Biocontrol of Monilinia laxa by Aureobasidium pullulans strains Insights on competition for nutrients and space

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Abstract

Two Aureobasidium pullulans strains (L1 and L8), able to prevent postharvest fruit decay,were evaluated in order to elucidate how the competition for nutrients and spacewas involved in their activity against Monilinia laxa, the causal agent of peach brown rot. The competition for nutrients was studied by co-culturing pathogen conidia and antagonists in different conditions of nutrient availability and avoiding contact between them. Both antagonists prevented the germination of conidia ofM. laxa inwater, reducing germination rate by N35%. However, L1 and L8 showed the lowest inhibition of conidial germination in peach juice at 5%, with a reduction of 12.6% and 13.9% respectively. HPLC amino acid analysis of peach juice revealed that the addition of the yeast suspension greatly modified their composition: asparaginewas completely depleted soon after 12 h of incubation andwas probably hydrolyzed to aspartic acid by the yeasts, as aspartic acid content markedly increased. Pure asparagine and aspartic acid were tested by in vitro trials at the concentrations found in peach juice: both influenced M. laxa growth, but in opposite ways. Asparagine stimulated pathogen growth; conversely, amended medium with aspartic acid significantly inhibited the conidia germination and mycelial development ofM. laxa. Scanning ElectronMicroscopy revealed that both strains showed a great capability to compete with M. laxa for space (starting 8 h after treatment), colonizing the wound surface and inhibiting pathogen growth. This study clearly showed that A. pullulans L1 and L8 strains could compete with M. laxa for nutrients and space; this mode of action may play an important role in the antagonistic activity,

especially in the first hours of tritrophic host-pathogen-antagonist interaction, although several other mechanisms can interact each other.

Keywords: Competition – Iron – Siderophores – Sugars – SEM micrographs

1. Introduction

Monilinia spp. causing brown rot of stone fruits are among the main pathogens of peaches and nectarines (Batra, 1991). The disease can be caused by at least three fungal species: M. fructicola, M. laxa and M. fructigena. All three pathogens are responsible for severe losses of fruits, more often after harvest, during storage and transport, than in the field. Brown rot control depends on an integrated strategy based on orchard fungicide applications and cultural practices, and maintenance of proper storage conditions in packinghouses and during distribution (Mari et al., 2012a). However, other control means have been investigated in order to reduce the impact of fungicides on human health and environment. Among the non-conventionalmethods, treatments based on biological control agents (BCAs) have been found effective in brown rot control and have been widely reviewed (Liu et al., 2013; Janisiewicz and Korsten, 2002). BCAs are living organisms and act using different strategies in relation to pathogen, host and environment. The knowledge of their modes of action is essential to increase their viability and potential in disease control (Di Francesco et al., 2016). Two Aureobasidium pullulans strains, L1 and L8, both isolated from the surface of peach fruit, previously showed good efficacy in the inhibition of all three Monilinia species (Mari et al., 2012a). The study of their modes of action revealed the production of volatile organic compounds (Di Francesco et al., 2015b) and hydrolytic enzymes (Di Francesco et al., 2015a) as some of their modes of action. However, the competition for nutrients and space is generally considered one of the main modes of action of BCAs because it involves the nutritional requirements of both antagonist and pathogen (Bautista-Rosales et al., 2013; Elad and Chet, 1987; Mekbib et al., 2011), and is also important mainly against wound pathogens that are typically dependent on exogenous nutrients for their development. In previous studies, A. pullulans

(Bencheqroun et al., 2007; Janisiewicz et al., 2000), Pichia caribbica (Xu et al., 2013) and P. guilliermondii (Chanchaichaovivat et al., 2008) showed antifungal activity respectively against Penicillium expansum, Rhizopus stolonifer and Colletotrichum capsici derived from competition for nutrients. The addition of exogenous nutrients influenced the biocontrol, showing different levels ofefficacy relating to their concentration (Bencheqroun et al., 2006; Druvefors et al., 2005; Liu et al., 2010; Nunes et al., 2001). The yeasts mostly act by consuming nutrients present in fruit and vegetable, depriving the pathogen of them and limiting its growth (Janisiewicz and Korsten, 2002). The study of the competition for nutrients is quite complicated, as direct contact between antagonist and pathogen is not required, unlike the competition for space. Both types of competition are often considered togetherwithout ascribing each the proper level of significance (Janisiewicz et al., 2000). Therefore a separation between the two aspects of competition (nutrients and space) could be appropriate in order to better understand their contribution to disease reduction. The main aim of this work was the study of the competition for nutrients and space in the biocontrol activity of two A. pullulans strains (L1 and L8) against M. laxa causing brown rot of peaches. Specifically, the ability of both strains to compete with the pathogen i) for nutrients was tested in peach juice; ii) for major amino acids was analyzed by HPLC and evaluated through in vitro trials, and iii) for space was studied by Scanning Electron Microscopy (SEM) observations.

