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1 **How siderophore production can influence the biocontrol activity of *Aureobasidium pullulans***
2 **against *Monilinia laxa* on peaches**

3
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8
9 **Abstract**

10 In the present study *Aureobasidium pullulans* strains L1 and L8 were shown both *in vitro* and *in*
11 *vivo* to compete for iron with *Monilinia laxa* through the secretion of siderophores (1.2 and 1.4 mg
12 ml⁻¹, respectively) and to prevent postharvest fruit decay of peaches. The two strains reduced
13 mycelial growth and conidial germination of *M. laxa* specially in presence of lower iron
14 concentrations (5 and 10 µg l⁻¹ FeCl₃), confirming a better efficacy when nutrients are scarce. In *in*
15 *vivo* assay, *A. pullulans* L1 and L8 strains inhibited pathogen virulence, reducing by 83.5% and
16 84.4% on average respectively the peach lesion diameter for each tested iron solutions (5, 10, 20 µg
17 l⁻¹ FeCl₃). The highest iron solution slowed down the antagonists' action and conversely increase
18 the pathogen aggressiveness. Results shows that *A. pullulans* L1 and L8 strains compete with *M.*
19 *laxa* for iron, so revealing new biocontrol aspects. Both strains showed the capability to decrease
20 the accumulation of iron competing with some fungal pathogens and reducing their virulence. These
21 results provide new perspectives for the use of biocontrol agents in agriculture.

22
23 **Keywords:** Yeast - Iron – Stone fruit – *Monilinia laxa* - Siderophore

24
25 **1. Introduction**

26 *Monilinia laxa* is the most common species in European stone fruit orchards, and able to cause
27 postharvest losses that reach high values (59%) (Larena et al., 2005). *Monilinia* control depends on
28 an integrated strategy based on fungicide use and cultural practices. However, to overcome the
29 issues related to the use of fungicides, alternative pathogen control strategies have been
30 investigated, such as the use of biocontrol agents (BCAs) (Di Francesco et al., 2016).
31 *Aureobasidium pullulans* L1 and L8 strains showed a high capability to control *Monilinia* spp. on
32 stone fruit as previously reported by Mari et al. (2012).

33 Among the various modes of action involved in the control of postharvest diseases by antagonistic
34 yeasts, competition for nutrients and space is considered one of the most important, particularly to
35 control wound pathogens for which the availability of exogenous nutrients is crucial during the
36 early stage of infection (Elad and Chet 1987, Mekbib et al., 2011, Bautista-Rosales et al., 2013, Di
37 Francesco et al., 2016, 2017, 2018a).

38 In fact, previous studies reported how the antifungal ability of *A. pullulans* (Janisiewicz et al., 2000;
39 Bencheqroun et al., 2006), *Pichia caribbica* (Xu et al., 2013), and *Pichia guilliermondii*
40 (Chanchaichaovivat et al., 2008) against *Penicillium expansum*, *Rhizopus stolonifer*, and
41 *Colletotrichum capsici* respectively, can be influenced by the presence or the absence of exogenous
42 nutrients (Druvefors et al., 2005, Bencheqroun et al., 2006, Liu et al., 2010).

43 Besides the role of competing for nutrients, iron may also play a role in the Biocontrol Agents
44 (BCAs) modes of action (Raaska and Mattila-Sandholm 1995) through the production of chelating
45 molecules (siderophores).

46 These are molecules able to chelate low weight ferric compounds enhancing the effectiveness of
47 BCAs by subtracting iron to pathogen inhibiting its growth and metabolic activity (Riquelme,
48 1996). Microorganisms can produce a variety of chelating agents, which solubilize ferric iron and
49 transport it into the cell (Liu et al., 2013, Calvente et al., 2001a, Sanz Ferramola et al., 2013). Under
50 competitive conditions when nutrient availability is a limiting factor, the production of siderophores
51 can represent a distinctive feature for a microorganism. It is known that yeasts produce only a type

52 of siderophore: hydroxamate (Riquelme, 1996). The siderophores production could play an
53 important role in the biocontrol of pathogens because by iron sequestering their growth and
54 metabolic activity were inhibited or slowed down (Riquelme 1996). Calvente et al. (2001a)
55 demonstrated that rhodotorulic acid, a siderophore produced by *Rhodotorula glutinis* improved the
56 biological control of blue rot of apples caused by *P. expansum*. Also, *Bacillus subtilis* produced
57 siderophores playing an important role in the control of *Fusarium oxysporum* (Yu et al., 2011).
58 Other studies reported the siderophores ability to inhibit the growth of certain pathogenic fungi, like
59 *Pythium ultimum* and *Sclerotinia sclerotiorum* (Hamdan et al., 1991, McLoughlin et al., 1992),
60 establishing that these molecules could be considered as a potential mechanism of action against
61 several fungal pathogens. Parafati et al. (2015) hypothesized that competition for iron was as one of
62 the main mechanisms of action for *Metschnikowia pulcherrima* against *Botrytis cinerea*.
63 In a previous study, *A. pullulans* L1 and L8 strains yet showed competition for nutrients like amino
64 acids and space (Di Francesco et al., 2017, 2018a) against *Monilinia laxa* and *B. cinerea*.
65 In the present work, we investigated for the first time the involvement of iron in the biocontrol
66 activity of both strains against the brown rot agent of peaches. Specifically, the aims of this study
67 were to determine the ability of L1 and L8 to produce siderophores through i) the chrome azurol S
68 (CAS) assay, ii) the spectrophotometer quantification, and iii) the *in vitro* and *in vivo* competition
69 for iron with *M. laxa* by testing three different concentrations of FeCl_3 (5, 10 and 20 $\mu\text{g ml}^{-1}$).

