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ISOLATION AND SELECTION OF EPIPHYTIC YEAST FOR BIOCONTROL OF *Botrytis cinerea* PERS. ON TABLE GRAPES

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Botrytis cinerea Pers., the causal agent of gray mold, infects more than 200 plant species. This pathogen has traditionally been controlled by fungicides. However, with the increasing demand for pesticide-free foods new control strategies are needed. The objective of this study was to isolate and select grapevine (*Vitis vinifera* L.) epiphytic yeasts for the biocontrol of *B. cinerea* in table grapes. Of the total isolated yeasts (n = 256), 32 exhibited mycelial growth inhibition in dual cultures with a halo > 4 mm, and eight of these isolates inhibited > 90% of conidial germination. When evaluating increasing concentrations on conidial germination inhibition, a dose-dependent response was observed with EC_{90} values from 0.45×10^5 to 0.22×10^8 cells mL^{-1}. The antagonistic activity of six yeasts against *B. cinerea* in table grape berries 'Flame Seedless' increased as the yeast colonization time increased from 1 to 24 h on the berries, resulting in a higher biocontrol activity on *B. cinerea*. These results show the effectiveness of grapevine epiphytic yeasts as biocontrol agents of *B. cinerea* on table grapes.

Key words: Gray mold, antagonistic yeast, biocontrol agents, *Vitis vinifera*.

Botrytis cinerea Pers., the causal agent of gray mold, is a fungus affecting more than 200 plant species worldwide (Elad *et al*., 2007), and this disease is considered a limiting factor for exporting table grapes (*Vitis vinifera* L.) in Chile (Latorre, 2007). *Botrytis cinerea* affects the vine's non-lignified aerial organs, such as leaves, buds, rachis, and flowers, causing tissue necrosis and soft rot of the berries (Benito *et al*., 2000; Holz *et al*., 2003; Latorre, 2004; Agrios, 2005; Elad *et al*., 2007; Williamson *et al*., 2007). This fungus generates abundant mycelia and produces a great quantity of conidia at the end of branched conidiophores. In adverse conditions also generates survival structures, known as sclerotia. Furthermore, it can survive as saprophyte on plant residues during the winter. Conidia can persist as latent inoculum in floral residues, such as stamens and calyptrae causing postharvest rot (Latorre, 2004; Viret *et al*., 2004).

Traditionally, the control of *B. cinerea* has been based on synthetic fungicides (Latorre *et al*., 2001; Serey *et al*., 2007). However, pathogen resistance has appeared because of the irrational use of these products (Latorre *et al*., 2002; Esterio *et al*., 2007) which leads to less control effectiveness. Furthermore, there is public concern about decreasing fungicide residues in foods and the environment, which is a limiting factor for this type of pesticide.

The use of biopesticides based on beneficial microorganisms appears as an interesting alternative to control *B. cinerea* (Zahavi *et al*., 2000; Spadaro and Guillino, 2003; Elmer and Reglinski, 2006; Chanchaichaovivat *et al*., 2007; Sharma *et al*., 2009). Yeasts are leading the advances to control pathogens causing fruit rot (McLaughlin *et al*., 1990; Droby *et al*., 1998; Spadaro *et al*., 2002; Vero *et al*., 2002; Chanchaichaovivat *et al*., 2007). Several studies have demonstrated an efficient antagonistic activity of yeast against *B. cinerea* (Saligkarias *et al*., 2002; Santos *et al*., 2004; Elmer and Reglinski, 2006; Dal Bello *et al*., 2008).

Given the importance of gray mold in Chile and the associated management problems, the objective of this study was to isolate and select grapevine epiphytic yeasts to evaluate its effectiveness to control *B. cinerea* on table grapes.

MATERIALS AND METHODS

Grape sample collection

The plant material was collected in March 2009 in the Coquimbo Region, Chile, at three commercial orchards with traditional management. 'Thompson Seedless' grapes were picked from the Almendro 4 sector of "La Granja" farm belonging to Del Monte company (30º40'41.5" S, 71º20'31.2" W, 200 m a.s.l.); 'Red Globe' grapes from the Almendro 11 sector in the same farm (30º44'37.8" S, 71º20'01.7" W, 217 m a.s.l.); and 'Moscatel de Alejandría' grapes from Limarí Bajo (30º37'25.7" S, 71º17'36.3" W, 166 m a.s.l.). To collect samples, 40 plants were selected from each sector; two healthy clusters were cut from

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each plant, placed into plastic bags, labeled, and stored in cooled thermal containers for transport. Fruit infected with *B. cinerea* were also obtained from the same sectors and stored under the above mentioned conditions.

