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Biocontrol of *Botrytis cinerea* in table grapes by non-pathogenic indigenous *Saccharomyces cerevisiae* yeasts isolated from viticultural environments in Argentina

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ABSTRACT

Botrytis cinerea, the causal agent of gray mold, is an important disease of grapes. Yeasts are members of the epiphytic microbial community on surfaces of fruits and vegetables and because some yeasts inhibit fungi they are used as biocontrol agents. The major objective of the present work was to isolate yeasts from grapes, vineyard soil, and grape must and select them for their ability to prevent gray mold onset after harvest. Yeasts that were found effective against the fungus were also assayed for their possible pathogenicity in humans. Two antagonism experiments were performed to study the effect of yeasts on *B. cinerea*, an *in vitro* study with Czapeck Yeast Extract Agar and an *in vivo* study with grape berries at 2 °C and 25 °C; both experiments were conducted at different yeast concentrations (10^5, 10^6 and 10^7 cfu/mL). Antagonists were subsequently assayed for their ability to colonize and grow in fruit wounds. The biocontrol yeasts were also examined for their possible pathogenicity in humans: phospholipase and proteolytic activity, growth at 37 °C and 42 °C, pseudohyphal formation and invasive growth. A total of 225 yeasts belonging to 41 species were isolated from must and grape berries and 65 of them, representing 15 species, exhibited *in vitro* inhibition of *B. cinerea* at 25 °C. These 65 biocontrol yeasts were subsequently assayed *in vivo* and 16 of them (15 *Saccharomyces cerevisiae* and 1 *Schizosaccharomyces pombe*) showed antagonistic properties against *B. cinerea* at 25 °C. Only one isolate (*S. cerevisiae* BSc68) was able to inhibit mycelial growth of *B. cinerea* on grape berries at both 2 °C and 25 °C. The biomass of this strain in grape wounds increased 221.5-fold at 25 °C after 3 d and 325.5-fold at 2 °C after 10 d of incubation. An increase in the concentration of certain yeasts significantly enhanced their antagonistic activity. All yeast isolates determined as biocontrol agents under *in vivo* conditions were isolated from fermenting musts. Twelve biocontrol agents (*S. cerevisiae*) revealed one or more phenotypical characteristics associated with pathogenicity in humans but none of them showed all characteristics together. The fact that there exist few reports on *S. cerevisiae* and none on *Sch. pombe* as biocontrol agents against *B. cinerea* makes our results even more relevant.

1. Introduction

San Juan province is the main producer and exporter of Argentine table grapes and in 2010 export totaled about 51,776 metric tons. In 2008–2009, table grapes grown in San Juan were negatively affected by multiple factors, which included adverse weather conditions and fungal diseases (Battistella, 2009). Postharvest fungal diseases might be considered a minor problem for local markets with short periods between harvest and selling of vegetables and fruit, however, when fruit is exported to foreign countries prolonged periods of postharvest disease control are required. Red globe table grapes are harvested in summer and preserved in a refrigerated storage room for a period of 1 month before exportation.

Botrytis cinerea, the causal agent of gray mold or botrytis bunch rot, is an important disease of grapes and causes heavy losses in table and wine grapes around the world (Masih et al., 2001). In general, *Botrytis* is an important problem to fruit and vegetables in

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cold storage and subsequent shipment, because the fungus is able to grow effectively at temperatures just above freezing (Droby and Lichter, 2004). The pathogen can be controlled on grapes with pre and postharvest fungicidal treatments (Rosslenbroich and Stuebler, 2000). The emergence of fungicide resistance and increasing consumer demands for reduction in residues on fruit emphasize the need for alternative disease control strategies (Pyke et al., 1996).

Biological control of postharvest diseases of fruits and vegetables by antagonistic microorganisms seems promising in replacing or reducing the use of synthetic fungicides (Lima et al., 1999; Janisiewicz and Korsten, 2002). Among other potential antagonists, yeasts have been extensively studied because they possess many features that make them suitable as biocontrol agents in fruits. Many yeast species have simple nutritional requirements, they are able to colonize dry surfaces for long periods of time and they can grow rapidly on inexpensive substrates in bioreactors, characteristics that are relevant in the selection of biocontrol agents (Chanchaichaovivat et al., 2007). In addition, they are a major component of the epiphytic microbial community on surfaces of fruits and vegetables and they are also phenotypically adapted to this niche. Therefore, they are able to effectively colonize fruit surfaces and compete for nutrients and space (Suzzi et al., 1995). However, yeast antagonists show a protective effect that diminishes with fruit ripening and senescence, and this process has no curative activity (El-Ghaouth, 1997; Yu et al., 2007; Droby et al., 2009).

Currently, there is only one biofungicide available on the commercial market for postharvest use: "Shemer", based on *Metschnikowia fructicola* (Droby et al., 2009; Wang et al., 2010). However, three more products will soon be launched onto the market: "Candifruit", based on *Candida sake* and developed in Spain, "Boni-Protect", based on *Aureobasidium pullulans* and developed in Germany and "NEXY", based on *Candida oleophila* and developed in Belgium. All these products have been registered for control of postharvest diseases of pome fruits (Janisiewicz, 2009). Other non-*Saccharomyces* yeasts have also been reported to effectively reduce *Botrytis* on grapes: *Hanseniaspora uvarum* (Rabosto et al., 2006), *Candida guilliermondii*, *Acremonium cephalosporium* (Zahavi et al., 2000), *Pichia anomala* (Masih et al., 2001) and *Metschnikowia pulcherrima* (Nigro et al., 1999). Although several researchers have described the biocontrol capacity of *Saccharomyces cerevisiae* against a range of phytopathogenic fungi (Attyia and Youssry, 2001; Zhou et al., 2008), there are few reports about *S. cerevisiae* as antagonist of *B. cinerea* on table grapes (Salmon, 2009). Suzzi et al. (1995) found two *S. cerevisiae* strains that showed a broad spectrum of *in vitro* antagonistic activity against 10 fungal pathogens isolated from soil and fruit, including *Botrytis squamosa*.