70

71 2. Materials and methods

72

73 2.1 Fruit

74 Peaches (*Prunus persica* (L.) Batsch) cv 'Redhaven' were harvested at commercial maturity in
75 orchards located in Cadriano (Bologna, Italy). Fruit-were stored at 0 °C and used within 5 days from
76 harvest and wounded by a sterile nail (3x3x3 mm) on opposite sides of the equatorial area.

77

78 2.2 Pathogen and antagonists

79 *Monilinia laxa* (ML4 strain from CRIOF-DipSA collection) (Di Francesco et al., 2017) was chosen
80 for the present study for its aggressiveness and grown as by Martini et al. (2016). Conidia from
81 pathogen colonies 10 days-old, grown on tomato agar (20 g of Agar Technical, Oxoid Basingstoke,
82 Hampshire, UK; 750 ml distilled water to which 250 ml tomato sauce was added after sterilization)
83 at 25 °C, were collected and suspended in sterile distilled water containing 0.05 %-(v/v) Tween 80.
84 The concentration of each conidia suspension was quantified with haematocytometer and adjusted
85 to a concentration of 10^5 conidia ml^{-1} with sterile distilled water. *Aureobasidium pullulans* L1 and
86 L8 strains (Di Francesco et al., 2018b), were maintained on nutrient yeast dextrose agar (NYDA: 8
87 g of nutrient broth, 5 g of yeast extract, 10 g of dextrose and 15 g of agar in 1 L of distilled water) at
88 4 °C until use. Two days before trials, each antagonist was inoculated on NYDA and incubated at
89 25 °C for 2 days. The yeast cells were collected in sterile distilled water and adjusted with
90 haematocytometer to a concentration of 10^8 cells ml^{-1} .

92 2.3 CAS assay

93 CAS-blue agar was prepared according to Schwyn and Neilands (1987). Dishes were prepared with
94 30 ml of culturing medium for each antagonist and fungus: NYDA for L1 and L8 and PDA for *M.*
95 *laxa*. Then the solidified growth media was cut in two halves, one of which was replaced by CAS-
96 blue agar. The dishes were inoculated **placing in the centre of the borderline**, between the two
97 media, a 6-mm-plug of L1, L8 or *M. laxa* derived from a colony in active growth. The plates were
98 incubated in the dark at 25 °C until the agar colour-change. The CAS reaction was determined by
99 measuring the advance of the colour-change in the CAS-blue agar from blue to different shades of
100 red. The control plates were not inoculated but incubated under the same conditions described
101 above. The sample unit was represented by 10 dishes and the experiment was repeated twice.

102

103 2.4 Spectrophotometer siderophore assay

104 For the siderophore production by yeasts and *M. laxa*, one loop of L1 and L8 cells and one of *M.*
105 *laxa* conidia were transferred in a siderophore solution as described in Wang et al. (2009) and
106 incubated at 25 °C at 200 rpm for 24 h. In addition, L1 or L8 cells and *M. laxa* were co-cultured in
107 the siderophore culture solution and incubated in the same conditions above mentioned. Aliquots of
108 5 ml of each sample's suspension were inoculated into 45 ml of new siderophore solution and
109 suddenly incubated for 120 h at 25 °C at 200 rpm. The supernatant of the centrifuged (5.000 rpm
110 for 20 min) cultures (500 µl) was added to 2.5 ml of a solution containing 5 mM FeCl₃ and used for
111 quantitative determination of siderophore by a spectrophotometer at 440 nm (Infinite® 200 PRO-
112 Tecan) after 30 min of incubation at 25 °C. A standard curve (0, 0.05, 0.1, 0.25, 0.5, 0.75 and 1.0
113 mg ml⁻¹) was prepared with deferoxamine mesylate (Sigma-Aldrich) (one type of hydroxamates) as
114 chelating agent standard (Calvente et al., 2001). The siderophore amount was extrapolated from the
115 deferoxamine mesylate standard curve. **The sample unit was represented by three flasks (replicates)**
116 **for each condition.** The experiment was repeated twice.

