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

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1 **REVIEW**

2  
3 **DEVELOPMENT OF BIOCONTROL PRODUCTS FOR POSTHARVEST DISEASES OF**  
4 **FRUIT: THE IMPORTANCE OF ELUCIDATING THE MECHANISMS OF ACTION OF**  
5 **YEAST ANTAGONISTS**  
6

7 Davide Spadaro<sup>1</sup>, Samir Droby<sup>2</sup>

8  
9 <sup>1</sup>Dept. Agricultural, Forestry and Food Sciences (DISAFA) and AGROINNOVA Centre of  
10 Competence for the Innovation in the Agroenvironmental Sector, University of Torino, Largo  
11 Braccini 2, 10095 Grugliasco (TO), Italy.

12  
13 <sup>2</sup>Department of Postharvest Science, ARO, The Volcani Center, P.O. Box 6, Bet Dagan, 50250, Israel.

14  
15 Corresponding author:

16 Davide Spadaro, phone: +39-0116708942; fax: +39-0112368942; email: [davide.spadaro@unito.it](mailto:davide.spadaro@unito.it)

18 **Abstract**

19

20 **Background**

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

25

26 **Scope and Approach**

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

32

33 **Key Findings and Conclusions**

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

44

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

46

## 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, Macarasin, & 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 (Janisiewicz & Peterson, 2004). Among the first products based on yeasts, Aspire™ (based on *Candida oleophila*) (Liu, Wisniewski, 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

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

94

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

102

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

114

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

119

## 120 **2. Fruit surface and wound environment**

121

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

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

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

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

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

164

### 165 **3. Studying the mechanism of action of postharvest biocontrol agents**

166

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

181

#### 182 **3.1 Competition for nutrients and space**



183

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

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

194 Competition for nutrients was evident for *Pichia guilliermondii* against *P. digitatum* co-cultivated on  
195 synthetic medium: the addition of exogenous nutrients resulted in a reduced efficacy and the  
196 antagonists was more effective in inhibiting the pathogen when nutrients were scarce (Droby,  
197 Chalutz, Wilson, & Wisniewski, 1989). Yeast can satisfactorily use a wide range of carbohydrates,  
198 which include disaccharides and monosaccharides, and nitrogen sources (Spadaro *et al.*, 2010).  
199 Nitrogen is likely to represent a limiting factor in the carbon-rich environment of pear wounds  
200 (Janisiewicz & Korsten, 2002). Exogenous amino acids, applied at high concentrations on apple  
201 wounds, significantly decreased the efficacy of *A. pullulans* against *P. expansum*, provide evidence  
202 that competition for nutrients may have major role in the mechanism of biocontrol activity  
203 (Bencheqroun *et al.*, 2007). Competition for sugars and nitrates plays a key role also in the  
204 interactions of *P. guilliermondii* with *B. cinerea* on apple (Zhang, Spadaro, Garibaldi, & Gullino,  
205 2011a) or *Colletotrichum* spp. on pepper (Chanchaichaovivat, Panijpan, & Ruenwongsa, 2008).

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

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

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

228

### 229 **3.2 Iron: a key source for competition**

230

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

240 In most microbial habitats, including the acidic pH of plant cells,  $Fe^{2+}$  is oxidized to  $Fe^{3+}$  to form  
241 stable ferric oxide hydrate complexes in the presence of oxygen and water. Strategies exhibited by  
242 yeast to respond to iron depletion consists of: (i) activation of systems of iron uptake, (ii) mobilization  
243 of intracellular stores of iron, and (iii) metabolic adaptations to iron limitation (Philpott & Protchenko,  
244 2008). Yeasts express two genetically distinct systems for iron uptake, namely, a reductive and a non-  
245 reductive system. Ferric salts and ferric chelates are substrates for the reductive system, while the  
246 nonreductive system exclusively recognizes siderophore-iron chelates.

