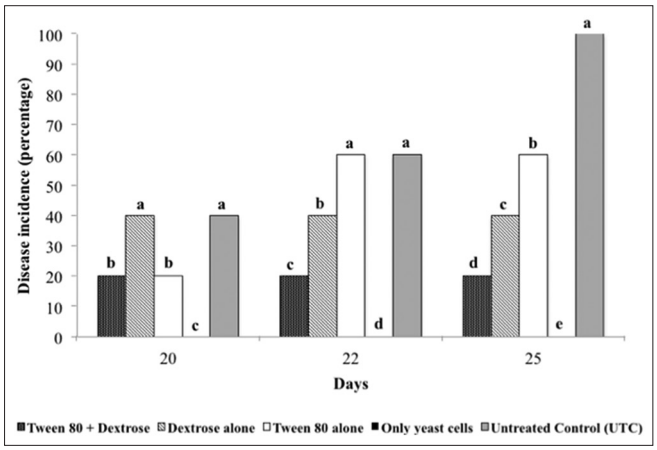

Fig 4. Disease incidence produced by *P. citrinum* in wounded Persian limes. Bars with the same letter are not significantly different (LSD, $p<0.05$).

a surface (Anderson et al., 2004). The EPS substances provide protection to microorganisms by concentrating nutrients, enhancing resistance to stresses and preventing desiccation (Chmielewski and Frank, 2003; Prakash et al., 2003; Droby et al., 2009; Elias and Banin, 2012). The ability to form biofilms on the inner surface of wounds was indicated as a possible mechanism of biocontrol (Giobbe et al., 2007). However, little is known about the role of biofilms in the biocontrol activity of antagonistic yeast used to manage postharvest diseases and mechanisms involved in its formation (Droby et al., 2009). Giobbe et al. (2007) observed that the biocontrol efficiency of *Pichia fermentans* (Lodder, Zentralblatt) against *M. fructicola* ((G. Winter) Honey) could therefore be due to the ability of yeast cells to remain adhered one to the other and to create a mechanical barrier (biofilm) interposing between the wound surface and the pathogen spore and germ tube, thus hampering infection by *M. fructicola.*

Population dynamics of biological control agent

Treatments with the antagonistic suspension containing the adjuvants Tween 80 + Dextrose, Dextrose alone and Tween 80 alone favored the permanency of yeast on flavedo with initial population of $3.0x10^8$ CFU/fruit (Dextrose alone) to a final population at 25th day of $3.2x10^8$ CFU/fruit (Tween 80 alone). On the other hand, a decrease in the yeast population on fruits treated with yeast suspension without adjuvants was observed ($p<0.05$): initial population (time zero) was $1.1x10^8$ CFU/fruit and the final population was $3.3x10^5$ CFU/fruit (25th day). No growth was observed in UTC, which was sprayed only with distilled water (Fig. 6). The differences in the colonization reached by *D. hansenii* on Persian lime could be explained by the use of dextrose and Tween 80. It was observed by SEM that yeast are covered by a film formed by the Tween 80, acting as a barrier at surface, and probably limiting yeast colonization on fruit wounds (Fig. 7A). Attachment to the fruit surface is probably an

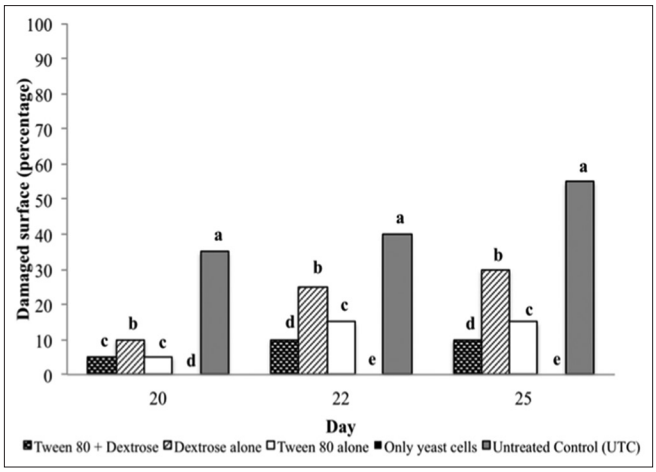

Fig 5. Percentage of damaged surface produced by *P. citrinum* in wounded Persian lime. Bars with the same letter are not significantly different (LSD, p<0.05).

