Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE E TECNOLOGIE AGRARIE, AMBIENTALI E ALIMENTARI

Ciclo XXVII

Settore Concorsuale di afferenza: 07/D1 - Patologia Vegetale ed Entomologia

Settore Scientifico disciplinare: AGR/12 - Patologia Vegetale

"Aureobasidium pullulans as biological control agent: modes of action"

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Esame finale anno 2015

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It's a long way to the top (if you wanna rock'n'roll)

Abstract

The postharvest phase has been considered an environment very suitable for successful application of biological control agents (BCAs). However, the tri-interaction between fungal pathogen, host (fruit) and antagonist is influenced by several parameters such as temperature, oxidative stresses, oxygen composition, water activity, etc. that could be determining for the success of biocontrol. Knowledge of the modes of action of BCAs is essential in order to enhance their viability and increase their potentialities in disease control.

The thesis focused on the possibility to explain the modes of action of a biological control agent (BCA): *Aureobasidium pullulans,* in particular the strains L1 and L8, control effective against fruit postharvest fungal pathogen.

In particular in this work were studied the different modes of action of BCA, such as:

i) the ability to produce volatile organic compounds (VOCs), identified by SPME-gas chromatography-mass spectrometry (GC-MS) and tested by *in vitro* and *in vivo* assays against *Penicillium* spp., *Botrytis cinerea*, *Colletotrichum acutatum;*

ii) the ability to produce lytic enzymes (exo and endo chitinase and β-1,3-glucanase) tested against *Monilinia laxa*, causal agent of brown rot of stone fruits. L1 and L8 lytic enzymes were also evaluated through their relative genes by molecular tools;

iii) the competition for space and nutrients, such as sugars (sucrose, glucose and fructose) and iron; the latter induced the production of siderophores, molecules with high affinity for iron chelation. A molecular investigation was carried out to better understand the gene regulation strictly correlated to

the production of these chelating molucules. The competition for space against *M. laxa* was verified by electron microscopy techniques;

iv) a depth bibliographical analysis on BCAs mechanisms of action and their possible combination with physical and chemical treatments was conducted.

Riassunto

La fase del post-raccolta è considerata un ambiente molto adatto per la riuscita applicazione degli agenti di controllo biologico (BCAs).

Tuttavia, la tri-interazione tra patogeno fungino, frutto ed antagonista è influenzata da diversi parametri quali temperatura, stress ossidativi, composizione dell'ossigeno, attività dell'acqua, ecc. che potrebbero essere determinanti per il successo del controllo biologico.

La conoscenza sui modi d' azione dei BCA è essenziale al fine di migliorare la loro vitalità e per aumentare le loro potenzialità nel controllo delle malattie.

La tesi è incentrata sulla possibilità di spiegare i modi d' azione di un agente di controllo biologico (BCA): l'*Aureobasidium pullulans*, in particolare i ceppi L1 e L8, efficaci contro i patogeni fungini della frutta del post-raccolta. In particolare, in questo lavoro sono stati studiati i diversi modi d' azione del BCA, quali:

i) la capacità di produrre composti organici volatili (VOCS) identificati mediante SPME-(GC-MS) spettrometria di massa e testati *in vitro* e *in vivo* contro *Penicillium* spp., *Botrytis cinerea*, *Colletotrichum acutatum;*

ii) la capacità di produrre enzimi litici (eso ed endo chitinasi e β-1,3-glucanasi) testati contro *Monilinia laxa*, agente causale del marciume bruno delle

drupacee. Gli enzimi litici prodotti da L1 e L8 sono stati valutati anche attraverso i loro relativi geni attraverso tecniche molecolari;

iii) la competizione per lo spazio e i nutrienti, come gli zuccheri (saccarosio, glucosio e fruttosio) e ferro; quest'ultimo induce la produzione di siderofori, molecole con elevata affinità chelante del ferro. Un' indagine molecolare è stata effettuata per comprendere meglio la regolazione dei geni strettamente correlata alla produzione di queste molecole chelanti. La competizione per lo spazio nei confronti di *M. laxa* è stata verificata mediante tecniche di microscopia elettronica;

iv) è stata condotta una approfondita analisi bibliografica sui meccanismi d'azione dei BCAs e la loro possibile combinazione con trattamenti fisici e chimici.

Preface

Aureobasidium pullulans, strains L1 and L8, were originally isolated from the surface of 'Redhaven' peaches collected from an organic orchard of the Agricultural Faculty (University of Bologna, Italy) and their preliminary selection was based on the activity against pathogens directly on fruit showing a effectiveness greater than 50%. After these preliminary trials, two antagonists (L1 and L8) were selected and subsequently identified through microscopic observation of cell and colony morphology and by sequencing of domain D1/D2 of 26S ribosomal DNA and the Internal Transcribed Spacers (ITS 1 and 2 region) according to White et al. (1990).

The sequencing of D1/D2 domain and ITS rDNA regions recognized the two antagonist yeast strains (L1 and L8) as *A. pullulans*, with a sequence homology percentage of 100% with *A. pullulans* CBS 584.75T (accession number FJ150906) (Fig. 1).
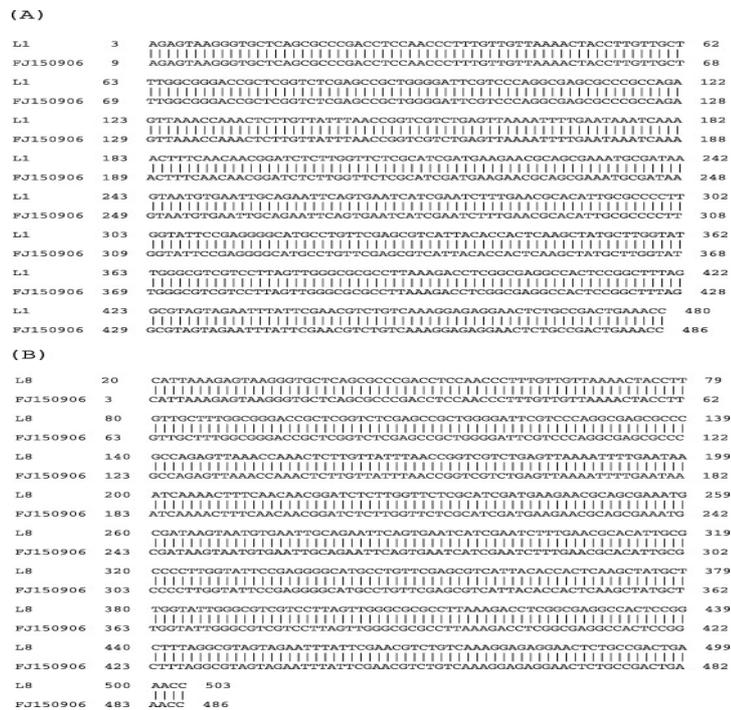
Fig. 1 Nucleotide sequences comparison of the internal transcribed spacer ITS 1 and 2 region of *Aureobasidium pullulans* yeast (strains L1 and L8) with the sequence of *A. pullulans* CBS 584.75T, accession number FJ150906, identification in BLASTN search. The alignment was performed using ClustalX program.

The results of microscopic observations of both fungal conidia and yeast colony morphology were compared with the identification keys of Batra (1991) and Barnett *et al.* (2000) respectively and were complementary to the molecular analysis. Purified colonies were maintained on specific culture substrate slants at 4 °C until use to assay their antagonistic capabilities.

References

Barnett J.A., Payne R.W., Yarrow D. Yeasts: Characteristics and Identification, 2000. Cambridge University Press, UK pp 1139.

Batra L.R. World Species of *Monilinia* (fungi) Their Ecology, Biosystematics and Control, 1991. Mycologia Memoir No. 16, Journal Cramer, Berlin.

Mari M., Martini C., Guidarelli M., Neri F. Postharvest biocontrol of *Monilinia laxa*, *Monilinia fructicola* and *Monilinia fructigena* on stone fruit by two *Aureobasidium pullulans* strains, 2012. Biological Control, 60: 132–140

White T.J., Bruns T., Lee S., Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, 1990, M.A. Innis, D.H. Gelfans, J.J. Sninsky, T.s. White (Eds.), PCR Protocols. A Guide to Methods and Applications, Academic Press, San Diego, CA, (USA), pp 315–322.

__Introduction__

Biological control of postharvest diseases by microbial antagonists: how many mechanisms of action?

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Abstract

The postharvest phase has been considered an environment very suitable for successful application of biological control agents (BCAs). However, the tri-interaction between fungal pathogen, host (fruit) and antagonist is influenced by several parameters such as temperature, oxidative stresses, oxygen composition, water activity, etc. that could be determining for the success of biocontrol. Knowledge of the modes of action of BCAs is essential in order to enhance their viability and increase their potentialities in disease control. The antagonists display a wide range of modes of action: antibiosis, competition for nutrients and space, parasitism and induction of resistance are considered the main ones. Their efficacy, however, is related to the host and the pathogen; sometimes, different modes act simultaneously, and it is therefore difficult to establish the contribution of a single mechanism to a specific antifungal action. The current review presents a brief overview of the research that has led to a better

understanding of the mode of action of BCAs with particular emphasis on the most recent literature.

1. Introduction

Since the appearance of the first work on the biological control of a postharvest disease (brown rot of stone fruit) (Pusey and Wilson, 1984), the postharvest phase has been considered an environment very suitable for successful application of biological control agents (BCAs). In fact, its features of constant temperature, high humidity, absence of UV-rays, etc. seem to offer more chances to BCAs, increasing their antifungal activity. However, the interactions between fungal pathogen, host (fruit) and antagonist in a multivariate system where several parameters such as oxidative stresses, oxygen composition, water activity, etc. play an important role, could be determining for the success of biocontrol. BCAs are living organisms and act following different antagonistic strategies depending on pathogens, host and environment; knowledge of their modes of action is therefore essential to enhance their viability and increase their potentialities in disease control. For example, understanding the mode of action can facilitate the registration for commercial use and can be useful in optimizing the formulation and delivery systems (Chanchaichaovivat et al., 2008).

In general, antagonists used for biocontrol of postharvest diseases are yeasts and bacteria, and to a lesser extent fungi, and they have been widely reviewed (Liu et al., 2013; Nunes, 2012; Sharma et al. 2009; Spadaro and Gullino, 2010). Considerable

information is available with respect to their efficacy, their application under storage conditions, their mixture with safe substances or according to the formulation, etc. However, the mechanisms by which BCAs exert their activity against pathogens have not yet been fully elucidated (Sharma et al., 2009). Antagonists can display a wide range of modes of action, at different stages of their activity, relating to different hosts, pathogens; sometimes different modes act simultaneously, and it is therefore difficult to establish the contribution of a single mechanism to a specific antifungal action.

Antibiosis (production of antimicrobial compounds), competition for nutrients and space, parasitism and induction of resistance are considered the main modes of action by which microbial antagonists control the principal pathogens including fruit postharvest pathogens. However, other mechanisms such as volatile organic compounds (VOCs) and siderophores production or induction of ROS production in the host are also under investigation.

In the current review, a brief overview of the research, that has led to better understanding of the BCAs' mode of action, is presented and discussed with particular emphasis on the most recent literature.

2. Mechanisms of action of BCAs

2.1 Antibiosis

In general, antibiosis is considered a biological process by which antagonists produce substances that inhibit or kill potential pathogens (bacteria or fungi) occurring in close proximity. Antibiosis is found more in bacteria than yeasts and fungi (Bull et al., 1998). The most common antibiotic compounds effective against postharvest fungal pathogens are: iturin produced by *Bacillus* spp. (Dimkic et al., 2013; Gueldner et al., 1988; Pusey,

1989; Waewthongrak et al., 2015); pyrrolnitrin produced by *Pseudomonas* spp. (Janisiewicz et al., 1991) and syringomycin produced by *P. syringae* (Bull et al., 1998). However, other metabolites such as bacillomycin, surfactin, and fengycin showing antibacterial and antifungal activity are synthetized by *Bacillus* spp. (Arrebola et al., 2010). The cyclic lipopeptide (LPs) families including iturin, surfactin and fengycin are well-recognized compounds with interesting potential applications because of their surfactant properties that improve the efficacy; in addition, different groups of LPs can confer an advantage for *Bacillus* strains, stimulating the resistance response in fruit (Ongena and Jacques, 2010). There is some unequivocal evidence that the substances produced by antagonists play a key role in the control of plant diseases, although there are doubts on the involvement of antibiosis in postharvest disease control. Iturin A produced by *B. amyloliquefaciens* PPCB004 was found to be active against three postharvest fungal pathogens (*Alternaria citri*, *Colletotrichum gloeosporioides* and *Penicillium crustosum*), while an iturin deficient mutant of *B. amyloliquefaciens* (called PPCB004itu-) displayed a higher disease incidence in comparison with the wild type PPCB004 (Arrebola et al., 2010). The production of antimicrobial compounds by BCAs could be influenced by several abiotic factors such as oxygen, temperature, specific carbon and nitrogen sources, and microelements. For example, the production of syringomycin *in vivo* is dependent on the physiological state of the *P. syringae* and on the nutrient availability, but also on the presence of plant signal molecules (Bull et al., 1998) for the activation of genes responsible for syringomycin biosynthesis (Mo and Gross, 1991). Therefore, the antibiosis observed *in vitro* trials is not necessarily correlated with inhibition of the pathogens *in vivo* (Fravel, 1988). The production of antimicrobial compounds was not generally detected in fruit, and in addition Bull et al.

(1998) found that the treatment of wounds with pure syringomicyn to control *P. digitatum* of lemons required 1000 times the concentration needed for pathogen inhibition in *in vitro* trials, suggesting the participation of other mechanisms of action not dependent on syringomycin production.

