Microbiological Research 188-189 (2016) 72–79

Contents lists available at ScienceDirect

Microbiological Research

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

Biocontrol ability and putative mode of action of yeasts against *Geotrichum citri-aurantii* in citrus fruit

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ARTICLE INFO

Article history:
Received 12 January 2016
Received in revised form 6 April 2016
Accepted 27 April 2016
Available online 28 April 2016

Keywords:
Citrus spp.
Hydrolytic enzymes
Killer factor
Sour rot

ABSTRACT

Sour rot is a major postharvest disease of citrus fruit and is caused by the fungal pathogen *Geotrichum citri-aurantii*. A lack of chemicals certified for the control of this disease has led to the consideration of alternative methods and strategies, such as the use of yeasts as biocontrol agents. The purpose of the present study was to test the ability of yeasts isolated from leaves, flowers, fruit, and soil, and six *Saccharomyces cerevisiae* isolates to control citrus sour rot, to assess the mechanisms of action of the yeast isolates that were demonstrated to be effective for biocontrol, and to identify the most effective yeast isolates for the biocontrol of *G. citri-aurantii.* In *in vivo* assays, three yeast isolates (ACBL-23, ACBL-44, and ACBL-77) showed a potential for controlling sour rot in citrus fruits, both preventatively and curatively. Most of the eight yeast isolates that were assessed for a mechanism of action did not produce antifungal compounds in an amount sufficient to inhibit the growth of the pathogen. Additionally, nutrient competition among the yeast strains was not found to be a biocontrol strategy. Instead, *killer* activity and hydrolytic enzyme production were identified as the major mechanisms involved in the biocontrol activity of the yeasts. Isolates ACBL-23, ACBL-44, and ACBL-77, which controlled sour rot most effectively, were identified as *Rhodotorula minuta*, *Candida azyma, and Aureobasidium pullulans*, respectively. To our knowledge, this is the first report of the potential of *C. azyma* as a biological control agent against a postharvest pathogen and its ability to produce a *killer* toxin.

1. Introduction

Citrus spp. are the most widely produced fruits in the world. Fruit production is mainly devoted to juice extraction, but a considerable proportion is traded as fresh fruit for direct consumption (Palou et al., 2015). Citrus fruits are usually quite acidic, and for this reason, the most common decay process that occurs in harvested fruits is caused by fungi (Talibi et al., 2014).

Sour rot is a major disease that occurs in postharvest citrus and is caused by the fungus *Geotrichum citri-aurantii* (Ferraris) Butler et al. (1988). This disease is widespread in all citrus-producing countries and affects all citrus species and cultivars. However, mandarin varieties generally develop the disease more readily than orange and grapefruit varieties (Brown, 2003).

Sour rot cannot be efficiently controlled by registered fungicides such as imazalil, thiabendazole, pyrimethanil, or fludioxonil, which can adequately control the incidence of green or blue molds (Liu et al., 2009; Zhou et al., 2014). The synthetic fungicides guazatine (Brown, 1988) and propiconazole (McKay et al., 2012) can control sour rot, but these fungicides are not registered in Brazil. A lack of chemicals certified for the control of this disease has led to the consideration of alternative methods. In this context, the use of yeast species represents a promising strategy in light of their ability to colonize and survive on the fruit surface for an extended period (Lahlali et al., 2011; Spadaro and Droby, 2016). Specifically, yeasts have phenotypic adaptations for colonizing leaf, fruit, and vegetable surfaces that allows them to compete for space and nutrients, giving them an advantage as biocontrol agents for postharvest pathogens (Filonow, 1998).

When used as biocontrol agents, different yeast species can prevent infection, decrease host tissue colonization, and reduce pathogen survival and sporulation with varying degrees of efficiency (Punja and Utkhede, 2003; Kupper et al., 2013). Recent

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http://dx.doi.org/10.1016/j.micres.2016.04.012
0944-5013/

research has shown the potential of yeasts to control postharvest fungal diseases (Platania et al., 2012; Kupper et al., 2013; Moretto et al., 2014; Liu et al., 2013).

Despite the known biological roles of yeasts in the environment, much remains to be discovered regarding yeast modes of action of in specific environments and their antagonistic behavior toward other organisms (Rosa-Magri et al., 2011). According to Droby et al. (1989), nutrient competition between *Candida guilliermondii* (strain US 7) and *Penicillium digitatum* may play a role in the biocontrol of green mold of citrus fruit. Some yeast species produce hydrolytic enzymes such as β-glucanases and chitinases, which destroy the cell walls of other fungi (Saligkarias et al., 2002). Santos et al. (2004) showed that the yeast *Pichia membranifaciens* (CYC 1106) controlled *Botrytis cinerea,* a causal agent of grey mold disease in apples, by producing a *killer* toxin.

In this context, the purpose of the present study was to test the ability of yeasts isolated from leaves, flowers, fruits, and soil, and six isolates of *Saccharomyces cerevisiae* to control citrus sour rot, to assess the mechanisms of action of the yeast isolates that were shown to be effective for biocontrol, and to identify the most effective yeast isolates for the biocontrol of *Geotrichum citri-aurantii.*

2. Material and methods

2.1. Pathogen

The pathogen (*Geotrichum citri-aurantii*) used in this study was obtained from the collection of micro-organisms at the Center Citrus "Sylvio Moreira"/IAC, Cordeirópolis, São Paulo, Brazil.

2.2. Yeasts isolation

The yeast biological control agents were isolated from citrus leaves, flowers, fruits and citrus-growing soils. They were obtained from 14 citrus producing regions in the state of São Paulo, Brazil. Yeast isolation was carried out according to Azeredo et al. (1998) using the decimal dilution technique, in which an 0.85% saline solution was used for the soil, and a sterile wash solution was used for leaves, flowers and fruits with agitation at 250 rpm for 30 min. Plating was performed in triplicate using YEPD culture media (Yeast Extract Peptone Dextrose-1% yeast extract, 2% peptone, 2% glucose, 2% agar, 0.01% ampicillin and 0.01% nalidixic acid dissolved in 1 L of distilled water), YM (Malt Extract-0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 1% glucose, 2% agar dissolved in 1 L of distilled water) and WLN [Wallerstein Nutrient Broth-80 g of WL Nutrient medium (Acumedia®) dissolved in 1 L of medium, 0.01% of ampicillin and 0.01% nalidixic acid]. The cultures were incubated in a BOD (Biochemical Oxygen Demand) chamber at 25 °C for 3–7 days with a 12 h photoperiod. Yeast colonies that had distinct morphologies were isolated and observed under an ordinary optical microscope. The isolates were then confirmed as yeasts by colony morphology and examination of the vegetative cells. They were purified and kept in test tubes on slopes of YEPD culture medium for later testing.

2.3. Screening of the yeast isolates for antifungal activity in vitro

To conduct the antagonism tests, in addition to the 95 yeast isolates obtained, six isolates of *Saccharomyces cerevisiae* (ACB-BG1, ACB-CAT1, ACB-CR1, ACB-K1, ACB-KD1 and ACB-PE2) were also tested. These isolates were obtained from an ethanol fermentation, characterized by electrophoretic karyotyping and stored in the collection of the Laboratory of Biochemistry and Plant Pathology at the University if São Paulo (ESALQ), Piracicaba-São Paulo, Brazil (Fialho et al., 2010), and later stored in the microorganism collection of the Apta Center Citrus "Sylvio Moreira"/IAC, Cordeirópolis, São Paulo State, Brazil.

