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Antifungal activity and action mechanisms of yeasts isolates from citrus against *Penicillium italicum*

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ABSTRACT

Penicillium italicum (Blue mold) is a major postharvest disease of citrus. An alternative to controlling the disease is through the use of yeasts. The purpose of the present study was to screen effective yeast antagonists against *P. italicum*, isolated from soil, leaves, flowers, and citrus fruits, to assess the action mechanisms of the yeast isolates that were demonstrated to be effective for biocontrol, and to identify the most effective yeast isolates for the biocontrol of blue mold. The *in vitro* assays showed that six yeast strains inhibited up to 90% of the pathogen's mycelial growth. *In vivo* assays, evaluating the incidence of blue mold on sweet oranges, the strains ACBL-04, ACBL-05, ACBL-10 and ACBL-11 were effective, demonstrating the potential for the blue mold control when preventively applied, whereas the ACBL-08 strain showed a high potential to preventive and curative applications. Additional studies on the modes of action of these yeast strains showed that most of the evaluated yeast strains did not produce antifungal substances, in sufficient quantities to inhibit the pathogen growth. Competition for nutrients was not a biocontrol strategy used by the yeast strains. The '*killer*' activity might be the main action mechanism involved in *P. italicum* biocontrol. This study indicated that the multiple modes of action against the pathogen presented by yeasts may explain why these strains provided *P. italicum* control under *in vitro* and *in vivo* conditions. However, further studies in future might be able to elucidate the '*killer*' activity and its interaction with pathogen cells and the bioproduct production using *Candida stellimalicola* strains for control postharvest diseases.

1. Introduction

Citrus spp. is one of the most cultivated fruits worldwide. In 2015, the world citrus production was around 121 million tons (Strano et al., 2017), which was 20.0% of total fruit production. Harvested fruits are usually stored before they reach the market for fresh consumption. During this period, approximately 20% of harvested fruits undergo decay during postharvest storage (Fu et al., 2014; Hodges et al., 2011; Tao et al., 2014). It has been reported that *Penicillium italicum* Wehmer cause blue mold, one of the most economically important citrus postharvest disease (García-Cela et al., 2014; Hernández-Montiel et al., 2010; Tian et al., 2011; Zhang et al., 2005). The fruits are infected through wounds, caused during harvesting and transportation, leading to quick losses in the yield and in the fruits quality (Lahlali et al., 2006; Palou et al., 2002).

Currently, the blue mold is primarily controlled by the synthetic fungicide applications, such as thiabendazole and imazalil (Sun et al., 2013). However, problems related to the development of pathogen resistance to many site-specific fungicides and to the potentially harmful effects on human safety and environment have stimulated researches related to alternative methods for disease control in postharvest (Lahlali et al., 2014; Palou et al., 2002; Zhou et al., 2014).

Biological control using microbial agents is considered to be a viable alternative to the use of synthetic fungicides. Among microbial agents, yeasts have several properties that make them ideal antagonists, including the ability to survive in adverse environmental conditions, having few nutritional requirements and being amenable to formulation with a long shelf-life. Recent researches showed the use of yeasts in the control of fungal diseases in postharvest (Ferraz et al., 2016; J. Liu et al., 2013; Liu et al., 2014; Moretto et al., 2014; Platania et al., 2012).

Understanding the action mechanism of antagonistic microorganisms is essential for using them in a safe and effective manner. The biocontrol activity exhibited by yeasts used to manage postharvest diseases has been reported to be associated with their ability to compete with fungal pathogens for nutrients and space, to adhere to host and pathogen tissues, by the production of *exo*-β-1,3-glucanase and

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chitinase, to induce host resistance, to secrete organic acids and lytic enzymes, including some that produce '*killer*' toxins (J. Liu et al., 2013; Parafati et al., 2016; Spadaro and Droby, 2016).

In this context, the objectives of this work were: (1) to select yeast isolates obtained from citrus crops with potential to *Penicillium italicum* control, (2) to assess the mechanisms of action of the yeast isolates that showed potential for biocontrol and, (3) to identify the most effective yeast isolates for the biocontrol of *P. italicum*. These results could be contributing for yeast-based-products as an alternative against fruits post-harvest pathogens management.

2. Material and methods

2.1. Pathogen

The pathogen (*P. italicum*) used in this study was obtained from microorganism's collection of the APTA Center Citrus "Sylvio Moreira"/ IAC, Cordeirópolis, São Paulo State, Brazil.

2.2. Yeasts isolation

The yeast biological control agents (ACBL) were isolated from leaves, flowers, fruits, and soils. They were obtained from 14 citrus producing regions in the São Paulo State, Brazil. The yeast isolation was carried out according to Azeredo et al. (1998) and Ferraz et al. (2016), using the decimal dilution technique, in which a 0.85% saline solution was used for the soil, and a sterile wash solution was used for leaves, flowers, and fruits. 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].

