Preventive and curative effects of native yeasts on different *Botrytis cinerea* strains in "Superior Seedless" (*Vitis vinifera* L.) table grape cultured in Argentina

Efectos preventivos y curativos de levaduras nativas sobre diferentes cepas de *Botrytis cinerea* en uva de mesa "Superior Seedless" (*Vitis vinifera* L.) cultivada en Argentina

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Originales: *Recepción:* 14/02/2023 - *Aceptación:* 09/12/2024

Abstract

The fermenting grape must is a dynamic, stressful, and selective habitat where many yeast species compete. Specific yeasts isolated from this habitat can play a fundamental role in table grape biocontrol of fungal diseases. The present study evaluated 225 grapevine yeasts against four *Botrytis cinerea* strains isolated from "Superior Seedless" grapes, considering the possible antifungal action mechanisms. Eighteen enological yeasts (13 *Saccharomyces* and 5 non- *Saccharomyces*) showed preventive antifungal activity against the four native *B. cinerea* strains, with disease severity varying between 0 and 49.91%. These 18 strains also presented curative activity against at least one of the *B. cinerea* strains assayed (severity values between 0 and 45.99%). Considering action mechanisms, thirteen yeast strains inhibited mycelial growth of at least one *B. cinerea* strain during dual plating (antibiosis), "*killer*" activity, and volatile antifungal assays. Our results showed that 7 yeast strains affected conidial germination (CG) and germinal tube length (GTL) of at least one *B. cinerea* isolate. Two yeast strains occupied the same niche as 4 *B. cinerea* strains (NOI values > 0.90). All yeast strains exhibited at least two inhibitory action mechanisms against gray rot, except for BSc140 with one mechanism. The possibility of more than one mechanism per yeast strain makes biocontrol an effective tool to prevent and cure gray rot in table grapes.

Keywords
preventive • curative • oenological yeasts • *Botrytis cinerea* • table grape • modes of action

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Resumen

El mosto de uva en fermentación es un hábitat dinámico, estresante y selectivo donde compiten diferentes especies de levaduras. Levaduras enológicas pueden desempeñar un papel fundamental en el biocontrol de las enfermedades fúngicas de la uva de mesa. El presente estudio evaluó la eficacia de 225 levaduras vitícolas para controlar cuatro cepas nativas de *Botrytis cinerea* aisladas de uvas "Superior Seedless" y los posibles mecanismos de acción antifúngica. Dieciocho levaduras enológicas (13 *Saccharomyces* y 5 no *Saccharomyces*) mostraron actividad antifúngica preventiva frente a las cuatro cepas de *B. cinerea* presentando una severidad de la enfermedad que varía entre 0 y 49,91%. Estas 18 cepas presentaron actividad curativa contra al menos una de las cepas de *B. cinerea* ensayadas (valores de severidad entre 0 y 45,99%). Posibles mecanismos de acción: 13 cepas de levadura inhibieron el crecimiento micelial de al menos una de las cepas de *B. cinerea* ensayadas durante los ensayos dual (antibiosis), actividad "killer" y volátiles antifúngicos. Nuestros resultados mostraron que 7 cepas de levadura afectaron la germinación de los conidios (CG) y la longitud del tubo germinativo (GTL) de al menos uno de las cepas patogénicas. Dos cepas de levadura ocuparon el mismo nicho que las 4 cepas de *B. cinerea* (valores NOI > 0,90). Los presentes hallazgos indican que todas las cepas de levadura exhibieron al menos dos mecanismos de acción antifúngico para controlar la pudrición gris, excepto BSc140 (un mecanismo). La posibilidad de que las cepas de levadura puedan ejercer más de un mecanismo de acción hace que el biocontrol sea una herramienta más eficaz para prevenir y curar la pudrición gris en uva de mesa.

Palabras clave

preventivo • curativo • levaduras enológicas • *Botrytis cinerea* • uva de mesa • modos de acción

Introduction

San Juan is Argentina's main producer of *Vitis vinifera* L. table grapes. In 2023, the province produced 41264 tons of grapes for fresh consumption or raisins (6). Indoor and outdoor grapes suffer from a variety of fungal diseases. *Botrytis cinerea*, causing "gray mold", is one important postharvest decay of fresh fruit and vegetables (4). Treatments against fungal diseases can be preventive or curative. In Argentina, chemical products such as azoxystrobin + difenoconazole, benomyl boscalid + pyraclostrobin, and carbendazim have a preventive effect against this fungus in grapes. In Argentina, boscalid is registered as preventive and curative fungicide against *B. cinerea* (2). However, excessive use of synthetic fungicides concerns human health and environmental well-being. *B. cinerea* is a polycyclic pathogen that can develop resistance against chemical fungicides (1).

Grape must is a dynamic habitat with high selection pressure (stress), resulting from physical (osmolarity, low pH) and chemical conditions (limited nitrogen availability, high ethanol concentrations) rich in different competing microbial species (18). Yeasts isolated from environments subjected to various stresses, such as grape fermentation, are more likely to be effective antagonists (16). Unfortunately, information on yeasts with antifungal curative activity as novel biocontrol technique is limited. Only a few reports mention curative activity (24 h after pathogen infection) of yeasts like *Saccharomyces cerevisiae* (8), *Candida stellimalicola* (3), *Candida inconspicua*, *Pichia kluyveri* (22), *Pichia kudriavzevii* and *Rhodotorula glutinis* (9) against different fungal pathogens in various vegetables. No reports consider applying curative yeasts in white table grapes. Most studies assessed yeast preventive effects against only one *B. cinerea* strain (13, 19). Possible modes of action against reported pathogenic fungi include competition for nutrients and space, reduction in spore germination and germ tube length, and inhibition of fungal mycelial growth by diffusible and volatile metabolites (14, 20). Few reports describe preventive and curative effects against fungi (8, 9). We aimed to assess efficacy of 225 viticultural yeasts against four native *B. cinerea* strains, conducting preventive and curative assays. Possible preventive or curative antifungal mechanisms of selected yeasts against four *B. cinerea* isolates were also evaluated *in vitro.*

Materials and methods

Yeast isolates

The present study assayed two hundred and twenty-five grapevine yeasts belonging to 41 species (13). Seventeen native yeasts were previously isolated from table grapes, 9 from vineyard soil (Caucete, San Juan), and 199 from fermenting musts of different varieties from San Juan, Argentina. These yeasts were previously identified by morphological and molecular techniques (13).

