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# Evaluation of Native Wine Yeast as Biocontrol Agents Against Fungal Pathogens Related to Postharvest Diseases

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**Abstract**

Changes in consumer expectations have led to increasing demand for novel plant protection strategies, in order to reduce the application of chemical products, reduce the occurrence of new pests and the impact that all these actions generate in the environment. In recent years there have been numerous investigations related to biological control and the use of microorganisms as new control strategies. As part of integrated disease management, antagonistic microorganisms have been investigated lately and presented great interest. Such microorganisms can be applied in conventional and in organic farming as biological control agents (BCA). Many of these microorganisms are present in the microbial ecology generating interactive associations between surrounding microorganisms. For these reasons, it has become necessary to search new natural antimicrobial agents as alternatives to synthetic and chemical products. It has been discovered that there are microorganisms, particularly yeasts, that have antagonistic activity and different mechanisms of action, indicating that they could be interesting candidates for the development of BCA. Here, we evaluate the antagonist effect of four endophytic yeast, *Cryptococcus antarcticus*, *Aureobasidium pullulans*, *Cryptococcus terrestris* and *Cryptococcus oeirensis* over the growth of *Botrytis cinerea*, *Monilinia laxa*, *Penicillium expansum* and *Geotrichum candidum* in *in vitro* assays (inhibition zone diameter assay and confrontation assay).The results revealed that the four yeast strains evaluated showed antagonistic activity against the phytopathogens tested, suggesting that these yeasts produce compounds capable of inhibiting the growth of fungi and, depending on the assay, the evaluated antagonist-yeasts have differential biocontrolling-effect against the postharvest pathogens tested.

**Keywords:** native yeast; biocontrol; fungal pathogens; VOCs

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## 1. Introduction

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., Cholletotrichum spp., among others [1-4].

The control of fungal diseases is mainly based on the use of synthetic fungicides [5,6]. In 2015, Spain, France, Italy, and Germany together made up 70.5 % of the European Union pesticide sales, increasing the level of hazardous residues in the environment; also, fungicides are becoming less effective due to the presence of resistant fungal strains [7,8].

Yeasts are unicellular fungi that are present in different ecosystems and sources, both natural and, in connection with human activities. They can be found on/in fruits, plants, insects, animal intestinal tracts, soils, and marine environments [9]. There has been extensive research to explore and develop the potential of yeasts as antagonists to biologically control harvest pathogens and as an alternative to chemical pesticides [10-12], representing an eco-friendly alternative to synthetic pesticides [13,16]. However, yeasts often show lower and non-comparable effectiveness against pathogenic fungi in comparison to chemical fungicides [10]. This reduces their practical applications and leaving the problem of plant-fungal disease still unsolved. On the other hand, the effects of environmental factors on biocontrol systems, especially the viability and efficacy of antagonistic yeast species, still need to be thoroughly investigated [11].

In general, interactions between the microorganism and the host also involve environmental factors (i.e., variation of climatic conditions and other abiotic factors) and, to successfully inhibit the pathogen infection and development, several possible mechanisms operate in a tritrophic host-pathogen-antagonist interaction system, where more than one mechanism is involved. The modes of action of yeast strains against pathogenic fungi have been reported, and these mechanisms include antibiosis, mycoparasitism, induced resistance [16-20]. nutrient or space competition [16-19,21], iron depletion [17,22], extracellular lytic enzymes production [23], volatile organic compounds [24,25], reactive oxygen species (ROS) tolerance [19,26], and biofilm formation [13,27].

We evaluated the inhibitory activity of four native yeast isolates *Cryptococcus antarcticus*, *Aureobasidium pullulans*, *Cryptococcus terrestris* and *Cryptococcus oeirensis* over the growth of four phytopathogenic fungi involved in postharvest diseases *Botrytis cinerea*, *Monilinia laxa*, *Penicillium expansum* and *Geotrichum candidum in vitro*, as potential biocontrol agents.

