

The action of yeast strains as biocontrol agents against *Penicillium digitatum* in Lima sweet oranges

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SUMMARY

The green mold caused by *Penicillium digitatum* is the most important disease affecting citrus in the postharvest period. An alternative to control the disease lies on the use of yeasts. The purpose of the present study was to assess the ability of 101 yeasts strains to control *P. digitatum* *in vitro* and *in vivo*. The assay followed a randomized design with five repetitions and the evaluation took into consideration the mean pathogen colony diameter in Petri dish paired culture. In addition, the effect of the fungus on yeast strain growth was assessed. Orange fruits (*Citrus sinensis* cv. Lima) were injured in two equidistant points and inoculated with *P. digitatum* conidia suspension 24 hours before and after treatments, in the assays conducted *in vivo*. Treatments consisted in the application of eight yeast suspensions, fungicide and distilled water (control treatment); they followed a randomized design with three repetitions (20 fruits per repetition). The best antagonist results *in vitro* were recorded for five strains (*Saccharomyces cerevisiae* ACBL-76 and ACBL-82 strains; *Candida stellimalicola* ACBL-84 strain; and ACBL-87), which presented mycelial growth inhibition values higher than 80%. Two yeast strains (*S. cerevisiae* ACBL-82 strain and *Meyerozyma caribbica* ACBL-86 strain) have successfully controlled the progress and incidence of green mold in orange fruits when they were applied in a preventive manner.

Index terms: green mold, antagonist yeasts, biological control, postharvest, *Citrus sinensis*.

Utilização de isolados de leveduras em laranjas Lima para o biocontrole de *Penicillium digitatum*

RESUMO

O bolor verde ocasionado pelo *Penicillium digitatum* é a doença mais importante que afeta frutos de citros na pós-colheita. Uma alternativa para controlar a doença é por meio da utilização de leveduras. O objetivo desse estudo foi avaliar a capacidade de 101 isolados de leveduras no controle *in vitro* e *in vivo* de *P. digitatum*. O *screening in vitro* foi conduzido por meio da técnica de cultivo pareado em placa de Petri. O ensaio foi realizado com delineamento inteiramente casualizado, com cinco repetições por tratamento e a avaliação feita por meio da aferição do diâmetro médio da colônia do fitopatógeno. Além disso, também foi avaliado o efeito do fungo no crescimento

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dos isolados de leveduras. Para os ensaios conduzidos *in vivo*, frutos de laranja (*Citrus sinensis* cv. Lima) foram feridos em dois pontos equidistantes e inoculados com uma suspensão de conídios de *P. digitatum*, 24 horas antes e após os tratamentos. Os tratamentos consistiram na aplicação de suspensões de levedura, fungicida (tratamento negativo) e água destilada (tratamento controle); com três repetições por tratamento (20 frutos por repetição). Cinco isolados de leveduras obtiveram os melhores resultados *in vitro* (*Saccharomyces cerevisiae*- ACBL-76 e ACBL-82; *Candida stellimalicola*- ACBL-84 e ACBL-87), com valores de inibição do crescimento micelial superiores a 80%. Dois isolados de levedura (*S. cerevisiae*- ACBL-82 e *Meyerozyma caribbica* - ACBL-86) diminuíram o progresso e a incidência de bolor verde em frutos de laranja quando aplicados preventivamente.

Termos de indexação: bolor verde, levedura antagonista, controle biológico, pós-colheita, *Citrus sinensis*.

INTRODUCTION

Citrus fruits are the top fruit crop in international trade; China and Brazil have the largest citrus production in the world (Liu et al., 2017). Injuries caused in fruits during harvesting and transportation processes are gateways to the fungus *Penicillium digitatum*, which is the causal agent of green mold and the most economically important postharvest disease affecting citrus. Green mold reduces the quality and quantity of citrus fruits, besides damaging nutritional and market values (Plaza et al., 2004).