We used a paired cultivation technique in Petri dishes (Dennis and Webster, 1971) to evaluate the antagonistic capacity of all 101 yeast isolates against *G. citri-aurantii*. Disks of 5 mm in diameter were removed from the active colonies of *G. citri-aurantii* grown in potato dextrose agar (PDA) medium for 7–10 days and placed in Petri dishes containing PDA culture medium 3 cm away from disks of the same size containing an isolate of each yeast cultured for 48 h in YEPD medium. The control contained *G. citri-aurantii* without the yeast. To evaluate the inhibition, the average diameter of the *G. citri-aurantii* colony was measured after seven days. A completely randomized design with four replications for each of the 5 trials was used. The data were subjected to an analysis of variance (ANOVA). The means were tested by Tukey's test at 5% probability.

2.4. In vivo antagonistic activity assays

Fruits of commercially mature 'Pera' orange (*Citrus sinensis* (L.) Osbeck) were obtained from a packinghouse in the city of Limeira, São Paulo, Brazil. The untreated oranges were washed, superficially disinfected with 0.2% (v/v) sodium hypochlorite for 3 min and rinsed in distilled water to eliminate the sodium hypochlorite. Then, the fruits were wounded to a depth of 3 mm at two equidistant points on the median region with sterile needles, and 20 μL of a *G. citri-aurantii* conidial suspension (1×10^5 conidia/mL) was inoculated in the wounded area, which was treated 24 h later with the yeast (the curative treatment). For the preventive treatment, the fruits were wounded, treated with the yeast and inoculated with the pathogen 24 h later.

Eight yeast strains (ACBL-23; ACBL-42; ACBL-44; ACBL-50; ACBL-52; ACBL-68; ACBL-77 and ACB-K1) were selected based on *in vitro* screening to compose *in vivo* assay treatments. The treatments were prepared by transferring each isolate from a 48-h plate culture into an Erlenmeyer flask containing 25 mL of liquid YEPD. The cultures were incubated at 25 °C on a shaker at 250 rpm for 48 h. After incubation, the cell number was determined by a direct microscopic count using a hemacytometer, and the suspensions were adjusted to 1×10^7 cells/mL. A 20 μL aliquot of each treatment was applied with an automatic pipette directly into the wounds in both the curative and preventive treatments. The fungicide imazalil (2.0 mLL^{-1}) was included as a treatment; although it is not effective in controlling the disease, this product is widely used in packinghouses in Brazil. The positive control was fruit treated with sterile distilled water instead of another treatment.

After inoculation with *G. citri-aurantii* and treatment application, the fruits were stored for 15 days at room temperature (25 ± 5 °C and 90% RH). Disease severity was assessed on the 9th, 11th, 13th and 15th days after pathogen inoculation by measuring the average diameter of sour rot lesions using a caliper. The incidence was expressed as the percentage of infected fruits observed at day 15. To analyze the *in vivo* tests, the treatments were arranged in a completely random design, with each treatment consisting of 3 replicates with 20 fruits per replicate. To analyze the effect of the treatments on the average size of the lesions caused by *G. citri-aurantii*, the area under the disease progress curve (AUDPC) was calculated and expressed by plotting the proportion of disease as a percentage versus time. According to Shaner and Finney (1977), the AUDPC can be calculated using the formula:

$$\text{AUDPC} = \sum^{n-1}[(Y_{i+1} + Y_i)/2] * [(T_{i+1} - T_i)],$$

where n—the number of observations/evaluations; Yi—proportion of the disease in the ith observation/evaluation; Ti—the time in days in the ith observation/evaluation; The results were analyzed by

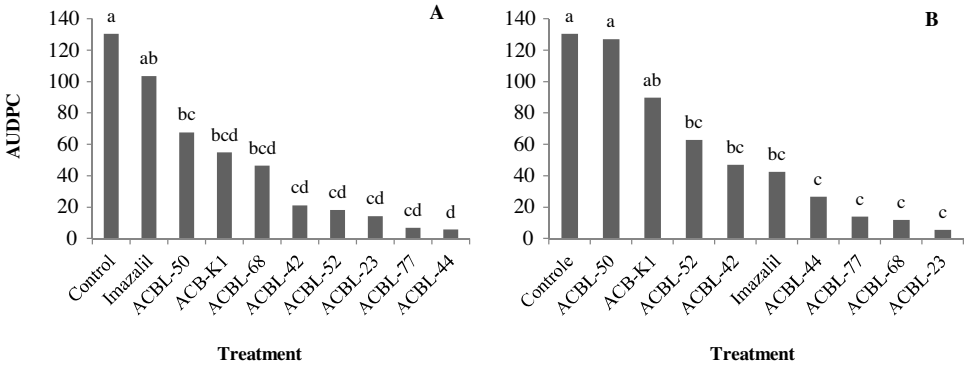

Fig. 1. Area under the disease-progress curve (AUDPC) for average diameter of lesion caused by *Geotrichum citri-aurantii*, after preventive (A) and curative (B) treatment with different yeast isolates, stored (25 ±2 °C and 90% RH) for 15 days. Mean values marked with the same lowercase letter are not significantly different according to Tukey's test ($P < 0.05$).

ANOVA, and the means were compared using the Tukey's test at 5% probability.

2.5. Study of the mechanisms of action

Eight yeast isolates (ACBL-23; ACBL-42; ACBL-44; ACBL-50; ACBL-52; ACBL-68; ACBL-77 and ACB-K1) that were shown to be effective for the *in vivo* biocontrol of sour rot were used.

2.5.1. Production of volatile antifungal compounds

Each of the yeast isolates was simultaneously cultivated with *G. citri-aurantii* on split plates, which prevented nonvolatile compounds produced by the yeasts from reaching the plant pathogen. A *G. citri-aurantii* culture disk (5 mm in diameter) was placed on PDA medium on one side of the split plate. On the other side, a yeast strain culture disk (5 mm) was placed on YEPD medium. After incubating the fungus and yeast strains at 25 °C for 10 days, fungal growth was measured as the mycelial diameter in the presence of the yeast compared to the mycelial diameter in the absence of the yeast. This methodology was according to Lopes et al. (2015).

2.5.2. Production of cell-free antifungal compounds in yeast

For each yeast isolate tested, a loopful of a 48 h culture was transferred to a 250 mL Erlenmeyer flask containing 50 mL of YEPD medium, followed by incubation at 150 rpm for 72 h in the dark.

Each yeast culture was filtered through Whatman N °4 filter paper and a 0.45 μm Millipore® membrane after incubation to remove the yeast cells according to a protocol adapted from Frighetto and Melo (1995). For each yeast cell-free filtrate, a 10 mL aliquot was added to 90 mL of melted PDA, then poured into a Petri dish. After solidification, a 5 mm *G. citri-aurantii* culture disk was placed in the center of each Petri dish. For the control, *G. citri-aurantii* was grown on PDA medium without a yeast filtrate. The cultures were incubated in a BOD chamber at 25 °C for 7 days with a 12 h photoperiod, and the growth of *G. citri-aurantii* was assessed by measuring the mean diameter of each colony in two perpendicular directions.