Although the antibiotic producing bacteria represent an important opportunity for the biological control of postharvest diseases, the development of non-antibiotic producing antagonists could be more accepted by public opinion, also avoiding the possibility of a rapid appearance of pathogen resistance towards these antimicrobial substances (El-Ghaout et al., 2004).

Among the substances produced by BCA, the volatile organic compounds (VOCs) are frequently involved in the biological control of several postharvest fungal diseases of fruit. Phenethyl alcohol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-methyl-1-propanol are some of the VOCs produced by *Aureobasidium pullulans* were found to be active against *B. cinerea*, *C. acutatum* and *Penicillium* spp.in *in vitro* and *in vivo* trials (Di Francesco et al., 2014). However, the VOCs emitted by these BCAs provide only a limited contribution to the biological control of pathogens, since their inhibitory activity is frequently not completed and fungistatic, while it is known that fungi produce a wide range of VOCs (Miyazawa et al., 2008), studied for their biotechnological potential in food or agricultural industries (Morath et al., 2012). For example, VOCs produced by a *P. expansum* (R82) strain (Rouissi et al., 2013) or by *Muscodor albus* (Mercier and Jimenez, 2004) were most active against some fungal postharvest diseases and their application appears promising. Similarly, other antagonists such as *Candida intermedia* (Huang et al., 2011) and *Sporidiobolus pararoseus* (Huang et al., 2012) inhibited the *B. cinerea* growth of strawberries by the emission of volatile compounds. In these studies,

the BCAs were considered biofumigants, representing a particular application of biological control, since the antagonists are not in direct contact with the pathogen and VOC production is their only mechanism of action.

2.2 Competition for nutrients and space

Competition is intended as the request for the same nutrients or space by two or more microorganisms. This mode of action is considered a key factor by which BCAs suppress postharvest pathogens (Nunes, 2012) and it has been demonstrated in several studies for different antagonists as reviewed by Sharma et al. (2009). Among the BCAs, it is mainly the yeasts that compete for space and some nutrients with the pathogens (Spadaro et al., 2004), being able to grow rapidly during the first 24 hours after treatment, deplete quickly available nutrients and physically occupy the wounds. The antagonists are frequently reported with a fungistatic activity, the competition effects result in an inhibition of fungal spore germination, leaving them alive and able to germinate after the addition of other nutrients (Janisiewicz et al., 2000). For this reason, from the first day after treatment, other modes of action could come into play and become determining for the success of disease control (Droby et al., 2009).

As the main postharvest fungal pathogens are wound parasites, the ability of antagonists to colonize these niches and rapidly increase their population appears strategic for the success of control. In addition, nutrient disposal is correlated with host species since different fruits can support different nutrients, explaining for example how the activity of some antagonists on citrus was less effective than on strawberry (Lima et al., 1999). A study on the population dynamics of two *A. pullulans* strains showed different rates of development with respect to fruit species; in apple wounds, the population size

increased the initial concentration 7-fold, while in peach wounds only a weak growth was observed, although it was enough to control *Monilinia* rot (Mari et al., 2012). The competition for nutrients was widely investigated; however, the non-destructive method adopted by Janisiewicz et al. (2000), that used tissue culture plates with cylinder inserts and excluded the competition for space avoiding any contact between antagonist and pathogen conidia, appears a most suitable tool for this kind of study. In fact, with this technique, it was possible to observe how *Pantoea agglomerans* CPA-2 prevented conidia germination at low but not at higher nutrient concentrations, indicating that the competition for nutrients can play a role in disease control at low but not at high nutrient concentrations (Poppe et al., 2003). Similar findings were observed with *P. expansum* and the antagonist bacterium *Erwinia herbicola*. A high inhibition of pathogen spore germination was reported in the presence of diluted but not undiluted apple juice, showing that at high nutrient concentrations both antagonist and pathogen can find adequate amounts of substances for growth, while when the nutrients are low, the competition can be between two microorganisms favouring the BCA (Bryk et al., 1998).

In fruit wounds, the antagonists can also remove essential elements from the pathogen, such as nitrogen, oxygen or amino acids, vitamins, etc. present in low concentrations, although it is difficult to test in *in vitro* assays what is the main element essential for both, antagonist and pathogen. *In vivo* trials showed that a strain of *A. pullulans* (Ach1-1) active against *P. expansum* assimilated apple amino acids better than the pathogen, most particularly serine, glycine and glutamic acid; among these amino acids, especially serine appeared to be the most limited nutrient (Bencheqroun et al., 2007).

Although the competition for nutrients and space has often been implied in biological control, direct proof of the importance of this mechanism in the fruit system is still lacking (Janisiewicz et al., 2000) and both (nutrient and space) are often considered together for a given antagonist without ascribing the proper level of significance to each factor. As reported, the majority of postharvest diseases derive from wounds, a niche where an abundant availability of nutrients occurs. The rapid colonization of this site is dependent on antagonist concentration but also on host species that could be more favourable for certain BACs than others.

Among nutrients, iron is one of the most critical for fungal development, to the point that it can be a limiting factor for fungi growth; for example, conidia require a large intake of iron to germinate (Charlang et al., 1981), so it is understandable that the presence of siderophores, the low molecular weight ferric chelating agents, may delay or reduce conidial germination. Competition for iron may thus play an important role in the antagonist-pathogen interactions (Raaska and Mattila-Sandholm, 1995) although it is still not well understood. There is substantial evidence that the siderophores produced by fluorescent pseudomonas have a role in biocontrol by sequestering iron. In addition, yeasts such as *Rhodotorula glutinis* are frequently siderophore producers and their concentration is closely associated with the iron concentration in the medium. High siderophore concentrations could help to control disease without increasing the population of the antagonist applied (Calvente et al., 1999). Otherwise, other antagonists, like a yeast *Cystofilobasidium infirmominiatum,* produced siderophores when grown in a suitable medium like CAS, but such compounds did not seem to affect the radial growth of *P. expansum* (Vero et al., 2013).

2.3 Induced resistance

Induced resistance in harvested fruits by BCAs as the mode of action for controlling postharvest decay has been widely investigated (Ippolito et al., 2000; Yao and Tian, 2005; Droby et al., 2009). There is accumulated evidence that shows how some antagonists could induce disease resistance by activating PR proteins (β-1-3glucanase, chitinase, etc.) or defence-related enzymes (phenylalanine ammonia-liase-PAL, peroxidase, polyphenoloxidase, etc.). A preharvest treatment of mandarin orange with *Rhodosporidum paludigenum* induced a significant reduction of postharvest *Penicillium* spp. disease incidence; since the β-1-3glucanase activity increased significantly in response to BCA preharvest application, a correlation with induced resistance was presumed (Lu et al., 2013). Similarly, other antagonists like *Cryptococcus saitona* and *A. pullulans* increased β−1-3 glucanase activity, although it is not clear whether the enzymatic activity detected in the yeast-treated wounds is mainly of host origin or derived from BCAs, since *A. pullulans* like other antagonists is capable of producing β 1−3−glucanse (Castoria et al., 1997). Once again, the line between the different modes of action of BCAs appears very tenuous and a clear understanding of these mechanisms could be difficult. The development of DNA microarrays and high-throughput sequencing technologies now makes it possible to study the changes in gene expression in host tissue and antagonist, providing insight on the regulation and origin of these enzymes (Liu et al., 2013).

The phenylpropanoid pathway is known to be influenced significantly by both biocontrol agents and postharvest pathogens (Ballester et al., 2013); in this pathway, PAL is responsible for the biosynthesis of p-cumaric acid derivates, phytoalexins, and lignin, and an increase in PAL activity in fruit tissue wounds treated with antagonists

can favour the cicatrisation processes, reducing the possibility for the pathogen to become established in wounds (Droby and Chalutz, 1994). In addition, several yeasts have been shown to stimulate the production of ethylene, a hormone able to activate PAL (Wisniewski et al., 1991).

One of the first barriers against invading pathogens is the accumulation of reactive oxygen species (ROS) that also work as signals for plant defence reactions. When yeasts are applied to fruit wounds, the ROS-generated oxidative stress can affect their viability and performance, and their tolerance to oxidative stress is consequently important for biocontrol (Liu et al., 2012). On the other hand, antagonist yeasts can influence ROS production and defence signalling in fruit tissues, stimulating both antioxidant gene expression and antioxidant enzyme activity in peach fruit tissues (Xu et al., 2008). A better understanding of the role of ROS in biocontrol systems could lead to a selection of more efficient antagonists and enhance their activity (Liu et al., 2013).

2.4 Parasitism

Antagonist and pathogen can also interact through a direct parasitism depending on close contact and recognition between the two microorganisms, on the secretion of lytic enzyme and on the active development of the antagonist in the fungal pathogen (Spadaro and Gullino, 2004).

Scanning electronic microscope (SEM) and transmission electron microscope (TEM) observations performed on wounded fruit tissues treated with cells of antagonists showed not only a consistent colonization of fruit wounds but also strong interactions with morphological abnormalities in a hypha pathogen (Zhou et al., 2011) resembling more a direct parasitism than a simple space competition.

Although there is little information on this mechanism in the literature, some authors observed that lytic enzyme production (Wisniewski et al., 1991) by BCAs could play an important role in their biocontrol activity. Since chitin, β-glucans, and chitosans are the principal components in the fungal cell walls, the antagonists showing high enzyme activity like β-1,3-glucanase, chitinases (endo- and exo-) etc. could represent promising BCAs, being able to breaking down the cell walls of pathogens (Masih and Paul, 2002). *P. membranefaciens* had strong capability of attachment to hyphae of pathogens such as *B. cinerea*, *P. expasnum* and *Rhizopus stoloniefer*; its attachment ability was found to be higher than that of *C. albidus* (Chan and Tian, 2005), showing the possibility of differences in parasitic activity for each antagonist. The chitinolytic microorganisms such as *Bacillus* spp. and *Pseudomonas* spp. are considered to be more effective antagonists of fungal pathogens because of the direct action of chitinase alone (Yu et al., 2008) or in combination with other antifungal compounds produced by the antagonist (De Boer et al., 1998). Moreover, the presence of fungal cell wall significantly increased the β-1,3-glucanase activity compared to that observed in media containing only laminarin. Different levels of activity of this enzyme were observed with respect to the incubation time; for example, the level of the exo−β-1,3-glucanase reached a maximum after 24 h of incubation at 25°C, while endo- β -1,3-glucanase peaked at 48 h (Masih and Paul, 2002). *P. anomala* produced an exo−β−1,3-glucanase (Exoglc1) that, when purified, caused morphological changes in *B. cinerea* germ tube growth, like leakage of cytoplasm and cell swelling (Jijakli and Lepoivre, 1998). Similarly, the hyphal cells of *B. cinerea* in close contact with the antagonist *C. saitoana* cells showed various degrees of alteration of cell walls and cytoplasm; however, data

showed that the attachment may enhance nutrient competition interfering with the ability of the pathogen to initiate infection (El-Ghaouth et al., 1998).

The ability of BCAs to form a biofilm, creating a mechanical barrier interposed between the wound surface and pathogen, can be considered a sort of parasitism. Biofilm formation has been proposed as a possible mechanism of biocontrol in some antagonist yeasts (Vero et al., 2013). One of the main issues of biofilm is the initial attachment of microorganisms, since it is the necessary first step of biofilm formation. A positive relationship was found in grape berry between biofilm formation and wound colonization capacity of *M. pulcherrima* and *W. anomalous,* which were found to be effective against gray mould decay, while *S. cerevisiae* showed a lower wound colonization, no film-forming ability and no *B. cinerea* reduction (Parafati et al., 2015).

3. Conclusion

Although fungicides remain a primary method of controlling postharvest diseases, the global trend shifts towards a reduction of their use. Postharvest disease management needs the development of alternative strategies, like biofungicides. Although a large number of studies are focused on their modes of action, they need to be more developed to better understand the activity and the interaction between them and the environment, in particular postharvest conditions. The importance of each mode of action can vary relating to yeast-pathogen-host. The basic mode of action for yeast is competition for space and nutrients, while for bacteria it could be antibiotic production; however, several other modes of action can interact with each other, supporting the efficacy of BCAs in determining innovative solutions for sustainable fruit production.

References

Arrebola, E., Jacobs, R., Korsten, L., 2009. Iturin A is the principal inhibitor in the biocontrol activity of *Bacillus amyloliquefaciens* PPCB004 against postharvest fungal pathogens. Journal of Applied Microbiology 108, 386-395.

Ballester, A.R., Lafuente, M.T., de Vos, R.C.H., Bovy, A.G., Gonzales-Candela,L., 2013. Citrus phenylpropanoids and defence against pathogens. Part I: Metabolic profiling in elicited fruits. Food Chemestry 136, 178-185.

Bencheqroun, S.M., Bajji, M., Massart, S., Labhilili, M., El-Jaafari, S., Jijakli, M.H., 2007. *In vitro* and *in situ* study of postharvest apple blue mold biocontrol by *Aureobasidium pullulans*: evidence for the involvement of competition for nutrients. Postharvest Biology and Technology 46, 128–135.

Bryk,H., Sobiczewski, P., Dyki, B., 1998. Antagonistic effect of *Erwinia herbicola* on *in vitro* spore germination and germ tube elongation of *Botrytis cinerea* and *Penicillium expansum.* BioControl 43, 97-106.

Bull, C.T., Wadsworth, M.L.K., Sorenson, K.N., Takemoto, J., Austin, R., Smilanick, J.L., 1998. Syringomycin E produced by biological agents controls green mold on lemons, Biological Control 12, 89–95.

