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Biological control of postharvest phytopathogenic molds promoted by food-isolated yeasts

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CHAPTER 1. STATE OF THE ART

1.1. MAJOR POSTHARVEST DISEASES

Postharvest decay of fruit and vegetable has been identified as a major factor causing large economic losses. Reports of Food and Agriculture Organization (FAO, 2011) indicated that average postharvest losses in Europe, North America and Oceania is nearly 29%, while in Asia, South East Asia, Africa and Latin America is about 38% (Spadaro and Drobry 2015). Due to the perishability of these products, development of postharvest fungal diseases may occur during the different stages of the postharvest chain, which include harvesting, field handling, packing operations, transportation and storage.

Fungal pathogens cause visible symptoms on the infected products; in addition some of them produces a number of toxic secondary metabolites (micotoxin), which can induce neurological disorders and are potential carcinogens (Andersen et al., 2004).

The most important pathogenic fungi responsible of the mainly significant post harvest disease belong to *Penicillium* spp., *Botrytis* spp., *Aspergillus* spp and *Monilinia* spp.

Among the genus *Penicillium* the most notable species are *P. digitatum* and *P. italicum* causing green and blue mold decay, respectively, on citrus fruit and *Penicillium expansum* causing significant spoilage in pears and apples and becoming a health hazard due to the production of patulin (Neri et al., 2010).

Botrytis cinerea is casual agent of gray rot in grapes, strawberries, tomatoes, raspberries, blueberries, onions, potatoes. *Botrytis* spp. is an important postharvest problem for fruits and

vegetables also in cold storage due to the ability of this fungus to grow effectively at low temperatures (Droby and Lichter, 2004).

Aspergillus spp. grows on carbon-rich substrates and is able to contaminate vegetables, cereals, dried fruits and nuts. *A. parasiticus* and *A. flavus* produce aflatoxin with high carcinogenic activity. Aflatoxins contamination is a very common problem in developing countries causing severe economic loss. In addition, *A. fumigatus* and *A. clavatus* are the most common species causing allergic disease (Williams et al., 2004).

Monilinia spp. is responsible of brown rot in stone fruits such as peach, apricot, plum, cherry and almond, and is able to attack pomes fruit such as apple and pear. The most important species are *M. laxa, M. fructicola* and *M. fructigena*, able to infect the fruit just before harvesting.

Furthermore, other important postharvest pathogens responsible for significant losses belong to *Geotricum* spp., *Fusarium* spp., *Rhizopus* spp., *Alternaria* spp., *Colletotricum* spp., and *Gloeosporium* spp. (Barkari-Golan 2001).

1.1.1. Gray mold

Among postharvest pathogens the causal agent of "gray mold", *B. cinerea*, is one of the most important, causing decay of a wide range of fruits and vegetables, such as table grape, strawberries, stone fruit, pome fruit, onion, tomato, potato, eggplant, etc., infecting more than 200 crop species worldwide (Elad et al., 2007).

B. cinerea infects fruits in mature or senescence period, but also in early stage; in the latter case, it can remain quiescent for long period until favorable conditions of environment and physiology (Williamson et al., 2007). The difficulty to control this pathogen is due to the

massive production of mycelium or conidia for long period, the genetic adaptability and the wide range of hosts as inoculum source (Mari et al., 2014, Williamson et al., 2007).

The optimum temperature condition is from 20 °C to 25 °C but mycelial growth occur also at - 4 °C while conidial germination need a minimum of 0 °C (De Cicco et al., 2009).

Control of *B. cinerea* is conducted during preharvest period with practices that have the purpose to reduce the inoculum and with different active molecules, such as thiophanate methyl, iprodione, cyprodinil, pyraclostrobin + boscalid, pyrimethanil, or fenhexamid; unfortunately, several reports indicated a common reduced sensitivity to all of the tested fungicides. Fungicide treatments to control gray mod decay are allowed in postharvest only on pome fruit and kiwi fruit (Mari et al., 2014).

1.1.2. Green and blue molds

Green and blue mold decays caused by *P. digitatum* and *P. italicum*, respectively, are problematic diseases during postharvest of citrus fruits that are very susceptible to infection due to their higher water content and nutrient composition (Pelser and Eckert, 1997).

The inoculum of the fungus is always present on the surface of fruit and after harvest can reach high levels unless appropriate packinghouse sanitization measures are adopted. At the beginning, the area of the infection appears as soft watery spot; as the lesion progresses a white mycelium, that produces cell wall-degrading enzymes causing break-down of fruit cell wall, develops within a few days and produces green or blue spores, if infected by *P. digitatum* or *P. italicum*, respectively.

The infection can spread from infected fruits to healthy fruits through the injured skin; in fact, pathogen contamination can occur only trough wound and may take place during preharvest

period, but are essentially postharvest diseases, which cause up to 90% of decay during transport, storage and commercialization (Agrios 2004).

The use of postharvest fungicides is subject to registration and permission in various countries; generally, the fungicides allowed are imazalil, thiabendazole, sodium orthophenyl phenate, fludioxonil, pyrimethanil or mixtures of these compounds.

The management of green and blue mold disease during postharvest is becoming difficult due to the development of multiple resistances to fungicides that has been detected. In addition, due to the increasing public concern for human health and environmental pollution, new potential alternatives to synthetic fungicides that limit the chemical residues in fruit are currently under investigation (Talibi et al., 2014).

1.2. FACTORS AFFECTING THE DEVELOPMENT OF MOLDS DURING POSTHARVEST

The storage phase is an unfavorable state for fruit and vegetables due to the high volumes of product in intimate contact in a limited space. These unnatural conditions predispose them to different kinds of diseases, often caused by fungal infection. In addition, the natural ripening process increases the susceptibility of the stored products to decay caused by microbial pathogens. Postharvest decay is dependent on many factors including the variety of the fruits and the cultural practices. Good cultural practices such as fertilization, pest control and use of herbicides to decrease wetness can prevent and reduce disease development during preharvest stage. Adequate pre- and postharvest management procedures limiting the physical damage of products with a control of storage parameters such as temperature, pH, humidity and oxygen.

In fact, several species of molds grow at temperatures between 15 and 30 °C, with optimum between 20 and 25 °C and are strongly influenced by pH (optimum pH around 5) and relative humidity up 75%. Reduction of oxygen is a practice generally used to limit the development of fungi since they are aerobic organisms (De Cicco et al., 2009).

An integrated approach is necessary to control, prevent and reduce pathogen infection and disease development during pre- and postharvest period.

1.3. PRIMARY POSTHARVEST DECAY CONTROL STRATEGIES

Within disease management of postharvest fruit decay, treatments can be classified in physical, chemical and biological.

The use of different physical agents, such as temperature, ultraviolet light (UV-C) and modification of storage atmosphere is a common and effective practice to control postharvest diseases.

Refrigeration condition, based on the use of low temperature, is a great success defense method to extend the shelf life of products (influencing ripening and senescence of vegetable and fruit) and to inhibit the growth of a wide range of microorganisms (De Cicco et al. 2009).

The use of high temperatures is another technique to prevent diseases of fruit during storage, mostly by immersion or spraying of hot water for variable times and temperatures (45-55 °C) and of water steam.

Ultraviolet light acts on DNA of microorganisms as mutagenic agent and, at the same time, determines an increase of resistance stimulating the production of phytoalexins in plant tissues. The ability to reduce disease of fresh fruit is influenced by a multiplicity of factors such as fruit,

cultivar, ripeness and position of the product in relation to the irradiation source (Janisiewicz et al., 2010).

Common chemical strategies to control postharvest diseases include application of fungicides and natural compounds.Use of fungicides is the most widely used and effective method to reduce postharvest fruit losses. Nevertheless, the number of fungicides usable for controlling postharvest decays is decreasing due to the increased demand to reduce potentially harmful chemical for human safety and environment and due to the development of pathogenic strains resistant to the common fungicides. Besides, number and type of registered fungicides change depending on the considered country.

Recently, use of natural compounds with antimicrobial properties is becoming a suitable alternative to fungicides. Food preservatives such as carbonates, bicarbonates, potassium sorbate and naturally occurring antimicrobial substance such as methyl jasmonate, salicylic acid, chitosan and essential oils have been found to be a valid alternative for the control of postharvest pathogens (Panebianco et al., 2014; Sivakumar and Bautista-Banos, 2014; Youssef et al., 2012,2014).

Biological control is a developing strategy based on the use of microbial antagonists, application of naturally derived bioactive metabolites and induction of natural resistance in the host (Talibi et al., 2014). In particular, several fungi and bacteria, isolated from fruit surfaces as epiphytic microbial population, have been employed for the control of the postharvest pathogens (Chan and Tian, 2005; Cirvilleri, 2008; Cirvilleri et al., 2005; Filonow et al., 1996; Scuderi et al.2009; Liu et al., 2013; Platania et al., 2012; Parafati et al., 2015; Saravanakumar et al., 2008; Zang et al., 2010), alone or in mixture (Panebianco et al., 2015). Screened antagonists should not be pathogenic for plants and vegetable and must not produce secondary metabolites hazardous for human health.

Several studies demonstrated that integrating physical, biological and chemical methods extends the spectrum of activity for an effective control of postharvest pathogens and provides pathogen-free and chemical-free products appreciated by consumers (Janisiewicz and Conway, 2010).

1.4. POTENTIAL USE OF YEASTS IN POSTHARVEST DISEASE CONTROL

Biological control using antagonistic yeasts has been reported to be an efficacy approach to manage postharvest fruit decay (Liu et al., 2013).

Yeasts are eukaryotic microorganisms commonly defined as unicellular fungi, although many types of yeast can grow by forming pseudo-hyphae. Use of yeasts as biocontrol agents has been emphasized because they possess many features that make them suitable: they possess a variety of mechanism of action against plant pathogenic microorganisms and they do not produce allergenic spores or mycotoxins such as many bacteria and fungi. Different yeast species colonize an extremely wide range of ecosystems thanks to their ability to grow and survive in different and stressful environments.

In addiction they have simple nutritional requirements, are able to colonize dry surfaces and have capacity to grow for long time and rapidly on inexpensive substrates.

The major groups of antagonistic yeasts utilized to manage postharvest diseases were isolated from fruit surfaces and plants. However, yeast population of extreme environment condition, as Antarctic soil and sea water, have been recently explored with the aim to isolate yeast strains with high resistance to stressful storage condition such as low temperature, lack of nutrients, oxidative stress, adverse pH and humidity (Liu et al., 2013).

1.4.1. Mechanisms of action

Improving the knowledge about the mechanism of action of yeast, to manage postharvest diseases, is required to better understand the microbial interaction between host, pathogen and antagonist (tritrophic interaction) and for the potential development of yeast-based products.

Various mechanism involved in the biocontrol activities have been attributed to yeast, including:

- competition for nutrients and space
- ability to form biofilm
- parasitism
- production of lytic enzymes
- production of volatiles organic compounds (VOCs)
- induction of host defense.

Niche exclusion and competition for nutrients is mainly involved during the first 24 h when yeast cells come in contact with fruit surface and is considered a basic feature for the selection of a good BCA. Yeast can compete for sugar, as reported for *Sporobolomyces roseus* by Folonow et al., (1996) and for nitrogenous compounds as reported by Scherm et al., (2003) for the specie *Candida guilliermondi.* Moreover, several different yeast species belonging to the genus *Mestchnikowia* demonstrated the ability to deplete iron and to produce siderophores, iron-chelating molecules that strongly inhibit the growth of various filamentous fungi (Saravanakumar et al., 2008; Spizcki et al., 2006; Parafati et al., 2015)

Parasitism is defined as the ability of the yeast to adhere and become firmly attached to pathogen hyphae; this mechanism often depends on the yeast ability to produce biofilm. Furthermore, parasitism is generally associated with secretion of lytic enzyme as observed in *R. glutinis* and *P. guillermondii* by Wisniewski et al. (1991) and Allen et al. (2004). Lytic enzymes such as glucanase, chitinase and proteinases act degrading cell wall of pathogenic fungi. In particular, inhibition of β-glucan synthesis or hydrolysis of β-glucans is carried out by yeast producing killer toxin, also named mycocins (Muccilli and Restuccia 2015). Antagonistic activities of some yeast species such as *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* (Platania et al., 2012) have been correlated to killer toxins identified as exoglucanases (Muccilli et al., 2013).

Another biocontrol mechanism of a wide range of yeasts regards the production of VOCs, substances with antimicrobial activities mainly identified as alcohols (ethanol, 3-methyl- 1-butanol, 2-methyl-1-butanol, 2-phenylethanol), esters (ethyl acetate, ethyl octanoate) and aldehydes (2-methyl-2-hexenal and 2-isopropyl-5-methyl-2-hexenal) (Fialho et al., 2010; Buzzini et al., 2003; Di Francesco et al., 2015).

Since that antagonistic activity of yeast is involved in a tritrophic system, as mentioned above, considerable importance plays the ability of yeast to activate host defense mechanism, called resistance induction. Fruits response to yeast application includes production of wall-degrading enzymes, production of antifungal compound such as phytoalexins, active oxygen species and thickening of the cell wall of the host. Resistance induction is reported by different studies, which indicated a significantly change in gene expression of wounded peel fruit in response to the application of antagonistic yeast strains (Jiang et al., 2009; Hershokovitz et al., 2012).

1.4.2. Main yeasts studied for their antagonistic activity against postharvest pathogens

Over the past two decades a wide number of yeast have been isolated from a multiplicity sources such as fruit surfaces, soil, water and phyllospere. Many species have proved to be effective in controlling different postharvest pathogens. Table 1 shows some rapresentative antagonistic yeast species and their mode of action as postharvest biocontrol agents.

Table 1. Representative yeast species and their mechanism of action against postharvest pathogens

Antagonistic yeast	Mode of action	Application	References
Aureobasidium pullulans (PL5, PI1)	Production of β-1,3-glucanase, exochitinase, endochitinase and competition for nutrient and space	*B. cinerea* and *P. expansum* on apples. *M. laxa* on stone fruits, *B. cinerea* on table grape	Zhang et al., 2010 Zhang et al., 2012 Parafati et al., 2015
Aureobasidium pullulans (L1, L8)	Production of VOCs	*B. cinerea*, *Colletotricum acutatum*, *P. expansum* on apple, *P. digitatum* and *P. italicum* on orange	Di Francesco et al., 2015
Candida intermedia (235)	competition for space and nutrients, release of diffusible antifungal compounds, production of VOCs	*A. carbonarius* on grape	Fiori et al., 2014
Candida oleophila (I-182)	Induction of host defences	*P.digitatum* on grapefruit	Droby et al., 2002
Cryptococcus laurentii (LS-28)	n.s	*B. cinerea*, *P. expansum* on apple	Lima et al., 2011
Lachancea thermotolerans (751)	competition for space and nutrients, release of diffusible antifungal compounds, production of VOCs	*A. carbonarius* on grape	Fiori et al., 2014

Metschnikowia pulcherrima (GS37, GS88, GA102, BIO126)	Competition for nutrient and spaces	*B. cinerea* and *P. expansum* on apple	Spadaro et al., 2002
Metschnikowia pulcherrima (MACH1)	Iron depletion	*B. cinerea*, *P. expansum* and *A. alternata* on apple	Saravanakumar et al., 2008
Metschnikowia pulcherrima (MPR3, Disva 267)	n.s.	*B. cinerea* on table grape and *M. laxa* on cherrie	Parafati et al. 2015 Oro et al., 2014
Metschnikowia fructicola (MfCHI)	production of endochitinase	*M. laxa*, *M. fructicola* on peach	Banani et al., 2015
Meyerozyma guilliermondii (443)	Production of β-1,3-glucanase	*C.gloeosporioides* on papaya	Lima et al., 2013
Pichia guilliermondi (M8)	Production of β-1,3-glucanase and chitinase,	*B. cinerea* on apple	Zhang et al., 2011
Pichia membraneafaciens	Induction of host defense	*P. expansum* on peach	Chan et al., 2007
Pichia membranaefaciens	Parasitism and production of exochitinase, endochitinase and β-1,3-glucanase	*M.fructicola, P.expansum* and *R. stolonifer* on apple	Chan and Tian, 2005
W. anomalus (422) (Strain k) (BS91, BS92, BCA15)	β-glucanase	*C.gloeosporioides* on papaya, *B. cinerea* on apple and grape berries, *P. digitatum* on orange	Lima et al., 2013 Friel et al., 2007 Parafati et al., 2015 Platania et al., 2012

1.4.3. Commercial biological products

During commercial preparation, yeasts are exposed to stressful situation such as high temperature, low nutrients, desiccation and oxidative stress. Unfavorable conditions can improve the efficacy of the biocontrol product; for this reason pretreatment practices are often used, such as heat shock and oxidative stress, to improve the tolerance of the microorganism to environmental stress. The primary requirement for a commercial product is an adequate shelf life of the biocontrol agent both in dry and in liquid formulations, commonly used to commercialize yeast biocontrol products. Dry formulation reveals some advances in maintaining viability of yeast also under non-refrigerated condition but it requires higher production cost in comparison

to liquid formulation. Moreover different studies reported that biocontrol ability of antagonistic yeasts can be enhanced using antimicrobial compounds such as salicylic acid, methyl jasmonate and chitosan or mineral salts such as calcium chloride and sodium carbonate (Liu et al. 2013).

Actually, even if many different BCAs have been reported to inhibit a wide spectrum of pathogenic fungi, only few products based on antagonistic yeasts are commercially available to manage postharvest diseases: Shemer™ (based on *Metschnikowia fructicola*), Candifruit™ (based on *Candida sake*), Boni-protect™ (based on *Aureobasidium pullulans*), Nexi™ (based on *Candida oleophila*), other products such as Aspire™ (based on *Candida oleophila*) and Yieldplus™ (based on *Cryptococcus albidus*) were commercialized only for some years and then withdrawn (Liu et al., 2013; Spadaro and Droby 2015).

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CHAPTER 2. OBJECTIVES OF THE THESIS

In order to find alternative strategy to chemical fungicide different approach have been investigated to control biotic postharvest diseases. Actually great attention has been devoted to the study of mechanism of action and *in vivo* use of antagonistic yeasts for the control of some of the most important phytopathogenic fungi, *B. cinerea, P. digitatum* and *P. italicum*, on table grapes, mandarins and strawberries.

The present research investigated antagonistic yeast strains belonging to 4 different species, *Wickerhamomyces anomalus*, *Metschnikowia pulcherrima*, *Aureobasidium pullulans* and *Saccharomyces cerevisiae.*

The main objectives of this study are illustrated in the four following chapters:

CHAPTER 3: *In vitro* and *in vivo* evaluation of the mode of action of food-isolated yeast strains and their efficacy in the controlling botrytis bunch rot on table grapes.

CHAPTER 4: Assessment of the efficacy of antagonistic yeasts in combination with natural edible coating (locust bean gum, LBG).

CHAPTER 5: Evaluation of the efficacy and potential commercial carrier of antifungal volatile organic compounds (VOCs) produced *in vitro* and *in vivo* by antagonistic yeasts.

CHAPTER 6: Characterization of the gene expression level of *W. anomalus* exoglucanases (*EXG1* and *EXG2*) in different yeast-host-pathogen interactions.

CHAPTER 3. BIOCONTROL ABILITY AND ACTION MECHANISM OF FOOD-ISOLATED YEAST STRAINS AGAINST BOTRYTIS CINEREA CAUSING BUNCH ROT OF TABLE GRAPE IN POSTHARVEST

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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

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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 postharvest 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 postharvest 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,

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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) × 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 mL 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 (SigmaeAldrich, 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 (SigmaeAldrich). 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

20 μg/mL of $FeCl_3$ before autoclaving, and then added with a *B. cinerea* conidial suspension to obtain 3×10^5 CFU/mL density. After drying, a loopful of yeast cells were streaked onto the center of each plate. The width of reddish halos developing around the yeast colonies were measured after 6 days of incubation at 25 °C in presence and absence of *B. cinerea*. The role of competition for iron on the antagonistic activity of the yeasts was investigated on the same media described above measuring the width of inhibition zones around the yeast colonies 6 days after inoculation. Five plates for each concentration of iron were used. Unsupplemented PDA plates were used as control.