2. Materials and methods

Fruit

'Redhaven' peaches (*Prunus persica* (L.) Batsch) 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. Fruit were wounded by a sterile nail (3x3x3 mm) on opposite sites of the the equatorial area (two wounds per fruit).

Pathogens

M. laxa (strain from our collection) was grown on tomato agar (20 g of Agar Technical, Oxoid Basingstoke, Hampshire, UK; 750 mL distilled water and after sterilization added with 250 mL tomato sauce) for 10 days at 25°C. Conidia from pathogen were collected and suspended in sterile

distilled water containing 0.05 %–(v/v) Tween 20. The concentration of each conidia suspension was quantified with a haematocytometer and adjusted with sterile distilled water..

Antagonists

A. pullulans strains L1 and L8 were previously identified (Mari *et al.* 2012) and 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 to a final concentration of 10^8 CFU mL^{-1}.

Competition for nutrients

The nutrient depletion by L1 and L8 was tested on germination and germ tube elongation ofM. laxa conidia in peach juice. The juicewas obtained by homogenizing 100 g of peach pulp and centrifuging the homogenate for 15 min at 4800 rpm. The supernatant was then diluted with distilled water at the required concentrations and sterilized by filtration with a sterile microfilter (pore size 0.45 μm). Tissue culture plates with 24 wells per plate (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. The inserts consisted of a polystyrene cylinder and a hydrophilic polytetrafluoroethylene (PTFE)membrane (pore size 0.45 μm). Aliquots (120 μL) of peach juice at 5%, 0.5% and water were dispensed in the wells of culture plates with 40 μL of the pathogen conidia suspension (105 conidia/mL), while the same aliquots of L1 or L8 cell suspensions (108 CFU/mL)were dispensed inside the cylinder inserts. The system allows for the interchange of nutrients and metabolites in the peach juice without physical contact between antagonist cells and pathogen conidia. The plates with the cylinders 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 juice was transferred to a glass slide for microscope (Nikon Eclipse TE2000–E) observations. The percentage of M. laxa conidia germination and germ tube elongation (μm) was determined on 90 conidia per treatment (3 microscopic fields, replicates, with 30 conidia each). The growth rate of the germ tube was evaluated from the slope of the straight line. Cylinder inserts, without L1 and L8 strains, were considered as a control. The experiment was performed twice.

Spectrophotometer siderophore assay

In order to determine the siderophore production by the two BCAs and *M. laxa* , one loop of L1 and L8 cells and one of *M. laxa* conidia were transferred in a siderophore solution containing sucrose

(3%), ammonium nitrate (0.3%), K_2HPO_4 (0.3%), citric acid (0.1%), $MgSO_4$ (0.008%), $ZnSO_4$ (0.0002%), L-ornithine (10 mM) and incubated at 25°C at 200 rpm for 24h. Aliquots of 5 mL of the cell suspension were inoculated into 45 mL of the same screening medium and further incubated at 28°C and 200 rpm for 120 h. The culture was centrifuged at 10.000 rpm at 4°C for 5 min, the supernatant (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. A standard curve was prepared with deferrioxamine mesylate (0.0, 0.05, 0.1, 0.25, 0.5, 0.75 and 1.0 mg/mL^{-1}). The amount of siderophore in the supernatant was extrapolated from the standard curve of deferoxamine mesylate. The production of siderophores by L1 and L8 in the same culture solution with pathogen was also determined.

Amino acid analysis by HPLC

Amino acids were determined by reversed phase HPLC analysis and automated pre-column derivatization with o-phtalaldehyde-3-mercapto propionic acid (OPA) for primary and 9-fluorenylmethylchloroformate (FMOC) for secondary amino acids, according to Schuster (1988). A Hewlett-Packard Model series 1100 system, coupled with a diode array detector, a Syncronics C18 column (3 × 250 mm, 5 µm, Thermo Scientific) and corresponding guard column, was used. Quantitative analysis was performed by using norvaline and sarcosine as internal standards for primary and secondary amino acids respectively, and calculating the single response factor for twenty amino acids. Amino acid standard preparations followed Agilent procedures (Application note 5990-4547EN 2010). Samples of peach juice (5%) alone and amended with L1 and L8 suspension (108 CFU/mL) and M. laxa (104 conidia/mL) as described before were diluted in 0.1MHCl and filtered prior to injection, after 12 h of incubation at 25 °C.

Effect of amino acids on Monilinia laxa growth

In order to investigate the influence of asparagine and aspartic acid on pathogen growth, plates containing malt extract agar (MEA, Oxoid, Cambridge, UK) amended with different concentrations of asparagine (Sigma Aldrich, St. Louis, MO, USA) (40 mg/L, 80 mg/L, and 120 mg/L) and aspartic acid (Sigma Aldrich) (15 mg/L, 30 mg/L, and 60 mg/L) were prepared. The concentrations used were similar to those found in peach juice (5%) by HPLC analysis. Plates were inoculated with an agar plug (6 mm in diameter) obtained from an actively growing M. laxa colony or 100 µL of conidia suspension (5 × 103 conidia/mL) prepared as described above. The control was represented

by MEA plates without amendments. The colony growth and conidia germination (as colony forming unit) were assessed after 4 and 2 days of incubation at 25 °C, respectively. The sample unitwas represented by five plates (replicates) for each treatment. The experiment was performed twice.