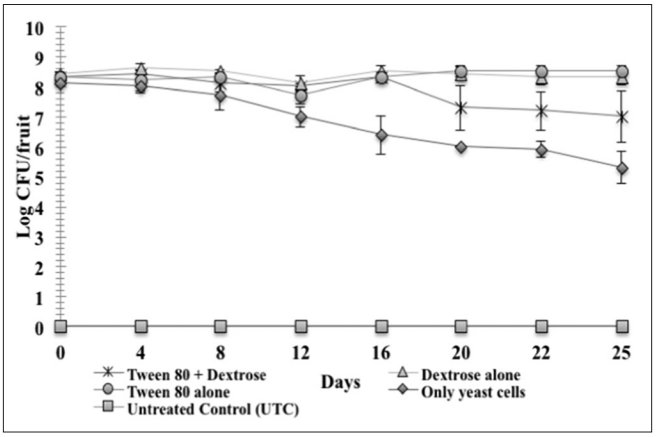

Fig 6. Population dynamics of *D. hansenii* on Persian lime treated with *D. hansenii* (10^8 CFU/mL) plus other additives.

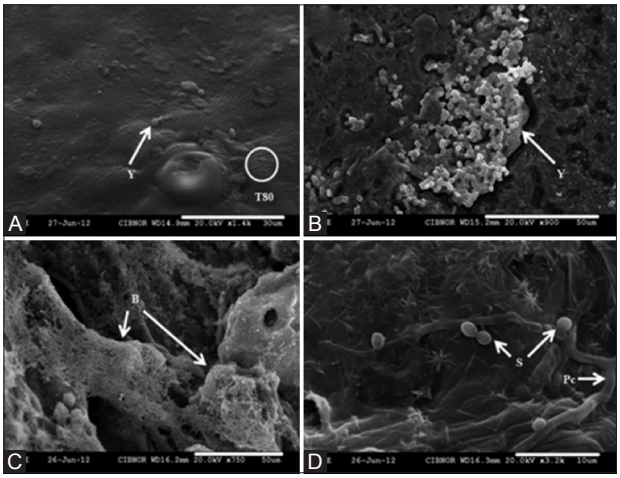

Fig 7. Population of *D. hansenii* on Persian lime (A and B) and interaction of *D. hansenii* with *P. citrinum* in wounds (C and D). A) Film of Tween 80 with yeast entrapped, B) Flocculation of yeast (Y), C) Biofilm (B) formed by yeast and D) Spore (S) and mycelium penetrating wounds infected by *Penicillium citrinum* (Pc).

important trait for the antagonists to possess for successful applications, since persistent attachment would contribute to a better colonization and avoids dislodging due to wind, rain or water level fluctuations (Ippolito and Nigro, 2000). The use of surfactants as part of a formulation technology may overcome environmental conditions limiting colonization, they have slow evaporation, aid in coverage, attract and bind water to the surface to prevent rapid drying and create films on the surface that hold in moisture (Bailey et al., 2007). The results suggest that formation of a film on flavedo by Tween 80 could have a protective effect of biological control agent of storage conditions. Cañamás, et al. (2008) obtained a similar result by using of Fungicover as adjuvant, which seems to bring a protective effect to the biocontrol agent against adverse environmental conditions. Surfactants as Tween 80 are generally used into the solution to improve its performance and effectiveness, those compounds cause reduction in surface tension and alter the energy relationships at interfaces, due to presence of both hydrophobic and hydrophilic group within the same molecule, these compounds adjust themselves as interfaces (Singh and Khan, 2012). On the other hand, a simple and efficient method to improve antagonistic activity is the use of additives like sucrose and yeast extract to increase the population and enhance the survival rate of the microbial antagonist (Ippolito and Nigro, 2000). De Cal et al. (2012) found that the population of biocontrol agent *Penicillium frequentans* Westling on surface peach was higher when treatments had additives (dextrose) than those treatments where no additives were used. In this study, fruits applied with only yeast, antagonistic yeast was unable to maintain high population levels on surface's fruit, which may be due to their sensitivity to storage conditions used (25°C and 60-75% HR) and to low nutrient availability. The lack of moisture limits the development of biocontrol agents (Lahlali, 2004). The population of *Candida oleophila* Montrocher on apples 'Golden delicious' (*Malus* × *domestica* Borkh) increased quickly when water was periodically applied on fruit, which could explain the low population dynamics in the result obtained by applying the treatment with only yeast (10^8 cells/mL). In this study, it shows that the treatment with surfactant Tween 80 promotes the maintenance of humidity resulted in a higher population of *D. hansenii*. Some yeasts can colonize plant surfaces or wounds for long periods under dry conditions and produce extracellular polysaccharides that enhance their survival and restrict pathogen colonization sites (Benbow and Sugar, 1999). Then it is necessary to deep in the knowledge of conditions that promote the formation of biofilm in Persian lime by *D. hansenni* DhhBCS03 in order to improve the colonization of this strain. For further applications it could be useful to consider the use of adjuvants as dextrose