2.5.3. Production of thermostable antifungal compounds

To evaluate the production of thermostable compounds, a yeast culture disk was transferred to a 250 mL Erlenmeyer flask containing 50 mL YEPD and incubated at 150 rpm for 72 h in the dark, as previously described. A 10 mL aliquot of each isolate was transferred to an Erlenmeyer flask containing 90 mL PDA medium and sterilized at 121 °C for 20 min. The sterilized medium was poured into a Petri dish, and after it solidified, a 5-mm *G. citri-aurantii* culture disk was placed in center of each plate. For the control, *G. citri-aurantii* was grown on PDA medium without metabolites. The cultures were incubated in a BOD chamber at 25 °C for 7 days, and the growth *of G. citri-aurantii* was assessed by measuring the mean diameter of each colony in two perpendicular directions.

2.5.4. Detection of killer activity

According to the methodology described by Ceccato-antonini et al. (2004), a 1.0×10^5 cell/mL suspension of *S. cerevisiae* NCYC 1006 (*killer*-factor sensitive) was cultured in YEPD at 28 °C for 24 h. A 100 μL aliquot was transferred to a Petri dish containing YEPD-methylene blue medium buffered at pH 4.3–4.7. To evaluate the presence of a *killer* factor, each yeast isolate was spotted on a separate Petri dish with a sterile toothpick, and the cultures were incubated at 28 °C for 3 days. The production of a *killer* factor and the death of sensitive cells were indicated by the presence of a growth inhibition zone and an adjacent blue zone.

2.5.5. Nutrient competition

To evaluate nutrient competition between *G. citri-aurantii* and antagonistic yeasts, agar-coated microscope slides were prepared with varying glucose concentrations (0%, 0.5%, 1%, 1.5%, 2%, and 2.5%) according to methodology of Kupper et al. (2013). Ten microliters of a *G. citri-aurantii* suspension (1×10^5 conidia/mL) and 10 μL of a yeast suspension (1×10^8 cells/mL) were spotted onto premarked locations on the slides, and the cultures were incubated in a BOD chamber at 25 °C for 16 h. Nutrient competition was assessed by counting the number of germinated conidia among 100 randomly selected conidia. Conidia were considered fully germinated when the length of the germ tube was at least the size of the swollen conidia.

2.5.6. Evaluation of the mycelial growth of G. citri-aurantii collected from the antagonism zone

G. citri-aurantii was co-cultured on a Petri dish with each yeast isolate studied, according to the methodology described by Dennis and Webster (1971). The test was performed on Petri dishes containing PDA medium by placing a 0.5 cm *G. citri-aurantii* mycelial disk on one side of the plate and a 0.5 cm yeast isolate culture disk 3 cm away. To provide a detailed characterization of the inhibition zone formed after the co-culture of the antagonistic yeast isolates and fungus, the hyphal viability and the mycelial growth of the pathogen in the antagonism zone (i.e., the area adjacent to the fungal inhibition zone) were quantified. To measure the mycelial growth, 0.5 cm mycelial disks were cut from the following three sections of each plate and compared: the inhibition zone, the zone adjacent to the inhibition zone (the intermediate zone), and the zone furthest from the inhibition zone where mycelia were not inhibited by the yeast (the distant zone). The cultures were incubated in a BOD chamber at 25 °C for 10 days with a 12 h photoperiod,

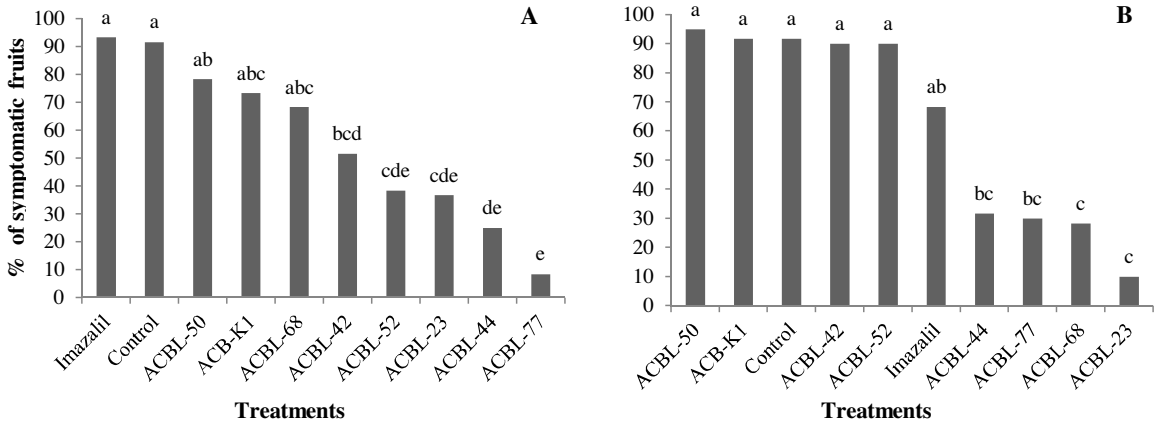

Fig. 2. Percentage of 'Pera' orange fruits with sour rot symptoms after preventive (A) and curative (B) treatment with different yeast isolates, stored (25 ± 2 °C and 90% RH) for 15 days. Mean values marked with the same lowercase letter are not significantly different according to Tukey's test ($P < 0.05$).

after which the mycelial growth diameter of *G. citri-aurantii* was measured in two perpendicular directions.

2.5.7. Production of hydrolytic enzymes

The production of hydrolytic enzymes by the yeast isolates was analyzed according to the methodology of Fialho (2005). A loopful of each yeast isolate was transferred to 20 mL liquid YEPD and incubated at 150 rpm for 72 h in the dark. Thereafter, 1 mL of each suspension was transferred to 15 mL Falcon tubes containing 10 mL of YEPD or modified YEPD medium (containing a *G. citri-aurantii* cell wall preparation in the place of 1% glucose). The cultures were prepared in triplicate and incubated at 150 rpm. After 24 h incubation, a 1.5 mL volume was taken and centrifuged at 3000 rpm for 10 min, and the supernatant was recovered and used for the quantification of β-1, 3-glucanase and chitinase.

For the cell wall preparation, 1 mL of a *G. citri-aurantii* suspension (1.0×10^5 conidia/mL) was cultured in 50 mL PDA medium at 150 rpm for 8 days. Mycelia were recovered by filtration through Whatman N°. 1 filter paper, then washed three times with distilled water, homogenized in 0.1 M phosphate buffer (pH 7.2) for 2 min, and stored at −20 °C overnight. After thawing and homogenization, approximately 20 mL of the fungal mycelia was transferred to a vial and macerated in liquid nitrogen, and the fungal cell wall preparation was stored at −80 °C until use (this protocol was adapted from Bar-Shimon et al., 2004).

2.5.7.1. Quantification of reducing sugars. The reducing sugars released during the enzymatic activity assays were quantified using the 3, 5-dinitrosalicylic acid (DNS) method described by Miller (1959).