117

118 2.5 *In vitro* competition for iron

119 The competition for nutrient exerted by L1 and L8 *A. pullulans* strains on *M. laxa* mycelial growth
120 was investigated through the dual interaction between the pathogen and the yeast strains. The
121 experiment conducted with the two strains and the pathogen was assessed on Minimal Medium
122 Agar (MMA: Na₂HPO₄ 6 g, KH₂PO₄ 3 g, NaCl 0.5 g, NH₄Cl 1 g, MgSO₄ 1M, CaCl₂ 0.1M, Sucrose
123 30 g, Agar technical 20 g) amended with 0, 5, 10 and 20 µg ml⁻¹ of FeCl₃ (Saravanakumar et al.,
124 2008). *Monilinia laxa* mycelial plugs (6 mm of diameter) from 7-day-old colony were inoculated at
125 30 mm of distance from the plate edge. *Aureobasidium pullulans* L1 and L8 cells were taken from
126 48 h culture on NYDA by sterile loop and were streaked at **30 mm from the edge of the other side**
127 **of the plate.** Plates were incubated at 25 °C for 5 days and the radial growth of pathogen mycelium
128 was measured. Plates inoculated only with *M. laxa* plug represented the control. **The sample unit**
129 **was represented by five plates (replicates) for each condition.** The experiment was performed twice.

130

131 2.6 Influence of iron concentrations on antagonists' biocontrol activity against *Monilinia laxa*
132 germination and germ tube elongation

133 The competition activity for iron of L1 and L8 was assayed on the germination and germ tube
134 elongation of *M. laxa* conidia. Tissue culture plates (Costar, Corning Inc., Corning, NY) and culture
135 plate inserts Millicell-CM (Millipore Corp., Bedford, MA) were used as reported by Janisiewicz et
136 al., (2000) with some modifications (Di Francesco et al., 2017). Aliquots (120 μl) of minimal
137 medium broth (without agar) amended with 0, 5, 10 and 20 $\mu\text{g ml}^{-1}$ of FeCl_3 were dispensed in the
138 wells of culture plates with 40 μl of the pathogen conidia suspension (10^5 conidia ml^{-1}), while the
139 same aliquots of L1 or L8 cell suspensions (10^8 cells ml^{-1}) were dispensed inside the cylinder
140 inserts, without physical contact between antagonist and pathogen. The plates were placed at 25 °C
141 on a rotary shaker at 50 rpm; after 6 h of incubation, cylinders were removed from the wells and 20
142 μl of the medium were transferred to a glass slide for microscope (Nikon Eclipse TE2000–E)
143 observations. The percentage of conidia germination and the germ tube elongation (μm) were
144 determined (90 conidia per treatment, 3 microscopic fields with 30 conidia each). Cells without iron
145 solutions **addition** were considered as a control. The experiment was performed twice.

146

147 2.7 *In vivo* competition for iron

148 Peach fruits (20) cv 'Redhaven' were wounded and inoculated with 20 μl of each yeast suspension
149 10^8 cell ml^{-1} ; after 1 h at room temperature fruit were inoculated with 20 μl of the pathogen conidia
150 suspension (10^5 cells ml^{-1}) and right after its drying, 20 μl of iron solutions, at concentrations above
151 mentioned, were added. Fruit treated with sterile distilled water instead of yeast cell suspensions
152 represented the control. Fruit were stored at 20 °C **and 90% of relative humidity (RH)** for 7 days
153 and the lesion diameters determined by the pathogen were recorded. **The sample unit was**
154 **represented by 45 peaches (15 for replicate) for each antagonist, iron concentration, and control.**
155 The experiment was repeated twice.

156

157 2.8 Statistical analysis

158 Data were statistically handled by one-way analysis of variance (ANOVA). Statistical comparison
159 of means was carried out to reveal the differences between treatments using Tukey's HSD Test
160 ($\alpha = 0.05$). Data were reported as mean values \pm standard error (SE) of two experiments. Before
161 analysis of data, homogeneity of variance was tested by the Kruskal–Wallis test. All analyses were
162 performed with Statgraphics software (version centurion 15.0).

163

164 3. Results

165

166 3.1 *Aureobasidium pullulans* L1, L8 strains siderophore production

167 In order to verify the siderophore production by the antagonists and *M. laxa*, CAS-blue agar was
168 used as screening assay for the target microorganisms. Our results showed that both L1 and L8
169 induced a colour change of CAS-blue agar from blue to dark orange indicating that both *A.*
170 *pullulans* strains are siderophore producers. However, despite the two strains required the same
171 time (twelve days) to change the CAS-blue-agar from blue to dark orange, the colour change halo
172 produced by both yeasts was different in size, being 20 mm for L1, and 27 mm for L8 (Table 1)
173 (Fig. 1). In fact, L1 strain after 12 days of incubation did not determined a total colour change of
174 CAS-blue agar half, as instead L8. Conversely, *M. laxa* produced a barrage between the two media
175 without any colour change of the indicative agar. Control plates, without plug inoculation, not
176 changed colour after incubation conditions.