2.3. Screening of the yeast strains for antifungal activity in vitro against P. italicum

To conduct the yeasts screening against *P. italicum* in addition to the 97 yeast strains obtained, six strains of *Saccharomyces cerevisiae* (ACB-BG1, ACB-CAT1, ACB-CR1, ACB-K1, ACB-PE2, and ACB-KD1) were also tested. These strains were obtained from an ethanol fermentation, characterized by electrophoretic karyotyping and stored in the yeast collection of the Laboratory of Biochemistry and Plant Pathology at the University of São Paulo (ESALQ), Piracicaba, São Paulo, Brazil (Fialho et al., 2010).

We used the pour plate methodology, adapted from Coelho et al. (2011) with the aim to evaluate the antagonistic capacity of all 103 yeasts strains against *P. italicum*. Suspension of 100 mL containing 1×10^5 conidia/mL of pathogen was added to 900 mL PDA (potato-dextrose-agar) around 45 °C. The culture media with the pathogen was poured into the Petri dish. After solidification the medium was perforated, leaving a 5 mm diameter well in the center of the plate. Then, 100 μL aliquots of a suspension containing 1×10^7 cells/mL of the yeasts strains were added into the wells. The suspensions of the yeasts were previously prepared by growing each strain, transferred from 48 h plate culture, into the Erlenmeyer flasks containing 25 mL of liquid YEPD medium. The cultures were incubated at 25 °C on a shaker at 250 rpm for 48 h and 12 h photoperiod.

The Petri dishes containing the pathogen and the yeasts were incubated at 22 °C photoperiod of 12 h for 7 days. The control corresponded to the growth of the fungus without the yeast strains. After the incubation period, the medium diameter of the halo inhibition was measured. A completely randomized design with five replications for each treatment was used. The assay was replicated. The data were subjected to analysis of variance (ANOVA). In cases of significant differences between treatments, multiple comparisons with the Tukey test ($P < 0.05$), with adjusted P values.

2.4. Antifungal activity in vivo

'Valência' sweet orange fruits [*Citrus sinensis* (L.) Osbeck (Rutaceae)] in the commercial mature stage were obtained from a packinghouse in the city of Limeira, São Paulo, Brazil. We used the methodology from Ferraz et al. (2016). 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 at two equidistant points, and 20 μL of a *P. italicum* 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; the treatments were applied, and inoculated with the pathogen 24 h later. Eight yeast strains (ACBL-04; ACBL-05; ACBL-06; ACBL-07; ACBL-08; ACBL-10; ACBL-11 and ACBL-14) were selected based on *in vitro* screening to compose *in vivo* assay treatments. A 20 μL aliquot of each treatment (yeast suspension at 1×10^7 cells/mL) was applied directly into the wounds in both the curative and preventive treatments. The fungicide imazalil (2.0 mL/L) was included as chemical treatment. The positive control was fruit treated with sterile distilled water instead of another treatment. Uninoculated and untreated fruits were kept in the room to verify the possible occurrence of symptoms.

After inoculation with *P. italicum* and treatment application, the fruits were stored for 10 days in a cold chamber (10 ± 2 °C and 90% RH). Disease severity was assessed on the 8th, 9th and 10th days after inoculation by measuring the average diameter of blue mold lesions using a caliper. The incidence was expressed as the percentage of infected fruits observed at day 10. To analyze the *in vivo* assays, treatments were arranged as 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 *P. italicum*, 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 – is the number of observations/evaluations;

Yi – proportion of the disease in the "**i**"-th observation/evaluation;

Ti – It's the time in days in the "**i**"-th observation/evaluation.

The results were analyzed by variance analysis (ANOVA) and comparisons of means were performed using the Tukey test ($P < 0.05$).

2.5. Study of action mechanisms

In this study, eight yeast strains (ACBL-04; ACBL-05; ACBL-06; ACBL-07; ACBL-08; ACBL-10; ACBL-11 and ACBL-14) that were shown to be effective for the *in vivo* biocontrol of blue mold were used.

2.5.1. Production of volatile antifungal compounds

Each of the yeast strain was simultaneously cultivated with *P. italicum* on split plates, which prevented non-volatile compounds produced by the yeasts from reaching the fungus. A *P. italicum* culture disk (5 mm of the 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 different yeast strains at 22 °C for 10 days, fungal growth was measured as the mycelial diameter in the presence of yeast relative to the mycelial diameter in the absence of the yeast (control) (Lopes et al., 2015).

