Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists

REVIEW

DEVELOPMENT OF BIOCONTROL PRODUCTS FOR POSTHARVEST DISEASES OF FRUIT: THE IMPORTANCE OF ELUCIDATING THE MECHANISMS OF ACTION OF YEAST ANTAGONISTS

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Abstract

Background

Impressive progress was made in the last decade in development, registration and commercialization of biocontrol products based on yeast to manage postharvest pathogens of fruit. To successfully inhibit the pathogen infection and development, several possible mechanisms operate in a tritrophic host-pathogen-antagonist interaction system.

Scope and Approach

The current reviews focuses on the recent knowledge on the mechanisms by which yeast biocontrol agents (BCAs) interact with pathogens and fruit tissues. The main mechanisms of action explored include antibiosis, mycoparasitism, production of lytic enzymes, induced resistance, competition for limiting nutrients and space, and the role of oxidative stress. Omics techniques can provide a powerful tool to study complex fruit host-pathogen-antagonist-native microflora interactions.

Key Findings and Conclusions

Various aspects relevant to mechanisms of action of yeast antagonists have been discussed, including unique environment of surface wounds, iron competition, biofilm formation, cell wall degrading enzymes, and involvement of oxidative stress. Outstanding advancement in molecular and omics technologies revolutionized the research about the physiological status of BCAs and the global effect of the application of BCAs on the transcriptome and/or proteome of fruit. Microbial communities on plant surfaces could impact disease control through their interactions with host plants, pathogens, and BCAs, in a quadritrophic interaction system, hence microbiome research opens new research opportunities. The complex modes of action make antagonistic performance and efficacy more dependent on production, formulation, packing, application, and storage. A deep understanding of the mode of action is essential to develop appropriate formulation and methods of application.

Keywords: biofungicide; biological control; fruit; omics; rots; yeast.

1. Introduction

Postharvest fungal pathogens are considered the main cause of losses of fresh fruits and vegetables at the postharvest, distribution, and consumption levels. While reports on the level of these losses are conflicting, a report by the Food and Agriculture Organization (FAO, 2011) indicated that global average loss in Europe, North America and Oceania is about 29%, compared to an average of about 38% in industrialized Asia, South East Asia, Africa and Latin America. Efforts have been made to minimize these losses through developing a better understanding of the biology and aetiology of postharvest diseases, as well as by developing adequate postharvest handling technologies and control strategies (Prusky & Gullino, 2010). While several approaches were suggested for managing postharvest decay, chemical control of postharvest diseases, applied in orchard or after harvesting, is still the most widely used method. Increasing concerns, however, regarding residues of fungicides in the fruit, development of resistant biotypes of the pathogens, as well as risks associated with their continuous use have prompted the search for safe and effective alternative strategies. Among these strategies, biological control based on naturally occurring microorganisms, has been the most studied (Liu, Sui, Wisniewski, Droby, & Liu, 2013a).

In the past thirty years, there have been extensive research activities to explore and develop strategies based on microbial antagonists to biologically control postharvest pathogens (Spadaro & Gullino, 2004; Droby, Wisniewski, Macarisin, & Wilson, 2009; Sharma, Singh, & Singh, 2009). By using the key words "biocontrol" OR "biological control" AND "postharvest" OR "post-harvest" in the Scopus search engine, 879 documents were retrieved (search performed on April 3, 2015), most of them (609; 69%) published in the last ten years. Impressive progress was made in development, registration and commercialization of biocontrol products to manage key postharvest pathogens, such as *Penicillium expansum, Penicillium digitatum, Penicillium italicum, Fusarium sambucinum, Rhizopus stolonifer* and *Botrytis cinerea*. Different products reached advanced stages of development and commercialization (Table 1). Biosave™ (*Pseudomonas syringae* Van Hall) was originally registered in the USA for postharvest application on pome and citrus fruits, and it was later extended to cherries, potatoes and sweet potatoes (Janisiewcz & Peterson, 2004). Among the first products based on yeasts, Aspire™(based on *Candida oleophila*) (Liu, Wisniewsi, Artlip, Sui, Droby, & Norelli, 2013b) and Yieldplus™ (based on *Cryptococcus albidus*) (Janisiewicz & Korsten, 2002) were commercialized for some years but they were withdrawn due to various reasons, including low and inconsistent efficacy under commercial conditions, low profitability and difficulties in market penetration and perception of the customers/industry, and small size companies with low available resources to maintain development and commercialization. Other products have been more successful, including

Shemer™, based on the yeast *Metschnikowia fructicola* (Droby *et al.*, 2009), initially registered in Israel for both pre- and postharvest application on various fruits and vegetables, including apricots, citrus fruit, grapes, peaches, peppers, strawberries, and sweet potatoes. Shemer™ was later acquired by Bayer CropScience (Germany) and recently sublicensed to Koppert (Netherlands). A commercial formulation of *Candida sake* has been developed for use on pome fruit and grapevine and registered in Spain under the name Candifruit™ (Calvo-Garrido *et al.*, 2014), however, it is not yet used due to constrains of the distribution company. In South Africa, Avogreen™ has been introduced for the control of Cercospora spot, a postharvest disease of avocado, but its use has been limited due to inconsistent results (Demoz & Korsten, 2006). Furthermore, Nexy, based on another strain of *C. oleophila* was developed in Belgium and is now registered throughout the European Union (Lahlali, Raffaele, & Jijakli, 2011). Finally, BoniProtect™, developed in Germany and based on two antagonistic strains of *Aureobasidium pullulans*, is used as preharvest application to control wound pathogens developing on apples during storage.

In practice, however, the acceptance and widespread use of postharvest biocontrol products is still limited. This can be attributed to several shortcomings related to reduced and inconsistent performance when biocontrol agents (BCAs) are used under commercial conditions, as well as to limited market and small size companies involved in their development and commercialization. Host, pathogen and environment variables affecting the decreased efficacy of postharvest BCAs and strategies for their improvement were the subject of several reviews (Janisiewicz & Korsten, 2002; Droby *et al.*, 2009).

Among the antagonistic microorganisms used as BCAs against postharvest pathogens, a relatively high number of yeast was reported (Table 1) and this is related to their features that make them effective as BCAs on fresh agricultural commodities as well as other foods. Yeasts are tolerant to extreme environmental conditions prevailing before and after harvest (low and high temperatures, desiccation, wide range of relative humidity, low oxygen levels, pH fluctuations, UV radiation). Furthermore, yeast are uniquely adapted to the fruit micro-environment (high sugar concentration, high osmotic pressure and low pH). Yeast can grow rapidly on inexpensive substrates in fermenters and are therefore easy to produce in large quantities (Spadaro, Ciavorella, Zhang, Garibaldi, & Gullino, 2010). In addition, they do not produce allergenic spores or mycotoxins, in contrast to filamentous fungi, and they have simple nutritional requirements that enable them to colonize dry surfaces for long periods of time.

The current review focuses on presenting recent knowledge on the mechanisms by which postharvest yeast BCAs interact with the pathogen and fruit tissue while discussing the importance of these interactions to effectively explore new antagonists, improve efficacy, develop effective formulations and proper application of the commercial products.

2. Fruit surface and wound environment

Interactions between the antagonist, the pathogen, the host and the fructoplane resident microflora have been extensively studied and suggested to play critical role in various biocontrol systems (Chan, Qin, Xu, Li, & Tian, 2007; Jiang, Zheng, & Chen, 2009; Hershkowitz *et al.*, 2013; Kwasiborski, Bajji, Renault, Delaplace, & Jijakli, 2014). In this regard, the wound site, the court of infection of most necrotrophic postharvest pathogens, is of particular interest when exploring the mechanisms of action of microbial antagonists.

In general, at the initial stages of the bitrophic plant-pathogen interaction (Figure 1), the fungal pathogen can release pathogen-associated molecular patterns (PAMPs) that can be recognized by specific plant recognition receptors, leading to trigger the first innate immunity response associated with a slight oxidative burst (Jones & Dangl, 2006). The response of the fruit is depending on the fruit species and/or cultivar and on its physiological/senescent stage (Cantu *et al.*, 2009; Prusky, Alkan, Mengiste, & Fluhr, 2013). The pathogen can then overcome this first line of defence by releasing effectors to supress further plant defence mechanisms, making the tissue susceptible to infection. In case the pathogen is unable to manipulate fruit defences to its advantage, the fruit can respond by triggering a stronger oxidative burst (Heller & Tudzinski, 2011), accompanied by the biosynthesis of phytoalexins and the production of pathogenesis-related proteins (PRPs). Certain pathogens, such as *B. cinerea*, are capable of actively stimulating oxidative burst, leading to cell death, necrosis, and colonization of the dead tissue (Finiti *et al.*, 2014). This is accompanied by the release of cell wall degrading enzymes and/or phytotoxins, that are regulated by host pH modification (Prusky *et al.*, 2013).