Hitherto, chemical treatments, such as synthetic fungicides Imazaliland Thiabendazole, have customarily been used to control the disease (Sun et al., 2013). Environmental and human health concerns, as well the emergence of pathogen strains resistant to these chemicals, have boosted the search for alternative control measures, such as biocontrol based on yeasts, to prevent or reduce citrus diseases (Zhu et al., 2006; Kinay et al., 2007; Spadaro & Droby, 2016; Liu et al., 2017).

The purpose of expanding citrus fruit exports and growing market lies on the acquisition of pesticide residue-free fruits; thus, biological control based on the application of antagonist microorganisms plays a major role among the alternative methods adopted to control several diseases affecting fruits and vegetables. Recent studies have investigated the use of yeasts to control fungal diseases in the postharvest period (Platania et al., 2012; Kupper et al., 2013; Moretto et al., 2014; Ferraz et al., 2016; Liu et al., 2017).

With respect to the microbial control field, yeasts have characteristics that make them promising pathogenic fungi-biocontrol agents, mainly because they rarely produce mycotoxins or antibiotic substances that could leave residues in fruits (Droby et al., 2002; Gamagae et al., 2004; Zhang et al., 2005). The use of yeasts in biological control programs has encouraged the development of commercial yeast-based biocontrol

products to treat postharvest diseases affecting fruits; among them, one finds *Candida oleophila* (I-182 strain), which is used to treat postharvest citrus diseases (Liu et al., 2013).

Nevertheless, the quest for identifying new yeast strains capable of thriving in different environments through new action modes remains because food security requires new biocontrol alternatives to help managing postharvest citrus diseases. Therefore, the current study has investigated yeast strains with potential to control green mold disease affecting citrus fruits by assessing their antagonist behavior towards *P. digitatum* *in vitro* and *in vivo*. The current results make a relevant contribution to the growing scientific literature about the use of yeast-based products as alternative sources to protect fruits against pathogens in post-harvest management processes.

MATERIAL AND METHODS

Pathogen and growth conditions

The pathogen (*Penicillium digitatum*) used in the current study was obtained in the microorganism collection of Centro de Citricultura “Sylvio Moreira” (IAC), Cordeirópolis County, SP, Brazil. The fungus was grown in potato-dextrose-agar (PDA) for 7-10 days and cultures were incubated in BOD (Biochemical Oxygen Demand) chamber at 25 °C for 3-7 days, under 12 h photoperiod, and kept in PDA medium within test tubes for further testing. Before it was use, the pathogen was monitored under bright field (Leica Microsystems Co., Wetzlar, Germany) microscope, at 400 × magnification, in order to observe vegetative conidia. Standardized conidia suspension concentrations were adjusted in Neubauer chamber, after serial dilutions.

Yeast strains

The herein performed assays used 101 yeast strains. Six *Saccharomyces cerevisiae* strains (ACB-BG1, ACB-CAT1, ACB-CR1, ACB-K1, ACB-KD1, and ACB-PE2) were obtained from ethanol fermentation; they were characterized by karyotyping electrophoresis and kept in the Collection of the Biochemistry and Plant Pathology Laboratory at University of São Paulo (ESALQ), Piracicaba County, SP, Brazil (Fialho et al., 2010). The others yeasts (95 strains) were obtained from citrus leaves, flowers, fruits and soil from São Paulo citrus belt; seven strains were identified by our group based on the sequencing of ITS regions of the rDNA (five strains of *Saccharomyces cerevisiae* - ACBL-76, ACBL-78, ACBL-80, ACBL-81, and ACBL-82 strains; one strain of *Candida stellimalicola* - ACBL-84 strain; and one strain of *Meyerozyma caribbica* - ACBL-86 strain) (Ferraz et al., 2018) and they were kept in the Microorganism Collection of Centro de Citricultura “Sylvio Moreira” (IAC), Cordeirópolis County, SP, Brazil.

Cultures were grown in YEPD medium (yeast extract peptone dextrose – 1% yeast extract, 2% peptone, 2% glucose, 2% agar, 0.01% ampicillin, and 0.01% nalidixic acid dissolved in 1 liter of distilled water), incubated in BOD chamber at 25 °C, for 3-7 days, under 12 h photoperiod, and kept in YEPD medium within test tubes for further testing.