Botrytis cinerea strains

Four *B. cinerea* strains (B11, B14, B15, B24) were previously isolated from "Superior Seedless" table grape from Mendoza, Argentina. Previous molecular identification was carried out using molecular markers based on PCR-RFLP. Amplification of the ribosomal intergenic spacer (IGS) was performed with PCR, and product restriction was carried out with CfoI, HaeIII, and HinfI enzymes (12).

Curative and preventive *in vivo* assays

Grape

Untreated grapes with chemical pesticides were washed, superficially disinfected with sodium hypochlorite 0.2% (v/v) for 3 min, and subsequently rinsed with distilled water to eliminate sodium hypochlorite. A single wound (3 mm diameter and 3 mm deep) was made at the equator of each fruit.

Preventive treatments

First, the antifungal effect of the 225 yeast strains against 4 *B. cinerea* strains was evaluated in *preventive* bioassays in the "Superior Seedless" table grape. A 20 µL of a yeast cell suspension (10^6 cells/mL) was inoculated in the artificially wounded area and treated 24 h later with 20 µL of a *B. cinerea* conidial suspension (10^4 conidia/mL).

Curative treatments

Wounded grapes were initially inoculated with *B. cinerea* suspension and 24 h later with yeast suspension with preventive antifungal activity (severity 50% or less). Microbial concentrations were as mentioned above.

Controls: a- A wounded grape initially inoculated with *B. cinerea* suspension (10^4 conidia/mL), b- A wounded grape initially inoculated with water, and c- A wounded grape inoculated with yeast alone.

Treatments were arranged in a completely random design, with 3 replicates and 9 grapes per replicate. Assays were performed three times. The fruit was stored for 10 d at 25°C and 90% RH.

After the bioassays, disease severity (%) considered as the average diameter of gray rot lesions (cm, using a digital caliper) was calculated as follows:

$$\textit{Disease Severity}\ (\%) = \frac{\textit{Average lesion diameter in grape wound inoculated with fungus and yeast}\ (cm)}{\textit{Average lesion diameter in fungal control}\ (cm)}$$

In preventive and curative assays, antagonistic yeasts reduced disease severity by 50% or more.

Evaluation of possible antifungal mechanisms of the selected preventive and curative yeasts against four *B. cinerea* strains (B11, B14, B15, B24) (18)

Dual culture assays (antibiosis)

Mycelial agar disks (5mm) obtained from the margin of 7-day-old fungus cultures were placed in the center of the dishes containing Czapeck-Agar (Sigma-Aldrich ®). Around the fungus, four aliquots (20 µL) of a yeast cell suspension (10^6 cells/mL) were spot-inoculated, 3 cm from the center. Plates were incubated at 25°C for 5 d, and subsequently, mycelial growth (cm) was measured with a digital caliper. Results are expressed as % of *B. cinerea* mycelial growth inhibition compared to control (100%) (21).

Detection of *killer* activity

Plates with YMB-MB-Phosphate Citrate Buffer- Agar (Britania®) at pH 4.5 were inoculated with 100 µL of *B. cinerea* conidia (10^4 conidia/mL) as lawn. After the plates solidified, spot inoculation of 20 µL of each yeast (10^6 cells/mL) was performed with an automatic pipette. Plates were incubated at 25 °C for 5 d in the dark. A clear zone around yeast colonies was recorded as positive (**+**).

Antifungal activity of volatile organic compounds (VOCs)

B. cinerea mycelial discs (5 mm diameter) were taken from margins of 7-day-old cultures. The mycelial disc was inoculated on the center in the base plate with Potato Dextrose Agar (PDA) (Britania ®) medium. Another base plate with YEPD-Agar medium was inoculated superficially with 100 µL of a yeast suspension (10^6 cells/mL). The two base plates were faced and sealed with plastic Parafilm® (13). Controls were performed by inoculating *B. cinerea* (without yeast cells) on PDA. The sealed plates were incubated at 25°C for 5 d. At the end of the assay, mycelial growth (diameter, cm) was measured with a digital caliper. Results are expressed as the % of mycelial growth inhibition of *B. cinerea* compared with fungal control.

Yeast effect on conidial germination (CG) and germinal tube length (GTL) of four *B. cinerea* strains in low-nutrient medium

A suspension of 100 µL yeast cells (10^6 cells/mL), 25 µL fungal conidia (10^4 conidia/mL), and 100 µL of 1% diluted (v/v) white grape must (Superior Seedless) were inoculated on sterile excavated slides (13). Controls consisted of *B. cinerea* conidia without yeast. In Petri dishes, the excavated slides were incubated at 25°C for 24 h in the dark at 80% RH. The CG results are expressed as percentage of germinated conidia compared with the control (observation of 100 conidia). Conidia were considered germinated when the germ tube length was larger or equal to the conidia. GTL (µm) was measured with an ocular micrometer calibrated in a 40X light microscope objective (observation of 30 conidia).