Scanning electron microscopy (SEM)

In order to investigate on the competition for the space between BCAs (L1 and L8) and *M. laxa*, some Scanning Electron Microscopy (SEM) observations were performed. Peach fruit were wounded once with a sterile nail (2x2x2 mm), treated with 20 μL of L1 and L8 suspension (10^8 cell mL^{-1}) and inoculated with 20 μL of *M. laxa* suspension (10^5 cell mL^{-1}). The control was inoculated only with 20 μL of pathogen suspension. From treated fruit, a little portion (2x2x2 mm) of inoculated pericarp tissue at different time of inoculation (0, 4, 8, 16 and 32 h) was excited and observed under SEM (Zeiss DSM 962 microscope at 30 kV). From each fruit two rind samples derived from the wound in equatorial zone of each fruit were fixed in a phosphate buffer (pH 7.4) containing gluteraldehyde (3%). Before observation, fixed tissues were rinsed three times with phosphate buffer (pH 7.4), dried by washing with increasing concentration of ethanol, stuck on aluminium stubs, and coated with gold palladium (Schirra *et al.* 2011). Samples from five fruit were examined for each strain during each evaluation; however only images from a single fruit are shown, as this sample most represented features seen in the others.

Statistical analysis

All data were analysed using one-way ANOVA and the least significant differences test (LSD) was used to separate the means; statistical significance was considered at $P<0.05$. All analyses were performed with Statgraphics software (version centurion 15.0). The experiments were carried out in a completely randomized block design.

3. Results

3.1. Competition for nutrients

Conidia of M. laxa dipped in water showed a low percentage of germination(26%),whereas in peach juice at 0.5% and 5% the rate of conidial germination substantially increased, reaching 60% and 80% respectively (Table 1) after 6 h of incubation. Similarly, the germ tubelength was longer in

peach juice at 5% than the control and 0.5% (16.4 μm and 12.5 μm respectively) (Table 2). Both antagonists prevented the conidial germination of M. laxa in water, reducing the rate of germination by N35%. However, L1 and L8 showed the lowest inhibition of conidial germination in peach juice at 5%, with 12.6% and 13.9% respectively. The highest juice concentration also influenced the efficacy of the antagonists against germ tube elongation, showing the lowest reduction (15.6% and 15.4% for L1 and L8 respectively).

3.2. HPLC analysis

Peach juice (5%) was analyzed for amino acid content in the presence of M. laxa or antagonist strains L1 or L8, in order to verify the microorganism amino acid consumption and metabolism after 12 h and 24 h of incubation at 25 °C and their possible role in the competition for nutrients. After 12 h of incubation M. laxa did not influence the amino acid composition in diluted juice; aspartic acid and asparagine were detected as major amino acids, with similar concentrations (about 7 mg/L and 107mg/L of diluted juice, for aspartic acid and asparagine respectively) either in the presence or absence of the fungus (Fig. 1 A). On the contrary, the addition of the yeasts greatly modified the amino acid composition: asparagine was completely depleted after 12 h of incubation and probably hydrolyzed to aspartic acid by both yeasts; actually aspartic acid content markedly increased (83 mg/L and 67 mg/L respectively with L1 and L8). At the same time, in the presence of the yeasts other amino acids such as glutamic acid, serine, glycine and alanine appeared, albeit in small amounts (b10 mg/L), indicating the ongoing metabolism of the antagonists (Fig. 1 B and C). After 24 h of incubation, the amino acid distribution did not markedly change; aspartic acid started to quantitatively decline while the other amino acids increased a little (15–30%) (data not shown). In general, the metabolism of L8 appeared slower than that of L1, as all amino acids were qualitatively the same in the presence of the two yeasts but in L8 quantitatively lower than L1 (data not shown).

3.3. Effect of amino acids on Monilinia laxa growth

The two amino acids, asparagine and aspartic acid, influenced the growth ofM. laxa in opposite ways (Table 3). The addition of asparagine in the medium stimulated the colony diameter and the conidial germination. The concentrations significantly more effective were 120 mg/L and 80 mg/L respectively. Conversely, on the mediumto which aspartic acid had been added, the development of the pathogen, the colony diameter and the germination of conidia were significantly inhibited by aspartic acid concentrations of 15 mg/L and 30 mg/L respectively.