2.5.7.2. Quantification of β-1, 3-glucanase enzymatic activity. To assess the production of β-1, 3-glucanases, a colorimetric assay was used to quantify the glucose released from the laminarin substrate along with the method for the quantification of reducing sugars. The reaction was performed using 200 μL of McIlvaine buffer (pH 6.0), 100 μL of the culture sample, and 100 μL of laminarin (4 mg/mL). The reaction was incubated at 50 °C for 1 h then stopped with 200 μL of DNS for the reducing sugar quantification. Absorbance readings at 540 nm were subtracted from the absorbance of the reaction mixture in the presence of a buffer solution in place of the culture medium. Additionally, the absorbance of the negative control (a buffer solution in place of the substrate) was subtracted from each experimental reading. The absorbance values were compared to a glucose standard curve, and the enzymatic activity was expressed as U/L, where a unit of activity (U) was defined as 1.0 g of reducing sugar (glucose) released from laminarin under the assay conditions used.

2.5.7.3. Quantification of chitinase enzymatic activity. The production of chitinase was quantified as the amount of N-acetyl glucosamine (NAG) released from a glycol chitin substrate. A 100 μL aliquot of each yeast culture was mixed with 200 μL of McIlvaine buffer (pH 6.0) and 100 μL of 0.01% glycol chitin (w/v) in the same buffer. After incubation at 50 °C for 60 min, the reaction was stopped with 200 μL of DNS, and the reducing sugars were quantified as previously described. A solution containing the reaction mixture combined with a buffer solution (in place of the culture medium) was used as a reagent blank. Absorbance readings were also subtracted from a negative control reading (a buffer solution in place of the glycol chitin substrate). The enzymatic activity was expressed as U/L, where U was defined as 1.0 g of reducing sugar (N-acetylglucosamine) enzymatically released from glycol chitin under the assay conditions used.

2.5.8. Statistical analysis

A completely randomized design with five replicates was used to quantify the production of antifungal compounds and to evaluate the mycelial growth of *G. candidum* collected from the antagonism zone. The data were analyzed by ANOVA, and the mean values were compared with Tukey's test at the 5% significance level. A factorial design with eight replicates per treatment was used to assess nutrient competition. The mean values for each treatment were compared using Tukey's test at the 5% significance level. All assays were performed in duplicate, and the ASSISTAT software was used for the statistical analysis.

2.6. Identification of yeast isolates by amplification of the ITS regions of the rDNA

Seven yeast isolates (ACBL-23, ACBL-42, ACBL-44, ACBL-50, ACBL-52, ACBL-68 and ACBL-77) were identified based on their genetic material using molecular biology techniques. DNA was extracted from the yeasts using the Wizard Genomic DNA Purification Kit by Promega®. The quantity and quality (purity) of the extracted DNA was determined by optical density using a spectrophotometer (NanoDrop 2000c). The amplification of the ITS regions (Internal Transcribed Spacer) of the rDNA including the 5.8S gene was performed by PCR using the following primers: forward-ITS1 (TCCGTAGGTGAACCTGCGG) and reverse-ITS4 (TCCTCCGCTTATTGATATGC), according to White et al. (1994). PCR cycling conditions consisted of an initial denaturation step at 94 °C for

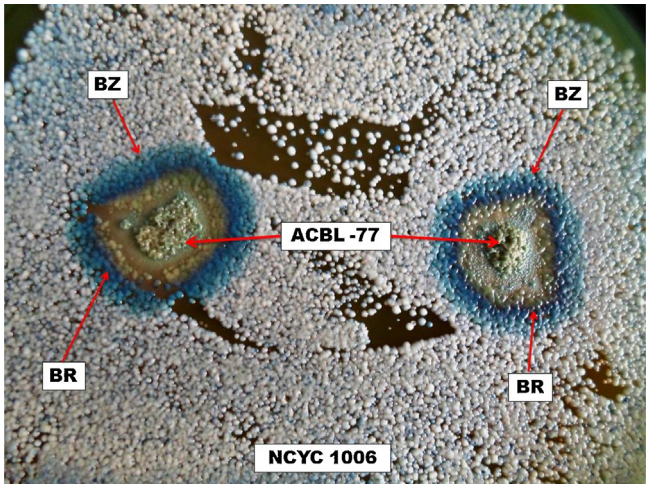

Fig. 3. *Killer* activity of the *Aerobasidium pullulans* ACBL-77 as shown by a blue halo and blue ring (indicative of *Saccharomyces cerevisiae* NCYC 1006 cell death) on YEPD-methylene blue medium (pH 4.5) at 28 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and extension at 72 °C for 2 min; and a final extension at 72 °C for 5 min. The PCR product was purified using a "PureLink™ Quick Gel Extraction and PCR Purification Combo Kit" (Invitrogen™), which is commercially available (Thompson et al., 1994). The sequences were aligned and compared with the NCBI database by the Internet using Basic Local Alignment Search Tool (Altschul et al., 1997).

3. Results

3.1. Isolation of yeasts

Ninety-five isolates were obtained, of which 55.8% were from citrus leaves, 21% from fruits, 13.7% from flowers and 9.5% from soil. The highest percentage of isolates was obtained on YEPD medium (49.5% of the total), followed by WLN (28.4%) and YM (22.1%).

3.2. Screening of the yeasts isolates by antifungal activity in vitro

Of the 101 yeast strains (95 yeast strains plus 6 strains of *S. cerevisiae*) tested *in vitro* for *G. citri-aurantii* antagonism, 21.8% significantly inhibited fungal mycelial growth (ranging from 13 to 35% of inhibition). The highest values for fungal inhibition were obtained with the yeast isolates ACBL-23 (28.7%), ACBL-42 (23.5%), ACBL-44 (23.3%), ACBL-50 (25.2%), ACBL-52 (28.4%), ACBL-68 (26.7%), ACBL-77 (35.0%) and ACB-K1 (24.1%). These eight yeast isolates were used in subsequent *in vivo* assays.

3.3. In vivo assays of antagonistic activity

3.3.1. Effect of yeast treatment on the progress of sour rot in oranges

When 'Pera' orange fruits were treated preventively, only the average size of the lesions caused by *G. citri-aurantii* of the fruits treated with a fungicide did not differ from the control. The progress of sour rot on the inoculated citrus fruits was significantly reduced when fruits were treated with ACBL-44, ACBL-77, ACBL-23, ACBL-52, ACBL-42, ACBL-68 and ACB-K1 isolates, which showed an average lesion size ranging from 5.8 to 54.9 mm (Fig. 1-A). For the curative treatment, the most effective treatment was ACBL-23, with an average lesion size of 5.6 mm, followed by ACBL-68 (12.1 mm), ACBL-77 (14.2 mm), ACBL-44 (26.9 mm), imazalil (26.9 mm), ACBL-42 (47 mm) and ACBL-52 (62.9 mm) (Fig. 1-B).

Table 1
Killer activity of yeast isolates against the sensitive yeast *Saccharomyces cerevisiae* NCYC 1006 cultivated on YEPD-methylene blue medium (pH 4.5) at 28 °C.