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

251 pathogen germination, growth and virulence. In iron starvation conditions, fungi have a lower catalase  
252 (CAT) activity and a lower ROS protection (Oberegger, Schoeser, Zadra, Abt, & Haas, 2001).  
253 Siderophores are designed to form tight and stable complexes with ferric iron and they can be divided  
254 into three main classes depending on the chemical nature of the moieties donating the oxygen ligands  
255 for Fe<sup>3+</sup> coordination, which are either catecholates (*sensu stricto*, catecholates and phenolates; better  
256 termed as “aryl caps”), hydroxamates, or (hydroxy-)carboxylates (Miethke & Marahiel, 2007). Yeasts  
257 produce hydroxamate-type compounds, while bacteria produce hydroxamate as well as catecholate  
258 siderophores. Rhodotorulic acid (Figure 4) is a dihydroxamate-containing siderophore produced by  
259 *Rhodotorula glutinis*, essential to improve the control of blue mold caused by *P. expansum* in apples  
260 (Calvente, Benuzzi, & de Tosetti, 1999).  
261 *Metschnikowia pulcherrima* and *Metschnikowia fructicola* are able to produce the red pigment  
262 pulcherrimin (Figure 4 and 5), formed non enzymatically from pulcherriminic acid and ferric ions,  
263 which is involved in the control of *B. cinerea*, *Alternaria alternata* and *P. expansum* on apple  
264 (Saravanakumar *et al.*, 2008). Iron depletion by the *M. pulcherrima* in the medium inhibited the  
265 mycelial growth and conidial germination of *B. cinerea*, *A. alternata* and *P. expansum*. When iron  
266 was added at higher concentrations, the pathogen inhibition activity of *M. pulcherrima* disappeared  
267 and the colonies turned brown red. Furthermore, hyphae cracked when entering the pigmented zones  
268 around the *M. pulcherrima* streaks, demonstrating that iron starvation elicits complex physiological  
269 changes in the fungal cells (Saravanakumar *et al.*, 2008). Also *Metschnikowia fructicola* is able to  
270 produce pulcherrimin and to inhibit the growth of both *B. cinerea* and *P. digitatum in vitro* (Figure  
271 5).

272

### 273 **3.3 The role of biofilm formation and Quorum sensing**

274

275 To successfully colonize intact and injured fruit surfaces, the antagonist should have the ability to use  
276 specific features facilitating its adherence, colonization and multiplication. In most cases this feature  
277 is associated with the formation of a biofilm, where micro colonies are enclosed in a hydrated matrix  
278 of microbe produced proteins, nucleic acids, and polysaccharides (Bassam, Annous Pina, Fratamico,  
279 & Smith, 2009). The importance of biofilm formation and quorum sensing (QS) in biocontrol systems  
280 was reported. Yeast cell attachment is often mediated by specific cell wall adhesive  
281 glycoposphatidyl inositol (GPI)-anchored proteins (Finkel & Mitchell, 2011). Environmental  
282 sensing and signal transduction pathways regulating morphogenetic transformations have been  
283 studied in depth in *Candida albicans*. Two families of adhesin genes (HWP1 and ALS) showed to  
284 play a critical role in host cell recognition, adhesion, invasion, and biofilm formation (Biswas, Van