177

178 3.2 Spectrophotometer quantification of siderophores

179 To quantify the siderophore production by *A. pullulans* L1 and L8 strains and the influence of *M.*
180 *laxa* on this activity, a spectrophotometer assay with deferoxamine mesylate standard curve was
181 conducted. Both strains showed a considerable activity, producing 1.2 and 1.4 mg ml⁻¹ of

182 siderophore by L1 and L8 respectively, while *M. laxa* produced only 0.64 mg ml⁻¹ of siderophore, a
183 quantity not detectable by CAS-blue-agar.

184 Siderophore quantity was not influenced by *M. laxa* showing the same amount with respect to the
185 yeasts grown alone (Fig. 2).

186

187 3.3 *In vitro* competition for iron

188 Competition for iron was tested co-culturing in MM plates amended with FeCl₃ (0, 5, 10, or 20 µg
189 ml⁻¹) L1 and L8 strains with *M. laxa* isolate. As shown by Table 2, both *A. pullulans* L1 and L8
190 strains reduced *M. laxa* mycelium diameter by 41.3% and 43.3%, respectively when iron
191 concentration was not present in MM.

192 L1 and L8 competition was mainly stimulated by the presence of 10 µg ml⁻¹ and 20 µg ml⁻¹ of FeCl₃,
193 both leading to an inhibition of fungal pathogen mycelial growth corresponding to 45% and 47.6%
194 and 43% and 45%, respectively.

195 *Aureobasidium pullulans* strain L8 was slightly more effective with respect to L1 in competing for
196 iron, determining a higher inhibition of *M. laxa* mycelial growth on average by 44.7% and 42.9%,
197 respectively.

198

199 3.4 Influence of iron concentrations on *M. laxa* germination and germ tube elongation

200 After 6 h of incubation at 25 °C in MM broth without iron and in MM broth with 5 µg ml⁻¹ of FeCl₃,
201 *M. laxa* conidial germination was reduced by both L1 and L8 strain of 50%, as showed in Table 3.

202 The presence of 10 µg ml⁻¹ of FeCl₃ in the culture medium induced a higher antagonistic activity by
203 both strains against *M. laxa* conidial germination with respect to the other iron concentrations (5
204 and 20 µg ml⁻¹ of FeCl₃). In effect, conidial germination of *M. laxa* was reduced by 62.5% and
205 68.7%, respectively by L1 and L8. Conversely, 20 µg ml⁻¹ of FeCl₃ inhibited the strains antagonistic
206 activity, that showed a reduction of *M. laxa* conidia germination of ~35%.

207 As regards to *M. laxa* germ tube length, the highest inhibition values exerted by L1 and L8 were
208 mainly showed with 5 and 10 $\mu\text{g ml}^{-1}$ of FeCl_3 solution by 59.5%, 63.1% and by 65.9%, 67.6%,
209 respectively. Nevertheless, 10 $\mu\text{g ml}^{-1}$ of FeCl_3 stimulated mostly pathogen germ tube elongation
210 (10.4%) with respect to the other concentrations (Table 4). Anyway, L8 strain was always slightly
211 more effective with respect to L1 in determining a higher inhibition of *M. laxa* conidial germination
212 and germ tube elongation on average by 52.1%, 50.2%, and by 57.7%, 57.1%, respectively.

213

214 3.5 *In vivo* competition for iron

215 The influence of the addition of aliquots of iron solutions at different concentrations on fungal
216 virulence and yeasts antagonistic activity on peach fruit was measured. *Monilinia laxa* growth on
217 peach fruit in absence of antagonists was slightly enhanced by 6.8% only at FeCl_3 concentrations of
218 10 or 20 $\mu\text{g ml}^{-1}$ whereas no influence was observed on the pathogen aggressiveness at 5 $\mu\text{g ml}^{-1}$,
219 the lowest FeCl_3 concentrations (Fig. 3).

220 However, L1 and L8 *A. pullulans* strains showed a great capability to inhibit *M. laxa*
221 aggressiveness, reducing the peach lesion diameters by 83.5% and 84.4% on average respectively,
222 at each iron concentration. Furthermore, the highest concentration of iron (20 $\mu\text{g ml}^{-1}$) seemed to
223 slow down the antagonists action and conversely increase the pathogen aggressiveness. In fact, both
224 L1 and L8 controlled *M. laxa* incidence in presence of 20 $\mu\text{g ml}^{-1}$ FeCl_3 on average by 88% with
225 respect to 92%, in presence of the other iron concentrations (data not reported).

226

227 4. Discussion

228 *Aureobasidium pullulans* (L1 and L8) are considered promising alternatives to fungicides in the
229 reduction of the stone fruits postharvest diseases' incidence caused by *Monilinia* spp. (Mari et al.,
230 2012; Di Francesco et al., 2017). In our work, competition for nutrients was studied and in
231 particular the competition for iron, a nutrient that played a significant role in biocontrol interactions
232 (Raaska and Mattila-Sandholm 1995).