2.5.2. Production of cell-free antifungal compounds in yeast

For each yeast strain, a loopful of a 48 h culture inoculum was transferred to 250 mL Erlenmeyer flasks containing 50 mL 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, and then poured into Petri dishes. After solidification, a 5 mm *P. italicum* culture disk was placed in the center of each Petri dish. For the control, *P. italicum* was grown on PDA medium without a yeast filtrate. The cultures were incubated in a B.O.D. chamber at 22 °C for 7 days with a 12 h photoperiod, and *P. italicum* growth was assessed by measuring the mean diameter of each colony in two perpendicular directions.

2.5.3. Production of thermostable antifungal compounds

For each yeast strain tested, a yeast culture disk was transferred to 250 mL Erlenmeyer flasks containing 50 mL YEPD medium and, incubated at 150 rpm for 72 h in the dark. A 10 mL aliquot of each strain was transferred to Erlenmeyer flasks containing 90 mL PDA medium and sterilized (at 121 °C for 20 min). Each sterilized medium was poured into a Petri dish, and after it solidified, a 5 mm *P. italicum* culture disk was placed in the center of each plate. For the control, *P. italicum* was grown on PDA medium without metabolites. The cultures were kept in B.O.D. chamber at 22 °C for 7 days, and the growth of *P. italicum* growth was assessed by measuring the mean diameter of each colony in two perpendicular directions. This methodology was according to Ferraz et al. (2016).

2.5.4. Detection of 'killer' activity by yeast isolates

This assay was conducted according the methodology described by Ceccato-Antonini et al. (2004) methodology. A 1.0×10^5 cell/mL suspension of *S. cerevisiae* NCYC 1006 ('*killer*' factor sensitive) was prepared by culturing in YEPD medium at 28 °C for 24 h. A 100 μL aliquot of the suspension was transferred to a Petri dish containing YEPD-methylene blue medium buffered at pH 4.3–4.7. To evaluate the presence of '*killer*' factor, each yeast strain was spotted on separate Petri dishes with sterile toothpicks, and the cultures were incubated at 28 °C for 3 days. The production of '*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

This assay was conducted according to Ferraz et al. (2016) methodology. To evaluate nutrient competition between *P. italicum* and antagonistic yeasts, agar-coated microscope slides were prepared with varying glucose concentrations (0%, 0.5%, 1%, 1.5%, 2%, and 2.5%). For each yeast strain tested, 10 μL of a *P. italicum* suspension (1×10^5 conidia/mL) and 10 μL of a yeast suspension (1×10^8 cells/mL) were spotted onto pre-marked locations on the slides, and the cultures were incubated in a B.O.D. chamber at 22 °C for 17 h. The nutrient competition was assessed by counting the number of germinated and no-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. The factorial design with eight replicates per treatment was used for this assay.

2.5.6. Evaluation of the mycelial growth of Penicillium italicum collected from the antagonism zone

P. italicum was co-cultured on Petri dishes with each yeast strain 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 *P. italicum* mycelial disk on one side of the plate and a 0.5 cm yeast strain culture disk 3 cm away. For a more detailed characterization of the inhibition zone formed after co-culture of the antagonistic yeast strains and fungus, hyphal viability and mycelial growth of the pathogen was quantified in the antagonism zone (*i.e.*, the area adjacent to the fungal inhibition zone). To measure 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 (intermediate zone), and the zone furthest from the inhibition zone where mycelia were not inhibited by yeast (distant zone). The cultures were incubated in a B.O.D. chamber at 22 °C for 10 days with a 12 h photoperiod, after which the mycelial growth diameter of *P. italicum* was measured in two perpendicular directions.

2.5.7. Production of hydrolytic enzymes

The production and secretion of hydrolytic enzymes by the yeast strains were analyzed according to the methodology of Fialho (2005). A loopful for each strain was transferred to 20 mL liquid YEPD medium 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 YEPD or modified YEPD medium (containing *P. italicum* cell wall preparation in place of 1% glucose). The cultures were prepared in triplicate and incubated at 150 rpm. After a 24 h incubation period, a 1.5 mL volume was aliquoted and centrifuged at 3000 rpm for 10 min. The supernatant was recovered and used for the quantification of β-1, 3-glucanase and chitinase.

A protocol adapted from Bar-Shimon et al. (2004) was used for the cell wall preparation. Briefly, 1 mL of a *P. italicum* suspension (1.0×10^5 conidia/mL) was cultured in 50 mL PD medium at 150 rpm for 8 days. Mycelial contents 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 were transferred to a vial and macerated in liquid nitrogen, and the fungal cell wall preparation was stored at −80 °C until use.

