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Alessandra Di Francesco, Elena Baraldi

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

Alessandra Di Francesco*, Elena Baraldi

Department of Agricultural Sciences, University of Bologna, Viale G. Fanin, 46, 40127 Bologna, Italy

**corresponding author:* alessand.difrancesc3@unibo.it

Abstract

In the present study *Aureobasidium pullulans* strains L1 and L8 were shown both *in vitro* and *in vivo* to compete for iron with *Monilinia laxa* through the secretion of siderophores (1.2 and 1.4 mg ml^{-1}, respectively) and to prevent postharvest fruit decay of peaches. The two strains reduced mycelial growth and conidial germination of *M. laxa* specially in presence of lower iron concentrations (5 and 10 µg l^{-1} $FeCl_3$), confirming a better efficacy when nutrients are scarce. In *in vivo* assay, *A. pullulans* L1 and L8 strains inhibited pathogen virulence, reducing by 83.5% and 84.4% on average respectively the peach lesion diameter for each tested iron solutions (5, 10, 20 µg l^{-1} $FeCl_3$,). The highest iron solution slowed down the antagonists' action and conversely increase the pathogen aggressiveness. Results shows that *A. pullulans* L1 and L8 strains compete with *M. laxa* for iron, so revealing new biocontrol aspects. Both strains showed the capability to decrease the accumulation of iron competing with some fungal pathogens and reducing their virulence. These results provide new perspectives for the use of biocontrol agents in agriculture.

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

1. Introduction

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

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

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

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

These are molecules able to chelate low weight ferric compounds enhancing the effectiveness of BCAs by subtracting iron to pathogen inhibiting its growth and metabolic activity (Riquelme, 1996). Microorganisms can produce a variety of chelating agents, which solubilize ferric iron and transport it into the cell (Liu et al., 2013, Calvente et al., 2001a, Sanz Ferramola et al., 2013). Under competitive conditions when nutrient availability is a limiting factor, the production of siderophores can represent a distinctive feature for a microorganism. It is known that yeasts produce only a type of siderophore: hydroxamate (Riquelme, 1996). The siderophores production could play an important role in the biocontrol of pathogens because by iron sequestering their growth and metabolic activity were inhibited or slowed down (Riquelme 1996). Calvente et al. (2001a) demonstrated that rhodotorulic acid, a siderophore produced by *Rhodotorula glutinis* improved the biological control of blue rot of apples caused by *P. expansum.* Also, *Bacillus subtilis* produced siderophores playing an important role in the control of *Fusarium oxysporum* (Yu et al., 2011).

Other studies reported the siderophores ability to inhibit the growth of certain pathogenic fungi, like *Pythium ultimum* and *Sclerotinia sclerotiorum* (Hamdan et al., 1991, McLoughlin et al., 1992), establishing that these molecules could be considered as a potential mechanism of action against several fungal pathogens. Parafati et al. (2015) hypothesized that competition for iron was as one of the main mechanisms of action for *Metschnikowia pulcherrima* against *Botrytis cinerea*.

In a previous study*, A. pullulans* L1 and L8 strains yet showed competition for nutrients like amino acids and space (Di Francesco et al., 2017, 2018a) against *Monilinia laxa* and *B. cinerea.*

In the present work, we investigated for the first time the involvement of iron in the biocontrol activity of both strains against the brown rot agent of peaches. Specifically, the aims of this study were to determine the ability of L1 and L8 to produce siderophores through i) the chrome azurol S (CAS) assay, ii) the spectrophotometer quantification, and iii) the *in vitro* and *in vivo* competition for iron with *M. laxa* by testing three different concentrations of $FeCl_3$ (5, 10 and 20 μg ml^{-1}).