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

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

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

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

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

317

### 318 **3.4 Production of diffusible and volatile antimicrobial compounds**

319

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

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

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

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

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

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

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

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

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

381

### 382 **3.5 Parasitism and release of hydrolases**

383

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

387 Such adhesion was blocked by exposure to compounds able to alter the protein integrity and the  
388 respiration process. When *Candida saitoana* cells were cultivated together with *B. cinerea* mycelium,  
389 the fungal hyphae showed cytological damages, such as formation of papillae and other protuberances  
390 in the cell wall, as well as cytoplasm degeneration (El-Ghaouth, Wilson, & Wisniewski, 1998).  
391 In the fungal cell walls, chitin as a structural backbone is arranged in regularly ordered layers, and  $\beta$ -  
392 1,3-glucan as a filling material is arranged in an amorphous manner. Glucan is the major structural  
393 polysaccharide of the fungal cell wall, constituting approximately 50–60% by dry weight of the wall.  
394  $\beta$ -1,3-glucan is considered the main structural constituent to which other cell wall components are  
395 covalently attached, providing the cell wall with mechanical strength and integrity. Chitin is a linear,  
396 insoluble homopolymer composed of beta-1,4-linked subunits of the acetylated amino sugar N-  
397 acetylglucosamine. The cell walls of filamentous fungi consist of up to 20% or more chitin (Seidl,  
398 2008). In addition, proteins represent approximately 20–30% of the cell wall. Most of the cell wall  
399 proteins are glycoproteins extensively modified with O-linked and N-linked oligosaccharides. Cell  
400 wall proteins play an important role in maintaining cell shape, mediating adhesion for cell migration  
401 and fusion, protecting the cell against foreign substances, mediating the absorption of molecules and  
402 synthesizing and remodelling cell wall components. Breakdown of fungal cell wall requires the  
403 participation of different enzymes, especially  $\beta$ -1,3-glucanase and chitinase, but also proteases.  
404 Glucanases, chitinases and proteases can be directly or indirectly involved in the mechanism of  
405 several yeast antagonists of postharvest pathogens (Table 2).

406

### 407 **3.5.1 Glucanases**

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

412 Masih & Paul (2002) showed that exo- $\beta$ -1,3-glucanase secreted by *P. membranifaciens* had a role in  
413 the biocontrol activity against *B. cinerea* on grapevine. Due to the assumed potential role of exo- $\beta$ -  
414 1,3-glucanase in biocontrol systems (Daguerre, Siegel, Edel-Hermann, & Steinberg, 2014), glucanase  
415 genes have been cloned and characterized from different yeast BCAs, including *C. oleophila*, *P.*  
416 *anomala*, and *P. guilliermondii* (Grevesse, Lepoivre, & Jijakli, 2003; Bar-Shimon *et al.*, 2004; Zhang,  
417 Spadaro, Valente, Garibaldi, & Gullino, 2011b). The contribution of exo- $\beta$ -1,3-glucanase to the  
418 biocontrol activity of *C. oleophila* was investigated by generating *CoEXG1*-knockouts and double-  
419 *CoEXG1* transformants: the control activity of the transformants against *P. digitatum* on kumquat  
420 fruit did not differ, however, from that of the wild-type strain (Yehuda, Droby, Bar-Shimon,

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

426

### 427 **3.5.2 Chitinases**

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

440

### 441 **3.5.3 Proteases**

442 Proteases are divided into four major groups according to the character of their catalytic active site  
443 and conditions of action (Barrett, Rawlings, & Woessner, 2003): serine proteinases (EC 3.4.21),  
444 cysteine proteinases (EC 3.4.22), aspartic proteinases (EC 3.4.23), and metalloproteinases (EC  
445 3.4.24). *A. pullulans* in apple and peach wounds releases extracellular glucanases, chitinases and  
446 proteases, that presumed to play a role in the antagonistic activity (Zhang *et al.*, 2010). An alkaline  
447 serine protease gene (ALP5) was cloned from *A. pullulans* and expressed in *Escherichia coli* (Zhang,  
448 Spadaro, Valente, Garibaldi, & Gullino, 2012) and in *P. pastoris* (Banani *et al.*, 2014). When the  
449 efficacy of ALP5 was evaluated against postharvest pathogens on apples, the protease was more  
450 efficient in controlling *Monilinia fructicola*, *B. cinerea* than *P. expansum* and *A. alternata* (Banani *et*  
451 *al.*, 2014). However, the extent of the activity was dependent on the enzyme concentration and the  
452 length of fruit storage.