3.4 Scanning electron microscopy (SEM)

Scanning electron microscopy micrographs of control peaches inoculated with *M. laxa* revealed that at 4 h of incubation at 25 °C the pathogen started to germinate. After 8 h, the majority of pathogen conidia showed a germ tube and, by the cross section (Fig. 6.C 8h), the pathogen appeared entering into the fruit tissue. In presence of the antagonist strains, *M. laxa* conidia were germinated at 8 h, 16 h and 32 h but remained on fruit surface. Both strains colonized the fruit surface and the fruit wounds (Fig. 6.T 8h cross section) preventing the pathogen attachment and competing for space. At 32 h of growth the fruit surface was totally covered by the yeast cells and only above them there were pathogens hyphae. Only L1 strain micrographs were showed because L8 strain exhibited the same behaviour towards *M. laxa.*

4. Discussion

Two strains of A. pullulans (L1 and L8) are considered potential biocontrol agents active against different fruit postharvest pathogens (Di Francesco et al., 2016) representing a promising alternative to common fungicides in the control of postharvest diseases. In the present work, competition for nutrients and space was studied as being one of the mechanisms of action displayed by BCAs. The possibility of physically separating the pathogen from the antagonist cells and keeping both in the same medium represents a formidable task in understanding the competition for nutrients. The technique was set up by Janisiewicz et al. (2000) and subsequently widely used, in particular with necrotrophic pathogens such as P. expansum (Bencheqroun et al., 2007) and P. italicum (Liu et al., 2013), both pathogens that require nutrients, derived fromfruit tissues, for conidial germination and the initiation of pathogenic processes. In our study, the germination rate of M. laxa conidia was low when the pathogen was dipped in water; this is in agreementwith previous observations on germination requirements of Monilinia conidia (Byrde and Willetts, 1977) and confirmed that Monilinia, like other necrotrophic pathogens, is nutrient-dependent and needs sufficient nutrients for conidial germination and hyphal development (Bencheqroun et al., 2007). Nevertheless, both L1 and L8 strains, in water, further reduced the rate of germination, probably producing volatile organic compounds (Di Francesco et al., 2015a) or other metabolites diffusible in the liquidmedium(Di Francesco et al., 2015b). When the test was performed using peach juice at 5%, 80% of the pathogen conidia germinated after 6 h of incubation at 25 °C and BCAs proved less active against M. laxa probably because of a greater availability of nutrients for the pathogen. Analogous results were obtained with ‘Navel’ oranges, using P. italicum and Kloeckera apiculata

as pathogen and antagonist respectively (Liu et al., 2013). Increased concentrations of orange juice stimulated the conidial germination of the pathogen and reduced the inhibitory effect of BCA. As the concentration of juice increased, from 0.5% to 10%, the efficacy of the antagonist decreased from 80.9% to 4.3% respectively. However, competition for nutrients is only one of the numerous modes of action of a BCA; in fact antagonists can display a wide range of mechanisms of biocontrol sometime acting simultaneously (Di Francesco et al., 2016). The major amino acid present in peach is asparagine; however, its concentration changes during fruit development with a peak in the second stage, between 55 and 85 days after bloom (Moing et al., 1998). In the present study, asparagine and aspartic acid were detected by reversed phase HPLC analysis in diluted peach juice, andwhile asparaginewas depleted by antagonists after 12 h of incubation, the aspartic acid content increased significantly. Peaks of other amino acids, such as glutamic acid, serine, glycine and alanine appeared, although at low concentrations. It is believed that yeasts use asparagine as their source of nitrogen by the deamination of amino acid to aspartic acid. In contrast, in apple juice Janisiewicz et al. (2000) found a depletion of aspartic acid, serine and glutamic acid caused by a strain of A. pullulans (STI-A24) antagonist of P. expansum. Supposing that the depletion of the amino acids alone may be partially responsible for inhibition of conidial germination, the observed behavior, in apple and peach juice, could explain the differences in efficacy of the same antagonists on different hosts. Competition for nutrients was involved for other BCAs such as Pichia guilliermondii against P. digitatum, co-cultivated on synthetic media (Droby et al., 1989); the addition of exogenous nutrients resulted in a reduced efficacy of the BCA thatwas able to competewith the pathogen mainlywhen nutrients were scarce. Bautista-Rosales et al. (2013) showed how Colletotrichum gloesporioides in mangoes increased its pathogenicity after the addition of sucrose and fructose. Other authors (Filonow, 1998; Sharma et al., 2009; Spadaro et al., 2010) reported that yeasts generally have the ability to successfully assimilate a wide variety ofmono- and di- saccharides, such as fructose and sucrose, making these nutrients unavailable to pathogens and allowing the yeasts to proliferate rapidly. In effect, competition for nutrients is believed to play a significant role in biocontrol interactions (Raaska and Mattila-Sandholm, 1995).