and Tween 80 in order to improve the colonization of the yeast in fruits.

Scanning electron microscopy

A film formation on fruit surface in treatments added with Tween 80 (Fig. 7A) and extensively population of yeast (Fig. 7B) were observed. Biofilm formation was observed in fruits where the disease had not progressed (Fig. 7C). Spore germination and penetration of mycelium of *P. citrinum* into the fruits were extensive in infected fruits (Fig. 7D).

CONCLUSION

The application of *Debaryomyces hansenii* was found to be effective in controlling infection by *P. citrinum*. The population dynamics of the antagonist was enhanced with the application of adjuvants Tween 80 and dextrose on fruit surface. The application of *D. hansenii* as biological control agent can be an alternative of postharvest control of *P. citrinum* in Persian lime.

ACKNOWLEDGMENTS

The authors are grateful for the financial support of SAGARPA-CONACYT for the project "Validación de un paquete tecnológico para el control de enfermedades precosecha en cítricos 2009-10-98-79" and CONACYT for the fellowship granted to Ramsés Ramón González Estrada.

Authors´ contributions

MCS: Design, formulation and supervision of experiment with writing and review of manuscript. RRGE: Collection of experimental data and writing of manuscript. FJAV: Design and supervision of strains isolation and identification. JARS: Supervision and statistical analysis of data.

REFERENCES

Alvindia, D. G. and K. T. Natsuaki. 2007. Control of crown rot-causing fungal pathogens of banana by inorganic salts and a surfactant. Crop Prot. 26: 1667-1673.

Anderson, P. K., A. A. Cunningham, N. G. Patel, F. J. Morales, P. R. Epstein and P. Daszak. 2004. Emerging infectious diseases of plants: Pathogen pollution, climate change and agrotechnology drivers. Trends Ecol. Evol. 19: 535-544.

Bailey, K. L., O. Carisse, M. Leggett, G. Holloway, F. Leggett, T. Wolf, M. Shivpuri, A. Derby, J. A. B. Caldwell and H. J. Geissler. 2007. Effect of spraying adjuvants with the biocontrol fungus *Microsphaeropsis ochracea* at different water volumes on the colonization of apple leaves. Biocontrol Sci. Technol. 17: 1021-1036.

Barhoom, S. and A. Sharon. 2004. cAMP regulation of "pathogenic" and "saprophytic" fungal spore germination. Fungal Genet. Biol. 41: 317-326.

Bautista-Rosales, P. U., M. Calderon-Santoyo, R. Servín-Villegas, N. A. Ochoa-Álvarez and J. A. Ragazzo-Sánchez. 2013. Action mechanisms of the yeast *Meyerozyma caribbica* for the control of the phytopathogen *Colletotrichum gloeosporioides* in mangoes. Biol. Control. 65: 293-301.

Benbow, J. M. and D. Sugar. 1999. Fruit surface colonization and biological control of postharvest diseases of pear by preharvest yeast applications. Plant Dis. 83: 839-844.

Brown, G. E. and J. W. Eckert. 1988. Green mold. Compendium of Citrus Diseases, APS Press, St. Paul, Minnesota.

