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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