Calvente, V., Benuzzi, D., de Tosetti, M.I.S., 1999. Antagonistic action of siderophores from *Rhodotorula glutinis* upon the postharvest pathogen *Penicillium expansum.* International Biodeterioration and Biodegradation 43, 167-172.

Castoria, R., De Curtis, F., Lima, G., De Cicco, V., 1997. *â*-1,3-glucanase activity of two saprophytic yeasts and possible mode of action as biocontrol agents against postharvest diseases. Postharvest Biology and Technology 12, 293-300.

Chan, Z., Thian, S., 2005. Interaction of antagonistic yeasts against postharvest pathogens of apple fruit and possible mode of action. Postharvest Biology and Technology 36, 215-223.

Chanchaichaovivat, A., Panijpan, B., Ruenwongsa, P., 2008. Putative mode of action of *Pichia guilliermondii* strain R13 in controlling chilli anthracnose after harvest. Biological control 47, 207-215.

Charlang, G., Ng B, Horowitz, N.H., Horowitz, R.M., 1981. Cellular and extracellular siderophores of *Aspergillus nidulans* and *Penicillium chrysogenum*. Molecular and Cellular Biology 1, 94-100.

De Boer, W., Gunnewiek, P.J.A.K., Lafeber, P., Jans, R., Spit, J.D., 1998. Woldendorp B.E. Anti-fungal properties of chitinolytic dune soil bacteria. Soil Biology Biochemistry 30, 193–203.

Di Francesco, A., Ugolini, L., Lazzeri, L., Mari, M., 2014. Production of volatile organic compounds by *Aureobasidium pullulans* as a potential mechanism of action against postharvest fruit pathogens. Biological Control 81, 8-14.

Dimkic, I., Živkoviæ,S., Beriæ, T., Ivanoviæ, Z., Gavriloviæ, V., Stankoviæ, S., Fira, D., 2013. Characterization and evaluation of two *Bacillus* strains, SS-12.6 and SS-13.1, as potential agents for the control of phytopathogenic bacteria and fungi. Biological Control 65, 312-321.

Droby, S., Chalutz, E., 1994. Mode of action of biological agents of postharvest diseases. C.L. Wilson, M.E. Wisniewski (Eds.), Biological Control of Postharvest Diseases – Theory and Practice, CRC Press, Boca Raton, pp. 63–75.

Droby, S., Wisniewski, M., Macarisin, D., Wilson, C., 2009. Twenty years of postharvest biocontrol research: Is it time for a new paradigm? Postharvest Biology and Technology 52, 137-145.

El-Ghaouth, A., Wilson, C.L., Wisniewski, M.E., 1998. Untrastructural and cytochemical aspects of biocontrol activity of *Candida saitona* in apple fruit. Phytopathology 88, 282–291.

El-Ghaouth, A., Wilson, C.L., Wisniewski, M.E., 2004. Biologically based alternatives to synthetic fungicides for the postharvest diseases of fruit and vegetables, S.A.M.H.

Naqvi (Ed.), Diseases of Fruit and Vegetables, vol. 2 Kluwer Academic Publishers, The Netherlands, pp. 511–535.

Fravel, D.R., 1988. Role of Antibiosis in the Biocontrol of Plant Diseases. Annual Review of Phytopathology 26, 75-91.

Gueldner, R.C., Reilly, C.C., Pusey, R.L., Costello, C.E., Arrendale, R.F., Cox, R.H., 1988. Himmelsbach D.S., Crumley F.G., Cutler H.G. Isolation and identification of iturins as antifungal peptides in biological control of peach brown rot with *Bacillus subtilis.* Journal of Agricultural and Food Chemestry 36, 366–370.

Ippolito, A., El Ghaouth, C.L., Wilson, M., Wisniewski, M., 2000. Control of postharvest decay of apple fruit by *Aureobasidium pullulans* and induction of defense responses. Postharvest Biology and Technology, 19: 265–272.

Janisiewicz, W.J., Yourman, L., Roitman, J., Mahoney, N., 1991. Postharvest control of blue mold and gray mold of apples and pears by dip treatment with pyrrolnitrin, a metabolite of *Pseudomonas cepacia.* Plant Disease 75, 490–494.

Janisiewicz, W.J., Tworkoski, T.J., Sharer, C., 2000. Characterizing the mechanism of biological control of postharvest diseases on fruit with a simple method to study competition for nutrients. Phytopathology 90, 1196–1200.

Jijakli M.H., Lepoivre, P., 1998. Characterization of an exo–1, 3-glucanase produced by *Pichia anomala* strain K, antagonist of *Botrytis cinerea* on apples. Phytopathology 88, 335–343.

Lima, G., Arru, S., De Curtis, F., Arras, G., 1999. Influence of antagonist, host fruit and pathogen on the biological control of postharvest fungal diseases by yeasts. Journal of Industrial Microbiology and Biotechnology 23, pp 223-229.

Liu, J., Wisniewski, M., Droby, S., Norelli, J., Hershkovitz, V., Tian, S., Farrell, R., 2012. Increase in antioxidant gene transcripts, stress tolerance and biocontrol efficacy of *Candida oleophila* following sublethal oxidative stress exposure. FEMS Microbiology Ecology 80, 578–590.

Liu, J., Sui, Y., Wisniewski, M., Droby, S., Liu, Y., 2013. Review: utilization of antagonistic yeasts to manage postharvest fungal diseases of fruit. International Journal of Food Microbiology 167, 153–1

Lu, L., Lu, H., Wu, C., Fang, W., Yu, C. Ye, C., Shi, Y., Yu, T., Zheng, X., 2013. *Rhodosporidium paludigenum* induces resistance and defense-related responses against *Penicillium digitatum* in citrus fruit. postharvest Biology and Technology 85, 196-202.

Mari, M., Martini, C., Spadoni, A., Rouissi, W., Bertolini, P., 2012. Biocontrol of apple postharvest decay by *Aureobasidium pullulans.* Postharvest Biology and Technology 73, 56-72.

Mari, M., Martini, C., Guidarelli, M., Neri, F., 2012. Postharvest biocontrol of *Monilinia laxa, Monilinia fructicola* and *Monilinia fructigena* on stone fruit by two *Aureobasidium pullulans* strains. Biological Control 60, 132-140.

Masih, E.I, Paul, B., 2002. Secretion of □-1,3-glucanases by the yeast *Pichia membranifaciens* and Its possible role in the biocontrol of *Botrytis cinerea* causing grey mold disease of the grapevine. Current Microbiology 44, 391-395.

Mercier, J., Jiménez, J.I., 2004. Control of fungal decay of apples and peaches by the biofumigant fungus *Muscodor albus.* Postharvest Biology and Technology 31, 1–8.

Miyazawa, M., Kimura, M., Yabe, Y., Tsukamoto, D., Sakamoto, M., Horibe, I., Okuno, Y., 2008. Use of Solid Phase Microextraction (SPME) for profiling the volatile metabolites produced by *Glomerella cingulata.* Journal of Oleo Science 57, 585-590.

Mo, Y.Y., Gross, D.C., 1991. Plant signal molecules activate the syrB gene, which is required for syringomycin production by *Pseudomonas syringae* pv. syringae. Journal of Bacteriology 173, 5784-5792.

Morat, S.U., Hung, R., Bennet, J.W., 2012. Fungal volatile organic compounds: A review with emphasis on their biotechnological potential. Fungal Biology Reviews 26, 76-83.

Nunes, C.A., 2012. Biological control of postharvest diseases of fruit, European Journal of Plant Pathology 133, 181-196.

Ongena, M., Jacques, P., 2008. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends in Microbiology 16, 115-125.

Parafati, L., Vitale, A, Restuccia, C, Cirvilleri, G., 2015. Biocontrol ability and action mechanism of food-isoalted yeast strains against *Botrytis cinerea* causing post-harvest bunch rot of table grape. Food Microbiology 47, 85-92.

Poppe, L., Vanhoutte, S., Höfte, M., 2003. Modes of Action of *Pantoea agglomerans* CPA-2, an Antagonist of Postharvest Pathogens on Fruits. European Journal of Plant Pathology 109, 963-973.

Pusey, P.L., Wilson, C.L. 1984. Postharvest biological control of stone fruit brown rot by Bacillus subtil*is.* Plant Disease 68, 753-756

Pusey, P.L., 1989. Use of *Bacillus subtilis* and related organisms as biofungicides. Pesticide Science 27, 133–140.

Raaska, L., Mattila-Sandholm, T., 1995. Effects of iron level on the antagonistic action of siderophores from non-pathogenic *Staphylococcus* spp.. Journal of Microbiology and Biotechnology 15, 480-485.

Rouissi, W., Ugolini, L., Martini, C., Lazzeri, L., Mari, M., 2013. Control of Postharvest Fungal Pathogens by Antifungal Compounds from*Penicillium expansum*. Journal of Food Protection 11, 1824-1993.

Sharma, R.R, Singh, D., Singh, R., 2009. Biological control of postharvest diseases ofd fruits and vegetables by microbial antagonists: A review, Biological Control 50, 205-221.

Spadaro, D., Gullino, M.L., 2004. State of the art and future prospects of the biological control of postharvest fruit diseses. International Journal of Food microbiology 91, 185-194.

Vero, S., Garmendia1, G., Gonzalez, N.B, Bentancur, O., Wisniewski, W., 2013. Evaluation of yeasts obtained from Antarctic soil samples as biocontrol agents for the management of postharvest diseases of apple (*Malus x domestica*). FEMS Yeast Research 13, 189-199

Xu, X., Tian, S., 2008. Salicylic acid alleviated pathogen-induced oxidative stress in harvested sweet cherry fruit. Postharvest Biology and Technology 49, 379-385.

Waewthongrak, W., Pisuchpen, S., Leelasuphakul, W., 2015. Effect of *Bacillus subtilis* and chitosan applications on green mold (*Penicilium digitatum* Sacc.) decay in citrus fruit. Postharvest Biology and Technology 99, 44-49.

Wisniewski, M., Biles, C., Droby, S., 1991. The use of yeast *Pichia guilliermondii* as a biocontrol agent: characterization of attachment to *Botrytis cinerea*. In: Wilson, C.L., Chalutz, E. (Eds.), Biological Control of Postharvest Diseases of Fruit and Vegetables. Proc. Workshop, US Department of Agriculture, pp. 167–183.

Yao, H., Tian, S., 2005. Effects of pre- and post-harvest application of salicylic acid or methyl jasmonate on inducing disease resistance of sweet cherry fruit in storage. Postharvest Biology and Technology 35, 253-262.

Yu, T., Wang, L., Yin, Y., Wang, Y., Zheng, X., 2008. Effect of chitin on the antagonistic activity of *Cryptococcus laurentii* against *Penicillium expansum* in pear fruit. International Journal of Food Microbiology 122, 44–48.

Zhou, Z.X., Wei, D.F., Guan, Y., Zheng, A.N., Zhong, J.J., 2011. Damage of *Escherichia coli* membrane by bactericidal agent polyhexamethylene guanidine hydrochloride: micrographic evidences. Journal of Applied Microbiology 108, 898-907.

Aims of the thesis

The objective of this research was to study the mechanisms of action of *Aureobasidium pullulans*, strains L1 and L8, used in biocontrol of some fruit postharvest fungal pathogens as *Botrytis cinerea, Monilinia* spp.*, Penicillium* spp.*, Colletotrichum acutatum.*

In particular was examined the role of volatile organic compounds (VOCs) with the following objectives:

   i) to evaluate the antifungal activity of VOCs produced by L1 and L8 strain against some postharvest pathogens by *in vitro* and *in vivo* tests;

   ii) to identify the compounds emitted by both *A. pullulans* strains with the solid-phase microextraction (SPME) - gas chromatographic technique;

   iii) to test the antifungal effect of pure compounds on target pathogens by the determination of EC_{50} values.

The *A. pullulans* capability to produce lytic enzymes (N-β-acetylglucosaminidase, endochitinase and β-1,3-glucanase) was examined by spectrophotomeric assays and qRT-PCR with the objectives to:

   i) evaluate the reduction of brown rot incidence on artificially infected peach fruits;

   ii) size up the inhibition of *Monilinia laxa* conidia germination and germ-tube elongation.

Competition aspects were also examined against *Penicillium expansum* and *Monilinia laxa* with the objectives to:

   i) determine the ability of L1 and L8 to compete for sugars and space in apple and peach fruit;

ii) examine the potential of siderophores in the *A. pullulans* biocontrol activity;

iii) by RT-qPCR analyze the gene expression of L-ornithine-N^5-monooxygenase gene, precursor of siderophore biosynthesis.

Also an objective of the research was analyzed in depth by a bibliographical study the modes of action of BCAs and their possible combined use with physical and chemical treatments.

<u>Chapter I</u>

Contents lists available at ScienceDirect

Biological Control

Production of volatile organic compounds by *Aureobasidium pullulans* as a potential mechanism of action against postharvest fruit pathogens

CrossMark

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HIGHLIGHTS

- *Aureobasidium* VOCs controlled five fruit postharvest pathogens.
- Significant pathogen inhibition was obtained in vitro and in vivo tests.
- VOCs produced in NYDA medium were identified by solid phase micro extraction (SPME).
- Authentic standard chemicals were used to determined EC_{50} values.