Yeast and *B. cinerea* isolation and inoculum production

Yeast isolates were obtained from the healthy fruit epidermis following the methodology described by Rabosto *et al*. (2006). Five berries from each cluster were washed for 30 s with 10 mL of saline solution (9 g NaCl in 1 L sterile distilled water). The resulting solution was diluted (1:10), and 50 μL were sown on Petri dishes containing a minimal sterile yeast-extract peptone dextrose (YPD min) medium (5 g dextrose, 3 g yeast extract, 5 g peptone, 18 g agar, and 1 L distilled water) supplemented with 0.05 g L^{-1} streptomycin (Sigma-Aldrich), 0.05 g L^{-1} chloramphenicol (Sigma-Aldrich), and adjusted to pH 4.6. These were incubated at 25 ºC until the development of the microorganism colonies in the culture medium was observed. Yeast colonies were isolated and cultured in YPD medium (20 g dextrose, 20 g peptone, 10 g yeast extract, 20 g agar, and 1 L distilled water). Finally, isolates were inoculated in tubes with inclined YPD medium and stored at 4 ºC for subsequent analysis.

For inoculum production, yeasts were activated in 10 mL of glucose-yeast extract-peptone broth medium (GYP broth; 5 g glucose, 3 g yeast extract, 5 g peptone, and 18 g agar per liter) in 250 mL flasks on a rotary shaker at 100 rpm at 25 ºC for 72 h. Cells were harvested by centrifugation (5000 rpm, 10 min), washed twice with sterile deionized water, and resuspended in water. Suspensions were adjusted to the desired concentration in a Neubauer chamber.

Botrytis cinerea was isolated from grape berries 'Red Globe'. Pathogen conidia were sown on dishes with potato dextrose agar medium (PDA) (Difco) and incubated at 25 ºC. Pure cultures were obtained with hyphal tips from the edges of mycelial growth and monosporic cultures were obtained from reproductive structures. The monosporic cultures were placed in tubes with PDA medium and kept at 4 ºC. For the inoculum production, a 5 mm diameter disk of actively-growing mycelia was cut from the monosporic culture and placed on a dish containing YPD medium, 12 to 15 d before each assay. When the reproductive structure formation was observed in the medium, 10 mL of saline solution was added to each dish, the conidia were removed with a glass rod, and the contents filtered through cloth gauze. A suspension of the pathogen conidia was prepared with a 1×10^6 conidia mL^{-1} concentration in a Neubauer chamber.

Selection of yeast with inhibitory activity on *B. cinerea* mycelial growth

The *in vitro* dual culture technique was employed to determine the degree of inhibition of the yeast isolates against *B. cinerea* mycelial growth. Mycelial disks of 5 mm diameter from actively growing culture were cut from the *B. cinerea* monosporic isolate. Each disk was placed in the center of a Petri dish with malt extract agar (MEA) (Difco) medium and incubated at 25 ºC for 24 h. Yeast isolates were inoculated on four perpendicular lines to the center of the fungus growth. A mycelial disk of the pathogen growing without yeast was the control. Cultures were incubated at 25 ºC for 7 d.

The level of mycelial growth inhibition was determined by the Swadling and Jeffries (1996) scale: 0 = without any visible signs of *B. cinerea* inhibition, mycelium surpasses antagonist colony; 1 = both organisms stop growing on contact; 2 = inhibition zone between pathogen and antagonist is < 2 mm; 3 = inhibition zone is 2 to 4 mm; and 4 = inhibition zone is > 4 mm. All yeast isolates reaching a value of 4 on this scale were selected for the next bioassay.

Inhibitory effect of yeast isolates on *B. cinerea* conidial germination

An assay was established to select yeasts with antagonistic activity on *B. cinerea* conidial germination at two temperatures (5 and 25 ºC) according to the methodology described by Zhang *et al*. (2007). A volume of 100 μL of suspended yeast (1×10^8 cells mL^{-1}) and 100 μL suspended pathogen conidia (1×10^6 conidia mL^{-1}) were added to an Eppendorf tube containing 800 μL malt extract broth (MEB) (Difco) medium. The suspended yeast was replaced by 100 μL of sterile distilled water in the control treatment. Three replicates were carried out for each treatment (yeast). Tubes were incubated for 24 h at 25 ºC and 72 h at 5 ºC. Subsequently, 100 conidia were counted for each replicate and the number of germinated conidia was determined. The germination criterion considered that conidium was germinated when the length of the germination tube was more than twice the greatest spore diameter. The conidial germination inhibition index (GII %) was calculated from the results according to the formula described by Manici *et al*. (1997), where GII (%) = (conidia germinated in control – conidia germinated in treatment) × 100/conidia germinated in control. Yeast isolates with a GII (%) greater than 90% at 5 and 25 ºC were selected for the next bioassays.