Yeast isolates	*Killer* activity
ACBL-23	Clear zone[a]
ACBL-42	Clear zone/Blue ring/Inhibition zone
ACBL-44	Blue zone
ACBL-50	Clear zone
ACBL-52	Clear zone/Blue ring
ACBL-68	Blue zone
ACBL-77	Blue zone/Blue ring/Inhibition zone
ACB-K1	Blue zone/Inhibition zone

[a] Blue inhibition, ring and zone-indicative of cell death; clear zone-indicative of inhibitory activity without cell death.

3.3.2. Treatment effectiveness on the incidence of sour rot in 'Pera' orange fruits

The most effective isolates for preventive treatment were ACBL-52, ACBL-23, ACBL-44 and ACBL-77, for which 38, 37, 25 and 8% of fruits showed sour rot symptoms, respectively (Fig. 2-A). Lower values of diseased fruits were obtained by curative treatment with ACBL-44 (32%), ACBL-77 (30%), ACBL-68 (28%) and ACBL-23, which had only 10% diseased fruit (Fig. 2-B).

3.4. Study of mechanisms of action

3.4.1. Production of volatile antifungal compound

None of the yeast isolates evaluated produced antifungal compounds that inhibited the development of the pathogen. The only yeast isolate that produced volatile metabolites was ACBL-52, which inhibited the pathogen colony at a rate lower than 10%. All yeast isolates stimulated *G. citri-aurantii* mycelial growth via the production of cell-free, thermostable antifungal compounds.

3.4.2. Detection of killer activity

Most yeast isolates tested exhibited *killer* activity. As summarized in Table 1, six of the isolates produced a blue inhibition ring or zone (indicative of cell death), and two of the isolates produced a clear zone (indicative of inhibitory activity without cell death) around each colony (Fig. 3).

3.4.3. Nutrient competition

All of the yeast isolates tested inhibited the germination of *G. citri-aurantii* conidia, regardless of the glucose concentration used. The isolates ACBL-68, ACB-K1, and ACBL-23 exhibited the greatest inhibition of conidial germination, with 94.4%, 92.7%, and 90.5% inhibition, respectively (Fig. 4).

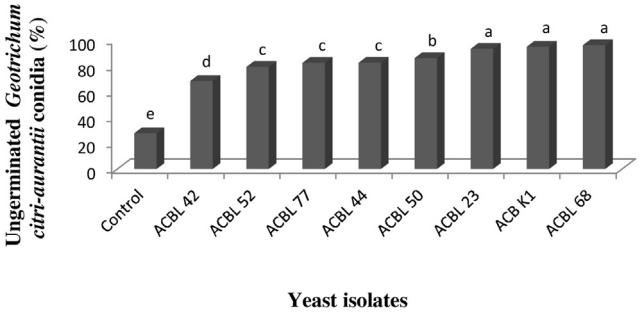

Fig. 4. Effect of yeast isolates on the conidial germination of *Geotrichum citri-aurantii* grown on agar medium with different glucose concentrations at 25 °C. Mean values (represented by bar height) marked with the same lowercase letter are nor significantly different according to Tukey's test. ($P < 0.05$).

Table 2
Colony size (cm) of *Geotrichum citri-aurantii* collected from the inhibition, intermediate, and distant zones following co-culture with the different yeast isolates.

Yeast isolates	Inhibition zone	Intermediate zone	Distant zone
Control	8.66a[a]	8.25a	8.39a
ACBL-23	8.28a	8.52a	8.50a
ACBL-42	8.73a	8.49a	8.60a
ACBL-44	8.92a	8.48a	8.50a
ACBL-50	8.31a	8.57a	8.28a
ACBL-52	8.18a	8.71a	8.67a
ACBL-68	8.30a	8.35a	8.38a
ACBL-77	8.23a	7.61a	8.18a
ACB-K1	7.28b	8.20a	8.22a

[a] Means followed by the same lowercase letter are not significantly different according to Tukey's test ($P < 0.05$).

3.4.4. Evaluation of the mycelial growth of G. citri-aurantii collected from the antagonism zone

The mycelial growth analysis indicated that none of the yeast isolates affected the development of hyphae removed from the antagonism zone, except for ACB-K1, which reduced fungal growth by 16% as compared to the control. The growth of *G. citri-aurantii* collected from the intermediate and distant zones was unaffected (Table 2).

3.4.5. Production of hydrolytic enzymes

The findings indicated that ACBL-23 and ACB-K1 were the only isolates that exhibited β-1, 3-glucanase activity. However, all of the isolates except ACBL-23 and ACBL-44, exhibited chitinase activity (Table 3).

3.5. Identification of yeast isolates by amplification of the ITS regions of rDNA

The most effective yeast isolates for the control of citrus sour rot were identified as *Rhodotorula minuta*-ACBL-23, *Sporobolomyces koalae*—ACBL-42, *Candida azyma*—ACBL-44, *Saccharomyces* sp.—ACBL-50, *S. cerevisiae*—ACBL-52, *Rhodotorula mucilaginosa*—ACBL-68, *Aureobasidium pullulans*—ACBL-77, and *S. cerevisiae* —ACB-K1.

4. Discussion

Biological control using antagonistic yeasts has been reported and is considered an alternative to synthetic fungicides (Droby et al., 2002; Geng et al., 2011; Zhang et al., 2009). The development of a biocontrol agent is a long process that involves several steps, starting with the isolation and selection of microorganisms that have the potential for biocontrol that have desirable characteristics for the development of a commercial product (Droby et al., 2009). Leaf and fruit surfaces are the most appropriate potential locations for the isolation of antagonistic yeast isolates because of their high sugar content and the rapid colonization of this environment by yeasts (Sharma et al., 2009). In this study, we isolated and selected yeasts from citrus leaves, flowers and fruits as well as from citrus soils and determined the most effective yeast strains for the biocontrol of sour rot in 'Pera' oranges.

In this study, the phylloplane was the primary source from which yeast isolates (90.5%) were obtained, and soil accounted for only 9.5% of the isolates. These results were similar to the obtained by other authors, who obtained a lower number of antagonists from soil (Cabral et al., 2009; Chanchaichaovivat et al., 2007; Wang et al., 2009).

In vitro assays indicated that 21.8% of the yeast isolates significantly inhibited the pathogen, with inhibition values ranging between 13% and 35%. The most effective isolates were ACBL-23, ACBL-68, ACBL-77, and ACB-K1. Working with *Candida sp*. and *Saccharomyces sp*. isolates, Abd-Alla et al. (2007) found antagonism to *G. citri-aurantii*, with pathogen inhibition percentages of up to 51.1%, however, those authors observed moderate inhibition by *Cryptococcus sp*. and *Candida sp*. of up to 31.5%, similar to the inhibition observed with ACBL-77, which was the only isolate obtained in the present study. Maldonado et al. (2010) used a strain of *Streptomyces* and found that the antagonist inhibited *G. citri-aurantii* by 29% on solid medium.

Although these isolates did not show high inhibition values for the pathogen *in vitro* when directly tested in citrus fruits, the results for disease control were more satisfactory, with inhibition values of up to 91% (ACBL-77) for the incidence of the disease and with values of 95% inhibition of lesion development (ACBL-44 and ACBL-23).