2. Materials and methods

2.1 Fruit

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

2.2 Pathogen and antagonists

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

2.3 CAS assay

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

2.4 Spectrophotometer siderophore assay

For the siderophore production by yeasts and *M. laxa*, one loop of L1 and L8 cells and one of *M. laxa* conidia were transferred in a siderophore solution as described in Wang et al. (2009) and incubated at 25 °C at 200 rpm for 24 h. In addition, L1 or L8 cells and *M. laxa* were co-cultured in the siderophore culture solution and incubated in the same conditions above mentioned. Aliquots of 5 ml of each sample's suspension were inoculated into 45 ml of new siderophore solution and suddenly incubated for 120 h at 25 °C at 200 rpm. The supernatant of the centrifuged (5.000 rpm for 20 min) cultures (500 µl) was added to 2.5 ml of a solution containing 5 mM $FeCl_3$ and used for quantitative determination of siderophore by a spectrophotometer at 440 nm (Infinite® 200 PRO-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 mg ml^{-1}) was prepared with deferoxamine mesylate (Sigma-Aldrich) (one type of hydroxamates) as chelating agent standard (Calvente et al., 2001). The siderophore amount was extrapolated from the deferoxamine mesylate standard curve. The sample unit was represented by three flasks (replicates) for each condition. The experiment was repeated twice.

2.5 *In vitro* competition for iron

The competition for nutrient exerted by L1 and L8 *A. pullulans* strains on *M. laxa* mycelial growth was investigated through the dual interaction between the pathogen and the yeast strains. The experiment conducted with the two strains and the pathogen was assessed on Minimal Medium Agar (MMA: Na_2HPO_4 6 g, KH_2PO_4 3 g, NaCl 0.5 g, NH_4Cl 1 g, $MgSO_4$ 1M, $CaCl_2$ 0.1M, Sucrose 30 g, Agar technical 20 g) amended with 0, 5, 10 and 20 µg ml^{-1} of $FeCl_3$ (Saravanakumar et al., 2008). *Monilinia laxa* mycelial plugs (6 mm of diameter) from 7-day-old colony were inoculated at 30 mm of distance from the plate edge. *Aureobasidium pullulans* L1 and L8 cells were taken from 48 h culture on NYDA by sterile loop and were streaked at 30 mm from the edge of the other side of the plate. Plates were incubated at 25 °C for 5 days and the radial growth of pathogen mycelium was measured. Plates inoculated only with *M. laxa* plug represented the control. The sample unit was represented by five plates (replicates) for each condition. The experiment was performed twice.

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

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

2.7 *In vivo* competition for iron

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

2.8 Statistical analysis

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

3. Results

3.1 *Aureobasidium pullulans* L1, L8 strains siderophore production

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

3.2 Spectrophotometer quantification of siderophores

To quantify the siderophore production by *A. pullulans* L1 and L8 strains and the influence of *M. laxa* on this activity, a spectrophotometer assay with deferoxamine mesylate standard curve was conducted. Both strains showed a considerable activity, producing 1.2 and 1.4 mg ml^{-1} of siderophore by L1 and L8 respectively, while *M. laxa* produced only 0.64 mg ml^{-1} of siderophore, a quantity not detectable by CAS-blue-agar.

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

3.3 *In vitro* competition for iron

Competition for iron was tested co-culturing in MM plates amended with $FeCl_3$ (0, 5, 10, or 20 µg ml^{-1}) L1 and L8 strains with *M. laxa* isolate. As shown by Table 2, both *A. pullulans* L1 and L8 strains reduced *M. laxa* mycelium diameter by 41.3% and 43.3%, respectively when iron concentration was not present in MM.

L1 and L8 competition was mainly stimulated by the presence of 10 µg ml^{-1} and 20 µg ml^{-1} of $FeCl_3$, both leading to an inhibition of fungal pathogen mycelial growth corresponding to 45% and 47.6% and 43% and 45%, respectively.

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

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

After 6 h of incubation at 25 °C in MM broth without iron and in MM broth with 5 µg ml^{-1} of $FeCl_3$, *M. laxa* conidial germination was reduced by both L1 and L8 strain of 50%, as showed in Table 3. The presence of 10 µg ml^{-1} of $FeCl_3$ in the culture medium induced a higher antagonistic activity by both strains against *M. laxa* conidial germination with respect to the other iron concentrations (5 and 20 µg ml^{-1} of $FeCl_3$). In effect, conidial germination of *M. laxa* was reduced by 62.5% and 68.7%, respectively by L1 and L8. Conversely, 20 µg ml^{-1} of $FeCl_3$, inhibited the strains antagonistic activity, that showed a reduction of *M. laxa* conidia germination of ~35%.