S. cerevisiae is widely distributed in nature and has recently become increasingly important to biotechnology. It is now one of the most studied microorganisms and it is used as a model eukaryote. However, numerous cases of clinical infections caused by *S. cerevisiae* and other yeasts have been reported in the literature in recent years, particularly in immunocompromised patients (Okawa and Yamada, 2002; de Llanos et al., 2006). In Europe, *S. cerevisiae* has been reclassified from GRAS to Biosafety level 1, indicating its ability to cause superficial or mild systemic infections. Hence, this microorganism should now be regarded as an opportunistic pathogen rather than non-pathogenic yeast (Murphy and Kavanagh, 1999; Mc Cusker et al., 1994). This shows once more the importance to study the possible pathogenicity of biocontrol yeasts in humans and animals. Some fungal properties are frequently associated with pathogenesis, e.g. the ability to grow at high temperatures, to adhere to and invade host cells and secrete degradative enzymes such as proteinases and phospholipases. In order to facilitate the invasion of host tissues, microbial cells possess constitutive and inducible hydrolytic enzymes that destroy or disturb certain constituents of the cell membranes in the host, resulting in membrane dysfunction and/or physical disruption. Since membranes are composed of lipids and proteins, these macromolecules are the target of enzyme attack (de Llanos et al., 2006).

In the current study, different concentrations of yeasts of viticultural origin were assessed as biocontrol agents against *B. cinerea*. Survival and growth of yeasts that were effective against the fungus were assayed at 25 °C and under storage conditions (2 °C) on grapes. Finally, the *in vitro* active biocontrol strains were tested for their pathogenicity in humans.

2. Materials and methods

2.1. Microorganisms

2.1.1. Yeasts

2.1.1.1. Isolation of yeast strains. Yeasts were isolated from three viticultural environments (fermenting musts, vineyard soil and healthy berries of Red Globe table grapes) in the Zonda district, San Juan, Argentina.

Isolations from fermenting must were carried out as follows: samples of spontaneous fermenting musts were taken aseptically, diluted and streaked onto YEPD-Agar medium (10 g/L Yeast Extract, 20 g/L Peptone, 20 g/L Dextrose, 20 g/L Agar; pH 4.5) (Sipiczki et al., 2001).

Soil was sampled from the top layer (0–10 cm) under vine canopy and placed in sterile vials, which were transported to the laboratory on ice and stored in the refrigerator. Then, portions of 1 g were suspended in 10 mL of sterile distilled water and incubated on a shaker (3.3 s^{-1}) for 12 h. Serial dilutions of 0.1 mL were spread in triplicate on acidified YM agar (10 g/L Glucose, 3 g/L Malt Extract, 3 g/L Yeast Extract, 5 g/L Peptone, 20 g/L Agar) supplemented with 400 mg/L of chloramphenicol and also on acidified Yeast Nitrogen Base (YNB) Agar, pH 4.5, supplemented with 5 g/L of glucose and 400 mg/L of chloramphenicol (Pan et al., 2009).

Epiphytic yeasts were isolated from grapes by washing the berries (10 per sample) in 50 mL of sterile distilled water on a rotary shaker at 3.3 s^{-1} for 30 min. Sample dilutions from 10^{-1} to 10^{-4} were seeded on YEPD-Agar, pH 4.5 (Bleve et al., 2006).

Samples from the three different sources were incubated at 25 °C for 5 d. Individual colonies were isolated from each plate and submitted to biochemical and molecular assays for identification and afterwards, yeasts were kept on YEPD-Agar at 4 °C.

2.1.1.2. Identification. Taxonomic identification of the isolates was first carried out by conventional yeast identification methods based on morphology, sporulation, fermentation and assimilation of carbon sources (Kurtzman and Fell, 1998) and then confirmed by PCR amplification and partial sequencing of internally transcribed spacer (ITS) regions and 5.8S ribosomal DNA (rDNA), using ITS1 (5′- CGTAGGTGAACCTGCGG-3) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3) primers. PCR cycling conditions consisted of an initial denaturation step at 95 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 55.5 °C for 2 min and extension at 72 °C for 2 min; and a final extension at 72 °C for 10 min. PCR products were digested without further purification with CfoI, HaeIII and HinfI restriction endonucleases (Boehringer Mannheim) (Esteve-Zarzoso et al., 1999).

2.1.1.3. Preparation of the inoculum. A loopful of pure isolated yeast was transferred to a 250 mL Erlenmeyer flask containing 100 mL of YEPD (prepared as above, without agar) and agitated on a rotary shaker for 12 h. Yeast cells were pelleted by centrifugation, re-suspended in sterile distilled water and centrifuged again. The resulting pellets were re-suspended in sterile distilled water and the yeast concentration was adjusted to10^6 cfu/mL using a

Neubauer chamber (El- Ghaouth et al., 1998). This preparation was used throughout the study.

2.1.2. Fungus

2.1.2.1. Botrytis cinerea. Virulent *B. cinerea* was isolated from rotten grapes collected in the Zonda district near the city of San Juan, Argentina. Each sample consisted of, at least, one infected berry, which was placed in a plastic sterile bag and transferred as quickly as possible to the laboratory (Forster and Staub, 1996). Fungi were purified by monospore isolation and maintained on Czapeck-Agar (20 g/L Sucrose, 2 g/L $NaNO_3$, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.5 g/L KCl, 0.01 g/L $FeSO_4$, 20 g/L Agar).

2.1.2.2. Identification. B. cinerea Pers.: Fr. was identified by morphological characteristics (Pitt and Hocking, 1997), amplification of the ribosomal intergenic spacer (IGS) by PCR and restriction of the product with the following restriction enzymes: HindIII, BamHI, HaeIII and RsaI (Giraud et al., 1997).