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 glucose released from the laminarin substrate along with the method for the quantification of reducing sugars (Section 2.5.7.1). The reaction was performed using 200 μL McIlvaine buffer (pH 6.0), 100 μL culture sample, and 100 μL laminarin (4 mg/mL). The reaction was incubated at 50 °C for 1 h then stopped using 200 μL 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 a negative control (a buffer solution in place of the substrate) was subtracted from each experimental reading. Absorbance values were plotted on a standard curve of glucose, and enzymatic activity was expressed in 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 chitinase production was quantified as the amount of *N*-acetyl glucosamine (NAG) released from glycol chitin substrate. Briefly, 100 μL of each yeast culture was mixed with 200 μL McIlvaine buffer (pH 6.0) and 100 μL 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 DNS, and reducing sugar quantification was performed as previously described. A solution containing reaction mixture combined with buffer solution (in place of culture medium) was used as the reagent blank. Absorbance readings were also subtracted from a negative control reading (buffer

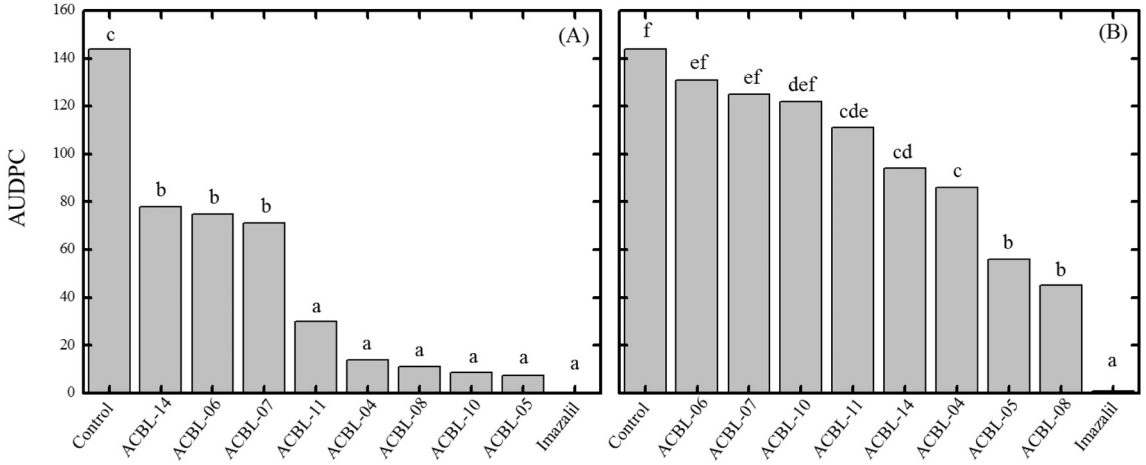

Fig. 1. The area under the disease progress curve (AUDPC) for lesion in 'Valência' fruits caused by *Penicillium italicum* after preventive (A) and the curative (B) treatment, kept in cold chamber (10 ± 2 °C) for 10 days. Statistical significance determined at $P < 0.05$ according to Tukey test.

solution in place of the glycol chitin substrate). Enzymatic activity was expressed in 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 *P. italicum* 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 the 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. Molecular identification of the most effective yeast strain

Eight yeast strains (ACBL-04, ACBL-05, ACBL-06, ACBL-07, ACBL-08, ACBL-10, ACBL-11 and ACBL-14) were identified by genetic material using molecular biology techniques. Yeast isolates were growth in YEPD medium at 28 °C for 24 h and the DNA extraction were performed using the Wizard Genomic DNA Purification Kit - Promega® according recommendation DNA quantity and purity was measured at A260, A280 and A260/A280 using a NanoDrop 2000c.

The amplification of the ITS region (Internal Transcribed Spacer) of the rRNA gene including the 5.8S gene was performed by PCR using the primers: forward-ITS1 (TCCGTAGGTGAACCTGCGG) and reverse-ITS4 (TCCTCCGCTTATTGATATGC), according to the White et al. (1994).

PCR products were purified from agarose gel using a "PureLink ™ Quick Gel Extraction and PCR Purification Combo Kit" (Invitrogen™) which is commercially available. For the sequencing of the ITS region of the rRNA gene, the reaction consisted of 3 μL of purified PCR product, 0.5 μL (100 μM) of the primer (ITS-1 or ITS-4), 0.4 μL of Big Dye Terminator Kit (Applied Biosystems), 2 μL of SM buffer and sterile Milli-Q water to complete 10 μL. Sequencing reactions for the rRNA gene were carried out using the ABI3730 automated sequencer (Applied Biosystems). The sequences obtained were aligned and compared with the at the National Centre of Biotechnology Information (NCBI) database by the website using Basic Local Alignment Search Tool (BLAST) (Thompson et al., 1994) and after identification, the sequences were deposited in the GenBank.

3. Results

3.1. Yeasts isolation

Ninety-seven 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%). The isolation-source of the yeasts selected to *in vivo* assays - ACBL-04, ACBL-05, ACBL-06, ACBL-07, ACBL-08, and ACBL-10 – were obtained from the citrus leaves, and strains ACBL-11 and ACBL-14 were isolated from citrus flowers in the same citrus-crop, localized in Leme, SP, Brazil.