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

3.5 *In vivo* competition for iron

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

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

4. Discussion

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

As known, iron is an essential nutrient for all organisms (Wang et al., 2009) acting as a cofactor for enzymes and regulatory proteins involved in many cellular processes (Miethke and Marahiel 2007; Li et al., 2018) but not promptly available due to the low solubility in alkaline environments. One of the main strategy used by microorganisms (such as yeasts) and by plants to obtain iron is the secretion of siderophores (Li et al., 2018), which are low-molecular-weight compounds that chelate iron in the extracellular phase re-entering it in the cells by specific membrane transporters (Hider and Kong 2010). More than 500 siderophores have been discovered from microorganisms and plants and their chemical diversity often renders a variety of biological functions beyond capture iron (Ho et al., 2019). Yeasts produce only hydroxamate-type siderophore (Riquelme 1996) derived from the amino acid ornithine and classified into four structural families: fusarines, coprogens, ferrichromes and rhodotorulic acid (Johnson 2008). Wang et al., (2009) showed that *A. pullulans* could produce 1.1 mg ml^{-1} hydroxamate type siderophore. In our experiments, both L1 and L8 were evaluated for their capability to produce siderophore by CAS screening assay (Schwyn and Neilands 1987). This assay was used for the screening of siderophore producing microorganisms by the induction of a colour change of the growth medium: from blue to orange, red, or purple.

Our results showed that L1 and L8 strains produced siderophore, displaying after twelve days of incubation a colour change of the CAS agar, from blue to red. Moreover, L8 strain induced a more intense agar colour change and extended halo with respect to L1. Conversely, *M. laxa* was not able to produce siderophores in solid medium but produced a clear barrage between the common fungal culture medium (PDA) and the siderophore screening agar (CAS). In fact, CAS agar assay is mainly useful for the identification of microorganisms capable to produce siderophores in high concentrations and characterized by a high affinity for iron (III). Most likely, the assay was not so sensitive to detect the low siderophores concentration produced by *M. laxa*.

In effect, these results were supported by the quantitative analysis conducted with the spectrophotometer. Both yeasts were found to produce high levels of chelating molecules (1.2 and 1.4 mg ml^{-1}, respectively) whit respect to the pathogen that seemed instead to be a poor siderophores producer (0.64 mg ml^{-1}). Probably siderophores role vary among different fungal pathosystems (Chen et al., 2013) and according to the strain intrinsic characteristics. In fact, the importance of siderophores for fungal pathogenesis was firstly demonstrated for the pathogens such as *Cochliobulus heterostrophus* (Lee et al., 2005; Oide et al., 2006), *Alternaria* spp. (Chen et al., 2013), *Ustilago maydis* (Mei et al., 1993) and for the human pathogen *Aspergillus fumigatus* (Hissen et al., 2005; Schrettl et al., 2007; Chen et al., 2013).

In our study, the siderophore production by the yeasts was not affected by the presence of *M. laxa*, probably for the nutritional and biochemical preferences of the fungus. According to these results, we can suppose that *M. laxa* probably mainly compete for other nutrients rather than for iron; on the other side, *A. pullulans* strains L1 and L8 showed a high effective antagonistic action against *M. laxa* in presence of iron (10 µg l^{-1} $FeCl_3$), displaying a great capability to create critical conditions for the pathogen growth.

As other microorganisms (Droby et al., 1989, Bencheqroun et al., 2006) both L1 and L8, reduced their antagonistic efficacy with the addition of high concentrations of exogenous nutrients, e.g. with 20 µg l^{-1} of $FeCl_3$ both in *in vitro* and in *in vivo* assays.

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

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

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

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

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

Conflict of Interest

The authors declare that they have no conflict of interests.

Authors contribution

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

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Figure 1

CAS assay performed with *Aureobasidium pullulans* L1*,* L8 strains and *Monilinia laxa* (isolate ML4).

