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Non-conventional yeasts as biocontrol agents against fungal pathogens related to postharvest diseases

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There are numerous investigations related to biological control and the use of microorganisms as new control strategies. In this sense, yeasts have been reported to have biocontrol activity and could be interesting candidates for BCA development. The main goal of this study was to evaluate the antagonist effect of four endophytic yeast, *Naganishia antarctica* YCPUC12, *Aureobasidium pullulans* YCPUC14, *Cryptococcus terrestris* YCPUC16 and *Filobasidium oeirense* YCPUC41 over the growth of *Botrytis cinerea* MPC40, *Monilinia laxa* MPC71, *Penicillium expansum* MPC102 and *Geotrichum candidum* MPC115. Three experimental approaches were used to evaluate biocontrol effect of yeast. Two *in vitro* assays (agar plug diffusion and dual-culture) and one *in vivo* assay. The results obtained here revealed that the four yeast strains evaluated showed inhibitory effect against the phytopathogens tested. Furthermore, it is indicated that the inhibitory effect by the yeasts over the fungi is differential, suggesting that there could be involved more than one antagonist-mechanism on the part of yeasts. Yeasts produce compounds capable of inhibiting the growth of fungi and, depending on the assay, the evaluated antagonist-yeasts have differential biocontrol effect against these pathogens. Their potential as biocontrol agents for postharvest diseases are interesting, and further investigation is needed to verify the effectiveness of these antagonists.

Keywords: Native yeast, inhibitory activity, mycelial growth, non-chemical control methods, antagonist-mechanism.

In the postharvest process, there are many losses in the productive chain, up to 25 % of total production in industrialized countries and more than 50 % in developing countries. This phenomenon is attributed to decay fungi, such as *Botrytis* spp., *Penicillium* spp., *Aspergillus* spp., *Colletotrichum* spp., among others (Barkai-Golan 2001, Janisiewicz & Korsten 2002, Gustavsson et al. 2011, Francesco et al. 2015).

The control of fungal diseases is mainly based on the use of synthetic fungicides (Sundh & Melin 2011, Villalba et al. 2016) which can impact human health, increase the level of hazardous residues in the environment and induce the occurrence of resistant strains due to unrestrained use (Bencheqroun et al. 2007, European Parliament 2009, Simionato et al. 2017)

There has been extensive research to explore and develop non-chemical control methods to reduce postharvest decay of fruits and postharvest physiological disorders. In this sense, yeasts are unicellular fungi that are present in different ecosystems and sources, both natural and in connection with human activities. They are the major component of the epiphytic microbial community on surfaces of fruits and vegetables and they are also phenotypically adapted to this ecological niche (Kurtzman et al. 2011, Parafati et al. 2015). The potential of yeasts as antagonists to biologically control harvest pathogens has emerged as an eco-friendly alternative, with a low environmental impact as part of integrated disease management (IDM) to reduce synthetic fungicide application (Droby et al. 2009, Kurtzman, Fell & Boekhout 2011, Sui et al. 2015, Sipiczki 2016, Spadaro & Droby 2016, Amina et al. 2018, Pretscher et al. 2018, Wisniewski & Droby 2019).

However, yeasts often show lower and non-comparable effectiveness against pathogenic fungi compared to chemical fungicides (Liu et al. 2013), reducing their practical application and leaving the problem of fungal diseases still unsolved. Bioprospecting of bioactive substances for commercial purposes takes into account genetic and biochemical resources. In contrast, the effects of environmental factors on biocontrol systems, especially the vi-

ability and efficacy of antagonistic yeast species that could be used in the control of diseases in agriculture, still need to be investigated (Sui et al. 2015, Simionato et al. 2017, Mukherjee et al. 2020)

In general, to successfully inhibit the pathogenic infection and development, several possible mechanisms operate in a tritrophic host-pathogen-antagonist interaction system (Spadaro & Droby 2016, Wisniewski & Droby 2019). Different modes of action of yeast strains against pathogenic fungi have been reported, and include antibiosis, mycoparasitism, induced resistance (Sipiczki 2006, Sharma et al. 2009, Jamalizadeh et al. 2011, Spadaro & Droby 2016); nutrient or space competition (Schena et al. 2000, Sipiczki 2006, Sharma et al. 2009, Jamalizadeh et al. 2011, Parafati et al. 2015, Spadaro & Droby 2016); iron depletion (Sipiczki 2006, Parafati et al. 2015); extracellular lytic enzymes production (Bar-Shimon et al. 2004), volatile organic compounds (Fredlund et al. 2004, Contarino et al. 2019); reactive oxygen species (ROS) tolerance (Jamalizadeh et al. 2011, Liu et al. 2015); and biofilm formation (Giobbe et al. 2007, Wisniewski et al. 2007).

