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Postharvest biocontrol ability of killer yeasts against *Monilinia fructigena* and *Monilinia fructicola* on stone fruit

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ABSTRACT

The antagonistic effects of *Debaryomyces hansenii* KI2a, *D. hansenii* MI1a and *Wickerhamomyces anomalus* BS91 were tested against *Monilinia fructigena* and *Monilinia fructicola* in *in vitro* and *in vivo* trials.

All yeast strains demonstrated antifungal activity at different levels depending on species, strain and pathogen. *D hansenii* KI2a and *W. anomalus* BS91 showed the highest biocontrol activity *in vitro*; the production of hydrolytic enzymes, killer toxins and volatile organic compounds (VOCs) were hypothesized as their main mechanisms of action against pathogens. *D hansenii* KI2a and *W. anomalus* BS91 significantly reduced brown rot incidence and severity on peach and plum fruits artificially inoculated with *M. fructigena* and *M. fructicola*, especially when applied 24 h before pathogen inoculation. On the opposite, *D. hansenii* MI1a exhibited weak antagonistic activity towards *M. fructigena* on peach and plum fruits and was ineffective against *M. fructicola*. The noticeable ability of *W. anomalus* BS91 to control brown rot could be also correlated with its high capacity to colonize the wound tissue and to increase its population density.

Accordingly, the antagonistic strains of *D. hansenii* and *W. anomalus* could be proposed as active ingredients for the development of biofungicides against *Monilinia* species that are responsible for considerable economic losses in stone fruit crops.

1. Introduction

Brown rot caused by *Monilinia laxa* (Aderhold and Rulhland) Honey, *Monilinia fructigena* (Aderhold and Ruhland) and *Monilinia fructicola* (Winter) Honey is one of the main causes of losses in all stone fruit growing areas (Byrde and Willetts, 1977; De Cal and Gell, 2009; De Cal and Melgarejo, 1999). These pathogens infect blooms, twigs and fruit in the field with a variety of symptoms, including blighting of blossoms, cankers on woody tissues and fruit rotting, but prevalent damage occurs in the postharvest phase during storage and transport (Martini and Mari, 2014).

Among the three *Monilinia* species, *M. fructicola* is considered the most destructive pathogen on both pome and stone fruits. In Europe this species is included in the European and Mediterranean Plant Protection Organization (EPPO) A2 list of quarantine pests, being still limited to some areas after its recent introduction (EFSA, 2011). Consequently, the possibility of further spread through infected fruit and plant material makes it necessary to promptly define effective control measures.

At present, chemical treatments such as triazoles, dicarboximides, hydroanilide fenhexamid, and succinate dehydrogenase inhibitors (Miessner and Stammler, 2010) are not sufficient to control brown rot in the orchard, and no chemical fungicides are allowed for postharvest treatment of stone fruit.

Fungicide resistance, lack of new effective fungicides, latent infections, and the ability to grow at low temperatures are among the factors that challenge the management of brown rot. Moreover, the public demands to reduce pesticide use on fruit and to improve environmental protection and human health have increased the need to develop alternative control methods (Lopez-Reyes et al., 2013; Sisquella et al., 2014).

In the last decade, several scientific studies demonstrated the efficacy of biocontrol agents (BCAs) against many postharvest phytopathogenic fungi including species of *Penicillium*, *Botrytis* and *Monilinia* on several commodities (Bautista-Rosales et al., 2013; Manso and Nunes, 2011; Mari et al., 2012; Oro et al., 2014;

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Panebianco et al., 2015; Parafati et al., 2015; Platania et al., 2012; Restuccia et al., 2006; Scuderi et al., 2009; Spadaro et al., 2002; Zhang et al., 2010a,b).

The use of yeasts as BCAs is particularly encouraged since they possess important characteristics, such as a faster growth compared to molds, simple nutritional requirements and the ability to colonize dry surfaces of several niches and to compete for nutrients and space (Liu et al., 2013; Parafati et al., 2015; Spadaro and Droby, 2016). The biological control process of yeasts involves several biochemical mechanisms including the yeast ability to adhere itself on specific sites of both host and pathogen cells, to secrete specific enzymes and antimicrobial substances, to induce host resistance and the capacity to form biofilms on the inner surface of wounds (Lu et al., 2013; Lutz et al., 2013).

Among a wide variety of antagonistic yeasts, particular attention has been directed toward the use of yeasts that exhibit a killer phenotype (K+) for controlling postharvest decay in fruits, due to their ability to secrete extracellular proteic toxins designated as killer proteins or killer toxins. These proteins have the potential to kill other species of yeasts, molds and pathogenic bacteria through different mechanisms, including the hydrolysis of the major cell wall component β-1, 3-glucans (Izgü and Altinbay, 2004; Muccilli et al., 2013), the ion leakage by ion channel formation on the cytoplasmic membrane of the target cell, and the inhibition of DNA synthesis (Schmitt and Breinig, 2002).

Killer phenotypes were first found in brewing strains of *Saccharomyces cerevisiae* and since then have been shown to occur in a large number of yeast isolates of environmental, clinical, industrial and agronomic interest (Santos et al., 2000; Sawant et al., 1989). Killer toxin production has been then demonstrated among many yeast genera, including *Saccharomyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Kluyveromyces*, *Pichia*, and *Wickerhamomyces* (Magliani et al., 1997; Muccilli et al., 2013; Schmitt and Breinig, 2002).