GRAPHICAL ABSTRACT
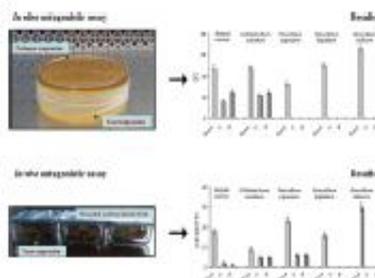
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ABSTRACT

Two *Aureobasidium pullulans* strains (L1 and L8), effective against some fruit postharvest pathogens were evaluated for VOCs production as a part of their modes of action towards five pathogens (*Botrytis cinerea*, *Colletotrichum acutatum*, *Penicillium expansum*, *Penicillium digitatum* and *Penicillium italicum*). The VOCs were assayed with a double petri dish assay against conidia germination of target pathogens. Results obtained showed that the VOCs generated by the antagonists inhibited significantly the conidia germination of all pathogens compared to the control. In particular, the conidia germination of all *Penicillium* was completely inhibited by VOCs produced by L1 and L8. In in vivo tests, apples and oranges were artificially inoculated with pathogen conidia and then biofumigated with VOCs emitted by both antagonists. The antagonistic treatment controlled significantly pathogen infection, confirming the results obtained in vitro tests. The best L1 and L8 VOCs activity was observed on apple inoculated with *B. cinerea* where the lesion diameter reduction observed was greater than the 88%. The compounds emitted by L1 and L8 strains were identified with the solid-phase microextraction (SPME)–gas chromatographic technique. Compounds as 2-phenyl, 1-butanol-3-methyl, 1-butanol-2-methyl and 1-propanol-2-methyl belonging to the group of alcohols were mainly produced for both strains, in the first 96 h of growth. These compounds were confirmed by comparison with standards. The pure compounds of VOCs cited above were used to determine the EC_{50} values for conidia germination of pathogens. The 1-propanol-2-methyl was the VOC least active against all tested fungi, with the EC_{50} values over $0.8\ \mu l\ ml^{-1}$, while the 2-phenethyl alcohol was the most active with EC_{50} values lower than $0.8\ \mu l\ ml^{-1}$, except for the *C. acutatum* ($1.97\ \mu l\ ml^{-1}$). The present study demonstrated, for the first time, that the production of VOCs could play an essential role in the antagonistic activity of two *A. pullulans* strains against five fruit postharvest pathogens.

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Production of volatile organic compounds by *Aureobasidium pullulans* as a potential mechanism of action against postharvest fruit pathogens

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Abstract

Two *Aureobasidium pullulans* strains (L1 and L8), effective against some fruit postharvest pathogens, were evaluated for production of volatile organic compounds (VOCs) as a part of their modes of action towards five pathogens (*Botrytis cinerea*, *Colletotrichum acutatum*, *Penicillium expansum*, *P. digitatum* and *P. italicum*). The VOCs were assayed with a double Petri dish assay against conidia germination of target pathogens and mycelium growth of *P. digitatum* and *P. italicum*. The results obtained showed that the VOCs generated by the antagonists significantly inhibited the conidia germination of all pathogens compared to the control. In particular, the conidia germination of all *Penicillium* was completely inhibited by VOCs produced by L1 and L8. In *in vivo* tests, apples and oranges were artificially inoculated with pathogen conidia and biofumigated with VOCs emitted by both antagonists. The treatment significantly controlled the infections, confirming the results obtained in *in vitro* tests. The best activity was observed on apple inoculated with *B. cinerea* and exposed to

VOCs emitted by L1 and L8; in fact, in treated fruits the lesion diameter reduction was over 88%. Furthermore, the compounds emitted by L1 and L8 strains were identified with the solid-phase microextraction (SPME)- gas chromatographic technique. Compounds such as 2-phenyl, 1-butanol-3-methyl, 1-butanol-2-methyl and 1-propanol-2-methyl belonging to the group of alcohols were mainly produced for both strains, in the first 96 h of growth. These compounds were confirmed by comparison with standards. The pure compounds of VOCs cited above were used to determine the EC_{50} values for conidia germination of pathogens. 1-propanol-2-methyl was the VOC least active against all tested fungi, with EC_{50} values over 0.8 µl ml^{-1}, while 2-phenethyl alcohol was the most active with EC_{50} values lower than 0.8 µl ml^{-1}, except for the *C. acutatum* (1.97 µl ml^{-1}). The present study demonstrated, for the first time, that the production of VOCs could play an essential role in the antagonistic activity of two *A. pullulans* strains against five fruit postharvest pathogens.

1. Introduction

Fruits are highly perishable products and after harvest they undergo considerable quantitative losses. Since they are rich in water and nutrients, they are an ideal substrate for the development of pathogenic microorganisms during storage, which are able to cause extensive losses, such as *Penicillium expansum, Botrytis cinerea* and *Colletotrichum acutatum* on apple (Mari et al., 2012b), *P. digitatum* and *P. italicum* on citrus (Lahlali *et al.*, 2004). Waste during the postharvest phase has an important

economic cost, which is greater the closer it is to the fruit sale, considering the added value of produce after harvest, storage, transport and commercialization. In addition, some postharvest pathogens represent a serious concern to human health since they produce toxic compounds like *P. expansum,* a producer of patulin with cytotoxic, genotoxic and immunosuppressive activity (Baert *et al*., 2007). In the citrus industry, *P. digitatum* and *P. italicum* are responsible for considerable fruit rot (Macarisin *et al.*, 2007) since harvested fruits are very susceptible to these pathogens, particularly during storage, transport and marketing (Eckert and Eaks, 1989). Imazalil, thiabendazole, and sodium ortho-phenyl phenate are old postharvest fungicides, generally used as the first line of defense against *P. digitatum* and *P. italicum*. However, because of their intense use, resistant isolates developed and became widespread within packinghouses (Kinay *et al.,* 2007). Recently, new pathogens, such as *C. acutatum* that appeared in Italy on apple (Mari *et al.,* 2012b), have increased concern about fruit postharvest losses.

In order to find alternative means to control these biotic diseases, different strategies were investigated and the use of biological control agents (BCAs) gained considerable attention and promising results. Among the BCAs used to control apple postharvest pathogens, *Aureobasidium pullulans* (Zhang *et al*. 2010; Mari *et al.,* 2012a), *Metschnikowia fructicola* (Spadaro *et al.,* 2013) and *M. pulcherrima* (Janisiewicz *et al.,* 2003) showed a high degree of efficacy. Two *A. pullulans* strains (L1 and L8), isolated from the carposphere of peach fruits and tested against *Monilinia laxa*, *M. fructicola* and *M. fructigena* (Mari *et al*. 2012a), reduced the incidence of brown rot by more than 89%. In addition, in preliminary *in vitro* trials conducted to evaluate the mechanisms of action of antagonists, the antifungal effects on pathogen mycelium growth of *B. cinerea*, *C. acutatum* and *P. expansum* were attributed to the production of volatile organic

compounds (VOCs) by L1 and L8 strains (Mari *et al.,* 2012b). In the past, studies on the mode of action of BCAs revealed that antibiotic production (Bull *et al.*, 1998), competition for nutrients and space (Bencheqroun *et al.*, 2007) and induced host resistance (El-Ghaouth *et al.,* 1998) are the main ones. Regarding antibiotics, these are divided into non-volatile antibiotic compounds (Bull *et al.*, 1998; Vinale *et al.,* 2006) and volatile antibiotic compounds (Fialho *et al.*, 2011; Huang *et al.*, 2012) with a different role (partial or essential) in their antimicrobial activity. Some VOCs produced by antagonists such as *Bacillus subtilis* (Chen *et al.,* 2008), *Streptomyces* spp. (Li *et al.,* 2011), *Candida intermedia* (Huang *et al.,* 2011) and *Muscodor albus* (Mercier and Jimenez, 2004) could appear essential in suppressing the spore germination and the hyphal growth of the main fruit pathogens, while others, produced by *A. pullulans* (Mari *et al.,* 2012b) and *Bacillus* spp. (Solanki *et al.*, 2013), showed a complementary role in the inhibition of the same and other plant pathogens. VOCs are chemicals with low molecular weights (<300Da), high vapour pressure and low water solubility; they are terpenoids, phenylpropanoids, fatty acid derivates, etc., and are mostly identified by GC-MS analysis (Chen *et al.,* 2008; Huang *et al.,* 2011). To collect VOCs, SPME has in recent years become a popular method, as it is a simple and rapid technique used for sampling volatile compounds at low concentration in headspace (HS) analysis. SPME has in fact been successfully used, coupled with GC-MS, to characterize VOC production profile by fungi and yeasts (Miyazawa *et al.,* 2008; Mauriello *et al.,* 2009; Fialho *et al.,* 2010; Kluger *et al.,* 2013), thanks to its non-invasive feature which makes it possible to monitor volatile metabolite production over time, thus achieving a direct profiling of living microorganism culture (Stoppacher *et al.*, 2010).

There are few data in the literature concerning the inhibition of plant pathogens by VOCs produced by yeasts (Fialho *et al.,* 2010; Huang *et al.*, 2011; 2012). From these results, the assayed yeasts seem to act as antagonists primarily by VOC production. In contrast, VOCs emitted by *A. pullulans* showed a capacity to reduce the mycelium growth of three postharvest pathogens to a lower level than that observed in washed cell trials (Mari *et al.*, 2012b), showing that VOC production could be a secondary aspect of antagonistic activity of *A. pullulans.* However, the identification of these compounds and the study of the dynamic process of production could improve BCAs efficacy.

The objectives of this study were: (i) to evaluate the antifungal activity of VOCs produced by L1 and L8 against some postharvest pathogens by *in vitro* and *in vivo* tests; (ii) to identify the compounds emitted by both *A. pullulans* strains with the solid-phase microextraction (SPME)- gas chromatographic technique and (iii) to test the antifungal effect of pure compounds on target pathogens by the determination of EC_{50} values.

2. Materials and methods

2.1. Fruit

'Golden Delicious' apples (*Malus domestica* L. Borkh.) were obtained from an orchard located in Forlì (North Italy) and used shortly after harvest. 'Navel' oranges (*Citrus sinensis* L. Osbeck) were obtained from an orchard located in Sicily (South Italy); on the same day as the harvest, the fruits were shipped by a refrigerated truck (5°C and 85-90 % relative humidity), arrived in the lab in a couple of days and were immediately processed.

2.2. Pathogens

P. expansum was isolated from rotted stored apple and grown on potato dextrose agar (PDA, Sigma, St. Louis, MO, USA) for 7 days at 20°C. *B. cinerea,* derived from decayed apple, was grown on oat meal agar (60 g of oat meal, 10 g of sodium nitrate, 30g of sucrose, 12 g of agar per 1000 ml of distilled water), and incubated at 25°C for 10 days. *C. acutatum* was isolated from rotted apple (Mari *et al*., 2012a) and grown on PDA at 20°C for 10 days. *P. italicum* and *P. digitatum* were isolated from oranges showing evident symptoms of blue and green mould respectively and were grown on malt extract agar (MEA, Oxoid, Cambridge, UK) for 7 days at 20°C. Conidia from the strains were collected and suspended in sterile distilled water containing 0.05% (v/v) Tween 80. The suspension concentration was quantified with a hematocytometer and adjusted with sterile distilled water relating to trials.

2.3. Antagonists

A. pullulans strains L1 and L8 were previously identified (Mari *et al*., 2012a) 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 grown on NYDA at 25°C, and the yeast cells were collected in sterile distilled water containing 0.05% (v/v) Tween 80 and adjusted to a final concentration of 10^8 CFU ml^{-1}.

2.4. In vitro antagonistic assay

The VOCs produced by L1 and L8 strains were tested against the conidia germination of pathogens cited above by a double Petri dish assay as reported by Rouissi et al.

(2013) with modifications. For this purpose, NYDA plates were inoculated by spreading 100 µl of antagonist cell suspension (10^8 CFU ml^{-1}). The lid of the plate was replaced, after 48 h of incubation at 25°C, by a base plate of MEA inoculated with 100 µl of conidia suspension (10^3 conidia ml^{-1}) of each pathogen. In addition, using the same dual culture technique, the activity of VOCs was assayed on the mycelium growth of *P. digitatum* and *P. italicum*. For this purpose, the lid of plate previously inoculated with yeasts as described above was removed and replaced with a base plate of MEA inoculated with a mycelium 6 mm agar-plug derived from the margin of a growing fungal culture of *P. digitatum* and *P. italicum*. The two base plates were sealed immediately with a double layer of parafilm and incubated at 25°C for 3-7days depending pathogens. The control was represented by plates inoculated only with the pathogens. The sample unit was represented by ten plates (replicates) for each pathogen and antagonist interaction. The experiment was repeated once. The percentage of inhibition of conidia germination was calculated using the formula: 100 – [(treatment value/control value) x 100] (Gamliel *et al*., 1989).

2.5. In vivo antagonistic assay

Fruits were wounded (3 x 3 x 3 mm) three times with a sterile needle and inoculated with 20 µl of suspension 10^5 conidia ml^{-1} of *P. expansum*, *B. cinerea* and *C. acutatum* for apples, and 10^4 conidia ml^{-1} of *P. digitatum* and *P. italicum* for oranges. Subsequently they were placed in sterile glass boxes (24 x 18 x 8 cm. *L* x *W* x *H*) containing at the bottom a thin layer of NYDA (250 ml) inoculated two days before with 500 µl of an L1 and L8 suspension of 10^8 CFU ml^{-1} and incubated at 25°C. Fruits were separated from the substrate with a sterile grid to avoid any physical contact

between antagonists and fruit surface. The boxes were closed with a plastic lid and sealed immediately with a Parafilm double layer. The control consisted of inoculated fruits placed in boxes without yeast suspensions. The boxes containing fruits inoculated with *P. expansum, P. digitatum*, *P. italicum* were kept at 20°C, while boxes containing fruits inoculated with *B. cinerea* and *C. acutatum* were kept at 25°C. The lesion diameters were measured after 7 days of incubation. The sample unit was represented by three boxes (containing 5 fruits with 3 wounds each) per each pathogen and antagonist. The experiment was performed three times.

