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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

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17

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

47 1. Introduction

48

49 Postharvest fungal pathogens are considered the main cause of losses of fresh fruits and vegetables
50 at the postharvest, distribution, and consumption levels. While reports on the level of these losses are
51 conflicting, a report by the Food and Agriculture Organization (FAO, 2011) indicated that global
52 average loss in Europe, North America and Oceania is about 29%, compared to an average of about
53 38% in industrialized Asia, South East Asia, Africa and Latin America. Efforts have been made to
54 minimize these losses through developing a better understanding of the biology and aetiology of
55 postharvest diseases, as well as by developing adequate postharvest handling technologies and control
56 strategies (Prusky & Gullino, 2010). While several approaches were suggested for managing
57 postharvest decay, chemical control of postharvest diseases, applied in orchard or after harvesting, is
58 still the most widely used method. Increasing concerns, however, regarding residues of fungicides in
59 the fruit, development of resistant biotypes of the pathogens, as well as risks associated with their
60 continuous use have prompted the search for safe and effective alternative strategies. Among these
61 strategies, biological control based on naturally occurring microorganisms, has been the most studied
62 (Liu, Sui, Wisniewski, Droby, & Liu, 2013a).

63 In the past thirty years, there have been extensive research activities to explore and develop strategies
64 based on microbial antagonists to biologically control postharvest pathogens (Spadaro & Gullino,
65 2004; Droby, Wisniewski, Macarasin, & Wilson, 2009; Sharma, Singh, & Singh, 2009). By using the
66 key words “biocontrol” OR “biological control” AND “postharvest” OR “post-harvest” in the Scopus
67 search engine, 879 documents were retrieved (search performed on April 3, 2015), most of them (609;
68 69%) published in the last ten years. Impressive progress was made in development, registration and
69 commercialization of biocontrol products to manage key postharvest pathogens, such as *Penicillium*
70 *expansum*, *Penicillium digitatum*, *Penicillium italicum*, *Fusarium sambucinum*, *Rhizopus stolonifer*
71 and *Botrytis cinerea*. Different products reached advanced stages of development and
72 commercialization (Table 1). Biosave™ (*Pseudomonas syringae* Van Hall) was originally registered
73 in the USA for postharvest application on pome and citrus fruits, and it was later extended to cherries,
74 potatoes and sweet potatoes (Janisiewicz & Peterson, 2004). Among the first products based on yeasts,
75 Aspire™ (based on *Candida oleophila*) (Liu, Wisniewski, Artlip, Sui, Droby, & Norelli, 2013b) and
76 Yieldplus™ (based on *Cryptococcus albidus*) (Janisiewicz & Korsten, 2002) were commercialized
77 for some years but they were withdrawn due to various reasons, including low and inconsistent
78 efficacy under commercial conditions, low profitability and difficulties in market penetration and
79 perception of the customers/industry, and small size companies with low available resources to
80 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, Ciavarella, 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 Fe³⁺ 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 β -
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 β -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 β -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- β -1,3-glucanase
409 (EC3.2.1.58) that hydrolyse β -glucans by sequentially cleaving glucose residues from the non-
410 reducing end, and (2) endo- β -1,3-glucanases (EC3.2.1.39) that cleave β -linkages at random sites
411 along the polysaccharide chain, releasing smaller oligosaccharides and glucose.

412 Masih & Paul (2002) showed that exo- β -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- β -
414 1,3-glucanase in biocontrol systems (Daguere, 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- β -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- β -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- β -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 β -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 β -
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 β -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 β -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, β -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- β -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, β -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, β -1,3-glucanase, PAL and POD activity. When *C. laurentii* was applied to jujube fruit, β -
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 *Rhodosporidium*
506 *paludigenum* induced resistance against postharvest diseases in mandarins through the activation of
507 defence-related enzymes, such as β -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 H₂O₂, 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 O₂⁻ 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 O₂⁻, 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 FeCl₃ 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 FeCl₃.