2.1.2.3. Preparation of the inoculum. Spores from 10-d-old cultures grown at 22 °C were collected in sterile water containing 0.1% (v/v) Tween 20. The suspension was filtered through a double layer of lens cleaning tissue (Whatman 105) to remove mycelial fragments and then centrifuged at 11,000 × *g* (2 min, 4 °C). The supernatant was decanted and the spore pellet re-suspended in 0.01% (v/v) Tween-20 to remove nutrients from the medium. This procedure was repeated twice. Conidia were re-suspended in sterile water and their concentration was adjusted (Neubauer chamber) by dilution to10^4 conidia/mL (Commenil et al., 1999). The pathogenicity of *B. cinerea* was maintained by inoculating grape berries every 6 months (Utkhede et al., 2001).

2.2. Table grapes

Red Globe grapes (*Vitis vinifera* cv. Red Globe) were harvested during the commercial ripening period from a local vineyard (Zonda district, San Juan) and immediately transferred to the laboratory. Homogeneous bunches were selected according to size, shape, color, weight and absence of injuries (Martínez- Romero et al., 2007). Before each assay, fruit were washed with sodium hypochlorite solution (1% active chlorine), rinsed with distilled water and left to dry at room temperature.

2.3. Antagonism assays

2.3.1. In vitro yeast–pathogen direct interaction

A preliminary *in vitro* screening of all isolated yeasts was conducted to assess inhibition of *B. cinerea* (Kloepper, 1991). The potential biocontrol yeasts were co-cultured with the pathogen on Petri dishes containing Czapeck-Yeast extract-Agar, to test antagonistic activity. A 5-mm mycelial disc, obtained from the edges of a 5-d-old culture of the fungus, was placed in the center of the dish. Ten microliters of yeast cell suspension (10^6 cfu/mL) were added at four sites at about 3 cm from the center. The effect of yeasts on pathogen growth was compared with a control (pure *B. cinerea*). The dishes were incubated at 25 °C and 80% RH. After 5 d, cultures were examined for inhibition zones (IZ) between the fungus and the yeast. When an IZ (0.2–0.7 cm) was formed the antagonist yeast was selected for *in vivo* assays (Capdeville et al., 2007).

2.3.2. Antagonistic action of different concentrations of yeast isolates against gray mold on grape berries

All isolates that inhibited the fungus during *in vitro* screening were evaluated for biocontrol activity at 2 °C and 25 °C, for 30 and 5 d, respectively. A single wound (3 mm diameter and 3 mm deep) was made at the equator of each fruit using the tip of a sterile dissecting needle. Twenty μL of the yeast suspension in water (10^5, 10^6 and 10^7 cfu/mL) were pipetted into each wound. After 2 h, 20 μL of 10^4 *B. cinerea* conidia/mL of sterile distilled water were poured into each wound. Treated grapes were air dried and placed in plastic bags (with wet paper towels to maintain high humidity). At the end of the experiment, the incidence of gray mold on each infected grape was calculated as follows: Incidence (%) = (number of decayed wounds/number of total wounds) x 100%. Positive controls (wounded grapes with 20 μL of fungal spore suspension and 20 μL of sterile distilled water) were included as well as two different negative controls: wounded grapes with 40 μL of sterile distilled water and wounded grapes with 20 μL of yeast suspension and 20 μL of sterile water. Each experiment used eighteen berries per replicate and three replicates per treatment in a randomized complete block design. A reduction in disease incidence of 70% or more was considered the selection criterion of antagonistic yeasts at both temperatures (Garmendia et al., 2005). The experiment was repeated 3 times to confirm reproducibility of the results.

2.4. Population profiles of S. cerevisiae BSc68 at 2 °C and 25 °C

The ability of *S. cerevisiae* BSc68 to survive and multiply in grape wounds was determined as follows: grape berries were rinsed with water, wounded, inoculated with 20 μL of a washed cell suspension (10^6 cfu/mL), placed on plastic trays (80% RH) and incubated at 2 °C and 25 °C. The population of *S. cerevisiae* BSc68 was examined after 0, 3 and 5 d on fruit stored at 25 °C, and after 0, 3, 5, 10, 15, 20, 25 and 30 d at 2 °C. The entire wound was excised from the grape with a sterilized 3 mm (internal diameter) cork borer and the resultant cylinder was trimmed to about 8 mm in length. The sample was placed in 10 mL of sterile 0.05 M phosphate buffer, pH 7.0, mashed thoroughly with a glass rod and vortexed. Then, portions of a 10-fold dilution series in sterile distilled water were spread onto YEPD-Agar and incubated at 25 °C. Colony counts were carried out after 3 d. Population densities of *S. cerevisiae* BSc68 were expressed as $\log_{10}$ cfu per wound. Control fruit were treated with sterile distilled water. Individual fruit wounds served as one replicate in a randomized complete block design, and three replicates were sampled at each sampling time and temperature (Zhang et al., 2007). The experiment was repeated 3 times to confirm reproducibility of the results.

2.5. Phenotypical assays associated to pathogenicity of biocontrol yeasts

2.5.1. Growth at 37 °C and 42 °C

Growth at 37–42 °C has been reported to be an important characteristic of pathogens (Mc Cusker et al., 1994; de Llanos et al., 2006). Ten μL of appropriate dilutions (10^6 cfu/mL) of the biocontrol yeast cultures were plated onto YEPD-Agar. Plates were incubated at 37 °C, 42 °C and 25 °C (control) for 3 d (de Llanos et al., 2006). Colony development was registered as positive.