2.6. VOC analysis

Yeast VOC composition was qualitatively evaluated by headspace (HS)-SPME coupled with gas chromatography-mass spectrometry (GC-MS). For selection of the appropriate fibre coating, the following materials were tested and compared at 20 min exposure time: 100 µm and 7 µm polydimethylsiloxane (PDMS), 50/30 µm divinylbenzene (DVB)/carboxene (CAR)/PDMS, 85 µm polyacrylate (PA), 75 µm CAR/PDMS (Supleco, Bellefonte, PA, USA). For all subsequent experiments, an SPME fiber with 50/30 µm DVB/CAR/PDMS coating was used. Fibres were conditioned prior to analyses according to the manufacturer's recommendations. Headspace sampling was performed in Petri dishes as described by Roussi *et al*. (2013) and the fibre was exposed to the gas phase for 5 min at room temperature. Analyses were performed between 48 and 72h of yeast growth, after Petri dish opening at 48h until pathogen growth evaluation. Trapped compounds were thermally desorbed into the GC injection port at 250°C for 2 min, and separated in a Bruker GC 451 gas cromatograph equipped with an HP-5 fused silica capillary column (30m by 0.25 mm inside diameter; 0.25 µm film

thickness, J%W Scientific Inc, Folsom, CA) connected to a Bruker Scion SQ Premium quadrupole mass detector (Bruker Daltonics, Macerata, Italy). The oven temperature was set at 40°C for 3 min and then programmed to rise from 40 to 90°C at 10°C/min, from 90°C to 180°C at 5°C/min and from 180°C to 260°C at 20°C/min. The transfer line was heated at 250°C and the ion source at 220°C. Helium carrier gas had a flow of 1 ml min^{-1}. The splitless injection mode was used. The mass spectrometer was operated in electron impact mode at 70 eV, scanning the range of 35/500 m/z, in a full scan acquisition mode. The identification of VOCs was achieved by comparing the GC retention times and mass spectra with those of pure standard compounds. All mass spectra were also compared with the data system library (NIST 11 MS Library). Blank sample analyses (growth medium not inoculated with antagonists) were performed under the same conditions in order to exclude interfering substances. All measurements were made with three replicates, each replicate representing the analysis of a different Petri dish. GC peak area data were used to estimate the relative abundance (relative peak area, RA) of each volatile compound.

2.7. Effect of pure volatile organic compound on conidial germination of fungal pathogens

Pure standards of four VOCs, (3-methyl-1-butanol; 2-methyl-1-btanol; 2-methyl-1-propanol and 2-phenylethanol) produced by L1 and L8 and identified by HS-GC-MS analysis, were obtained from Sigma Aldrich (St. Louis, MO) and tested in order determine their effect against conidia germination of target pathogens. For this purpose, different aliquots of pure compounds: 25, 50 and 100 µl were placed with a microsyringe on a paper filter (Whatmann No.1, 90 mm diameter) and positioned inside the cover of an MEA dish previously spread with 100 µl of a conidia suspension (10^3

conidia ml^{-1}) of the five pathogens. The aliquots of pure compounds introduced in the Petri dishes corresponded to 2.25, 1.12 and 0.56 µl ml^{-1} headspace. The dishes were quickly closed and sealed with Parafilm and incubated at 20°C and 25°C, depending on the pathogen, as described above. The activity of each pure compound against *P. expansum, P. digitatum* and *P. italicum* was evaluated after 2 days of incubation and against *C. acutatum*, *B. cinerea* after one day. In the control test, pure compounds were substituted by equivalent amounts of distilled water. The sample unit was represented by three plates for each VOC dose and pathogen. The experiment was performed twice. Conidia germination was expressed as number of colony forming units (CFU). EC$_{50}$ values were calculated as the headspace concentrations (µl ml^{-1}) that inhibit conidia germination by 50% compared with the control.

2.8. Data analysis

Data regarding infected fruits were subjected to a one-way analysis of variance (ANOVA) using the statistical package Statistica for Windows (Statsoft Inc.). Separation of means was performed using the least significant difference (LDS) test, at *P*<0.05. Data are reported as mean values ± standard error (SE). The EC$_{50}$ of each substance was calculated using the probit analysis applied to the percentage of conidia germination (Lesaffre and Molenberghs, 1991). All experiments were carried out in a completely randomized design.

3. Results

3.1. In vitro antagonistic assay

In the double Petri dish assay, there was no contact between the two cultures, L1 or L8 strains and pathogens, so the antifungal effect on conidia germination and mycelium growth could be attributed to VOCs produced by the BCAs used in the present assay. The VOCs generated by the antagonists significantly inhibited the conidia germination of all pathogens compared to the control but with different efficacy levels (Table 1). The conidia germination of *Penicillium* spp. was completely inhibited by VOCs produced by L1 and L8 (100% of inhibition), while the control of the other two pathogens, *B. cinerea* and *C. acutatum,* ranged between 65% and 54% of efficacy respectively for L1, and 49% and 47% respectively for L8. The L1 strain appeared more effective than L8, although there were no significant differences between the two BCAs. Data from *in vitro* trials showed that *Penicillium* spp. was the most sensitive tested fungus to VOCs emitted by L1 and L8 strains, since its conidia germination was completely inhibited. Lower inhibition was found against *B. cinerea* (65% - L1; 47.4% - L8) and *C. acutatum* (54.6 - L1; 49.5% - L8).

With respect to mycelial growth inhibition of *P. digitatum* and *P. italicum*, after 5 d at 25°C, VOCs emitted by L1 and L8 significantly reduced the colony diameter of the two pathogens compared to the control. The reduction was higher in the case of *P. italicum* (48% for L1 and 45% for L8) than *P. digitatum* (22% for L1 and 23% for L8) (Table 2).

3.2. In vivo antagonistic assay

In vivo assay results are shown in Table 3. The biofumigation of apple with L1 and L8 VOCs was effective in controlling all pathogens tested, confirming the results obtained

in vitro tests. The best antifungal activity was observed on fruits inoculated with *B. cinerea* and exposed to L1 and L8 VOCs with a reduction of 88.9% and 94.4% respectively. The VOCs emitted by both strains reduced the incidence of blue mould by 73.9%, and that of bitter pit, caused by *C. acutatum*, by 44.4%. On orange, *P. italicum* was more aggressive than *P. digitatum*, producing wider lesion diameters (30 mm) in untreated fruits than those produced by *P. digitatum* (16 mm). The exposure of fruits for 7 days to VOCs emitted by L1 and L8 strains inhibited pathogen activity almost completely, showing an efficacy of more than 96%. L1 and L8 VOCs showed a similar activity, without significant difference between them as observed in *in vitro* tests.

3.3 SPME GC-MS analyses of L1 and L8 VOCs

The headspace profile of L1 and L8 VOCs was investigated using SPME combined with GC-MS technique. Preliminary evaluations were performed by exposing different types of SPME fibres to VOCs produced by yeast in Petri dishes for 20 min: absorbent film coatings at different polarity and thickness, 100 μm and 7 μm PDMS and 75 μm PA, and adsorbent porous coatings with different size and distribution of particles, 50/30 μm DVB/CAR/PDMS and 85 μm PA, were tested. The fibre which made it possible to extract the greatest number of compounds was the dual coated fibre DVB/CAR/PDMS (data not shown), which adsorbs molecules at low concentrations, with a large range of MW, due to its physical-chemical characteristics suitable for complex mixtures. Furthermore, the time of analysis was reduced, as a shorter extraction time (5 min) was established to be able to equally detect volatiles, at the same time avoiding VOC competition for adsorption to the fibre (Tuduri *et al.,* 2001).

L1 and L8 *A. pullulans* VOC production was then followed by DVB/CAR/PDMS-GC-MS analysis from 48 to 72h of growth after Petri lid opening (see M&M). Maximum relative peak areas (RA) of detected and tentatively identified compounds are shown in Table 4. Compounds belonging to the group of alcohols were mainly produced for both L1 and L8, with 2-phenylethanol with the highest RA, followed by 3-methyl-1-butanol, 2-methyl-1-butanol and 2-methyl-1-propanol alcohols. These compounds were confirmed by comparison with standards and used for subsequent experiments. Quantitative considerations could not be extrapolated as peak areas depend not only on compound concentration but also on the fibre affinity and the detector sensitivity to the different analytes. Regarding the kinetics of VOC production, this was found to be highly variable. The maximum production of VOCs was observed from 54h to 74h of yeast growth, after which all RA of compounds started to decrease until they were no longer detectable.

3.4 Assay of pure VOCs identified in L1 and L8

Pure VOCs, previously identified by SPME GC-MS, were tested in *in vitro* trials against the conidia germination of the target pathogens: *B. cinerea, C. acutatum*, *P. expansum, P. italicum* and *P. digitatum* (Table 5) in order to determine the EC_{50} values. The values ranged between 0.48 μl ml^{-1} of 1-butanol-2-methyl against *P. digitatum* and 1.97 μl ml^{-1} of 2-phenethyl alcohol against *C. acutatum*. 1-propanol-2-methyl was the VOC least active against all tested pathogens, with EC_{50} values over 0.8 μl ml^{-1}, while 2-phenethyl alcohol was the most active with EC_{50} values lower than 0.8 μl ml^{-1}, expect for *C. acutatum* (1.97 μl ml^{-1}). In addition, *C. acutatum* appeared the most resistant pathogen to VOCs produced by tested BCAs; in fact, all the EC_{50} values were over 1.25

µl ml^{-1}, while *B. cinerea* was the most sensitive with EC$_{50}$ values below 1 µl ml^{-1}, except for 1-butanol-2-methyl (1.38 µl ml^{-1}).

4. Discussion

Two *A. pullulans* strains (L1 and L8), effective against some fruit postharvest pathogens (Mari et *al.*, 2012a,b), were evaluated for VOC production as part of their modes of action against *B. cinerea, C. acutatum, P. expansum. P. digitatum* and *P. italicum*. In the double Petri dish assays and in *in vivo* trials, both antagonists showed a significant reduction of conidia germination and disease severity respectively. Since in the trials, performed under air-tight conditions, there was no contact between antagonist and pathogen, an antifungal activity of VOCs, emitted by BCAs, was persumed. Similarly, in previous works, other antagonists such as *C. intermedia* (Huang *et al.,* 2011) and *Sporidiobulus pararoseus* (Huang *et al.*, 2012) inhibited the *B. cinerea* growth of strawberries by the emission of volatile compounds. Moreover, *B. subtilis* strain JA produced volatiles inhibiting spore germination and tube elongation of *B. cinerea* in *in vitro* trials (Chen *et al.,* 2008). At the same time, the fumigation with *M. albus* gave excellent control of blue mould and grey mould of apples as well as brown rot of peaches (Mercier and Jimenez, 2004) and green mould of citrus (Mercier and Smilanick, 2005), showing that VOCs could represent a new frontier in the biological control of fruit diseases (Morath *et al.,* 2012).

Data from *in vitro* trials showed that conidia of *Penicillium* spp. were the most sensitive to volatile antifungal compounds emitted by L1 and L8 strains, since their germination was completely controlled. On the other hand, the mycelium growth of *P. digitatum* and *P. italicum* was only partially inhibited, confirming previous results obtained in similar

trials with the same antagonists against *P. expansum* (Mari *et al.,* 2012b). In contrast, VOCs produced by a *P. expansum* strain (R82) were the most active against *B. cinerea*, *C. acutatum* and *M. laxa,* completely inhibiting their conidia germination, while it reduced the conidia germination of another *P. expansum* strain by only 18.1% (Rouissi *et al.*, 2013). The poor antifungal activity of *P. expansum* strain (R82) against another *P. expansum* strain could be attributed to a low competition between strains belonging to the same species, although preliminary investigations on VOCs produced by L1 and L8 against themselves showed a complete inhibition of antagonists growth (data not reported). The growth inhibition observed *in vitro* was partially confirmed in *in vivo* trials. Orange fruit inoculated with *P. digitatum* and *P. italicum* and exposed to VOCs produced by L1 and L8 strains showed an almost complete control of green and blue mould respectively (Table 3). Similarly, *P. digitatum* of lemons was controlled by a fumigation of *M. albus* for 48 or 72 hr (Mercier and Smilanick, 2005). Apples artificially infected by *P. expansum* and treated with VOCs of L1 and L8 strains showed a significant reduction (-73.9%) of lesion diameters with respect to untreated fruit; however, the antifungal activity was lower than that observed in apples treated with *M. albus* by Mercier and Jimenez (2004) in similar conditions. The efficacy of *M. albus* is mainly derived from volatile compound production while in the case of *A. pullulans* strains, L1 and L8, the emission of VOCs could be considered as a contribution limited to biological control of pathogens.

Each antagonist can produce a high number of VOCs: *C. intermedia* (strain C410) released 49 VOCs after one day of incubation (Huang *et al.*, 2011), while a total of 39 volatile compounds were determined in cultures of *S. pararoseus* (strain YCXT3) (Huang et al. 2012).