In our trials, analogous concentrations of asparagine, found in peach juice,were tested by in vitro assays againstM. laxa growth. The development of the pathogen was stimulated by the presence of asparagine, confirming previous data reported by Hall (1967) that showed that asparaginesupported the M. fructicola growth more than the other sources of nitrogen such as glycine, ammonium chloride or potassium nitrate. In the same way, aspartic acid, at similar concentrations detected in juice after 12 h of antagonist incubation, revealed an antifungal effect evaluated against themyceliumgrowth and conidial germination of M. laxa. To our knowledge, this is the first study

demonstrating the activity of aspartic acid against the pathogen causing brown rot, despite being obtained through in vitro experiments. Fromour data, the hypothesis of the competition for nutrients is only partially confirmed, revealing that the interactions between pathogen, host and antagonist are complex and not yet fully elucidated; for example, the role of aspartic acid in the inhibition of M. laxa growth has to be further investigated. Together, competition for nutrients and space is considered the main mode of action of yeasts in biocontrol (Zhang et al., 2011). The study of the competition for space requires observations with light microscopy or SEM able to reveal the attachment of fungal hyphae or the occupancy of wound space by BCAs. Previously, several authors studied the physical interaction between antagonist and pathogen, revealing tenacious attachment of P. guilliermondii to the hyphae of Botrytis cinerea despite the extensive rinsing of samples with distilled water during sample preparation (Wisniewski et al., 1991). Similarly, P. membranifaciens and C. albidus showed different capability to attach to the hyphae of three pathogens in both in vivo and in vitro trials (Chan and Tian, 2005). In the latter case, the attachment of antagonist cells to pathogen hyphae was correlated with the secretion of hydrolytic enzymes inducing the degradation of fungal mycelium. Although A. pullulans L1 and L8 strains showed enzymatic activity (Di Francesco et al., 2015b) in the first 24 h after treatment, in the present work they also exhibited a great ability to colonize the wound in the presence of M. laxa, starting immediately after treatment. Moreover, to successfully compete with the pathogen, the microbial antagonist needs to quickly adapt to various environmental and nutritional conditions of the host (Barkai-Golan, 2001; El-Ghaouth et al., 2004). A. pullulans showed good adaptation in apple and peach wound environments, not only being able to survive but also to increase its population, remaining high over time (Mari et al., 2012a, 2012b). Scanning electron microscopy confirmed the considerable ability of both A. pullulans strains to colonize the peach wound and consequently to prevent the pathogen growth at each time of inoculation. In contrast with other BCAs such as P. guillermondi that parasitized B. cinerea in apple (Wisniewski et al., 1991) or Meyerozyma caribbica active against C. gloesporioides of mangoes (Bautista-Rosales et al., 2013), our preliminary data fromSEMobservations showed that both strainswere unable to adhere to the hyphae of M. laxa and parasitize them. On the other hand, they were able to make the wound environment critical for the pathogen occupying the space, also probably depleting the nutrients such as asparagine, synthesizing aspartic acid and producing VOCs (Di Francesco et al., 2015a). In conclusion, this study clearly showed that A. pullulans L1 and L8 strains can compete with M. laxa for nutrients and space. Although this mode of action plays an important role in antagonistic activity, especially

in the first hours of tritrophic host-pathogen-antagonist interaction (Spadaro and Droby, 2016), several other modes of action can interactwith each other. Further research is required to obtainmore information that may increase our understanding of BCA activity in view of their formulation.

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Figure 1.

Influence of L1 and L8 strains and sugars (glucose, sucrose and fructose) (2%) on *Monilinia laxa* (B) mycelium dry weight (MDW) (mg) determined after 7 days of incubation at 20°C. Within the same sugar, different letters indicate significant differences according to LSD test ($P<0.05$). Vertical bars represent the standard deviation (n=3).

Table 1
Percentage of germinated *Monilinia laxa* conidia after 6 h of incubation without or with *Aureobasidium pullulans* strains (L1 and L8) in 0 (water), 0.5, and 5% of peach juice.

	Peach juice (%)		
	0	0.5	5
Pathogen	26.1 ± 3.00a*	60.3 ± 1.46a	80.1 ± 1.00a
Pathogen + L1	10 ± 2.94b	33.4 ± 1.92b	70 ± 1.11b
Pathogen + L8	11 ± 1.44b	16 ± 2.91c	69 ± 2.11b

* Values are the means of three repetitions (30 conidia each) per treatment. Means within the same column followed by different letters are significantly different ($P<0.05$) according to the LSD test.

Table 2
Germ tube length (μm) of *Monilinia laxa* conidia after 6 h of incubation without or with *Aureobasidium pullulans* strains (L1 and L8) in 0 (water), 0.5, and 5% of peach juice.

	Peach juice (%)		
	0	0.5	5
Pathogen	16.4 ± 1.64a*	12.5 ± 1.04a	19.5 ± 1.58a
Pathogen + L1	5.2 ± 0.78b	10.3 ± 0.88b	16.4 ± 0.64b
Pathogen + L8	6.3 ± 0.36b	7.2 ± 0.90c	16.5 ± 0.86b

* Values are the means of germ tube length of 90 germinated conidia. Means within the same column followed by different letters are significantly different ($P < 0.05$) according to the LSD test.