2.2.5. Biofilm formation

Biofilm formation was evaluated following a previously described procedure (Jin et al., 2003) partially modified. Yeast strains were grown overnight at 28 °C in yeast nitrogen base (YNB) medium supplemented with 100 mM glucose. Cells were then harvested, washed twice with phosphate-buffered saline (PBS, pH 7.2) and re-suspended in YNB þ 100 mM glucose to 10^7 cells/mL. Aliquots (100 μL) were inoculated in triplicate into wells of a 96-well polystyrene plate and incubated for 3 h at 28 °C in a shaker at 75 rpm. As control, three wells were handled in an identical fashion, except that no yeast suspensions were added. Following the adhesion phase, each well was washed twice with 150 μL of PBS, and 100 mL of YNB were transferred into each washed well. The plates were incubated at 28 °C at 75 rpm for up to 72 h. The medium was replenished daily by aspiration of the spent medium and the addition of the fresh medium. All assays were carried out twice. After incubation (3, 48 and 72 h) wells were washed twice with PBS and stained with 100 μL of 0.4% aqueous crystal violet solution for 45 min. Afterwards, wells were washed four times with sterile distilled water (SDW), and immediately destained with 200 mL of 95% ethanol. After 45 min, 100 μL of destaining solution was transferred to a new polystyrene 96-well plate and the amount of crystal violet in the solution was measured at 590 nm. The absorbance values for the controls were subtracted from the values for the test well to minimize background interference.

2.3. In vivo antagonistic activity

2.3.1. Efficacy of the yeast cells in controlling botrytis bunch rot of grape

In order to assess the efficiency of the yeasts as BCAs, the method described by Scuderi et al. (2009), with slight modifications, was used. Representative strains of each species (*M. pulcherrima* MPR3, *A. pullulans* PI1, *W. anomalus* BS91 and *S. cerevisiae* BCA61) were used for subsequent *in vivo* test. Table grape fruits were obtained from commercial orchards in Sicily, Italy. Grape fruits had not received any pre-harvest fungicide treatment. Healthy and homogenous grape berries were selected and randomly assigned to different treatments. Before inoculation and treatment, fruits were washed with tap water, surface-disinfected by dipping for 1 min in 1% (w/v) of sodium hypoclorite (NaOCl) solution, rinsed with SDW and then air-dried. Artificial wounds were performed using a sterile needle to make 3 mm deep and 3 mm wide wounds (four wounds for each berry) along the berry equatorial areas. Each wound was inoculated with 20 mL drop of 1×10^6 conidia/mL of *B. cinerea.* After air drying (~2 h), 20 mL drop of 1×10^8 cells/mL of yeast were added to each wound. The grape berries were placed on plastic packaging trays. To create a humid environment, a wet paper was placed on cavity trays coated with a plastic bag. The trays were incubated at 25 °C and 95% relative humidity (RH) for 5 days after inoculation to provide favorable conditions for the postharvest onset of the disease.

Data concerning the disease incidence (DI) were transformed into arcsine square-root values to normalize the distribution before performing the analysis of variance. The disease severity (DS) was previously evaluated by using an empirical 1-to-4 rating scale: 1 = no visible symptoms (0%); 2 = soft rot (35%); 3 = mycelium (65%); 4 = sporulation (90%) before analysis of variance. Average fruit severity was calculated by the following formula:

$$D = \frac{\sum (Ci)}{N} \times 100$$

where *DS* is the average severity index, *C* the number of fruits in each class, *i* (1-to-4) the numerical values of classes, *N* the total number of fruits examined.

Lesion diameter (LD) was also assessed by measuring the average diameter of the damaged area five days after pathogen inoculation. The same amount of SDW (20 mL) was used as negative control. Three replicate of ten grape fruits were used for each treatment (30 fruit/treatment). The experiment was repeated twice.

2.3.2. Wound site colonization

The determination of *M. pulcherrima* MPR3, *A. pullulans* PI1, *W. anomalus* BS91 and *S. cerevisiae* BCA61 populations on the surface of grape berries wounds was assessed following a previously described procedure (Cirvilleri et al., 2005) partially modified. Artificial wounds were performed using a sterile needle on superficially sanitized grape berries (four wounds/berry). Yeast suspensions (20 mL, 1×10^6cells/mL) of each yeast were individually inoculated into wounds, and grape berries were placed on plastic packaging trays and incubated at 25 °C and 95% RH as previously described. For each treatment, 30 fruits were arranged in a randomized complete block design. The yeast growth at the wound site was monitored during the incubation time. Tissue samples containing the whole wound were extracted, using a sterile knife, at various times (0, 24, 48, 72 and 96 h) after inoculation. Tissue samples were weighed, placed in tubes containing 1 mL of buffered peptone water (Biolife Italiana S.r.l., Milano, Italy), homogenized by vortexing, and plated onto Sabouraud Dextrose Agar (Oxoid, Basingstoke, UK) added with chloramphenicol (Oxoid) (100 mg/L) using a Spiral Plater Eddy Jet (IUL Instruments, Barcelona, Spain). Yeast colonies were counted after 48 h at 25 °C to calculate the means of colonies ($\log_{10}$ CFU) based on fresh weight. Three replicate of ten grape fruits were used for each treatment (30 fruit/treatment). The experiment was repeated twice.

2.3.3. Efficacy of the VOCs in controlling botrytis bunch rot of grape

The experiments were done in polypropylene packaging closed in plastic bags. Table grape fruits were surface-disinfected and inoculated with *B. cinerea* as previously described and subsequently placed in above mentioned packaging. The antagonists were inoculated separately on YPDA pH 4.5 and incubated 48 h at 25 °C. Thereafter, the plates were transferred into the polypropylene packaging (six plates per packaging) containing ten inoculated fruits and sealed on both sides and on the cover. Thus obtained packaging was closed in plastic bags to prevent air leakage. The inoculated fruits were held at 25 °C for 5 days. Disease incidence (DI), disease severity (DS) and lesion diameter (LD) were measured for each treatments as describe above. The same packaging with inoculated berries and with agar plates without the antagonists served as controls. The treatments *B. cinerea* þ MPR1, *B. cinerea* þ PI1, *B. cinerea* þ BS91, *B. cinerea* þ BCA61 and *B. cinerea* without the antagonists were replicated three times and the experiment was repeated twice.

Table 1
In vitro antagonistic activity of yeast strains and their volatile organic compounds (VOCs) referred to mycelial growth reduction of *Botrytis cinerea*.

Species	Growth inhibition (%) induced by yeast cells		Growth inhibition (%) induced by VOCs	
	pH 6.0	pH 4.5	pH 6	pH 4.5
M. pulcherrima[a, e]	47.67 ± 0.93 b	72.67 ± 2.13 a	30.95 ± 2.18 b	47.48 ± 3.84 a
A. pullulans[b, e]	70.89 ± 1.55 a	71.33 ± 0.93 a	31.33 ± 6.72 a	38.00 ± 3.34 a
W. anomalus[c, e]	63.75 ± 2.06 b	81.50 ± 0.36 a	33.08 ± 0.56 b	99.67 ± 0.19 a
S. cerevisiae[d, e]	63.00 ± 0.58 a	64.00 ± 0.58 a	20.00 ± 0.88 b	71.00 ± 0.58 a

[a] Data regarding *Metschnikowia pulcherrima* are referred to seven strains.
[b] Data regarding *Aureobasidium pullulans* are referred to three strains.
[c] Data regarding *Wickerhamomyces anomalus* are referred to four strains.
[d] Data regarding *Saccharomyces cerevisiae* strain.
[e] Data presented as mean ± standard error of the mean. In each row, values followed by different letter within the same assay (activity of yeasts or their VOCs) are significantly different according to Fisher's least significant difference test ($p = 0.01$).

2.4. Statistical analyses

Data from in *vitro* and *in vivo* experiments were analyzed separately by using the Statistica package software (Version 10; Statsoft Inc). In all repeated experiments, the arithmetic means were calculated and analyzed by using one-way analysis of variance (ANOVA).

Percentage data concerning DI were previously transformed using the arcsine transformation (sin-1 square root x). Fisher's least significant difference test was used to compare the significance of differences among *in vitro* and *in vivo* (DI and LD) data at $p = 0.01$ and 0.05, respectively.

Since an ordinal scale was adopted for DS, the data were analyzed according to the Kruskal Wallis non-parametric one-way analysis of variance followed by the Mann-Whitney test ($P < 0.05$) for all possible pairwise comparisons.

3. Results

3.1. In vitro antagonistic activity and inhibitory effects of VOCs

Data from the dual culture assays, performed at two different pH values, showed that all tested yeasts inhibited the mycelial growth of *B. cinerea* with variable efficacy (Table 1). On the whole, the mycelial growth inhibition of *B. cinerea* by *M. pulcherrima* and *W. anomalus* strains was higher at pH 4.5 than pH 6.0. Otherwise, *A. pullulans* and *S. cerevisiae* strains maintained the same biocontrol activity (data not significant) at both pH values.

Similarly the greatest inhibition values of *B. cinerea* by VOCs was observed at pH 4.5 (Table 1). In detail, *W. anomalus* and *S. cerevisiae* strains showed the highest values of growth inhibition (99.67 ± 0.19% and 71.00 ± 0.58% respectively after 9 days of incubation). A significantly higher fungal growth inhibition of pathogen by *M. pulcherrima* was also observed at pH 4.5 than pH 6.0 (Table 1), with an average efficacy of 47% within the seven strains tested. Among these strains the highest inhibition activity was detected for the strain MPR3, with 67% of fungal growth inhibition (data not shown). Only for *A. pullulans* the production of antifungal VOCs did not significantly differ at the two pH values.

3.2. Enzymatic assays

All *A. pullulans* and *W. anomalus* strains were able to hydrolyze laminarin (β-*1,3-glucanase* activity). Moreover, all *A. pullulans* strains showed pectinolytic and proteolytic (skim milk and gelatin) activities. On the opposite, no extracellular enzyme activity was detected for *M. pulcherrima* and *S. cerevisiae* strains. No strain showed chitinase activity.

3.3. Effect of iron concentration on antagonistic activity of yeasts

Antagonistic activity of each strains belonging to *A. pullulans, W. anomalus* and *S. cerevisiae* did not significantly influence by tested iron concentrations showing that the inhibition activity of these yeasts is not depending by iron competition. Otherwise, activity of *M. pulcherrima* was iron-dependent.

M. pulcherrima strains were always able to synthesize pigments at both pH values. In detail, they significantly produced the widest halo zones (>7.6 mm) in absence of $FeCl_3$ (Fig. 1). The increasing of iron concentration significantly reduced the width of pigmented halo around the colonies (decreasing from 5.6-6.6 mm to 0.1e0.6 mm at 5 and 20 μg/mL $FeCl_3$, respectively). The increasing of iron concentration also affected the pigment coloration of yeast colonies, that turned from pale to dark red.

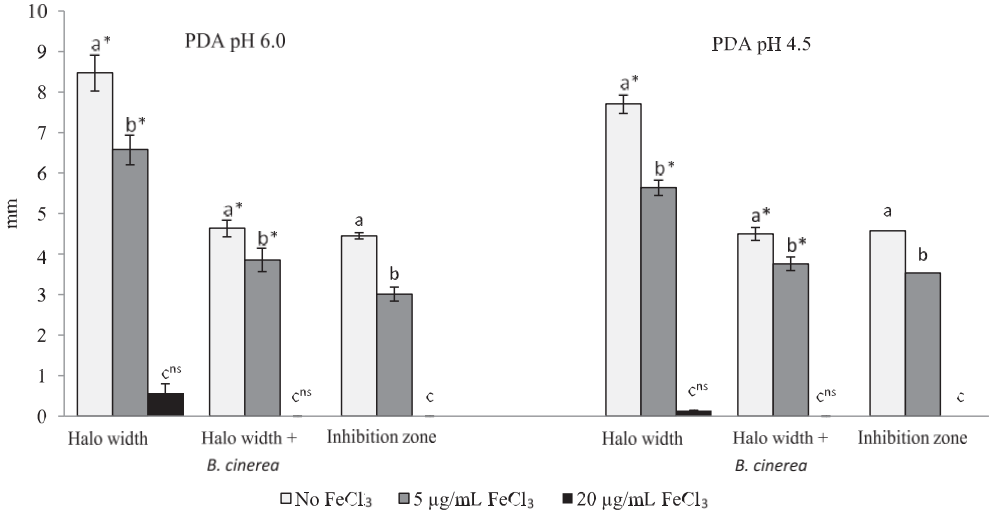

Fig. 1. Effect of iron on width of pigmented halo and of inhibition zone produced by *Metschnikowia pulcherrima* strains on PDA pH 6.0 and PDA pH 4.5. Values are mean of seven *Metschnikowia pulcherrima* strains. Vertical bars indicate the standard error of the mean. Different letters over the bars within each parameter (halo width, halo width þ *Botrytis cinerea* and inhibition zone) are significantly different according to Fisher's least significant difference test ($p = 0.01$). Columns comparing halo width in absence and in presence of *Botrytis cinerea* followed by * are significantly different according to Fisher's least significant difference test ($p = 0.01$); ns = not significant data.

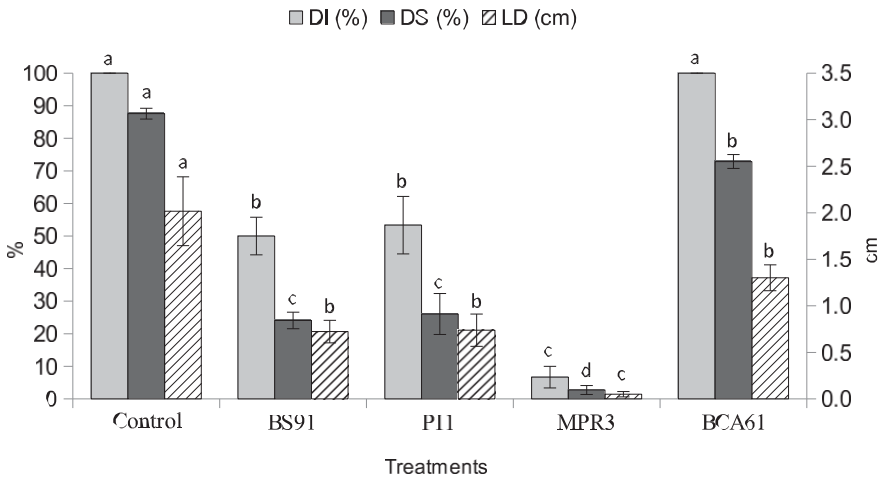

Fig. 2. *In vivo* antagonistic activity of *Wickerhamomyces anomalus* BS91, *Aureobasidium pullulans* PI1, *Metschnikowia pulcherrima* MPR3 and *Saccharomyces cerevisiae* BCA61 yeast strains in inhibiting gray mold decay on grape berries. Effect of yeasts are referred to Disease incidence (DI), disease severity (DS) and lesion diameter (LD) caused by *Botrytis cinerea* 5 days after incubation at 25 ° C. Vertical bars indicate the standard error of the mean. Columns representing the same disease parameter followed by different letters are significantly different according to Fisher's least significant difference test (p = 0.05). Differences related to DS data within treatments were previously analyzed on disease rating classes with Kruskal Wallis one way analysis of variance by ranks (x^2 = 99.17; dF = 4; p < 0.001) followed by all pairwise multiple comparisons with Mann-Whitney test (z > 2.56; p < 0.01). Percent DS data are shown.

The presence of *B. cinerea* has significantly affected the halo width of *M. pulcherrima* both in absence and presence of 5 μg/mL $FeCl_3$ whereas it was not able to influence the halo at 20 μg/mL $FeCl_3$ (Fig. 1). Similarly, the width of inhibition zones always decreased with increasing of iron concentration. At 20 μg/mL $FeCl_3$ all strains lost their inhibition activity.

Comprehensively, pigmented halos and inhibition zones were never significantly affected by pH value, since the lowering of the pH from 6.0 to 4.5 did not produce significant variations (Fig. 1).

3.4. Biofilm formation

M. pulcherrima and *W. anomalus* strains after 3 h were able to adhere to the polystyrene plates subsequent to repeated washes (OD 1.85 ± 0.16 and 1.77 ± 0.08, respectively). On the contrary, *A. pullulans* and *S. cerevisiae* strains were unable to adhere to polystyrene surface (OD 0.06 and 0.19, respectively). Experiments also demonstrated that *M. pulcherrima* and *W. anomalus* strains maintained high film-forming capacity also after 48 h (OD 1.15 ± 0.08 and 0.73 ± 0.02, respectively) and 72 h of incubation (OD 1.12 ± 0.16 and 0.79 ± 0.18, respectively), compared with *S. cerevisiae* that showed very low values (OD 0.16).

3.5. In vivo efficacy of the yeast cells in controlling botrytis bunch rot of grape

The efficacy of different tested yeasts in reducing botrytis bunch rot is reported in Fig. 2. In detail, DI values of gray mold decay were significantly lower (p = 0.05) in grape berries treated with *W. anomalus* strain BS91, *A. pullulans* strain PI1, and *M. pulcherrima* strain MPR3 than ones detected in the control and berries treated with *S. cerevisiae* strain BCA61 (all infected berries). In particular, MPR3 showed significantly the lowest DI among all representative yeasts. All BCA strains reduced significantly (x^2 = 99.17; dF = 4; p < 0.01) DS value on grape berries if compared to control. Once again, the lowest DS value was recorded for the MPR3 application, followed by BS91 and PI1 yeasts (Figs. 2 and 3). Although at different levels, all tested yeasts significantly decreased the size of decay (LD) caused by *B. cinerea* if compared untreated grape berries (Fig. 2).

Comprehensively, *M. pulcherrima* MPR3 showed the highest efficacy in reducing DI (6.7%), DS (2.7%) and LD (0.05 cm) caused by *B. cinerea* on grape berries. *W. anomalus* BS91 and *A. pullulans* PI1 reached a certain activity against the pathogen, whereas *S. cerevisiae* strain BCA 61 registered the worst efficacy in controlling gray moldmold decay on grape berries (Fig. 2).

3.6. Colonization of berry wounds

The population dynamics of *W. anomalus* BS91, *A. pullulans* PI1, *M. pulcherrima* MPR3 and *S. cerevisiae* BCA61 strains on artificially wounded grape berries are displayed in Fig. 4. The population trend, starting from the same concentration for all yeasts (log_{10} CFU/g of tissue= 4.27), rapidly increased for BS91, PI1 and MPR3 already within 24 h, whereas population of *S. cerevisiae* BCA61 remained quite constant, reaching a maximum population peak of 6.2 log at 48 h. After 48 h the population dynamics appeared enough stable for all tested yeasts. At 96 h, population densities of *W. anomalus* BS91, *A. pullulans* PI1, *M. pulcherrima* MPR3

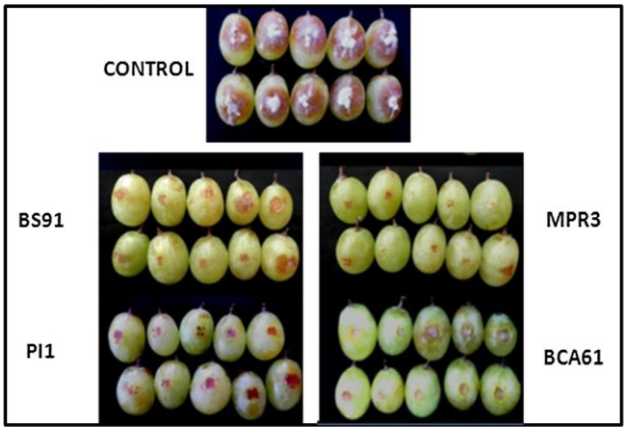

Fig. 3. Biocontrol activity of *Wickerhamomyces anomalus* BS91, *Aureobasidium pullulans* PI1, *Metschnikowia pulcherrima* MPR3 and *Saccharomyces cerevisiae* BCA61 yeast strains in inhibiting gray mold caused by *Botrytis cinerea* on grape berries. Results 5 days after treatments are shown.