In order to understand the nature of VOCs produced by L1 and L8 antagonists on pathogen development, it was necessary to identify the components of the atmosphere produced by the two strains. Thus, VOCs were preliminary identified by the SPME-GC-MS technique using a DVB/CAR/PDMS fibre, which showed, in our conditions, a good extracting performance with small polar volatiles. VOCs were detected starting from 48 h of yeast incubation. Both L1 and L8 strains produced the same compounds but with different peak areas and especially at different times of growth (not shown). In addition, the relative percentage of compounds detected in the headspace analyses varied in the course of time depending on the current physiological state of the yeast. The principal group of L1 and L8 *A. pullulans* strain metabolites detected was formed by alcohols. The main compounds such as 1 butanol-2-methyl, 1-butanol-3-methyl, 1-propanol-2-methyl, phenethyl alcohol were previously observed for other fungi like *M. albus* (Strobel *et al.,* 2001; Mercier and Jimenez, 2004), *Trichoderma atroviride* (Stoppacher *et al.,* 2010), *P. expansum* (Rouissi *et al.,* 2013), *Glomerella cingulata* (Miyazawa *et al.*, 2008), yeasts like *Saccharomyces cerevisiae* (Fialho *et al.*, 2010), *S. pararoseus* (Huang *et al.*, 2012), *C. intermedia* (Huang *et al.,* 2011) and endophitic strains of *Phaeosphaeria nodorum* (Pimenta *et al.,* 2012). However, the methodology applied to collect and detect VOCs can strongly influence the results and often hinder the comparison between different studies. Huang *et al.* (2012), for instance, evaluated VOC production by the yeast *S. pararoseus* and their inhibition of *B. cinerea* on strawberries at 20°C. They detected a large number of compounds by SMPE GC-MS, even if the analysis was carried out at 40°C, thus at temperature condition quite different from inhibition experiments, maybe favouring the presence of compounds in vapour phase not responsible for the observed effect on fruit. Pimenta et al. (2012) identified five

inhibitory volatile compounds (2- propenenitrile, 2-propyn-1-ol, 3-methyl-1-butanol, acetic acid, ethyl acetate) produced by endophitic strains of *P. nodorum* able to alter *M. fructicola* hyphae, and the isolate 30-1B, the most inhibitory, produced only two volatile compounds (ethyl acetate and acetic acid). Moreover, VOC production varies among species and it is highly influenced by different parameters, like microorganism growth stage, culture mode, temperature, air exchange, media composition, etc. VOC quantity and quality, for instance, could be enhanced by nutrient addition to culture media (amino acids and sugars), not only as a consequence of microorganism increased biomass but also thanks to the availability of new media constituents essential to produce secondary metabolites like VOCs (Fielder *et al*., 2001; Bruce *et al*., 2004, Martins *et al*., 2010).

Alcohols were connected to microorganism metabolism, specifically called Microbial Volatile Organic Compounds (MVOCs). Alcohols, aldehydes, amines, ketones, terpenes, aromatic and chlorinated hydrocarbons and sulphur–based compounds are the principal products of the primary (break down food) and secondary (competition for resources) microorganism metabolism (Santo-Pietro, 2006). 1-butanol-3-methyl and 1-butanol-2-methyl alcohols, for instance, derive from leucine and isoleucine metabolism in bacteria and have been correlated with aroma production in meat products (Montel *et al.,* 1998). The antimicrobial properties of alcohols are widely documented, justifying their use as disinfectants or preservatives. In general, the activity of alcohols appears more related to their physico-chemicals properties than to specific receptors. For instance, lipophilic compounds such as 1-butanol, 2-methyl and 1 butanol, 3-methyl with a high affinity for plasma membrane show a higher toxicity than ethanol, a less

lipophilic compound, toxic for microorganisms only at high concentrations (Weber and Bont, 1996).

Few works reported the EC_{50} values of pure VOCs against fungal pathogens. *Sclerotinia sclerotiorum* was completely inhibited by 2-methyl-1-butanol and 3-methyl-1 butanol at 0.8 µl $^{-1}$ (Fialho *et al.,* 2011). In our experiments, the two compounds showed values ranging between 0.48 µl ml^{-1} (2-methyl-1-butanol against *P. digitatum*) and 1.38 µl ml^{-1} (2-methyl-1-butanol against *B. cinerea*). However, VOCs are produced by microorganisms at very low concentrations (Rouissi *et al.*, 2013) and their effect is presumed to be due to synergic or additive action and not to a single component activity (Mercier *et al.,* 2004; Strobel *et al.,* 2001). A mixture of commercial available compounds, identified as *M. albus* VOCs, was tested against different plant pathogenic fungi and bacteria by Strobel *et al.* (2001) and IC_{50} values ranging from 0.08 to 1.13 µl mL^{-1} were found. Nevertheless, the authors did not find any correspondence between *M. albus* atmosphere and artificial test atmosphere, with inhibitory and lethal effects respectively, on some pathogens, and different treatment modes and/or absence in the artificial mixture of not identified critical compounds were suggested as possible reasons. Furthermore, VOC proportions in the artificial test mixtures were obtained taking into account the compound relative peak area from GC-MS analyses that, especially using the SPME technique, may not reflect the real situation, as a calibration with standards would have been necessary to quantify real VOC atmosphere concentration.

The present study demonstrated, for the first time, that the production of VOCs could play an essential role in the antagonistic activity of two *A. pullulans* L1 and L8 strains against five fruit postharvest pathogens. However, the variety of volatiles emitted by

BCAs as well as the proportion of the various components of the headspace produced could also be strongly influenced by the growth substrates relating to their capacity of assimilating and fermenting carbohydrates (Nout and Barlett, 1998). More investigations are necessary to understand the exact contribution of L1 and L8 VOCs in the control of tested fungal pathogens, in order to improve the efficacy of antagonists.

References

Baert, K., Devlieghere, F., Flyps, H., Oosterlinck, M., Ahmed, M.M., Rajkovic, A., Verlinden, B., Nicolai, B., Debevere, J., De Meulenaer, B., 2007. Influence of storage conditions of apples on growth and patulin production by *Penicillium expansum*. Int. J. Food Microbiol. 119, 170-181.

Bencheqroun, S.K, Bajji Massart, S., Labhilili, M., El-jafari, S., Jijakli, M.H., 2007. In *vitro* and in *situ* study of postharvest apple blue mold biocontrol by *Aureobasidium pullulans*: evidence for the involvement of competition for nutrients. Postharvest Biol. Tech. 46, 128–135.

Bruce, A., Verrall, S., Hackett, C.A., Wheatley, R.E., 2004. Identification of volatile organic compounds (VOCs) from bacteria and yeast causing growth inhibition of sapsatain fungi. Holzforschung 58, 193-198.

Bull, C.T., Wadsworth, M.L., Sorensen, K.N., 1998. Syringomycin E produced by biological control agents controls green mold on lemons. Biol. Control 12, 89–95.

Chen, H., Xiao, X., Wang, J., Wu, L.J., Zheng, Z.M., Yu, Z.L., 2008. Antagonistic effects of volatile genereated by *Bacillus subtilis* on spore germination and hyphal growth of the plant pathogen, *Botrytis cinerea*. Biot. Lett. 30, 919-923.

Eckert, J.W., Eaks, I.L., 1989. Postharvest disorders and diseases of citrus fruits, in: Reuther, W., Calavan, E.C., Carman, G.E. (Eds.), The Citrus Industry, University of California Press, Berkeley, pp. 179–260.

El-Ghaouth, A., Wilson, C.L., Wisniewski, M., 1998. Ultrastructural and cytochemical aspects of the biological control of *Botrytis cinerea* by *Candida saitoana* in apple fruit. Phytopathology 88, 282–291.

Fialho, M.B., Toffano, L., Pedroso, M. P., Augusto, F., Pascholati S.F., 2010. Volatile organic compounds produced by *Saccharomyces cerevisiae* inhibit the in vitro development of *Guignardia citricarpa,* the casual agent of citrus black spot. World J. Microb. Biot. 26, 925-932.

Fialho, M.B., Duarte de Moraes, M.H., Tremocorti, A.R., Pascholati, S.F., 2011. Potential of antimicrobial voaltile organic compounds to control *Sclerotinia sclerotiorum* in bean seeds. Pesq. Agropec. Bras. 46, 137-142.

Fielder, K., Schütz, E., Geh, S., 2001. Detection of microbial volatile organic compounds (MVOCs) produced by moulds on various materials. Int. J. Hyg. Environ. Health 204, 111-121.

Gamliel, A., Katan, J., Cheh, Y., Grinstein, A., 1989. Solarization for the recycling of container media. Acta Hort. 255, 181-188.

Huang , R., Che, H.J., Zhang, J., Yang, L., Jiang, D.H., Li, G.Q., 2012. Evaluation of *Sporidiobolus pararoseus* strain YCXT3 as biocontrol agent of *Botrytis cinerea* on post-harvest strawberry fruits. Biol. Control 62, 53-63.

Huang, R, Li, G.Q., Zhang, J., Yang, L., Che, H.J, Jiang, D.H., Huang, H.C., 2011. Control of postharvest *Botrytis* fruit rot of strawberry by volatile organic compounds of *Candida intermedia.* Phytopathology 101, 859-869.

Janisiewicz, W.J., Leverentz, B., Conway, W.S., Saftner, R.A., Reed, A.N., Camp, M.J., 2003. Control of bitter rot and blue mold of apples by integrating heat and antagonist treatments on 1-MCP treated fruit stored under controlled atmosphere conditions. Postharvest Biol. Tech. 29, 129–143.

Kinay, P., Mansour, M.F., Gabler, F.M., Margosan, D.A., Smilanick, J.L., 2007. Characterization of fungicide-resistant isolates of *Penicillium digitatum* collected in California. Crop Prot. 26, 647-656.

Kluger, B., Zeilinger, S., Wiesenberger, G., Schöfbeck, D., Schuhmacher, R., 2013. Detection and identification of fungal microbial volatile organic compounds by HS-SPME-GC-MS. V.K. Gupta et al. (eds), Laboratory protocols in fungal biology:

current methods in fungal biology, Fungal Biology. DOI 10.1007/978-1-4614-2356-0_42. Springer New York.

Lahlali, R., Serrhini, M.N., Jijakli, M.H., 2004. Efficacy assessment of *Candida oleophila* (strain O) and *Pichia anomala* (strain K) against major post-harvest diseases of citrus fruits in Morocco. Commun. Agric., Appl. Biol. Sci. 69, 601-609.

Lesaffre, E., Molenberghs, G., 1991. Multivariate probit analysis: A neglected procedure in medical statistics. Stat. Med. 9, 1391-1403.

Li, F, Jiang, P., Zheng, H., Wang, S., Zhao, G., Qin, S., Liu, Z., 2011. Draft genome sequence of the marine bacterium *Streptomyces griseoaurantiacus* M045, which produces novel manumycin-type antibiotics with a pABA core component. J. Bacteriol. 193, 3417–3418.

Macarisin, D., Cohen, L., Eick, A., Rafael, G., Belausov, E., Wisniewski, M, Droby, S., 2007. *Penicillium digitatum* suppresses production of hydrogen peroxide in host tissue during infection of citrus fruit. Phytopathology 97, 1491-1500.

Mari, M., Martini, C., Guidarelli, M., Neri, F., 2012a. Postharvest biocontrol of *Monilinia laxa*, *Monilinia fructicola* and *Monilinia fructigena* on stone fruit by two *Aureobasidium pullulans* strains. Biol. Control 60, 132-140.

Mari, M., Martini, C., Spadoni, A., Roussi, W., Bertolini, P., 2012b. Biocontrol of apple postharvest decay by *Aureobasidium pullulans*. Postharvest Biol. Tech. 73, 56-62.

Martins, M., Henriques, M., Azeredo, J., Rocha, S.M., Coimbra, M.A., Oliveira, R., 2010. *Candida* species extracellular alchols: production and effect in sessile cells. J. Basic Microbiol. 50, S89-S97.

Mauriello, G., Capece, A., DiAuria, M., Garde-Cèrdan, T., Romano, P., 2009. SPME-GC method as a tool to differenziate VOC profiles in *Saccharomyces cerevisiae* wine yeasts. J. Food Microbiol. 26, 246-252.

Mercier, J., Jimenez, J.I., 2004. Control of fungal decay of apples and peaches by the biofumigant fungus *Muscodor albus.* Postharvest Biol. Tech. 31, 1-8.

Mercier, J., Smilanick, J.L., 2005. Control of green mold and sour rot of stored lemon by biofumigation with *Muscodor albus.* Biol. Control 32, 401-407.

Miyazawa, M., Kimura, M., Yabe, Y., Tsukamoto, D., Sakamoto, M., Horibe, I., Okuno, Y., 2008. Use of solid phase microextraction (SPME) for profiling the volatile metabolites produced by *Glomerella cingulata.* J. Oleo Sci. 57, 585-590.

Montel, M.C., Masson, D., Talon, R., 1998. Bacterial role in flavour development. Meat Sci. 49, S111-S123.

Morath, S.U., Hung, R., Bennet, J.W., 2012. Fungal volatile organic compounds: A review with emphasis on their biotechnological potential. Fungal Biol. Rev. 26, 73-83.

Nout, M.J.R., Barlett, R.J., 1998. Attraction of a flying nitidulid (*Carpophilus humeralis*) in volatiles produced by yeasts grown on sweet corn and a corn-based medium. J. Chem. Ecol. 24, 1217-1239.

Pimenta, R.S., Moreira da Silva, J.F., Buyer, J.S., Janisiewicz, W.J., 2012. Endophytic fungi from plums (*Prunus domestica*) and their antifungal activity against *Monilinia fructicola*. J. Food. Protect. 75, 1883-1889.

Rouissi, W., Ugolini, L., Martini, C., Lazzeri, L., Mari, M., 2013. Control of postharvest fungal pathogens by antifungal compounds from *Penicillium expansum*. J. Food. Protect. 11, 1879-1886.