***In vivo* yeast pathogenicity**

To determine if yeast selected in the previous assay are pathogenic in grapes, 15 μL suspended yeast (1×10^8 cells mL^{-1}) were inoculated in a micro-wound made on grape berries 'Flame Seedless' that were homogeneous in size and 16.5 to 17.7 ºBrix. These berries were previously disinfected with a commercial sodium hypochlorite solution (Clorinda®) at 5% v/v for 5 min. They were placed in 250 cm^3 plastic containers at 25 ºC for 7 d and incubated. It was determined that the yeast was pathogenic when an alteration of the berry tissue was observed.

Effect of yeast concentration and colonization time

Yeast selected in the previous assay, which were non-pathogenic were activated in the above mentioned culture medium and adjusted in 1×10^5, 1×10^6, 1×10^7, and 1×10^8 cells mL^{-1} concentrations. The methodology was the same as the one described for the conidial germination inhibition assay. Three replicates per treatment were established. The GII (%) and 90 (EC_{90}) effective concentration of *B. cinerea* conidial germination inhibition were determined.

To determine the effect of yeast colonization time on grape berry wounds on the biocontrol activity of gray mold, homogeneous-sized grape berries 'Flame Seedless' were disinfected as described above and four evenly spaced micro wounds were made in the equatorial zone with a sterile needle. They were then submerged in a selected 1×10^8 cells mL^{-1} yeast suspension for 20 s. Each treatment (yeast) consisted of 56 berries separated into two groups of 28 berries. In the first group, the pathogen was applied 1 h after the yeast suspension, and the second group was inoculated 24 h after the yeast was applied. In both cases, pathogen inoculation was carried out by aspersion with 2.5 mL of suspended conidia (1×10^6 conidia mL^{-1}) at 30 cm from the berries using a manual sprayer. Yeast suspension in the control treatment was replaced by sterile distilled water. Three replicates per treatment were established. Berries were placed homogeneously on plastic trays with paper moistened with sterile distilled water, covered with plastic bags to maintain a high relative humidity, and incubated at 25 ºC for 7 d. After incubation, berries were checked with a stereomicroscope to verify the presence of *B. cinerea*. Disease incidence was calculated as a percentage of infected berries divided by the total berries analyzed per treatment.

Identification of yeast with greater biocontrol activity

To identify yeast showing greater antagonistic activity against *B. cinerea*, DNA was extracted from pure cultures with the Qiagen DNeasy Plant Mini Kit (Quiagen, Hilden, Germany) following manufacturer's instructions. One micro liter of DNA was suspended in a final volume of 50 μL Polymerase Chain Reaction (PCR) reaction mixture containing 0.5 μM ITS1 (5` TCCGTAGGTGAACCTGCGG 3`) primer, 0.5 μM of ITS4 (5` TCCTCCGCTTATTGATATGC 3`) primer, 0.2 μM deoxynucleotide, 1.5 mM $MgCl_2$ and 1x buffer, and one unit of polymerase DNA (Invitrogen). PCR conditions were: 95 ºC for 7 min; 35 cycles at 94 ºC for 1 min, 55.5 ºC for 30 s, and 72 ºC for 1 min, as well as a final step at 72 ºC for 10 min. The PCR products were separated in a 1% agarose gel with 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA, and pH 8). After electrophoresis, the gel was dyed with ethidium bromide and visualized under UV light. The PCR products were sequenced by Macrogen Inc. (Seoul, Korea). Sequence analysis results of antagonistic yeast were aligned with the published full length sequences in the Basic Local Alignment Search Tool (BLAST) databases (National Center for Biotechnology Information [NCBI], US National Library of Medicine, Bethesda, Maryland, USA).

Experimental design and statistical analysis

The experiments were conducted in a complete randomized design with three replicates. All data were analyzed by ANOVA, multiple comparisons with the Duncan Test, and evaluated at $\alpha = 0.05$. Analyses were performed with Statgraphics Plus® 5.0. To determine EC_{90}, data were subjected to a Probit analysis with the Polo Plus v2.0 computer program.