Fruit surface injuries, inflicted during harvest and subsequent handling, represent ideal infection court for necrotrophic pathogens. These wound sites are generally rich in nutrients (e.g. glucose derived molecules) that are readily available for the pathogen. In addition, damaged fruit tissue can release damage-associated molecular patterns (DAMPs, i.e. oligogalacturonides; Bove, Kim, Gibson, & Assmann, 2008), which can be recognized by plant cell receptors triggering downstream host defence mechanisms that are regulated by the jasmonate signalling pathway (Robert-Seilaniantz, Grant, & Jones, 2011). Activation of these mechanisms will eventually result in accelerating wound healing

processes where strong oxidative burst, synthesis of phenolics, and the formation of corky cells serve as means of protection against pathogen invasion. If a fungus gains entry to the wounded fruit surface, its growth may be inhibited by plant substances which are either present or induced in response to injury or infection. Moreover, in the wound microenvironment, oxygen level can be depleted, due to plant cell respiration and rapid colonization of various epiphytic microorganisms that are able to tolerate hypoxia or anoxia (Fredlund, Druvefors, Olstorpe, Passoth, & Schnurer, 2004).

To successfully inhibit the pathogen infection and development, several possible mechanisms operate in a tritrophic host-pathogen-antagonist interaction system (Figure 2), including antibiosis, mycoparasitism, production of lytic enzymes, induced resistance, biofilm formation, and competition for limiting nutrients and space. Often, more than one mechanism is involved. Furthermore, the role of BCAs in modulating the oxidative state of the wound is essential. A successful BCA is generally equipped with several attributes which often work in concert and may be crucial for controlling disease development. Understanding the modes of action of antagonists is one of the parameters for biofungicide product development and is relevant for marketing purposes, because it permits to improve biocontrol performance and reliability through the development of appropriate formulations.

3. Studying the mechanism of action of postharvest biocontrol agents

Advanced microbiological, microscopic, biochemical and molecular techniques are currently available and can be utilized effectively to improve our knowledge about mechanisms of action of microbial antagonists (Liu *et al.*, 2013a). When studying mechanisms of action, the quadritrophic interactions taking place between the antagonist, the pathogen, the host, and the resident epiphytic microflora should be taken into consideration (Figure 3). Epiphytic microflora studies should be integrated into the traditional biocontrol approach, since microbial communities on plant surfaces could impact disease control through their interaction with host plants, pathogens, and BCAs (Massart, Martinez-Medina, & Jijakli, 2015). Until now, the scientific approaches focused on looking at different components of such interactions separately. This conceptualization, however, raises some critical questions: (1) what are the effects of antagonists on wound healing and host resistance? (2) how important and widespread are the direct effects of antagonists on pathogens? (3) how do incidental or resident microorganisms or mixtures of antagonists affect pathogen/antagonist interactions? and (4) how does the nutrient/chemical composition at the wound site affect the antagonist, other microflora, the infection process, and the wound response?

3.1 Competition for nutrients and space

Competition for nutrients (e.g. carbohydrates, nitrogen, oxygen) and space has been considered the primary mode of action of yeast antagonistic microorganisms against postharvest fungal pathogens. Competition can be an effective biocontrol mechanism when the antagonist is present in sufficient amounts at the right time and location, and it is able to use limited resources more efficiently than the pathogen.

Yeast and some bacteria can successfully compete with the pathogen at the wound site or *in vitro* for limiting nutritional factors, inhibiting its growth, but often leaving it alive (Janisiewicz, Tworkoski, & Kurtzman, 2001; Zhang, Spadaro, Garibaldi, & Gullino, 2010). In the competition for space, yeasts usually have the advantage of rapid growth and formation of an extracellular polysaccharide capsule that can promote adhesion to fruit surface forming biofilms covering the entire wound area.

Competition for nutrients was evident for *Pichia guilliermondii* against *P. digitatum* co-cultivated on synthetic medium: the addition of exogenous nutrients resulted in a reduced efficacy and the antagonists was more effective in inhibiting the pathogen when nutrients were scarce (Droby, Chalutz, Wilson, & Wisniewski, 1989). Yeast can satisfactorily use a wide range of carbohydrates, which include disaccharides and monosaccharides, and nitrogen sources (Spadaro *et al.*, 2010). Nitrogen is likely to represent a limiting factor in the carbon-rich environment of pear wounds (Janisiewicz & Korsten, 2002). Exogenous amino acids, applied at high concentrations on apple wounds, significantly decreased the efficacy of *A. pullulans* against *P. expansum*, provide evidence that competition for nutrients may be have major role in the mechanism of biocontrol activity (Bencheqroun *et al.*, 2007). Competition for sugars and nitrates plays a key role also in the interactions of *P. guilliermondii* with *B. cinerea* on apple (Zhang, Spadaro, Garibaldi, & Gullino, 2011a) or *Colletotrichum* spp. on pepper (Chanchaichaovivat, Panijpan, & Ruenwongsa, 2008).

A biological sensor, composed of a nutrient-responsive promoter fused to a reporter gene, could be used to assess the spatial distribution and availability of nutrients in fruit wounds at critical times for pathogen infection and colonization. Reporter genes encoding the Green Fluorescent Protein (GFP) are especially useful for studies evaluating gene expression by bacterial antagonists on and in plant tissues (Smith & Lindow, 2013). Studies on the repartition of radiolabelled glucose between the antagonistic yeasts *Sporobolomyces roseus* and *Cryptococcus laurentii* and the pathogen *B. cinerea* point out a strong sugar consumption by the BCAs, that blocks fungus conidial germination due to carbon source deprivation (Filonow, 1998). The yeast antagonists *C. laurentii* and *S. roseus* used the volatile compound butyl acetate as a food source and reduced its stimulatory effects on the pathogen *in vitro*. In fruit wounds, however, competition for nutrients is likely extended to additional sources, such as nitrogen compounds found in low concentrations.

Recently, Kwasiborski *et al.* (2014) reported that during the exponential growth phase of *Pichia anomala*, and in presence of *B. cinerea*, the pentose phosphate pathway seems to be enhanced and would provide the needed nucleic acids and energy for wound colonization by the antagonist. These findings would suggest that the pentose phosphate pathway (PPP) may supply the yeast with an efficient consumption of apple nutrient, favouring the competitive colonization of apple wounds by the yeast against *B. cinerea*.

Determining the role, the components and the factors involved in competition for nutrients and space in the biocontrol system is crucial for enhancing biocontrol efficacy of the antagonist. This information would be useful during upscale production and formulation. The supplementation of a limiting factor or essential nutrient for improved growth of the BCA may significantly contribute to its consistent performance against the pathogen on wide range of fruits and vegetables.

3.2 Iron: a key source for competition

Iron is essential for fungal growth and pathogenesis, and competition for iron is believed to play a significant role in the biocontrol of postharvest pathogens (Saravanakumar, Ciavorella, Spadaro, Garibaldi, & Gullino, 2008). Iron is essential for growth of nearly all microbes and is present in heme-cofactored proteins (such as catalase and cytochromes), iron sulphur cluster (Fe/S) containing electron carriers proteins, and di-iron and mononuclear enzymes, required for the activity of numerous cellular enzymes involved in a wide range of cellular processes. Furthermore, several transcriptional and posttranscriptional regulators interact with iron to sense its intracellular level or current status of oxidative stress in order to efficiently control the expression of a broad array of genes involved mainly in iron acquisition or protection against reactive oxygen species (ROS).

In most microbial habitats, including the acidic pH of plant cells, Fe^{2+} is oxidized to Fe^{3+} to form stable ferric oxide hydrate complexes in the presence of oxygen and water. Strategies exhibited by yeast to respond to iron depletion consists of: (i) activation of systems of iron uptake, (ii) mobilization of intracellular stores of iron, and (iii) metabolic adaptations to iron limitation (Philpott & Protchenko, 2008). Yeasts express two genetically distinct systems for iron uptake, namely, a reductive and a non-reductive system. Ferric salts and ferric chelates are substrates for the reductive system, while the nonreductive system exclusively recognizes siderophore-iron chelates.

Transcriptome analyses in human pathogenic fungi demonstrated that hypoxia adaptation and iron homeostasis are involved in regulation of several common genes responsible for fungal virulence (Chung, Haas, & Cramer, 2012). Yeast could profit from the fruit wound, which is a low oxygen and low iron microenvironment, by producing siderophores to compete for iron and interfere with the

pathogen germination, growth and virulence. In iron starvation conditions, fungi have a lower catalase (CAT) activity and a lower ROS protection (Oberegger, Schoeser, Zadra, Abt, & Haas, 2001). Siderophores are designed to form tight and stable complexes with ferric iron and they can be divided into three main classes depending on the chemical nature of the moieties donating the oxygen ligands for Fe^{3+}coordination, which are either catecholates (*sensu stricto*, catecholates and phenolates; better termed as "aryl caps"), hydroxamates, or (hydroxy-)carboxylates (Miethke & Marahiel, 2007). Yeasts produce hydroxamate-type compounds, while bacteria produce hydroxamate as well as catecholate siderophores. Rhodotorulic acid (Figure 4) is a dihydroxamate-containing siderophore produced by *Rhodotorula glutinis*, essential to improve the control of blue mold caused by *P. expansum* in apples (Calvente, Benuzzi, & de Tosetti, 1999).

