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Performance evaluation of volatile organic compounds by antagonistic yeasts immobilized on hydrogel spheres against gray, green and blue postharvest decays

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ABSTRACT

Wickerhamomyces anomalus, *Metschnikowia pulcherrima*, *Aureobasidium pullulans* and *Saccharomyces cerevisiae* yeasts were tested for their ability to survive and synthesize antifungal volatile organic compounds (VOCs) both *in vitro* and *in vivo* conditions when immobilized on commercial hydrogel spheres. The results showed a good survival of all yeasts on hydrogel spheres up to 10 days of incubation. Moreover, VOCs produced *in vitro* by tested yeasts inhibited *Botrytis cinerea, Penicillium digitatum* and *P. italicum* radial growth and conidial germination, with the highest antagonistic activity reported for *W. anomalus* and *A. pullulans* strains.

Experimental *in vivo* trials performed on strawberry and mandarin fruits proved the ability of VOCs to reduce significantly postharvest decays on artificially wounded tissues. Comprehensively, the best efficacy was detected for *W. anomalus*, which totally inhibited gray mold decay on strawberry fruits and significantly reduced green mold infections on mandarin fruits. On the other hand, blue mold decay on mandarin fruits was more effectively managed by *A. pullulans* VOCs.

Accordingly, hydrogel spheres used as a support for VOC-generating yeasts could open a new way for the employment of this polymeric material as a bio-emitter in postharvest packaging.

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1. Introduction

Application of biocontrol agents (BCAs) for postharvest disease management has been recently oriented to the use of volatile organic compounds (VOCs) produced by the BCAs as a promising and sustainable approach in controlling postharvest decays of fruits, vegetables, grains and seeds to replace chemicals and implement integrated control programs.

VOCs are low molecular weight metabolites (usually < 300 Da), with low polarity and high vapor pressure (Vespermann et al., 2007), which were used for pathogen suppression (Fialho et al., 2010). Their activity usually depends on the targeted pathogen (Mari et al., 2012). As well known, VOCs have negligible residual effects and do not require spray or drench application (Mercier and Jiménez, 2004).

So far different microorganisms have been investigated for their production of antimicrobial VOCs and potential suppression of phytopathogenic fungi. VOCs produced by bacteria such as *Bacillus subtilis* (Chen et al., 2008), *Streptomyces* spp. (Wan et al., 2008), filamentous fungi as *Muscodor albus* (Mercier and Manker, 2005), higher plants as 'Isabella' grapes (*Vitis labrusca*) (Kulakiotu et al., 2004) and yeasts as *Candida intermedia* and *Sporidiobolus pararoseus* (Huang et al., 2011, 2012) were reported to be effective in suppressing both conidial germination and mycelial growth of *Botrytis cinerea* on agar media and on plant tissues.

VOCs produced by *S. pararoseus* are mainly consisted of 2-ethyl-1-hexanol (Huang et al., 2012), while VOCs produced by *C. intermedia* have been identified as 1,3,5,7-cyclooctatetraene, 3-methyl-1-butanol, 2-nonanone, and phenylethyl alcohol (Huang et al., 2011).

Also *Meyerozyma guilliermondii* (formerly *Pichia guilliermondii*) revealed VOC production with antifungal activity, mainly based on the ethyl-acetate release (Coda et al., 2013) and helvolic acid, which exerted a marked inhibitory activity on the spore germination of *Magnaporthe oryzae* (Zhao et al., 2010). Otherwise, antifungal activity of *Wickerhamomyces anomalus* (formerly *Pichia anomala*) has been correlated with the capacity to synthesize ethyl-acetate, which mainly prevents the intracellular accumulation of toxic

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acetic acid, contributing to the biocontrol activity during airtight storage of grain (Fredlund et al., 2004; Druvefors et al., 2005). More recently Hua et al. (2014) stated that the biocontrol ability of *W. anomalus* can be attributed to the production of 2-phenyl ethanol, which affects spore germination, mycelial growth, toxin production, and gene expression in *Aspergillus flavus.* Moreover, Di Francesco et al. (2015) demonstrated the essential role of VOCs produced by two strains of *Aureobasidium pullulans* in inhibiting some causal agents of postharvest diseases (*B. cinerea, Colletotrichum acutatum, Penicillium expansum, P. digitatum* and *P. italicum*) and tested the effect of pure VOCs against the same pathogens. Parafati et al. (2015) also reported that biocontrol ability of *W. anomalus, Metschnikowia pulcherrima, A. pullulans* and *Saccharomyces cerevisiae* strains is related to the production of VOCs, effective in reducing mycelial growth of *B. cinerea* and its infections on table grape berries.

Favorable yeast growth conditions induce a modification in variety and amount of volatile compounds emitted (Nout and Barlett, 1998), and recent studies showed how yeast grown under acidic condition, pH 4.5, produced VOCs that significantly reduced the mycelial growth of *B. cinerea* if compared to pH 6.0 (Parafati et al., 2015).

Thus, the present paper aimed at evaluating the efficacy of antifungal VOCs from antagonistic yeasts using a potential commercial carrier having the multiple function to immobilize yeast cells, prolong their viability, and release antifungal VOCs.

