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Biocontrol ability and action mechanism of food-isolated yeast strains against *Botrytis cinerea* causing post-harvest bunch rot of table grape

Lucia Parafati, Alessandro Vitale, Cristina Restuccia*, Gabriella Cirvilleri

Dipartimento di Agricoltura, Alimentazione e Ambiente, University of Catania, via S. Sofia 100, 95123, Catania, Italy

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ABSTRACT

Strains belonging to the species *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, *Metschnikowia pulcherrima* and *Aureobasidium pullulans*, isolated from different food sources, were tested *in vitro* as biocontrol agents (BCAs) against the post-harvest pathogenic mold *Botrytis cinerea*. All yeast strains demonstrated antifungal activity at different levels depending on species and medium. Killer strains of *W. anomalus* and *S. cerevisiae* showed the highest biocontrol *in vitro* activity, as demonstrated by largest inhibition halos. The competition for iron and the ability to form biofilm and to colonize fruit wounds were hypothesized as the main action mechanisms for *M. pulcherrima*. The production of hydrolytic enzymes and the ability to colonize the wounds were the most important mechanisms for biocontrol activity in *A. pullulans* and *W. anomalus*, which also showed high ability to form biofilm. The production of volatile organic compounds (VOCs) with *in vitro* and *in vivo* inhibitory effect on pathogen growth was observed for the species *W. anomalus*, *S. cerevisiae* and *M. pulcherrima*. Our study clearly indicates that multiple modes of action may explain as *M. pulcherrima* provide excellent control of postharvest botrytis bunch rot of grape.

1. Introduction

Botrytis cinerea, the causal agent of gray mold or botrytis bunch rot on grapes, is responsible for significant economic damage in vineyards worldwide depending on the environmental conditions of temperature and humidity. In general, *Botrytis* spp. is also an important postharvest problem for fruits and vegetables in cold storage and subsequent shipment, because the fungus is able to grow effectively at temperatures just above freezing (Droby and Lichter, 2004). The approach to use pre- and post-harvest fungicidal treatments for controlling the pathogens causing bunch rot of grape is not considered anymore as sustainable, because of the emergence of fungicide-resistant strains of *B. cinerea* within vineyard populations (Latorre et al., 2002; Leroux, 2004; Vitale and Panebianco, unpublished data; Sergeeva et al., 2002), increasing public interest about hazards for human and environmental health (Janisiewicz and Korsten, 2002; Spadaro and Gullino, 2005; Vitale et al., 2012) and high sustained costs to synthesize new chemicals. In Italy, no commercial fungicides are authorized for the control of decay of table grapes after harvest; sulfur dioxide (SO_2) is permitted as an adjuvant and is effective in reducing gray mold development during storage. However, alternatives to SO_2 are required in view of hazards for human health and of the difficulties in using SO_2 with colored grapes (Nelson and Richardson, 1967) or with grapes stored into cardboard boxes because of SO_2 absorption by the cardboard (Lichter et al., 2008). Therefore, developing non-chemical control methods to reduce postharvest decay of fruits is becoming more important. Biological control with microbial antagonists has emerged as a promising alternative, with a low environmental impact, either alone or as part of integrated pest management to reduce synthetic fungicide application (Droby et al., 2009; Wilson and Wisniewski, 1994). Among the potential antagonists, yeasts have been extensively studied because they possess many features that make them suitable as biocontrol agents (BCAs) in fruits (Liu et al., 2013; Santos et al., 2004). 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 BCAs (Chanchaichaovivat et al., 2007). Moreover, they do not produce allergenic spores or mycotoxins as many mycelial fungi or antibiotics which might be produced by bacterial antagonists (El-Tarabily and Sivasithamparam,

* Corresponding author. Tel.: +39 095 7580219.
E-mail address: crestu@unict.it (C. Restuccia).

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2006; Nunes, 2012). 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. Actually, yeast-based biocontrol products are available in the market, and are registered on several commodities against rots caused by genera *Penicillium*, *Aspergillus*, *Botrytis*, *Rhizopus* (Liu et al., 2013). The biocontrol abilities of *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* strains have been recently proved to be correlated with killer phenotype (Lima et al., 2013; Platania et al., 2012), while the biocontrol abilities of *Metschnikowia pulcherrima* and *Aureobasidium pullulans* have been mainly attributed to competition for nutrients or to production of volatile organic compounds (VOCs). In particular, the competition of *M. pulcherrima* for iron was reported to play a significant role in biocontrol interactions (Saravanakumar et al., 2008), while the antagonistic activity of *A. pullulans* mainly includes nutrient competition (Bencheqroun et al., 2007), and production of glucanase, chitinase, protease and extracellular proteases (Castoria et al., 2001; Zhang et al., 2012).

The first step in developing BCAs is the isolation and screening process (Droby et al., 2009), and the best sources of antagonistic microorganisms are their natural environments in which they compete with plant pathogens (Janisiewicz and Korsten, 2002). The second step is to clarify the mechanism of action, as well as the understanding of biocontrol systems represents a crucial point to know the interactions among environment, pathogen and BCA and, therefore, the expected biocontrol efficacy.

The aim of this study was to ascertain whether food-isolated yeasts possessed biocontrol activity against the pathogenic fungus *B. cinerea*. Thus, the biocontrol activity of different yeast species and strains isolated from fruit and olive brine was evaluated *in vitro* conditions and on grapes. Furthermore, the effects of iron (Fe^{3+}) availability, the production of extracellular lytic enzymes, VOCs and biofilm formation, as well as the ability to colonize the fruit wounds were investigated for each species.