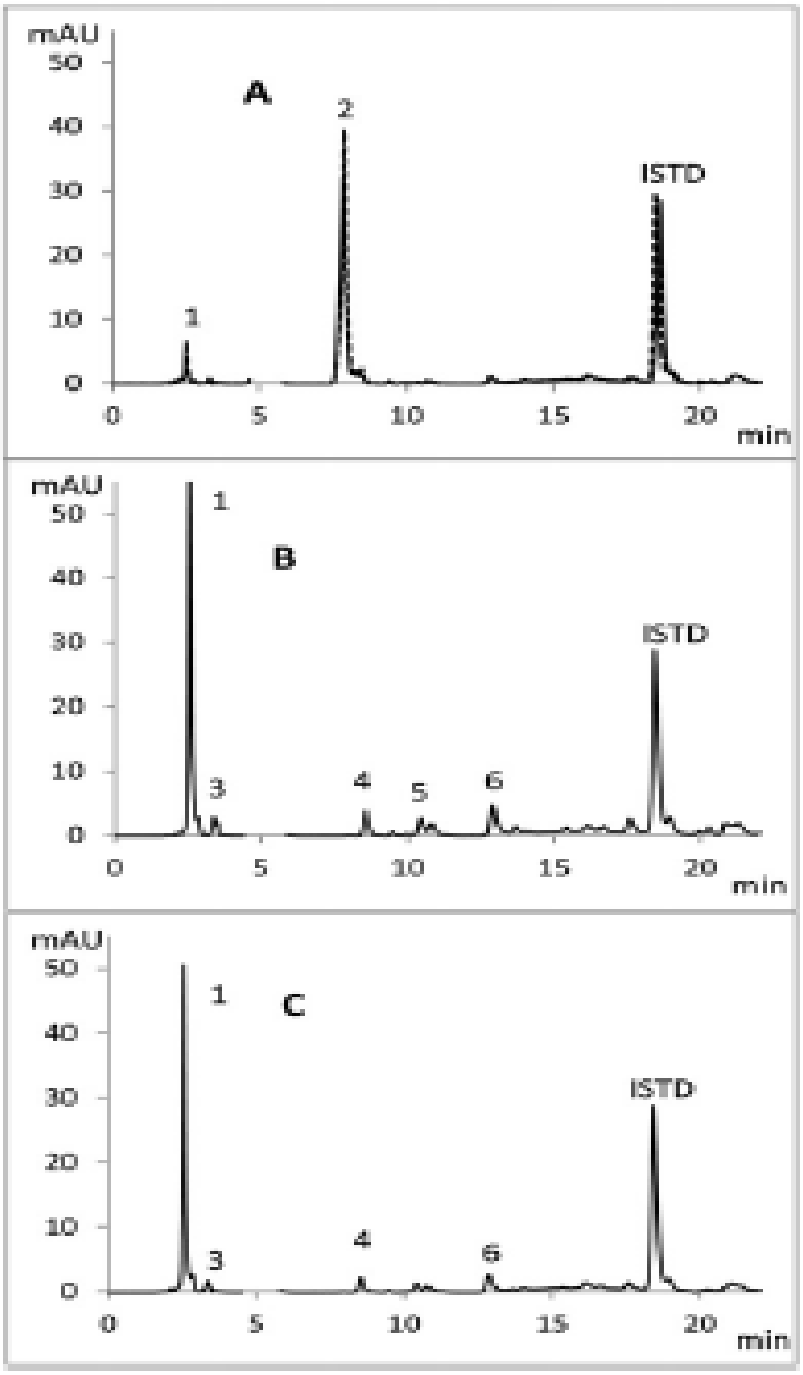


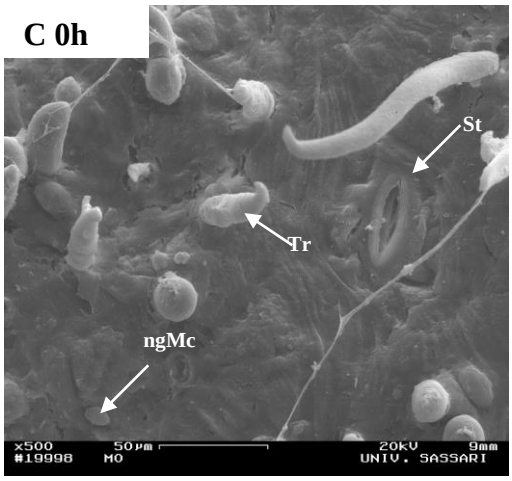
Fig. 1. HPLC chromatograms of samples analyzed after 12 h of incubation at 25 °C: **A**, 5% peach juice with and without *M. laxa* (continuous and dotted line respectively); **B**, 5% peach juice and L1; **C**, 5% peach juice and L8. 1, aspartic acid; 2, asparagine; 3, glutamic acid; 4, serine; 5, glycine; 6, alanine; ISTD, internal standard, norvaline.

Table 3
Effect of pure asparagine and aspartic acid on *Monilinia laxa* growth.