2. Materials and methods

2.1. Microorganisms and culture conditions

Yeast strains used in this study belong to Di3A (Dipartimento di Agricoltura, Alimentazione e Ambiente, University of Catania, Italy) collection and were previously isolated from naturally fermented olive brine and minimally processed pomegranate. The strains used were previously identified as *Wickerhamomyces anomalus* BS91, *Metschnikowia pulcherrima* MPR3, *Aureobasidium pullulans* PI1 and *Saccharomyces cerevisiae* BCA61 by sequencing the D1/D2 region of the 26S rRNA gene. *W. anomalus* BS91 was selected for its good antagonistic ability related to β-glucanase production (Muccilli et al., 2013). In addition, *W. anomalus* BS91 showed excellent performances in reducing postharvest decay caused by *P. digitatum* on Tarocco oranges (Platania et al., 2012). More recently *W. anomalus* BS91, *M. pulcherrima* MPR3 and *A. pullulans* PI1 exhibited a good control of *P. digitatum* and *P. italicum* on mandarin fruit (Parafati et al., 2016) and *B. cinerea* on table grape (Parafati et al., 2015).

B. cinerea was recovered from diseased table grape in Sicily (Italy) and selected for virulence by artificial inoculation in wounded berries (Panebianco et al., 2015a; Vitale et al., 2016). *P. digitatum* and *P. italicum* mold strains were isolated from infected mandarin fruit in Sicily (Italy) and tested for pathogenicity as previously described (Oliveri et al., 2007). The yeast and mold stock cultures were respectively maintained 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 water] and Potato Dextrose Agar (PDA, CM0139, Oxoid, Basingstoke, UK).

2.2. Preliminary effects of volatile organic compounds (VOCs) on conidial germination

The effect of VOCs produced by BS91, MPR3, PI1, and BCA61 strains on conidial germination of *B. cinerea, P. digitatum* and *P. italicum* was verified according to the method reported by Panebianco et al. (2015b) with slight modifications. In all trials, aliquots of 50 μL for each conidial suspension (1×10^6 spores/mL) were placed onto U-shaped glass microscope slides (three drops per slide) and kept in Petri dishes with moistened filter paper. The plates supporting glass microscope slides were individually covered face-to-face under dishes containing 48-h-old yeast strain grown at 25 °C on YPDA pH 4.5. The plates were wrapped together girdling two times with Parafilm™ around the edges to prevent air leakage, and incubated at 22 °C for 12–14 h in darkness. The control consisted of un-inoculated YPDA plates. The number of germinated conidia was counted under an optical light microscope (Olympus, Hamburg, Germany) with a 40 × magnification.

To this aim three observations (replicates), each containing 200 spores of the fungi, were performed to determine germinated conidia (germ tube length equal to or greater than the transverse diameter of the spore). The mean percent germination and standard error of the means were calculated. The experiments were performed twice.

2.3. Immobilization and viability determination of yeast strains on commercial hydrogel spheres

Due to their properties as appropriate water holding capacity, softness, flexibility and biocompatibility (Caló and Khutoryanskiy, 2015), hydrogel spheres were used as support for the immobilization of BS91, MPR3, PI1 or BCA61 yeast strain. One liter of Yeast Extract Peptone Dextrose Broth [YPDB: yeast extract, 10 g; peptone, 10 g; dextrose, 20 g (Oxoid, Basingstoke, UK) per liter of distilled water] pH 4.5 was inoculated with 10 g of hydrogel spheres and 1 mL of each yeast suspension (1×10^9 cells/mL).

Three days after incubation at 25 °C and 200 rpm, the spheres that have absorbed the culture broth containing the yeasts and increased their diameter approximately up to 1 cm were rinsed twice with sterile distilled water (SDW) and incubated at 25 °C in an empty Petri plate. The colonization and survival ability of each yeast strain on hydrogel spheres were assessed after 0, 1, 3, 7 and 10 days of incubation.

Hydrogel spheres were weighted and placed in tubes containing proportional amount of sterile Ringer solution (Oxoid, Basingstoke, UK), homogenized, sonicated for 5 min and subsequently plated onto Sabouraud Dextrose Agar (SDA, Oxoid) supplemented with chloramphenicol (100 mg/L) using a Spiral Plater Eddy Jet (IUL Instruments, Barcelona, Spain). Yeast viability (CFU/g) was evaluated after 48 h at 25 °C to calculate the means of colonies ($\log_{10}$ CFU) per gram of hydrogel. Three replicates each formed by 10 sample (spheres) were used for each treatment. The experiment was performed twice.

2.4. In vitro VOC efficacy of yeasts inoculated on hydrogel spheres against Botrytis cinerea, Penicillium digitatum and Penicillium italicum

The efficacy of VOCs produced by the four yeast strains, inoculated in hydrogel spheres, was evaluated by means of dual culture method against *B. cinerea*, *P. digitatum* and *P. italicum*. Aliquots (20 μL) of each spore suspensions (10^6 conidia/mL) of above mentioned pathogens were inoculated on PDA and allowed to dry at room temperature for 2 h. Hydrogel spheres, yeast-inoculated as described above, were placed into Petri plates (approximately 50 spheres for plate). Three replicates (plates) were used for each yeast strain. Plates inoculated with each fungal spore suspension were individually placed face to face over Petri plates containing yeast-inoculated hydrogel spheres. The control was prepared with hydrogel spheres that absorbed sterile YPDB. The two plates were sealed together with Parafilm™, two times around the edges to prevent air leakage. Ten days after incubation, at 20 °C for *B. cinerea*

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