RESULTS AND DISCUSSION

Yeast isolation and selection

A total of 256 grapevine epiphytic yeasts were obtained. These were placed in the microorganism collection of the Faculty of Agronomy of the Universidad de Concepción and kept at 4 ºC. Figure 1 shows the number of yeast isolates obtained from each table grape cultivar classified according to the scale proposed by Swadling and Jeffries (1996). Of the total yeast isolates, 32 were able to generate an inhibitory halo > 4 mm with the pathogen in dual cultures, which represent 12.5% of the total isolates. Yeasts with antagonistic activity on *B. cinerea* were obtained from the three analyzed table grape cultivars (Figure 1). Inhibition zones in the dual cultures could be due to the production of antibiotics, siderophores, toxic or antifungal metabolites used by these organisms as biological control mechanisms, and the size of the observed inhibition zones would represent the concentration and diffusivity of the inhibitory compounds secreted by each isolate (Swadling and Jeffries, 1996). However, production of these compounds in the culture media is not indicative of its production in action sites on the fruits (Dal Bello *et al*., 2008).

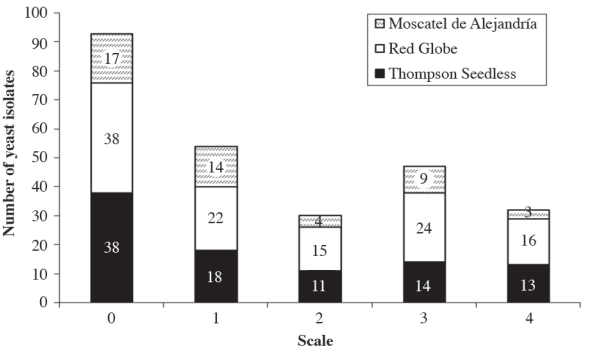


Figure 1. Antagonistic activity of yeasts isolated from grape cuticles against *Botrytis cinerea* in dual culture according to the scale: 0 = no visible signs of *B. cinerea* inhibition and mycelium surpassed the yeast colony; 1 = both organisms stopped growing on contact; 2 = inhibition zone between pathogen and antagonist < 2 mm; 3 = inhibition zone 2 to 4 mm; 4 = inhibition zone > 4 mm.

Inhibitory effect of yeast isolates on *B. cinerea* conidial germination

Eight of the 32 selected yeast isolates in the previous experiment (032a1, 058a3, 087a2, 100b1, 101b2, 156a3, 156a5, and 174b1) were able to inhibit *B. cinerea* conidial germination with a GII greater than 90% at both 5 and 25 ºC (Figure 2). This inhibition could be due to different action mechanisms exerted by the yeasts. One of them could be the competition for nutrients since it has been reported that *B. cinerea* conidial germination is dependent on the amount of nutrients from the environment (Filonow *et al*., 1996). Another mechanism could be parasitism and/or production of enzymes that degrade the pathogen wall, such as glucanases; these are responsible for the degradation of cellulose and hemicellulose that are polymers making up the conidial walls (Masih and Paul, 2002).

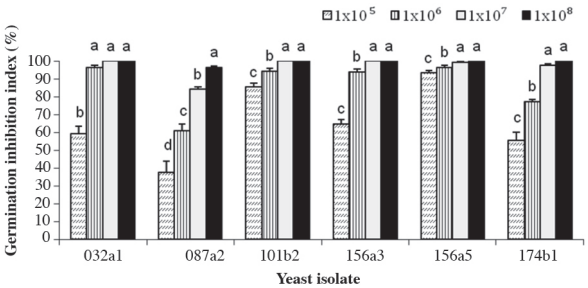
Yeast pathogenicity on table grape berries

Of the eight previously selected yeast isolates, two of them (058a3 and 100b1) exhibited a damaging effect when they were inoculated on table grape berries cv. Flame Seedless by observing a soft consistency and tissue disintegration. This pathogenic characteristic contrasts with the objectives of this study; these isolates were therefore discarded for the following bioassays.

Yeast pathogenic aggressiveness can be related to the ability that some exhibit to develop forms of mycelial growth because they can invade plant tissues more rapidly and allow greater surface contact with the substrate (Gognies *et al*., 2001). There are various yeast species described as pathogenic for table grapes being *Pichia*, *Brettanomyces*, *Cryptococcus*, and *Rhodotorula* the most studied (Gognies *et al*., 2006). Even *Saccharomyces cerevisiae*, the most researched fermentative yeast, can also act as a pathogen on plants causing delay in their growth or death (Gognies *et al*., 2001).