Niche Overlap Index (NOI)

Fungal conidia (20 µL, 10^4 conidia/mL) and yeast cells (20 µL, 10^6 cells/mL) were inoculated on separate plates containing distilled water agar (2% agar, pH 4.5) with different nutritional sources (10 mM). Carbon sources assayed are generally present in table grapes and represent niche size: proline, asparagine, rhamnose, alanine, melibiose, glycine, malic acid, glutamic acid, tyrosine, raffinose, arginine, lysine, fructose, methionine, mannitol, glucose, saccharose, citric acid, galactose and tartaric acid (14). Plates were incubated at 25°C for 7 d in the dark. NOI values were obtained as follows:

$$NOI = \frac{number\ of carbon\ sources\ used\ by\ both\ microorganisms\ (yeast\ and\ B.cinerea)}{number\ of\ compounds\ used\ by\ B.cinerea}$$

NOI values >0.90 represent occupation of the same niche (competitive exclusion), and values < 0.90 represent occupation of separate niches (coexistence) (23).

Statistical analysis

All experiments were performed in triplicate and thrice. SPSS® software was used for statistical analysis. ANOVA assumptions were examined before statistical analyses, and mean values were compared with Tukey's test at a p-value = 0.05. Percentages of wounds infected by *B. cinerea* and germination conidia were arcsine-square-root transformed before ANOVA. When ANOVA assumptions were not met, the non-parametrical Kruskal-Wallis test was used.

Results

Table grape bioassays (preventive and curative)

In preventive assays with artificially wounded grapes, 18 isolates of the 225 enological yeasts assayed reduced disease severity by 50% or more in 4 *B. cinerea* strains: B11, B14, B15, and B24 (table 1). Yeast strains belonged to different species, including *Saccharomyces cerevisiae* (BSc14, BSc16, BSc27, BSc60, BSc90, BSc96, BSc97, BSc102, BSc103, BSc112, BSc140, BSc206), *Saccharomyces chevalieri* (BSch26), *Torulaspora delbrueckii* (BTd156, BTd165), *Candida sake* (BCs54), *Hanseniaspora vineae* (BHv86), and *Debaryomyces vanrijiae* (BDv197). All 18 strains had been isolated from fermented musts. Antifungal preventive treatments with these yeasts reduced disease severity between 50.14 and 100%. Notably, *S. cerevisiae* BSc103 and *S. chevalieri* BSch26 inhibited total fungal growth of B14 and B15, respectively, during preventive assays (table 1 and figure 1, page 108).

Table 1. Disease severity (%) caused by *B. cinerea* strains (B11, B14, B15, and B24) in white table grapes treated with yeast strains in preventive and curative assays.

Tabla 1. Severidad de la enfermedad (%) causada por cepas de *B. cinerea* (B11, B14, B15 y B24) en uva de mesa blanca tratadas con cepas de levadura en ensayos preventivos y curativos.

Treatments	Severity							
	Preventive assay (%) *B. cinerea* strains				Curative assay (%) *B. cinerea* strains			
	B11	B14	B15	B24	B11	B14	B15	B24
S. cerevisiae BSc14	49.50 ^{b}	48.70 ^{b}	45.23 ^{c}	45.65 ^{b}	• ^{d}	• ^{c}	34.32 ^{a}	23.42 ^{a}
S. cerevisiae BSc16	48.36 ^{b}	45.36 ^{b}	47.85 ^{c}	12.42 ^{a}	• ^{d}	23.56 ^{a}	• ^{c}	12.98 ^{a}
S. cerevisiae BSc27	47.70 ^{b}	44.32 ^{b}	49.50 ^{c}	45.76 ^{b}	• ^{d}	23.77 ^{a}	• ^{c}	34.21 ^{b}
S. cerevisiae BSc60	48.9 1 ^{b}	45.98 ^{b}	34.65 ^{c}	45.32 ^{b}	• ^{d}	45.11 ^{b}	• ^{c}	34.37 ^{b}
S. cerevisiae BSc90	45.76 ^{b}	12.22 ^{a}	49.25 ^{c}	49.86 ^{b}	39.88 ^{c}	• ^{c}	• ^{c}	• ^{c}
S. cerevisiae BSc96	48.30 ^{b}	43.45 ^{b}	23.87 ^{b}	46.75 ^{b}	• ^{d}	23.99 ^{a}	• ^{c}	• ^{c}
S. cerevisiae BSc97	45.44 ^{b}	49.24 ^{b}	45.89 ^{c}	45.33 ^{b}	23.11 ^{b}	• ^{c}	45.93 ^{b}	• ^{c}
S. cerevisiae BSc102	48.57 ^{b}	45.13 ^{b}	49.86 ^{c}	45.34 ^{b}	• ^{d}	23.85 ^{a}	• ^{c}	23.96 ^{a}
S. cerevisiae BSc103	48.96 ^{b}	47.94 ^{b}	0 ^{a}	12.34 ^{a}	• ^{d}	• ^{c}	45.99 ^{b}	45.22 ^{b}
S. cerevisiae BSc112	45.44 ^{b}	49.20 ^{b}	49.11 ^{c}	45.65 ^{b}	0 ^{a}	• ^{c}	• ^{c}	• ^{c}
S. cerevisiae BSc140	48.91 ^{b}	45.63 ^{b}	45.99 ^{c}	49.83 ^{b}	• ^{d}	34.45 ^{b}	34.89 ^{a}	• ^{c}
S. cerevisiae BSc206	23.44 ^{a}	48.71 ^{b}	49.33 ^{c}	45.11 ^{b}	0 ^{a}	• ^{c}	• ^{c}	12.96 ^{a}
S. chevalieri BSch26	49.31 ^{b}	0 ^{a}	45.33 ^{c}	48.56 ^{b}	• ^{d}	34.56 ^{b}	45.94 ^{b}	• ^{c}
C. sake BCs54	49.91 ^{b}	45.63 ^{b}	23.32 ^{a}	47.84 ^{b}	• ^{d}	23.44 ^{a}	45.84 ^{b}	• ^{c}
H. vineae BHv86	49.32 ^{b}	33.92 ^{a}	47.32 ^{c}	45.80 ^{b}	• ^{d}	23.21 ^{b}	• ^{c}	23.93 ^{a}
T. delbrueckii BTd156	49.51 ^{b}	34.7 ^{a}	45.98 ^{c}	34.20 ^{b}	• ^{d}	23.12 ^{b}	• ^{c}	45.92 ^{b}
T. delbrueckii BTd165	48.22 ^{b}	45.21 ^{b}	49.31 ^{c}	45.41 ^{b}	• ^{d}	23.19 ^{b}	• ^{c}	45.95 ^{b}
D. vanrijiae BDv197	47.8 ^{b}	45.35 ^{b}	48.33 ^{c}	45.54 ^{b}	• ^{d}	34.4 ^{b}	• ^{c}	• ^{c}
Control ($H_2$0)	100 ^{c}	100 ^{c}	100 ^{d}	100 ^{c}	100 ^{d}	100 ^{c}	100 ^{c}	100 ^{c}