We evaluated the antifungal activities of four native yeast *Naganishia antarctica* YCPUC12, *Aureobasidium pullulans* YCPUC14, *Cryptococcus terrestris* YCPUC16 and *Filobasidium oeirense* YCPUC41 over the growth of four phytopathogenic fungi (*Botrytis cinerea* MPC40, *Monilinia laxa* MPC71, *Penicillium expansum* MPC102 and *Geotrichum candidum* MPC115) as potential biocontrol agents, using *in vitro* and *in vivo* approaches.

Materials and methods

Microorganisms

Native wine yeast strains were obtained from the yeast collection (YCPUC) of the Microbiology and Yeast Genetics Laboratory of Pontificia Universidad Católica de Chile (Tab.1). The fungi evaluated were obtained from the collection of the Molecular Phytopathology Laboratory. Strains were identified by amplification and sequencing of the ribosomal Internal Transcribed Spacer (ITS) region according to Schoch et al. (2012).

The yeasts were grown in yeast extract–peptone–dextrose (YPD) medium (0.5 % peptone, 0.5 % yeast extract, and 2 % glucose) at 28 ºC with constant agitation for one to three days, as per the strain's growth conditions. Then, they were maintained on YPD agar (0.5 % peptone, 0.5 % yeast extract, 2 % glucose, and 2 % agar) at 4 °C until use. The fungi were grown on potato-dextrose-agar (PDA) (2 % dehydrated potato, 2 % dextrose, and 2 % agar) acidified with 250 µl of 1N lactic acid (APDA) and incubated for seven days at 22 °C. Then, they were maintained at 4 °C until use.

Agar Plug Diffusion method

Using a classic qualitative method, the ability of each yeast strain to inhibit growth of the four fungi from the collection was tested. The yeasts were grown individually for 48 h at 28 ºC with agitation to 200 rpm in YPD medium until a concentration of 1×10^8 cells/ml. A dilution 1:10 was used as inoculum, and an aliquot of 100 µl was plated on each APDA agar. When the lawn was dry, disc of the fungus was placed face down in the middle of the agar plate. The fungi were grown individually on APDA plates for 7 days. Then a disc of the fungus was taken using sterile toothpick/forceps and put upside down in the middle of the plate, in direct contact with the yeast lawn previously prepared (Fig. 1A). Every fungus yeast combination was tested in triplicate.

Inhibition zone around the disc was used as a measure of inhibition activity; this measurement was recorded in centimeters (cm). To determine the percentage of inhibition of the assays, the calculation was performed according to the following formula (Eq. 1).

Dual-Culture Assay

Dual-culture assay was made to assess the production of volatile organic compounds (VOCs) by

Tab. 1. Isolates of native wine yeasts and fungi used in this study.

Code	Yeasts	Code	Fungi
YCPUC12	*Naganishia antarctica*	MPC40	*Botrytis cinerea*
YCPUC14	*Aureobasidium pullulans*	MPC71	*Monilinia laxa*
YCPUC16	*Cryptococcus terrestris*	MPC102	*Penicillium expansum*
YCPUC41	*Filobasidium oeirense*	MPC115	*Geotrichum candidum*