Metschnikowia pulcherrima and *Mestschnikowia fructicola* are able to produce the red pigment pulcherrimin (Figure 4 and 5), formed non enzymatically from pulcherriminic acid and ferric ions, which is involved in the control of *B. cinerea*, *Alternaria alternata* and *P. expansum* on apple (Saravanakumar *et al.*, 2008). Iron depletion by the *M. pulcherrima* in the medium inhibited the mycelial growth and conidial germination of *B. cinerea*, *A. alternata* and *P. expansum*. When iron was added at higher concentrations, the pathogen inhibition activity of *M. pulcherrima* disappeared and the colonies turned brown red. Furthermore, hyphae cracked when entering the pigmented zones around the *M. pulcherrima* streaks, demonstrating that iron starvation elicits complex physiological changes in the fungal cells (Saravanakumar *et al.*, 2008). Also *Metschnikowia fructicola* is able to produce pulcherrimin and to inhibit the growth of both *B. cinerea* and *P. digitatum in vitro* (Figure 5).

3.3 The role of biofilm formation and Quorum sensing

To successfully colonize intact and injured fruit surfaces, the antagonist should have the ability to use specific features facilitating its adherence, colonization and multiplication. In most cases this feature is associated with the formation of a biofilm, where micro colonies are enclosed in a hydrated matrix of microbe produced proteins, nucleic acids, and polysaccharides (Bassam, Annous Pina, Fratamico, & Smith, 2009). The importance of biofilm formation and quorum sensing (QS) in biocontrol systems was reported. Yeast cell attachment is often mediated by specific cell wall adhesive glycophosphatidyl inositol (GPI)-anchored proteins (Finkel & Mitchell, 2011). Environmental sensing and signal transduction pathways regulating morphogenetic transformations have been studied in depth in *Candida albicans*. Two families of adhesin genes (HWP1and ALS) showed to play a critical role in host cell recognition, adhesion, invasion, and biofilm formation (Biswas, Van

Dijck, & Datta, 2007). Different yeast species carry different families of adhesins that reflect their species lifestyle. In *Saccharomyces cerevisiae*, five FLO (flocculation) genes are responsible for adhesion (Smukalla *et al.*, 2008). Different aromatic alcohols exert different effects on morphogenesis in *S. cerevisiae* and *C. albicans* (Chen & Fink, 2006). Two QS regulatory molecules, tyrosol and farnesol, coordinating phenotype switching (yeast-to-hypha and vice versa), have been identified in *C. albicans*. Recently, the aromatic alcohol phenylethanol was identified as a QS molecule stimulating pseudohyphal growth in *S. cerevisiae* and *Debaryomyces hansenii* (Gori, Knudsen, Nielsen, Arneborg, & Jespersen, 2011).

However, little is known about the role of biofilms in the biocontrol activity of yeast antagonists used to manage postharvest diseases and the mechanisms involved in their formation. Experiments carried out on *S. cerevisiae,* capable of forming a biofilm in liquid culture, demonstrated its effectiveness against some postharvest pathogens. The biocontrol activity of a biofilm-forming *S. cerevisiae* strain is tightly related to the morphological phase of cell harvesting after growing in liquid culture. Only yeast cells collected from the biofilm phase are effective in limiting pathogen growth, apparently being able to colonize more efficiently the inner surface of artificial wounds. In this relation, the ability to form biofilms and filamentous growth are often correlated (Ianiri *et al.*, 2013).

The ability to form biofilms has been also proposed as an effective mechanism of action in some biocontrol yeasts (Fiori, Fadda, Giobbe, Berardi, & Migheli, 2008). Interestingly, a strain of *Pichia fermentans*, which controls brown rot on apple fruit, showed to become a destructive pathogen when applied to peach fruit. On apple surfaces and within apple wounds, the antagonist retained its yeast-like shape whereas colonization of peach fruit tissue was always characterized by a transition from budding growth to pseudohyphal growth, suggesting that pseudohyphal growth could play a major role in governing the potential pathogenicity of *P. fermentans* on peaches (Giobbe *et al.*, 2007).

Phenylethanol, as a QS molecule, can induce *Kloeckera apiculata* to adhere and form biofilm on citrus fruit and embed in an extracellular matrix, by creating a mechanical barrier interposed between the wound surface and the pathogen (Pu, Jingfan, Kai, Chao-an, & Yunjiang, 2014). Lutz, Sosa, Rodriguez, Lopez, & Sangorrin (2013) suggested that biofilm formation is one of the main features of yeast antagonists against pear postharvest decay, involved in their efficacy and adaptation to low temperatures.

We suggest that understanding the mechanisms of biofilm formation as well as the environmental cues regulating morphogenetic transformations in postharvest BCAs will lead to the selection of more effective antagonists and new methods of optimizing their activity.

3.4 Production of diffusible and volatile antimicrobial compounds

Yeasts can produce antifungal compounds, such as killer toxins, peptides and antibiotic metabolites (Selitrennikoff, 2001). Certain yeast strains with a killer phenotype produce extracellular protein toxins designated as killer toxins or killer proteins, which are lethal to sensitive microbial cells belonging to either the same or a different species. Producers of these toxins are able to kill each other, but are immune to killer toxins of their own class. The most studied examples are the toxins K1, K2 and K28 of *S. cerevisiae* (Breinig, Tipper, & Schmitt, 2002). The killer toxins confer an ecological advantage to yeast cells over their competitors. Most killer toxins are stable and active at pH values ranging from 3 to 5.5 (Marquina, Santos, & Peinado, 2002), typical of wounded or injured fruits, and they are protease-sensitive and heat labile (the killer toxin K1 is unstable at temperatures above 25°C). *Pichia membranifaciens* can produce two killer toxins (PMKT and PMKT2) that are active against spoilage yeast and fungi (Santos, San Mauro, Bravo, & Marquina, 2009). Though there is diversity in the mode of action of killer toxins, several killer toxins (K1, PMKT) seem to be membrane pore forming-related toxins.

Among the antibiotic metabolites, the most thoroughly studied example is farnesol from *C. albicans*, which can inhibit *in vitro* various bacteria and fungi. Another antifungal volatile substance, 2-phenylethanol, was isolated from *K. apiculata* and demonstrated to have inhibitory activity against green and blue mould on citrus caused by . *P. digitatum and P. italicum* (Liu *et al.*, 2014).

A. pullulans may produce aureobasidin A, a cyclic depsipeptide, with antifungal and antibiotic properties, particularly against *Botrytis* spp., *Monilinia* spp., and *Penicillium* spp. both *in vitro* and *in vivo* (Liu *et al.*, 2007). Aureobasidin A is able to block the activity of inositol phosphorylceramide synthase, an essential enzyme for fungal sphingolipid biosynthesis.

The main concern, related to the use of antifungal and antibiotic compounds in food products, is the development of human pathogens resistant to these compounds and the possible development of resistance in fruit pathogens. Particular care should be taken in using BCAs producing antimicrobials on fruit, though antibiotic producers may be able to control also wound infections established before antagonist application.

Many fungal species, such as *Trichoderma harzianum, Fusarium oxysporum,* and *A. pullulans,* are known to produce low concentrations of volatile antifungal substances (Mari, Martini, Spadoni, Rouissi, & Bertolini, 2012). Fungi capable of producing antifungal volatile organic compounds (VOCs) have the potential of being used as biofumigants and to be potential new BCAs for controlling postharvest diseases. The production of VOCs, including 2-phenethyl alcohol, could play an essential role in the antagonistic activity of *A. pullulans* against fruit postharvest pathogens both *in vitro* and *in vivo* (Di Francesco, Ugolini, Lazzeri, & Mari, 2015). *P. anomala,* when applied in fruit wounds,

initially may consume a large part of the available oxygen, but later, during the stationary phase, it could use alcoholic fermentation to produce antifungal VOCs, such as ethanol or ethyl acetate (Kwasiborski *et al.*, 2014), which could be involved in the antimicrobial activity against *B. cinerea* (Fredlund *et al.*, 2004). The main issue, when elucidating the role of diffusible or volatile antimicrobial compounds, is the assessment of the real impact of VOCs on biocontrol efficacy. Based on the results obtained *in vitro*, it is difficult to relate with the antagonistic activity on fruit surfaces under real situations. When tests are conducted *in vivo*, however, it is imperative to know the relevance of VOC concentration applied to that produced under commercial conditions.

Muscodor albus and *Muscodor crispans* are endophytic fungi that produce mixtures of VOCs with antimicrobial activity (Mitchell, Strobel, Moore, Robison, & Sears, 2010). The potency and types of VOCs, that include 3-methyl-1-butanol, 2-nonanone, and phenylethyl alcohol, vary among isolates. An isolate of *M. albus*, obtained from a cinnamon tree, was able to kill a wide spectrum of plant pathogens and other microorganisms both *in vitro* and *in vivo* (Schnabel & Mercier, 2006). The fungus was further developed as a commercial product, but later it was dropped by the company, since some of these VOCs pose carcinogenic risks (personal communication).

Another antifungal volatile-producing species, *Oxyporus latemarginatus*, was able to inhibit the mycelial growth of *A. alternata*, *B. cinerea* and *Colletotrichum gloeosporioides*, by mycofumigation (Lee *et al.*, 2009). Mycofumigation with solid cultures of this strain effectively reduced also the development of postharvest apple decay caused by *B. cinerea*, due to the production of 5-pentyl-2-furaldehyde. *Candida intermedia* and *Sporodiobolus pararoseus* were able to suppress conidial germination and mycelial growth of *B. cinerea* and control grey mould of strawberry due to the release of a variety of VOCs, including 2-nonanone (Huang *et al.*, 2011) and 2-ethyl-1-hexanol (Huang *et al.*, 2012).