The evaluation of severity and incidence in this study showed that the yeast isolates ACBL-23 (*Rhodotorula minuta*), ACBL-44 (*Candida azyma*) and ACBL-77 (*Aureobasidium pullulans*) had the potential to control sour rot in citrus as a preventive and curative control (Figs. 1 and 2). Hernández-Montiel et al. (2011); found that the incidence and size of the lesions caused by *G. citri-aurantii* in Mexican lime fruits (*Citrus aurantifolia*) were significantly reduced when the fruits were treated with two isolates of the epiphytic yeast *Debaryomyces hansenii*. Additionally, Ren et al. (2012) demonstrated that the incidence and the average size of the lesions caused by *G. aurantii citri* in citrus fruits were reduced with the use of a recombinant isolate of *Pichia pastoris*.

Understanding the mode of action of a biocontrol agent is essential for developing more effective antagonistic isolates and may contribute to the improvement of formulations and production methods. The present findings indicate that all of the yeast isolates analyzed exhibited at least two inhibitory mechanisms for the control of *G. citri-aurantii*, except for ACBL-44, which exhibited only one mechanism of action.

The findings also revealed that the yeast isolates analyzed did not produce volatile, cell-free, or thermostable antifungal compounds in quantities sufficient to markedly impair the development of *G. citri-aurantii*. Although ACBL-52 produced enough volatile metabolites to inhibit fungal growth, the inhibition of fungal colony size relative to the control was only 9.3%. The lack of production of antifungal compounds by most of the yeast isolates tested did not does not imply that these organisms do not have antifungal action, but the yeast isolates analyzed in this work probably do not produce antifungal substances active against *G. citri-aurantii*. However, different results have been obtained by other authors when yeast species were tested against fungal pathogens. For example, Bruce et al. (2003) reported that *S. cerevisiae* grown in tryptone soy medium produced volatile metabolites that inhibited the growth of wood rot fungi, such as *Sclerophoma pithyophila*, by approximately 75%. Similarly, Fialho et al. (2010) reported that volatile compounds produced by *S. cerevisiae* inhibited the growth of the fungus *Phyllosticta citricarpa* by up to 83%. Parafati et al. (2015) verified the production of volatile organic compounds on

Table 3
Production of chitinases and β-1,3-glucanases by the yeast isolates as measured by the amount of reducing sugars released.

Yeast isolates	Chitinase RS[a] (g/L)	β-1,3-glucanase RS(g/L)
ACBL-23	0	0.004
ACBL-42	0.089	0
ACBL-44	0	0
ACBL-50	0.011	0
ACBL-52	0.001	0
ACBL-68	0.017	0
ACBL-77	0.089	0
ACB-K1	0.067	0.039

[a] RS: reducing sugar.

PDA at pH 4.5 for *Wickerhamomyces anomalus* strains that inhibited the mycelial growth of *B. cinerea*.

Some yeast species can produce *killer* toxins and other antimicrobial compounds that are lethal to filamentous fungi. Six of the yeast isolates tested in this study produced *killer* toxins, supporting prior reports that these compounds are the main mode of action for the biocontrol of *G. citri-aurantii* (Table 1 and Fig. 3). For example, Walker et al. (1995) were the first to observe pathogen susceptibility to mycocinogenic yeasts. Weiler and Weiler and Schmitt (2003) obtained similar results for the control of *Fusarium oxysporum* by the *killer* yeast *Zygosaccharomyces bailii*. *Pichia membranifaciens* was found to inhibit the growth of the fungus *Botrytis cinerea* via the action of a *killer* toxin (Santos et al., 2004), and Coelho (2005) demonstrated that the *killer* yeasts *Candida guilliermondii* and *Pichia ohmeri* could be used for the control *in vitro* of the fungus *Penicillium expansum* in apples.

Competition among microorganisms for essential environmental resources such as nutrients and space is considered as a fundamental biocontrol mechanism in yeast-pathogen interactions (Bleve et al., 2006; Spadaro and Droby, 2016). In this context, our findings revealed that nutrient competition was not a biocontrol strategy used by the yeast isolates studied: the decrease of *G. citri-aurantii* conidial germination was not dependent on an increase in the glucose concentration in the culture medium, indicating that these isolates did not compete with the fungus for nutrients in the culture (Fig. 4). Nevertheless, this result is inconsistent with some previously reported findings. For example, Chanchaichaovivat et al. (2008) investigated the possible mechanisms of action of the yeast *Pichia guilliermondii* against the fungus *Colletotrichum capsici* for the control of anthracnose in pepper and found that increasing the sugar concentration suppressed the conidial germination of *C. capsici*, indicating competition for nutrients and inhibition of the fungal development in culture. Saravanakumar et al. (2008) studied the role of the yeast *Metschnikowia pulcherrima* for the control of the phytopathogens *B. cinerea*, *P. expansum*, and *Alternaria alternata* and observed that fungal development was controlled of the yeast by competition for iron in culture.

In *G. citri-aurantii*, hyphal alterations occurred when the colonies were collected from the inhibition zone during co-culture with ACB-K1 and then grown in fresh culture medium, leading to the suppression of pathogen development (Table 2). These hyphal alterations may have resulted from the production of *killer* toxins or hydrolytic enzymes, such as β-1, 3-glucanases and chitinases, by the antagonist. Although the nature of the *killer* toxins from the studied isolates has not been elucidated, it has been proposed that the cell wall components of pathogens are the primary recognition sites for these enzymes and toxins (Gooday, 1995). This hypothesis was supported by the findings of Masih et al. (2000) in a study of the mode of action of the yeast *Pichia anomala* isolated from the surface of apples for the control of *B. cinerea*. The authors observed deformation of the fungal hyphae, such as the coagulation and leakage of the cytoplasmic contents when in contact with the yeast cells.

All eight yeast isolates except for ACBL-23 and ACBL-44 produced chitinases in the presence of the pathogen cell wall. In contrast, only ACBL-23 and ACB-K1 produced β-1,3-glucanases (Table 3). Similar results were reported by Zhang et al. (2011), who investigated the antagonistic potential of the yeast *Pichia guilliermondii* against *Botrytis cinerea* and found that control of the pathogen was mediated by the production β-1,3-glucanases and chitinases by the yeast in a medium containing fungal cell wall and sucrose or glucose as the carbon source. Saravanakumar et al. (2009) observed higher chitinase production by the yeast *M. pulcherrima* compared to *Rhodotorula* sp. and even higher production in the presence of *B. cinerea* cell wall. Ultimately, the increased production of chitinases in *M. pulcherrima* led to more efficient control of gray mold in apple.

The findings of this study indicate that multiple modes of action are important for the control of *G. citri-aurantii*, as evidenced by the ABC-K1 strain, which produced two classes of hydrolytic enzymes (chitinases and β-1,3-glucanases), exhibited *killer* activity, and inhibited conidial germination. These activities led to a reduction in the colony size of *G. citri-aurantii* collected from the inhibition zone, along with possible hyphal alterations. Moreover, *killer* activity was found to be a common biocontrol mechanism because it was observed in all of the yeast isolates tested. Accordingly, improvement of the control of *G. citri-aurantii* will require the identification of the *killer* toxins produced by these isolates, in particular, of the isolates ACBL23, ACBL-44, and ACBL-77, which most effectively controlled sour rot both preventively and curatively.