Cañamás, T. P., I. Viñas, J. Usall, R. Torres, M. Anguera and N. Teixidó. 2008. Control of postharvest diseases on citrus fruit by preharvest applications of biocontrol agent *Pantoea agglomerans* CPA-2: Part II. Effectiveness of different cell formulations. Postharvest Biol. Technol. 49: 96-106.

Chalutz, E. and C. L. Wilson. 1990. Postharvest biocontrol of green and blue mold and sour rot of citrus fruit by *Debaryomyces hansenii*. Plant Dis. 74: 134-137.

Chmielewski, R. and J. Frank. 2003. Biofilm formation and control in food processing facilities. Compr. Rev. Food Sci. Food Saf. 2: 22-32.

d'Enfert, C. 1997. Fungal spore germination: Insights from the molecular genetics of aspergillus nidulans and *Neurospora crassa*. Fungal Genet. Biol. 21: 163-172.

De Cal, A., I. Larena, B. Guijarro and P. Melgarejo. 2012. Use of biofungicides for controlling plant diseases to improve food availability. Agriculture. 2: 109-124.

Droby, S., S. Lischinski, L. Cohen, B. Weiss, A. Daus, T. Chand-Goyal, J. W. Eckert and S. Manulis. 1999. Characterization of an epiphytic yeast population of grapefruit capable of suppression of green mold decay caused by *Penicillium digitatum*. Biological Control. 16: 27-34.

Droby, S., V. Vinokur, B. Weiss, L. Cohen, A. Daus, E. E. Goldschmidt and R. Porat. 2002. Induction of resistance to *Penicillium digitatum* in grapefruit by the yeast biocontrol agent *Candida oleophila*. Phytopathology. 92: 393-399.

Droby, S., M. Wisniewski, D. Macarisin and C. Wilson. 2009. Twenty years of postharvest biocontrol research: Is it time for a new paradigm? Postharvest Biol. Technol. 52: 137-145.

Eckert, J. W. and I. Eaks. 1989. Postharvest disorders and diseases of citrus fruits. Citrus Ind. 5: 179-260.

Elias, S. and E. Banin. 2012. Multi-species biofilms: Living with friendly neighbors. FEMS Microbiol. Rev. 36: 990-1004.

Fernández, E. 2000. Microbiología e Inocuidad de Los Alimentos, Universidad Autónoma de Querétaro, México.

Giobbe, S., S. Marceddu, B. Scherm, G. Zara, V. L. Mazzarello, M. Budroni and Q. Migheli. 2007. The strange case of a biofilm-forming strain of *Pichia fermentans*, which controls *Monilinia brown* rot on apple but is pathogenic on peach fruit. FEMS Yeast Res. 7: 1389-1398.

Guijarro, B., P. Melgarejo and A. De Cal. 2007. Effect of stabilizers on the shelf-life of *Penicillium frequentans* conidia and their efficacy as a biological agent against peach brown rot. Int. J. Food Microbiol. 113: 117-124.

Guyot, J. P., M. Calderon and J. Morlon-Guyot. 2000. Effect of pH control on lactic acid fermentation of starch by *Lactobacillus manihotivorans* LMG 18010T. J. Appl. Microbiol. 88: 176-182.

Hernández-Montiel, L. G., R. J. Holguin-Pena, C. P. Larralde-Corona, R. Zulueta-Rodriguez, E. Rueda-Puente and M. Moreno-Legorreta. 2012. Effect of inoculum size of yeast *Debaryomyces hansenii* to control *Penicillium italicum* on Mexican lime (*Citrus aurantiifolia*) during storage. CyTA J. Food. 10: 235-242.

Hernández-Montiel, L. G., C. P. Larralde-Corona, S. Vero, M. G. López-Aburto, J. L. Ochoa and F. Ascencio-Valle. 2010a. Characterization of yeast *Debaryomyces hansenii* for the biological control of blue mold decay of Mexican lemon. CyTA J. Food. 8: 49-56.

Hernández-Montiel, L. G., J. L. Ochoa, E. Troyo-Diéguez and C. P. Larralde-Corona. 2010b. Biocontrol of postharvest blue mold (*Penicillium italicum* Wehmer) on Mexican lime by marine and citrus *Debaryomyces hansenii* isolates. Postharvest Biol Technol. 56: 181-187.