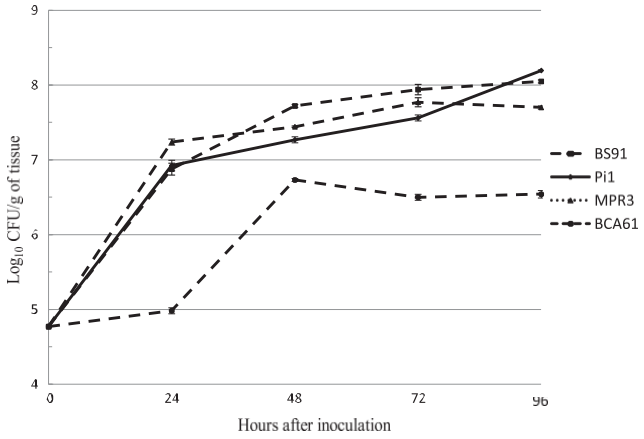

Fig. 4. Population dynamic of the four selected yeasts *Wickerhamomyces anomalus* BS91, *Aureobasidium pullulans* PI1, *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* BCA61 in the wounds of grape barriers at 25 °C. Vertical bars indicate the standard error of the mean.

and *S. cerevisiae* BCA61 reached levels of 7.5, 7.9, 7.2 and 6.04, respectively.

3.7. In vivo efficacy of the VOCs in controlling botrytis bunch rot of grape

The efficacy of VOCs produced by tested yeasts in reducing botrytis bunch rot is reported in Fig. 5. On the whole, disease parameters (DI, DS and LD) recorded in this experiment were averagely higher than ones reported for the *in vivo* activity of yeast cells. Once again, *M. pulcherrima* MPR3 confirmed the best efficacy in controlling gray mold decay of grape berries since the DI, DS and LD were significantly reduced, followed by *W. anomalus* BS91. *A. pullulans* PI1 and *S. cerevisiae* BCA61 averagely showed the less efficacy in controlling disease on grape berries (Fig. 5).

4. Discussion

A selection strategy for microbial antagonists to control post-harvest diseases of fruits and vegetables usually start off with the isolation from natural environments in which they compete with epiphytic microorganisms. In addition, for successful biocontrol, the conditions that favor a potential antagonist should be the same or similar to those that favor the pathogen. For this reason we evaluated 15 yeast strains belonging to 4 species isolated from minimally processed and fermented vegetables for their biocontrol activities against *B. cinerea.* The results of the preliminary antagonistic activity *in vitro* assays demonstrated that yeast isolates were able to suppress causal agent of gray moldmold disease at different levels, although acidic conditions (PDA medium at pH 4.5) emphasized the biocontrol efficacy of *M. pulcherrima* and *W. anomalus* strains. Since several mechanisms have been reported to play a significant role in the biocontrol activity of antagonistic yeasts, in this study we ascertained within four yeast species the role of the main biocontrol modes of actions, as iron competition, production of VOCs, production of cell wall-degrading enzymes, biofilm formation, in controlling the *in vitro* growth of *B. cinerea*.

Only antagonistic activity of *M. pulcherrima* was strictly correlated with iron availability. In fact, the inhibitory activity as well as the diameter of the pigmented halo decreased with the increase of iron concentration in nutrient media. These results are in accordance with the findings of Sipiczki (2006) who suggested that the cells do not secrete the pigment but instead secrete a soluble, diffusible precursor that forms the pigment in the medium when it encounters iron; this observation is supported by the reduction in halo size observed when the medium is supplemented with $FeCl_3$. The production of VOCs was mainly observed on PDA at pH 4.5 for *W. anomalus* strains which totally inhibited the mycelial growth of *B. cinerea* up to 9 days. Also *S. cerevisiae* produced VOCs which significantly reduced the fungal growth under acidic condition compared to pH 6.0. Fungal inhibition by VOCs produced by *M. pulcherrima* was significantly higher at pH 4.5, but the efficacy was strain-dependent, as the inhibition of pathogen growth (%) varied from 24.67 to 66.67%. Although antimicrobial VOCs produced by yeasts, such as *Candida intermedia* (Huang et al., 2011) and *Sporidiobolus pararoseus* (Huang et al., 2012), were reported to be effective in suppressing both the conidial germination and the mycelial growth of *B. cinerea* on agar media and in suppressing *Botrytis* diseases on plant tissues, to our knowledge the effect of VOCs produced by *W. anomalus*, *M. pulcherrima* and *S. cerevisiae* has not been investigated against *B. cinerea*. In this regard, ethyl acetate

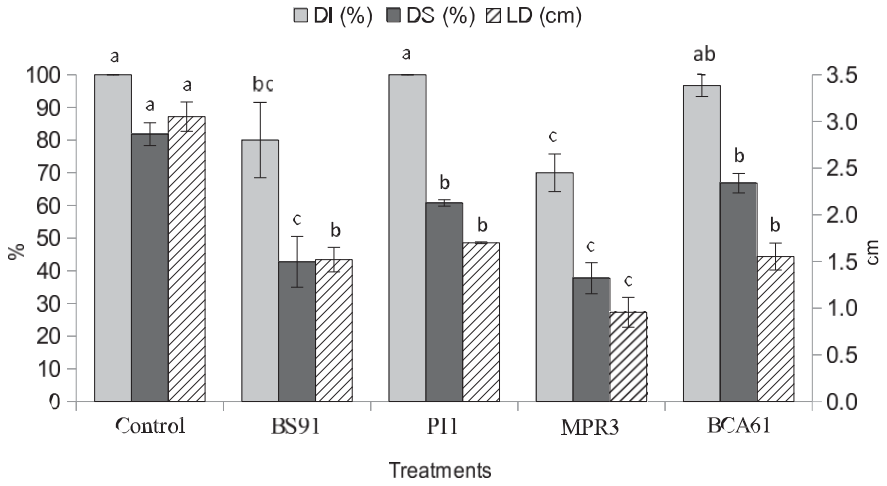

Fig. 5. *In vivo* antagonistic activity of volatile organic compounds (VOCs) produced by *Wickerhamomyces anomalus* BS91, *Aureobasidium pullulans* PI1, *Metschnikowia pulcherrima* MPR3 and *Saccharomyces cerevisiae* BCA61 yeast strains in inhibiting gray mold decay on grape berries. Effects of VOCs are referred to Disease incidence (DI), disease severity (DS) and lesion diameter (LD) caused by *Botrytis cinerea* 5 days after incubation at 25 ° C. Vertical bars indicate the standard error of the mean. Columns representing the same disease parameter followed by different letters are significantly different according to Fisher's least significant difference test ($p = 0.05$). Difference related to DS within treatments were previously analyzed with Kruskal Wallis one way analysis of variance by ranks ($x^2 = 56.54$; dF = 4; $p < 0.001$) followed by all pairwise multiple comparisons with Mann-Whitney test ($z > 2.56$; $p < 0.01$).

has been shown to have an antifungal effect and might contribute to the biocontrol activity of *W. anomalus* during airtight storage of grain (Fredlund et al., 2004; Druvefors et al., 2005) while more recently Hua et al. (2014) stated that the biocontrol ability of *W. anomalus* can be attributed to the production of 2-phenylethanol, which affects spore germination, growth, toxin production, and gene expression in *Aspergillus flavus*.

No enzymatic activity was detected both for *S. cerevisiae* and *M. pulcherrima* species, while b-1,3-glucanase was produced by *W. anomalus* strains, as already demonstrated by Muccilli et al. (2013), and by *A. pullulans* strains, as previously reported by Castoria et al. (2001). Additionally, *A. pullulans* was the only species exhibiting a broad spectrum of diffusible hydrolytic enzymatic activities, as it was positive also for pectinase and protease activities, as previously proved by Buzzini and Martini (2002).

Positive relation between biofilm formation and wound colonization capacity was observed for *M. pulcherrima* and *W. anomalus*; on the opposite, *A. pullulans* PI1 showed high wound colonization capacity *in vivo* but no biofilm-formation in *in vitro* assays. *S. cerevisiae* evidenced the lowest wound colonization ability and no film-forming ability.

The inhibitory effect of the yeasts and their VOCs was further proven on wounded grape berries artificially inoculated with *B. cinerea*. *M. pulcherrima* exhibited, in both experiments, the best efficacy in reducing *B. cinerea* gray mold decay, followed by *W. anomalus* and *A. pullulans*. Regarding the ability of each yeast to survive and multiply in artificial wounds made on grapes, *W. anomalus* BS91, *M. pulcherrima* MPR3 and *A. pullulans* PI1 reached high population levels in grape wounds after 96 h under storage conditions, indicating that these yeasts are able to fit in well with the wound environment in grape berries and have a considerable colonizing potential. Surprisingly, the environment of the wound site seemed to be less favorable to the growth of *S. cerevisiae* which showed a longer lag phase and the lowest number of cells at the end of experiment.

These results demonstrate that the cumulative effects of several control mechanisms detected *in vitro* are not always sufficient to explain the high biocontrol efficacy observed when these yeasts were applied to the wounds against the pathogen in *in situ* assays. In fact, despite *M. pulcherrima* strains evidenced lack of enzymatic activity and moderate volatiles production *in vitro*, they demonstrated an excellent antagonistic behavior *in vivo* by VOCs production and, probably, by starving the pathogen of iron.

On the other hand, *W. anomalus* and *A. pullulans* which evidenced the best performances in the *in vitro* assays, based respectively on VOCs and on extracellular enzymes production, but lack of iron competition, were less effective in reducing gray mold incidence, disease severity and lesion diameter on artificially wounded grapes.

Therefore, the hypothesis that the involvement of other indirect antagonistic mechanisms, as the reduction of virulence of *B. cinerea* under iron-starvation conditions (Saravanakumar et al., 2008; Kieu et al., 2012) or stimulation of plant host defense pathways may contribute to increase efficacy of this BCA, could not be excluded. It has been demonstrated, in fact, that antagonist yeasts are capable to induce resistance responses in various fruits, as in the case of *Pichia guilliermondii* strain US-7 in apples (Wisniewski and Wilson, 1992), *A. pullulans* and *Candida saitoana* in apples (Ippolito et al., 2000; El-Ghaouth et al., 2003) and *A. pullulans* in strawberries (Adikaram et al., 2002). In the antagonist-fruit interaction, expression of genes involved in oxidative stress, iron homeostasis, zinc homeostasis, and lipid metabolism can be induced, as recently demonstrated by Hershkovitz et al. (2013), providing new insight into the biology of the tritrophic interactions that occur in the biocontrol system yeast-pathogen-fruit.

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CHAPTER 4. COMBINATION OF SELECTED YEASTS AND LOCUST BEAN GUM, FOOD ADDITIVE, TO ENHANCE PREVENTIVE EFFECT ON POSTHARVEST DECAY OF MANDARINS

4.1. INTRODUCTION

In fresh citrus industry, the most economically important postharvest diseases are green and blue molds, caused by *Penicillium digitatum* (Pers.:Fr.) Sacc and *Penicillium italicum* Wehmer, respectively (Eckert and Eaks, 1989). However, prolonged and extensive use of synthetic fungicides lead to the development of resistant strains of the pathogens and a consequent loss of fungicide effectiveness (Sánchez-Torres and Tuset, 2011). Moreover, accumulation of chemical residues in fruit peel represents a treat for human health and a limiting factor for citrus production since essential oils and peels are widely used by the food industry. Most of the commercially harvested citrus fruits undergo a wax coating process in packing houses, usually based on oxidised polyethylene, organic solvents, surfactants (Porat et al., 2005) and preservatives as propyl paraben (Moscoso-Ramírez et al., 2013). Consequently, the development of non-toxic, safe alternatives to control phytopathogenic fungi and prevent postharvest decay is of the utmost importance.

Nowadays, great public awareness and international regulations have emphasized the application of materials that show health benefits and are environmentally friendly, especially in food products. This results in additional research to develop alternative materials for the coating of citrus fruits (Dhall, 2013). Edible films and coatings have been developed as natural or nonpolluting materials to replace commonly used waxes, to extend shelf-life of fruits, to improve fruit appearance, to reduce moisture losses, and eventually to incorporate antimicrobial food additives (Aloui et al., 2014; Arnon et al., 2015; Debeaufort et al., 1998; Embuscado and Huber, 2009; Valencia-Chamorro

et al., 2011). The antifungal activity of a low molecular chitosan-based coating with potassium sorbate maintained postharvest fruit quality and showed good performance against *P. digitatum* and *P. italicum* on "Murcott" tangors stored at 15 °C (Chien et al., 2007). Valencia-Chamorro et al. (2008) developed HPMC (hydroxypropylmethylcellulose)-lipid edible composite films and coatings with food additives, such as mineral salts, organic acid salts, salts of parabens, and some mixtures that presented antifungal properties *in vitro* against *P. digitatum* and *P. italicum*. In subsequent papers, selected coatings reduced *in vivo* the incidence and severity of green mold and blue mold on "Clemenules" clementine mandarins, "Ortanique" hybrid mandarins, and "Valencia" oranges (Valencia-Chamorro et al., 2009a). Further studies confirmed, under long-term cold storage conditions, the performances of HPMC-lipid coatings, in reducing incidence and severity of *P. digitatum* and *P. italicum* on Valencia oranges, without affecting fruit quality (Valencia-Chamorro *et al.* 2009b).

Several reports proposed the use of biocontrol agents (BCAs) in reducing fruit infections caused by *Penicillium* spp. (Chalutz and Wilson, 1990; Cirvilleri et al., 2005; El-Ghaouth et al., 2001; Lahlali et al., 2004; Panebianco et al., 2015; Platania et al., 2012; Restuccia et al., 2006; Taqarort et al., 2008). The use of yeasts is particularly encouraged since they are characterized by high inhibitory capacity, rapid colonization of fruit wounds, production of volatile organic compounds (Di Francesco et al., 2015; Parafati et al., 2015; Rosa-Magri et al., 2011) and simple nutritional requirements, and they are able to colonize dry surfaces for long time periods (El-Tarabily and Sivasithamparam, 2006). Although the efficacy of many antagonistic yeasts against a wide variety of phytopathogenic fungi has been well documented in literature (Liu et al., 2013), there are only few published data on their compatibility and efficacy when incorporated into coating formulations for fresh fruits (McGuire and Hagenmaier, 1996; McGuire and Dimitroglou, 1999; Fan et al., 2009) and on the effect of the coating on the population dynamics of BCAs over time (Potjewjid et al.,

1995; Mc Guire and Baldwin, 1994). To investigate these shortcomings, a very recent and comprehensive study of Aloui and co-workers (2015) demonstrated the efficacy of sodium alginate and locust bean gum (LBG) coatings containing *Wickerhamomyces anomalus* killer strain in preserving the postharvest quality of 'Valencia' oranges and in inhibiting green mold infection.

Therefore, the objective of the present paper was to determine the effect of LBG edible coating on the survival and biocontrol ability of different yeast species applied to reduce the postharvest decay caused by *P. digitatum* and *P. italicum* on mandarin. The induction of resistance by the biocontrol yeasts was also investigated on mandarin peel.

4.2. MATERIALS AND METHODS

4.2.1. Microorganisms and culture conditions

The yeasts used in this study were selected within the collection of Di3A (Dipartimento di Agricoltura, Alimentazione e Ambiente, University of Catania, Italy). The strains *Wickerhamomyces anomalus* BS91, *Metschnikowia pulcherrima* MPR3, and *Aureobasidium pullulans* PI1 were identified by sequencing the D1/D2 region of the 26S rRNA gene and selected for their biocontrol ability. *W. anomalus* BS91 has revealed a good antagonistic activity related to β-glucanase production (Muccilli et al., 2013). More recently, *M. pulcherrima* MPR3 and *A. pullulans* PI1 showed a strong efficacy in controlling botrytis bunch rot of table grape (Parafati et al., 2015).

P. digitatum and *P. italicum* were isolated from decayed mandarin fruits in Sicily (Italy), identified and tested for pathogenicity as described in Oliveri et al. (2007).

The yeast strains and fungal pathogens 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 of sterile distilled water (SDW)] and Potato Dextrose Agar (PDA, Oxoid), respectively.

4.2.2. In vitro efficacy of yeast strains against Penicillium digitatum and Penicillium italicum

BCAs and phytopathogenic molds were, respectively, grown on YPDA for 48 h and PDA for 7-14 days at 25 °C until sporulation. To evaluate the antagonistic activity against *P. digitatum* and *P. italicum*, 100 µL of yeasts cell suspensions (10^9 cells/mL) were 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 value were used. An amount of 20 µL *P. digitatum* and *P. italicum* conidial suspensions (about 10^5 conidia/mL) was placed on PDA plates (3 cm away from yeast), 1 h (co-inoculation experiment) and 48 h (differed inoculation experiment) after streaking of BCA. Thus, obtained dishes were incubated at 25 °C. PDA plates only inoculated with green and blue molds served as controls. After 6 days of incubation, radial growth reduction was calculated and referred to growth of relative controls according to following formula:

$$\%I = (C\text{-}T/C) \text{ X } 100$$

where %I represents the inhibition of mycelial growth, C represents mycelial growth of the pathogen alone and T the mycelial growth of the pathogen in the presence of yeast. The experiments were performed twice.

4.2.3. Yeast colonization of wounded mandarin fruit

The survival and colonization ability of *M. pulcherrima, W. anomalus* and *A. pullulans* yeast suspensions were assessed on wounded mandarins surface. Mandarin fruits (*Citrus reticulata*

Blanco) were obtained from commercial organic orchards in Sicily, Italy. Healthy and homogenous mandarins were selected and randomly assigned to different treatments. Before any treatment, fruits were washed with tap water, surface disinfected by dipping for 1 min in 1% (w/v) of sodium hypochlorite (NaOCl) solution, rinsed with SDW and then air-dried. Artificial wounds were performed using a sterile needle on superficially disinfected mandarins (4 wounds for fruit). Yeast suspensions (20 µL, 1×10^9 cells/mL) were individually inoculated into wounds; fruits were placed on plastic packaging trays and incubated at 25 °C and 95% RH. The yeast growth at the wound sites was monitored. Tissue samples containing the whole wound were cut, using a sterile knife, at different time intervals (0, 24, 48, 72 and 96 h) after treatment. Tissue samples were weighted and placed in tubes containing proportional amount of sterile quarter-strength Ringers solution (Oxoid). Serial dilutions of each sample were plated onto Sabouraud Dextrose Agar (SDA, Oxoid) added with chloramphenicol (Oxoid, 100 mg/L) using a Spiral Plater Eddy Jet (IUL Instruments, Barcelona, Spain). Yeast colonies were counted and the means (log CFU) calculated on fresh weight (FW). Three replicates, each including 10 fruits, were used for each treatment. The experiment was performed twice.

4.2.4. In vivo efficacy of yeasts in controlling green and blue mold decay on wounded mandarins

In order to assess the ability of the yeasts as BCAs, *W. anomalus* BS91, *M. pulcherrima* MPR3 and *A. pullulans* PI1 were used for subsequent *in vivo* assays, following the method described by recent papers (Panebianco et al., 2014; Parafati et al., 2015), with slight modifications. Healthy and homogenous mandarin fruits were selected and randomly assigned to different treatments. Before inoculation and treatment, fruits were washed with tap water, surface disinfected by dipping for 1 min in 1% (w/v) of NaOCl solution, rinsed with SDW and air-dried. Artificial wounds (4 for each

fruit) were performed using a sterile needle to make 3 mm deep and 3 mm wide wounds along the basal area. An aliquot of 30 µL of *W. anomalus*, *M. pulcherrima* or *A. pullulans* at 1×10^9 cells/mL was pipetted into each wound and allowed to dry at room temperature. After 4 h, each wound was inoculated with 20 µL of *P. digitatum* or *P. italicum* (1×10^5 conidia/mL) and air dried. Fruits were packaged on plastic trays. To create a humid environment, a wet paper was placed on cavity trays coated with a plastic bag. The trays were incubated at 25 °C and 95% relative humidity (RH) for 5 days after inoculation to provide favorable conditions for the postharvest onset of the disease. Data concerning the disease incidence (DI) were transformed into arcsine square-root values to normalize the distribution before performing the analysis of variance. The disease severity (DS) was evaluated by using an empirical 1-to-4 rating scale: 1 = no visible symptoms (0%); 2 = soft rot (35%); 3 = mycelium (65%); 4 = sporulation (90%). Average fruits severity was calculated by the following formula:

$$DS=\frac{\sum (Ci)}{N}\times 100$$

where DS is the average severity index, *C* the number of fruits in each class, *i* (1-to-4) the numerical values of classes, N the total number of fruits examined. Lesion diameter (LD) was also assessed 5 days after pathogen inoculation by measuring the average diameter of the damaged area. The same amount of SDW (20 µL) was used as negative control. Three replicates, each formed by 10 mandarin fruits, were used for each treatment. The experiment was done twice.