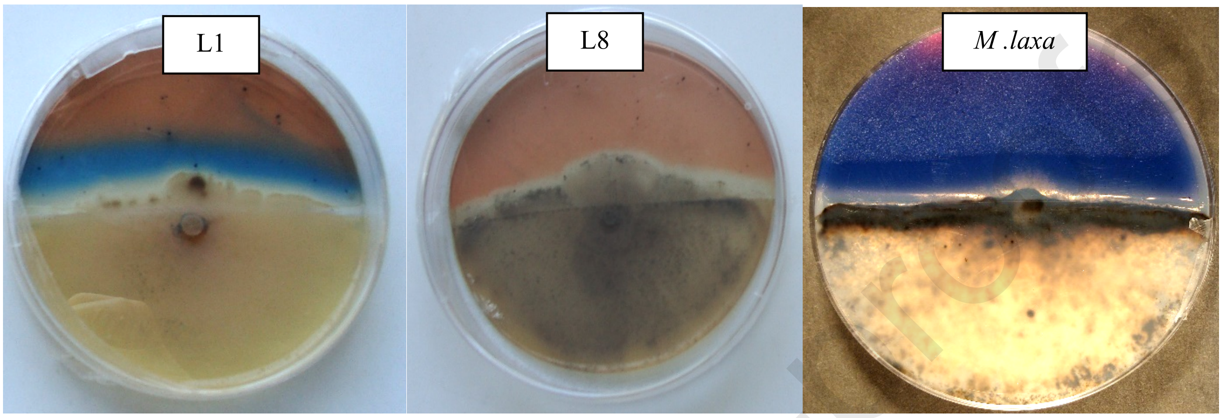

Figure 2

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

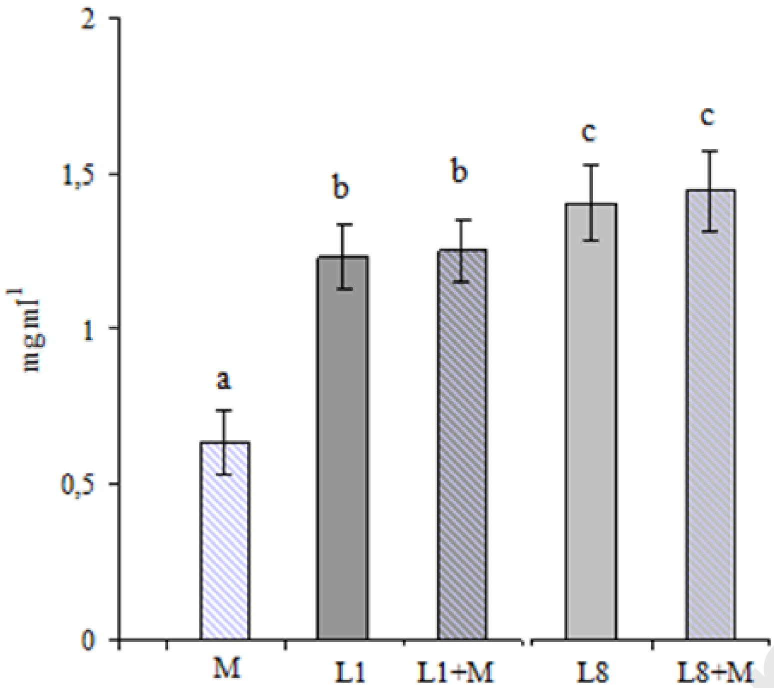

Figure 3

In vivo antagonistic effect of L1 and L8 *Aureobasidium pullulans* strains on *Monilinia laxa* in peach fruits. Fruits were artificially inoculated with 20 µl of yeasts cell suspensions (10^8 cells ml^{-1}); after air drying they were inoculated with 20 µl of *M. laxa* conidia suspension (10^5 conidia ml^{-1}) and finally with an iron suspension (0, 5, 10 and 20 µg ml^{-1} $FeCl_3$) (0, 5, 10, 20). Control consisted of peaches inoculated with water instead of yeasts suspensions. Data are the means of two independent experiments ± standard error, each consisting of 45 fruit per treatment. For each treatment group (Control, L1, and L8) different letters indicate significant differences at $\alpha = 0.05$ according to *Tukey's* HSD Test.