VOC-producing microorganisms open new possibilities to control microbial decays in the agro-food chain, as biofumigation does not require physical contact with the product or commodity to be treated. Volatile compounds are ideal antimicrobials because their spectrum of activity extends from proximal interaction through water diffusion to greater distances via air diffusion. In this regard, however, the safety of using such approach needs to be thoroughly evaluated.

3.5 Parasitism and release of hydrolases

Parasitism occurs when the antagonist feeds on the pathogen, resulting in a direct destruction or lysis of fungal propagules and structures. Wisniewski *et al.* (1991) observed a strong adhesion *in vitro* of *P. guilliermondii* antagonist cells to *B. cinerea* mycelium, perhaps due to a lectin like interaction.

Such adhesion was blocked by exposure to compounds able to alter the protein integrity and the respiration process. When *Candida saitoana* cells were cultivated together with *B. cinerea* mycelium, the fungal hyphae showed cytological damages, such as formation of papillae and other protuberances in the cell wall, as well as cytoplasm degeneration (El-Ghaouth, Wilson, & Wisniewski, 1998).

In the fungal cell walls, chitin as a structural backbone is arranged in regularly ordered layers, and β-1,3-glucan as a filling material is arranged in an amorphous manner. Glucan is the major structural polysaccharide of the fungal cell wall, constituting approximately 50–60% by dry weight of the wall. β-1,3-glucan is considered the main structural constituent to which other cell wall components are covalently attached, providing the cell wall with mechanical strength and integrity. Chitin is a linear, insoluble homopolymer composed of beta-1,4-linked subunits of the acetylated amino sugar N-acetylglucosamine. The cell walls of filamentous fungi consist of up to 20% or more chitin (Seidl, 2008). In addition, proteins represent approximately 20–30% of the cell wall. Most of the cell wall proteins are glycoproteins extensively modified with O-linked and N-linked oligosaccharides. Cell wall proteins play an important role in maintaining cell shape, mediating adhesion for cell migration and fusion, protecting the cell against foreign substances, mediating the absorption of molecules and synthesizing and remodelling cell wall components. Breakdown of fungal cell wall requires the participation of different enzymes, especially β-1,3-glucanase and chitinase, but also proteases. Glucanases, chitinases and proteases can be directly or indirectly involved in the mechanism of several yeast antagonists of postharvest pathogens (Table 2).

3.5.1 Glucanases

Glucanases can hydrolyse glucans by two possible mechanisms: (1) exo-β-1,3-glucanase (EC3.2.1.58) that hydrolyse β-glucans by sequentially cleaving glucose residues from the non-reducing end, and (2) endo-β-1,3-glucanases (EC3.2.1.39) that cleave β-linkages at random sites along the polysaccharide chain, releasing smaller oligosaccharides and glucose.

Masih & Paul (2002) showed that exo-β-1,3-glucanase secreted by *P. membranifaciens* had a role in the biocontrol activity against *B. cinerea* on grapevine. Due to the assumed potential role of exo-β-1,3-glucanase in biocontrol systems (Daguerre, Siegel, Edel-Hermann, & Steinberg, 2014), glucanase genes have been cloned and characterized from different yeast BCAs, including *C. oleophila*, *P. anomala*, and *P. guilliermondii* (Grevesse, Lepoivre, & Jijakli, 2003; Bar-Shimon *et al.*, 2004; Zhang, Spadaro, Valente, Garibaldi, & Gullino, 2011b). The contribution of exo-ß-1,3-glucanase to the biocontrol activity of *C. oleophila* was investigated by generating *CoEXG1*-knockouts and double-*CoEXG1* transformants: the control activity of the transformants against *P. digitatum* on kumquat fruit did not differ, however, from that of the wild-type strain (Yehuda, Droby, Bar-Shimon,

Wisniewski, & Goldway, 2003). Different results were obtained when two exo-ß-1,3-glucanase genes of *P. anomala* – *PaEXG1* and *PaEXG2* – were separately and sequentially disrupted (Friel, Pessoa, Vandenbol, & Jijakli, 2007). The resulting mutant strains showed a significantly reduced efficiency of grey mould control when applied to wounded apple fruit, demonstrating that exo-ß-1,3-glucanases play a role in antagonism.

3.5.2 Chitinases

Chitinases hydrolyse chitin, the unbranched homopolymer of N-acetyl glucosamine in a β-1,4 linkage, by two possible mechanisms: (1) exo-chitinase or N-acetyl-b-glucosaminidase (EC 3.2.1.52) sequentially cleave NAG residues from the end, and (2) endo-chitinase (EC 3.2.1.14) cleave β-linkages at random sites along the polymer chain (Stoykov, Pavlov, & Krastanov, 2015). In recent decades, a significant number of investigations were performed on chitinases produced by antagonistic yeast (Chan & Tian, 2005; Castoria *et al.,* 2001). Extracellular chitinase enzymes produced by strains of *M. pulcherrima* showed an inhibitory effect against *B. cinerea* (Saravanakumar, Spadaro, Garibaldi, & Gullino, 2009). *Metschnikowia fructicola* exhibited chitinase activity and the chitinase gene MfChi was highly induced in the presence of *Monilinia fructicola* cell wall, suggesting a possible primary role of MfChi chitinase in the antagonistic activity of the yeast (Banani *et al.,* 2015). The MfChi chitinase overexpressed in *Pichia pastoris* significantly controlled *Monilinia fructicol*a and *Monilinia laxa in vitro* and on peaches.

3.5.3 Proteases

Proteases are divided into four major groups according to the character of their catalytic active site and conditions of action (Barrett, Rawlings, & Woessner, 2003): serine proteinases (EC 3.4.21), cysteine proteinases (EC 3.4.22), aspartic proteinases (EC 3.4.23), and metalloproteinases (EC 3.4.24). *A. pullulans* in apple and peach wounds releases extracellular glucanases, chitinases and proteases, that presumed to play a role in the antagonistic activity (Zhang *et al.,* 2010). An alkaline serine protease gene (ALP5) was cloned from *A. pullulans* and expressed in *Escherichia coli* (Zhang, Spadaro, Valente, Garibaldi, & Gullino, 2012) and in *P. pastoris* (Banani *et al.,* 2014). When the efficacy of ALP5 was evaluated against postharvest pathogens on apples, the protease was more efficient in controlling *Monilinia fructicola*, *B. cinerea* than *P. expansum* and *A. alternata* (Banani *et al.,* 2014). However, the extent of the activity was dependent on the enzyme concentration and the length of fruit storage.

3.6 Induction of resistance

Yeast antagonists have the capability to interact with the host tissue, particularly the wounds, increasing the cicatrisation processes. These antagonistic were much more effective when applied before pathogen inoculation. This observation raised the assumption that yeast cells could induce resistance processes in fruit skin through elicitors that are either secreted or component of their cell wall.

Induction of several biochemical and molecular defence responses following the application of yeast BCAs to fruit surfaces have already been demonstrated in the past. One of the first studies in this relation showed that treatment of lemon wounds with *P. guilliermondii* enhanced the production of the phytoalexin scoparone (Rodov, Ben-Yehoshua, D'Hallewin, & Castia, 1994). Similarly, Arras (1996) showed that scoparone accumulation could be 19 times higher when the antagonist *C. famata* was inoculated 24 h prior to *P. digitatum*, and four times higher if inoculated 24 h after the pathogen. *C. saitoana* induced chitinase activity and caused deposition of papillae and protuberances on host cells in apple surface wounds (El-Ghaouth *et al.*, 1998). In apple wounds, *A. pullulans* caused transient increases in β-1,3-glucanase, chitinase, and peroxidase (POD) activities. These increases started 24 h after treatment and reached maximum levels at 48 h and 96 h after treatment. Wounding also increased β-1,3-glucanase, chitinase, and POD activity, but the increments were markedly lower than those detected in yeast-treated wounds (Ippolito, El Ghaouth, Wilson, & Wisniewski, 2000). Fajardo, McCollum, McDonald, & Mayer (1998) demonstrated that various biologically based elicitors, including a strain of *C. oleophila*, were capable of inducing resistance to *P. digitatum*, when it was inoculated 14 h after the application of the elicitors to whole, unwounded orange (*Citrus sinensis* cv. Valencia) fruits. This increased resistance was associated with differential temporal induction of chitinase, β-1,3-glucanase, and POD activities. Application of *C. oleophila* to surface wounds or to intact 'Marsh Seedless' grapefruit elicited systemic resistance against *P. digitatum*, the main postharvest pathogen of citrus fruit (Droby *et al.*, 2002). The induction of pathogen resistance in fruit was pronounced already 24 h after elicitation; it was distance, concentration, and time dependent, and it was restricted to the peel tissue closely surrounding the yeast application site. The induction of pathogen resistance required viable yeast cells at concentrations of 10^8 to 10^9 cells/ml. Nonviable autoclaved or boiled yeast cells or lower yeast concentrations were ineffective in enhancing fruit disease resistance. Application of *C. oleophila* cell suspensions to grapefruit peel tissue increased ethylene biosynthesis, phenylalanine ammonia lyase activity, and phytoalexin accumulation, and increased chitinase and endo-β-1,3-glucanase protein levels, as indicated by western immunoblotting analysis. Scanning electron microscope observations revealed that spore

germination and germ tube growth of *P. digitatum* were markedly inhibited in wounds made near the yeast-treated sites (Droby *et al.,* 2002).