Santo-Pietro, K.A., 2006. Microbial Volatile Organic Compounds (MVOC's). http://www.emlab.com.

Solanki, M.K., Singh, R.K., Srivastava, S., Kumar, S., Kashyap, P.L., Srivastava, A.K., 2013. Characterization of antagonist-potential of two Bacillus strains and their biocontrol activity against *Rhizoctonia solani* in tomato. J. Basic Microbiol. 53, 1-9.

Spadaro, D., Lorè, A., Garibaldi, A., Gullino, M.L., 2013. A new strain of *Metschnikowia fructicola* for postharvest control of *Penicillium expansum* and patulin accumulation on four cultivars of apple. Postharvest Biol. Tech. 75, 1-8.

Stoppacher, N., Kluger, B., Zeilinger, S., Krska, R., Schuhmacher, R., 2010. Identification and profiling of volatile metabolites of the biocontrol fungus *Trichoderma atroviride* by HS-SPME-GC-MS. J. Microbiol. Methods 81, 187-193.

Strobel, G.A., Dirkse, E., Sears, J., Markworth, C., 2001. Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. Microbiology 147, 2943-2950.

Tuduri, D., Desauziers, V., Fanlo, J.L., 2001. Potential of solid-phase microextraction fibers for the analysis of volatile organic compounds in air. J. Chromatog. Sci. 39, 521-529.

Vinale, F., Marra, R., Scala, F., Ghisalberti, E.L., Lorito, M., Sivasithamparam, K., 2006. Major secondary metabolites produced by two commercial *Trichoderma* strains active agaisnt diferent phytopathogens. Lett. Appl. Microbial. 43,143-149.

Weber, F.J., Bont, J.A., 1996. Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on memebranes. Biochim. Biophys. Acta 1286, 225-245.

Zhang, D., Spadaro, D., Garibaldi, A., Gullino, M.L., 2010. Efficacy of the antagonist *Aureobasidium pullulans* PL5 against postharvest pathogens of peach, apple and plum and its modes of action. Biol. Control 54, 172-18.

Table 1

Effect of organic volatile compounds produced by two strains of *Aureobasidium pullulans* (L1 and L8) on the conidia germination of five fruit postharvest pathogens on MEA plates

Treatment	*Botrytis cinerea*	*Colletotrichum acutatum*	*Penicillium expansum*	*Penicillium digitatum*	*Penicillium italicum*
Control	95[a]±4.66a[b]	97±1.71a	66±4.98 a	100±3.26 a	133±5.6 a
L1	33±2.35b	44±2.65b	0±0 b	0±0 b	0±0 b
L8	50±3.83b	49±4.42b	0±0 b	0±0 b	0±0 b

[a]Conidia germination was expressed as colony forming units after 3 days of incubation at 25°C.
[b]Each value is the means of 10 plates (replicates) ± standard error. In the same column, data followed by the same letters are not significantly different according to the least significant difference test (*P*≤0.05).

Table 2

Effect of organic volatile compounds produced by two strains of *Aureobasidium pullulans* (L1 and L8) on the mycelium growth of *Penicillium* spp. of citrus on MEA plates

Treatment	*Penicillium digitatum*	*Penicillium italicum*
Control	32±1.03 a	30±1.02 a
L1	22±2.12 b	16±0.98 b
L8	23±1.09 b	17±0.94 b

[a]Colony diameter (mm) was measured after 5 d at 25°C.
[b]Each value is the means of 10 plates (replicates) ± standard error. In the same column, data followed by the same letters are not significantly different according to the least significant difference test (*P*≤0.05).

Table 3

Effect of organic volatile compounds produced by two strains of *Aureobasididum pullulans* (L1 and L8) on fungal pathogen infections on fruit. Lesion diameters (mm) [a].

	'Golden Delicious' apples			'Navel' oranges	
Treatment	*Botrytis cinerea*	*Colletotrichum acutatum*	*Penicillium expansum*	*Penicillium digitatum*	*Penicillium italicum*
Control	$18^{b}\pm 0.95$a	9 ± 0.41a	23 ± 1.41a	16 ± 1.32a	30 ± 2.24a
L1	2 ± 0.87b	5 ± 0.43b	6 ± 0.82b	0 ± 0b	0 ± 0b
L8	1 ± 0.62b	5 ± 0.54b	6 ± 0.97b	0 ± 0b	1 ± 0.65b

[a]'Golden Delicious' apples were wounded and inoculated with 20 µl of *B. cinerea*, *C. acutatum* and *P. expansum* conidia suspension (10^3 conidia ml^{-1}). 'Navel' oranges were wounded and inoculated with 20 µl of *P. digitatum* and *P. italicum* conidia suspension (10^5 conidia ml^{-1}). Fruits were kept at 20°C for 7 days and lesion diameters were recorded.

[b] Each value is the means of 15 lesions (replicates) $\pm$ standard error. In the same column, data followed by the same letters are not significantly different according to the least significant difference test ($P \leq 0.05$)

Table 4

Analyses of volatile organic compounds produced by *Aureobasidium pullulans* (L1 and L8) strains with HS-SMPE and GC-MS gas phase. DVB/CAR/PDMS SPME fibre was used. Retention time (RT) and molecular weights (MW) of tentatively identified compounds by comparison with the NIST database.

RT (min)	Possible compound	MW	L1 RA (%)	L8 RA (%)
2.51	1-Propanol, 2-methyl-*	74	2.67	2.62
4.31	1-Butanol, 3-methyl-*	88	17.77	20.07
4.36	1-Butanol, 2-methyl-*	88	3.92	6.89
10.07	butanoic acid, 2-oxo-	102	4.58	9.29
10.28	3-heptene, 2,2,4,6,6, pentamethyl-	168	7.44	15.30
10.81	limonene	136	1.85	5.36
10.87	Unknown	132	4.24	9.58
11.14	3-Penten-1-ol, 2,2,4, trimethyl-	128	1.01	2.12
11.41	1-Nonene, 2,4,4,6,6,8,8, Heptamethyl-	224	3.37	6.95
12.88	Phenethyl alcohol*	122	53.15	21.82

* Confirmed by RT and mass spectra comparison with identical standard compounds

Table 5

Effect of four synthetic volatile organic compounds (VOCs) emitted by two strains of *Aureobasidium pullulans* (L1 and L8) on conidia germination of five fruit postharvest fungal pathogens. EC_{50} values (µl ml^{-1} headspace)*.

VOC	*Botrytis cinerea*	*Colletotrichum acutatum*	*Penicillium expansum*	*Penicillium digitatum*	*Penicillium italicum*
1-Butanol,2-methyl	1.38	1.27	0.89	0.48	0.85
1-Butanol, 3-methyl	0.78	1.35	1.01	0.73	1.13
1-Propanol,2-methyl	0.82	1.54	1.28	1.71	1.65
Phenethyl alcohol	0.57	1.97	0.79	0.61	0.62

*The evaluations were carried out after 2 days of incubation at 20°C for *Penicillium* spp. and 25°C for *B. cinerea* and *C. acutatum*.

<u>Chapter II</u>

Activities of *Aureobasidium pullulans* cell filtrates against *Monilinia laxa* of peaches

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Keywords

Biological Control Agent - Chitinolytic microorganism - Enzymes - Fungal pathogen

Abstract

Cell filtrates of *Aureobasidium pullulans* strains L1 and L8, grown at different times, were studied for their antagonistic activity against *Monilinia laxa* disease, conidia germination and germ tube elongation, and for their chitinase and glucanase activities (spectrophotometry / RT-qPCR).

Disease symptoms on peach fruit were reduced by L1 and L8 culture filtrates of 42.5% and 65% respectively. *M. laxa* conidia germination was inhibited 70% by both strains; the germ tube elongation was reduced 52% and 41% respectively. From 24 h onward, both culture filtrates showed β,1-3,glucanase and. chitinase activities, the most pronounced of which was N-β-acetylglucosaminidase. Genes expression level encoding for these enzymes in L1 decreased with the time of yeast growth;in L8, *N-β-acetylglucosaminidase* and *glucan β-1,3-glucosidase* genes decreased their expression

during the yeast growth, whereas the *endochitinase* expression increased at 96 h.. These results show that L1 and L8 are able to produce hydrolytic enzymes involved in their antagonistic activity.

1. Introduction

Monilinia laxa (Aderhold & Ruhland) Honey, *Monilinia fructigena* (Aderhold & Ruhland) and *Monilinia fructicola* (Winter) Honey are three important fungal pathogens of stone fruit (Byrde & Willetts 1977). These fungi infect aerial parts of host plants with a variety of symptoms, including blighting of blossoms, cankers on woody tissues and rotting of fruits, although the prevalent fruit losses are in the postharvest phase (Martini and Mari 2014). The disease is controlled by field chemical treatments such as triazoles, dicarboximides and, more recently, strobilurin-type fungicides, the hydroanilide fenhexamid, and succinate dehydrogenase inhibitors (Miessner and Stammler 2010), although their indiscriminate use caused environmental issues and the appearance of pathogen resistant strains. In this context, biological control agents (BCAs) could be considered a safe and environmentally friendly alternative, to manage brown rot (Zhang et al. 2010). Currently, yeasts and yeast-like microorganisms deserve particular attention as BCAs since they can exert an effective control of postharvest diseases, for example *Candida sake* and *Pantoea agglomerans* against *Penicillium expansum* (Morales et al. 2008), *Kloeckera apiculata* against postharvest pathogens of citrus fruit (Long et al. 2007), *Meyerozyma caribbica* against *Colletotrichum gloesporioides* (Bautista Rosales et al. 2013), *Aureobasidium pullulans* against *P. italicum* and *P. digitatum* on orange and against *Botrytis cinerea*, *C. acutatum* and *P. expansum* on apple (Di Francesco et al. 2015).

A. pullulans (De Bary) Arnaud, a yeast-like fungus, is one of the most promising BCAs; it resides in different environments such as the surface of fruit from the early development stages to maturity (Janisiewicz *et al.*, 2010), or in woody tissues and leaves (Gonzalez and Tello 2011). It can also survive under different conditions such as dry and wet conditions, controlled atmosphere and a wide range of temperatures (Kohl and Fokkema 1994). Previous works demonstrated the efficacy of *A. pullulans* against *P. expansum*, *B. cinerea M. laxa* and revealed that competition for nutrients (Bencheqron et al. 2007), induction of host defence (Ippolito et al. 2000), antibiosis, parasitism and production of lytic enzymes (esochitinase, endochitinase and β-1,3-glucanase) (Zhang et al. 2010) are among the main mechanisms which are responsible for its efficacy.

Promising results were also obtained with *A. pullulans* L1 and L8 strains, isolated from the surface of 'Redhaven' peaches, against brown rot of stone fruit (Mari et al. 2012a), but little is known about their mechanisms of action. The production of volatile organic compounds from the two *A. pullulans* strains could be considered a partial mode of action (Di Francesco et al. 2015).

Based on the above considerations, the main aim of this study was to evaluate the antifungal activity of *A. pullulans* L1 and L8 culture filtrates against *M. laxa*. In particular, i) the reduction of brown rot incidence on artificially infected peach fruits; ii) the inhibition of *M. laxa* conidia germination and germ-tube elongation, and iii) the display of chitinolytic (N-β-acetylglucosaminidase and endochitinase) and glucanolytic (β-1,3-glucanase) enzymatic activities were investigated. Finally, iv) the gene expression of key lytic enzymes in L1 and L8 cells was also analyzed by qRT-PCR.

2. Materials and methods

2.1 Antagonists

L1 and L8 strains used in the experiments, isolated from peaches and identified as reported by Mari et al. (2012), were grown on nutrient yeast dextrose agar (NYDA, 8 g l^{-1} of nutrient broth, 5 g l^{-1} of yeast extract, 10 g l^{-1} of dextrose, 25 g l^{-1} technical agar, Oxoid, UK) at 25°C and stored in a glycerol solution (10 %) at -80°C until use. In order to obtain a cell-free filtrate of both antagonists, they were cultured in NYDB (NYDA without agar) at 25°C in a rotary shaker (250 rpm) for 0 (1 hour), 24, 48, 72 and 96 h. The cultures of each incubation time were centrifuged at 5000 g for 20 min at 4°C and the supernatants were sterilized with Millex-GV 0.22 μm syringe filters (Millipore, UK). The supernatants (culture filtrates) were used for *in vivo* and enzyme assays and the yeast cells for gene expression test.

2.2 Pathogen

M. laxa strain was isolated from peaches showing evident symptoms of brown rot and identified by sequencing of ribosomal DNA ITS regions (Mari et al., 2012). The pathogen was grown and maintained on potato dextrose agar (PDA, 39 g l^{-1}, Oxoid, UK) at 25°C for the experiments. Spore suspension of *M. laxa* was prepared from a 7 days old colony by scraping and suspending conidia in sterile distilled water to which 0.05% (v/v) Tween 80 was added, and adjusted to a final concentration of 10^5 conidia ml^{-1} with a hemocytometer

2.3 Fruit

'Redhaven' peaches [*Prunus persica* (L.) Batsch] at commercial maturity were provided by the experimental orchard of the Agricultural Faculty located in Cadriano (Bologna, Italy); the orchard was under conventional management, but no fungicide treatments against *Monilinia* spp. were performed. Harvest fruits were stored at 0°C and used for experiments within 5 days from harvest. For inoculum, fruits were wounded by a sterile nail ($3\times3\times3$ mm) at the equator (one wound per fruit) before pathogen inoculation.