3.2. Screening of the yeast strains for antifungal activity in vitro against P. italicum

Of the 103 yeast strains tested *in vitro* (97 yeast isolates plus six strains of *S. cerevisiae*) for *P. italicum* antagonism, 61 were able to inhibit mycelial growth of the fungus (ranging from 10 to 100% of inhibition). Among the strains with the pour plate technique, seven strains inhibited > 80% of mycelial growth-in decreasing order of inhibition power: ACBL-08, ACBL-07, ACBL-11, ACBL-05, ACBL-06, ACBL-14 and ACBL-10. The ACBL-04 strain promoted 100% inhibition. These eight strains were therefore selected for the *in vivo* assays (Supplementary data).

3.3. Antifungal activity in vivo

When 'Valência' sweet orange fruits were treated preventively, all eight strains reduced the development of the average size of the fruits lesions. The best preventive treatments achieved with the yeasts strains were: ACBL-05, ACBL-10, ACBL-08, ACBL-04, and ACBL-11 in this order (Figs. 1-A and 2). For curative treatment trials, the best results were displayed with the five following yeasts strains: ACBL-08, ACBL-05, ACBL-04, ACBL-14, and ACBL-11 in this order (Fig. 1-B). The fungicide imazalil control samples promoted the best curative results and did not significantly differed from the strains ACBL-05, ACBL-10, ACBL-08, ACBL-04 and ACBL-11 in the preventive treatment. The fruits untreated and uninoculated did not show blue mold symptoms. Apparently, the yeasts influenced the blue mold and did not cause damages to the fruits.

For preventive treatments, the best results regarding the percentage of healthy fruits were achieved according to the following order: ACBL-05 (92%), ACBL-10 (83%), ACBL-08 (78%) and ACBL-04 (75%) (Figs. 3-A and 2). For curative treatment, the strain ACBL-08 was able

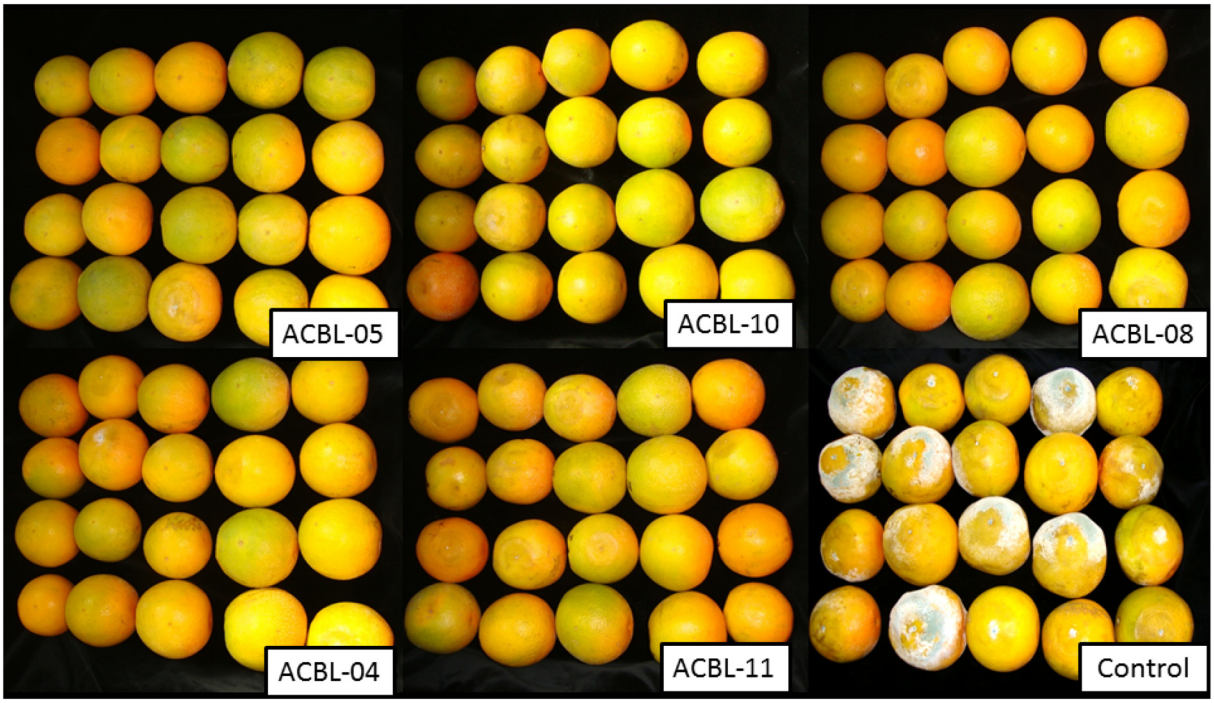

Fig. 2. Fruits of 'Valência' after preventive treatments application, they were kept for 10 days in a cold chamber (10 ± 2 °C and 90% RH). Yeast strains (treatments): ACBL-05, ACBL-10, ACBL-08, ACBL-04, and ACBL-11. Control: fruits treated with sterile distilled water with blue mold symptoms. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to reduce the occurrence of symptomatic fruits with blue mold by 57% of lesion fruits (Fig. 3-B). The fungicide imazalil control samples promoted the best curative results and did not significantly differed from the strains ACBL-05, ACBL-10, ACBL-08 and ACBL-04 in the preventive treatment.