Ippolito, A. and F. Nigro. 2000. Impact of preharvest application of biological control agents on postharvest diseases of fresh fruits and vegetables. Crop Prot. 19: 715-723.

Lahlali, R. and M. Jijakli. 2004. Evaluation of population density of *Pichia anomala* strain K and *Candida oleophila* strain O and their protection against *Penicillium expansum* link on apples. IOBC/ WPRS Bull. 27: 340-346.

Liu, J., Y. Sui, M. Wisniewski, S. Droby and Y. Liu. 2013. Review: Utilization of antagonistic yeasts to manage postharvest fungal diseases of fruit. Int. J. Food Microbiol. 167(2): 153-160.

Medina-Córdova, N., R. López-Aguilar, F. Ascencio, T. Castellanos, A. I. Campa-Córdova, and C. Angulo. 2016. Biocontrol activity of the marine yeast *Debaryomyces hansenii* against phytopathogenic fungi and its ability to inhibit mycotoxins production in maize grain (*Zea mays* L.). Biol. Control. 97: 70-79.

Nunes, C., J. Usall, N. Teixidó, M. Miró and I. Viñas. 2001. Nutritional enhancement of biocontrol activity of *Candida* sake (CPA-1) against *Penicillium expansum* on apples and pears. Eur. J. Plant Pathol. 107: 543-551.

Ochoa, J., L. Hernández-Montiel, H. Latisnere-Barragán, J. L. de La Luz and C. Larralde-Corona. 2007. Isolation and identification of pathogenic fungi from orange citrus sinensis L. Osbeck cultured in Baja California Sur, México. CyTA J. Food. 5: 352-359.

Pitt, J. and A. Hocking. 2009. Fungi and Food Spoilage, Springer Science, New York, NY, USA.

Prakash, B., B. M. Veeregowda and G. Krishnappa. 2003. Biofilms: A survival strategy of bacteria. Curr. Sci. 85: 1299-1307.

Sambrook, J. and D. W. Russell. 2001. Molecular Cloning. A Laboratory Manual, Cold Pring Harbor Laboratory Press, New York.

Santos, A., D. Marquina, J. Barroso and J. Peinado. 2002. ß-D-glucan as the cell wall binding site for *Debaryomyces hansenii* killer toxin. Lett. Appl. Microbiol. 34: 95-99.

Sharma, R., D. Singh and R. Singh. 2009. Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A review. Biol. Control. 50: 205-221.

SAGARPA, 2012. México, entre los líderes en producción de cítricos a nivel mundial. http://www.sagarpa.gob.mx/Delegaciones/sanluispotosi/boletines/Paginas/BOL1301112.aspx.[Last accessed on 2017 Jan 25].

Singh, Z. and A. S. Khan. 2012. Surfactant and nutrient uptake in citrus. In: Advances in Citrus Nutrition, Springer, New York, USA, pp. 157-167.

Spadaro, D. 2003. Biological Control of Postharvest Diseases of Pome Fruit using Yeast Antagonists, Università degli studi di Torino, Torino, Italia.

Spadaro, D. and S. Droby. 2016. Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists. Trends Food Sci. Technol. 47: 39-49.

Taqarort, N., A. Echairi, R. Chaussod, R. Nouaim, H. Boubaker, A. A. Benaoumar and E. Boudyach. 2008. Screening and identification of epiphytic yeasts with potential for biological control of green mold of citrus fruits. World J. Microbiol. Biotechnol. 24: 3031-3038.

Usall, J., N. Teixido, R. Torres, X. Ochoa de Eribe and I. Viñas. 2001. Pilot tests of *Candida* sake (CPA-1) applications to control postharvest blue mold on apple fruit. Postharvest Biol. Technol. 21: 147-156.

White, T. J., T. Bruns, S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: A Guide to Methods and Applications, Vol. 18. Academic Press, Inc., New York, NY, pp. 315-322.

Yao, H., S. Tian and Y. Wang. 2004. Sodium bicarbonate enhances biocontrol efficacy of yeasts on fungal spoilage of pears. Int. J. Food Microbiol. 93: 297-304.