453

### 454 **3.6 Induction of resistance**



455

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

461 Induction of several biochemical and molecular defence responses following the application of yeast  
462 BCAs to fruit surfaces have already been demonstrated in the past. One of the first studies in this  
463 relation showed that treatment of lemon wounds with *P. guilliermondii* enhanced the production of  
464 the phytoalexin scoparone (Rodov, Ben-Yehoshua, D'Hallewin, & Castia, 1994). Similarly, Arras  
465 (1996) showed that scoparone accumulation could be 19 times higher when the antagonist *C. famata*  
466 was inoculated 24 h prior to *P. digitatum*, and four times higher if inoculated 24 h after the pathogen.  
467 *C. saitoana* induced chitinase activity and caused deposition of papillae and protuberances on host  
468 cells in apple surface wounds (El-Ghaouth *et al.*, 1998). In apple wounds, *A. pullulans* caused  
469 transient increases in  $\beta$ -1,3-glucanase, chitinase, and peroxidase (POD) activities. These increases  
470 started 24 h after treatment and reached maximum levels at 48 h and 96 h after treatment. Wounding  
471 also increased  $\beta$ -1,3-glucanase, chitinase, and POD activity, but the increments were markedly lower  
472 than those detected in yeast-treated wounds (Ippolito, El Ghaouth, Wilson, & Wisniewski, 2000).  
473 Fajardo, McCollum, McDonald, & Mayer (1998) demonstrated that various biologically based  
474 elicitors, including a strain of *C. oleophila*, were capable of inducing resistance to *P. digitatum*, when  
475 it was inoculated 14 h after the application of the elicitors to whole, unwounded orange (*Citrus*  
476 *sinensis* cv. Valencia) fruits. This increased resistance was associated with differential temporal  
477 induction of chitinase,  $\beta$ -1,3-glucanase, and POD activities. Application of *C. oleophila* to surface  
478 wounds or to intact 'Marsh Seedless' grapefruit elicited systemic resistance against *P. digitatum*, the  
479 main postharvest pathogen of citrus fruit (Droby *et al.*, 2002). The induction of pathogen resistance  
480 in fruit was pronounced already 24 h after elicitation; it was distance, concentration, and time  
481 dependent, and it was restricted to the peel tissue closely surrounding the yeast application site. The  
482 induction of pathogen resistance required viable yeast cells at concentrations of  $10^8$  to  $10^9$  cells/ml.  
483 Nonviable autoclaved or boiled yeast cells or lower yeast concentrations were ineffective in  
484 enhancing fruit disease resistance. Application of *C. oleophila* cell suspensions to grapefruit peel  
485 tissue increased ethylene biosynthesis, phenylalanine ammonia lyase activity, and phytoalexin  
486 accumulation, and increased chitinase and endo- $\beta$ -1,3-glucanase protein levels, as indicated by  
487 western immunoblotting analysis. Scanning electron microscope observations revealed that spore

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

490 Yao & Tian (2005) showed that treatment of peach fruit with *C. laurentii* in combination with methyl  
491 jasmonic acid (MeJA) induced stronger activities of chitinase,  $\beta$ -1,3-glucanase, phenylalanine  
492 ammonia-lyase (PAL) and POD in peach fruit than the yeast or MeJA alone and the BCA significantly  
493 reduced the diameter of fruit lesions caused by *Monilinia fructicola* and *P. expansum*. The onset of  
494 the disease resistance against *Monilinia fructicola* and *P. expansum* paralleled closely the increase in  
495 chitinase,  $\beta$ -1,3-glucanase, PAL and POD activity. When *C. laurentii* was applied to jujube fruit,  $\beta$ -  
496 1,3-glucanase activity increased, and the expression of the corresponding *Glu-1* gene in fruit tissue  
497 was highly induced. Consequently it was concluded that the product of this gene may play a role in  
498 the defence response against infection by *A. alternata* and *P. expansum* (Tian *et al.*, 2007).

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

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

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

### 512 513 **3.7 The role of oxidative stress and alleviation of oxidative damage**

514  
515 The production of ROS in plants is an initial response to microorganisms, both pathogenic and non-  
516 pathogenic (Bolwell *et al.*, 2002). In the case of a non-compatible host–parasite interaction, an initial  
517 moderate increase in the production of ROS usually precedes a stronger oxidative burst, while in a  
518 compatible interaction no further increase in the level of reactive radicals in host tissue is observed.  
519 Oxidative burst at the injury site following the colonization of antagonist cells was suggested to have  
520 a role in the mechanism of action of antagonistic yeast and may be involved in signalling pathways  
521 resulting in activation of fruit resistance system (Chan & Tian, 2005; Macarisin, Droby, Bauchan, &