233 As known, iron is an essential nutrient for all organisms (Wang et al., 2009) acting as a cofactor for
234 enzymes and regulatory proteins involved in many cellular processes (Miethke and Marahiel 2007;
235 Li et al., 2018) but not promptly available due to the low solubility in alkaline environments. One of
236 the main strategy used by microorganisms (such as yeasts) and by plants to obtain iron is the
237 secretion of siderophores (Li et al., 2018), which are low-molecular-weight compounds that chelate
238 iron in the extracellular phase re-entering it in the cells by specific membrane transporters (Hider
239 and Kong 2010). More than 500 siderophores have been discovered from microorganisms and
240 plants and their chemical diversity often renders a variety of biological functions beyond capture
241 iron (Ho et al., 2019). Yeasts produce only hydroxamate-type siderophore (Riquelme 1996) derived
242 from the amino acid ornithine and classified into four structural families: fusarines, coprogens,
243 ferrichromes and rhodotorulic acid (Johnson 2008). Wang et al., (2009) showed that *A. pullulans*
244 could produce 1.1 mg ml⁻¹ hydroxamate type siderophore. In our experiments, both L1 and L8 were
245 evaluated for their capability to produce siderophore by CAS screening assay (Schwyn and
246 Neilands 1987). This assay was used for the screening of siderophore producing microorganisms by
247 the induction of a colour change of the growth medium: from blue to orange, red, or purple.
248 Our results showed that L1 and L8 strains produced siderophore, displaying after twelve days of
249 incubation a colour change of the CAS agar, from blue to red. Moreover, L8 strain induced a more
250 intense agar colour change and extended halo with respect to L1. Conversely, *M. laxa* was not able
251 to produce siderophores in solid medium but produced a clear barrage between the common fungal
252 culture medium (PDA) and the siderophore screening agar (CAS). In fact, CAS agar assay is mainly
253 useful for the identification of microorganisms capable to produce siderophores in high
254 concentrations and characterized by a high affinity for iron (III). Most likely, the assay was not so
255 sensitive to detect the low siderophores concentration produced by *M. laxa*.
256 In effect, these results were supported by the quantitative analysis conducted with the
257 spectrophotometer. Both yeasts were found to produce high levels of chelating molecules (1.2 and
258 1.4 mg ml⁻¹, respectively) whit respect to the pathogen that seemed instead to be a poor siderophores

259 producer (0.64 mg ml⁻¹). Probably siderophores role vary among different fungal pathosystems
260 (Chen et al., 2013) and according to the strain intrinsic characteristics. In fact, the importance of
261 siderophores for fungal pathogenesis was firstly demonstrated for the pathogens such as
262 *Cochliobulus heterostrophus* (Lee et al., 2005; Oide et al., 2006), *Alternaria* spp. (Chen et al.,
263 2013), *Ustilago maydis* (Mei et al., 1993) and for the human pathogen *Aspergillus fumigatus*
264 (Hissen et al., 2005; Schrettl et al., 2007; Chen et al., 2013).

265 In our study, the siderophore production by the yeasts was not affected by the presence of *M. laxa*,
266 probably for the nutritional and biochemical preferences of the fungus. According to these results,
267 we can suppose that *M. laxa* probably mainly compete for other nutrients rather than for iron; on the
268 other side, *A. pullulans* strains L1 and L8 showed a high effective antagonistic action against *M.*
269 *laxa* in presence of iron (10 µg l⁻¹ FeCl₃), displaying a great capability to create critical conditions
270 for the pathogen growth.

271 As other microorganisms (Droby et al., 1989, Bencheqroun et al., 2006) both L1 and L8, reduced
272 their antagonistic efficacy with the addition of high concentrations of exogenous nutrients, e.g. with
273 20 µg l⁻¹ of FeCl₃ both in *in vitro* and in *in vivo* assays.

274 In fact, the highest reduction of conidial germination, brown rot severity and incidence (data not
275 shown) in fruit was revealed in presence of lower iron concentrations, confirming that both strains
276 displayed their antagonistic efficacy when exogenous nutrients were scarce (Calvente et al., 1999,
277 Calvo et al., 2008, Sanz Ferramola et al., 2013). Plante and Labbè (2019) showed how in
278 *Schizosaccharomyces pombe* the siderophore synthetase expression remains upregulated under low
279 iron conditions.