Asparagine	Control	40 mg/L	80 mg/L	120 mg/L
Colony diameter (mm)[a]	47 ± 1.08b[*]	45 ± 2.89b	46 ± 1.35b	53 ± 1.47a
Colony forming units	79 ± 1.50b	81 ± 0.75b	96 ± 2.74a	99 ± 1.17a
Aspartic acid	**Control**	**15 mg/L**	**30 mg/L**	**60 mg/L**
Colony diameter (mm)	54 ± 0.88a	53 ± 1.45a	48 ± 1.20b	46 ± 3.72c
Colony forming units	83 ± 1.28a	79 ± 4.35a	66 ± 5.41b	50 ± 0.95c

[a] A mycelial plug (6 mm) from an actively growing pathogen culture or aliquots of 0.1 mL of conidia suspension (10^3 conidia mL) were placed or spread in the center of malt extract agar plates, incubated at 25 °C for 5 and 2 days respectively. The control was represented by plates without amendments.

[*] Data represent the mean of five petri dishes. Within a row, values followed by the same letters are not statistically significant per LSD test ($P < 0.05$).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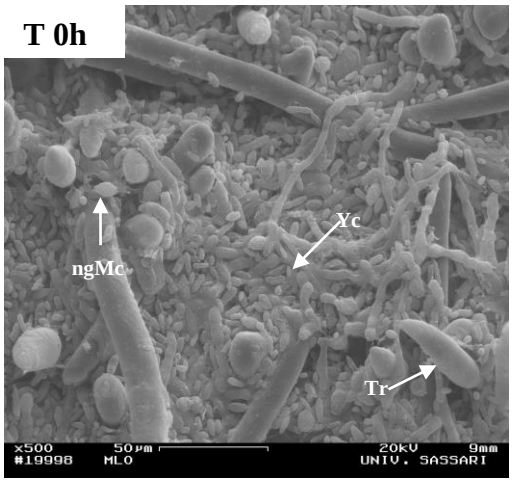
St
Tr
ngMc
x500 50µm 20kV 9mm
#19998 M0 UNIV. SASSARI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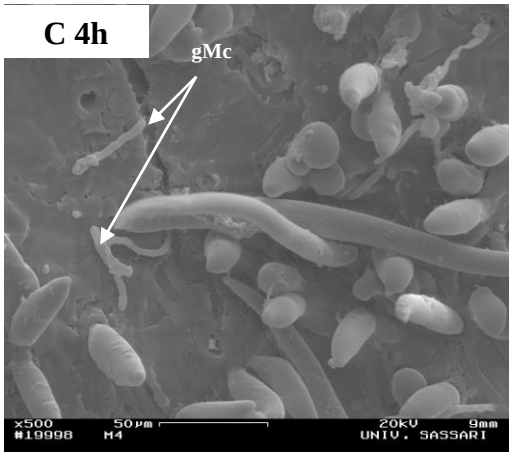
463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gMc
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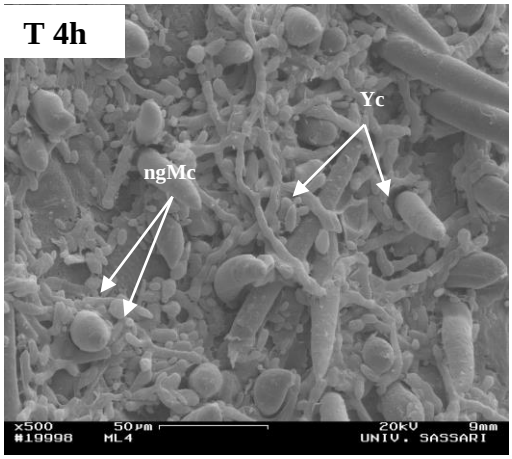
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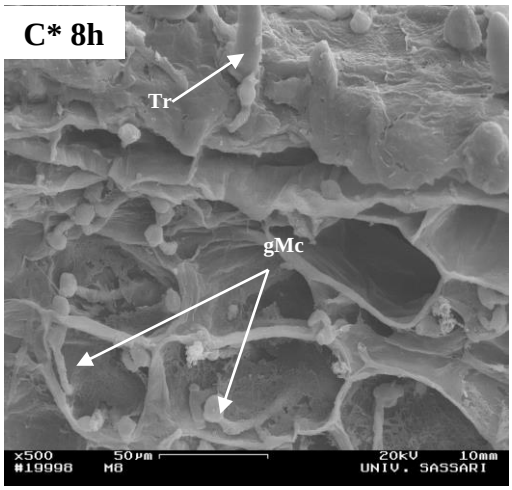
464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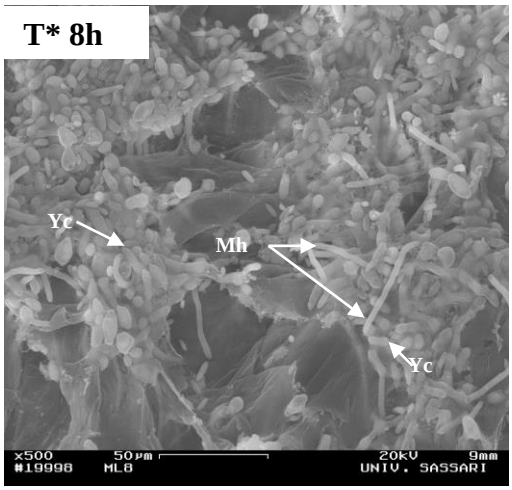
Tr
gMc
x500 50µm 20kV 10mm
#19998 M8 UNIV. SASSARI