2.5.2. Enzyme production

Phospholipase (lipolytic activity) was detected with Egg–Yolk medium. This medium consisted of 11.7 g NaCl, 0.1 g $CaCl_2$ and 10% (v/v) sterile egg yolk (Sigma Aldrich) in 184 mL of distilled water. Plates were inoculated with 10-μL drops of suspended yeast cells (10^6 cfu/mL) and incubated at 30 °C for 7–10 d. Activity was visualized as a precipitation area around each colony (Kantarcioglu and Yűcel, 2002; Samaranayake et al., 2005; Fotedar and Al-Hedaithy, 2005; de Llanos et al., 2006; Kumar et al., 2006; Lane and Garcia, 1991; Price et al., 1992).

Proteolytic activity was assayed according to Aoki et al. (1994). Sixty milliliter of bovine serum albumin (BSA) test medium

contained: 0.04 g $MgSO_4 \cdot 7H_2O$, 0.5 g K_2HPO_4, 1 g NaCl, 0.2 g Yeast Extract, 4 g Glucose and 0.5 g BSA; pH 5. The solution was filter-sterilized and mixed with 140 mL of melted agar (4 g Malt Extract, 4 g Agar). Twenty mL of this medium were poured into Petri dishes. Each plate was inoculated with 10 μL of suspended yeast cells (10^6 cfu/mL) and plates were incubated at 37 °C for 4 d (Kantarcioglu and Yűcel, 2002; Fotedar and Al-Hedaithy, 2005; de Llanos et al., 2006; Aoki et al., 1994). After incubation, plates were stained with 0.5% amido black (Sigma Aldrich) and a clearance zone around the colony was recorded as positive (Kumar et al., 2006).

2.5.3. Pseudohyphal formation

Synthetic low ammonia dextrose (SLAD, containing: 6.7 g/L of YNB without amino acids, 0.05 mM $(NH_4)_2SO_4$, 20 g/L Glucose, 20 g/L Agar) was used for assaying pseudohyphal formation. Biocontrol yeasts (10 μL, 10^6 cfu/mL) were streaked onto SLAD-Agar, and plates were incubated at 30 °C and observed after 4 d (de Llanos et al., 2006; Klingberg et al., 2008). Microscopic and macroscopic examinations were carried out to check pseudohyphal formation.

2.5.4. Invasive growth

Biocontrol yeasts were inoculated on YEPD-Agar, and plates were first incubated at 30 °C for 3 d and then at room temperature for an additional 2 d. Sterile distillated water was then used to rinse all the cells from the agar surface in order to observe the presence of cells growing below the agar surface (de Llanos et al., 2006; Klingberg et al., 2008). Microscopic examination of the remaining cells was used for invasiveness confirmation.

Human pathogenic yeasts (*Candida albicans* ATCC10231) were used as positive control in all pathogenicity assays.

All yeast samples were handled according to biosecurity standards of the World Health Organization (2004) and National Committee for Clinical Laboratory Standards (1997).

2.6. Statistical analysis

All experiments were carried out in triplicate. The percentages of wounds infected by *B. cinerea* were arcsine-square-root transformed before analysis of variance. Data (incidence %) were submitted to one-way univariate analysis of variance (ANOVA, SPSS release 17.0 for Windows; SPSS Inc., Chicago, IL). The threshold for statistical significance was set at $p < 0.05$. In the case of statistical significance, Duncan's multiple range tests were applied to separate the means (Lima et al., 1999). Linear regression analysis was applied to determine the relationship between yeast concentrations and rot incidence on berries. Values for R^2 (determination coefficient) were calculated based on treatment means (***SPSS*** *release* 17.0, SPSS Inc., Chicago IL).

3. Results

3.1. Isolation and identification of viticultural yeasts

Two hundred and twenty four yeasts belonging to 41 species were isolated from viticultural environments. Eighteen yeasts were isolated from Red Globe table grapes (Zonda, San Juan), 8 from vineyard soil (Caucete, San Juan) and 199 from fermenting musts of different grape varieties from San Juan, Argentina. All yeasts were classified into: 149 *Saccharomyces* and 75 non-*Saccharomyces*. Most of the isolates belonged to *S. cerevisiae and Torulaspora delbrueckii* species: 113 and 28, respectively. *S. cerevisiae* yeasts were isolated from fermenting must, soil samples and grape berries (Table 1), and *T. delbrueckii* yeasts were isolated from must and grape berries.

Table 1
Saccharomyces and non-*Saccharomyces* yeasts isolated from vineyard soil, fermenting must and grape berries.

Identified species	Non-*Saccharomyces* isolates		
	Must	Soil	Grapes
Candida apis	0	1	0
Candida cantarellii	1	0	0
Candida catenulata	0	2	0
Candida coliculosa	2	0	0
Candida intermedia	2	0	0
Candida famata	4	0	0
Candida galacta	1	0	0
Candida milleri	0	0	1
Candida parapsilosis	3	0	0
Candida rugosa	0	1	0
Candida sake	5	1	0
Candida steatolytica	1	0	0
Candida stellata	1	0	0
Candida versatilis	0	0	2
Crytococcus albidus	0	0	1
Debaryomyces vanrijiae	1	1	0
Debaryomyces hansenii	5	0	1
Dekkera anomala	1	1	0
Dekkera curtessiana	1	0	0
Dekkera bruxellensis	0	0	1
Hanseniaspora osmophila	1	0	0
Hanseniaspora vineae	1	0	0
Issatchenkia orientalis	2	0	0
Kluyveromyces marxianus	4	0	0
Kluyveromyces thermotolerants	2	0	0
Pichia anomala	0	0	1
Pichia guilliermondii	1	0	0
Pichia membranifaciens	3	0	0
Pichia stipitis	0	0	1
Saccharomycopsis fibuligera	0	0	1
Saccharomycopsis vini	1	0	0
Schizosaccharomyces pombe	1	0	0
Sporobolomyces roseus	1	0	0
Torulaspora delbrueckii	20	0	8
Zygosaccharomyces bailli	1	0	0
Identified species	*Saccharomyces* isolates		
	Must	Soil	Grapes
Saccharomyces bayanus	6	0	0
Saccharomyces cerevisiae	109	1	3
Saccharomyces chevalieri	8	0	0
Saccharomyces kluyveri	2	0	0
Saccharomyces steineri	4	0	0