Effect of yeast concentration and colonization time

When evaluating the effects of increasing antagonistic yeast concentrations on the inhibition of *B. cinerea* conidial germination, it was observed that there is a dose-dependent response. As yeast concentration increased, inhibition of *B. cinerea* conidial germination increased (Figure 3). The values of EC_{90} fluctuated between 0.45×10^5 and 0.22×10^8 cells mL^{-1} (Table 1), and isolate 156a5 was noted for the lowest EC_{90} (0.45×10^5 cells mL^{-1}).



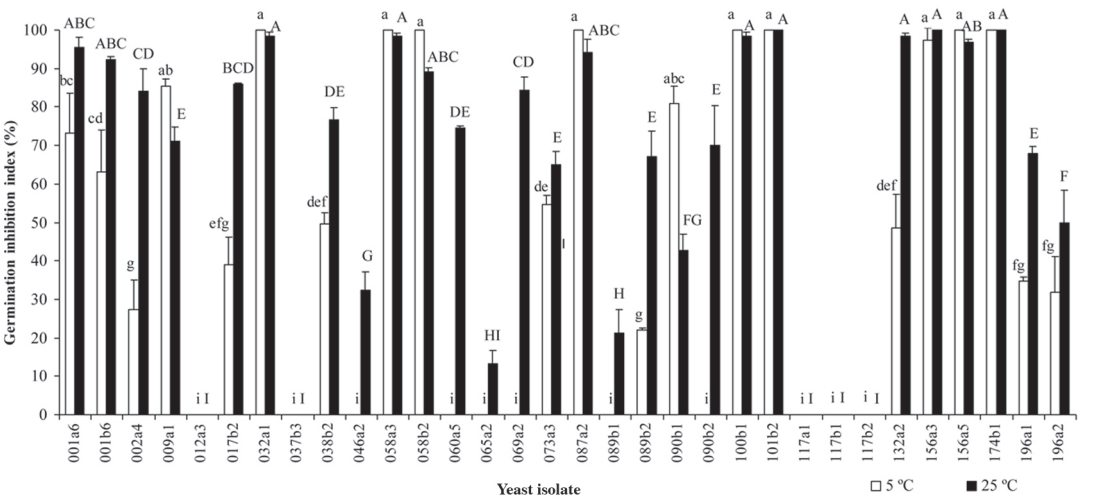
Different letters over the bars indicate significant differences between different concentrations for the same isolate according to Duncan test (P < 0.05).

Figure 3. Germination inhibition index of *Botrytis cinerea* treated with different concentrations of antagonistic yeast.

Table 1. Effective concentration (EC_{90}) values of yeast applied to *Botrytis cinerea* conidia.

Yeast isolate	EC_{90} (cell mL^{-1}) (95% FL)	Slope ± SE	Chi square (χ^2)	df
032a1	0.47×10^6 (0.37×10^6 to 0.63×10^6)	1.56 ± 0.131	24.99	22
087a2	0.22×10^8 (0.14×108 to 0.39×10^8)	0.66 ± 0.043	26.20	20
101b2	0.23×10^6 (0.14×10^6 to 0.34×10^6)	0.76 ± 0.095	23.10	22
156a3	0.54×10^6 (0.43×10^6 to 0.72×10^6)	1.24 ± 0.108	14.39	22
156a5	0.45×10^5 (0.10×10^5 to 1.01×10^5)	0.50 ± 0.091	17.75	22
174b1	0.24×10^7 (0.17×10^7 to 0.38×10^7)	0.89 ± 0.061	31.81	22

FL: fiducial limits; SE: standard error.



Different lower-case letters indicate significant differences according to Duncan test (P < 0.05). Different upper-case letters indicate significant differences according to Duncan test (P < 0.05).

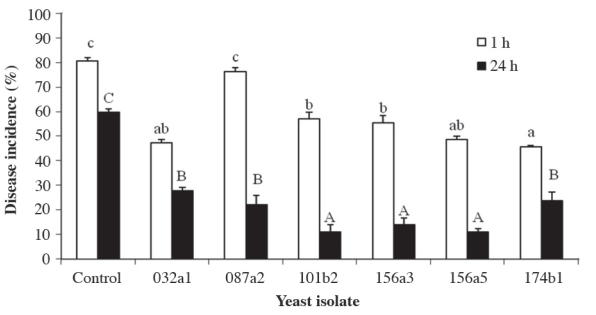
Figure 2. Germination inhibition index of *Botrytis cinerea* treated with different yeast isolates at 5 and 25 ºC.