Screening the antifungal activity *in vitro* of yeast strains

The paired cultivation technique in Petri dishes (Dennis & Webster, 1971) was used to assess the antagonistic capacity of all 101 yeast strains against *P. digitatum*. Disks (5mm diameter) were removed from active *P. digitatum* colonies grown in PDA for 7-10 days and placed in Petri dishes containing PDA culture medium, 3cm away from disks containing 1 strain of each yeast (5 mm diameter) grown in YEPD medium for 48 hours. The control group comprised fungi growth without yeast addition. The experiment followed a completely randomized design with four repetitions for each of the 5 trials. Data were subjected to analysis of variance (ANOVA). Means were tested through the Scott-Knott test ($P < 0.05$).

In addition, the effect of *P. digitatum* on the vegetative growth of 101 yeast strains colonies was assessed based on the paired cultivation technique applied in Petri dishes; however, for this assay, we assessed the colony growth of each yeast strain cultivated with the fungus. Control

treatment corresponded to yeasts strains colony growth without the fungus (adapted from Dennis & Webster, 1971). This assay followed a completely randomized design with two repetitions. The average of colonies diameter from yeasts strains were subjected to analysis of variance (ANOVA) with two-factor factorial design and means were compared to each other through the Scott-Knott test ($P < 0.05$).

Antifungal activity *in vivo*

Based on the methodology described by Ferraz et al. (2016), Lima sweet orange fruits [*Citrus sinensis* (L.) Osbeck] at commercial ripening stage were obtained in a packinghouse located in Limeira County, São Paulo State, Brazil. Orange fruits (without treatment) were superficially disinfected with 0.2% (v/v) sodium hypochlorite for 3 minutes and, later, they were washed with distilled water. Sterile needles were used to injure the fruits (3 mm-deep) at two equidistant points in their median region; next, the wounded area was inoculated with 20 µL of *P. digitatum* conidia suspension (1.0×10^5 conidia mL⁻¹) and treated with the yeast (curative treatment) 24 hours later. As for the preventive treatment, the fruits were wounded, treated with the yeast and inoculated with the pathogen, 24 hours later.

Eight yeast strains (*Saccharomyces cerevisiae* - ACBL-76, ACBL-78, ACBL-80, ACBL-81, and ACBL-82 strains; *Candida stellimalicola* - ACBL-84 strain; *Meyerozyma caribbica* - ACBL-86 strain; and ACBL-87) were selected based on screening *in vitro* in order to compose the treatments applied in assays conducted *in vivo*. Treatments were prepared based on the methodology by Ferraz et al. (2016); yeast strain suspensions were adjusted to 1.0×10^7 cells mL⁻¹. The fungicide Imazalil was used as negative control, based on the manufacturer's recommendations to control green mold (200 mL/L). Fruits treated with sterile distilled water were used as positive control.

Inoculated and treated fruits were stored for seven days at room temperature (22 °C ± 2, 80% RH, and 12 h photoperiod). Disease severity was assessed on the 5th, 6th and 7th day after inoculation by measuring the diameter of green mold lesions with a caliper. The incidence of the disease was assessed based on the percentage of infected fruits observed at the 7th day after inoculation. Treatments of the assays conducted *in vivo* followed a completely randomized design, with three repetitions per treatment and 20 fruits per repetition. The effect of the treatments on the mean size of the lesions caused by

P. digitatum was analyzed based on the area under the disease progress curve (AUDPC), which was calculated and expressed by plotting the disease proportion as percentage versus time. According to Shaner & Finney (1977), the AUDPC can be calculated based on the following formula:

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

wherein: n is the number of observations/evaluations; Y_i is the disease proportion in the “i”-th observation/evaluation; and T_i is the time (in days) in the “i”-th observation/evaluation;

Results were subjected to analysis of variance (ANOVA) in the *Assistat 7.7 beta* software and means were compared to each other through Tukey test ($P < 0.05$).