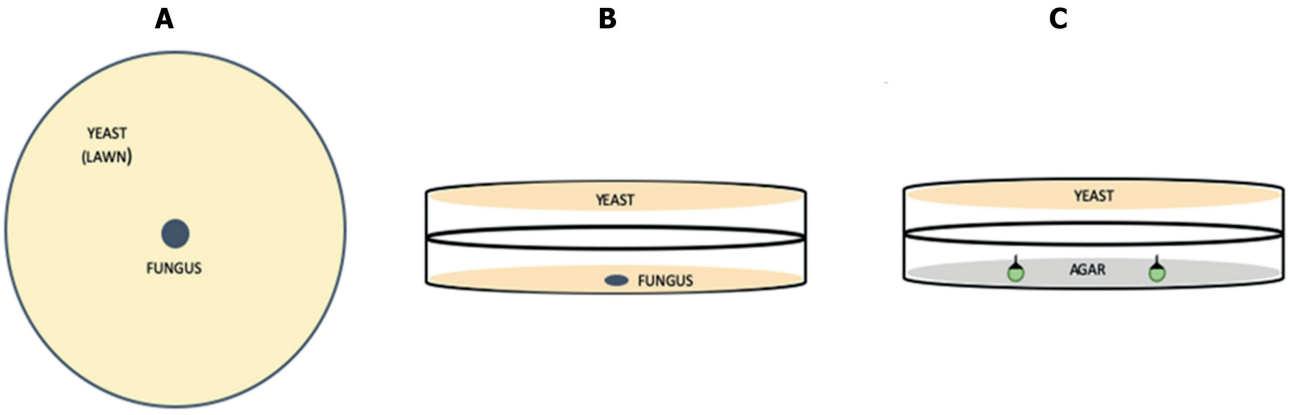

Fig. 1. Schematic representation of the different strategies to evaluate the antimicrobial activity of the microorganisms. **A.** Inhibition Zone Diameter Assay; **B.** Confrontation Assay; **C.** In vivo confrontational assay.

the yeasts. One plate contained a lawn of the yeast, and other plate contained a disc of the fungus previously grown. The yeast plate was inverted and placed on top of the other plate. The plate containing the fungus was the basal plate and the plate with the yeast, the cover. Control treatments were prepared using the same experimental setup, but the upper plates only contained APDA medium without the presence of the yeast. The plates were sealed with parafilm and incubated for 7–10 days at 22 °C (Fig. 1B). The experiments were made in triplicate. The inhibition rate of each yeast against the pathogenic fungi was calculated with Eq.1:

$$\text{Average percent inhibition of each treatment} = \left(\frac{C-T}{C}\right) \times 100 \quad (1)$$

Where:
C = Average of three replicates of the mycelial growth diameter of control treatment (C-)
T = Average of three replicates of the mycelial growth diameter in the presence of the selected treatment.

In vivo Dual-culture assay

This assay was performed using berries without any postharvest treatment. Two *Vitis vinifera* cv. 'Viognier' berries were used in each treatment and the experiment was made in triplicate. Berry size was selected between 1.5–2.0 cm in diameter and 1.8–2.2 g of weight. Berries were disinfected with 70 % (v/v) of ethanol for 5 min, washed with distilled sterile water for 5 min (this was repeated 3 times) and dried at room temperature. After this step, the corresponding treatments were applied. Wound treatments were made with a surgical blade N° 15. The size of the wound was about 4 mm deep and 4 mm wide (blade width). Each wound was inoculated with 20 µl droplet of 1×10^8 conidia/ml of *B. cinerea*. The yeast suspension was prepared in the same way as described in agar plug diffusion assay. Finally, the plates were assembled (Fig. 1C). For each treatment, two berries were placed, equidistant from each other, in a 2 % agar (to avoid movement). Next, the plate with the yeast lawn was placed on the top of the berry plate, and both plates were sealed and incubated for seven to ten days at 28–30 °C. In the case of the control treatments, an APDA plate without the yeast lawn was used. Four treatments were tested: *A. pullulans* YCPUC14 + healthy berries without Bc (T1), inoculated with Bc (T2), wounded berries without Bc (T3), wounded berries inoculated with Bc (T4). Control treatments were evaluated in the absence of the yeasts and named as C1-4.

The incidence and severity of the disease were determined using the Phytotoxicity Scale (Romanazzi et al. 2001) which includes 6 degrees of infection. This scale is based in McKinney's Disease Index (McKinney 1923) and it expresses the weighted means of the disease, as a percentage of the maximum possible level.