Yao & Tian (2005) showed that treatment of peach fruit with *C. laurentii* in combination with methyl jasmonic acid (MeJA) induced stronger activities of chitinase, β-1,3-glucanase, phenylalanine ammonia-lyase (PAL) and POD in peach fruit than the yeast or MeJA alone and the BCA significantly reduced the diameter of fruit lesions caused by *Monilinia fructicola* and *P. expansum*. The onset of the disease resistance against *Monilinia fructicola* and *P. expansum* paralleled closely the increase in chitinase, β-1,3-glucanase, PAL and POD activity. When *C. laurentii* was applied to jujube fruit, β-1,3-glucanase activity increased, and the expression of the corresponding *Glu-1* gene in fruit tissue was highly induced. Consequently it was concluded that the product of this gene may play a role in the defence response against infection by *A. alternata* and *P. expansum* (Tian *et al.,* 2007).

Using a proteomic approach, Chan *et al.* (2007) demonstrated that application of the yeast antagonist *P. membranifaciens* on peach fruits induced various proteins in fruit tissue including antioxidant proteins, such as glutathione peroxidase, CAT and peroxiredoxin, methionine sulfoxide reductase, polyphenol oxidase that are related to the repair of oxidative damage and to protect the tissue against oxidative damage and responsible for diseases resistance. In addition, *P. membranifaciens* increased activities of PR-9, PR-10, GTP-binding, and heat shock proteins.

Lu *et al.* (2013) reported that preharvest application of antagonistic yeast *Rhodosporidium paludigenum* induced resistance against postharvest diseases in mandarins through the activation of defence-related enzymes, such as β-1,3-glucanase, phenylalanine ammonia-lyase, POD and polyphenoloxidase.

Although all the results about induction of resistance responses in the host tissue following antagonist treatment are correlative, direct evidence for the ability of induced substances to inhibit pathogen infection and development has not yet been established.

3.7 The role of oxidative stress and alleviation of oxidative damage

The production of ROS in plants is an initial response to microorganisms, both pathogenic and non-pathogenic (Bolwell *et al.,* 2002). In the case of a non-compatible host–parasite interaction, an initial moderate increase in the production of ROS usually precedes a stronger oxidative burst, while in a compatible interaction no further increase in the level of reactive radicals in host tissue is observed.

Oxidative burst at the injury site following the colonization of antagonist cells was suggested to have a role in the mechanism of action of antagonistic yeast and may be involved in signalling pathways resulting in activation of fruit resistance system (Chan & Tian, 2005; Macarisin, Droby, Bauchan, &

Wisniewski, 2010). To play this role, antagonist cells must be able to tolerate alleviated levels of oxidative stress. In this regard, Castoria, Caputo, De Curtis, & De Cicco (2003) were the first to report that postharvest biocontrol fitness of the yeast antagonists *C. laurentii* LS-28 and *R. glutinis* was correlated with their ability to tolerate relatively high levels of ROS. These findings highlighted the role of oxidative stress in biocontrol systems and its possible direct and indirect effects either on the fruit tissue or on the antagonist cells at intercellular and intracellular level. In this relation, Liu *et al.* (2012), evaluated the response of several yeast BCAs (*Metschnikowia fructicola, C. oleophila and Cystofilobasidium infirmominiatum* PL1) to oxidative stress. Findings indicated that *C. infirmominiatum* was the most sensitive to exposure to exogenous H_2O_2, while *Metschnikowia fructicola* was the most tolerant.

Macarasin *et al.* (2010) demonstrated that yeast antagonists used to control postharvest diseases have the ability to produce relatively high amounts of super oxide anions. Interestingly, in this work, yeast applied to surface wounds of fruits produced higher amounts of super oxide anions than yeast grown *in vitro* in artificial media.

Superoxide anion production on the intact fruit surface could also serve as a QS signal to trigger aggregation into a biofilm which would increase yeast attachment and improve survival on the fruit surface by providing a microenvironment resistant to environmental stress. While the role of O_2^- in yeast cell multiplication, intercellular communication, or as an adaptive response to an unstable environment remains to be elucidated, results clearly show that, when yeasts sense host tissue, they are able to produce and apparently tolerate high levels of $O2^-$, regardless of the availability of nutrients.

4. The potential of Omics to study antagonist-pathogen-host interactions

The availability of more efficient DNA-based and proteomics technologies, along with bioinformatics, has provided new opportunities and tools to gain deeper and more accurate insights about the interactions already indicated (An, Chen, Li, Qin, & Tian, 2014). Though omics technologies have been widely used to elucidate the mechanisms of action of BCAs against soilborne and foliar pathogens, seldom they have been used to clarify the modes of action of postharvest BCAs (Massart, Perazzolli, Höfte, Pertot, & Jijakli, 2015). Developments in deep sequencing, transcriptomics, proteomics, metagenomics, comparative and functional genomics could be utilized to determine changes in the physiological status of BCAs, and the effect of environmental stress on its intracellular machinery (Herschkowitz *et al.*, 2013).

555 Global changes in gene expression both in host tissue and antagonist cells have been reported (Chan
556 *et al.*, 2007; Jiang *et al.*, 2009; Hershkovitz *et al.*, 2012; 2013). New omic tools can be used to evaluate
557 the global effect of the application of BCAs on the transcriptome and/or proteome of fruit. To obtain
558 an overview on transcript modification during the interaction of cherry tomato fruit with *C. laurentii*,
559 a microarray analysis, using Affymetrix Tomato Genechip arrays, representing approximately 10,000
560 genes, was performed (Jiang *et al.* 2009). The results showed that 194 and 312 genes were up- or
561 downregulated, respectively, more than ten time fold in BCA-treated tomato fruit as compared with
562 control fruit. Up-regulated genes included genes involved in metabolism, signal transduction, and
563 stress response. Conversely, genes related to energy metabolism and photosynthesis were generally
564 down-regulated. BCA treatment induces fruit resistance response and it suppresses energy
565 metabolism and photosynthesis.
566 In grapefruit surface wounds treated with *Metschnikowia fructicola* cells, there was significant
567 expression of PRPs genes and MAPK cascade genes involved in defence signalling, and down-
568 regulation in antioxidant genes, like POD, superoxide dismutase (SOD) and CAT. The genes up-
569 regulated by *Metschnikowia fructicola* in grapefruit were consistent with an induced resistance
570 response and it was suggested that the induced response played a role in the efficacy of *Metschnikowia*
571 *fructicola* against postharvest pathogens like *P. digitatum* (Hershkovitz *et al.*, 2012). Chan *et al.*
572 (2007) indicated that *P. membranifaciens* induced antioxidant and PR proteins in peach fruit, and it
573 was suggested that these proteins played an essential role in the control of *P. expansum* in this
574 biocontrol system. In an investigation to study the responses of cherry tomato to the yeast antagonist
575 *C. laurentii*, Jiang *et al.* (2009) showed that genes involved in metabolism, signal transduction, and
576 stress response were up-regulated while genes related to energy metabolism and photosynthesis were
577 suppressed. Hershkovitz *et al.* (2013) conducted a transcriptomic analysis, using RNA-Seq, to
578 examine changes in gene expression in *Metschnikowia fructicola* when it was exposed to citrus tissues
579 and the postharvest pathogen *P. digitatum*. Results indicated that more than 250 genes exhibited
580 expression responses specifically associated with the yeast-citrus vs. the yeast-pathogen interaction.
581 Genes related to transmembrane, multidrug transport, and amino acid metabolism were induced in
582 the yeast-pathogen interaction, while expression of genes involved in oxidative stress, iron
583 homeostasis, zinc homeostasis, and lipid metabolism were induced in the yeast-fruit interaction.
584 Collectively, these reports indicate that different gene/protein profiles are involved in different
585 antagonistic yeast–host–pathogen interactions, demonstrating the dynamics of different biocontrol
586 system and how “omic” technologies can provide insights into the modes of action of antagonistic
587 yeast. The above reported studies were the first to report molecular changes at the biocontrol system.
588 Determination of changes in the level of expression of “biocontrol genes" during mass production,

formulation and storage, or in response to exposure and contact with host plant tissue after application can be now easily studied. It is expected however, that many more results will be reported in the near future about interactions between antagonistic yeast, host tissue, the pathogen, and also the epiphytic microflora.

5. Conclusions

To date, there are hundreds of reports about using of yeast antagonists to biologically control postharvest diseases. Very few of these antagonists, however, have reached the commercial development stage and launched as commercial products. In most cases, there are inherit problems in the biocontrol systems related to poor performance and inconsistency under commercial conditions. Among the reasons for these shortcoming is the lack of understanding of mechanisms of actions of these BCAs. It is apparent that the performance of yeast BCA is the result of complex interactions taking place between all the components of the biocontrol system (plant host, the antagonist, the pathogen, and resident microflora). Although these interactions have been the subject of research for over thirty years, our understanding is still incomplete. This because of the difficulties associated with the study of complex interactions and the lack of appropriate research tools and technologies.