4.2.5. Preparation of yeast strains and film forming dispersion

Yeast cells were grown on YPDA pH 4.5 at 25 °C for 48 h, collected and re-suspended in SDW and in LBG (Sigma-Aldrich, Saint Louis, MO, USA) film forming solution up to reach final

concentration of 1×10^9 cells/mL. The LBG solution was previously prepared by dissolving LBG (0.5% and 1% w/v) in SDW heated at 70 °C with constant agitation until all particles were thoroughly dispersed.

4.2.6. Bioactive LBG dipping treatments

All fruits were surface-disinfected, air dried and wounded as described above. The mandarins were randomized in a complete random block design and immersed for 10 min in 10^9 CFU/mL cell suspension of *W. anomalus* BS91, *M. pulcherrima* MPR3, and *A. pullulans* PI1 suspended in SDW, LBG 0.5% and LBG 1%. Fruits treated with SDW were used as a control. After air drying at room temperature for 2 h, wounds of each fruit were inoculated with 20 µL of *P. digitatum* or *P. italicum* conidial suspensions (1×10^5 conidia/mL). Fruits were packaged on plastic trays. To create a humid environment, a wet paper was placed on cavity trays coated with a plastic bag. The inoculated fruit were held at 25 °C for 5 days. DI, DS and LD values were measured for each treatments as described above.

4.2.7. Viability of yeasts following LBG dipping treatments

The survival of each yeast strain was evaluated on mandarin fruits after dipping in different solution (SDW, LBG 0.5% and LBG 1%). Data were recorded immediately after treatments (2 h) and after 5 days of storage at 25 ±1 °C and 98% RH.

Tissue samples consisting of 1 cm^2 of mandarin peel were removed from fruits with a sterile knife and placed in tubes containing 5 mL of sterile Ringers solution. Serial dilutions of each sample were plated onto SDA added with chloramphenicol using a Spiral Plater Eddy Jet (IUL Instruments, Barcelona, Spain). Yeast colonies were counted and the means (log CFU) calculated

on FW. Three replicates, containing 10 fruits, were used for each treatment. The experiment was performed twice.

4.2.8. Influence of yeast treatments on enzyme activities of mandarins

To examine the effect of yeast strains on the oxidative reaction markers of mandarins, activities of peroxidase (POD) and superoxide dismutase (SOD) were tested according to Wang et al., 2011. Peel tissues (3 g) from 4 fruits for each yeast treatment were taken at various time intervals (0, 24, 48 and 72 h), mashed in 5 mL of 0.05 M sodium phosphate buffer (SPB) pH 7.8 containing 1% (w/v) polyvinyl-polypyrrolidone at 4 °C. After centrifuging at 12.000 g at 4 °C for 10 min, the supernatant was collected as crude enzyme extract and used for subsequent assays. POD activity was measured using guaiacol as a substrate, in a reaction mixture containing 3 mL of 50 mM SPB pH 7.8, 220 µL of 0.3% (v/v) guaiacol, and 20 µL of crude enzyme extract. The reaction started immediately by adding 0.3% (v/v) H_2O_2 and was allowed to proceed for 5 min while A_{470} was measured every 30 s. One unit (U) of POD activity was defined as the amount of crude enzyme extract producing an increase of A_{470} by 0.01 in 1 min and expressed as U/min g FW. SOD activity assay was carried out using 3 mL reaction mixture containing 50 mM SPB pH 7.8, 12.37 mM methionine, 1.33 mM EDTA, 71.3 µM nitroblue tetrazolium (NBT), 2 µM riboflavin and 50 µL crude enzyme extract. Samples were illuminated under two 15-W fluorescent lamps for 10 min and then the absorbance was measured at 560 nm. Identical solutions held in the dark served as blank. One unit of SOD was defined as the amount of enzyme that caused a 50% decrease of the SOD-inhibitable NBT reduction.

4.2.9. Statistical analyses

Data from *in vitro* and *in vivo* experiments were analyzed separately by using the Statistical package software (version 10; Statsoft Inc). Yeast × pH interaction was calculated in *in vitro* mycelial growth tests. In all repeated experiments, the arithmetic means were calculated and analyzed by using one-way analysis of variance (ANOVA). Percentage data concerning DI were previously transformed using the arcsine transformation (sin-1 square root x). Fisher's least significant difference test was used to compare the significance of differences among *in vitro* and *in vivo* (DI, DS and LD) data at $p = 0.01$ and 0.05, respectively.

4.3. RESULTS

4.3.1. In vitro antagonistic activity of yeasts

Data from the dual culture assays performed on PDA showed that all yeasts strongly inhibited the mycelial growth of *P. digitatum* and *P. italicum* although with variable effects (Table 1). Specifically for each fungal pathogen, the ANOVA data provided a strongly significant effect for yeast treatments and pH ($p < 0.001$) whereas not significant interactions between yeast × pH were detected for both pathogens.

Comprehensively, *A. pullulans* among all yeasts provided the best results in reducing mycelial growth of target fungi and especially *P. digitatum.* In addition, the higher effects of yeasts in reducing mycelial growth of pathogens were always detected at pH 4.5 if compared with pH 6.0.

Table 1. *In vitro* antagonistic activity of yeast strains against *Penicillium digitatum* and *Penicillium italicum*.

Yeast [b]	Inhibition of mycelial growth of target pathogens (%) [a]			
	P. digitatum		***P. italicum***	
	pH 6.0	**pH 4.5**	**pH 6.0**	**pH 4.5**
W. anomalus	55.84 ± 2.42 b	62.23 ± 1.36 b	51.11 ± 4.43	68.78 ± 1.06 a
M. pulcherrima	53.51 ± 1.09 b	65.88 ± 1.63 b	39.72 ± 5.53	60.18 ± 1.03 b
A. pullulans	66.89 ± 1.36 a	79.48 ± 0.52 a	59.29 ± 1.35	71.51 ± 0.92 a
F; p (yeast)	61.09; *< 0.001*		12.99; *< 0.001*	
F; p (yeast × pH)	3.47; 0.065[ns]		0.801; 0.471[ns]	

[a] Antagonistic activity of yeasts was referred to mycelial growth reduction on PDA as follows: %I = (C-T/C) * 100, where %I represented the inhibition of radial mycelial growth, C was radial growth measurement in control and T was the radial growth of *P. digitatum* or *P. italicum* in presence of yeast strains.

[b] Data are means of 3 replicates ± standard error of the mean (SEM). Values followed by different letter within each column (yeast effect against *P. digitatum* or *P. italicum*) are significantly different according to Tukey's honest significant difference test ($p = 0.01$).

4.3.2. Population dynamics of yeasts on mandarin fruits

The colonization trends over time of *W. anomalus* BS91, *M. pulcherrima* MPR3 and *A. pullulans* PI1 on artificially wounded mandarins are reported in Fig. 1. The population dynamics of *W. anomalus* BS91 and *M. pulcherrima* MPR3 (starting from 6.60 log CFU/g of tissue) rapidly increased already within 24 h. After 48 h they resulted approximately stable. Otherwise, *A. pullulans* PI1 population grew slowly with an irregular trend over time.

At 96 h, population densities of BS91, MPR3 and PI1 reached levels of 7.98, 8.10 and 6.90 log CFU/g, respectively.

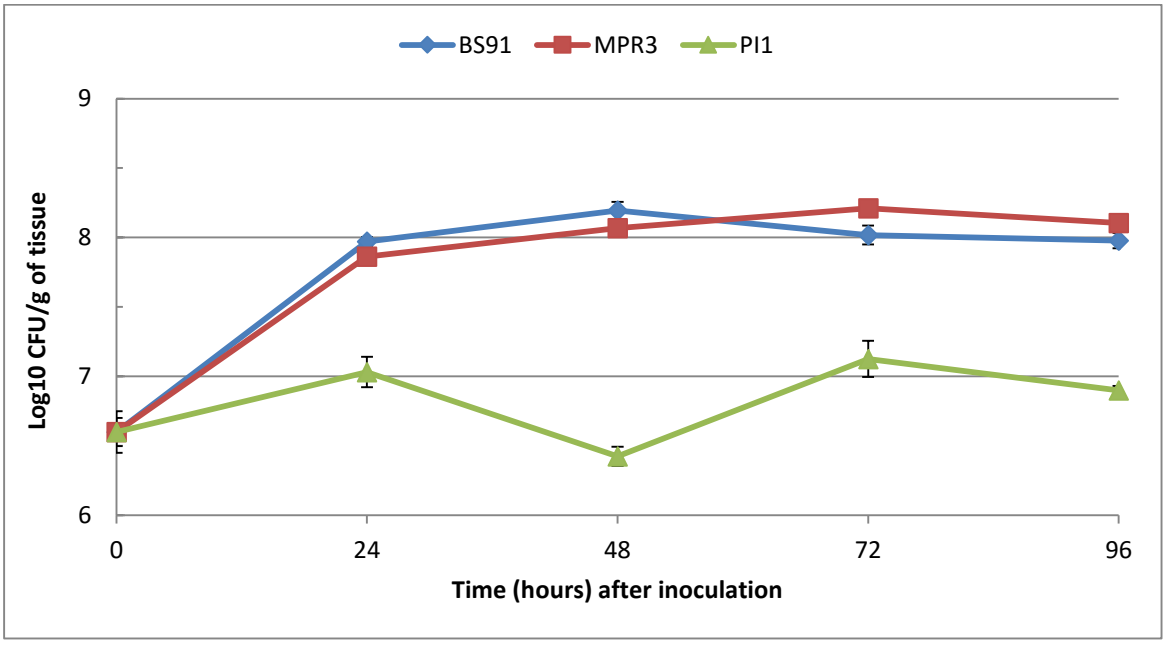

Fig. 1. Population dynamics of *Wickerhamomyces anomalus* BS91, *Metschnikowia pulcherrima* MPR3, and *Aureobasidium pullulans* PI1 assayed on artificially wounded and inoculated mandarin fruits.

4.3.3. Evaluation of yeast treatments in controling green and blue mold decays on wounded mandarin

Data of BCAs effects in controlling *P. digitatum* and *P. italicum* infections are reported in Fig. 2-a,b, respectively. Results clearly showed that tested yeast strains significantly reduced green and blue mold decays on wounded mandarins if compared with relative controls. Comprehensively, the best performances of BCA treatments in controlling penicillium infection were recorded for *P. italicum* (Fig. 2b).

In detail, the lowest DI, DS and LD caused by *P. digitatum* (Fig. 2a) were detected when mandarins were treated with *M. pulcherrima* MPR3 followed by *A. pullulans* and *W. anomalus*.

M. pulcherrima MPR3 also strongly inhibited blue mold decay caused by *P. italicum*, showing the significantly lowest DI value if compared with remaining treatments (Fig. 2b). Lowest DS and LD values were recorded for both *M. pulcherrima* MPR3 and *W. anomalus* BS91. Although with lower effect, also *A. pullulans* inhibited *P. italicum* infections showing significantly reduction of DI, DS and LD values if compared to the relative controls.

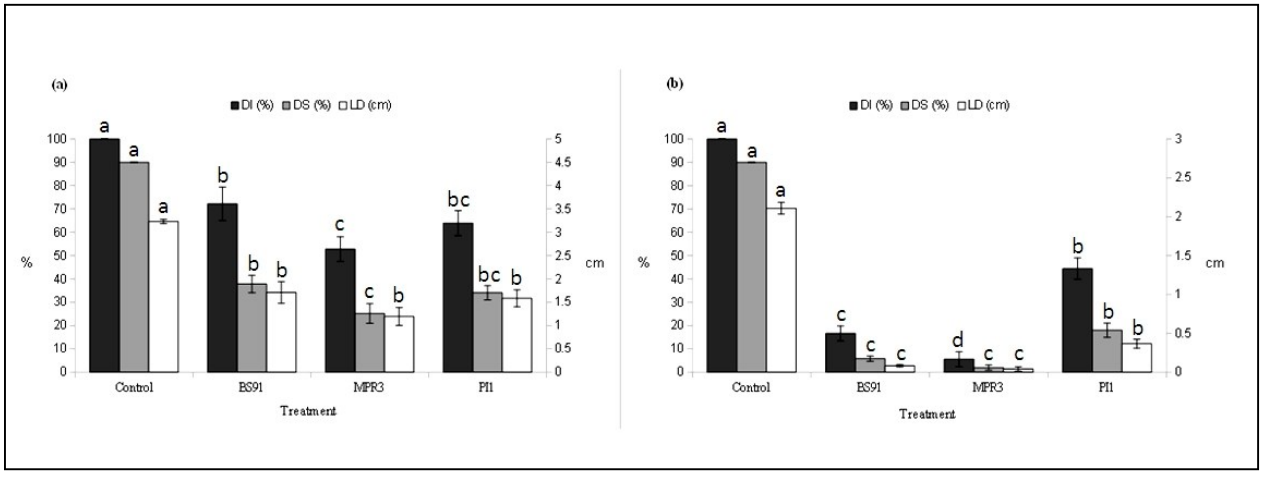

Fig. 2. Effect of *Wickerhamomyces anomalus* BS91, *Metschnikowia pulcherrima* MPR3, and *Aureobasidium pullulans* PI1 in controlling green and blue mold decays caused by *Penicillium digitatum* (a) and *Penicillium italicum* (b) on wounded mandarin fruits. BCAs efficacy is referred to disease incidence (DI), disease severity (DS) and lesion diameter (LD) caused by fungi 5 days after inoculation. Bars show the standard error of the mean. Columns (treatments) with the same color (i.e. disease parameter) followed by different letters are significantly different according to Fisher's least significant difference test ($p = 0.05$).

4.3.4. *Evaluation of BCAs and LBG coating efficacy in reducing green mold decay on mandarin fruit*

Data of yeast performances and LBG effects against *P. digitatum* are reported in Fig. 3 and Tab. 2, respectively.

All yeast strains (BS91, MPR3 and PI1) always reduced significantly disease parameters (DI, DS

and LD) in mandarin fruits within each dipping treatment (H_2O and LBG coatings) if compared with relative controls (Fig. 3). However, *M. pulcherrima* MPR3 showed averagely the best performances than those of *W. anomalus* BS91 and *A. pullulans* PI1, especially when *M. pulcherrima* MPR3 was dispersed into LBG 1%.

Overall, the use of BCAs + LBG reduced significantly the disease parameters on treated mandarin (Tab. 2). In detail, the reduction of DI, DS and LD values was further enhanced by LBG 1% in presence of *M. pulcherrima* MPR3. Otherwise, the increasing rate effect of LBG was not observed for *W. anomalus* BS91 and *A. pullulans* PI1. For these latter yeasts, LBG 0.5% induced lower LD values although not always significant.

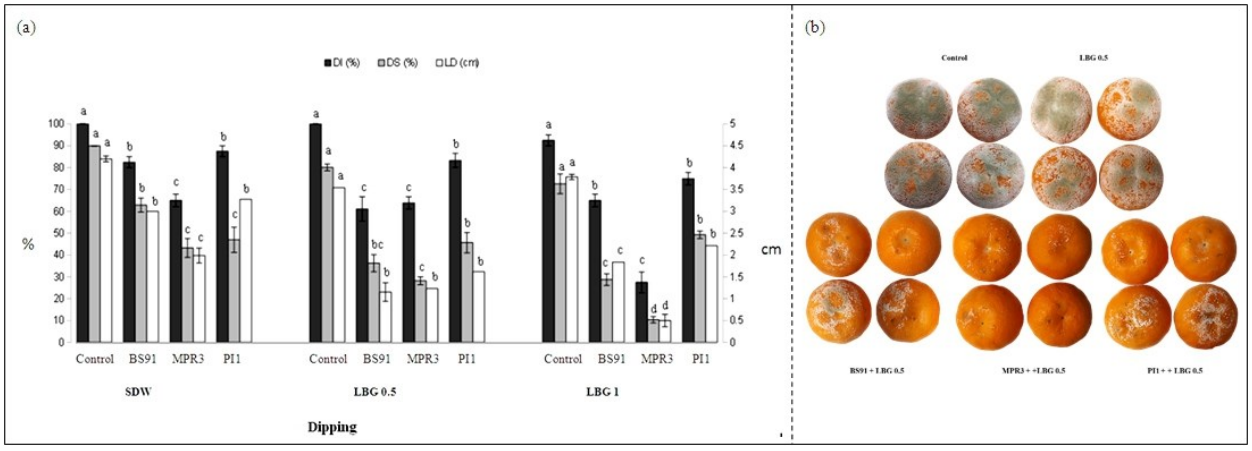

Fig. 3. (a) Effect of BCAs and LBG coating in reducing green mold decay caused by *Penicillium digitatum* on mandarin fruits. Efficacy of *Wickerhamomyces anomalus* BS91, *Metschnikowia pulcherrima* MPR3 and *Aureobasidium pullulans* PI1 suspended on SDW, LBG 0.5% and LBG 1%, is referred to DI, DS and LD parameters. Bars indicate standard error of the mean. Columns with the same color within each dipping treatment followed by same letters are not significantly different according to Fisher's least significant difference test ($p = 0.05$). (b) Activity of BCAs with LBG 0.5% against artificial infection caused by *P. digitatum* on mandarin fruits.

Table 2. Rate effects of LBG coating in enhancing reduction of green mold decay.

	Treatments											
	Control			*W. anomalus* BS91			*M. pulcherrima* MPR3			*A. pullulans* PI1		
	SDW	LBG 0.5	LBG 1	SDW	LBG 0.5	LBG 1	SDW	LBG 0.5	LBG 1	SDW	LBG 0.5	LBG 1
DI (%)	100 a	100 a	92.5 b	82.5 a	61.1 b	65.0 b	65.0 a	63.8 a	27.5 b	87.5 a	83.3 ab	75.0 b
DS (%)	90.0 a	80.1 b	72.6 b	62.8 a	36.2 b	28.7 b	43.2 a	28.2 b	10.4 c	47.0 a	45.7 a	49.2 a
LD(cm)	4.20 a	3.54 c	3.79 b	3.00 a	1.15 c	1.84 b	1.99 a	1.23 b	0.50 c	3.28 a	1.62 b	2.21 b

Data are means of 3 replicates. In each row, values followed by different letters within the same treatment (LBG rate effect) are significantly different according to Fisher's honest significant difference test ($p = 0.01$).

4.3.5. Evaluation of BCAs and LBG coating efficacy in reducing blue mold decay on mandarin fruit

Results of yeast and LBG effects against *P. italicum* are reported in Fig. 4 and Tab. 3, respectively.

W. anomalus BS91, *M. pulcherrima* MPR3 and *A. pullulans* PI1 always reduced significantly DI, DS and LD values of blue mold decay of mandarin fruits coated with different matrices (Fig. 4) inducing slightly higher reductions than those detected for *P. digitatum*. *M. pulcherrima* MPR3 partially confirmed the previous performances reported for *P. digitatum*.

Unlike to the previous experiment, the effect related to increase of LBG concentration in enhancing decay reduction was not detected for *M. pulcherrima* MPR3. Moreover, LBG 0.5% allowed a reduction of blue mold decay although statistically significant only for *A. pullulans* (Tab. 3).