3.2. In vitro assaying of antagonistic activity of yeasts against B. cinerea

Sixty-five of the 225 yeasts isolated showed antagonistic properties against *B. cinerea* under *in vitro* conditions (Fig. 1). Sixty-two of the 65 selected antagonistic yeasts were isolated from fermenting musts and the remaining 3 from table grapes (Red Globe) and identified as *Candida* (4), *Cryptococcus* (1), *Debaryomyces* (1), *Hanseniaspora* (1), *Pichia* (3), *Saccharomyces* (51), *Torulaspora* (3) and *Schizosaccharomyces* (1) (Table 2).

3.3. Preventative action of different concentrations of yeast isolates in control of gray mold on grape berries

The *in vitro* antagonistic effect of the 65 yeasts isolates was also assayed *in vivo* on grape berries at two storage temperatures: 2 °C and 25 °C. Room temperature (25 °C) normally favors pathogen growth and 2 °C is the most common temperature for commercial fruit storage.

The *in vivo* assays revealed that of the 65 yeasts with *in vitro* activity, 16 strains (15 *S. cerevisiae* and 1 *Sch. pombe*) significantly reduced progress of gray mold (70% or more) at a concentration of

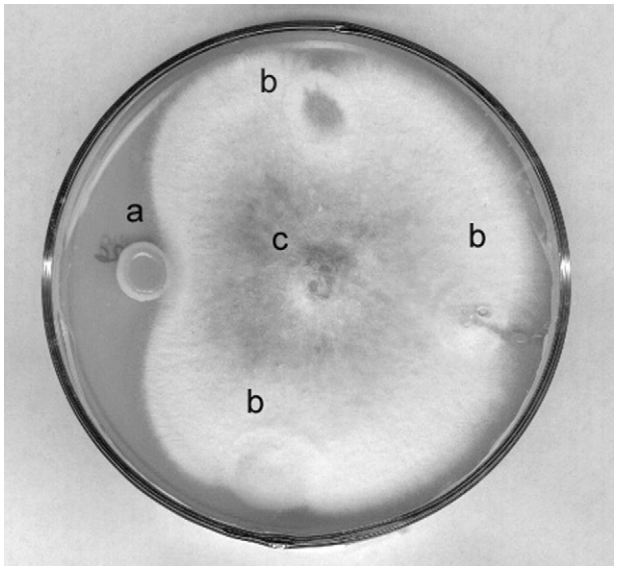

Fig. 1. Antagonistic interaction between *B. cinerea* and isolated yeasts. Co-cultures were incubated on Czapeck-Yeast Extract-Agar for 5 d at 25 °C. Note the inhibition zone between *B. cinerea* and yeasts isolated. (a) antagonistic yeast, (b) non-antagonistic yeasts, (c) *B. cinerea*.

10^7 cells/mL and 7 (*S. cerevisiae*) at a concentration of 10^6 cfu/mL both at 25 °C (Table 3). The 16 biocontrol yeasts with *in vivo* activity were isolated from grape must. No yeasts isolated from grape berries or vineyard soil (49) reduced the disease incidence produced by *B. cinerea*. Incidence of gray mold on control grapes at 25 °C was 100%.

The occurrence of gray mold rot caused by *B. cinerea* was completely inhibited by 9 of the 16 *S. cerevisiae* isolates at a concentration of 10^7 cfu/mL at 25 °C. The remaining 7 *Saccharomyces* yeasts protected grapes between 11 and 29% against disease incidence compared with control (100%). Two yeasts, *S. cerevisiae* BSc49 and BSc140 completely inhibited *B. cinerea* at a concentration of 10^6 cfu/mL. At this concentration, the other isolates reduced the disease incidence between 65 and 88%, approximately. No biocontrol activity against *B. cinerea* was observed by the 16 enological yeasts assayed at a concentration of 10^5 cfu/mL (Table 3).

Table 2
Origin of viticultural isolates of biocontrol yeasts that inhibited *B. cinerea* under *in vitro* conditions (Czapeck-Yeast Extract-Agar).

Biocontrol yeasts during *in vitro* assaying	Origin and number of isolates	
	Fermenting musts	Table grapes
C. famata	1	0
C. parapsilosis	1	0
C. sake	2	0
C. albidus	0	1
D. hansenii	0	1
H. vinae	1	0
P. anomala	0	1
P. membranifaciens	2	0
S. bayanus	2	0
S. cerevisiae	40	0
S. chevallieri	6	0
S. kluyveri	2	0
S. steineri	1	0
T. delbrueckii	3	0
Sch. pombe	1	0

An increase in yeast concentration of *S. cerevisiae* BSc16, *S. cerevisiae* BSc61 and *Sch. pombe* BSchp67 from 10^6 to 10^7 cfu/mL did not show any significant difference in the control of *B. cinerea* (Table 3).

From the 65 yeasts selected under *in vitro* conditions only 1 strain, *S. cerevisiae* BSc68, reduced the disease incidence to about 30% at a concentration of 10^6 cfu/mL at 2 °C and it completely inhibited *B. cinerea* at 10^7 cfu/mL at this temperature. At 25 °C this yeast also inhibited *B. cinerea* with 70% (incidence about 30%; Table 3). Incidence of gray mold on control grapes was 100% at 2 °C.