In recent years there has been a phenomenal advancement in the use of molecular techniques contributing to the development of innovative tools for improving knowledge on the antagonistic mechanisms of BCAs. In particular, the omics techniques, including genomics, transcriptomics, proteomics, metagenomics, and metabolomics are providing a powerful tool to dissect the complex interactions between the antagonist, the pathogen, the fruit host, the natural microflora, and the environmental conditions. Induced resistance has been suggested to be one of the mechanisms of action of postharvest BCAs. However, information about elicitors/effectors of the antagonist involved and our ability to genetically and physiologically manipulate them is still lacking. Fundamental knowledge on the physiology, genetic traits and molecular basis of colonization, survival and differentiation of BCAs on plant tissue is needed.

From a commercial point of view, complex modes of action make antagonistic performance and efficacy more dependent on production, formulation, packing, application, and storage. A deep understanding of the mode of action is essential to develop appropriate formulation and methods of application, and to obtain registration.

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627 **References**

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629 An, B., Chen, Y., Li, B., Qin, G., & Tian, S. (2014). Ca2+-CaM regulating viability of *Candida*
630 *guilliermondii* under oxidative stress by acting on detergent resistant membrane proteins. *Journal of*
631 *Proteomics, 109,* 38-49.

632 Arras, G. (1996). Mode of action of an isolate of *Candida famata* in biological control of *Penicillium*
633 *digitatum* in orange fruits. *Postharvest Bioloy and Technology, 8,* 191–198.

634 Banani, H., Spadaro, D., Zhang, D., Matic, S., Garibaldi, A., & Gullino, M.L. (2014) Biocontrol
635 activity of an alkaline serine protease from *Aureobasidium pullulans* expressed in *Pichia pastoris*
636 against four postharvest pathogens on apple. *International Journal of Food Microbiology, 182-183,*
637 1-8.

638 Banani, H., Spadaro, D., Zhang, D., Matic, S., Garibaldi, A., & Gullino, M.L. (2015). Postharvest
639 application of a novel chitinase cloned from *Metschnikowia fructicola* and overexpressed in *Pichia*
640 *pastoris* to control brown rot of peaches. *International Journal of Food Microbiology, 199,* 54-61.

641 Barrett, A.J., Rawlings, N.D., & Woessner, J.F. (2003). *The Handbook of Proteolytic Enzymes*, 2nd
642 ed. Academic Press: London.

643 Bar-Shimon, M., Yehuda, H., Cohen, L., Weiss, B., Kobeshnikov, A., Daus, A., et al. (2004).
644 Characterization of extracellular lytic enzymes produced by the yeast biocontrol agent *Candida*
645 *oleophila. Current Genetics, 45,* 140-148.

646 Bassam, A., Annous Pina, M., Fratamico, & Smith, J.L. (2009). Quorum Sensing in Biofilms: Why
647 Bacteria Behave the Way They Do. *Journal of Food Science, 74,* R24–R37.

648 Bencheqroun, S.K., Bajji, M., Massart, S., Labhilili, M., El Jaafari, S., Jijakli, M.H. (2007). In vitro
649 and in situ study of postharvest apple blue mold biocontrol by *Aureobasidium pullulans*: Evidence
650 for the involvement of competition for nutrients. *Postharvest Biology and Technology, 46,* 128–135.

651 Biswas, S., Van Dijck, P., & Datta, A. (2007). Environmental sensing and signal transduction
652 pathways regulating morphopathogenic determinants of *Candida albicans*. *Microbiology and*
653 *Molecular Biology Reviews, 71,* 348-376.

654 Bolwell, P.G., Bindschedler, L.A., Blee, K.A., Butt, V.S., Davies, D.R., Gardner, S.L., et al. (2002).
655 The apoplastic oxidative burst in response to biotic stress in plants: a three component system.
656 *Journal of Experimental Botany, 53,* 1367–1376.

657 Bove, J., Kim, C.Y., Gibson, C.A., & Assmann, S.M. (2008). Characterization of wound-responsive
658 RNA-binding proteins and their splice variants in Arabidopsis. *Plant Molecular Biology, 67,* 71-88.

659 Breinig, F., Tipper, D. J., & Schmitt, M.J. (2002). Kre1p, the plasma membrane receptor for the
660 yeast K1 viral toxin. *Cell, 108,* 395–405.

661 Calvente, V., Benuzzi, D., & de Tosetti, M.I.S. (1999). Antagonistic action of siderophores from
662 *Rhodotorula glutinis* upon the postharvest pathogen *Penicillium expansum*. *International*
663 *Biodeterioration and Biodegradation, 43*,167-172.
664 Calvo-Garrido, C., Viñas, I., Usall, J., Rodríguez-Romera, M., Ramos, M.C., & Teixidó, N. (2014).
665 Survival of the biological control agent *Candida sake* CPA-1 on grapes under the influence of abiotic
666 factors. *Journal of Applied Microbiology, 117,* 800-811.
667 Cantu, D., Blanco-Ulate, B., Yang, L., Labavitch, J.M., Bennett, A.B., & Powell, A.L.T. (2009).
668 Ripening-regulated susceptibility of tomato fruit to *Botrytis cinerea* requires *NOR* but not *RIN* or
669 ethylene. *Plant Physiology, 150,* 1434-1449.
670 Castoria, R., Caputo, L., De Curtis, F., & De Cicco, V. (2003). Resistance of postharvest biocontrol
671 yeasts to oxidative stress: A possible new mechanism of action. *Phytopathology, 93,* 564-572.
672 Castoria, R., De Curtis, F., Lima, G., Caputo, L., Pacifico, S., & De Cicco, V. (2001).
673 *Aureobasidium pullulans* (LS-30) an antagonist of postharvest pathogens of fruits: study on its modes
674 of action. *Postharvest Biology and Technology*, *22,* 7–17.
675 Chan, Z., Qin, G., Xu, X., Li, B., & Tian, S. (2007). Proteome approach to characterize proteins
676 induced by antagonist yeast and salicylic acid in peach fruit. *Journal of Proteome Research, 6,* 1677-
677 1678.
678 Chan, Z., & Tian, S. (2005). Interaction of antagonistic yeasts against postharvest pathogens of
679 apple fruit and possible mode of action. *Postharvest Biology and Technology, 36,* 215–223.
680 Chanchaichaovivat, A., Panijpan, B., & Ruenwongsa, P. (2008). Putative modes of action of *Pichia*
681 *guilliermondii* strain R13 in controlling chilli anthracnose after harvest. *Biological Control, 47,* 207–
682 215.
683 Chen, H., & Fink, G.R. (2006). Feedback control of morphogenesis in fungi by aromatic alcohols.
684 *Genes & Development, 20,* 1150–1161.
685 Chung, D., Haas, H., & Cramer, R.A. (2012). Coordination of hypoxia adaptation and iron
686 homeostasis in human pathogenic fungi. *Frontiers in Microbiology, 3,* 381. doi:
687 10.3389/fmicb.2012.00381
688 Daguerre, Y., Siegel, K., Edel-Hermann, V., & Steinberg, C. (2014). Fungal proteins and genes
689 associated with biocontrol mechanisms of soil-borne pathogens: a review. *Fungal Biology Reviews,*
690 *28,* 97-125.
691 Demoz, B.T., & Korsten, L. (2006). *Bacillus subtilis* attachment, colonization, and survival on
692 avocado flowers and its mode of action on stem-end rot pathogens. *Biological Control, 37,* 68–74.

693 Di Francesco, A., Ugolini, L., Lazzeri, L., & Mari, M. (2015). Production of volatile organic
694 compounds by *Aureobasidium pullulans* as a potential mechanism of action against postharvest fruit
695 pathogens. *Biological Control, 81*, 8-14.
696 Droby, S., Chalutz, E., Wilson, C.L., & Wisniewski, M.E. (1989). Characterization of the biocontrol
697 activity of *Debaryomices hansenii* in the control of *Penicillium digitatum* on grapefruit. *Canadian*
698 *Journal of Microbiology, 35*, 794-800.
699 Droby, S., Vinokur, V., Weiss, B., Cohen, L., Daus, A., Goldschmidt, E.E., et al. (2002). Induction
700 of Resistance to *Penicillium digitatum* in Grapefruit by the Yeast Biocontrol Agent *Candida*
701 *oleophila. Phytopathology, 92*, 393-399.
702 Droby, S., Wisniewski, M., Macarisin, D., & Wilson, C. (2009). Twenty years of postharvest
703 biocontrol research: Is it time for a new paradigm? *Postharvest Biology and Technology, 52*, 137-
704 145.
705 El-Ghaouth, A., Wilson, C., & Wisniewski, M. (1998). Ultrastructural and cytochemical aspects of
706 the biological control of *Botrytis cinerea* by *Candida saitoana* in apple fruit. *Phytopathology, 88*,
707 282–291.
708 Fajardo, J.E., McCollum, T.G., McDonald, R. E., & Mayer, R.T. (1998). Differential induction of
709 proteins in orange flavedo by biologically based elicitors and challenged by *Penicillium digitatum*
710 Sacc. *Biological Control, 13*, 143-151.
711 FAO report (2011). Global Food Losses and Food Waste: Extent, Causes and Prevention. FAO,
712 Rome. Available at: http://www.fao.org/docrep/014/mb060e/mb060e.pdf (Accessed on 7 April
713 2015).
714 Filonow, A.B. (1998). Role of competition for sugars by yeasts in the biocontrol of gray mould of
715 apple. *Biocontrol Science and Technology, 8*, 243-256.
716 Finiti, I., de la O. Leyva, M., Vicedo, B., Gómez-Pastor, R., López-Cruz, J., García-Agustín, P., et
717 al. (2014). Hexanoic acid protects tomato plants against *Botrytis cinerea* by priming defence
718 responses and reducing oxidative stress. *Molecular Plant Pathology, 15*, 550-562.
719 Finkel, J.S., & Mitchell, A.P. (2011). Genetic control of *Candida albicans* biofilm development.
720 *Nature Reviews Microbiology, 9*, 109–118.
721 Fiori, S., Fadda, A., Giobbe, S., Berardi, E., & Migheli, Q. (2008). *Pichia angusta* is an effective
722 biological control yeast against postharvest decay of apple fruit caused by *Botrytis cinerea* and
723 *Monilia fructicola. FEMS Yeast Research, 8*, 961–963.
724 Fredlund, E., Druvefors, U.A., Olstorpe, M.N., Passoth, V., & Schnurer, J. (2004). Influence of
725 ethyl acetate production and ploidy on the anti-mould activity of *Pichia anomala*. *FEMS*
726 *Microbiology Letters, 238*, 133–137.