3.4. Study of action mechanisms

3.4.1. Production of antifungal compounds

All the yeast strains evaluated did not produce cell-free and thermostable antifungal compounds in sufficient amounts to inhibit the mycelial growth of *P. italicum*.

3.4.2. Detection of 'killer' activity by yeast isolates

Three yeast strains (ACBL-05, ACBL-07 and ACBL-08) exhibited '*killer*' activity, they produced a blue inhibition ring or zone (indicative of cell death) and five strains produced a clear zone (indicative of inhibitory activity without cell death) around each colony, as illustrated in Fig. 4.

3.4.3. Nutrient competition

All of the yeast strains tested inhibited the germination of *P. italicum* conidia, regardless of the glucose concentration used (Fig. 5). ACBL-07 and ACBL-08 strains exhibited the greatest inhibition of conidial germination, with 78.18% and 68.20% inhibition, respectively.

3.4.4. Evaluation of the mycelial growth of Penicillium italicum collected from the antagonism zone

The mycelial growth analysis indicated that none of the yeast strains affected the viability of hyphae (*P. italicum*) removed from the antagonism zone. Additionally, the growth of pathogen collected from the intermediate and distal zones was unaffected.

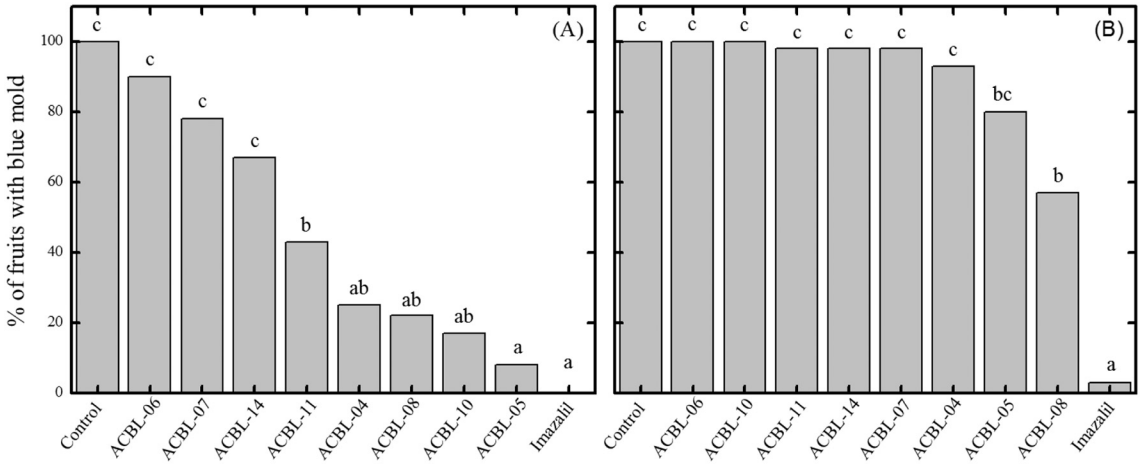

Fig. 3. Incidence (%) of 'Valência' fruits with blue mold symptoms after preventive (A) and curative (B) treatment with yeast isolates, kept in a cold chamber (10 ± 2 °C) for 10 days. Statistical significance determined at $P < 0.05$ according to Tukey test.