The determination coefficient (R^2) for the 16 yeasts was also calculated (Table 3). This coefficient, ranging from 0 to 1, represents the fraction of the response variation that is attributable to variations of the factors studied (yeast concentration and disease incidence) and their interactions. An R^2 value close to 1 means a high predictive power of the model, and the 7 antagonistic *S. cerevisiae* strains showed values between 0.92 and 0.99 (Table 3).

The 16 isolates that reduced the disease incidence to 30% or less were assayed for pathogenicity characteristics.

Table 3
Disease incidence by *B. cinerea* after simultaneous inoculation with different concentrations of yeast strains on Red Globe grapes. Disease incidence (% of infected wounds) was obtained by simultaneous inoculation of *B. cinerea* with yeasts after 5 d (25 °C) and 30 d (2 °C) of incubation. Means were obtained from three trials. Values followed by the same lower case letter in the same row are not significantly different at $p < 0.05$. Values followed by the same capital letter in the same column are not significantly different at $p \leq 0.05$ (ANOVA; Duncan's Multiple Range Test, SPSS).

	Disease incidence (%)				
	10^5 cells/mL	10^6 cells/mL	10^7 cells/mL	R^2 [c]	*p*
S. cerevisiae BSc5[a]	99.59 ± 0.7 a; A	33.48 ± 0.18 b; C	0 ± 0 c; G	0.97 C	<0.0001
S. cerevisiae BSc16[a]	95.33 ± 2.51 a; A	22.76 ± 0.31 b; E	22.46 ± 0.31 b; C	0.75 K	0.0026
S. cerevisiae BSc31[a]	96.07 ± 3.29 a; A	33.26 ± 0.37 b; C	11.82 ± 0.23 c; E	0.92 F	<0.0001
S. cerevisiae BSc47[a]	88.59 ± 0.6 a; B	36.29 ± 0.92 b; C	22.78 ± 0.22 c; C	0.9 G	<0.0001
S. cerevisiae BS49[a]	84.81 ± 3.69 a; B	0 ± 0 b; G	0 ± 0 b; G	0.75 K	0.0026
S. cerevisiae BSc56[a]	96.12 ± 3.53 a; A	33.89 ± 0.65 b; C	11.73 ± 0.2 c; D–E	0.93 F	<0.0001
S. cerevisiae BSc61[a]	99.17 ± 0.8 a; A	11.99 ± 0.54 b; F	11.15 ± 0.64 b; F	0.76 J–K	<0.0001
S. cerevisiae BSc64[a]	96.44 ± 3.09 a; A	34.03 ± 0.41 b; C	20.32 ± 1.08 c; D	0.88 H	0.0002
Sch. pombe BSchp67[a]	97.03 ± 2.64 a; A	34.01 ± 0.843 b; C	29.92 ± 0.45 c; C	0.76 J	0.0021
S. cerevisiae BSc68[a]	97.77 ± 3.74 a; A	30.02 ± 0.02 b; D	0 ± 0 c; G	0.96 D	<0.0001
S. cerevisiae BSc81[a]	88.65 ± 0.28 a; B	33.64 ± 0.46 b; C	0 ± 0 c; G	0.98 B	<0.0001
S. cerevisiae BSc92[a]	87.66 ± 1.12 a; B	23.55 ± 0.43 b; E	0 ± 0 c; G	0.93 E	<0.0001
S. cerevisiae BSc121[a]	63.41 ± 4.05 a; C	33.18 ± 0.43 b; C	0 ± 0 c; G	0.99 A	<0.0001
S. cerevisiae BSc140[a]	86.21 ± 3.46 a; B	0 ± 0 b; G	0 ± 0 b, G	0.75 K	0.0026
S. cerevisiae BSc175[a]	97.81 ± 2.29 a; A	33.13 ± 0.91 b; C	0 ± 0 c; G	0.97 C	0.0005
S. cerevisiae BSc203[a]	97.8 ± 1.99 a; A	11.76 ± 0.22 b; F	0 ± 0 c; G	0.84 I	<0.0001
S. cerevisiae BSc68[b]	98.67 ± 0.29 a; A	30 ± 0.03 b; D	0 ± 0 c; G	0.76 J	0.0021

References.
[a] Yeasts incubated at 25 °C.
[b] Yeast incubated at 2 °C.
[c] R^2: determination coefficient.

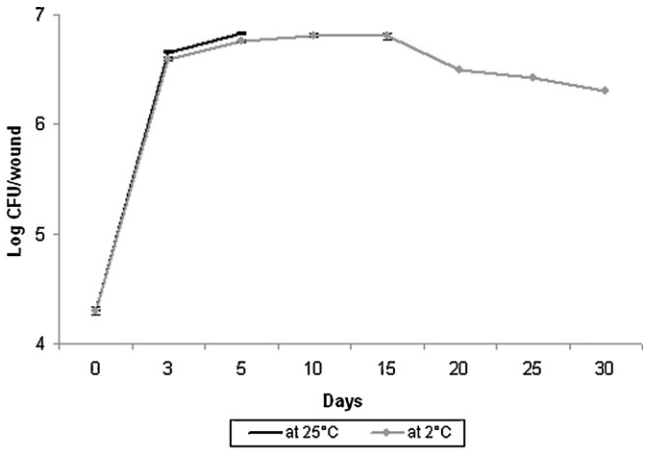

Fig. 2. Population profiles of *S. cerevisiae* BSc68 from wounds of grapes inoculated with the antagonist and incubated at 25 °C for 5 d (black line) and at 2 °C for 30 d (gray line). Fruit samples were removed after various incubation times to recover the antagonist from the wounds. Bars represent the standard deviation ($p \leq 0.05$).