727 Friel, D., Pessoa, N.M.G, Vandenbol, M., & Jijakli, M.J. (2007). Separate and combined disruptions
728 of two exo-β-1,3-glucanase genes decrease the efficiency of *Pichia anomala* (strain K) biocontrol
729 against *Botrytis cinerea* on apple. *Molecular Plant-Microbe Interactions, 20,* 371-379.
730 Giobbe, S., Marceddu, S., Scherm, B., Zara, G., Mazzarello, V.L., Budroni, M., et al. (2007). The
731 strange case of a biofilm-forming strain of *Pichia fermentans*, which controls *Monilinia* brown rot on
732 apple but is pathogenic on peach fruit. *FEMS Yeast Research, 7,* 1389-1398.
733 Gori, K., Knudsen, P.B., Nielsen, K.F., Arneborg, N., & Jespersen, L. (2011). Alcohol-based
734 quorum sensing plays a role in adhesion and sliding motility of the yeast *Debaryomyces hansenii*.
735 *FEMS Yeast Research, 11,* 643–652.
736 Grevesse, C., Lepoivre, P., & Jijakli, M.H. (2003). Characterization of the exo-glucanase encoding
737 gene PaEXG2 and study of its role in the biocontrol activity of *Pichia anomala* strain K.
738 *Phytopathology, 93,* 1145-1152.
739 Heller, J., & Tudzynski, P. (2011). Reactive oxygen species in phytopathogenic fungi: signaling,
740 development, and disease. *Annual Reviews of Phytopathology, 49,* 369–390.
741 Hershkovitz, V., Ben-Dayan, C., Raphael, G., Pasmanik-Chor, M., Liu, J., Belausov, E., et al.
742 (2012). Global changes in gene expression of grapefruit peel tissue in response to the yeast biocontrol
743 agent *Metschnikowia fructicola*. *Molecular Plant Pathology, 13,* 338–349.
744 Hershkovitz, V., Sela, N., Taha-Salaime, L., Liu, J., Rafael, G., Kessler, C., et al. (2013). De-novo
745 assemble and characterization of the transcriptome of *Metschnikowia fructicola* reveals differences
746 in gene expression following interaction with *Penicillium digitatum* and grapefruit peel. *BMC*
747 *Genomics, 14,* 168.
748 Huang, R., Che, H.J., Zhang, J., Yang, L., Jiang, D.H., & Li, G.Q. (2012). Evaluation of
749 *Sporidiobolus pararoseus* strain YCXT3 as biocontrol agent of *Botrytis cinerea* on post-harvest
750 strawberry fruits. *Biological Control, 62,* 53-63.
751 Huang, R., Li, G.Q., Zhang, J., Yang, L., Che, H.J., Jiang, D.H., et al. (2011). Control of postharvest
752 Botrytis fruit rot of strawberry by volatile organic compounds of *Candida intermedia*.
753 *Phytopathology, 101,* 859-869.
754 Ianiri, G., Idnurm, A., Wright, S.A.I., Duran-Patron, R., Mannina, L., Ferracane, R., et al. (2013).
755 Searching for genes responsible for patulin degradation in a biological control yeast provides insights
756 into the basis for resistance to this mycotoxin. *Applied and Environmental Microbiology, 79,* 3101-
757 3115.
758 Ippolito, A., El Ghaouth, A., Wilson, C. L., & Wisniewski, M. (2000). Control of postharvest decay
759 of apple fruit by *Aureobasidium pullulans* and induction of defense responses. *Postharvest Biology*
760 *and Technology, 19,* 265-272.

761 Janisiewicz, W.J., & Korsten, L. (2002). Biological control of postharvest diseases of fruits. *Annual*
762 *Reviews of Phytopathology, 40,* 411-441.
763 Janisiewicz, W.J., & Peterson, D.L. (2004). Susceptibility of the stem pull area of mechanically
764 harvested apples to blue mold decay and its control with a biocontrol agent. *Plant Disease, 88,* 662-
765 664.
766 Janisiewicz, W.J., Tworkoski, T.J., & Kurtzman, C.P. (2001). Biocontrol potential of
767 *Metschnikowia pulcherrima* strains against blue mold of apple. *Phytopathology, 91,* 1098-1108.
768 Jiang, F., Zheng, X., & Chen, J. (2009). Microarray analysis of gene expression profile induced by
769 the biocontrol yeast *Cryptococcus laurentii* in cherry tomato fruit. *Gene, 430,* 12-16.
770 Jones, J.D.G., & Dangl, J.L. (2006). The plant immune system. *Nature, 444,* 323-329.
771 Kwasiborski, A., Bajji, M., Renaut, J., Delaplace, P., & Jijakli, H. (2014). Identification of
772 Metabolic Pathways Expressed by *Pichia anomala* Kh6 in the Presence of the Pathogen *Botrytis*
773 *cinerea* on Apple: New Possible Targets for Biocontrol Improvement. *PLoS ONE, 9 (3),* e91434.
774 doi:10.1371/journal.pone.0091434
775 Lahlali, R., Raffaele, B., & Jijakli, M.H. (2011). UV protectants for *Candida oleophila* (strain O),
776 a biocontrol agent of postharvest fruit diseases. *Plant Pathology, 60,* 288-295.
777 Lee, S.O., Kim, H.Y., Choi, G.J., Lee, H.B., Jang, K.S., Choi, Y.H., et al. (2009). Mycofumigation
778 with *Oxyporus latemarginatus* EF069 for control of postharvest apple decay and Rhizoctonia root rot
779 on moth orchid. *Journal of Applied Microbiology, 106,* 1213-1219.
780 Liu, J., Sui, Y., Wisniewski, M., Droby, S., & Liu, Y. (2013a). Review: Utilization of antagonistic
781 yeasts to manage postharvest fungal diseases of fruit. *International Journal of Food Microbiology*
782 *167,* 153–160.
783 Liu, J., Wisniewski, M., Artlip, T., Sui, Y., Droby, S., & Norelli, J. (2013b). The potential role of
784 PR-8 gene of apple fruit in the mode of action of the yeast antagonist, *Candida oleophila*, in
785 postharvest biocontrol of *Botrytis cinerea*. *Postharvest Biology and Technology, 85,* 203–209.
786 Liu, P., Cheng, Y., Yang, M., Liu, Y., Chen, K., Long, C., et al. (2014). Mechanisms of action for
787 2-phenylethanol isolated from *Kloeckera apiculata* in control of *Penicillium* molds of citrus fruits.
788 *BMC Microbiology, 14,* 242.
789 Liu, J., Wisniewski, M., Droby, S., Norelli, J., Hershkovitz, V., Tian, S., et al. (2012). Increase in
790 antioxidant gene transcripts, stress tolerance and biocontrol efficacy of *Candida oleophila* following
791 sublethal oxidative stress exposure. *FEMS Microbiology Ecology, 80,* 578-590.
792 Liu, X., Wang, J., Gou, P., Mao, C., Zhu, Z.-R., & Li, H. (2007). *In vitro* inhibition of postharvest
793 pathogens of fruit and control of gray mold of strawberry and green mold of citrus by aureobasidin
794 A. *International Journal of Food Microbiology, 119,* 223-229.

Lu, L.,Ye, C., Guo, S., Sheng, K., Shao, L., Zhou, T., et al. (2013). Preharvest application of antagonistic yeast *Rhodosporidium paludigenum* induced resistance against postharvest diseases in mandarin orange. *Biological Control, 67,* 130–136.

Lutz, M.C., Sosa, M. C., Rodriguez, M.E., Lopez, C.A., & Sangorrín, M.P. (2013). Efficacy and putative mode of action of native and commercial antagonistic yeasts against postharvest rots of pear pathogens. International Journal of Food Microbiology, 164, 166-172.

Macarisin, D., Droby, S., Bauchan, G., & Wisniewski, M. (2010). Superoxide anion and hydrogen peroxide in the yeast antagonist-fruit interaction: A new role for reactive oxygen species in postharvest biocontrol? *Postharvest Biology and Technology, 58,* 194-202.

Mari, M., Martini, C., Spadoni, A., Rouissi, W., & Bertolini, P. (2012). Biocontrol of apple postharvest decay of *Aureobsidium pullulans. Postharvest Biology and Technology, 73,* 56-62.