2. Materials and methods

2.1. Microorganisms and culture conditions

The yeasts used in this study, belonging to Di3A (Dipartimento di Agricoltura, Alimentazione e Ambiente, University of Catania, Italy) collection, were isolated from naturally fermented olive brine and minimally processed pomegranate.

The tested yeasts were identified as *M. pulcherrima* (seven strains), *A. pullulans* (three strains), *W. anomalus* (four strains) and *S. cerevisiae* (one strain) by sequencing the D1/D2 region of the 26S rRNA gene. Moreover, *W. anomalus* strains were selected for their high killing capacity against sensitive *S. cerevisiae* strain and their toxic mechanism was identified as a β-glucanase (Muccilli et al., 2013). *B. cinerea* isolate was recovered from diseased table grape berries in Sicily (Italy) and selected for virulence by artificial inoculation in wounded grapes (Vitale and Panebianco, unpublished data). The yeast and mold stock cultures were respectively stored at 4 °C on Petri dishes containing Yeast Extract Peptone Dextrose Agar [YPDA; yeast extract, 10 g; peptone, 10 g; dextrose, 20 g; agar, 20 g (Oxoid, Basingstoke, UK) per liter of distilled H_2O] and Potato Dextrose Agar (PDA, CM0139, Oxoid, Basingstoke, UK).

2.2. In vitro assays

2.2.1. Antagonistic activity on agar plates

The yeast and mold strains to be tested were respectively grown on PDA for 48–72 h and for 7–12 days at 25 °C. To evaluate the antagonistic activity against *B. cinerea,* a loopful of yeasts strains was streaked orthogonally from the center of Petri dish, containing PDA pH 6.0 and pH 4.5 media. Three plates for each yeast strain and pH were used. After incubation at 25 °C for 48 h, mycelial discs (5-mm square plug) of *B. cinerea* were placed on agar plates 3 cm away from yeast inoculum. A control dish only inoculated with gray mold was also prepared. At the end of the incubation period (12 days at 25 °C) radial growth reduction was calculated in relation to growth of the control as follows: $\%I = (C - T/C) \times 100$, where %I represented the inhibition of radial mycelial growth, C was radial growth measurement in control and T was the radial growth of the pathogen in the presence of yeast strains. The experiments were repeated three times.

2.2.2. Effects of volatile organic compounds (VOCs)

A dual culture method was used to evaluate the efficacy of volatile compounds from yeasts against *B. cinerea*. Aliquots of 20 μL of yeast suspensions (10^8 cells/mL) were seeded on plates containing PDA pH 6.0 and pH 4.5 and incubated 48 h at 25 °C. Aliquots (20 μl) of the conidial suspension of *B. cinerea* (10^6 conidia/mL) were inoculated on PDA and dried at room temperature. The plates with *B. cinerea* conidia were individually covered face to face under dishes containing 48-h-old yeast strains. The control was prepared with un-seeded PDA plates. The two plates were wrapped together with Parafilm, two revs around the edges to prevent air leakage, and incubated a 25 °C. Radial growth reduction of *B. cinerea* was calculated after 9 days of incubation as previously described. All experiments were performed three times.

2.2.3. Extracellular lytic enzymes activity

In order to characterize the ability of all the selected yeasts to produce and secrete cell wall lytic enzymes (chitinase, glucanase, pectinase, protease), aliquots (10 μL) of 24 h yeast culture suspensions (10^7 cells/mL) were superficially spotted on solid media (agar 15 g/L) containing the corresponding substrates according to previously reported techniques: a) *chitinase* on colloidal chitin (Sigma–Aldrich, St. Louis, MO, USA) pH 7 amended with mineral salts following method 1 reported by Souza et al. (2009). Extracellular chitinase activity was detected daily after 1–7 days at 25 °C by the presence of clear zone around the inoculum zone; b) *β-1,3-glucanase* on solid medium containing 5 g/L laminarin, and 6.7 g/L Yeast Nitrogen Base (BD, Franklin Lakes, NJ, USA) (Lutz et al., 2013). After 72 h incubation at 25 °C the plates were stained with Congo Red (0.6 g/L) and left a room temperature for 90 min. The stain not absorbed was decanted and the plates were observed for hydrolysis of the glucan by a yellow-orange zone around the colonies; c) *pectinase* on solid medium containing 6.7 g/L YNB and 10 g/L citrus pectin (Sigma–Aldrich). After cell growth at 25 °C, the plates were flooded with hexadecyltrimethylammonium bromide (10 g/L). A clear halo around a colony in an otherwise opaque medium indicated degradation of the pectin (Buzzini and Martini, 2002); d) *protease* activity on 10% skim milk powder (Oxoid) and 2% agar (Gardini et al., 2006). Medium was autoclaved at 110 °C for 5 min and then poured into Petri dishes. Inoculated plates were incubated at 25 °C and examined daily for 1 week. Enzymatic activity was detected when a light halo surrounded the inoculum zone. Test of gelatine liquefaction was done on medium prepared dissolving 100 g/L of gelatin, 5 g/L of glucose, and pouring 9 mL of the medium into sterilized tubes (Gardini et al., 2006). Inoculated tubes were regularly examined during 3 weeks checking for sign of gelatin liquefaction. All experiments were carried out twice.

2.2.4. Effect of iron concentration on antagonistic activity of yeasts

The yeasts were streaked onto PDA plates pH 6.0 and pH 4.5 with different concentration of iron to test the pigment production and antagonistic activity. The media were supplemented with 5 and