Debaryomyces hansenii (teleomorph of *Candida famata*), a halotolerant species, commonly isolated from sea water, concentrated brines, dairy and salty food products, with several possible biotechnological applications (Brauer and Harms, 2006), is reported to be an efficient antagonist yeast against pathogenic fungi in various food products, such as dairy products (Liu and Tsao, 2009), dry-cured meat products (Andrade et al., 2014; Simoncini et al., 2014) and dry-fermented sausage (Núñez et al., 2015). Moreover, some strains exhibit several mechanisms of action in biocontrol of phytopathogenic fungi that involves killer activity, induction of host resistance and the competition for nutrients and space (Chalutz and Wilson, 1990; Droby et al., 1989; Hernández-Montiel et al., 2010; Santos et al., 2004). Interestingly, *D. hansenii* has been included in the list of qualified presumption of safety (QPS) for the European Food Safety Authority (BIOHAZ, 2012), supporting the industrial and commercial purposes.

Among the killer species, *Wickerhamomyces anomalus* (previously named *Pichia anomala*) has been reported to produce high levels of killer toxins with a wide spectrum of killing activity and a relatively high stability compared with toxins of other killer yeasts (Wang et al., 2008). In the last decade, several studies have demonstrated the efficacy of *W. anomalus* as a biological control agent against different postharvest phytopathogenic fungi, such as *Colletotrichum gloeosporioides* (Lima et al., 2013), *Penicillium digitatum*, *P. italicum* and *Botrytis cinerea* (Parafati et al., 2015, 2016; Platania et al., 2012). Furthermore, also *W. anomalus* has been granted Qualified Presumption of Safety (QPS) status by European Food Safety Authority (EFSA), which may authorize its use as a novel microorganism in food preservation (Sundh and Melin, 2011).

Despite these encouraging results, no studies have been reported on efficacy of *D. hansenii* and *W. anomalus* killer strains as biological control agents of *Monilinia* spp. pathogens causing brown rot on stone fruits.

The present study was aimed at assessing the *in vitro* and *in vivo* efficacy of three killer yeast strains, *Debaryomyces hansenii* strains KI2a and MI1a, and *Wickerhamomyces anomalus* strain BS91, on the control of *M. fructigena* and *M. fructicola*, two of the most important and devastating postharvest pathogens of stone fruits. *D. hansenii* and *W. anomalus* strains were evaluated for *in vitro* performance in reducing pathogen radial growth, for VOCs production and efficacy, and for extracellular lytic enzymes and killer toxin activity. In addition, the influence of inoculation time on the biocontrol potential was examined *in vivo* along with the evaluation of the progression of yeast population on stone fruit wounds.

2. Materials and methods

2.1. Microorganisms and culture conditions

Debaryomyces hansenii strains MI1a and KI2a used in this study were isolated from blue-veined Rokpol cheese, identified (Wojtatowicz et al., 2001) and deposited at the Department of Biotechnology and Food Microbiology Culture Collection (University of Environmental and Life Sciences, Wrocław, Poland). *Wickerhamomyces anomalus* BS91 yeast strain, belonging to the Department of Agricultural, Food and Environment (Di3A, University of Catania, Italy) Culture Collection, was isolated from naturally fermented olive brine (Muccilli et al., 2011). *D. hansenii* strains were selected for their high killing capacity against *S. cerevisiae* sensitive strain and their previously described antagonistic activity towards *Yarrowia lipolytica* (Żarowska et al., 2004; Żarowska, 2012). *W. anomalus* BS91 was selected for its high killing capacity (Muccilli et al., 2013) and for its high antagonistic activity against *Penicillium digitatum* on oranges (Platania et al., 2012), *Botrytis cinerea* on grapes (Parafati et al., 2015), and *P. digitatum* and *P. italicum* on mandarins (Parafati et al., 2016).

Monilinia fructigena strain 2138 was obtained from Bank of Plant Pathogens (Environmental Protection Institute in Poznań, Poland), and *M. fructicola* strain was recovered from decayed peach fruits in Sicily (Italy), identified and kept in the collection of the Department of Agricultural, Food and Environment (University of Catania). *M. fructigena* and *M. fructicola* pathogenic strains were routinely reisolated from artificially inoculated peach and plum fruits, and monosporic cultures of the pathogens were used in *in vitro* and *in vivo* experiments.

The yeast and mold stock cultures were 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, pH 4.5] and Potato Dextrose Agar (PDA, Oxoid, Basingstoke, UK), respectively.

2.2. In vitro antagonistic activity

The yeast and mold strains to be tested were grown at 25 °C on YPDA and PDA respectively for 48–72 h and for 7–12 days. To evaluate the antagonistic activity of *D. hansenii* MI1a, *D. hansenii* KI2a, and *W. anomalus* BS91against phytopathogenic molds, a loopful of each yeast strain was streaked orthogonally near the edge of Petri dish containing PDA pH 6.0 and pH 4.5. After incubation at 25 °C for 48 h, mycelial discs (5-mm square plugs) of actively growing fungal mycelium of *M. fructigena* and *M. fructicola* were individually placed 3 cm away from yeast inoculum. Three plates for each yeast strain and pH were used. A control dish only inoculated with each of tested molds was also prepared. Dual cultures were incubated for 10 days (pH 6.0) and 14 days (pH 4.5) at 25 °C. At the end of the incubation period, radial growth reduction was