Statistical analysis

The analyses were done using Statgraphics Plus, version 5.1. (StatPoint Technologies, USA). Comparisons were carried out using analysis of variance (ANOVA) and the mean values of the experiments were compared using the LSD test. The treatments were considered significant when the p-values ≤ 0.05.

Results

As a first experimental approach to evaluate the biocontrol effect of yeasts, the agar plug diffusion assay was done. This assay measures the ability of a

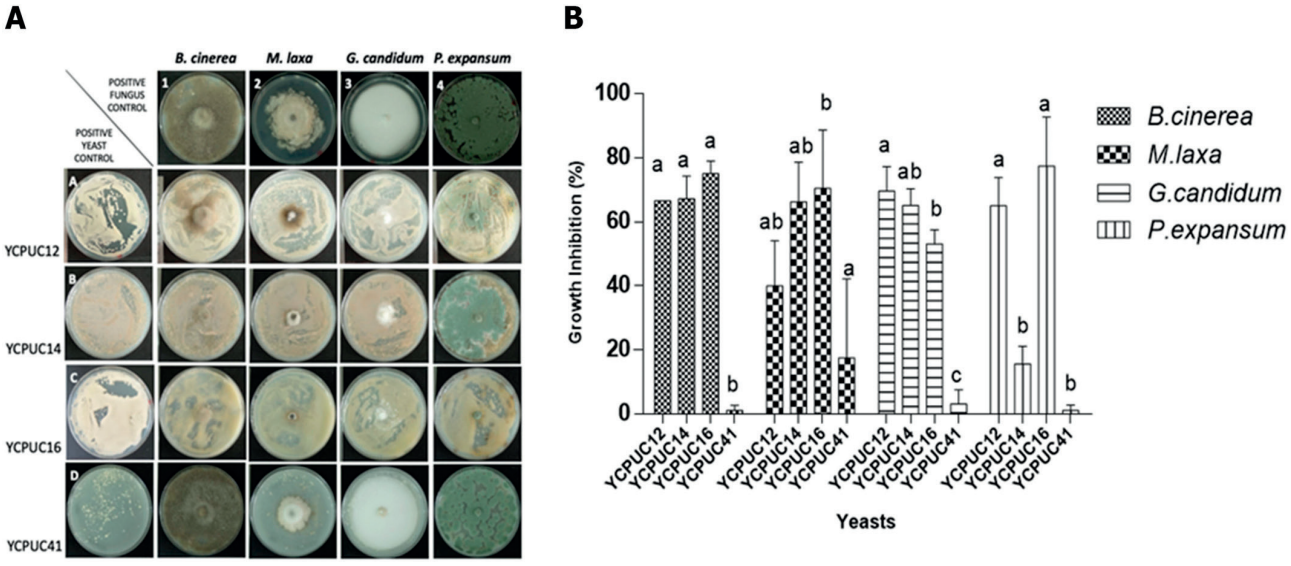

Fig. 2. Evaluation of the biocontrol activity of the yeast isolates selected. On the left side of the figure (A) Inhibition Zone Diameter Assay. On the top of the figure are the names of the fungi tested and in the first row are their positive controls (1–4). The first column corresponds to the positive control of the yeast isolates (A–D). (B) Percentage of growth inhibition growth. The experiments were performed in triplicate and results are the average. Different letters indicate significant difference at 95 % confidence level.

microorganism to inhibit the growth of another through the production of inhibitory compounds or through competition for space or nutrients.

The results obtained (Fig. 2 A, B) showed that *N. antarctica* YCPUC12 was able to reduce the mycelial growth of *B. cinerea* MPC40, *G. candidum* MPC115 and *P. expansum* MPC102 by 67, 70 and 65 % respectively, compared to the control. For *M. laxa* MPC71, the inhibition was only 42 %.

Aureobasidium pullulans YCPUC14 reduced mycelial growth of *B. cinerea* MPC40, *M. laxa* MPC71 and *G. candidum* MPC115 by 67, 68 and 65 %, and the lowest effect was observed for *P. expansum* MPC102 (16 %). *Cryptococcus terrestris* YCPUC16 was able to reduce the mycelial growth of *B. cinerea* MPC40, *M. laxa* MPC71, *G. candidum* MPC115 and *P. expansum* MPC102 by 75, 73, 53 and 77 % respectively. *Filobasidium oeirense* YCPUC41 presented the lowest inhibitory effect, with percentages below 20 % for all pathogens evaluated.