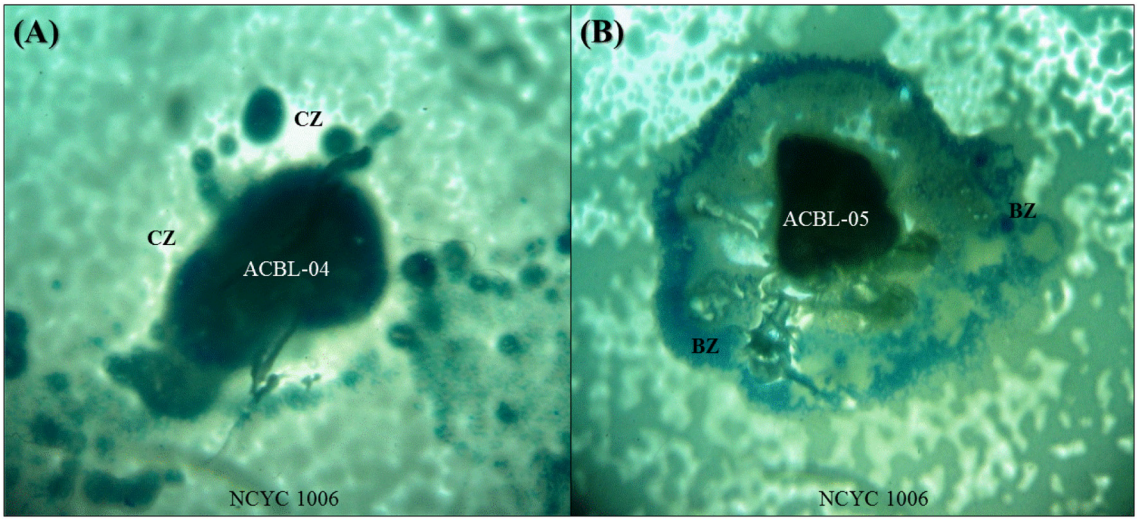

Fig. 4. (A) ACBL-04 '*killer*' activity showed clear zone (CZ) (indicative of inhibition of the susceptible yeast isolate *Saccharomyces cerevisiae* NCYC 1006). (B) ACBL-05 '*killer*' activity with a blue zone (BZ) (indicative of *S. cerevisiae* NCYC 1006 cell death) on YEPD-methylene blue medium (pH 4.5) at 28 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

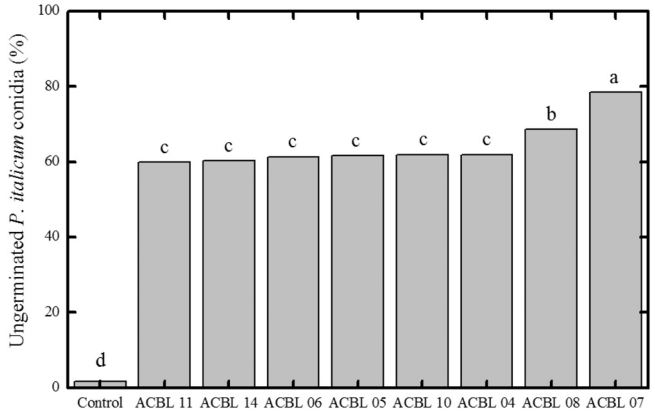

Fig. 5. Effect of yeast isolates on the conidial germination of *Penicillium italicum* grown on agar medium with different glucose concentrations at 22 °C. Mean values (represented by bar height) marked with the same letter are not significantly different according to Tukey's test ($P < 0.05$).

3.4.5. Production of hydrolytic enzymes

The findings indicated that ACBL-04, ACBL-7 and ACBL-11 were the only strains that exhibited chitinase activity (0.007, 0.003, and 0.031 g/L, respectively). The others strain no exhibited chitinase and β-1,3-glucanase activity.

3.5. Molecular identification of the most effective yeast strain

The most effective yeast isolates for the control of citrus blue mold were identified as: *Candida stellimalicola* to strains ACBL-04 (GenBank accession number: MH001971), ACBL-05 (MH001972), ACBL-06 (MH001973), ACBL-07 (MH001974), ACBL-08 (MH001975), and *Saccharomyces cerevisiae* to strains ACBL-11 (MH001976) and ACBL-14 (MH001970). The strain ACBL-10 was not identified by molecular technics.

Candida sp. strains were similar with 5.8S ribosomal RNA gene, ACBL-04 (95% of ident), ACBL-05 (97%), ACBL-06 (97%), ACBL-07 (96%), ACBL-08 with 99% of identity. The strains ACBL-11 and ACBL-14 were identified as *S. cerevisiae* with by partial sequence 5.8S rRNA gene, both with 99% of similarity.

4. Discussion

In our study, we isolated and selected yeasts from citrus leaves, flowers, fruits, and citrus soils. Also, we determined the most effective yeast strains for the biocontrol of blue mold of 'Valência' sweet oranges. Surfaces of leaves and fruits are suitable for the isolation of antagonistic microorganisms such as yeast strains, due to their quick colonization facilitated by the high sugar content of these environments (Sharma et al., 2009). Our results indicated that the phylloplane was the main source to obtain yeast strains (90.5%), they were similar by other authors (Cabral et al., 2009; Chanchaichaovivat et al., 2007; Ferraz et al., 2016; Rosa-Magri et al., 2011; Wang et al., 2009).

Evaluating the incidence and severity of blue mold on 'Valência' sweet oranges (Figs. 1 and 3), we demonstrated that the yeast strains ACBL-04, ACBL-05, ACBL-08, ACBL-10 and ACBL-11 were most effective when applied preventively rather than curatively. Other reports are in agreement with these results (Demirci, 2011; Guo et al., 2014; Lu et al., 2013).