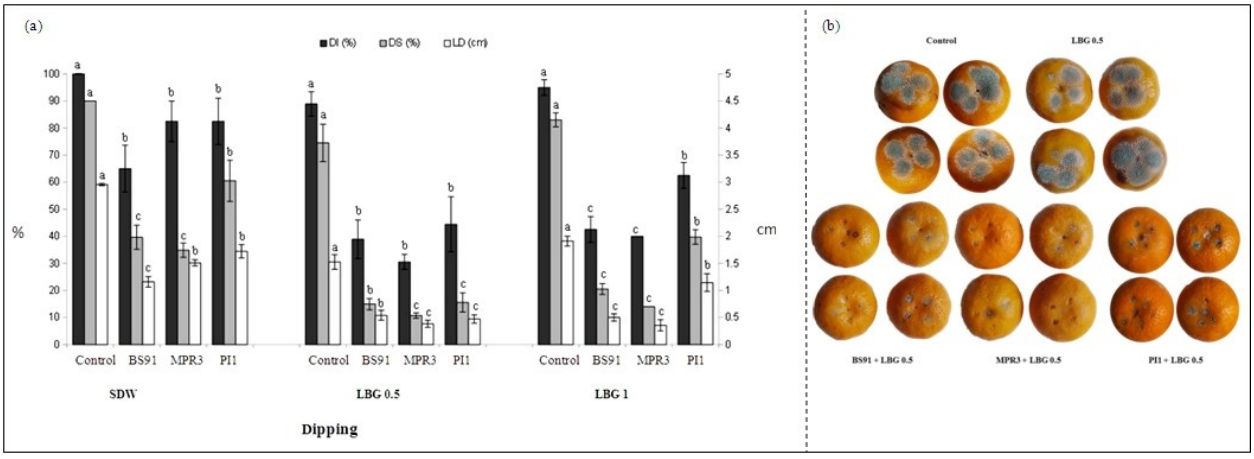

Fig. 4. (a) Effect of BCAs and LBG coating in reducing blue mold decay caused by *Penicillium italicum* on mandarin fruits. Efficacy of *Wickerhamomyces anomalus* BS91, *Metschnikowia pulcherrima* MPR3 and *Aureobasidium pullulans* PI1 suspended on SDW, LBG 0.5% and LBG 1%, is referred to DI, DS and LD parameters. Bars indicate standard error of the mean. Columns with the same color within each dipping treatment followed by same letters are not significantly different according to Fisher's least significant difference test ($p = 0.05$). (b) Activity of BCAs with LBG 0.5% against artificial infection caused by *P. italicum* on mandarin fruits.

Table 3

Rate effects of LBG coating in enhancing reduction of blue mold decay.

	Treatments											
	Control			***W. anomalus* BS91**			***M. pulcherrima* MPR3**			***A. pullulans* PI1**		
	SDW	**LBG 0.5**	**LBG 1**	**SDW**	**LBG 0.5**	**LBG 1**	**SDW**	**LBG 0.5**	**LBG 1**	**SDW**	**LBG 0.5**	**LBG 1**
DI (%)	100 a	88.8 b	95.0 ab	65.0 a	38.9 b	42.5 ab	82.5 a	30.5 b	40.0 b	82.5 a	44.4 b	62.5 ab
DS (%)	90.0 a	74.5 b	83.0 ab	39.6 a	14.9 b	20.5 b	34.9 a	10.7 b	14.0 b	60.5 a	15.5 c	39.7 b
LD(cm)	2.95 a	1.52 b	1.91 c	1.16 a	0.54 b	0.50 b	1.51 a	0.38 b	0.35 b	1.72 a	0.47 c	1.14 b

Data are means of 3 replicates. In each row, values followed by different letters within the same treatment (LBG rate effect) are significantly different according to Fisher's honest significant difference test ($p = 0.01$).

4.3.6. Viability of yeasts on mandarin fruit in different dipping treatment

Survival data of *W. anomalus* BS91, *M. pulcherrima* MPR3 and *A. pullulans* PI1 immediately following treatments and after 4 days of storage are reported in Fig. 5. Data clearly showed as LBG matrices are always able to significantly enhance the viability of all yeast strains. A significant effect of increasing the LBG concentration was always detected for each tested yeast. In addition, *M. pulcherrima* strain averagely showed the highest values of viability over time among tested strains, whereas *A. pullulans* PI1 revealed the least persistence on mandarin fruit (*significant data, not shown*).

4.3.7. Influence of yeast treatment on enzyme activities of mandarins

The POD activity in mandarin peel after treatments with *M. pulcherrima* MPR3 and *W. anomalus* BS91was greatly induced and markedly higher than the control, reaching a maximum at 24 h. In mandarin tissues treated with *A. pullulans* PI1, POD activity was slightly reduced up to 48 h and increased thereafter, reaching at 72 h the highest level (Fig. 6a).

The SOD activity of mandarin peel treated with *W. anomalus* BS91 and *A. pullulans* PI1 yeast strains declined at first, although enzyme levels at 24 h showed higher values compared to the control. Subsequently, SOD activity tended to be steady for *A. pullulans* PI1 and *M. pulcherrima* MPR3 and to increase for *W. anomalus* BS91 (Fig. 6b). A different trend was observed after *M. pulcherrima* MPR3 treatment within 24 h, as SOD activity was slightly induced. On the whole, SOD activity with *A. pullulans* PI1 treatment was higher than that of the other yeasts and the control from 0 to 48 h. All values of SOD activity became similar at 72 h after incubation.

4.4. DISCUSSION

This paper provided useful information investigating the potential use of some yeasts as BCAs in controlling green and blue mold postharvest decays on mandarin fruits. A successful biocontrol strategy depends on BCA adaptability to storage conditions, host fruit, target pathogen as well as on the application mode of antagonist. For these reasons, the effects of *M. pulcherrima* MPR3*, W. anomalus* BS91 and *A. pullulans* PI1 were preliminarily assessed *in vitro* and, subsequently, *in vivo* conditions by artificial inoculation of wounded mandarin fruits. Moreover, the performances of these BCAs were evaluated using an integrated approach based on dipping application of LBG incorporating yeast strains, resembling to commercial processing conditions. *In vitro* data clearly showed that all tested yeasts were able to reduce mycelial growth of *P. digitatum* and *P. italicum* at different pH conditions, although the best performances were always detected for *A. pullulans* PI1. Otherwise, *in vivo* findings revealed the best performances for *M. pulcherrima* MPR3 and *W. anomalus* BS91 on wounded mandarin artificially inoculated with green and blue mold molds. Localized application of BCAs on wounded mandarin fruits enhanced the yeast ability in controlling target pathogens, probably due to the higher concentration of BCAs on mandarin wound. These results were confirmed, but to a lesser extent, by dipping treatments with LBG carrying yeast strains. Comprehensively, dipping experiments revealed a higher efficacy of yeast strains when incorporated in LBG if compared with yeast strains suspended in SDW, due to the ability of the LBG matrix to enhance survival of the yeasts on mandarin peel. *M. pulcherrima* MPR3 resulted to be particularly promising when dispersed into LBG1% in controlling green mold decay. Moreover, both LBG concentrations resulted equally effective in enhancing MPR3 antagonistic activity against blue mold decay of mandarin. Otherwise, potential use of lower LBG rate can be suggested for *W. anomalus* BS91 and *A. pullulans* PI1 that showed the best efficacy at

LBG 0.5%. This could be due to the different biocontrol mechanism recently identified for the same strains (Parafati et al., 2015) and mainly based on the production of lytic enzymes, whose diffusion may be decreased by the higher concentration (1%) of the polysaccharidic matrix.

As biocontrol activity is related to the tri-trophic interaction "yeast-pathogen-fruit", the induction of resistance, like the stimulation of POD and SOD enzymes in mandarin peel, may be involved in yeast biocontrol mechanism. The induction of POD enzyme in mandarin may be involved in the biocontrol efficacy of *M. pulcherrima* MPR3 and *W. anomalus* BS91 against *P. italicum*, as they were the most effective in reducing the disease incidence and severity of blue mold *in vivo*. On the other hand, different response of POD activity to *W. anomalus* BS91 and *A. pullulans* PI1 application was not useful alone to explain comparable efficacy results in *in vivo* assays against *P. digitatum.* Otherwise, SOD response at 24 h of mandarin peel treated with all tested BCAs showed higher levels than the control, indicating a likely contribute to their biocontrol efficacy.

The results of this study indicated that the incorporation of yeast strains into safe and edible LBG coatings can represent an effective approach for the long-term maintenance of yeast cell viability and, at the same time, of the biocontrol efficacy in reducing green and blue mold decays under commercial conditions. However, further studies under semi-commercial and large-scale conditions should be addressed for a deeper evaluation of potential use of these BCAs.

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CHAPTER 5. PRODUCTION OF VOLATILE ORGANIC COMPOUNDS BY SELECTED YEAST STRAINS AS A POTENTIAL MECHANISM OF ACTION AGAINST POSTHARVEST FRUIT PATHOGENS

5.1. INTRODUCTION

Volatile organic compounds (VOCs) are low molecular weight substances (usually <300 Da) with low polarity, but with high vapor pressure (Vespermann et al., 2007).

Antimicrobial VOCs, produced by bacteria such as B*acillus subtilis* (Chen et al., 2008), *Streptomyces* spp. (Wan et al., 2008), filamentous fungi such as *Muscodor albus* (Mercier and Manker, 2005), higher plants such as 'Isabella' grapes (*Vitis labrusca*) (Kulakiotu et al., 2004) and yeasts such as *Candida intermedia* and *Sporidiobolus pararoseus* (Huang et al., 2011; Huang et al., 2012) were reported to be effective in suppressing both the conidial germination and the mycelial growth of *B. cinerea* on agar media and in suppressing Botrytis diseases on plant tissues.

VOCs produced by *S. pararoseus* were mainly represented by 2-ethyl-1-hexanol, effective in suppression of both conidial germination and mycelial growth of *B. cinerea* (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). More recently Parafati et al. (2015) reported that the biocontrol ability of *W. anomalus, M. pulcherrima, A. pullulans* and *S. cerevisiae* strains was also related to the production of VOCs, effective *in vitro* and *in vivo* against *B. cinerea*.

The production of VOCs has been observed also in other yeast species: *Meyerozyma guilliermondii* (formerly *Pichia guilliermondii*) has antifungal activity, based mainly on the production of ethyl-

acetate (Coda et al., 2013) and of helvolic acid, which was shown to exert a marked inhibitory activity on the spore germination of *Magnaporthe oryzae* (Zhao et al., 2010), while the 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 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 capacity ability of *W. anomalus* can be attributed to the production of 2-phenyl ethanol, which affects spore germination, growth, toxin production, and gene expression in *A. flavus.* Moreover, Di Francesco *et* al., (2015) demonstrated the essential role of VOCs produced by two strains of *A. pullulans* in inhibiting 5 post harvest pathogens (*B. cinerea, Colletotricum acutatum, P. expansum, P. digitatum* and *P. italicum*) and tested the effect of pure VOCs against the same pathogens.

Ethyl-acetate is the most important ester synthesized by yeasts during fermentation (Swiegers and Pretorius, 2005). Studies by Druvefors et al. (2005) and Coda et al. (2011) showed that ethyl-acetate is the key antifungal compound synthesized by *Pichia* spp. A marginal, but synergic, role was attributed to ethanol (Druvefors et al., 2005; Coda et al., 2011).

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

The aim of the present work was to evaluate the efficacy of antifungal volatile organic compounds (VOCs) produced *in vitro* and *in vivo* by antagonistic yeasts using a potential commercial carrier with the dual function to immobilize and at the same time to provide nourishment for the microorganism.

5.2. MATERIALS AND METHODS

5.2.1. Microorganisms and culture conditions

The four yeasts 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 tested yeasts were identified as *M. pulcherrima* MPR3, *W. anomalus* BS91, *A. pullulans* PI1 and *S. cerevisiae* BCA61 by sequencing the D1/D2 region of the 26S rRNA gene. Moreover, *W. anomalus* strain was selected for its high killing capacity against sensitive *S. cerevisiae* strain and the toxic mechanism was identified as a β-glucanase (Muccilli et al., 2013).

B. cinerea was isolated from infected strawberry fruits in Sicily (Italy) and were identified and tested for pathogenicity. *Penicillium digitatum* and *P. italicum* mold strains were isolated from infected mandarin fruits in Sicily (Italy) and were identified and tested for pathogenicity as previously described (Oliveri et al., 2007). 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).

5.2.2. Fruits

Strawberry and mandarin fruits were obtained from commercial organic orchards in Sicily (Italy). All fruits were immediately refrigerated after harvest and processed within 3 h.

5.2.3. Immobilization and survival of yeasts strains on commercial polyacrylamide hydrogel spheres

Commercial polyacrylammide hydrogels spheres were used as supports for the immobilization of the yeast strains, as they possess unique properties such as high water retention capacity, softness, flexibility and biocompatibility (Caló and Khutoryanskiy, 2015). 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 H_2O] broth (NYDB) was inoculated with 10 g of polyacrylamide hydrogel spheres and 1 mL 10^9 yeast cell suspension of *M. pulcherrima, W. anomalus, A. pullulans* or *S. cerevisiae* and incubated for 3 day at 20 °C and 200 rpm.

Three days after incubation the spheres, that have absorbed the culture broth containing the yeasts, were rinsed twice with sterile distilled water (SDW) and incubated at 20 °C in an empty Petri plate. The survival and colonization ability of *M. pulcherrima, W. anomalus, A. pullulans* and *S. cerevisiae* on polyacrylammide hydrogels were assessed after 0, 1, 3, 7 and 10 days of incubation.

Polyacrylammide hydrogel samples were weighted and placed in tubes containing proportional amount of sterile Ringers solution (Oxoid), homogenized and sonicated for 5 minutes. Samples were then plated onto Sabouroud Dextrose Agar (SDA, Oxoid) added with Chloramphenicol (Oxoid) (100 mg/L) using a Spiral Plater Eddy Jet (IUL Instruments, Barcelona, Spain). Yeast colonies were counted after 48-72 h at 25 °C to calculate the means of colonies (log CFU) per gram of hydrogel. Three replicate of ten samples were used for each treatment. The experiment was performed twice.

5.2.4. VOCs efficacy of yeast inoculated polyacrylamide hydrogel spheres in vitro condition

A dual culture method was used to evaluate the efficacy of volatile organic compounds (VOCs) produced by *M. pulcherrima* MPR3, *A. pullulans* PI1, *W. anomalus* BS91 and *S. cerevisiae* yeast strains against *B. cinerea*, *P. digitatum* and *P. italicum*. Aliquots (20 µL) of the conidial suspension (10^6 conidia/mL) of mentioned pathogens were inoculated on PDA and dried at room temperature. Polyacrylammide hydrogels spheres used as supports for the immobilization of the yeasts, as described above, were used to cover the surface of a blank Petri plate. Three plates for each yeast strain were used. The plates with *B. cinerea, P. digitatum* and *P. italicum* conidia were individually covered face to face above dishes containing inoculated hydrogels spheres (Fig. 1). The control was prepared placing in the plates polyacrylammide hydrogels spheres absorbed only with YPDB. The two plates were sealed together with Parafilm®, two revs around the edges to prevent air leakage. Plates containing *B. cinerea* conidial suspension were incubated at 20 °C while plate containing *P. digitatum* and *P. italicum* conidial suspension were incubated a 25 °C. The radial growth reduction of molds was calculated, after 10 days of incubation, in relation to growth of the control as follows: %I=(C-T/C) ×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. Three replicates for each mold–yeast combination were set and each experiment was repeated twice.

5.2.5. Microscopic observations of the pathogen mycelium development

The fungal mycelium was microscopically observed at 40× and 100 × magnification after 10-15 days of incubation on PDA to assess any hyphal damage caused by VOCs produced by yeast strains. Hyphal morphology of *B. cinerea*, *P. digitatum* and *P. italicum* was observed when

mycelial samples were removed from PDA plates exposed to VOCs produced by yeast strains entrapped into the hydrogel beads, as previously described. Hyphae were photographed, and their morphologies were compared with those of mycelia unexposed to VOCs (control).

5.2.6. Efficacy yeast inoculated in polyacrylamide hydrogel spheres in vivo condition

M. pulcherrima MPR3, *A. pullulans* PI1, *W. anomalus* BS91 and *S. cerevisiae* BCA61 yeast strains were used for subsequent *in vivo* test in order to assess the efficiency of the yeast VOCs in controlling gray, green and blue mold.

Healthy and homogenous fruits were selected and randomly assigned to different treatments. Before inoculation and treatment, mandarins and strawberry were washed with tap water, disinfected by dipping respectively for 2 min in 2% (wt/vol) and 1min in 1% (wt/vol) of sodium hypochlorite (NaOCl) solution, rinsed with sterile distilled water (SDW) and then air-dried.

Artificial wounds were performed using a sterile needle to make 3 mm deep and 3 mm wide wounds along the basal areas of mandarins (four for each fruit) and along the equatorial areas of strawberries (one for each fruit). Each wound was inoculated with 20 µL drop of 1×10^5 conidia/mL of *B. cinerea* for strawberries and 20 µL drop of 1×10^5 conidia/mL of *P. digitatum* or *P. italicum* for mandarin fruits.

The yeast antagonists were adsorbed as previously described in hydrogel beads, which were used to form an even layer in the lower part of experimental plastic packaging trays, endowed with a double bottom. Ten fruits were placed on the top layer of the trays, which were covered with a plastic lid. The trays containing mandarin and strawberry fruits were incubated respectively at 25 °C for 5 days and 20 °C for 10 day, after inoculation to provide favorable conditions for the postharvest onset of the disease. The same packaging with inoculated fruits and hydrogel beads without the antagonists served as controls. Three replicate of ten fruits were used for each treatment.

Data concerning the disease incidence (DI) were transformed into arcsine square-root values to normalize the distribution before performing the analysis of variance. The disease severity (DS) was previously evaluated by using an empirical 1-to-4 rating scale: 1= no visible symptoms (0%); 2= soft rot (35%); 3= mycelium (65%); 4= sporulation (90%) before analysis of variance. Average fruits severity was calculated by the following formula:

$$DS = \frac{\sum (Ci)}{N} \times 100$$

where *DS is* the average severity index, *C* the number of fruits in each class, *i (1-to-4)* the numerical values of classes, *N* the total number of fruits examined. Lesion diameter (LD) was also assessed by measuring the average diameter of the damaged area five days after pathogen inoculation.

5.2.7. Statistical analyses

Data from in vitro and in vivo experiments were analyzed separately by using the Statistica package software (Version 10; Statsoft Inc). In all repeated experiments, the arithmetic means were calculated and analyzed by using one-way analysis of variance (ANOVA).

Percentage data concerning DI were previously transformed using the arcsine transformation (sin-1 square root x). Fisher's least significant difference test was used to compare the significance of differences among in vitro and in vivo (DI, DS and LD) data at p = 0.01 and 0.05, respectively.

5.3. RESULTS

5.3.1. Colonization of polyacrylamide hydrogel spheres

The viability of yeast strains on polyacrylamide hydrogel spheres over ten days of incubation is reported in Fig 1. Analysis of spheres, immediately after incubation and subsequent repeated washes (time 0), showed higher concentration values of *M. pulcherrima* MPR3 and *W. anomalus* BS91 strains (6.65 and 6.62 log CFU/g, respectively) in comparison with *A. pullulans* PI1 and *S. cerevisiae* BCA61 strains (5.90 and 5.91 log CFU/g, respectively).

The population of the yeasts increased after 1 d, then resulting approximately stable. A lower cell concentration was detected for *A. pullulan* strain.

Only after 7 days of incubation, the population of all yeasts strains slowly started to decrease. At 10 days population densities of MPR3, BS91, PI1 and BCA61, reached levels of 7.27, 7.09, 6.53 and 6.97 respectively (Fig.1).

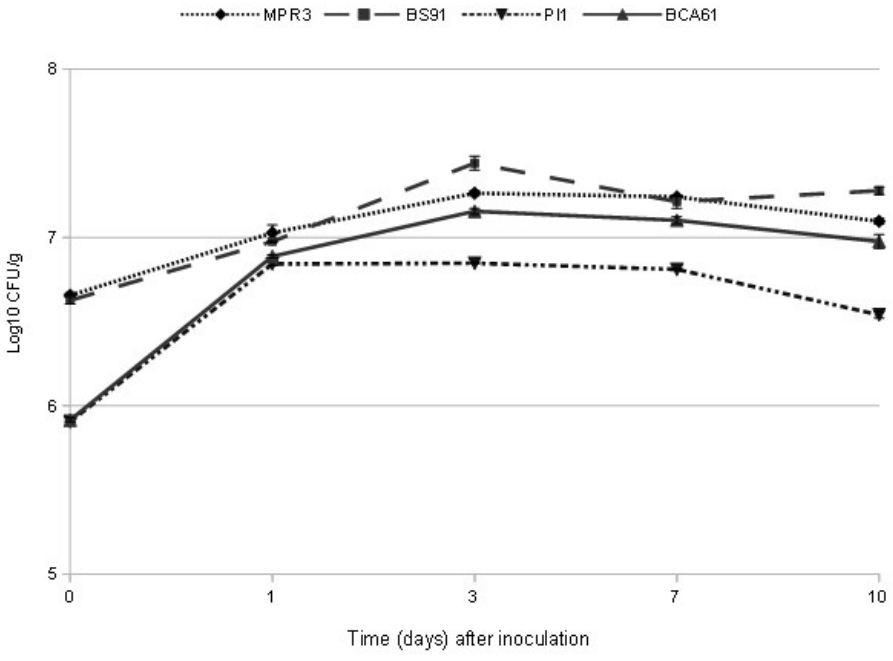

Fig. 1. Viability over time of yeast strains *M. pulcherrima* MPR3, *W. anomalus* BS91, *A. pullulans* PI1 and *S. cerevisiae* BCA61 on polyacrylamide hydrogel spheres.