In order to determine the ability of the yeast to produce volatile organic compounds (VOCs), the second experimental approach was dual-culture assay. The results showed that *A. pullulans* YCPUC14 reduced mycelial growth of *B. cinerea* MPC40 and *M. laxa* MPC71 with 72 and 64 % respectively, and *C. terrestris* YCPUC16 with 52 and 51 % for the same fungi. In the case of *P. expansum* MPC102, *N. antarctica* YCPUC12 and *F. oeirense* YCPUC41 reduced by 31 % the mycelial growth of the pathogen (Fig. 3).

The final experimental approach was to evaluate the biocontrol effect of yeasts against *B. cinerea* MPC40 through an *in vivo* confrontational assay performed on *V. vinifera* cv Viognier. For this assay, we evaluated the yeast *A. pullulans* YCPUC14 as it showed the best results in the *in vitro* assays. Activity was determined by calculating the degrees of infection using the McKinney's Index as a measure of the phytotoxicity caused by this pathogen (Fig. 4A,

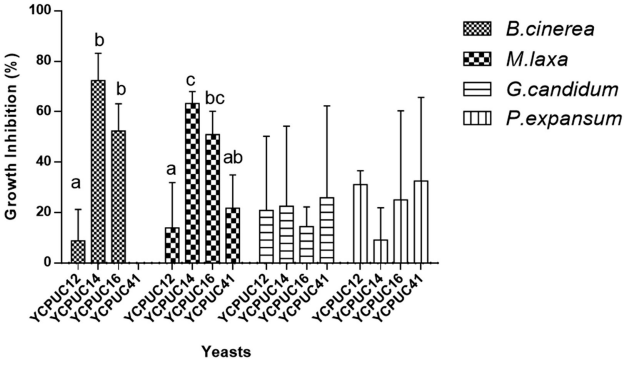

Fig. 3. Percentage of growth inhibition obtained by the confrontation assay after 7 days incubation at 22 °C. The experiments were performed in triplicate and results are the average. Different letters indicate significant difference at 95 % confidence level.