Different lowercase letters within the same column indicate significant differences among severity means and control according to Tukey's test ($p < 0.05$).

The • symbol indicates non-significant difference between disease severity and control (100%).

Gray highlight indicates 0% disease severity.

Diferentes letras minúsculas en la misma columna indican diferencias significativas entre las medias de severidad con respecto al control en relación al Test de Tukey ($p < 0,05$).

El símbolo • representa valores de severidad que no presentan diferencias significativas en relación con el control (100%)

Resaltado con gris indica 0% de severidad.

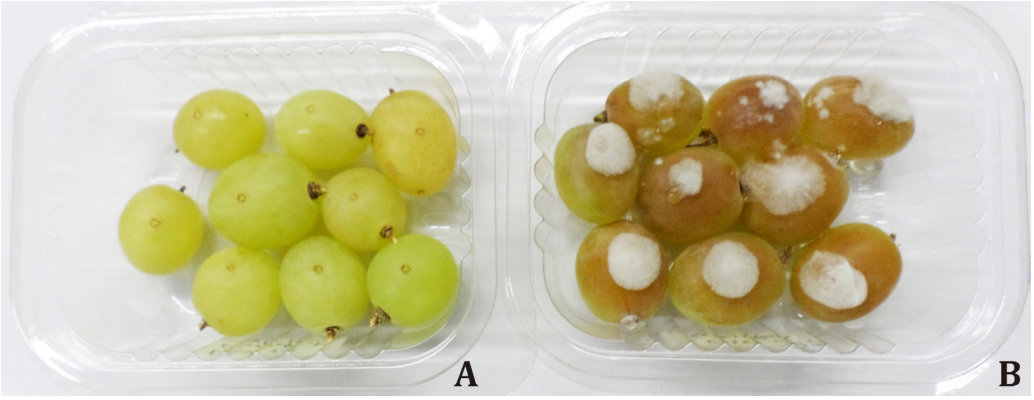


Figure 1. Grapes inoculated with *S. cerevisiae* BSc103- *B. cinerea* B15 (A) (0% severity) and inoculated with water- *B. cinerea* B15 (B) (100% severity), in preventive assays.

Figura 1. Uvas inoculadas con *S. cerevisiae* BSc103- *B. cinerea* B15 (A) (0% severidad) e inoculadas con agua- *B. cinerea* B15 (B) (100% severidad), en ensayos preventivos.

In curative *in vivo* experiments, 15 of the 18 preselected yeasts presented curative activity against two of the four *B. cinerea* strains assayed (9 *S. cerevisiae,* 1 *S. chevalieri*, 1 *C. sake*, 1 *H. vineae*, and 2 *T. delbrueckii*), reducing disease severity between 54.01 and 100%. Additionally, four isolates (3 *S. cerevisiae* and 1 *D. vanrijiae*) significantly reduced the rot halo caused by one *B. cinerea* strain with a severity between 0 and 39.88% (table 1, page 107).

S. cerevisiae BSc206 and BSc212 inhibited total growth of B11 during curative assays (table 1, page 107).

Possible mechanism of action

Eighteen yeast isolates that showed antifungal effectivity (table 1, page 107) were analyzed.

Dual culture assay

Five of the 18 yeast isolates did not inhibit any *B. cinerea* strain (B11, B14, B15, B24) in dual culture assays (table 2, page 109). Two yeasts (BSc27, BCs54) significantly inhibited three *B. cinerea* strains, whereas five isolates (BSc14, BSc16, BSc90, BSch26, BHv86) significantly reduced fungal development of two strains. Five yeast strains only inhibited one pathogenic strain (BSc96, BSc97, BSc103, BSc112, BSc140, BTd156). Table 2 (page 109), shows the highest inhibition percentages (50% or more) in *Saccharomyces* strains BSc27, BSch26, and BSc14 against B11, B15, and B24, respectively.

Yeast *'Killer'* activity

Thirteen yeast strains presented *'killer'* activity against at least one *B. cinerea* pathogenic strain**.** Seven yeasts (*5 S. cerevisiae, 1 T. delbrueckii, 1 D. vanrijiae*) showed *'killer'* activity against B11 strain, four *Saccharomyces* yeasts against B14, two yeasts against B15 and none yeast against B24 (table 3, page 109-110).