Inhibition of *B. cinerea* conidial germination could be due to the parasitism exerted by the yeast (Wisniewski *et al*., 1991) and enzyme action, such as quitinases and β-1,3 glucanases, which degrade the *B. cinerea* cell wall and produce cytological damage (El-Ghaouth *et al*., 1998; Rabosto *et al*., 2006).

In the berries treated with yeasts, whether 1 or 24 h before inoculation with the pathogen, the incidence of gray mold decreased in relation to the control (Figure 4). The lowest incidence was obtained when yeast were applied 24 h before inoculation of the pathogen and the 101b2, 156a3, and 156a5 isolates were able to reduce gray mold incidence up to approximately 80% as compared to the control; these were considered as the best controllers of gray mold under the study conditions. Similar results were reported by Raspor *et al*. (2010), who obtained a significant decrease in the degree of infection of *B. cinerea* on grapes treated with yeast 24 h before inoculation with *B. cinerea* as compared to grapes treated with yeast and immediately inoculated with the pathogen. This increase in yeast biocontrol activity as determined by a greater colonization time in the berry wounds could be due to a greater yeast proliferation with subsequent better colonization of the wounds enabling it to compete with the pathogen for space and nutrients (Filonow *et al*., 1996; Saligkarias *et al*., 2002).

Identifying yeast with greater biocontrol activity

In order to identity the 101b2, 156a3, and 156a5 antagonistic yeast, the PCR products were sequenced (Macrogen Inc.) and compared to the sequences deposited in the GenBank database. For the three antagonistic yeasts, 99% similarity was obtained with the published sequences for *Issatchenkia terricola* (Accession Nº AY235808.1). Antagonistic activity of *I. terricola* yeast isolated from grapes was reported by Bleve *et al*. (2006); in such study, *I. terricola* reduced *Aspergillus carbonarius* and *A. niger* colonization on grape berries.



Different lower-case letters indicate significant differences according to Duncan test (P < 0.05). Different upper-case letters indicate significant differences according to Duncan test (P < 0.05).

Figure 4. Gray mold incidence in grape berries treated with antagonistic yeast 1 or 24 h before *Botrytis cinerea* inoculation.

CONCLUSIONS

In accordance with the conditions established in this study, we can conclude that grapevine epiphytic yeasts exhibit antagonistic activity on *Botrytis cinerea*. There is a direct relationship between the concentration of the antagonist and the inhibition of *in vitro* germination of *B. cinerea* conidia; a longer yeast colonization time on the berries decreases the incidence of gray mold. Our results confirm the effectiveness of epiphytic yeasts as biocontrol agents of gray mold, which is one of the most damaging diseases for agricultural plants.

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Aislación y selección de levaduras epífitas para el biocontrol de *Botrytis cinerea* Pers. en uva de mesa. *Botrytis cinerea* Pers., agente causal de la pudrición gris, infecta a más de 200 especies vegetales. Tradicionalmente, este patógeno ha sido controlado con fungicidas; sin embargo, la creciente demanda de alimentos libres de pesticidas hace necesario el uso de nuevas estrategias de control. El objetivo de este estudio fue aislar y seleccionar levaduras epífitas de vid (*Vitis vinifera* L.) para el biocontrol de *B. cinerea* en uva de mesa. Del total de levaduras aisladas (n = 256), 32 presentaron inhibición del crecimiento micelial, en cultivos duales, con un halo > 4 mm y ocho de estos aislamientos inhibieron la germinación de conidias > 90%. Al evaluar concentraciones crecientes de levaduras sobre la inhibición de la germinación de conidias, se observó una respuesta dosis-dependiente, con valores de CE_{90} de $0{,}45 \times 10^5$ a $0{,}22 \times 10^8$ células mL^{-1}. Al evaluar la actividad antagonista de seis levaduras frente a *B. cinerea* en bayas de uva de mesa 'Flame Seedless' se determinó que al aumentar el tiempo de colonización de las levaduras en las bayas de 1 a 24 h, la actividad de biocontrol sobre *B. cinerea* fue superior. Estos resultados demuestran la efectividad de levaduras epífitas de vid como agentes de biocontrol de *B. cinerea* en uva de mesa.

Palabras clave: pudrición gris, levaduras antagonistas, agentes de biocontrol, *Vitis vinifera*.

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