To our knowledge, this is the first report of *Candida azyma* and *Sporobolomyces koalae* as biological control agents against a postharvest pathogen and their ability to produce a *killer* toxin. Therefore, in conclusion, *R. minuta, C. azyma* and *A. pullulans* are not pathogenic yeasts in plants, so they could be used against *G. citri-aurantii* as an alternative postharvest control in citrus fruits. However, the postharvest formulations must be further studied before it is practical and effective to use them as biological control agents.

Acknowledgements

The financial support of the Fapesp (The State of São Paulo Research Foundation) (Proceeding no. 2011/13006-1). Financial support of the CAPES which provided a scholarship to Luriany P. Ferraz, is gratefully acknowledged. The Citricola Lucato by the fruits citrus donated.

References

Abd-Alla, M.A., El-Mohamedy, R.S.R., El-Mougy, N.S., 2007. Control of sour rot disease of lime fruits using saprophytic isolates of yeast. Egypt. J. Phytopathol. 35, 39–51.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., et al., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acid Res. 25, 3389–3402.

Azeredo, L.A.I., Gomes, E.A.T., Mendonça-Hagler, L.C., 1998. Hagler yeast communities associated with sugarcane in campos, Rio de Janeiro Brazil. Int. Microbiol. 1, 205–208.

Bar-Shimon, M., Yehuda, H., Cohen, L., Weiss, B., Kobeshnikov, A., Daus, A., Goldway, M., Wisniewski, M., Droby, S., 2004. Characterization of extracellular lytic enzymes produced by the yeast biocontrol agent *Candida oleophila*. Curr. Genet. 45, 140–148.

Bleve, G., Grieco, F., Cozzi, G., Logrieco, A., Visconti, A., 2006. Isolation of epiphytic yeasts with potential for biocontrol of *Aspergillus carbonarius* and *A. niger* on grape. Int. J. Food Microbiol. 108, 204–209.

Brown, G.E., 1988. Efficacy of guazatine and iminoctadine for control of postharvest decays of oranges. Plant Dis. 72, 906–908.

Brown, G.E., 2003. Sour rot. Causal organism and disease cycle. University of Florida, Institute of Food and Agricultural Science Extension Service Fact Sheet, Gainesville, pp. 137.

Bruce, A., Douglas, S., Susan, V., Ron, E.W., 2003. Effect of volatiles from bacteria and yeast on the growth and pigmentation of *sapstain fungi*. Int. Biodeterior. Biodegrad. 51, 101–108.

Butler, E.E., Fogle, D., Miranda, M., 1988. Galactomyces citri-aurantii, a newly found téleomorphe of *Geotrichum citri-aurantii* the cause of sour rot of citrus fruit. Mycotaxon Ithaca 33, 197–212.

Cabral, A.S., Carvalho, P.M.B., Pinotti, T., Hagler, A.N., Mendonça-Hagler, L.C.S., Macrae, A., 2009. Yeast inhibit the growth of the phytopathogen *Moniliophthora perniciosa*, the causal agent of 'witches' broom disease. Braz. J. Microbiol. 40, 108–110.

Ceccato-antonini, S.R., Tosta, C.D., Silva, A.C., 2004. Determination of yeast *Killer* activity in fermentation sugarcane juice using selected ethanol-making strains. Braz. Arch. Biol. Technol. 47, 13–23.

Chanchaichaovivat, A., Ruenwongsa, P., Panijpan, B., 2007. Screening and identification of yeast strains from fruits and vegetables: potential for biological control of postharvest chilli anthracnose (*Colletotrichum capsici*). Biol. Control 42, 326–335.

Chanchaichaovivat, A., Bhinyo, P., Ruenwongsa, P., 2008. Putative modes of action of *Pichia guilliermondii* strain R13 in controlling chilli anthracnose after harvest. Biol. Control 47, 207–215.

Coelho A.R., Controle de *Penicillium expansum*/Biodegradação de Patulina: perfil cromatográfico de composto bioativo de leveduras *killer* visando aplicação