Antifungal activity of volatile organic compounds (VOCs)

From the 18 isolates, 13 yeast strains produced volatile compounds that significantly inhibited mycelial growth of at least one *Botrytis* strain (table 4, page 110). The most susceptible *B. cinerea* strain was *B. cinerea* B24 (7/18), followed by B11, B15 (6/18) and B14 (3/18).

Volatile compounds produced by *S. cerevisiae* BSc206 significantly inhibited mycelial growth of three *B. cinerea* strains, B14, B15, and B24, between 25.6 and 54.7% (table 4, page 110). Seven strains (4 *Saccharomyces* and 3 non- *Saccharomyces)* produced antifungal volatile compounds against 2 *B. cinerea strains* and 5 *Saccharomyces* yeasts against one *B. cinerea* strain. These 12 yeast strains inhibited mycelial growth between 21.4 and 76.9% (table 4, page 110).

Table 2. Mycelial growth inhibition (%) of *B. cinerea* strains after released dual culture assays with 18 yeast strains.

Tabla 2. Inhibición del crecimiento micelial (%) de cepas de *B. cinerea* luego de realizar ensayos de cultivos duales con 18 cepas de levaduras.

Treatments	Mycelial growth inhibition of *B. cinerea* strains (%)			
	B11	B14	B15	B24
S. cerevisiae BSc14	• [a]	• [a]	41.33 ± 2.52 [b]	52.33 ± 1.53 [c]
S. cerevisiae BSc16	• [a]	• [a]	40.00 ± 2.00 [b]	39.33 ± 1.53 [b,c]
S. cerevisiae BSc27	51.60 ± 1.5 [d]	24.0 ± 4.0 [a,b]	44.33 ± 1.53 [b]	• [a]
S. cerevisiae BSc90	37.60 ± 0.5 [b,c,d]	• [a]	• [a]	29.60 ± 1.5 [b,c]
S. cerevisiae BSc96	31.00 ± 1 [c,d]	• [a]	• [a]	• [a]
S. cerevisiae BSc97	• [a]	39.03 ± 3 [b]	• [a]	• [a]
S. cerevisiae BSc103	• [a]	• [a]	• [a]	31.30 ± 1.5 [b,c]
S. cerevisiae BSc112	35.30 ± 0.5 [b,c,d]	• [a]	• [a]	• [a]
S. cerevisiae BSc140	• [a]	• [a]	• [a]	29.30 ± 0.5 [b,c]
S. chevalieri BSch26	38.70 ± 2 [b,c,d]	• [a]	53.67 ± 0.58 [c]	• [a]
C. sake BCs54	47.30 ± 3.5 [c,d]	31.00 ± 1 [b]	• [a]	31.00 ± 1.51 [b,c]
H. vineae BHv86	47.00 ± 1 [c,d]	42.30 ± 3.5 [b]	• [a]	• [a]
T. delbrueckii BTd156	40.30 ± 2.5 [b,c,d]	• [a]	• [a]	• [a]
S. cerevisiae BSc60 *S. cerevisiae* BSc102 *S. cerevisiae* BSc206 *D. vanrijiae* BDv197 *T. delbrueckii* BTd165	• [a]	• [a]	• [a]	• [a]
Control	0.00 [a]	0.00 [a]	0.00 [a]	0.00 [a]

Different lowercase letters within the same column indicate significant differences among means and SD of mycelial growth according to Tukey's test ($p \leq 0.05$).

The •symbol represents values not significantly different from control (0%).

Gray highlight indicates a mycelial growth inhibition percentage of 50% or more.

Diferentes letras minúsculas en la misma columna indican diferencias significativas entre las medias y el DS del crecimiento fúngico micelial con respecto al control ($p \leq 0,05$).

El símbolo • representa valores que no difieren significativamente con el control (0%).

Resaltado con gris indica porcentajes de inhibición del crecimiento micelial de 50% o superior.

Table 3. Yeast *"Killer"* activity against four *B. cinerea* isolates (B11, B14, B15, B24) at 25°C.

Tabla 3. Actividad "killer" de las cepas de levaduras frente a los cuatro aislamientos de *B. cinerea* (B11, B14, B15, B24), a 25°C.

Treatments	*B. cinerea* strains			
	B11	B14	B15	B24
***S. cerevisiae* BSc16**	+	-	-	-
***S. cerevisiae* BSc27**	-	+	-	-
***S. cerevisiae* BSc60**	-	+	-	-
***S. cerevisiae* BSc90**	+	-	-	-
***S. cerevisiae* BSc102**	+	-	-	-
***S. cerevisiae* BSc103**	+	-	-	-
***S. cerevisiae* BSc112**	+	-	-	-
***S. cerevisiae* BSc206**	-	-	+	-

Positive signs (+) indicate killer activity (presence of a clear zone around the yeast colony), and negative signs (-) indicate no "killer" activity.

Signos positivos (+) indican la presencia de zona transparente alrededor de las colonias de levadura, y los signos negativos (-) indican ausencia de actividad "killer".

***S. chevalieri* BSch26**	-	+	-	-
***T. delbrueckii* BTd156**	+	-	-	-
***T. delbrueckii* BTd165**	-	-	+	-
***D. vanrijiae*BDv197**	+	-	-	-
***S. cerevisiae* BSc14**	-	+	-	-
***S. cerevisiae* BSc97**	-	-	-	-
***S. cerevisiae* BSc140**	-	-	-	-
***C. sake* BCs54**	-	-	-	-
***H. vineae* BHv86**	-	-	-	-

Positive signs (+) indicate killer activity (presence of a clear zone around the yeast colony), and negative signs (-) indicate no "killer" activity.