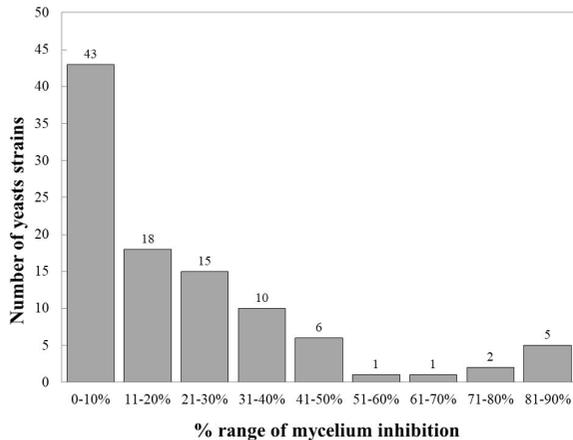


Figure 1. *In vitro* screening of the yeasts strains by antifungal activity against *Penicillium digitatum*. Number of yeasts strains assessed *in vitro* (y-axis). Range of mycelium inhibition (x-axis).

RESULTS

Antifungal activity *in vitro*

The screening *in vitro* of 101 yeast strains (Figure 1) has shown that 14 yeast strains were capable of inhibiting *P. digitatum* mycelial growth up to 45%. Five strains inhibited more than 80% of *P. digitatum* mycelial growth; they are herein presented based on their increasing order of inhibition power: ACBL-82, ACBL-86, ACBL-84, ACBL-76, and ACBL-87. Three strains (ACBL-81, ACBL-78, and ACBL-80) inhibited *P. digitatum* mycelial growth by 60-79% (Figure 2). Therefore, these eight best strains were selected for the assays conducted *in vivo*.