Marquina, D., Santos, A., & Peinado, J.M. (2002). Biology of killer yeasts. Int. Microbiol. 5, 65-71.

Masih, E.I., & Paul, B. (2002). Secretion of b-1,3-glucanase by the yeast *Pichia membranifaciens* and its possible role in the biocontrol of *Botrytis cinerea* causing grey mold disease of the grapevine. *Current Microbiology, 44,* 391–395.

Massart, S., Martinez-Medina, M., & Jijakli, M.H. (2015) Biological control in the microbiome era: Challenges and opportunities. *Biological Control, 89,* 98, 108.

Massart, S., Perazzolli, M., Höfte, M., Pertot, I., & Jijakli, M.H. (2015). Impact of the omic technologies for understanding the modes of action of biological control agents against plant pathogens. *BioControl*, DOI 10.1007/s10526-015-9686-z.

Miethke, M., & Marahiel, M.A. (2007). Siderophore-Based Iron Acquisition and Pathogen Control. *Microbiology and Molecular Biology Reviews, 71,* 413–451.

Mitchell, A.M., Strobel, G.A., Moore, E., Robison, R., & Sears, J. (2010). Volatile antimicrobials from *Muscodor crispans*, a novel endophytic fungus. *Microbiology, 156,* 270-277.

Oberegger, H., Schoeser, M., Zadra, I., Abt, B., & Haas, H. (2001). SREA is involved in regulation of siderophore biosynthesis, utilization and uptake in *Aspergillus nidulans*. *Molecular Microbiology, 41,* 1077-1089.

Philpott, C.C., & Protchenko, O. (2008). Response to Iron Deprivation in *Saccharomyces cerevisiae. Eukaryotic Cell, 7,* 20–27.

Prusky, D., Alkan, N., Mengiste, T., & Fluhr, R. (2013). Quiescent and Necrotrophic Lifestyle Choice During Postharvest Disease Development. *Annual Review of Phytopathology, 51,* 155-176.

Prusky, D., & Gullino, M.L. (2010). Post-harvest Pathology. Springer: Dordrecht.

828 Pu, L., Jingfan, F., Kai, C., Chao-an, L., & Yunjiang, C. (2014). Phenylethanol promotes adhesion
829 and biofilm formation of the antagonistic yeast *Kloeckera apiculata* for the control of blue mold on
830 citrus. *FEMS Yeast Research, 14,* 536–546.
831 Robert-Seilaniantz, A., Grant, M., & Jones, J.D.G. (2011). Hormone Crosstalk in Plant Disease and
832 Defense: More Than Just JASMONATE-SALICYLATE Antagonism. *Annual Reviews of*
833 *Phytopathology, 49,* 317-343.
834 Rodov, V., Ben-Yehoshua, S., D'hallewin, G., & Castia, T. (1994). Accumulation of phytoalexins
835 scoparone and scopoletin in citrus fruits subjected to various postharvest treatments. *Acta*
836 *Horticulturae, 381,* 517-523.
837 Santos, A., San Mauro, M., Bravo, E., & Marquina, D. (2009). PMKT2, a new killer toxin from
838 *Pichia membranifaciens,* and its promising biotechnological properties for control of the spoilage
839 yeast *Brettanomyces bruxellensis*. *Microbiology, 155,* 624–634.
840 Saravanakumar, D., Ciavorella, A., Spadaro, D., Garibaldi, A., & Gullino, M.L. (2008).
841 *Metschnikowia pulcherrima* strain MACH1 outcompetes *Botrytis cinerea*, *Alternaria alternata* and
842 *Penicillium expansum* in apples through iron depletion. *Postharvest Biology and Technology, 49,*
843 121-128.
844 Saravanakumar, D., Spadaro, D., Garibaldi, A., & Gullino, M.L. (2009). Detection of enzymatic
845 activity and partial sequence of a chitinase gene in *Metschnikowia pulcherrima* strain MACH1 used
846 as post-harvest biocontrol agent. *European Journal of Plant Pathology, 123,* 183-193.
847 Schnabel, G., & Mercier, J. (2006). Use of a *Muscodor albus* pad delivery system for the
848 management of brown rot of peach in shipping cartons. *Postharvest Biology and Technology, 42,*
849 121-123.
850 Seidl, V. (2008). Chitinases of filamentous fungi: a large group of diverse proteins with multiple
851 physiological functions. *Fungal Biology Reviews, 22,* 36–42.
852 Selitrennikoff, C.P. (2001). Antifungal proteins. *Applied and Environmental Microbiology, 67,*
853 2883-2894.
854 Sharma R.R., D. Singh, R. Singh. 2009. Biological control of postharvest diseases of fruits and
855 vegetables by microbial antagonists: A review. *Biological Control, 50,* 205-221.
856 Smith, A.P, & Lindow, S. (2013). Contribution of nitrate assimilation to the fitness of *Pseudomonas*
857 *syringae* pv. *syringae* B728a on plants. *Applied and Environmental Microbiology, 79,* 678-687.
858 Smukalla, S., Caldara, M., Pochet, N., Beauvais, A., Guadagnini, S., Yan, C., et al. (2008). FLO1 is
859 a variable green beard gene that drives biofilm-like cooperation in budding yeast. *Cell, 135,* 726–737.
860 Spadaro, D., & Gullino, M.L. (2004). State of art and future perspectives of biological control of
861 postharvest fruit diseases. *International Journal of Food Microbiology, 91,* 185-194.

Spadaro, D., Ciavorella, A., Zhang, D., Garibaldi, A., & Gullino, M.L. (2010). Effect of culture media and pH on the biomass production and biocontrol efficacy of a *Metschnikowia pulcherrima* strain to be used as a biofungicide for postharvest disease control. *Canadian Journal of Microbiology, 56,* 128–137.

Stoykov, Y.M., Pavlov, A.I., & Krastanov, A.I. (2015). Chitinase biotechnology: Production, purification, and application. *Engineering in Life Sciences, 15,* 30–38.

Tian, S.P., Yao, H.J., Deng, X., Xu, X.B., Qin, G.Z., & Chan, Z.L. (2007). Characterization and expression of beta-1,3-glucanase genes in jujube fruit induced by the microbial biocontrol agent *Cryptococcus laurentii*. *Phytopathology, 97,* 260–268.

Wisniewski, M.E., Biles, C., Droby, S., McLaughlin, R., Wilson, C.L., & Chalutz, E. (1991). Mode of action of the postharvest biocontrol yeast, *Pichia guilliermondii*. I. Characterization of attachment to *Botrytis cinerea*. *Physiology and Molecular Plant Pathology, 39,* 245-258.

Yao, H.J., & Tian, S.P. (2005). Effects of a biocontrol agent and methyl jasmonate on postharvest diseases of peach fruit and the possible mechanisms involved. *Journal of Applied Microbiology, 98,* 941–950.

Yehuda, H., Droby, S., Bar-Shimon, M., Wisniewski, M., & Goldway, M. (2003). The effect of under- and overexpressed *CoEXG1*-encoded exoglucanase secreted by *Candida oleophila* on the biocontrol of *Penicillium digitatum*. *Yeast, 20,* 771-780.

Zhang, D. Spadaro, D., Valente, S., Garibaldi, A., & Gullino, M.L. (2012). Cloning, characterization, expression and antifungal activity of an alkaline serine protease of *Aureobasidium pullulans* PL5 involved in the biological control of postharvest pathogens. *International Journal of Food Microbiology, 153,* 453-464.

Zhang, D., Spadaro, D., Garibaldi, A., & Gullino, M.L. (2010). Efficacy of the antagonist *Aureobasidium pullulans* PL5 against postharvest pathogens of peach, apple and plum and its modes of action. *Biological Control, 54,* 172-180.

Zhang, D., Spadaro, D., Garibaldi, A., & Gullino, M.L. (2011a). Potential biocontrol activity of a strain of *Pichia guilliermondii* against grey mould of apples and its possible modes of action. *Biological Control, 57,* 193-201.

Zhang, D., Spadaro, D., Valente, S., Garibaldi, A., & Gullino, M.L. (2011b). Cloning, characterization and expression of an exo-1,3-beta-glucanase gene from the antagonistic yeast, *Pichia guilliermondii* strain M8 against grey mold on apples. *Biological Control, 59,* 284-293.

Figure captions

Figure 1 – Bitrophic system: main host fruit-pathogen interactions, including the specificity of the wound environment.

Figure 2 – Tritrophic system: modes of action used by biocontrol agents, when interacting with the pathogen and the host-fruit.

Figure 3 – Quadritrophic system: a systemic approach to the study of the mechanisms of action involved in postharvest biological control should consider the interactions occurring between host fruit, pathogen, antagonist and the epiphytic (endophytic) microflora.

Figure 4 – Chemical structure of two siderophores produced by yeast biocontrol agents.

Figure 5 – Inhibition of mycelial growth of *Penicillium digitatum* and *Botrytis cinerea* as a result of production of pulcherrimin by *Metschnikowia fructicola*. In presence of $FeCl_3$ in the growth medium (PDA), *M. fructicola* produced the red pigment pulcherrimin sounding its colony (left panels). Red arrows (right panels) show inhibition zones of either *P. digitatum* or *B. cinerea* co-cultured with the yeast on a medium containing $FeCl_3$.