280 According with previous observations on nutritional requirements of *Monilinia* spp. germination
281 (Byrde and Willetts 1977), in the present research, the germination rate of the pathogen conidia
282 resulted low when grown in minimal medium. This confirmed that the target pathogen is nutrient-
283 dependent and needs an adequate amount of supplements for its growth: both conidial germination
284 and hyphal development (Bencheqroun et al., 2006, Di Francesco et al., 2017). Moreover, the two

285 BCAs notably reduced *M. laxa* germ tube elongation: a fungal characteristic directly related to the
286 pathogen aggressiveness (Fujii 2004). In fact, one critical step of germination is the germ tube
287 elongation that can be critically blocked by iron deficiency (Plante and Labbè 2019), in this case
288 caused by the antagonistic action of both L1 and L8 to compete for nutrients.

289 In conclusion, *A. pullulans* L1 and L8 strains can compete with *M. laxa* for nutrients such as amino
290 acids as previously reported by Di Francesco et al. (2017, 2018a) and in the present study for iron, a
291 vital element for cell proliferation and survival for almost all organisms. Therefore, the ability of *A.*
292 *pullulans* strains L1 and L8 to acquire iron can counteract the expression of fungal pathogen genes
293 required for the production of siderophores and virulence in fruits (Chen et al., 2013).

294 This information can be very important to exploit L1 and L8 activity in a commercial formulation
295 characterized by a right and efficient composition. More investigations are necessary to isolate and
296 characterize siderophores produced by both yeast strains and possibly verify their environmental
297 involvement in absorption and metabolization of metals. Nevertheless, in our study the strong
298 efficacy exerted by L1 and L8 strains can be attributed most likely to a synergic activity of different
299 mechanisms of action.

300

301 **Conflict of Interest**

302 The authors declare that they have no conflict of interests.

303

304 **Authors contribution**

305 Dr. Alessandra Di Francesco conducted all the experiments, interpreted data, and drafted the
306 manuscript. Prof. Elena Baraldi coordinated the experimental plan, has corrected, and approved the
307 final draft of the manuscript.

308

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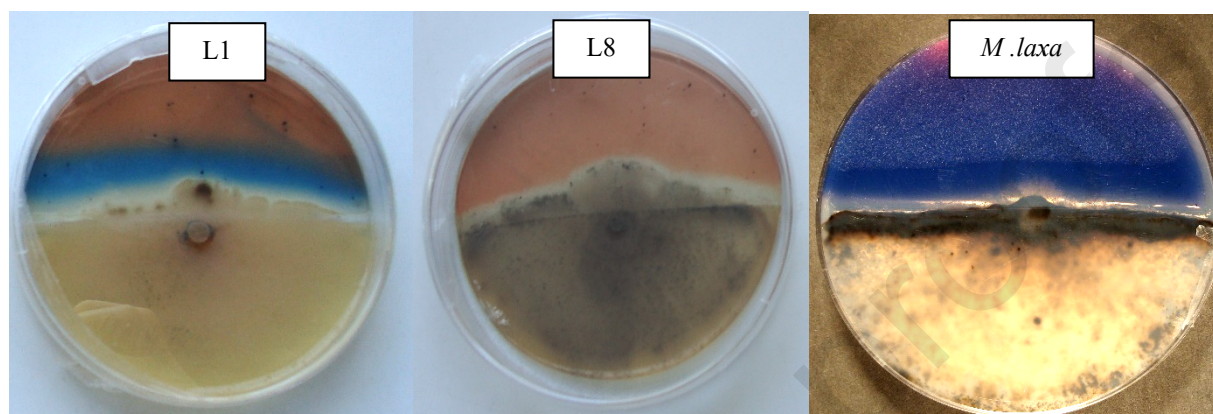
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477 Figure 1

478 CAS assay performed with *Aureobasidium pullulans* L1, L8 strains and *Monilinia laxa* (isolate

479 ML4).



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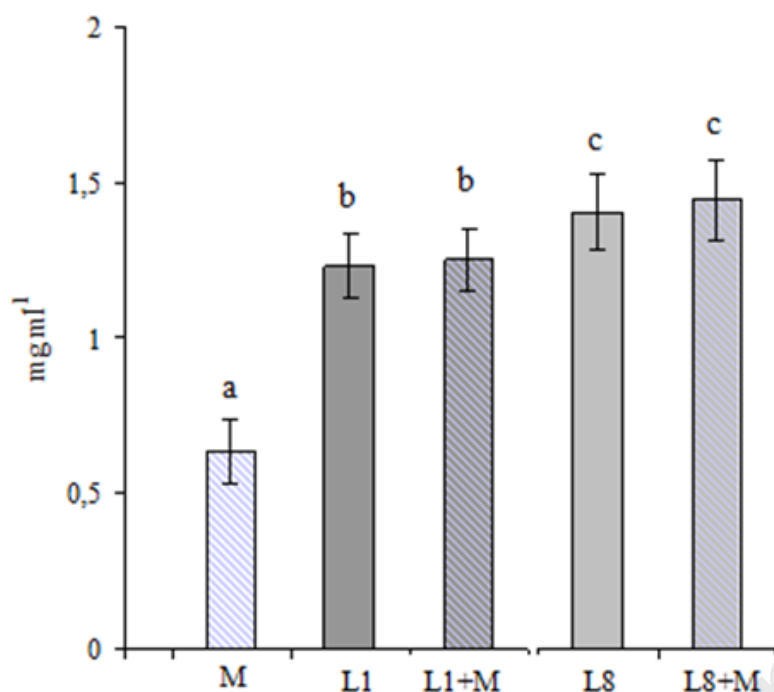
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486 Figure 2