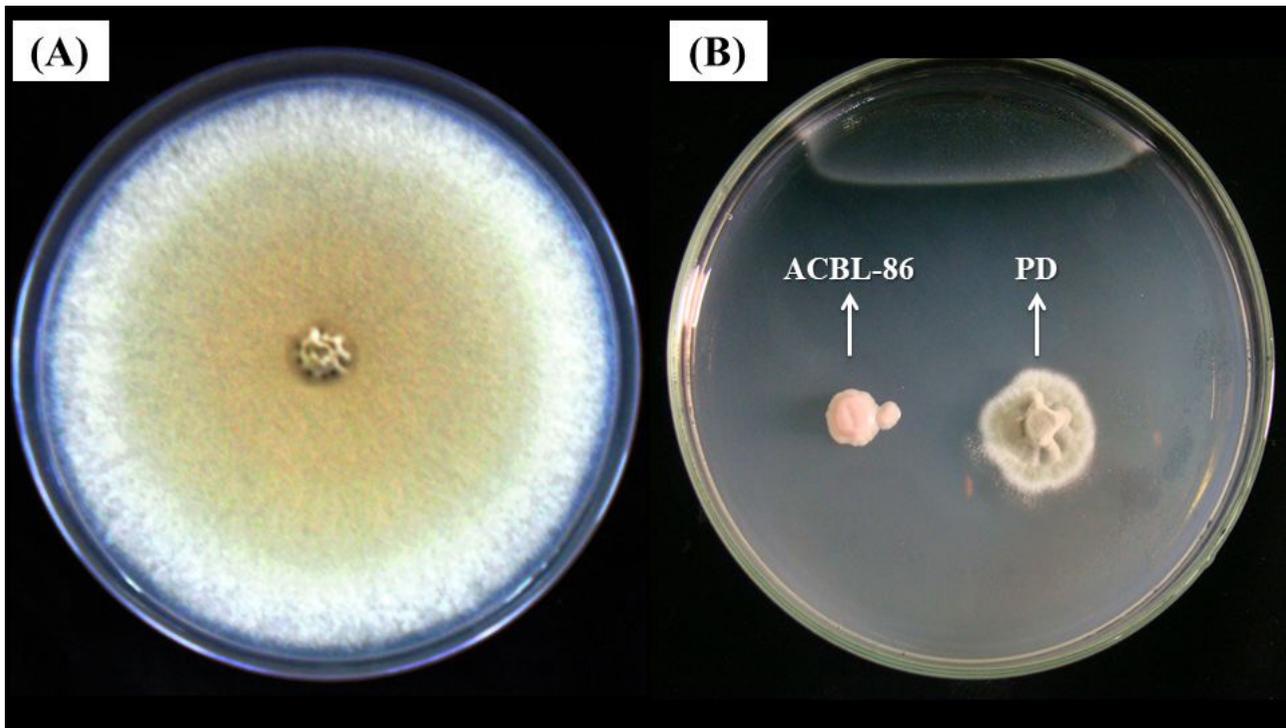


Figure 2. *In vitro* antagonism of *Saccharomyces cerevisiae* - ACBL-86 strain (B), with 83% inhibition of *P. digitatum* colony (PD) by paired cultivation technique in Petri dishes, after seven days storage at $25 \text{ }^\circ\text{C} \pm 2$ and 12h photoperiod. *P. digitatum* colony without yeast strain (control) (A).

The effect of *P. digitatum* on the growth of all 101 yeast strains was assessed and the fungus was capable of inhibiting the vegetative growth of 24 strains up to 35% (Figure 3). The colony growth of six strains (ACLB-39, ACLB-51, ACLB-76, ACLB-83, ACLB-84, and ACB-KD1) was stimulated when they were cultivated along with the fungus. Among these yeast strains, ACBL-76 was also capable of inhibiting pathogen growth in assays conducted *in vitro* -by up to 88%, as demonstrated in the above assay by evaluation of antifungal activity *in vitro* (Figure 1).

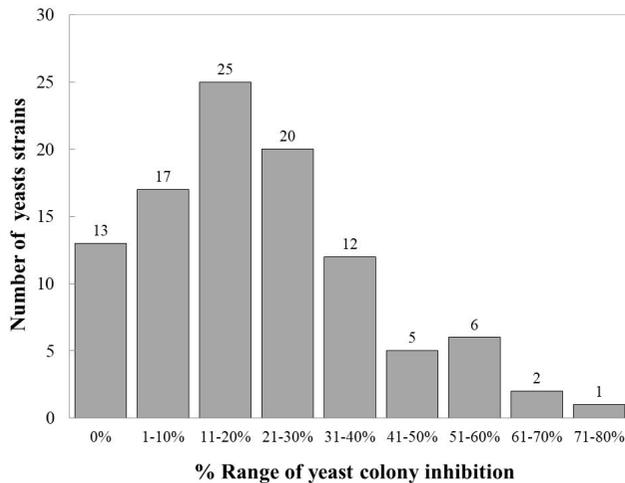


Figure 3. Influence of *P. digitatum* on the yeast isolates colony's growth in paired cultivation. Number of yeasts strains assessed *in vitro* (y-axis). Range of inhibition of yeasts colonies (x-axis).

Effect of yeast treatments on the progress of green mold disease affecting Lima sweet orange fruits

Three yeast strains-ACBL-86(11.9mm),ACBL-82(19.2mm) and ACBL-80 (22.8 mm) (Figure 4A) -reduced the development of the mean size of fruit lesions when Lima sweet oranges were preventively treated. Among the curative treatment trials, only the negative treatment (Imazalil) reduced the progress of green mold disease (Figure 4B). However, results recorded for strains ACBL-86, ACBL-82 and ACBL-80 were statistically similar to that of the fungicide, when these strains were preventively used (Figure 4A).

Effect of treatments on the incidence of green mold in Lima sweet orange fruits

Yeast strains ACBL-86 and ACBL-82 enabled lower disease incidence control when fruits were preventively treated (Figure 5); they inhibited by 42% and 28% the incidence of symptomatic fruits, respectively (Figure 6A). The negative control (Imazalil) enabled 100% healthy fruits when it was used in a preventive manner. Yeast strains used in the curative treatment trials was not effective in controlling the disease (Figure 6B).