Signos positivos (+) indican la presencia de zona transparente alrededor de las colonias de levadura, y los signos negativos (-) indican ausencia de actividad "killer".

Table 4. Effect of volatile compounds produced by 18 yeast isolates on mycelial growth of four *B. cinerea* strains (%).

Tabla 4. Efecto de los compuestos volátiles producidos por 18 levaduras sobre la inhibición del crecimiento micelial de las cuatro cepas de *B. cinerea* ensayadas (%).

Treatments	Inhibition of mycelial growth of *B. cinerea* strains (%)			
	B11	**B14**	**B15**	**B24**
S. cerevisiae BSc14	• ^{d}	62.60 ± 0.29 ^{b}	• ^{d}	64.20 ± 0.28 ^{a}
S. cerevisiae BSc16	• ^{d}	• ^{c}	66.80 ± 0.01 ^{c}	• ^{c}
S. cerevisiae BSc27	• ^{d}	• ^{c}	54.90 ± 0.06 ^{b}	69.70 ± 0.12 ^{a}
S. cerevisiae BSc60	• ^{d}	67.60 ± 0.1 ^{b}	• ^{d}	• ^{c}
S. cerevisiae BSc96	• ^{d}	• ^{c}	64.30 ± 0.02 ^{c}	• ^{c}
S. cerevisiae BSc97	77.60 ± 0.1 ^{c}	• ^{c}	• ^{d}	72.80 ± 0.06 ^{b}
S. cerevisiae BSc102	76.7 ± 0.05 ^{c}	• ^{c}	• ^{d}	• ^{c}
S. cerevisiae BSc112	78.60 ± 0.5 ^{c}	• ^{c}	• ^{d}	71.80 ± 0.49 ^{b}
S. cerevisiae BSc206	• ^{d}	50.20 ± 0.2 ^{a}	45.30 ± 0.26 ^{b}	74.40 ± 0.04 ^{b}
S. cerevisiae BSch26	• ^{d}	• ^{c}	• ^{d}	70.00 ± 0.32 ^{b}
C. sake BCs54	60.76 ± 0.5 ^{a}	• ^{c}	• ^{d}	65.80 ± 0.02 ^{a}
T. delbrueckii BTd165	72.70 ± 0.1 ^{b}	• ^{c}	23.10 ± 0.06 ^{a}	• ^{c}
D. vanrijiae BDv197	68.7 ± 0.05 ^{b}	• ^{c}	63.90 ± 0.1 ^{c}	• ^{c}
S. cerevisiae BSc90	• ^{d}	• ^{c}	• ^{d}	• ^{c}
S. cerevisiae BSc103				
S. cerevisiae BSc140				
H. vineae BHv86				
T. delbrueckii BTd156				
Control (H_2O)	**100** ^{d}	**100** ^{c}	**100** ^{d}	**100** ^{c}

Different lowercase letters within the same column indicate significant differences between means of mycelial growth according to Tukey's test ($p \leq 0.05$).

The •symbol represents mycelial growth not significantly different from the control (100%).

Letras distintas en de la misma columna indican diferencias significativas entre las medias para el crecimiento fúngico según la prueba de Tukey ($p \leq 0,05$).

El símbolo • representa valores de crecimiento micelial que no difieren significativamente con el del control (100%).

Yeast effect on conidial germination (CG) and germinal tube length (GTL) of *B. cinerea* in low-nutrient medium (diluted grape must)

In this assay, seven yeast strains (6 *Saccharomyces* and 1 non-*Saccharomyces*) significantly affected conidial germination (CG) and germinal tube length (GTL) of at least one *B. cinerea* strain. B24 was the most susceptible strain, inhibited by five yeasts (5/18), followed by B15 inhibited by 3 yeasts (3/18), B14 (2/18), and B11 inhibited by 1 yeast (1/18). Five *Saccharomyces* yeasts (*S. cerevisiae* BSc27, BSc102, BSc112, BSc206, and *S. chevalieri* BSch26) significantly inhibited conidial germination and reduced the germ tube length of one *B. cinerea* strain. *S. cerevisiae* Bsc60 significantly reduced two *B. cinerea* strains: B24 and B15 (table 5, page 111).

Table 5. Evaluation of conidial germination (CG; %) and germ tube length (GTL; µm) of *B. cinerea* strains in co-cultures with yeasts (excavated slides).

Tabla 5. Evaluación de la germinación de conidios (CG; %), y longitud del tubo germinal (GTL; µm) de cepas de *B. cinerea* en co-cultivos con levaduras (portaobjetos excavados).