487 Quantitative (mg ml^{-1}) determination of siderophore at 440 nm by spectrophotometer, on488 deferoxamine mesylate standard curve, of *Aureobasidium pullulans* L1 and L8 strains and489 *Monilinia laxa* (M), alone and co-cultured. Data are the means of two independent490 experiments \pm standard error, each consisting of three flasks per treatment. Different letters indicate491 significant differences at $\alpha=0.05$ according to Tukey's HSD Test.



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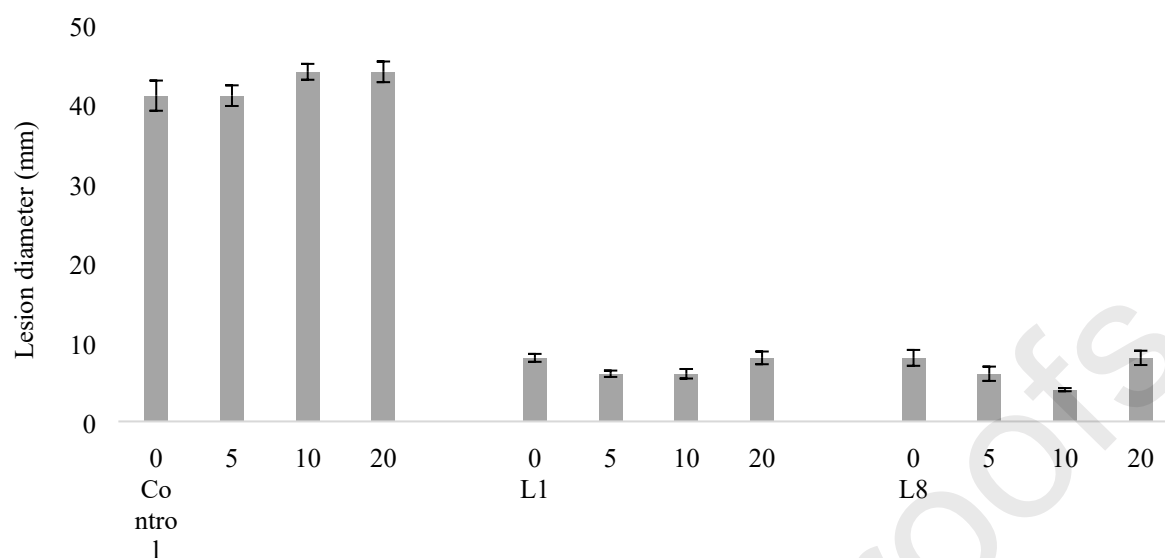
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495 Figure 3

496 *In vivo* antagonistic effect of L1 and L8 *Aureobasidium pullulans* strains on *Monilinia laxa* in peach
 497 fruits. Fruits were artificially inoculated with 20 μ l of yeasts cell suspensions (10^8 cells ml⁻¹); after
 498 air drying they were inoculated with 20 μ l of *M. laxa* conidia suspension (10^5 conidia ml⁻¹) and
 499 finally with an iron suspension (0, 5, 10 and 20 μ g ml⁻¹ FeCl₃) (0, 5, 10, 20). Control consisted of
 500 peaches inoculated with water instead of yeasts suspensions. Data are the means of two independent
 501 experiments \pm standard error, each consisting of 45 fruit per treatment. For each treatment group
 502 (Control, L1, and L8) different letters indicate significant differences at $\alpha = 0.05$ according to
 503 Tukey's HSD Test.

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511 Table 1

512 CAS assay for analysis of siderophore production by *Aureobasidium pullulans* (L1 and L8) and
 513 *Monilinia laxa* (isolate ML4). Data are the means of two independent experiments \pm standard error,
 514 each consisting of ten replicates per treatment. Different letters within the same column indicate
 515 significant differences at $\alpha = 0.05$ according to Tukey's HSD Test.

516

517

<i>Microorganism</i>	<i>Growth time</i> (days)*	<i>Color change</i>	<i>CAS reaction</i> **
<i>Monilinia laxa</i>	10 \pm 0.4b	-	0 \pm 0.0a
L1	12 \pm 0.0a	dark orange	20 \pm 1.9b
L8	12 \pm 0.0a	dark orange	27 \pm 1.5c

518

519 *Days required for the fungal mycelium to cover the non-CAS half of the plate.

520 ** mm of advance of colour change front in the CAS blue agar after three weeks of incubation.