5.3.2. In vitro antagonistic activity of VOCs against Botrytsis cinerea, Penicillium digitatum and Penicillium italicum

Data regarding the efficacy of VOCs in controlling *in vitro* growth of postharvest pathogens is reported in Table 1. Although with variable effects, VOCs produced by MPR3, BS91, PI1 and BCA61 were able to inhibit radial growth of *B. cinerea*, *P. digitatum* and *P. italicum* in comparison to the respective controls (Fig. 2).

In detail, *W. anomalus* BS91 showed the highest values of growth inhibition against all tested pathogens (100.0 ± 0.00 %, 97.30 ± 0.20 % and 100.0 ± 0.00% respectively against *B. cinerea*, *P. digitatum* and *P. italicum*).

Moreover, antifungal activity of VOCs generated by *A. pullulans* PI1 provided good result in reducing mycelial growth of *B. cinerea*. On the other hand, VOCs produced by *M. pulcherrima* MPR3 provided the greatest result in reducing *P. digitatum* and *P. italicum*.

Nonetheless, *P. digitatum* and *P. italicum* growth was less affected by *S. cerevisiae* BCA 61 VOCs.

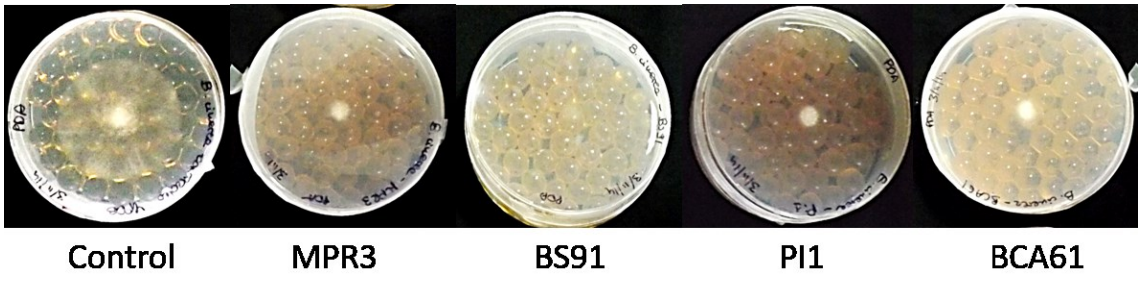

Fig. 2. Biocontrol activity of VOCs produced by *M. pulcherrima* MPR3, *W. anomalus* BS91, *A. pullulans* PI1, *S. cerevisiae* BCA61 (immobilized on polyacrylammide hydrogels beads) in inhibiting *B. cinerea* mycelia growth on PDA. Results 5 days after incubation are shown.

Table 1. *In vitro* antagonistic activity assay of the yeast strains

Inhibition of mycelial growth of target pathogens (%) [a] by VOCs			
Yeast [b]	***B. cinerea***	***P. digitatum***	***P. italicum***
M. pulcherrima	67.22 ± 0.03 c	65.54 ± 0.72 b	65.05 ± 0.51 b
W. anomalus	100.0 ± 0.00 a	97.30 ± 0.20 a	100.0 ± 0.00 a
A. pullulans	83.33 ± 0.00 b	23.31 ± 0.77 c	44.64 ± 0.14 c
S. cerevisiae	50.00 ± 0.06 d	10.14 ± 0.09 c	15.22 ± 0.16 d

[a] Antagonistic activity of yeasts was referred to mycelial growth reduction on PDA as follows: %I = (C-T/C) * 100, where %I represented the inhibition of radial mycelial growth, C was radial growth measurement in control and T was the radial growth of *B. cinerea*, *P. digitatum* or *P. italicum* in presence of VOCs produced by yeast strains.

[b] Data are means of 3 replicates ± standard error of the mean (SEM). Values followed by different letter within each column (yeast effect against *B. cinerea P. digitatum* or *P. italicum*) are significantly different according to Fisher's honest significant difference test ($p = 0.01$).

5.3.3. Microscopic observations of hyphal damage

Hyphal morphology of 10 days-old pathogen mycelia on PDA, exposed or unexposed to VOCs, was microscopically observed under 40× and 100× magnification. Microscopic analysis of pathogen mycelia exposed to yeast VOCs showed a less compact mycelium growth characterized by hyphal deformity if compared to the unexposed control. Hyphae appeared emptied of intracellular content, undefined, elongated and scattered whereas hypahea of control pathogens appeared turgid and well-extended (Fig. 3).

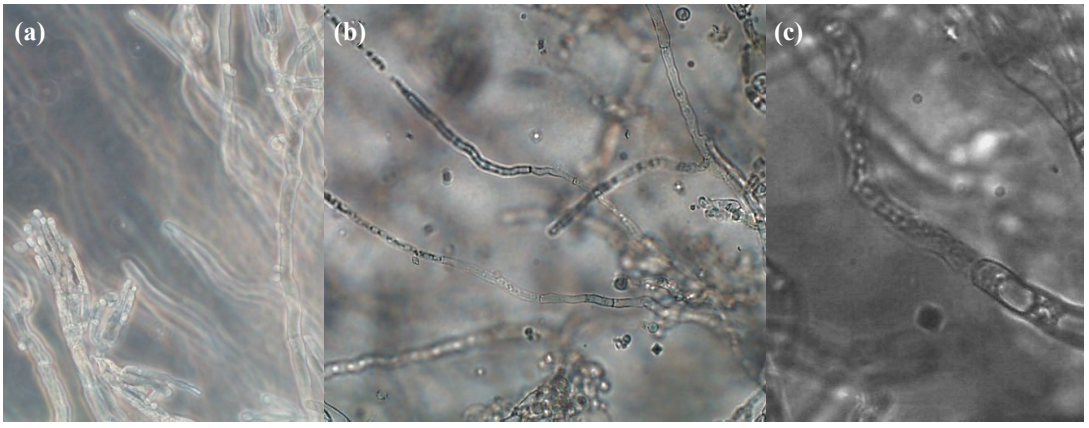

Fig. 3. Hyphal morphology of unexposed *P. digitatum* (control) under optical microscopy at 40 × magnification (a), hyphal morphology of *P. digitatum* exposed to VOCs produced by BS91 at 40 × magnification (b) and 100 × magnification (c).

5.3.4. In vivo efficacy of the VOCs in controlling gray mold decay of strawberry fruits

The effects of VOCs produced by tested yeasts against *B. cinerea* are reported in Fig. 4-a, b. VOCs produced by *W. anomalus* BS91 totally inhibited all diseases parameters of gray mold decay on strawberry fruits, if compared to the control. Moreover, data clearly showed as *A. pullulans* significantly reduced DI, DS and LD, while VOCs produced by *M. pulcherrima* and *S. cerevisiae* showed significant reduction of DS and LD parameters.

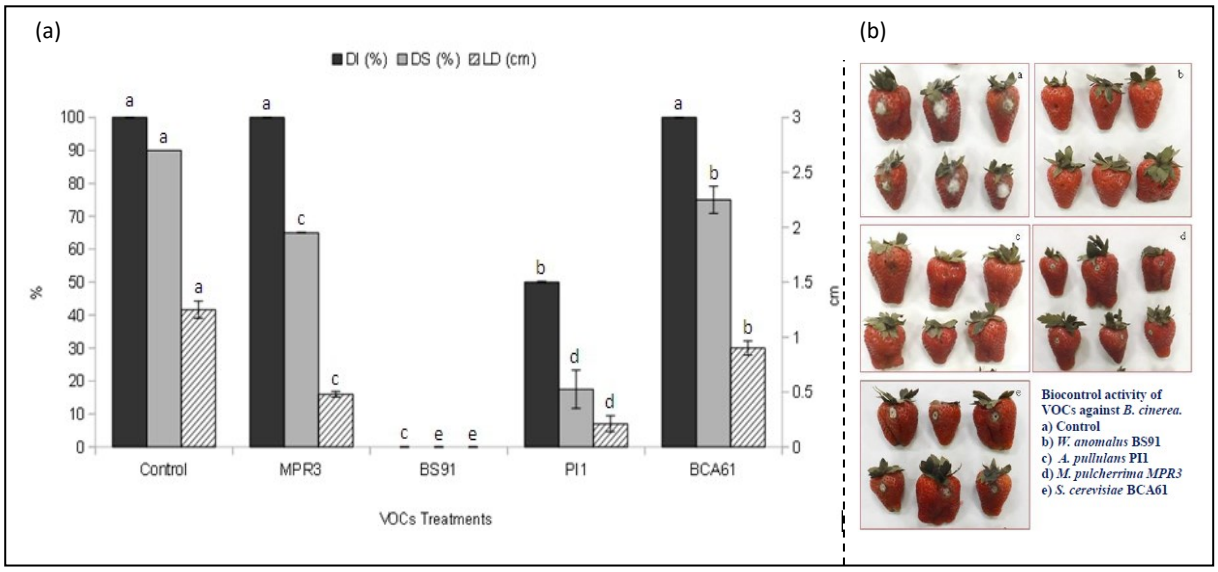

Fig. 4. Effect of VOCs produced by *Metschnikowia pulcherrima* MPR3, *Wickerhamomyces anomalus* BS91, *Aureobasidium pullulans* PI1 and *S. cerevsiae* BCA61 in controlling gray mold decays caused by *Botrytis cinerea* on wounded strawberry fruits. (a) BCAs efficacy is referred to disease incidence (DI), disease severity (DS) and lesion diameter (LD) caused by fungi 5 days after inoculation. Bars show the standard error of the mean. Columns (treatments) with the same color (i.e. disease parameter) followed by different letters are significantly different according to Fisher's least significant difference test ($p = 0.05$). (b) Activity of VOCs produced by different BCAs against artificial infection caused by *B. cinerea* on strawberry fruits.

5.3.5. In vivo efficacy of the VOCs in controlling green and blue molds decay on mandarin fruits

The effects of VOCs, produced by yeast strains, in controlling *P. digitatum* and *P. italicum* infection on mandarin fruits are reported in Fig. 5 and Fig. 6 respectively.

Comprehensively, the best performance in controlling *P. digitatum* infection was recorded for VOCs produced by *W. anomalus* BS91, showing the lowest DI, DS and LD, if compared with remaining treatments (Fig. 5). Even if with lower efficacy, also *M. pulcherrima* MPR3, *A. pullulans* PI1 and *S. cerevisiae* BCA6 inhibited *P. digitatum* infection, showing significant reduction of DS and LD values if compared to the control.

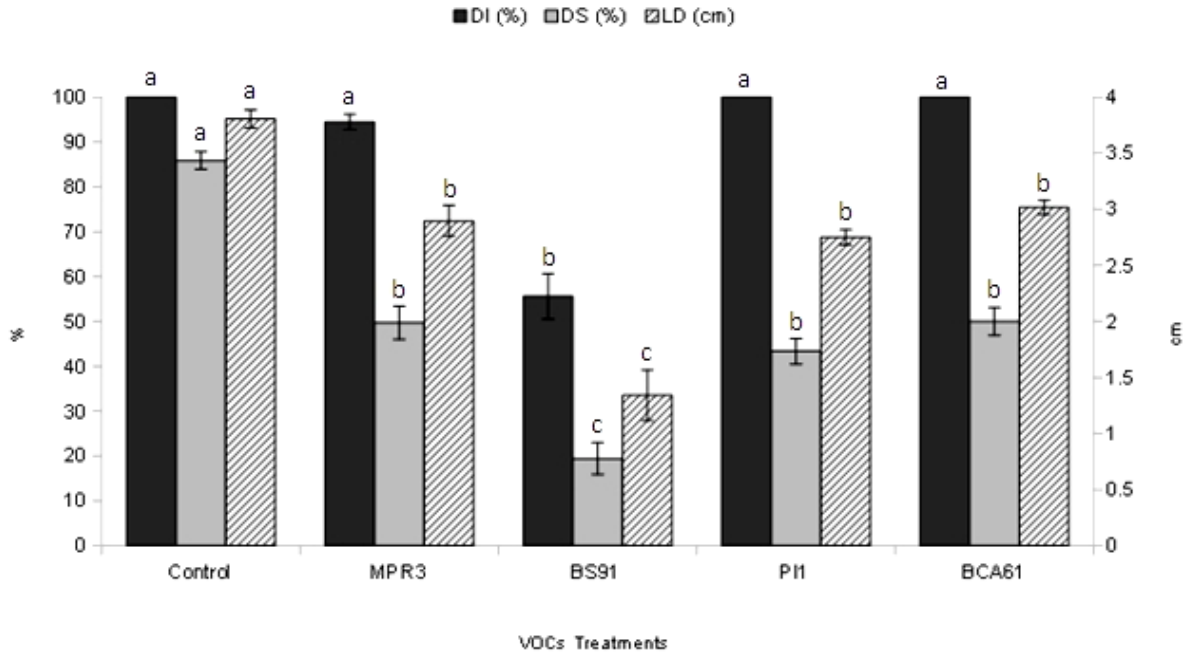

Fig. 5. Effect of VOCs produced by *Metschnikowia pulcherrima* MPR3, *Wickerhamomyces anomalus* BS91, *Aureobasidium pullulans* PI1 and *S. cerevsiae* BCA61 in controlling green mold decay caused by *Penicillium digitatum* on wounded mandarin fruits. BCAs efficacy is referred to disease incidence (DI), disease severity (DS) and lesion diameter (LD) 5 days after inoculation. Bars show the standard error of the mean. Columns (treatments) with the same color (i.e. disease parameter) followed by different letters are significantly different according to Fisher's least significant difference test ($p = 0.05$).

Reduction of diseases parameters caused by *P. italicum* were also observed when mandarins were treated with VOCs produced by yeast strains. In detail, significantly lowest DI, DS and LD were recorded by using *A. pullulans* PI1 followed by *W. anomalus* BS91. Otherwise significant reduction of diseases parameters were not observed by using *M. pulcherrima* MPR3 and *S. cerevisiae* BCA61 (Fig. 6).

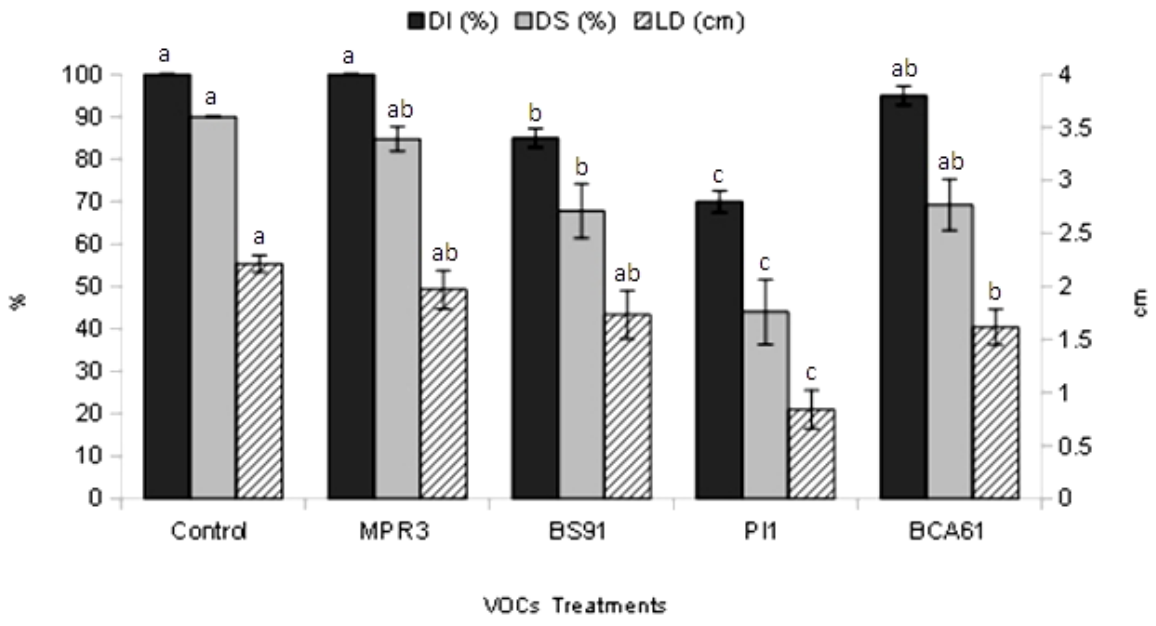

Fig. 6. Effect of VOCs produced by *Metschnikowia pulcherrima* MPR3, *Wickerhamomyces anomalus* BS91, *Aureobasidium pullulans* PI1 and *S. cerevsiaei* BCA61 in controlling blue mold decay caused by *Penicillium italicum* on wounded mandarin fruits. BCAs efficacy is referred to disease incidence (DI), disease severity (DS) and lesion diameter (LD) 5 days after inoculation. Bars show the standard error of the mean. Columns (treatments) with the same color (i.e. disease parameter) followed by different letters are significantly different according to Fisher's least significant difference test ($p = 0.05$).

5.4. DISCUSSION

Several mechanisms, such as competition for space and nutrients, parasitism, secretion of antifungal compounds, induction of resistance and biofilm formation, have been investigated as responsible for the antagonistic activity of yeast BCAs (Liu et al., 2013). Among these ones, the ability of yeasts to produce volatile organic compounds (VOCs) with antifungal activity may represent an important tool for biological control of a wide range of postharvest pathogens (Di Francesco et al., 2015, Fiori et al., 2014, Huang et al., 2012). Fungi are known to produce a broad

variety of VOCs, mainly identified as alcohols, aldehydes, esters, and many other compounds at very low concentration (Fialho et al., 2010, Buzzini et al., 2003). Therefore, efficacy of VOCs is not depending from a single component activity but seems to be correlated to the synergic effect of a large number of compounds (Mercier and Jimenez, 2004; Strobel et al., 2008).

Nowadays a potential limitation to the use of microorganisms as postharvest biocontrol agents is due to their adaptability and viability to fruit and storage environment. Developing strategy to enhance yeasts viability and stress tolerance is essential to improve their efficacy and potential commercial application. This work afforded useful information for investigating the potential use of commercial polyacrylammide hydrogels spheres as support for the immobilization of BCAs as well as for proposing a potential and inexpensive tool for releasing VOCs produced by yeasts. Selected yeast strains, *W. anomalus* BS91, *M. pulcherrima* MPR3, *A. pullulans* PI1 and *S. cerevisiae* BCA61, entrapped on polyacrylammide support, showed great ability to survive and to increase the cell number, maintaining high population levels up to 10 days.

The antifungal effect of VOCs produced by immobilized yeasts was tested *in vitro* and *in vivo* conditions against *B. cinerea*, *P. digitatum* and *P. italicum.*

In vitro conditions, all tested yeasts were able to reduce radial growth of tested pathogens. In particular, *W. anomalus* showed a total inhibition of *B. cinerea* and *P. italicum* (100%) and a strong inhibition of *P. digitatum* (97.30 %) up to 10 days.

In vivo findings also revealed good performances of all selected yeasts, in reducing disease parameters on strawberries artificially infected with *B. cinerea*. Also in such conditions, the best results were obtained with *W. anomalus* BS91, which totally inhibited gray mold decay. The inhibitory effect of VOCs was further proven on artificially inoculated mandarins. Once again *W. anomalus* BS91 showed the best efficacy in reducing green mold decay caused by *P. digitatum*, while *A. pullulans* PI1 was more effective in reducing blue mod decay caused by *P. italicum.*

The present study demonstrated as production of VOCs can play an essential role in the antagonistic activity of BCAs, although all yeasts were comprehensively more effective against gray mod decay, suggesting greater sensitivity of *B. cinerea* to the VOCs. Moreover future use of polyacrylammide hydrogel spheres can be suggested as a tool for VOCs release in packaging, as they are able to enhance viability and to support yeast growth. Future studies are necessary to investigate the compounds emitted by each yeast strain and to test the antifungal effect of pure compounds on target pathogens.