## 2. Materials and Methods

### *2.1 Microorganisms*

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Native wine yeast strains were obtained from the yeast collection (YCPUC) of the Microbiology and Yeast Genetics Laboratory of Pontificia Universidad Católica de Chile (Table 1). The fungus evaluated were obtained from the cepary of the Molecular Phytopathology Laboratory. The analysed yeasts were grown in yeast extract–peptone–dextrose (YPD) medium (0.5 % peptone, 0.5 % yeast extract, and 2 % glucose) at 28 ± 1 ºC with agitation (200 rpm) for 1–3 days, according to the strain. Then, they were maintained in YPD agar (0.5 % peptone, 0.5 % yeast extract, 2 % glucose, and 2 % agar) at 4 ± 1 °C until use. The analysed fungi were grown in potato-dextrose-agar (PDA) (2% dehydrated potato, 2% dextrose, and 2% agar) acidulated with 250 **µ**L of 1N lactic acid (APDA) and incubated for 7 days at 20 to 22 °C. Then, they were maintained at 4 ± 1 °C until use.

*2.2 Detection of antimicrobial activity*

2.2.1 Inhibition Zone Diameter Assay

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 for 48 h at 28 ± 1 ºC with agitation (200 rpm) in YPD liquid medium until a concentration of $1x10^8$ cells/mL was obtained. Then, an aliquot of the concentrated culture (100 **µ**L) was taken and transferred to a new tube with 900 **µ**L of sterile water. This solution was used as inoculum, and then, 100 **µ**L were spread over an APDA plate. When the lawn was dry, the disc of the fungus was placed.

The fungi were grown individually in APDA plates for 7 days. Then a disc of the fungus was taken using sterile toothpick/forceps and put upside down at the center of the plate, in direct contact with the yeast lawn previously prepared (Figure 1a). Every fungi and yeast tested was done in triplicate, considering every treatment to evaluate.

The diameter of the 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 (Equation 1):

2.2.2 Confrontation Assay

Confrontational assay was tested to assess the production of volatile compounds. 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 (Figure 1b). The experiments were made in triplicate. The inhibition rate of each yeast against the pathogenic fungi was calculated with the formula mentioned in the Inhibition Zone Diameter Assay

*2.3. Statistical analysis*

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The data were analysed using the Statgraphics Centurion XVI.I program (Statpoint Technologies, Warrenton, USA) by means of Student's t-test or analysis of variance as indicated.

## 3. Results and Discussion

The proper control of postharvest decay involves the integration of preharvest factors (soil preparation, spray programs, orchard hygiene, etc.) with postharvest crop management. To date, the principal means to control postharvest fungal diseases remains as the application of synthetic fungicides and, the chemicals that can be used to control decay, only a few are registered for postharvest use [28, 29].

As a first experimental approach to evaluate the biocontroller effect of yeasts, it was used Inhibition Zone Diameter Assay that measure the ability of a microorganism to inhibit the growth of another through the production of antifungal compounds or through competition for nutrients.

The results (Figure 2A) showed that *C. antarcticus* YCPUC12 was able to reduce the mycelial growth of *B. cinerea, G. candidum* and *P. expansum* in 67%, 70% and 65% compared to the positive control, respectively. For *M.laxa*, the effect was nearly to 40% (Figure 2A). *A. pullulans* YCPUC14 reduced mycelial growth of *B. cinerea, M. laxa* and *G. candidum* in 67%, 68% and 65% respectively, and a lowest effect was observed for *P. expansum* (16%). *C. terrestris* YCPUC16 was able to reduce mycelial growth of *B. cinerea, M. laxa, G. candidum* and *P. expansum* in 75%, 70%, 53% and 77%. On the contrary, *C. oeirensis* YCPUC41 presented the lowest effect inhibitory, with percentages below 20% for all pathogens evaluated (Figure 2B).

Using inhibition zone diameter assay, in general all yeasts evaluated were capable to inhibit growth of fungus over 50 %, with exception of *C. oeirensis* YCPUC41. Perez et al. [30], using the same method, 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*. They observed an inhibition equal to or greater than 40% over the three pathogens evaluated.

The yeasts that presented the highest inhibition percentages were *C. antarticus* 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 [31–36]. Also, the biocontroller effect of *A. pullulans* has been described by several authors. Schena et al. [37] 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. [8] identified that *A. pullulans* was able to inhibit the development of *P. expansum* on apples. Ippolito et al. [38] reported similar results for *B. cinerea* in apples.

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

Have been describe that antagonism phenomenon of yeasts occurs due to competition for nutrients, pH changes, and the production of organic acids [39,40] in addition to mechanisms based on the secretion of antimicrobial compounds such as killer toxins. Several mechanisms are involved in biological control processes based on the ability of biocontrol agents to adhere to specific sites, including both yeasts and pathogenic cells [13]; colonize wounds and compete for nutrients; secrete specific enzymes [41]; induce resistance [42]; regulate the population density at specific sites [43]; secrete antimicrobial substances (soluble or volatile) [2,30,31] and form a biofilm on the inner surface of wounds [27].