Treatments	*B. cinerea* strains							
	B11		B14		B15		B24	
	CG	GTL	CG	GTL	CG	GTL	CG	GTL
S. cerevisiae BSc14	100 [b]	15.42 [b]	100 [b]	20.06 [b]	100 [b]	11.43 [b]	100 [b]	25.0 [b]
S. cerevisiae BSc16	100 [b]	15.67 [b]	100 [b]	21.85 [b]	100 [b]	11.40 [b]	100 [b]	25.02 [b]
S. cerevisiae BSc27	100 [b]	13.21 [b]	67.52 [a]	5.75 [a]	100 [b]	9.45 [b]	100 [b]	25.11 [b]
S. cerevisiae BSc60	100 [b]	14.55 [b]	100 [b]	22.23 [b]	71.49 [a]	5.87 [a]	61.22 [a]	3.56 [a]
S. cerevisiae BSc90	100 [b]	14.52 [b]	100 [b]	21.96 [b]	100 [b]	11.49 [b]	100 [b]	25.01 [b]
S. cerevisiae BSc96	100 [b]	17.23 [b]	100 [b]	21.54 [b]	100 [b]	11.42 [b]	100 [b]	25.02 [b]
S. cerevisiae BSc97	100 [b]	16.32 [b]	100 [b]	21.85 [b]	92.4 [b]	11.12[b]	100 [b]	25.07 [b]
S. cerevisiae BSc102	100 [b]	17. 54 [b]	100 [b]	21.15 [b]	67.44 [a]	5.62 [a]	100 [b]	25.71 [b]
S. cerevisiae BSc103	100 [b]	13.64 [b]	100 [b]	20.54 [b]	100 [b]	11.40 [b]	100 [b]	25.03 [b]
S. cerevisiae BSc112	100 [b]	13.56 [b]	100 [b]	19.55 [b]	100 [b]	11.42 [b]	63.87 [a]	3.78 [a]
S. cerevisiae BSc140	100 [b]	15.46 [b]	100 [b]	21.56 [b]	100 [b]	11.43 [b]	100 [b]	25.01 [b]
S. cerevisiae BSc206	100 [b]	16.87 [b]	100 [b]	22.78 [b]	98.76 [b]	10.13 [b]	71.55 [a]	3.56 [a]
S. chevalieri BSch26	100 [b]	17.84 [b]	100 [b]	21.87 [b]	100 [b]	11.45 [b]	67.54 [a]	2.78 [a]
C. sake BCs54	100 [b]	17.24 [b]	100 [b]	19.84 [b]	100 [b]	11.44 [b]	100 [b]	25.21 [b]
H. vineae BHv86	70.65 [a]	3.14 [a]	71.43 [a]	3.98 [a]	67.38 [a]	5.81[a]	69.11 [a]	3.24 [a]
T. delbrueckii BTd156	100 [b]	16.44 [b]	100 [b]	21.56 [b]	100 [b]	11.42 [b]	100 [b]	25.03 [b]
T. delbrueckii BTd165	100 [b]	15.38 [b]	100 [b]	20.59 [b]	100 [b]	11.47 [b]	100 [b]	25.02 [b]
D. vanrijiae BDv197	100 [b]	16.57 [b]	100 [b]	21.65 [b]	100 [b]	11.43 [b]	100 [b]	25.0 [b]
Control (H_20)	**100 [b]**	**17.96 [b]**	**100 [b]**	**21.97 [b]**	**100 [b]**	**11.4 [b]**	**100 [b]**	**25.77 [b]**

Different lowercase letters within the same column indicate significant differences between means of germinated conidia (CG) expressed in % and germinal tube length (GTL) in µm, according to Tukey's test ($p \leq 0.05$).

Gray highligh indicates values significantly differing from the control ($p \leq 0.05$).

Letras distintas en la misma columna indican diferencias significativas entre los valores de conidios germinados (CG) expresados en %, y longitud del tubo germinal (GTL) en µm, en relación al Test de Tukey ($p \leq 0,05$).

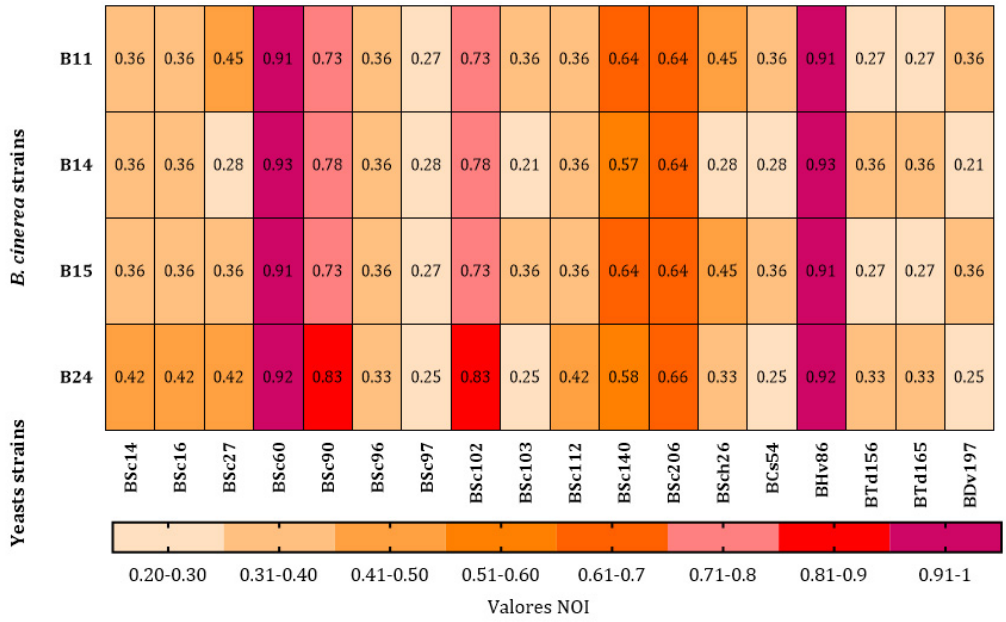
Resaltado con color gris indica valores que difieren significativamente del control ($p \leq 0,05$).

H. vineae BHv86 was the only isolate significantly inhibiting conidial germination and germinal tube length of all five *B. cinerea* B11, B14, B15, and B24. This yeast strain reduced conidial germination between 28.57 and 32.62%, and germ tube length between 12.57 and 50.96% (table 5).

Niche Overlap Index (NOI)

S. cerevisiae BSc60 and *H. vineae* BHv86 occupied the same niche as the 4 *B. cinerea* isolates. Both yeast strains competed with B11, B14, B15 and B24 for the nutritional sources. Four *S. cerevisiae*/four *H. vineae-B. cinerea* interactions presented NOI values between 0.91 and 0.93 (table 6, page 112). The remaining interacting pairs (64) presented NOI values between 0.21 and 0.83, indicating ecological coexistence (separate niches) (table 6, page 112).