pós-colheita. 2005. 130 f. Tese (Doutorado em Ciência de Alimentos)—Universidade Estadual de Londrina, Londrina, PR, Brazil.
Dennis, C.E., Webster, J., 1971. Antagonistic properties of species groups of *Trichoderma* III. Hyphal interactions. Transa. Br. Mycol. Soc. 57, 359–363.
Droby, S., Chalutz, E., Wilson, C.L., Wisniewski, M., 1989. Characterization of the biocontrol activity of *Debaryomyces hansenii* in the control of *Penicillium digitatum* on grapefruit. Can. J. Microbiol. 35, 794–800.
Droby, S., Vinokur, V., Weiss, B., Cohen, L., Daus, A., Goldschmidt, E.E., Porat, R., 2002. Induction of resistance to *Penicillium digitatum* in grapefruit by the yeast biocontrol agent *Candida oleophila*. Biol. Control 92, 393–399.
Droby, S., Wisniewski, M., Macarisin, D., Wilson, C., 2009. Twenty years of postharvest biocontrol research: is it time for new paradigm. Postharvest Biol. Technol. 52, 137–145.
Fialho, M.B., Toffano, L., Pedroso, M.P., Augusto, F., Pascholati, S.F., 2010. Volatile organic compounds produced by *Saccharomyces cerevisiae* inhibit the *in vitro* development of *Guignardia citricarpa*, the causal agent of citrus black spot. World J. Microbiol. Biotechnol. 26, 925–932.
Fialho M.B., 2005. Efeito *in vitro* de *Saccharomyces cerevisiae* sobre *Guignardia citricatrpa*, agente causal da pinta preta dos citros. 60 f. Dissertação (Mestrado em Microbiologia Agrícola) –Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Piracicaba.
Filonow, A.B., 1998. Role of competition for sugars by yeast in the biocontrol of gray mold of apple. Biol. Sci. Technol. 8, 243–256.
Frighetto, R.T.S., Melo, I.S., 1995. Produção de antibióticos por microrganismos. In: Melo, I.S., De; Sanhueza, R.M.V., (Coord.), Métodos de seleção de microrganismos antagônicos a fitopatógenos. Jaguariúna: EMBRAPA-CNPMA; 1995. p. 40–46. Manual Técnico.
Geng, P., Chen, S., Hu, M., Rizwan-ul-Haq, M., Lai, K., Qu, F., Zhang, Y., 2011. Combination of *Kluyveromyces marxianus* and sodium bicarbonate for controlling green mold of citrus fruit. Int. J. Food Microbiol. 151, 190–194.
Gooday, G.W., 1995. Cell walls. In: Gow, N.A.R., Gadd, G.M. (Eds.), The Growing Fungus. Chapman and Hall, London, pp. 41–62.
Hernández-Montiel, L.G., Holguín-Peña, J., López-Aburto, M.G., Troyo-Diéguez, E., 2011. Control poscosecha de *Geotrichum citri-aurantii* en limón mexicano (*Citrus aurantifolia* [Christm.] *Swingle*) mediante leveduras marinas y epífitas. Univ. y Cienci. 27, 191–198.
Kupper, K.C., Cervantes, A.L.L., Klein, M.N., Silva, A.C., 2013. Avaliação de micro-organismos antagônicos, *Saccharomyces cerevisiae* e *Bacillus subtilis* para o controle de *Penicillium digitatum*. Rev. Bras. de Frutic. 35, 020–031.
Lahlali, R., Hamadi, Y.E.L., Guilli, M., Jijakli, M.H., 2011. Efficacy assessment of *Pichia guilliermondii* strain Z1, a new biocontrol agent, against citrus blue mould in Morocco under the influence of temperature and relative humidity. Biol. Control 56, 217–224.
Liu, X., Wang, L.P., Li, Y.C., Li, H.Y., Yu, T., Zheng, X.D., 2009. Antifungal activity of thyme oil against *Geotrichum citri-aurantii in vitro* and *in vivo*. J. Appl. Microbiol. 107, 1450–1456.
Liu, J., Sui, Y., Wisniewski, M., Droby, S., Review, Liu Y., 2013. Utilization of antagonistic yeasts tomanage postharvest fungal diseases of fruit. Int. J. Food Microbiol. 167, 153–160.
Lopes, M.R., Klein, M.N., Ferraz, L.P., Silva, A.C., Kupper, K.C., 2015. Saccharomyces cerevisiae: a novel and efficient biological control agent for *Colletotrichum acutatum* during pre-harvest. Microbiol. Res. 175, 93–99.
Maldonado, M.C., Orosco, C.E., Gordillo, M.A., Navarro, A.R., 2010. In vivo and *in vitro* antagonism of *Streptomyces* sp: rO3 against *Penicillium digitatum* and *Geotrichum candidum*. Afr. J. Microbiol. Res. 4, 2451–2456.
Masih, E.I., Alie, I., Paul, B., 2000. Can the grey mold disease of grape-vine be controlled by yeast. FEMS Microbiol. Lett. 189, 233–237.
McKay, A.H., Förster, H., Adaskaveg, J.E., 2012. Efficacy and application strategies for propiconazole as a new postharvest fungicide for managing sour rot and green mold of citrus fruit. Plant Dis. 96, 235–242.
Miller, G.H., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–429.
Moretto, C.M., Cervantes, A.L.L., Filho, A.B., Kupper, K.C., 2014. Integrated control of green mold to reduce chemical treatment in post-harvest citrus fruits. Sci. Hortic. 165, 433–438.
Palou, L., Valencia-Chamorro, S.A., Pérez-Gago, M.B., 2015. Antifungal edible coatings for fresh citrus fruit: a review. Coatings 5, 962–986.
Parafati, L., Vitale, A., Restuccia, C., Cirvilleri, G., 2015. Biocontrol ability and action mechanism of food-isolated yeast strains against *Botrytis cinerea* causing post-harvest bunch rot of table grape. Food Microbiol. 47, 85–92.
Platania, C., Restuccia, C., Muccili, S., Cirvilleri, G., 2012. Efficacy of killer yeasts in the biological control of *Penicillium digitatum* on Tarocco orange fruits (*Citrus sinensis*). Food Microbiol. 30, 219–225.
Punja, Z.K., Utkhede, R.S., 2003. Using fungi and yeasts to manage vegetable crop diseases. Trends Biotechnol. 21, 400–407.
Ren, X., Kong, Q., Wang, H., Yu, T., Zhou, W., Zheng, X., 2012. Biocontrol of fungal decay of citrus fruit by *Pichia pastoris* recombinant strains expressing cecropin A. Food Chem. 131, 796–801.
Rosa-Magri, M.M., Tauk-Tornisielo, S.M., Cecato-Antonini, S.R.C., 2011. Bioprospection of yeasts as biocontrol agents against phytopathogenic molds. Braz. Arch. Biol. Technol. 54, 1–5.
Saligkarias, I.D., Gravanis, F.T., Epton, H.A.S., 2002. Biological control of *Botrytis cinerea* on tomato plants by use of epiphytic yeasts *Candida guilliermondii* strains 101 and US 7 and *Candida oleophila* strain I-182: II a study on mode of action. Biol. Control 25, 151–161.
Santos, A., Sanchez, A., Marquina, D., 2004. Yeasts as biological agents to control *Botrytis cinerea*. Microbiol. Res. 159, 331–338.
Saravanakumar, D., Ciavorella, A., Spadaro, D., Garibaldi, A., Gullino, M.L., 2008. Metschnikowia pulcherrima strain MACH1 outcompetes *Botrytis cinerea*, *Alternaria alternata* and *Penicillium expansum* in apples through iron depletion. Postharvest Biol. Technol. 49, 121–128.
Saravanakumar, D., Spadaro, D., Garibaldi, A., Gullino, M.L., 2009. Detection of enzymatic activity and partial sequence of a chitinase gene in *Metschnikowia pulcherrima* strain MACH1 used as post-harvest biocontrol agent. Eur. J. Plant Pathol. 123, 183–193.
Shaner, G., Finney, R.E., 1977. The effects of nitrogen fertilization on the expression of slowmildwing in knox wheat. Phytopathol 67, 1051–1055.
Sharma, R.R., Singh, D., Singh, R., 2009. Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: a review. Biol. Control 50, 205–221.
Spadaro, D., Droby, S., 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.
Talibi, I., Boubaker, H., Boudyach, E.H., Ait Ben Aoumar, A., 2014. Alternative methods for the control of postharvest citrus diseases. J. Appl. Microbiol. 117, 1–17.
Thompson, J.D., Higgins, D.G., Gibson, T.J., Clustal, W., 1994. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
Walker, G.M., Mcleod, A.H., Hodgson, V.J., 1995. Interactions between *killer* yeasts and pathogenic fungi. FEMS Microbiol. Lett. 127, 213–222.
Wang, X., Li, G., Jiang, D., Huang, H.C., 2009. Screening of plant epiphytic yeasts for biocontrol of bacterial fruit blotch (*Acidovorax avenae* subsp citrulli) of hami melon. Biol. Control 50, 164–171.
Weiler, F., Schmitt, M.J., 2003. Zygocin a secreted antifungal toxin of the yeast *Zygosaccharomyces bailii* and its effect on sensitive fungal cells. FEMS Yeast Res. 3, 69–76.
White, T.J., Bruns, T., Lee, S., Taylor, J., 1994. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: a Guide to Methods and Applications. Acad Press, New York, pp. 315–321.
Zhang, H.Y., Wang, L., Ma, L.C., Dong, Y., Jiang, S., Xu, B., Zheng, X.D., 2009. Biocontrol of major postharvest pathogens on apple using *Rhodotorula glutinis* and its effects on postharvest quality parameters. Biol. Control 48, 79–83.
Zhang, D., Spadaro, D., Garibaldi, A., Gullino, M.L., 2011. Potential biocontrol activity of a strain of *Pichia guilliermondii* against grey mold of apples and its possible modes of action. Biol. Control 57, 193–201.
Zhou, H., Tao, N., Jia, L., 2014. Antifungal activity of citral, octanal and α-terpineol against *Geotrichum citri-aurantii*. Food Control 37, 277–283.