521

522

523 Table 2

524 Mycelium diameter (mm) of *Monilinia laxa* growth in dual culture with *Aureobasidium pullulans*525 L1 and L8 strains in minimal medium agar plates amended with 0, 5, 10 and 20 $\mu\text{g ml}^{-1}$ FeCl_3 and

526 incubated at 25 °C for 5 days. Control plates were inoculated only with pathogen plug (6 mm).

527 Data are the means of two independent experiments \pm standard error, each consisting of five528 replicates per treatment. Within the same FeCl_3 treatment (column), the same lower-case letters529 represent no significant differences at $\alpha = 0.05$ according to Tukey's HSD Test. Within the same

530 yeast strains or pathogen isolate (control) (row), the same upper-case letters represent no significant

531 differences at $\alpha = 0.05$ according to Tukey's HSD Test.

532

533

	Treatment (FeCl_3)			
	0 $\mu\text{g ml}^{-1}$	5 $\mu\text{g ml}^{-1}$	10 $\mu\text{g ml}^{-1}$	20 $\mu\text{g ml}^{-1}$
Control	60.0 \pm 0.6cA	60.0 \pm b1.2bA	63.0c \pm 1.0cB	61.0 \pm 1.5cAB
L1	35.2 \pm 1.9bB	35.0a \pm 0.7aB	34.7b \pm 1.3bA	34.9 \pm 1.1bA
L8	34.0 \pm 1.1aB	34.4a \pm 1.0aB	33.0a \pm 0.8aA	33.6 \pm 0.8aAB

534

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538 Table 3

539 Effect of *Aureobasidium pullulans* (L1 and L8) on conidia germination (%) of *Monilinia laxa*540 grown in culture medium (minimal medium) amended with 5, 10 or 20 $\mu\text{g ml}^{-1}$ FeCl_3 and incubated

541 at 25°C for 6 hours. Data are the means of two independent experiments \pm standard error, each
 542 consisting of 90 conidia per treatment. Within the same FeCl₃ treatment (column), the same lower-
 543 case letters represent no significant differences at $\alpha = 0.05$ according to *Tukey's HSD Test*.

544 Within the pathogen isolate (control) and yeast strains (row), the same upper-case letters represent
 545 no significant differences at $\alpha = 0.05$ according to *Tukey's HSD Test*.

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	Treatment (FeCl ₃)			
	0 $\mu\text{g ml}^{-1}$	5 $\mu\text{g ml}^{-1}$	10 $\mu\text{g ml}^{-1}$	20 $\mu\text{g ml}^{-1}$
Control	40 \pm 1.3cA	41 \pm 1.8bA	48 \pm 0.8cC	43 \pm 1.5bB
L1	20 \pm 2.1bB	19 \pm 1.7aB	18 \pm 1.4bA	28 \pm 2.1aC
L8	19 \pm 0.9aB	19 \pm 1.5aB	15 \pm 1.2aA	29 \pm 1.8aC

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552 Table 4

553 Effect of *Aureobasidium pullulans* (L1 and L8) on *Monilinia laxa* conidia germ tube elongation
 554 (μm) grown in liquid culture medium (minimal medium) amended with 0, 5, 10 and 20 $\mu\text{g ml}^{-1}$
 555 FeCl₃ at 25 °C for 6 hours. Data are the means of two independent experiments \pm standard error,
 556 each consisting of 90 conidia per treatment. Within the same FeCl₃ treatment (column), the same
 557 lower-case letters represent no significant differences significant differences at $\alpha = 0.05$ according
 558 to *Tukey's HSD Test*. Within the pathogen isolate (control) and yeast strains (row), the same upper-
 559 case letters represent no significant differences significant differences at $\alpha = 0.05$ according to
 560 *Tukey's HSD Test*.

561

	Treatment (FeCl ₃)			
	0µg ml ⁻¹	5 µg ml ⁻¹	10 µg ml ⁻¹	20 µg ml ⁻¹
Control	16.2±1.3cA	16.3±1.6cA	18.2±2.1cB	16.5±1.8bA
L1	7.7±2.1aB	6.6±1.8bA	6.2±2.3bA	8.1±2.1aC
L8	8.3±1.5bC	6.0±1.6aB	5.7±3.5aA	8.2±1.8aC

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563

564 **Conflict of Interest**

565 The authors declare that they have no conflict of interests.

566

567 **Authors contribution**

568 Dr. Alessandra Di Francesco conducted all the experiments, interpreted data, and drafted the
 569 manuscript. Prof. Elena Baraldi coordinated the experimental plan, has corrected, and approved the
 570 final draft of the manuscript.

571

572

573 **Highlights**

574

575 - Competition for nutrients is one of the most important mechanism of action for BCAs

576 - Iron plays a role in BCAs modes of action through the production of siderophores

577 - Siderophores can inhibit the growth of certain pathogenic fungi

578 - *Aureobasidium pullulans* L1 and L8 strains compete for iron against *Monilinia laxa*

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580