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CHAPTER 6. POTENTIAL ROLE OF EXOGLUCANASE GENES (*WAEXG1* AND *WAEXG2*) INDUCED IN WICKERHAMOMYCES ANOMALOUS

6.1. INTRODUCTION

Biological approaches used to manage postharvest decay in fruit and vegetables include the use of antagonistic microorganisms as biocontrol agents (BCAs). Understanding the mechanism of action of BCAs is essential for using them in a safe and effective manner. The biocontrol activity associated with microbial antagonists used to manage postharvest diseases has been reported to be associated with their ability to compete with pathogens for nutrients and space, adhere to host and pathogen tissues, induce host resistance, secrete organic acids, lytic enzymes, and in some cases killer toxins (Muccilli and Restuccia 2015, Spadaro and Droby, 2015, Liu et al., 2013).

In general, killer toxins belong to the family of glucanase enzymes acting on glucan, a main structural constituent of the cell walls of yeast and other fungi (Satynarayana and Kunze, 2009). Killer toxins, also called mycocins, are increasingly being investigated due to the importance that these enzymes have in the postharvest and food industry (Serviene et al., 2012).

Wickerhamomyces anomalus, strain BS91*,* has shown potential to be used as a postharvest biocontrol agent against *Penicillium digitatum* on 'Tarocco' and 'Valencia' orange fruits (Platania et al., 2012; Aloui et al., 2015) and against *Botrytis cinerea* on grapes (Parafati et al., 2015). This strain is known to produce killer toxins that have been demonstrated to be exoglucanases, coded by the genes *WaEXG1* and *WaEXG2* (Mucilli et al., 2013). Glucanase production and secretion by antagonistic yeasts has been reported to be one of the mechanisms by which yeast biocontrol agents inhibit other fungi, especially postharvest pathogens. *WaEXG1* and *WaEXG2*, however, may have

variable levels of expression depending on nutrient conditions and/or the presence or absence of other fungi. This aspect, however, has not been investigated in any detail.

The objective of the present study was to characterize the expression level of these genes when the yeast was interacting with two different postharvest pathogens on two different fruit hosts (*W. anomalus*-orange-*P.digitatum*, *W. anomalus*-grape-*B. cinerea)*. Using RT-qPCR, gene expression during these interactions was compared to the expression of these genes when the yeast was grown in in NYDB (control).

6.2. MATERIALS AND METHODS

6.2.1. Yeast and pathogen cultures

W. anomalus, strain BS91, belonging to Di3A (Dipartimento di Agricoltura, Alimentazione e Ambiente, University of Catania, Italy) collection, was isolated from naturally fermented olive brine and identified by sequencing the D1/D2 region of the 26S rRNA gene (Muccilli et al., 2011). *B cinerea, P. digitatum, Penicillium expansum, Penicillium italicum* and *Monilinia fructicola* cultures were obtained from fungal collections at the US Department of Agricultural Research Service (USDA-ARS, 2217 Wiltshire Road, Kearneysville, WV 25430, USA).

Stock cultures of the yeast were maintained at 4 °C on Petri dishes containing, Yeast Extract Peptone Dextrose Agar (YPDA; yeast extract, 10 g/L; peptone, 10 g/L; dextrose, 20 g/L; agar, 20 g/L), while pathogen cultures were maintained on Potato Dextrose Agar (PDA, CM0139, Oxoid, Basingstoke, UK)

6.2.2. Fruits

USDA-certified oranges and grapes were obtained from a local supermarket and used within 24 h after purchase. Fruits without wounds or rot were selected based on uniformity of size, washed with tap water and detergent, rinsed, dried, and surface disinfected by wiping with 70% ethanol. These fruit were then used in subsequent experiments.

6.2.3. Wickerhamomyces anomalus-Penicillium digitatum-orange interactions (Wa-Pdig-orange)

W. anomalus BS91 was grown overnight in NYDB [nutrient broth (8 g/L), yeast extract (5 g/L), D-glucose (10 g/L)] at 25 °C. After 24 h, 1 mL of the yeast cell suspension was aseptically transferred to 250 mL of NYDB in an Ehrlenmeyer flask and placed on an orbital shaker at 160 rpm for 24 h at 26 °C.The yeasts culture was then centrifuged at 6000 rpm for 10 min and resuspended in sterile distilled water (SDW). The concentration was adjusted to 1×10^8 cells/mL using a hemocytometer.

Fungal spore suspensions were obtained from ten-day-old *P. digitatum* cultures. Condia were washed from the plates, and the resulting wash fluid was passed through cheesecloth in order to remove mycelia. The final spore concentration was adjusted to 1×10^5 conidia/mL. Surface washed and disinfected orange were wounded by making four wounds (4 mm deep × 3 mm wide) around the equator of each fruit. A 30 µLsuspension of *W. anomalus* (1×10^8 cells/mL) was pipetted into each wound that had been either non-inoculated or previously inoculated with 20 µL of conidia (1×10^5 conidia/mL of *P. digitatum* and incubated at 26 °C. At 5, 14, 24 and 48 h after inoculation, fruit tissues, consisting of both the skin (exocarp) and flesh (mesocarp), were removed from the fruit and transferred to 50 mL sterile conical tubes containing 10 mL of sterile distilled water (SDW) and

shaken on an orbital shaker at 180 rpm. Fruit tissues were then discarded and yeast cells were pelleted by centrifugation at 6000 rpm, washed twice with SDW and pelleted again by centrifugation. The pellet was frozen and stored at −80 °C for subsequent RNA extraction. Yeast cells grown in NYDB (1×10^8 cells/mL) for 5, 14, 24 and 48 h were used as controls following pelleting and washing with water.

6.2.4. Wickerhamomyces anomalus-Botrytis cinerea-grape interactions (Wa-Bc-grape)

W. anomalus cells (1×10^8 cell/mL) in SDW were prepared as described above. A 30 µL suspension of *W. anomalus* (1×10^8 cells/mL) was administered into the wounds (2 wound, 4 mm deep × 3 mm wide) made along the equator of individual grape berries that had been previously disinfected as described above. The wounds in the grape berries were then either inoculated with 20 µL of *B. cinerea* conidia (1×10^5/mL) or left uninoculated. Fruit were then incubated at 26 °C. At 5, 14, 24 and 48 hpi, fruit tissues, consisting of both skin (exocarp) and flesh (mesocarp), were removed and transferred to a 50 mL sterile conical tube containing 10 mL of SDW, shaken, pelleted, and washed as previously described The final pellet was then frozen in liquid nitrogen and stored at −80°C for subsequent RNA extraction. Yeast cells grown in NYDB (1×10^8 cells/mL) for 5, 14, 24 and 48 h were used as controls following pelleting and washing with water.

6.2.5. Wickerhamomyces anomalus-pathogen cell wall interactions

Cell walls of *B. cinerea, P. digitatum, P. expansum, P. italicum* and *M. fructicola* were prepared as described by Wisniewski et al. (1991). A conidial suspension of each pathogen (10^6/mL) was inoculated into 500 mL PDB [Potato Dextrose Broth, Liofilchem, Rose to degli Abruzzi (Te), Italy]

at 25 °C and placed on an orbital shaker. The mycelium was pelleted after 10 d, washed repeatedly with SDW, and finally dried at 40 °C until constant weight was reached.

Expression of *WaEXG1* and *WaEXG2* genes over 5, 14, 24 and 48 h was determined when yeast were grown in minimal salt media (MSM) containing: 7 mM KH_2PO_4, 10mM L-asparagine, 20 mM $MnSO_4$, 0.3 mM thiamin, 54 mM $ZnSO_4$, 0.65 M $FeSO_4$, 2 mM $MgSO_4 \cdot 7H_2O$ and 0.4 mM biotin and supplemented with 1% cell walls of *B. cinerea, P. digitatum, P. expansum, P. italicum,* or *M. fructicola* as the sole carbon source. *WaEXG1* and *WaEXG2* expression were also evaluated after *W. anomalus* was grown for 48 h in MSM with 1% cell walls of *B. cinerea or P. digitatum*, supplemented with different amounts of glucose (2.5, 5, and 10 g/L). Each analysis utilized three biological replicates at each time point, and each experiment was repeated three times.

6.2.6. RNA extraction and reverse transcriptase quantitative PCR (RT-qPCR)

Total RNA from frozen samples was extracted using a MasterPure™ Yeast RNA Purification Kit (Epicentre W Biotechnologies, Madison, WI, USA) according to the manufacture instructions. The quality and concentration of total RNA were analyzed by gel electrophoresis and with a Nano Drop spectrophotometer ND-1000 (Nano Drop, Wilmington, DE, USA). Extracted RNA was purified with an RNeasy Mini Kit (Qiagen Science, Germantown, MD, USA). RT-qPCR analysis was performed using 12.5 ng of total RNA, SuperScript III Platinum SYBRGreen One-Step RT-qPCR Kit with ROX (Invitrogen, Carlsbad, CA, USA), and 20 pmol of each primer per reaction. Primers used to analyze *WaEXG1* and *WaEXG2* expression are reported in Table 1. Primers for Actin C are also listed in Table 1. Actin was used as a housekeeping gene to normalize *WaEXG1* and *WaEXG2* expression values. Each sample consisted of 3 biological replicates, run in triplicate (technical replicates).

Table 1. Primers used in RT-qPCR analysis of *W. anomalus* EXG1 and EXG2

Gene name	Primer sequence	Tm (°C)
EXG1 D	F:TTGG GGT TGG TTT GTG R:GTC TAA CTG TTT CTG AGC TT	49.7 53.4
EXG2 C	F:GGG GTG CAA ACT CTA CTA AA R:AAC GAG AAC CAC GAC CAA	52.1 54.0
ACTIN C	F: ATG CTG GTT TCG CTT TAC R: AGC AGT AGT GGT GAA TGA A	50.6 51.0

An ABI Vii 7 (Applied Biosystems, Foster City, CA, USA) was set to cycle as follows: cDNA synthesis at 48 °C for 30 min; 95 °C denaturation for 10 min; 40 cycles of 95 °C for 15 s followed by a specific annealing temperature for 1 min; and a 50 °C for 1 min, dissociation step. The cycle threshold values (CT) were determined and the relative fold differences were calculated using the standard curve method with actin C serving as the endogenous references gene. Each sample consisted of three biological replicates and each bioreplicate had three technical repetitions. Each experiment was repeated twice.

6.2.7. Wickerhamomyces anomalus growth on minimal salt medium and pathogen cell walls

The viability of *W. anomalus* BS91 on MS medium alone or MS medium amended with pathogen cell walls was assessed. MS medium and MS medium containing 1% of *B. cinerea, P. digitatum, P. expansum, P. italicum,* or *M. fructicola* cell wall were prepared as described above and incubated at 26 °C. Yeast growth was monitored at 5, 14, 24 and 48 h after placing 1 mL of each sample in a conical centrifuge tube containing proportional amounts of sterile Ringers solution. The cultures were homogenized by vortexing and then plated onto YPDA. Yeast colony forming units (CFU) were counted after 48 h at 26 °C.

6.3. RESULTS

6.3.1. WaEXG1 and WaEXG2 expression in the Wa-Pdig-orange interaction

Expression of *WaEXG1* increased slightly over a 48 h period when *W. anomalus* BS91 was grown in NYDB, non-inoculated wounds in orange fruit, or in wounds inoculated with *P. digitatum* (Fig.1a). The greatest level of expression of *WaEXG1* was seen in wounded-inoculated fruit after 48 h.

In contrast, expression of *WaEXG2* was more responsive (greater than 2-fold induction) then *WaEXG1* when the yeast was grown in NYDB for 48 h (Fig. 1b). *WaEXG2* expression also did not exhibit any significant increase over the 48 h period when *W. anomalus* was grown on non-inoculated or wounded-inoculated orange fruit.

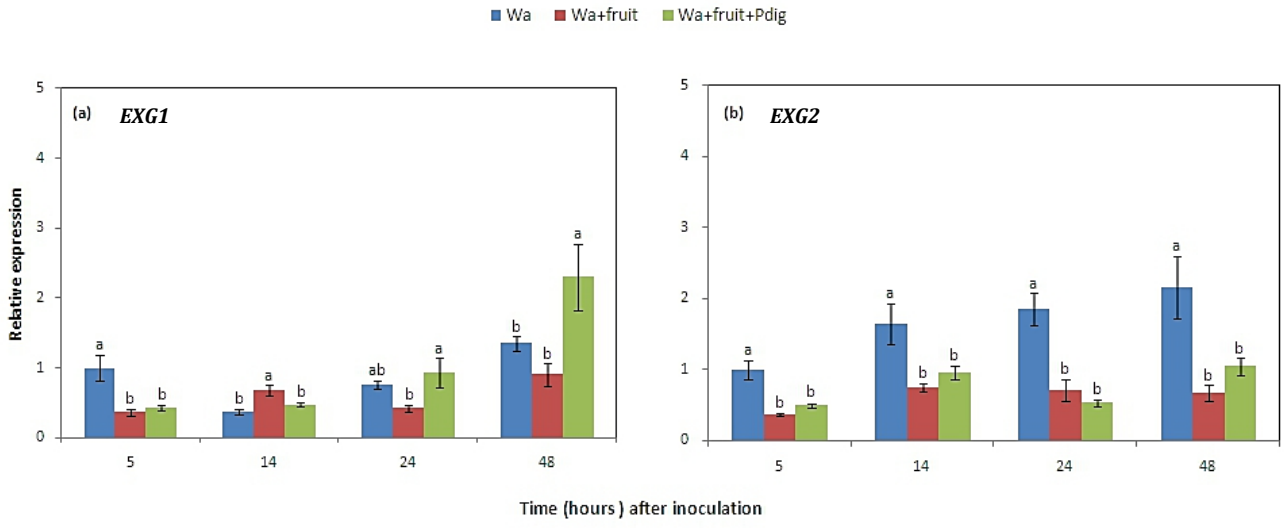

Figure 1. Expression of *EXG1* (a) and *EXG2* (b) in *W. anomalus* cells cultured in NYDB (Wa), in wounded orange fruit (Wa+fruit), and orange fruit wounded-inoculated with *P. digitatum* (Wa+fruit+Pdig). Values of gene expression are relative to the level of gene expression in Wa cultured in NYDB for 5 h, which was arbitrarily set to 1. Vertical lines represent standard error for an average of three biological replicates. Columns within each time (hours) followed by same letters are not significantly different according to Fisher's least significant difference test ($p = 0.05$).

6.3.2. WaEXG1 and WaEXG2expression in the Wa-Bc-grape interaction

Expression of *WaEXG1* (Fig. 2a) during the first 14 h increased most when the yeast was grown in non-inoculated grape berries or grape berries that had been wounded and inoculated with *B. cinerea*, relative to the expression of yeast grown in NYDB (control).

Significant differences in the expression of both *EXG1* and *EXG2* were detected at several time points when the yeast was grown in non-inoculated or wounded-inoculated grape berries. Both *WaEXG1* and *WaEXG2* expression at 48 h, however, was highest in yeast grown in NYDB.

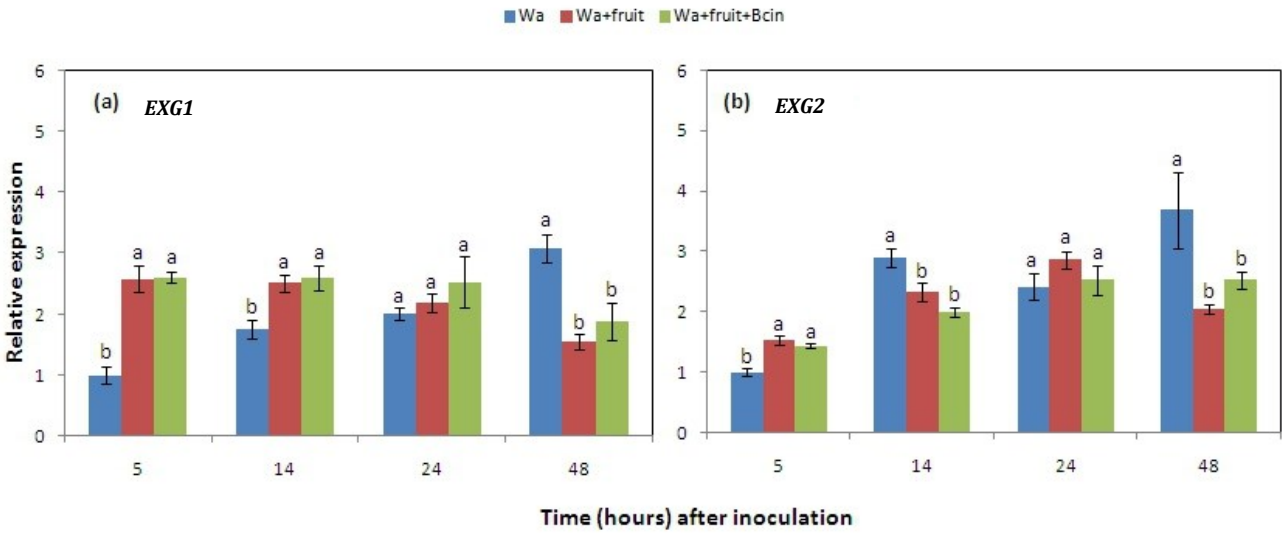

Fig.2 Expression of *EXG1* (a) and *EXG2* (b) in *W. anomalus* cells grown in NYDB (Wa), wounded grape berries (Wa+fruit) and wounded grape berries inoculated with *B. cinerea* (*Wa*+fruit+Bcin), at 5, 12, 24 and 48 h after inoculation. Values of gene expression are relative to the level of gene expression in Wa cultured in NYDB for 5 h, which was arbitrarily set to 1. Vertical lines represent standard error for an average of three biological replicates. Columns within each time (hours) followed by same letters are not significantly different according to Fisher's least significant difference test ($p = 0.05$).

6.3.3. WaEXG1 and WaEXG2 expression in Wickerhamomyces anomalus grown in MS medium with different pathogen cell walls

Little to no induction of *WaEXG1* expression was observed when the yeast was grown in MS media supplemented with 1% cell walls of various plant pathogens as a carbon source (Fig. 3a). The highest level of *WaEXG1* expression was observed at 14 h in MS medium supplemented with 1% *B. cinerea* cell walls. In contrast, a marked induction in *WaEXG2* expression was observed when the yeast was grown in MS medium supplemented with 1% cell walls of *P. digitatum*. An induction in *WaEXG2* expression was also observed in yeast cells were cultured in MS medium supplemented with 1% cell walls of *B. cinerea* (Fig. 3b), though to a much lower degree.