Lu et al. (2013) study the effect of preventive treatment of cold-stored (10 °C to 15 °C) lemon fruits with *Debaryomyces hansenii* showed that yeasts at 1×10^8 cells/mL were able to confer protection to *P. italicum* (Blue mold). The protection in a room (± 22 °C) and cold temperatures (10 °C) was similar to that obtained with imazalil when the yeasts were used preventively. Their results agree with our assays.

Most postharvest infections occur through wounds caused in the fruits during harvest or soon after in the packinghouses. Thus, the preventive control of blue mold through an application of yeasts during pre-harvest may allow better protection of fruits to the pathogens that occur in the post-harvest (Usall et al., 2008). Our *in vivo* results revealed that the *C. stellimalicola* (ACBL-05) produced the best control (above 90%) of blue mold on 'Valência' sweet orange fruits when it was applied preventively. This data demonstrates the potential use of this strain for the biocontrol of citrus blue mold disease in the packinghouse. Studies by Long et al. (2005) demonstrated high levels (up to 100%) of control of *P. italicum* induced blue mold disease in citrus using the yeast *Klockera apiculata*.

In our study, the *in vivo* assays were performed in cold chamber, suggesting that the antagonist yeasts studied endure storage at low temperatures. This characteristic turns them potential agents for the biocontrol of blue mold in citrus fruit because these fruits are commonly stored under refrigerated conditions to control diseases of fruits and vegetables after harvest (Zhang et al., 2005). However, before

reaching commercial use, further studies should be conducted to assess whether these yeast strains can keep their biocontrol abilities at different temperatures ranges to endure the needs of a supply chain of citrus fruit. Human health safety studies will also definitely be needed.

We explore the mechanisms which these antagonistic yeasts interact with *P. italicum* to promote the biocontrol of citrus blue mold disease. It was verified that three yeasts strains analyzed were able to produce '*killer*' toxin, supporting that this compound is one of the main modes of action for the biocontrol of *P. italicum*. These results corroborate with data reported by other authors that found the susceptibility of pathogens to the mycocinogenic yeasts, enhancing the prospects of application of this group of antagonists (Coelho, 2005; Lima et al., 2013; Platania et al., 2012; Santos et al., 2004; Walker et al., 1995; Weiler and Schmitt, 2003).

The '*killer*' activity in diverse genera and species of yeasts has been extensively studied and it has reached important industrial recognitions (Aladdin et al., 2018). Especially, the use of *killer* yeasts as biological control agents of fungi responsible for post-harvest diseases of fruits and vegetables are being investigated more in the last two decades (Parafati et al., 2016; Perez et al., 2018). For example, Grzegorczyk et al. (2017), reported the antagonist effects from '*killer*' yeasts *Debaryomyces hansenii* KI2a, *D. hansenii* MI1a and *Wickerhamomyces anomalus* BS91 against *Monilinia fructigena* and *M. fructicola*, when tested *in vitro* and *in vivo* conditions. Parafati et al. (2015), also observed high '*killer*' activity from *W. anomalus* and *S. cerevisiae* in the biocontrol of *Botrytis cinerea*.

The *in vitro* assay showed that 58% of the yeast strains inhibited the mycelial growth of the phytopathogen, and among the best were the strains ACBL-04, ACBL-05, ACBL-06, ACBL-07, ACBL-08, and ACBL-11 reaching inhibition values above 90%. Possibly these yeasts strains were the best in the biocontrol of *P. italicum* due to biocontrol mechanisms by which these yeasts interact with the pathogen such as chitinase production, toxin '*killer*' and inhibited the germination of conidia. *S. cerevisiae* strain ACBL-14 inhibited 86% of *P. italicum* mycelial growth *in vitro*. This data agree with the study by Zhang et al. (2005), which demonstrated that *Cryptococcus laurentii* was able to inhibit the conidial germination of *P. italicum* in postharvest oranges. Other studies have been implicated yeasts in the biocontrol of *P. italicum in vitro* (P. Liu et al., 2013; McKay et al., 2012).

To our knowledge, this is the first report of *C. stellimalicola* as biological control agents against a postharvest pathogen to citrus fruit when used preventively. Our results indicated the multiple modes of action against the pathogen presented by *C. stellimalicola* strains (ACBL-04, ACBL-05, and ACBL-08), as '*killer*' activity, production of chitinase and inhibition of conidial germination, may explain why these yeasts provided control of *P. italicum* under *in vitro* and *in vivo* conditions. Therefore, in conclusion, these strains of yeast are not pathogenic to plants and so have utility against *P. italicum* 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, and later studies will be required to elucidation the '*killer*' toxin produced by these strains.

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