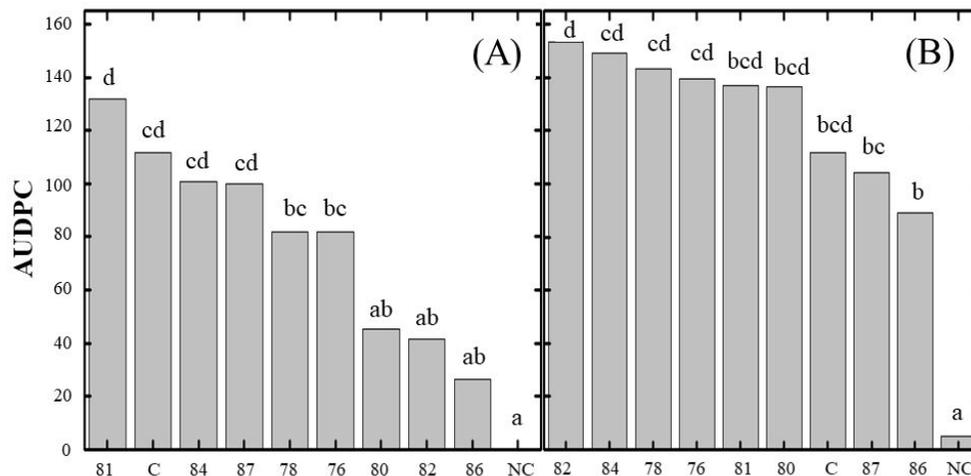


Figure 4. Area under the disease-progress curve (AUDPC) for average diameter of lesion caused by *P. digitatum*, after preventive (A) and curative (B) treatment with yeast strains, stored (22 °C ± 2 and 90% RH) for seven days (y-axis). Mean values marked with the same lowercase letter are not significantly different according to Tukey's test ($P < 0.05$). (x-axis): The number corresponds to the yeast biological control agents (ACBL) (x-axis); C = control: distilled water; NC = negative control – Imazalil.