Table 6. Niche overlap index (NOI) between 18 yeast and 4 *B. cinerea* strains.
Tabla 6. Índice de superposición de nichos (NOI) entre 18 cepas de levaduras y 4 cepas de *B. cinerea*.



Discussion

Yeast antifungal activity is strain-dependent, and therefore, screening numerous microorganisms becomes necessary for finding strains with broad inhibitory spectrums. Few reports have mentioned the potential use of yeasts of different species and genera with preventive and curative activity to control fungi on fruit tissues (5, 22). Our study first reports yeast species isolated from grape fermentation, like *S. cerevisiae*, *S. chevalieri*, *T. delbrueckii*, *D. vanrijiae*, *H. vineae* and *C. sake,* with preventive and curative antifungal activity against *B. cinerea* in table grapes and postharvest conditions. These enological yeasts demonstrated greater antifungal activity in preventive than in curative assays in table grape wounds (table 1, page 107). Pesce *et al.* (2018) determined that oenological yeasts have stronger antifungal activity than yeasts isolated from another habitat (olives), presenting competitive advantages against other microorganisms.

Commercially developing a product based on native microorganisms involves several steps, starting with isolation and selection of potential biocontrol strains exhibiting desirable characteristics, like different antifungal mechanisms of action (5). Our study examined 18 viticultural yeast strains promoting biocontrol of gray mold under preventive and curative conditions in white table grapes (table 2, page 109; table 3, page 109-110 and table 4, page 110; table 5, page 111 and table 6). We assessed biocontrol activity and modes of action of native yeasts against a pool of four native *B. cinerea* strains previously isolated from different vineyards, constituting a representative way to measure biocontrol activity. To the best of our knowledge, this is the first report on antifungal modes of action of *Saccharomyces, Torulaspora, Debaryomyces, and Candida* with preventive and curative effects against native *B. cinerea* strains isolated from Superior Seedless table grapes (table 1, page 107). The dual culture assay revealed that 72.2% (13/18) of the biocontrol yeasts inhibited mycelial growth of at least one *B. cinerea* strain in the PDA medium (table 2, page 109). Strains belonged to *S. cerevisiae*, *S. chevalieri*, *C. sake*, *H. vineae,* and *T. delbrueckii* species. Korres *et al.* (2011) state that yeasts synthesized and secreted suppressive antifungal substances like diffusible and volatile metabolites, and observed mycelial inhibition on

plates in dual cultures. "*Killer*" activity is a widespread characteristic among yeast species of different genera, including *Saccharomyces*, *Hansenula*, *Kluyveromyces*, and *Pichia*, conferring an ecological advantage over competitors (10). In this study, 13 yeasts belonging to *Saccharomyces*, *Torulaspora,* and *Debaryomyces* species showed positive *'killer'* activity against at least one *B. cinerea* strain at pH 4.5 (table 3, page 109-110). Fungal growth inhibition by volatile compounds avoids adverse environmental or toxicological effects (11). Parafati *et al.* (2015) found that the biocontrol activity of *S. cerevisiae* strains against *B. cinerea* on table grape berries was attributed to volatile organic compounds (VOCs) *in vitro* and *in vivo*. In our study, *Saccharomyces*, *Candida*, *Torulaspora,* and *Debaryomyces* isolates significantly inhibited mycelial diameter of different *B. cinerea* isolates through volatile compounds *in vitro* (table 4, page 110)**.** Primarily, biocontrol yeasts compete for nutrients and space (20). Co-cultures of *Saccharomyces* (BSc27, BSc60, BSc102, BSc112, BSc206, BSch26) and *Hanseniaspora* (BHv86) with *B. cinerea* conidia in liquid medium with low nutrients (excavated slides) showed that these yeasts significantly inhibited conidia germination and reduced germ tube length. In our study, *Hanseniaspora* yeast significantly reduced conidial germination (CG) and germinal tube length (GTL) of four *B. cinerea* strains (table 5, page 111). Qin *et al.* (2015) reported similar results in a co-culture of *H. uvarum* and *B. cinerea*, while Wilson and Lindow (1994) previously suggested that nutritional resources might mediate microorganism coexistence and competitive exclusion. To the best of our knowledge, our study first reports two yeast isolates, *S. cerevisiae* (BSc60) and *H. vineae* (BHv86) with NOI values of 0.91 and 0.93 when co-cultured with four *B. cinerea* strains (table 6, page 112).

S. cerevisiae (BSc27, BSc60, BSc112) and *S. chevalieri* (BSch26) presented the highest amount of possible antifungal mechanisms (four). Our results confirm multiple possible modes of action against pathogens like antibiosis (dual culture), *'killer'* activity, antifungal activity by volatile compounds, inhibition of conidial germination, reduction of germinal tube length (low nutrient medium) and competitive exclusion (NOI).

Conclusions

Under preventive and curative conditions, yeasts isolated from wine fermentation are key biocontrol agents against *B. cinerea* in white table grapes. Understanding yeast strategies against *B. cinerea* strains in preventive and curative assays is essential for selection and effective application. Our study highlights the importance of testing diverse mechanisms that native biocontrol yeasts apply against different *B. cinerea* isolates. In addition, considering that different isolates can exert more than one mechanism of action makes biocontrol an effective tool to prevent gray rot in white table grapes. Further studies should establish bio-antagonism effects on quality attributes of table grapes, the application of biofungicide yeast consortium and the effect of nonviable yeast cells against *B. cinerea*.

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