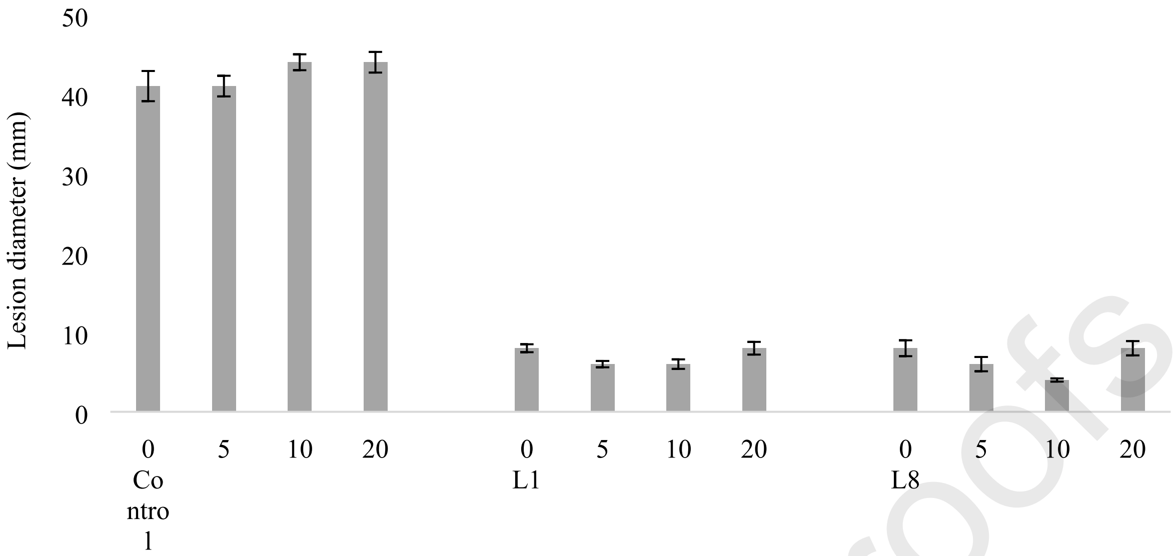

Table 1

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

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

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

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

Table 2

Mycelium diameter (mm) of *Monilinia laxa* growth in dual culture with *Aureobasidium pullulans* L1 and L8 strains in minimal medium agar plates amended with 0, 5, 10 and 20 µg ml^{-1} $FeCl_3$ and incubated at 25 °C for 5 days. Control plates were inoculated only with pathogen plug (6 mm). Data are the means of two independent experiments ± standard error, each consisting of five replicates per treatment. Within the same $FeCl_3$ treatment (column), the same lower-case letters represent no significant differences at α = 0.05 according to *Tukey's* HSD Test. Within the same yeast strains or pathogen isolate (control) (row), the same upper-case letters represent no significant differences at α = 0.05 according to *Tukey's* HSD Test.

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

Table 3

Effect of *Aureobasidium pullulans* (L1 and L8) on conidia germination (%) of *Monilinia laxa* grown in culture medium (minimal medium) amended with 5, 10 or 20 µg ml^{-1} $FeCl_3$ and incubated at 25°C for 6 hours. Data are the means of two independent experiments ± standard error, each consisting of 90 conidia per treatment. Within the same $FeCl_3$ treatment (column), the same lower-case letters represent no significant differences at $\alpha = 0.05$ according to *Tukey's* HSD Test. Within the pathogen isolate (control) and yeast strains (row), the same upper-case letters represent no significant differences at $\alpha = 0.05$ according to *Tukey's* HSD Test.

	Treatment ($FeCl_3$)			
	0µg ml^{-1}	5 µg ml^{-1}	10 µg ml^{-1}	20 µg ml^{-1}
Control	40±1.3cA	41±1.8bA	48±0.8cC	43±1.5bB
L1	20±2.1bB	19±1.7aB	18±1.4bA	28±2.1aC
L8	19±0.9aB	19±1.5aB	15±1.2aA	29±1.8aC

Table 4

Effect of *Aureobasidium pullulans* (L1 and L8) on *Monilinia laxa* conidia germ tube elongation (µm) grown in liquid culture medium (minimal medium) amended with 0, 5, 10 and 20 µg ml^{-1} $FeCl_3$ at 25 °C for 6 hours. Data are the means of two independent experiments ± standard error, each consisting of 90 conidia per treatment. Within the same $FeCl_3$ treatment (column), the same lower-case letters represent no significant differences significant differences at $\alpha = 0.05$ according to *Tukey's* HSD Test. Within the pathogen isolate (control) and yeast strains (row), the same upper-case letters represent no significant differences significant differences at $\alpha = 0.05$ according to *Tukey's* HSD Test.

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

Conflict of Interest

The authors declare that they have no conflict of interests.

Authors contribution

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

Highlights

- Competition for nutrients is one of the most important mechanism of action for BCAs
- Iron plays a role in BCAs modes of action through the production of siderophores
- Siderophores can inhibit the growth of certain pathogenic fungi
- *Aureobasidium pullulans* L1 and L8 strains compete for iron against *Monilinia laxa*