522 Wisniewski, 2010). To play this role, antagonist cells must be able to tolerate alleviated levels of  
523 oxidative stress. In this regard, Castoria, Caputo, De Curtis, & De Cicco (2003) were the first to report  
524 that postharvest biocontrol fitness of the yeast antagonists *C. laurentii* LS-28 and *R. glutinis* was  
525 correlated with their ability to tolerate relatively high levels of ROS. These findings highlighted the  
526 role of oxidative stress in biocontrol systems and its possible direct and indirect effects either on the  
527 fruit tissue or on the antagonist cells at intercellular and intracellular level. In this relation, Liu *et al.*  
528 (2012), evaluated the response of several yeast BCAs (*Metschnikowia fructicola*, *C. oleophila* and  
529 *Cystofilobasidium infirmominiatum* PL1) to oxidative stress. Findings indicated that *C.*  
530 *infirmominiatum* was the most sensitive to exposure to exogenous H<sub>2</sub>O<sub>2</sub>, while *Metschnikowia*  
531 *fructicola* was the most tolerant.

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

536 Superoxide anion production on the intact fruit surface could also serve as a QS signal to trigger  
537 aggregation into a biofilm which would increase yeast attachment and improve survival on the fruit  
538 surface by providing a microenvironment resistant to environmental stress. While the role of O<sub>2</sub><sup>-</sup> in  
539 yeast cell multiplication, intercellular communication, or as an adaptive response to an unstable  
540 environment remains to be elucidated, results clearly show that, when yeasts sense host tissue, they  
541 are able to produce and apparently tolerate high levels of O<sub>2</sub><sup>-</sup>, regardless of the availability of  
542 nutrients.

543

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

545

546 The availability of more efficient DNA-based and proteomics technologies, along with  
547 bioinformatics, has provided new opportunities and tools to gain deeper and more accurate insights  
548 about the interactions already indicated (An, Chen, Li, Qin, & Tian, 2014). Though omics  
549 technologies have been widely used to elucidate the mechanisms of action of BCAs against soilborne  
550 and foliar pathogens, seldom they have been used to clarify the modes of action of postharvest BCAs  
551 (Massart, Perazzolli, Höfte, Pertot, & Jijakli, 2015). Developments in deep sequencing,  
552 transcriptomics, proteomics, metagenomics, comparative and functional genomics could be utilized  
553 to determine changes in the physiological status of BCAs, and the effect of environmental stress on  
554 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,

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

593

594

## 595 **5. Conclusions**

596

597 To date, there are hundreds of reports about using of yeast antagonists to biologically control  
598 postharvest diseases. Very few of these antagonists, however, have reached the commercial  
599 development stage and launched as commercial products. In most cases, there are inherent problems in  
600 the biocontrol systems related to poor performance and inconsistency under commercial conditions.

601 Among the reasons for these shortcomings is the lack of understanding of mechanisms of actions of  
602 these BCAs. It is apparent that the performance of yeast BCA is the result of complex interactions  
603 taking place between all the components of the biocontrol system (plant host, the antagonist, the  
604 pathogen, and resident microflora). Although these interactions have been the subject of research for  
605 over thirty years, our understanding is still incomplete. This because of the difficulties associated  
606 with the study of complex interactions and the lack of appropriate research tools and technologies.

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

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

621

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623

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628

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893 **Figure captions**

894

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

897

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

900

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

904

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

906

907 **Figure 5** – Inhibition of mycelial growth of *Penicillium digitatum* and *Botrytis cinerea* as a result of  
908 production of pulcherrimin by *Metschnikowia fructicola*. In presence of FeCl<sub>3</sub> in the growth medium  
909 (PDA), *M. fructicola* produced the red pigment pulcherrimin surrounding its colony (left panels). Red  
910 arrows (right panels) show inhibition zones of either *P. digitatum* or *B. cinerea* co-cultured with the  
911 yeast on a medium containing FeCl<sub>3</sub>.