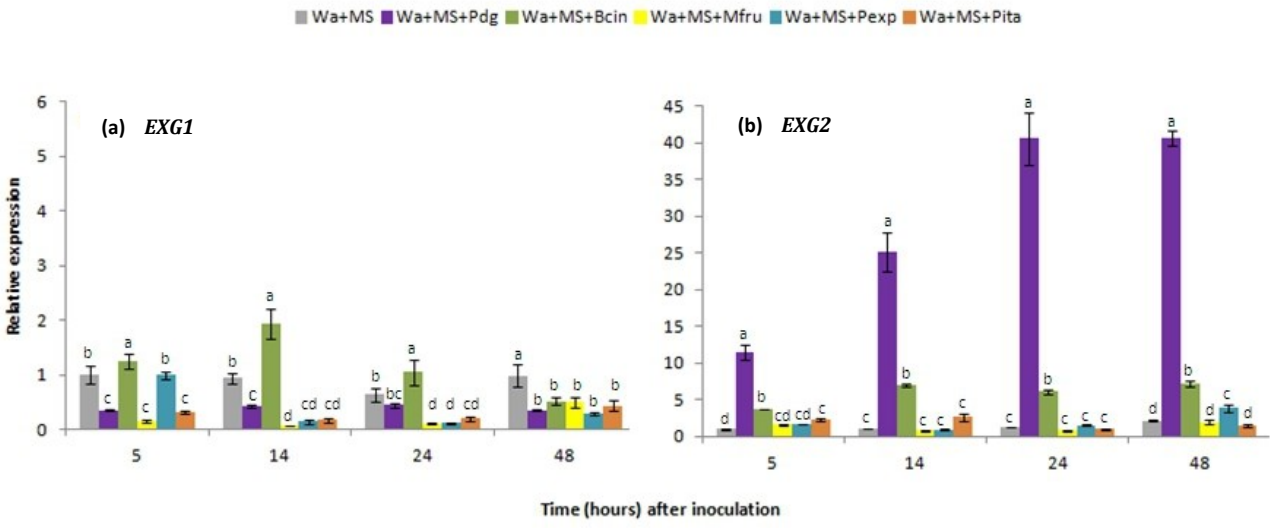

Figure 3. Expression of EXG1(a) and EXG2 (b) in *W. anomalus* grown in MS media (WaMS) and MS media supplemented with 1% cell walls (w:v) of *Penicillium digitatum* (Wa+MS+Pdg), *Botritys cinerea* (Wa+MS+Bcin), *Monilia fructicola* (Wa+MS+Mfru), *Penicillium expansum* (Wa+MS+Pexp), and *Penicillium italicum* (Wa+MS+Pita). Values represent levels of expression relative to the level of gene expression in *W. anomalus* grown on MSM for 5 h, which was arbitrarily set to 1. Vertical lines represent standard error for an average of three biological replicates. Columns within each time (hours) followed by same letters are not significantly different according to Fisher's least significant difference test ($p = 0.05$).

WaEXG1 expression (Fig. 4a) was not significantly affected by the presence of different amount of glucose (2.5, 5 and 10 g/L) added to the MS medium amended with 1% *P. digitatum* cell wall. In contrast, *WaEXG2* expression (Fig. 4b) significantly increased in MS medium amended with 2.5 g/L of glucose and higher.

The same general trend was observed for *WaEXG2* in MS medium containing 1% *B. cinerea* cell wall amended with increasing amounts of glucose (Fig. 5b). In the case of *WaEXG1*, expression was also induced by the addition of 5.0 and 10.0 g/L of glucose to MS medium containing 1% *B. cinerea* cell walls.

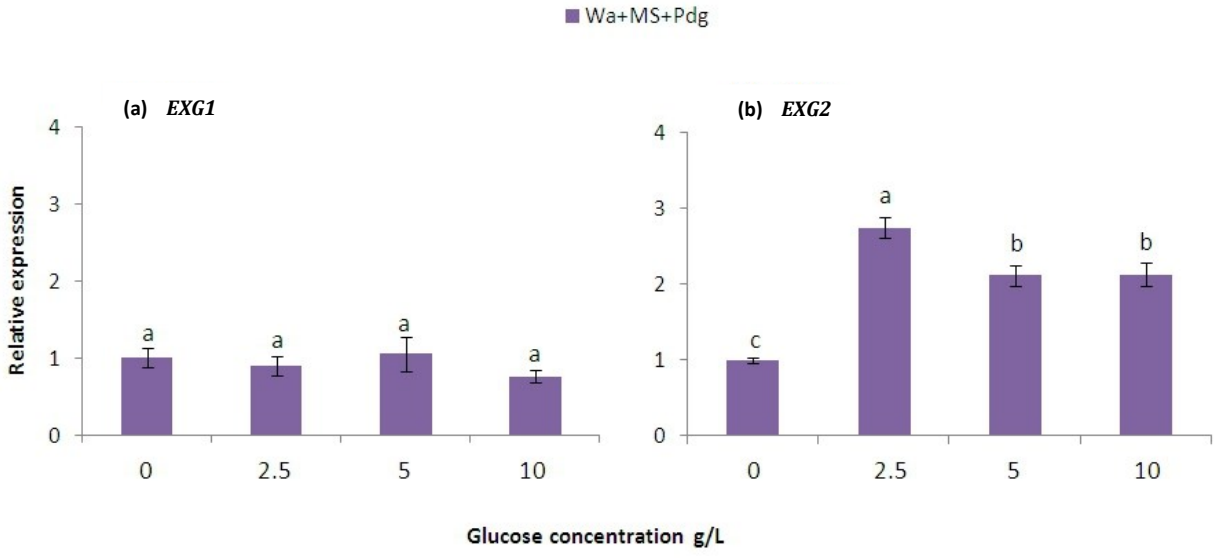

Figure 4. Expression of *EXG1* (a) and *EXG2* (b) in *W. anomalus* grown in MS media supplanted with 1% cell walls (w/vol) of *Penicillium digitatum* (Wa+MS+Pdg) and different amount of glucose 0, 2.5, 5 and 10 g/L. Values represent levels of expression relative to gene expression in *W. anomalus* grown in MS media supplemented with 1% cell walls of *P. digitatum* (Wa+MS+Pdg) at 48 h, which was arbitrarily set to 1. Vertical lines represent standard error for an average of three biological replicates.

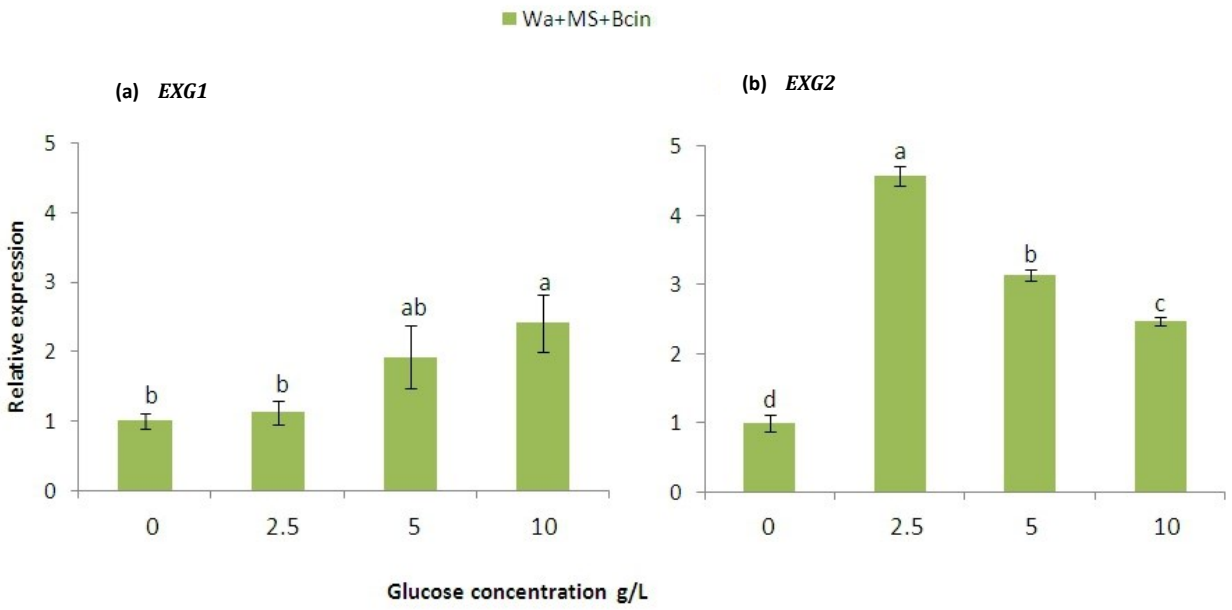

Figure 5. Expression of *EXG1* (a) and *EXG2* (b) in *W. anomalus* grown in MS media supplemented with 1% cell walls (w/vol) of *Botritys cinerea* (Wa+MS+Bcin) and different amount of glucose 0, 2.5, 5 and 10 g/L. Values represent expression relative to the expression of these genes in *W. anomalus* grown in MS media supplemented with 1% cell walls of *B. cinerea* (Wa+MS+Bcin) at 48h, which was arbitrarily set to 1. Data represent the mean ± se, n = 3. Columns followed by same letters are not significantly different according to Fisher's least significant difference test ($p = 0.05$).

6.3.4. Wickerhamomyces anomalus growth on minimal salt media and pathogen cell walls

Survival of *W. anomalus* BS91 over 48h in NYDB medium or MS medium containing different pathogen cell walls as a sole carbon source is shown in Fig. 6. The population *W. anomalus* BS91 initially decreased in the first five hours of culture, regardless of the carbon source. Subsequently, the population rose in the yeast cultures grown in NYDB reaching slightly over 1 x 10^8 cfu/mL. The yeast population in the MS medium continued to decline over the 48 h period, regardless of which pathogen cell wall served as a carbon source. Surprisingly, the most rapid decrease was observed in MS medium amended with 1% cell walls of *P. digitatum.*

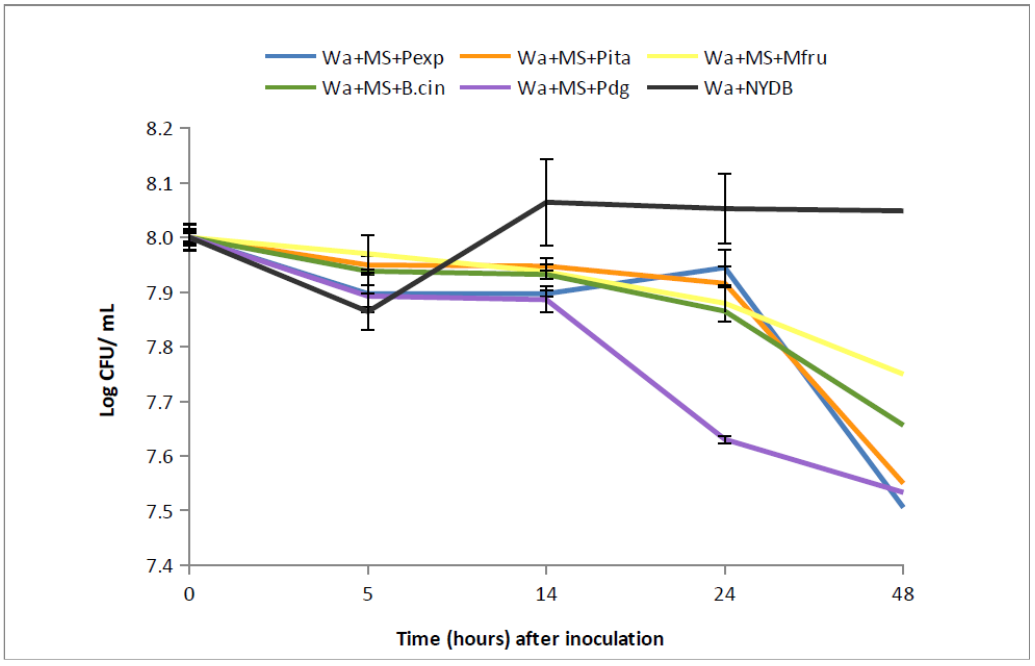

Fig. 6. Population dynamics of *W. anomalus* grown in NYDB (Wa+NYDB) and MS media supplemented with 1% cell walls (w/vol) of *Penicillium digitatum* (Wa+MS+Pdg), *Botritys cinerea* (Wa+MS+Bcin), *Monilia fructicola* (Wa+MS+Mfru), *Penicillium expansum* (Wa+MS+Pexp), and *Penicillium italicum* (Wa+MS+Pita). Bars represent the standard errors of the mean.

6.4. DISCUSSION

The production of killer toxins has been reported to play an important role in the biocontrol mechanism of BCAs that have been used to manage postharvest diseases (Lutz et al., 2013). Antagonistic activity of killer toxins seem to be mainly connect to the hydrolysis of β-1,3-glucans, a cell wall component of pathogenic fungi (Izgü and Altinbay, 2004; Muccilli et al., 2013).

Already in the nineties, a study on *Rhodotorula glutinis* (isolate LS-11) and *Cryptococcus laurentii* (isolate LS-28) (Castoria et al., 1997), showed different levels of antagonistic activity against *B.*

cinerea and *P. expansum*, hypothesizing in the microorganism a different induction of β -1,3-glucanase by hyphal cell walls. They demonstrated also how the addition of exogenous nutrients (NYDB) significantly reduced the antagonistic activity of both isolates.

Among the killer species, *Wickerhamomyces anomalus* revealed strong biocontrol activity against a wide range of mold diseases, such as green mold decays on Tarocco orange (Platania et al., 2012), anthracnose disease caused by *Colletotrichum gloeosporioides* in papaya (Lima et al., 2013), and botrytis bunch rot on table grape (Parafati et al., 2015).

Few studies have been conducted about the changes in gene expression that occur in yeast cells in response to the presence of pathogens or host tissues (Hershokovitz et al., 2013).

Therefore, more studies investigating the gene expression profile of BCAs are needed to better understand the molecular processes associated with mechanism of action of the yeast BCAs. More specifically, the analysis of genes associated with biocontrol activity, such as β-glucanases, will lay the foundation for understanding biocontrol systems and the molecular and biochemical processes that occur in the tritrophic interactions between the yeast, host, and pathogen.

Different responses of yeast to pathogens cell walls were already reported by Chan et al., (2005), that demonstrate how β-1,3-glucanase activity of *Pichia membranefaciens* and *Cryptococcus albidus* were strongly induced by the presence of *M. fructicola* cell walls as the sole carbon source, while lowest induction was observed in presence of *P. expansum* and *R. stolonifer*.

Other study conducted by Larralde-Corona et al. (2011) reported an induction of gene expression (increase of expressed sequence tags) in *P. guilliermondii* grown in direct contact with fungal cell walls of *P. digitatum*, hypothizing that metabolites excreted by the fungus are also important factors in inducing an antagonistic response in yeast that optimizes the limited carbon/energy source present in the MS medium and produces antifungal compounds.

Results of the present study indicate that between the two genes, *WaEXG1* and *WaEXG2,* encoding killer toxins, the latter gene, *WaEXG2*, is more responsive to the nutritional environment (including the addition of glucose to MS plus cell wall media) both *in vitro* and *in vivo*, and appears to play a greater role in the cellular metabolism of *W. anomalus*. *WaEXG2* expression also appeared to be more responsive to the presence of cell walls of *P. digitatum* and *B. cinerea* than other fungal species.

In addition, although the high levels of *EXG2* expression were observed in vitro on MS medium amended with cell walls, the same level of induction was not seen *in vivo* when the yeast was grown in wounded/inoculated orange fruit. The reason for this is still unclear, but it illustrates the complexity of the interaction of the yeast with the pathogen and the nutritional and metabolic environment presented by the host tissues. Other aspects of the tritrophic environment, such as pH, glucose levels, phytoalexins, etc. may have inhibited the yeast from responding to *P. digitatum* by elevating the expression of *EXG2*.

However, the basis for better understand this pathogen-specificity will require further study.

The nutritional studies performed *in vitro* also indicated that in a "nutrient poor" environment, such as MS medium is, the presence of 1% pathogen cell walls as a sole carbon source was simply not enough to maintain high population levels of yeast.

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CHAPTER 7. CONCLUSION

Over the last 20 years, the use of yeast in controlling postharvest diseases shows great potential as an alternative method to chemical fungicides. A successful selection of BCAs is strictly connected to their adaptability to host fruit and target pathogen and implies the investigation of the action mechanism involved in the biological control process.

In the present study, we evaluated the mechanisms of action associated with 15 food-isolated yeast strains belonging to the species *W. anomalus, M. pulcherrima, A. pullulans* and *S. cerevisiae.* The objective was also to determine the efficacy of selected yeast strains in controlling gray mold, green and blue mold decays, caused by *B. cinerea, P. digitatum* and *P. italicum,* respectively. Therefore, subsequent studies investigated the potential use of edible LBG coating enriched with yeast cells and a commercial carrier for their antifungal VOCs.

Among tested yeasts, *W.anomalus* BS91 showed great antagonistic activity both by direct application of yeast cells and by application of VOCs. This strain is known to produce killer toxins that have been demonstrated to be exoglucanases, coded by the genes *WaEXG1* and *WaEXG2* (Muccilli et al 2013). To better understand the involvement of these genes in the antagonist activity of *W. anomalus* in the last phase of the research activity characterization of the gene expression level of *EXG1* and *EXG2* in different yeast-host-pathogen interactions was carried out.

The main biocontrol modes of actions for each yeast species, such as iron competition, production of cell wall-degrading enzymes, production of VOCs, biofilm formation and the ability to control the causal agent of gray mold *in vitro* conditions were clarified (Parafati et al., 2015).

In detail, *M. pulcherrima* biocontrol ability was inversely correlated with iron availability as the best efficacy was detected in presence of low amount of iron. No enzymatic activity was detected

for *M. pulcherrima* and *S. cerevisiae* species, while *W. anomalus* showed the ability to produce β-1,3-glucanase. *A. pullulans* was the only species exhibiting a broad spectrum of diffusible hydrolytic enzymatic activities, as it was positive for β-1,3-glucanase, pectinase and protease.

In addition, positive correlation between biofilm formation ability and grape berries wound colonization was observed for *M. pulcherrima* and *W. anomalus*. In *in vitro* assays the best performances, based on VOCs production, were detected using *W. anomalus* and *A. pullulans* yeast strains.

Four selected yeasts, one for each species (*M. pulcherrima* MPR3, *W. anomalus* BS91, *A. pullulans* PI1 and *S. cerevisiae* BCA61), were further tested i*n vivo* conditions on wounded grape berries artificially inoculated with *B. cinerea*. Great results were obtained using *M. pulcherrima* MPR3, that exhibited the best efficacy in reducing *B. cinerea* disease parameters followed by *W. anomalus* BS91 and *A. pullulans* PI1.

Direct antifungal activity was evaluated *in vitro* and *in* vivo conditions against *P. digitatum* and *P. italicum.* In vitro assays showed how all yeasts were able to suppress the casual agents of green and blue mold, althought at different levels, although the best results were obtained with *A. pullulans* PI1 strain. The *in vivo* assays were carried out on wounded and artificially inoculated mandarin fruits. Once again *M. pulcherrima* MPR3 and *W. anomalus* BS91 revealed the best performance in controlling *P. digitatum* and *P. italicum* mold decays. After testing the efficacy of localized BCAs application, the biocontrol behaviors of the yeasts were evaluated using an integrated approach based on application by dipping of LBG (0.5 and 1 %) enriched with the tested yeast strains. This study proved the efficacy of the bioactive edible coatings (LBG 0.5 and 1 %), in comparison with SDW, to enhance viability of the yeasts on mandarin peel.

VOCs produced by the four yeast strains were evaluated *in vitro* and *in vivo* conditions by using a potential commercial carrier (polyacrylammide hydrogels spheres). *In vitro* conditions, all tested

yeasts were able to reduce radial growth of tested pathogens. In particular, *W. anomalus* showed a total inhibition of *B. cinerea* and *P. italicum* (100%) and a strong inhibition of *P. digitatum* (97.30 %) up to 10 days. *In vivo* findings also revealed good performances of all selected yeasts. In detail, *W. anomalus* BS91 totally inhibited gray mold decay on strawberries artificially infected with *B. cinerea*. The inhibitory effect of VOCs was further proven on artificially inoculated mandarins. Once again *W. anomalus* BS91 showed the best efficacy in reducing green mold decay caused by *P. digitatum*, while *A. pullulans* PI1 was more effective in reducing blue mod decay caused by *P. italicum.*

Characterization of expression levels of *WaEXG1* and *WaEXG2* revealed how the latter gene, *WaEXG2*, is more responsive to the nutritional environment both *in vitro* and *in vivo*, and appears to play a greater role in the cellular metabolism of *W. anomalus*. *WaEXG2* expression also appeared to be more responsive to the presence of cell walls of *P. digitatum* and *B. cinerea* than other fungal species.

Overall, the present study hypothesized the use of yeasts as potential antifungal agents both using direct yeast application and incorporation in an edible coating (LBG). Moreover, it has been demonstrated as production of VOCs can play an essential role in the antagonistic activity of BCAs, and suggests a future use of polyacrylammide hydrogel spheres as a tool for VOCs release in packaging or storage conditions, as they are able to enhance viability and to support yeast growth.

In brief, present research activity aimed to increase the knowledge of various aspects involved in the biocontrol efficacy of these beneficial microorganisms, that are being used and can be successfully exploited in future for safeguarding food productions, environment and human health.

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