3.4. Population profiles of *S. cerevisiae* BSc68 at 2 °C and 25 °C

Growth of *S. cerevisiae* BSc68 was evaluated after 0, 3 and 5 d of incubation at 25 °C and 0, 3, 5, 10, 15, 20, 25 and 30 d at 2 °C. Cell population profiles of *S. cerevisiae* BSc68 showed that this yeast was able to colonize grape wounds at both temperatures (Fig. 2). The population in wounded fruit stored at 25 °C increased approximately 221-fold (6.64 $\log_{10}$ cfu/wound) during the first 3 d (Fig. 2) and maximum population was obtained 5 d after inoculation (6.82 $\log_{10}$ cfu/wound). On fruit stored at 2 °C, the population increased 325-fold after 10 d of inoculation (6.81 $\log_{10}$ cfu/wound).

3.5. Phenotypical assaying for pathogenicity of biocontrol yeasts

3.5.1. Growth at 37 °C and 42 °C

The ability of biocontrol yeasts to grow at high temperatures, 37 °C and 42 °C, was assayed on YEPD-Agar plates. Eleven of the 16 biocontrol yeasts grew at 37 °C and one strain, *S. cerevisiae* (BSc56), at 42 °C (Table 4).

3.5.2. Enzyme production (phospholipases and proteinases)

Qualitative methods developed for *Candida* (Lane and Garcia, 1991) and *Cryptococcus neoformans* (Chen et al., 1997) were applied to determine phospholipase production in the 16 strains. Phospholipase activity was associated to six yeasts (*S. cerevisiae:* BSc16, BSc31, BSc47, BSc49, BSc56 and BSc61) and only 1 strain, *S. cerevisiae* BSc5, produced proteinases (Table 4).

3.5.3. Pseudohyphal formation

Some yeasts are able to switch from single cells to filamentous hyphal or pseudohyphal forms. This switching from normal colonial growth to hyphal formation has been associated with pathogenesis and virulence in *C. albicans* and in clinical isolates of *S. cerevisiae* (Gognies and Belarbi, 2002). Non-pseudohyphal formation was observed after 4 d of incubation except BSc49 (Table 4).

3.5.4. Invasive growth

Various yeasts invade YEPD-Agar plates, producing filaments that penetrate into the agar and make them resistant to vigorous washing of the agar surface (de Llanos et al., 2006). From the biocontrol yeasts assayed only *S. cerevisiae* BSc175 revealed invasive growth (Table 4).

Thirteen isolates out of 16 presented at least one characteristic associated with pathogenicity (Table 4). *S. cerevisiae* BSc49 was the most virulent yeast and presented three virulence factors: growth at 37 °C, phospholipase and protease production and pseudohyphal formation.

4. Discussion

Kloepper (1991) recommended the use of a rapid prescreening technique (widespread *in vitro* culture techniques) to test a large number of strains when looking into potential biocontrol agents.

In the present study, *in vitro* co-culture assays indicated that yeast strains belonging to 8 different genera (*Candida, Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Pichia, Saccharomyces*, *Torulaspora* and *Schizosaccharomyces*) and isolated from fermenting musts and table grapes, produced inhibitory substances against mycelial growth of *B. cinerea* (Fig. 1 and Table 2). However, production of antibiotics in culture medium does not necessarily imply their production on the fruit surface (Dal Bello et al., 2008). Therefore, the initial *in vitro* screening of isolates only provides preliminary

Table 4
Phenotypical assaying for yeast pathogenicity in humans.

Yeast	Growth at		Phospholipases	Proteases	Pseudohyphal formation	Invasive growth
	37 °C	42 °C				
S. cerevisiae BSc5	–	–	–	–	–	–
S. cerevisiae BSc16	+	–	+	–	–	–
S. cerevisiae BSc31	+	–	+	–	–	–
S. cerevisiae BSc47	–	–	+	–	–	–
S. cerevisiae BSc49	+	–	+	+	+	–
S. cerevisiae BSc56	+	+	+	–	–	–
S. cerevisiae BSc61	+	–	+	–	–	–
S. cerevisiae BSc64	+	–	–	–	–	–
Sch. pombe BSchp67	–	–	–	–	–	–
S. cerevisiae BSc68	+	–	–	–	–	–
S. cerevisiae BSc81	+	–	–	–	–	–
S. cerevisiae BSc92	+	–	–	–	–	–
S. cerevisiae BSc121	+	–	–	–	–	–
S. cerevisiae BSc140	+	–	–	–	–	–
S. cerevisiae BSc175	–	–	–	–	–	+
S. cerevisiae BSc203	–	–	–	–	–	–
C. albicans ATCC10231[a]	+	+	+	+	+	+

References: Growth at 37 °C and 42 °C: (+) growth; (–) no growth. Enzymatic activity (phospholipases, proteases): (+) activity; (–) no activity. Pseudohyphal formation: + present; (–) absent. Invasive growth: (+) present; (–) absent.

[a] Positive control yeast.

information on the possible modes of action used by each strain to control fungi (Capdeville et al., 2007). Volatile metabolites, extracellular enzymes and/or antibiotics are considered to be involved in antibiosis (Verma et al., 2007; El-Tarabily and Sivasithamparam, 2006). Several of the yeasts assayed in the present study showed *in vitro* control of the fungus but *in vivo* results were negative: 65 isolated yeasts produced detectable inhibition zones on PDA-agar plates (Table 2), but only 16 inhibited *B. cinerea* on grapes (Fig. 2).

Several reports have mentioned the potential use and applications of different genera and species of antagonist yeasts to control *B. cinerea* on grape tissues (Lima et al., 1999; Castoria et al., 2001; Mc Laughlin et al., 1992; Zahavi et al., 2000; Schena et al., 2000, 2004; Kurtzman and Droby, 2001; Keren-zur et al., 2002; Nigro et al., 1999; Masih et al., 2001; Sesan et al., 1999; Salmon, 2009). Other researchers have reported the biocontrol capacity of *S. cerevisiae* against fungi like *Penicillium roqueforti* in stored wheat (Petersson and Schnurer, 1995), *Macrophomina phaseolina* and *Fusarium solani* in tomato (Attyia and Youssry, 2001), *Monilia fruticola* in apples (Zhou et al., 2008) and *Alternaria alternata* in *Pinus silvestris* (Payne et al., 2000). So far, no reports have mentioned *Sch. pombe* as biocontrol agent against *B. cinerea* on grapes. The present study is the first that reports on several *S. cerevisiae* isolates (15) and 1 *Sch. pombe* strain with antifungal activity against *B. cinerea* on grapes.