T* 8h
Yc
Mh
Yc
x500 50µm 20kV 9mm
#19998 ML8 UNIV. SASSARI

465

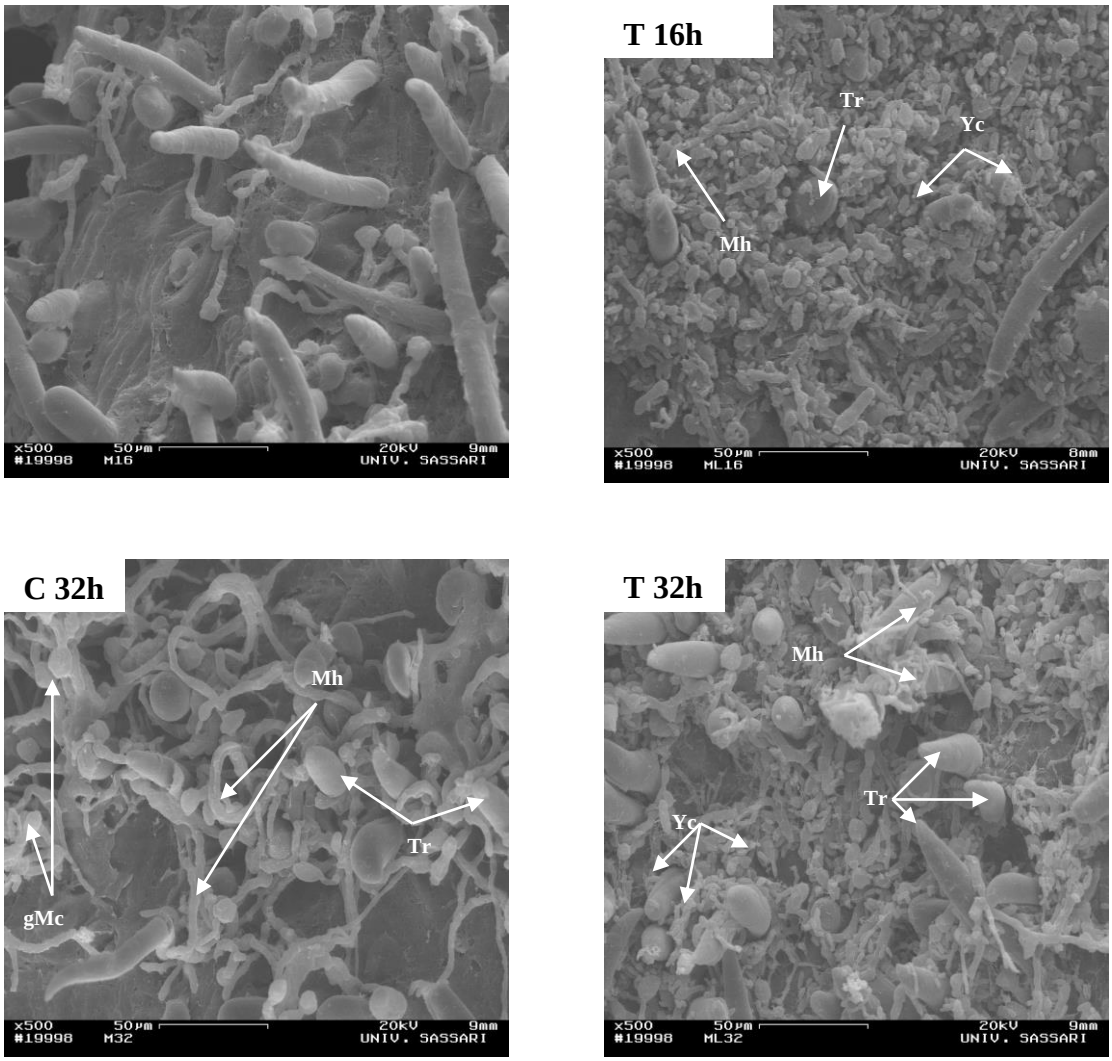


Figure 6.
Scanning electron micrographs (SEM) of "Redhaven" peach (surface and cross section) inoculated with *Monilinia laxa* (Control=C), *M. laxa* and *Aureobasidium pullulans* (L1 strain) (Treatment=T) at different times from the inoculum (0, 4, 8, 16, 32 h).