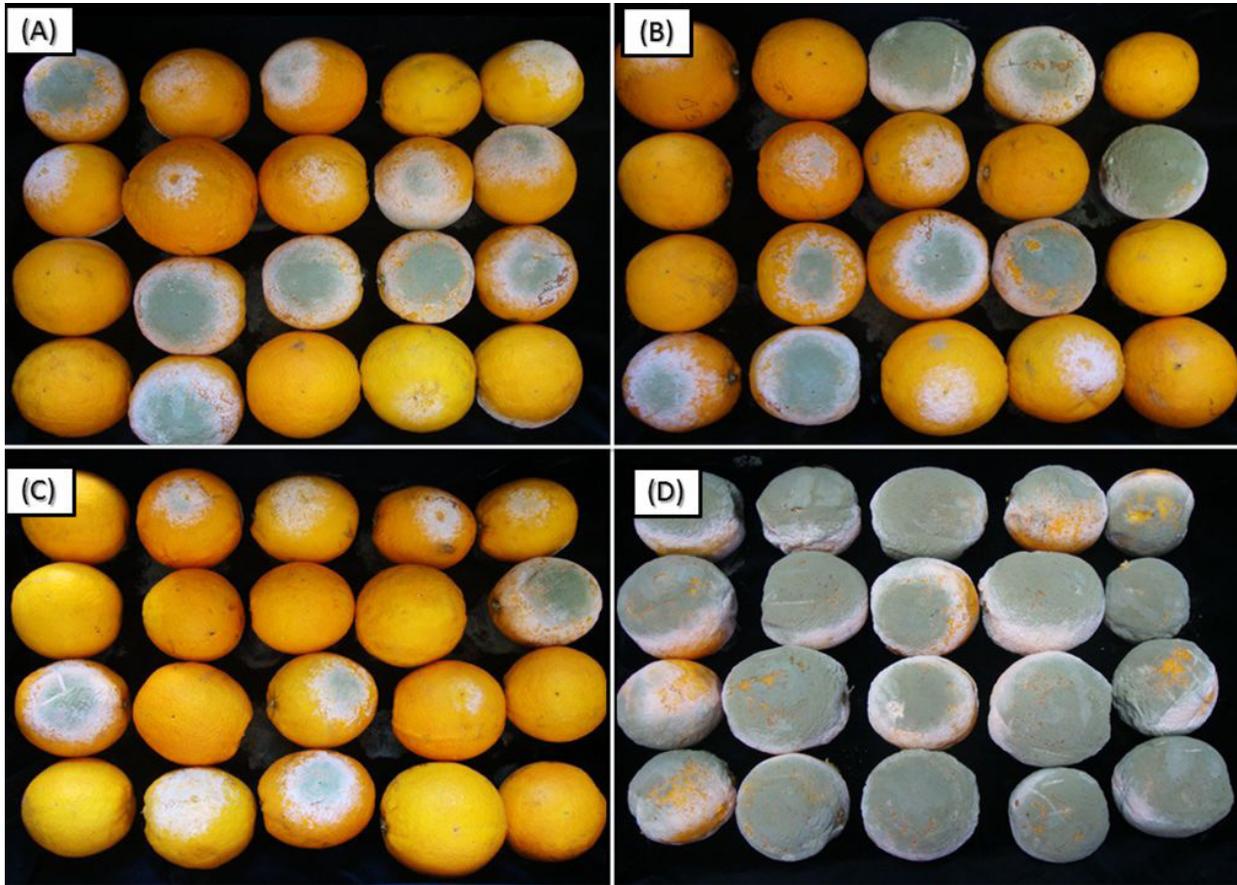


Figure 5. Lima sweet oranges after preventive treatments application and inoculation with *P. digitatum*. Fruits stored for seven days at 22 °C ± 2 and 90% RH. Yeast strains treatments (A) ACBL-80 (*Saccharomyces cerevisiae*); (B) ACBL-82 (*S. cerevisiae*) and (C) ACBL-86 (*Meyerozyma caribbica*); (D) Control: fruits treated with sterile distilled water with green mold symptoms

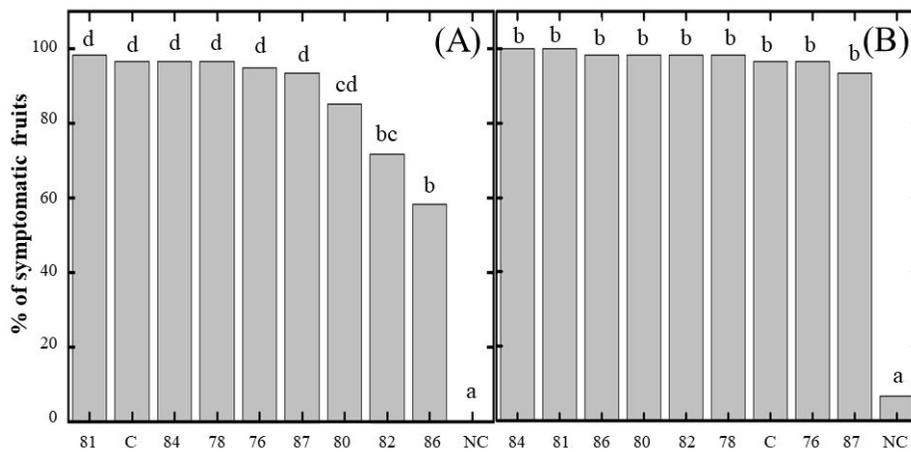


Figure 6. Percentage of Lima orange fruits with green mold symptoms after preventive (A) and curative (B) treatment with different yeast isolates, stored (22 °C ± 2 and 90% RH) for seven days (y-axis). Means values marked with the same lowercase letter are not significantly different according to Tukey’s test ($P < 0.05$). (x-axis): The number corresponds to the yeast biological control agents (ACBL) (x-axis); C = control: distilled water; NC = negative control – Imazalil.

DISCUSSION

Several steps are required to obtain a biological control agent, which involve the isolation and selection of different microorganisms in order to find strains with desirable features used to formulate commercial products (Droby et al., 2009). The current study selected yeasts with potential to enable the biocontrol of *P. digitatum*.