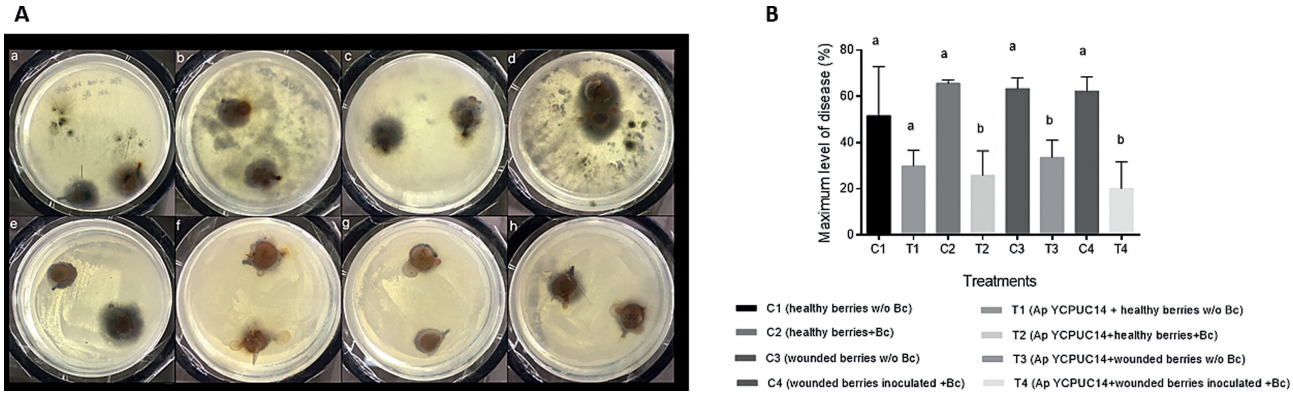

Fig. 4. A. In vivo evaluation of *A. pullulans* YCPUC14 against *B. cinerea* tested in berry grapes. a-d corresponds to control, where (a) APDA plates+ berries w/o damage w/o Bc; (b) Berries w/o damage + Bc; (c) Berries damage w/o Bc; (d) Berries damage + Bc; e-h are the treatments with *A. pullulans* YCPUC14 lawn, where (e) APDA plates with *A. pullulans* YCPUC14 lawn and berries w/o damage w/o Bc; (f) Berries w/o damage + Bc; (g) Berries damage w/o Bc; (h) Berries damage + Bc. – **B.** Maximum level of disease (%) obtained by the *in vivo* confrontation assay after 7 days incubation at 22 °C. Three replicates were evaluated per treatment. C1-4 are the control treatments and T1-4 are the treatments with the yeast antagonist *A. pullulans* YCPUC14. Different letters indicate significant difference at 95 % confidence level.

B). The results obtained were expressed as the average percentage of three replicates per treatment.

In control treatments C2, C3 and C4, we observed 100 % of infection and C1 showed a 59% of infection on berries. On the other hand, in the treatments (T) where the yeast was present, we could observe infection at lower percentages: T1 (20 %), T2 (35 %) T3 (60 %) and T4(20 %). These results suggest a biocontrol effect of *A. pullulans* YCPUC14.

In those treatments without inoculum of the pathogen (C1, C3, T1, T3), mycelial fungal growth was observed, possibly because of the inoculum load that the berries bring from the harvest was not totally eliminated in the disinfection process.

Discussion

The proper control of postharvest decay involves the integration of preharvest factors (e.g. soil preparation, spray programs, orchard hygiene) with postharvest crop management. To date, the main strategy to control postharvest fungal diseases remains is the application of synthetic fungicides and of the chemicals that can be used to control decay, only a few are registered for postharvest use (Hansmann & Combrink 2003, Hua et al. 2018).

In this study, all yeasts evaluated using agar plug diffusion assay were capable to inhibit the growth of fungi over 50 %, except for *F. oeirense* YCPUC41. Similar results have been reported by Perez et al. (2016) who evaluated the biocontrol activity of 13 yeasts belonging to the species *Saccharomyces cerevisiae*, *Pichia fermentans*, *Kazachstania exigua* and *Candida catenulata* against *Penicillium digitatum*, *P. italicum* and *P. citri*. The results showed, using the same method, an inhibition equal to or greater than 40 % over the three pathogens evaluated.

The yeasts that presented the highest inhibition percentages were *N. antartica* YCPUC12 and *C. terrestris* YCPUC16, with percentages above 60 %, followed by *A. pullulans* YCPUC14. In this regard, it has been reported that yeasts belonging to *Cryptococcus* genera have antifungal properties (Tian et al. 2002, 2004; Leyva Salas et al. 2017; Shi & Sun 2017; Abdallah et al. 2018; Amina et al. 2018). Also, the biocontrol effect of *A. pullulans* has been described by several authors. Schena et al. (1999) reported its effect on the growth of *P. digitatum*, *B. cinerea*, *Rhizopus stolonifer* and *Aspergillus niger* in grapes and *R. stolonifer* in cherry tomatoes. On the other hand, Bencheqroun et al. (2007) identified that *A. pullulans* was able to inhibit the development of *P. expansum* on apples. Ippolito et al. (2000) reported similar results for *B. cinerea* in apples.

Our results indicate that the inhibitory effect by the yeasts over the fungi is differential, suggesting that there could be involved more than one antagonist-mechanism on the part of yeasts. Likewise, we evaluate fungi of different genera, which may explain the differential inhibitory effect observed.

Also, our results suggest that yeasts evaluated can inhibit the mycelial growth through production of VOCs. Similar results have been reported by Parafati et al. (2015) who evaluated biocontrol activity of *S. cerevisiae*, *Wickerhamomyces anomalus*,

Metschnikowia pulcherrima and *A. pullulans* against *B. cinerea*. The results showed that *W. anomalus* and *S. cerevisiae* strains presented highest values of growth inhibition (99.67 and 71 %, respectively). Mari et al. (2012) reported the biocontrol effect of two *A. pullulans* strains over brown rot diseases on peaches and nectarines. The yeasts were selected for their activity (*in vitro* and *in vivo*) against three species of *Monilinia* (*M. laxa*, *M. fructicola* and *M. fructigena*). *In vitro* antagonistic activity assays showed that two *A. pullulans* strains selected (L1 and L8) presented the highest levels of activity in the control of *M. laxa* growth in peaches and nectarines with 93 and 60 %, respectively.