Our results show minimum inhibitory concentrations (MICs), defined as the lowest concentration of yeasts that resulted in complete growth inhibition of *B. cinerea*, of 10^6 cfu/mL (*S. cerevisiae* BSc49, BSc140) and 10^7 cfu/mL (*S. cerevisiae* BSc5, BSc68, BSc81, BSc92, BSc121, BSc140, BSc175, BSc203) in wounded grapes and at 25 °C (Table 3). These concentrations are lower than those found for other biocontrol yeasts (Zhang et al., 2007; Tian et al., 2002; Chanchaichaovivat et al., 2007; Zheng et al., 2005). An increase in the concentrations of some yeasts significantly improved their biocontrol activity with higher R^2 values (Table 3). This suggests that the main mode of action of these yeasts is competition for space and nutrients giving a feasible explanation for the lack of inhibition at 10^5 cfu/mL (Table 3). This kind of competition between yeasts and fungi was previously reported for grapes (Mc Laughlin et al., 1992), apple (Filonow et al., 1996; Ippolito et al., 2000) and tomato (Kalogiannis et al., 2006).

It is known, that *Botrytis* can grow and cause decay in fruits and vegetables in refrigerated chambers and at room temperature (Tian et al., 2002). Therefore, it is important to find biocontrol agents that can effectively control the mold at both temperatures. In previous studies, strains of *P. anomala, C. sake* and *Metschnikowia pulcherrima* were reported as biocontrol agents against *B. cinerea* in apples at room temperature and in a refrigerated chamber (Chand-Goyal and Spotts, 1997; Spadaro et al., 2004). In the present work, one isolate, *S. cerevisiae* BSc68, inhibited *B. cinerea* on grapes stored at both 2 °C and 25 °C. Although several researchers do not consider *S. cerevisiae* a biocontrol agent that grows at low temperatures, *Saccharomyces* species like *S. bayanus*, *S. pastorianus* and *S. uvarum* are commonly considered cryophilic (or cryotolerant) microorganisms because they are associated with low-temperature fermentation processes in the production of wines, beers and ciders (Sampaio and Goncalves, 2008).

It is important to protect wounds that inevitably occur during harvest, transport and handling, because they not only directly spoil the harvested fruit (Zhang et al., 2007), but also provide pathways for pathogens, especially wound-invading necrotrophic fungi (Janisiewicz and Korsten, 2002). In this work, the number of viable BSc68 yeasts on wounded grapes after 30 d of incubation at 2 °C and 5 d at 25 °C was larger than that originally introduced (Fig. 2). This indicates that a single application of the antagonist was enough to prevent gray mold rot at both experimental temperatures (Table 3). Similar results were obtained by Vero et al. (2002) and Viñas et al. (1998) using non-*Saccharomyces* yeasts. The adaptation of *S. cerevisiae* BSc68 to different temperatures makes it a useful tool to control gray mold on grapes during storage (both at low temperatures and at room temperature) and transportation.

Many factors have to be taken into account regarding yeast pathogenicity in humans and growth at temperatures above 37 °C is one of them (Mc Cusker et al., 1994). When studying laboratory and industrial *S. cerevisiae* strains, growth was observed within the range of 37–42 °C, but only pathogenic isolates were able to grow at 42 °C (Murphy and Kavanagh, 1999; de Llanos et al., 2006). In addition, pathogenic *C. albicans* can be distinguished from the closely related, non-pathogenic *Candida stellatoidea* by its ability to grow at 42 °C (Mc Cusker et al., 1994). Proteinase secretion is another important pathogenic factor in many *Candida* and *S. cerevisiae* species (de Llanos et al., 2006). Phospholipases have only been detected in opportunistic *C. albicans*, *Cryptococcus neoformans*, *Candida glabrata, S. cerevisiae*, *Malassezia furfur* and *Rhodotorula rubra* yeasts (Kantarcioglu and Yűcel, 2002; Chen et al., 1997; de Llanos et al., 2006). Pseudohyphal formation and invasive growth are two more pathogenicity factors (de Llanos et al., 2006). *S. cerevisiae* strains isolated from a systemic infection have been reported to produce more pseudohyphal and invasive growth than the food and industrial strains (de Llanos et al., 2006). In this work biocontrol yeasts presented phenotypical characteristics of pathogenicity in humans similar to those assayed in previous reports, but none of the strains showed all the pathogenic characteristics at the same time. Four yeasts that did not present these phenotypical characteristics could be used as possible biocontrol agents in different enological environments, alone or in integrated fungal disease management.

5. Conclusions

Yeasts from fermenting musts were found effective as *in vivo* biocontrol agents against *B. cinerea*. The occurrence of gray mold rot was completely inhibited by *S. cerevisiae* isolates at inoculations of 10^6 and 10^7 cfu/mL at 25 °C, and one isolate of this species also inhibited the mold at 2 °C. Four biocontrol yeasts (3 *S. cerevisiae* and 1 *Sch. pombe*) did not present phenotypical characteristics associated to pathogenicity in humans. To the best of our knowledge this is the first report that studied human pathogenicity of *S. cerevisiae* and *Sch. pombe* biocontrol yeasts against *Botrytis*. Our laboratory is currently doing research to explain the antagonistic process and to determine the relationship between antagonism mechanisms of yeasts and their biocontrol potential. This work represents an initial step for further research to be conducted in other pathosystems in which *B. cinerea* is a serious pathogen.

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