Previous studies have demonstrated that phylloplane was the main source used to obtain yeast isolates; most yeast strains used in the current study were isolated from citrus leaves, flowers and fruits (Ferraz et al., 2018). Similar to other studies conducted with yeasts isolated from citrus phylloplane, antagonist yeasts have shown potential to control *Penicillium italicum* (Liu et al., 2017; Cunha et al., 2018) and *P. digitatum* (Perez et al., 2017; Ghosh et al., 2018; Habiba et al., 2019). Indeed, leaf and fruit surfaces are suitable for the isolation of antagonistic microorganisms such as yeast strains, due to fast colonization enabled by high sugar contents found in these environments (Sharma et al., 2009; Liu et al., 2013).

The dual culture plate method was applied to all yeast strains in order to investigate their antagonistic behavior towards *P. digitatum*; 15% of the strains inhibited the mycelial growth of the fungus by at least 40%. Five strains were capable of inhibiting more than 80% of pathogen mycelial growth (*S. cerevisiae* ACBL-82 strain, *M. caribbica* ACBL-86 strain, *C. stellimalicola* ACBL-84 strain, *S. cerevisiae* ACBL-76 strain, and ACBL-87). The current results are in compliance with other studies conducted *in vitro*, such as the one conducted by Mekbib et al. (2011), who found that three yeast strains (two *Cryptococcus laurentii* and one *Candida sake*) were capable of rapidly colonizing the surface of *P. digitatum* colonies, where they competed with the pathogen for nutrients and space.

Based on the effect of *P. digitatum* on the growth of yeast strains, it was possible seeing that ACBL-76 had its growth boosted by the presence of the fungus. In addition, the aforementioned yeast strain was capable of inhibiting the growth of the pathogen colony by up to 88%. This outcome may indicate that the action mode of ACBL-76 against *P. digitatum* is based on factors such as the inhibition of fungal conidia germination, nutrient competition, *killer* activity, and chitinase production, as reported in previous studies conducted by our research group (Ferraz et al., 2018).

Yeast strains ACBL-86 (*M. caribbica*) and ACBL-82 (*S. cerevisiae*) decreased the severity, and prevented the incidence of green mold in Lima sweet

oranges subjected to assays *in vivo*. Taqarort et al. (2008) reported fifteen yeast strains capable of reducing by more than 50% the incidence of *P. digitatum* in citrus fruits after seven incubation days at 25°C. Yeast strain ABCL-86 inhibited by at least 42% the incidence of symptomatic fruits and reduced the development of green mold lesions, when it was used in a preventive manner in the current study. However, biocontrol agents alone cannot assure consistency and protection effect in comparison to the negative control (synthetic fungicide Imazalil). Overall, antagonistic microorganisms have shown great effectiveness variability, since their protective effect decreased as fruit ripening progressed. In addition, these microorganisms cannot often eradicate pre-existing or incipient fungal pathogens and/or do not prevent their sporulation (Moretto et al., 2014). Thus, the use of antagonistic microorganisms in combination with other biological, chemical or physical methods is one of the main alternatives to improve the biocontrol of postharvest diseases (Usall et al., 2008).

Based on the evaluation of yeast ability to control green mold in orange fruits assessed in the present study, two strains (ACBL-82 and ACBL-86) were capable of decreasing the progress, and the incidence, of the disease when they were used in a preventive manner. Physical postharvest treatments such as short hot water brushing, and low ultraviolet light irradiation doses, have demonstrated potential to control postharvest fruit diseases (Terao et al., 2015). In addition, the combination between antagonistic yeasts and different disease control methods has been more effective in reducing postharvest fruit decay, besides compensating limitations resulting from their individual use. Thus, this combination is a promising way to minimize the use of synthetic fungicides in postharvest treatments (Wisniewski et al., 2016; Terao et al., 2017). Therefore, yeast strains could be preventively used against *P. digitatum* as an alternative, or complementary, control method in citrus postharvest management processes.

Finally, the postharvest biocontrol of green mold based on epiphytic yeasts appears to be a promising alternative to the use of synthetic fungicides, since it has less negative impact on human health and on the environment. Hence, it is necessary conducting further studies with yeast strains ACBL-82 and ACBL-86 in order to assess their ability to prevent *P. digitatum* colonization in fruit wounds treated with commercial formulations, as well as to investigate whether it is necessary combining the use of these yeasts with other methods in order to control green mold disease.

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