VOCs production has been described recently as a mechanism for biocontrol. *W. anomalus*, *M. pulcherrima*, *A. pullulans*, *Pichia anomala* and *S. cerevisiae* species have been identified as capable to produce VOCs as ethyl alcohol, 3-methyl-1-butanol and phenylethyl alcohol and acetate esters (Contarino et al. 2019). Francesco et al. (2015) reported that the compounds emitted by these two *A. pullulans* strains (L1 and L8) were identified as 2-phenyl, 1-butanol-3-methyl, 1-butanol-2-methyl belonging to the group of alcohols. The production of VOCs is species-specific and acts as a chemical communication signal among cells, as a carbon release mechanism and, as a promoter or inhibitor of microbial growth (Kai et al. 2009).

Our results indicate that using both methodologies it is possible to observe fungal growth inhibition, suggesting that yeasts analysed have at least two inhibitory mechanisms for the control of phytopathogenic fungi studied (Figs. 2B, 3). *N. antartica* YCPUC12 is the exception because it inhibits the growth of *B. cinerea* MPC40 by 10 % using the confrontational assay methodology, and by 70 % in the inhibition zone diameter assay. This suggests the existence of only one inhibitory mechanism for this yeast.

Parafati et al. (2015) demonstrated the inhibitory effects of the yeast *W. anomalus*, *M. pulcherrima* and *S. cerevisiae* against *B. cinerea in vitro* and *in vivo* on table grapes, suggesting these yeast antimicrobial activities through multiple modes of action, including the production of VOCs. Cañamás et al. (2011) and Calvo-Garrido et al. (2013) performed treatments with *Candida sake* and the botrycide Fungicover® against *B. cinerea*, applied on the surface of *V. vinifera* cv. 'Cabernet Sauvignon'. Results showed a significant reduction on the severity of the infection caused by the pathogen in 75 %, compared to the control. Chen et al. (2018) observed similar results using *Galactomyces candidum* JYC1146 as a biocontrol agent in strawberries, inhibiting the mycelial growth of *B. cinerea* under *in vitro* conditions and significantly reduced rot severity on *in vivo* assays. Elmer & Hoyte (2020) made several treatments with the yeast strain *A. pullulans* YBCA5, their results showed that the evaluated yeast was effective at controlling and reducing postharvest fruit rot due to phytopathogenic fungi *Botrytis* spp., *Penicillium* spp., *Alternaria* spp., *Monilinia* spp. among others. The incidence of *Botrytis* spp. fruit rot was reduced between 24 and 49 % among *A. pullulans* YBCA5 treatments.

In our study, *N. antarctica* YCPUC12, *A. pullulans* YCPUC14, *C. terrestris* YCPUC16 and *F. oeirense* YCPUC41 yeasts, were capable of inhibiting the growth of selected phytopathogenic fungi *in vitro*. These results suggest that these compounds could be volatile and, depending on the assay, the evaluated yeasts have differential biocontrol effect over these phytopathogens. To our knowledge, this is one of the first reports on the biocontrol potential of *F. oeirense*. These exploratory results are not enough to attribute the biocontrol activity to a specific compound or mechanism. It is necessary to identify the compounds produced and mechanisms to strengthen and enhance their effect for in situ applications in the field.

The biocontrol effect of yeast strains on four fungi mycelial growth was evaluated *in vitro*. Most of yeasts were able to inhibit the mycelial growth of the evaluated fungi strongly.

Additionally, the biocontrol capacity of *A. pullulans* YCPUC14 over *B. cinerea* MPC40 was evaluated *in vivo* assay, and we observed a significantly reducing the damage caused by *B. cinerea*.

The use of yeasts as BCA may constitute an important alternative to replace synthetic fungicides, and their potential as biocontrol agents for postharvest diseases is interesting. Further investigation is needed to verify the effectiveness of these antagonists.

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