The confrontation assay was made in order to determine the ability of the yeast to produce volatile compounds. The results showed that *A. pullulans* YCPUC14 reduced mycelial growth of *B. cinerea* and *M. laxa* with 72 % and 64 % respectively, and *C. terrestris* YCPUC16 with 52% and 51%. In the case of *P. expansum*, *C. antarcticus* YCPUC12 and *C. oeirensis* YCPUC41 reduced in 31% the mycelial growth of the pathogen (Figure 3).

Our results suggest that yeasts evaluated can inhibit the mycelial growth through production of volatile compounds. Parafati et al. [22] evaluated biocontrol activity of *S. cerevisiae*, *Wickerhamomyces anomalus*, *Metschnikowia pulcherrima* and *A. pullulans* against the postharvest pathogenic mold *B. cinerea*. The results showed that *W. anomalus* and *S. cerevisiae* strains presented the highest values of growth inhibition (99.67 and 71%, respectively). Seven strains of *M. pulcherrima* showed an average efficacy of 47%, where the strain MPR3 present the highest inhibition activity, with 67% of fungal growth inhibition. Mari et al. [44] 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.

The volatile organic compounds (VOCs) production has been described recently as a mechanism biocontrol yeast. *W. anomalus, M. pulcherrima, A. pullulans, P. anomala* and *S. cerevisiae* species have been identified as capable to produce volatile compounds as ethyl alcohol, 3-methyl-1-butanol and phenylethyl alcohol and acetate esters [25]. Di Francesco et al. [3] 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 [45].

The results indicate that with both methodologies (inhibition zone diameter assay and confrontational assay) it is possible to observe fungal growth inhibition, suggesting that yeasts analysed have at least two inhibitory mechanisms for the control of the phytopathogenic fungi studied (Figure 2B and 3). *C. antarticus* YCPUC12 is the exception due inhibits the growth of *B. cinerea* 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 in this yeast.

In our study, *C. antarcticus* YCPUC12, *A. pullulans* YCPUC14, *C. terrestris* YCPUC16 and *C. oeirensis* YCPUC41 yeasts, were capable of inhibiting the growth of phytopathogenic fungi. Results suggest that these compounds could be volatile. Depending on the assay, the evaluated yeasts have differential biocontrolling-effect on the phytopathogenic fungi tested. To our knowledge, this is one of the first reports on the biocontrol potential of *C. oeirensis.* These exploratory results are not enough to attribute the biocontrol activity to a specific compound or mechanism. Is necessary to clarify how these yeasts can inhibit the growth of the fungi, to strengthen and enhance their effect.

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

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**Equation 1.**

$$\text{Average per cent inhibition of each treatment} = \frac{C-T}{C} \times 100$$

Where:

C = average of 3 replicates of the mycelial growth diameter of the control treatment

T= average of 3 replicates of the mycelial growth diameter in the presence of the selected treatment

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

| Code | Name | Code | Name |
|---|---|---|---|
| **YCPUC12** | *Cryptococcus antarcticus* | BC | *Botrytis cinerea* |
| **YCPUC14** | *Aureobasidium pullulans* | GT | *Geotrichum candidum* |
| **YCPUC16** | *Cryptococcus terrestris* | PE | *Penicillium expansum* |
| **YCPUC41** | *Cryptococcus oeirensis* | ML | *Monilinia laxa* |

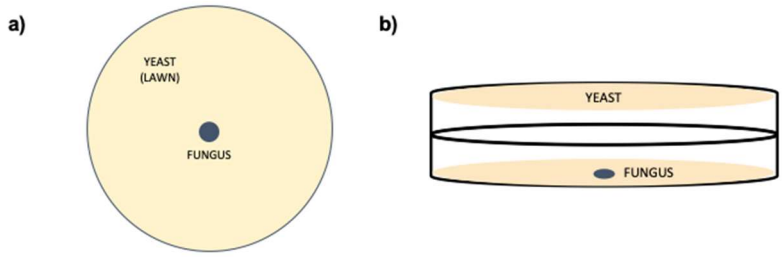


**Figure 1.** Schematic representation of the different strategies to evaluate the antimicrobial activity of the microorganisms. In a) Inhibition Zone Diameter Assay; b) Confrontation Assay.

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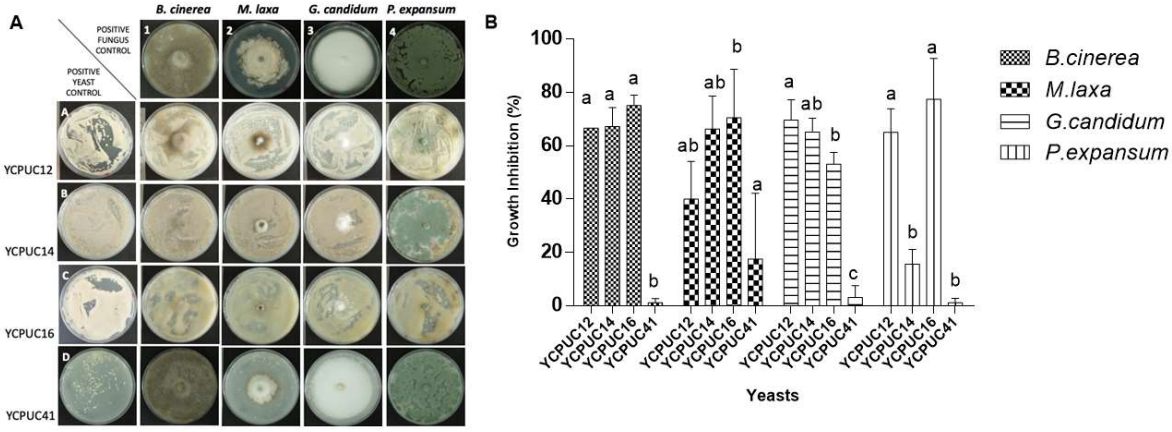


**Figure 2**. Evaluation of the biocontrol activity of the yeast isolates selected. On the left side of the figure (A) Inhibition Zone Diameter Assay. On the top of the figure are the names of the fungi tested and in the first row are their positive controls (1-4). The first column corresponds to the 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 significance difference at 95% confidence level.

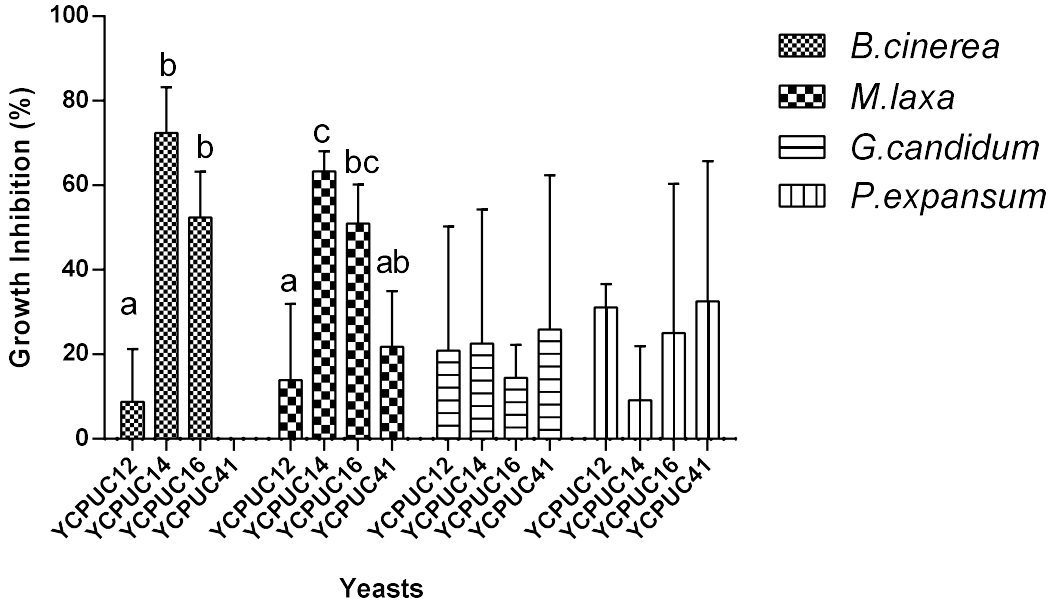


**Figure 3**. Percentage of growth inhibition obtained by the confrontation assay after seven days incubation at 22 °C. The experiments were performed in triplicate and results are the average. Different letters indicate significance difference at 95% confidence level.