2.4 Yeast growth test

A. pullulans strains, from a 2 days old culture, were grown in 50 mL of Minimal Medium (MM) broth (30 g of sucrose, 2 g of $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4x7H_2O$, 0.5 g KCl, 0.2 ml of mineral solution in 1000 ml of distilled H_2O) at 25°C in a rotary shaker (250 rpm) for 6 d. Every 24 h, an aliquot of cell suspension (50 μl) was serially diluted. One hundred μl of each dilution were spread with a sterile spatula on the surface of NYDA in Petri dishes. Dishes were incubated at 25°C for 2 days and the colony forming units (CFUs) were recorded with a hemocytometer. The sample unit was represented by three flasks for each strain and the experiment was repeated once.

2.5 In vivo *antifungal activity*

L1 and L8 free cell-filtrates of *in vitro* trials were also utilized for *in vivo* assays. Twenty μl of each yeast cell-filtrate derived from 0, 24, 48, 72 and 96 h of incubation were introduced in the fruit wounds. After air drying at room temperature, the wounds were inoculated with 20 μl of pathogen conidia suspension (10^5 conidia ml^{-1}). Fruits were incubated at 20 °C for 7 days. Wounds treated with fresh cell-filtrate obtained

from 0 h of incubation and inoculated with pathogen conidial suspension were considered as the control. The sample unit was represented by five fruits for each incubation time and each antagonist; the experiment was performed twice.

2.6 In vitro *antifungal activity*

L1 and L8 cell-filtrates were obtained as described above. The antifungal activity tests were performed as follows: on a microscope slide, aliquots of 25 µl of *M. laxa* 10^5 cfu/ml^{-1} were added to the same aliquots of yeast sterile culture filtrates of 0, 24, 48, 72 and 96 h of incubation. The filtrates contained 2 µg µl^{-1} of total proteins determined according to the Bradford method (1976). Slides were introduced in a sterile Petri dish and the dish was immediately sealed with a layer of Parafilm to maintain high humidity. After 6 h of incubation at 25°C, the slide was observed through a microscope (Nikon Eclipse TE2000-E) and the conidia germination and the germ tube elongation (30 conidia per microscopic field) of each sample were determined. The growth rate of the germ tube was evaluated from the slope of the straight line. The germination time was the time at which the length of the germ tube equalled the conidia diameter. Cell filtrate obtained from 0 h of incubation was considered as the control. For each treatment, three microscope slides (replicates) were observed. The experiment was repeated once.

2.7 Quantification of enzymatic activity

Liquid cultures of BCAs, harvested after 0, 24, 48, 72 and 96 h of incubation, were centrifuged to remove cells and salts with Ultrafree -4 Centrifugal Filter Unit (Millipore) at 5000 *g* for 20 min at 4°C. Protein concentration in the supernatant was determined by the protein-dye binding method as described above (Bradford, 1976), using bovine serum albumin (Sigma, USA) as the standard. The supernatant was

recovered for enzymatic assays of N-β-acetylglucosaminidase (Nagase), endochitinase and β-1,3-glucanase. The activity of N-β-acetylglucosaminidase and endochitinase enzymes was assayed following a modified procedure by Tronsmo & Harman (1993). Both chitinase assays were based on colorimetric determination of *p*-nitrophenyl cleaved from the chitin-analogous substrates, p-nitrophenyl-N-acetyl-β-D-glucosaminide and p-nitrophenyl-β-D-N,N',N''-triacetylchitotriose, respectively (Sigma, USA). Aliquots of 15 µl of each substrate stock solution (2 mg ml^{-1}) were dissolved in 50 mM potassium phosphate buffer (pH 6.7) and added to 30 µl of the yeast culture filtrate from each sample. After incubation for 2 h in a water bath at 37°C, the reaction was stopped by adding 0.05 ml of 0.2 M Na_2CO_3 and the absorbance was measured at 405 nm with a spectrophotometer (Infinite® F50 Tecan, Männedorf,CH). Chitinase activities were calculated using an absorption coefficient for the p-nitrophenyl of 18.5/mM/cm. Each treatment was performed in three replicates and the assay was repeated three times.

β-1,3-glucanase activity was evaluated by measuring the rate of production of reducing sugars, employing laminarin (Sigma) as the substrate and following a modified version of the procedure of Kauffman et al. (1987). The reaction mixture consisted of 0.4 ml of 0.1 M sodium acetate buffer (pH 5.2) containing 1 mg ml^{-1} laminarin and 100 µl of the yeast culture filtrate. After incubating for 2 h at 37°C, 0.3 ml of alkaline copper reagent was added and the mixture was heated at 100°C for 20 min. After cooling, 0.2 ml of Nelson's chromogenic reagent was added and absorbance was measured at 660 nm with a spectrophotometer (Ashwell 1957). Glucose, enzyme standards and substrate blanks were included. One unit of β-1,3-glucanase activity was defined as the amount of

enzyme that releases 1 mg of glucose per min. Each treatment was performed in three replicates and the assay was repeated three times.

2.8 Gene expression analysis

The total RNA of *A. pullulans* L1 and L8 strains was extracted from yeast cells grown in NYDB medium at 25°C on a rotary shaker (250 rpm) for 0, 24, 48, 72 and 96 h of incubation. The RNA was extracted from three biological replicates using the Spectrum Plant Total RNA Kit (Sigma). The extraction was completed according to the protocol described by the kit manufacturer, and DNAse treatment was carried out using TURBO DNA-free^{TM} Kit (Life Technologies, USA). RNA quality and concentration were assessed spectrophotometrically (260 nm) and by electrophoresis in 1% agarose gel. One μg of RNA was retro-transcribed with ImProm-II Reverse Transcriptase^{TM} (Promega, USA) and oligo(dT) as primers. The primers used in this study were designed using the free Primer 3 software (Life Technologies). Primers were purchased from PRIMM BIOTECH (Milan, Italy). Each primer was previously tested in PCR reaction and the amplified products were sequenced (MWG) to verify the specificity. The primer sequences and the gene accession number of the corresponding gene used in this study are reported in Table 1.

qRT-PCR was performed in 96-well plates on a MX3000 thermocycler (Stratagene, USA) using the SYBR Green reaction system kit (Life Technologies). Each reaction contained 1X of Platinum Sybr-Green Master mix (Invitrogen, Milan, Italy), 5 μM of each primer, 3.25 μl of nuclease-free water and 2.5 μl of 1:10 dilution of cDNA, in a total volume of 12.5 μl. The following cycling conditions were used: 95°C for 10 min; 40 cycles of 95°C for 15 sec, 58°C for 30 sec, and 72°C for 30 min; and a final

extension at 95°C for 1 min. Fluorescence was monitored at the end of each annealing step. To assess the amplification specificity, melting curve analysis was always performed at the end of each experiment, by monitoring the fluorescence from 55°C to 95°C, every 0.1°C. Data were analyzed using MXPro QPCR Software version 3.0 (Stratagene, USA). Quantification was carried out using the relative standard curve method (Applied Biosystems 1997) based on the threshold cycle (Ct) determination for each amplification. Briefly, for each target gene, a standard curve was generated by serially diluting a randomly chosen first strand cDNA. The Ct values of each dilution were plotted against the log of the dilution values. The cDNA target quantity in each experimental sample was determined by interpolation of its Ct value on the standard curve to find the log of its relative quantity value. This quantity value was then normalized against that of the β-tubulin house-keeping gene calculated with same method. For each sample, three independent biological replicates were made and each replicate was run three times.

2.9 Statistical analysis

All data were analysed using one-way ANOVA and the least significant differences test (LSD) was used to separate differences among the means; statistical significance was considered as $P<0.05$. 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 Yeast growth test

A. pullulans L1 and L8 strains showed similar trends of growth in MMB at 25 °C (Fig. 1). The population of both strains increased significantly from 0 h until 96 h of incubation, with a peak of 2×10^5 CFU. After that, the population remained constant until 120 h of incubation and then it started to decrease to 1×10^5 CFU for L1 and 9×10^4 for L8. These data were useful to decide the times of incubation most suitable for the evaluation of enzymatic activity of L1 and L8 strains.

3.2 In vivo *antifungal activity*

The cell-filtrates, obtained from the liquid culture of *A. pullulans* L1 and L8 strains, were applied on peach fruit artificially inoculated with conidia suspension of *M. laxa* (Fig. 4). After incubation for 7 days at 20°C, the disease severity, evaluated as lesion diameters, was significantly reduced by L1 and L8 strain culture filtrates from 48 h onward. A similarly high reduction was obtained in fruit treated with the 72 h and 96 h filtrates of each strain; the disease was reduced by the 96 h filtrate to 37.5% (L1) and 47.1% (L8) levels compared to the control. In general, the reduction of lesion diameters obtained with L8 strain cell-filtrate was higher than that obtained with L1 strain cell-filtrate.

3.3 In vitro *antifungal activity*

Conidia germination of *M. laxa* was reduced significantly by L1 and L8 strain cell-filtrates from 24 h of incubation, compared to the control (Fig. 2). Conidia germination was markedly reduced by the 48, 72 and 96 h-cell-filtrates of both strains. Filtrates of 48

h of incubation caused inhibition of 70% and 67% for L1 and L8 respectively. No significant differences were shown by cell-filtrates of L1 or L8 strains from 48 h, 72 h and 96 h of incubation.

With respect to the length of *M. laxa* germ tube, the culture filtrates of the two yeasts caused significant reductions from 24 h (Fig. 3) onward. The highest germ tube reduction was observed when conidia were treated with the 72 h-filtrate of L1 and with the 48 h-filtrate of L8 (52% and 41%, respectively, compared to the control).

3.4 Quantification of enzymatic activities in L1 and L8 culture filtrates

L1 and L8 culture filtrates showed N-β-acetylglucosaminidase, endochitinase and β,1-3,glucanase (Fig. 5 a, b, c) enzymatic activities which varied with the sampling times. Both yeast culture filtrates showed increased chitinase activities from 24 h onward (Fig. 5 a, b). The highest N-β-acetylglucosaminidase activity was shown by the 48 h L8 filtrate (1.11 mU mg^{-1}); after this sampling time, the enzymatic activity of the yeast culture filtrate decreased. L1 strain showed a peak of N-β-acetylglucosaminidase activity at 72 h (0.738 mU mg^{-1}). The endochitinase activity increased gradually with time and reached maximum values in L1 culture filtrate at 96 h sampling (2.316 mU mg^{-1}), peaked in L8 filtrate at 48 h (1.843 mU mg^{-1}), and then slowly decreased.

The highest β,1-3,glucanase activity was exhibited by 48h culture filtrates for both strains; however in L1 strain this activity was much higher than in L8 (9.367 mU mg^{-1} vs 26.903 mU mg^{-1}, respectively). After this time, this activity decreased until 96 h (Fig.5 c).

3.5 Gene expression analysis

Relative expression levels of L1 and L8, *N-β-acetylglucosaminidase, endochitinase* and *β-1,3-glucanase* genes were determined by qRT-PCR. In both L1and L8 strains, their expression decreased drastically from 0 to 24 h and onwards (Fig. 6 a). Similarly, the expression of the *endochitinase* gene in L1 decreased by 2.5 times soon after the first 24 h of growth and by 1.6 times at 96 h with respect to 0 h. In L8, this gene expression slightly increased at the last examined time point by 0.51 times with respect to 0 h. Similarly to the other genes, the *β-1,3-glucanase* gene expression decreased during the early phase of culturing time (0-24 h by 31 times) and gradually increased again thereafter up to 96 h by 0.08 times (Fig 6 a, b, c).

4. Discussion

A. pullulans, a ubiquitous, polymorphic and black fungus, produces a variety of important metabolites including enzymes, polysaccharides and single cell protein (Dake and Beniwal 2014).

A previous study on the population dynamics of the two strains showed different rates of development according to the fruit species; in apple wounds, the population size increased to 7-fold the initial concentration, while in peach wounds only a weak growth was observed, although it was enough to control *Monilinia* rot (Mari et al. 2012a and b). The growth curve of L1 and L8 strains showed, instead, 96 h at 25°C to be the most representative time of growth in MMB for antagonist strains; after that, the population of both BCAs declined, in agreement with previous data obtained with the same BCAs in peach wound (Mari et al. 2012 a).

Cell filtrates of *A. pullulans* L1 and L8 strains were able to inhibit *M. laxa* conidial growth. L1 and L8 cell-filtrates, obtained after 24 h of incubation, inhibited *M. laxa* conidia germination by 9% and 33.3% for L1 and L8, respectively. By extending the incubation time for both BCAs, the conidial germination inhibition was higher (Fig. 2). These results are consistent with *in vivo* trials, where the percentage of lesion reduction in fruits treated with cell-filtrates and inoculated artificially with pathogen conidia was greater in fruit treated with cell-filtrates derived from over 48 h of incubation than those treated with cell-filtrates of only 24 h. Probably, the synthesis of active antimicrobial compounds occurred towards the end of the logarithmic phase of growth when nutrients become limited (McCormack et al. 1994). The involvement of these compounds in the control of fungal pathogens such as *M. laxa* is still not completely elucidated. In contrast, Zhang et al. (2010) found that 48h- cell-filtrates of *A. pullulans* did not have effects on spore germination and germ tube length of some postharvest fungal pathogens including *M. laxa*. The highest antifungal activity was observed using 72 h- and 96 h-cell-filtrates of both BCAs (Fig. 4). Since the cell-filtrate activity increased as the incubation time increased (from 48 to 96 h), the active antifungal compounds produced by the two strains may have had to increase their concentration in the filtrates to be active against the pathogen; however, their efficacy remained very limited showing only a fair, but significant reduction of lesion diameters. A complete inhibition of brown rot incidence was observed by Mari et al. (2012a) using the washed cells of the same BCAs. In this case, competition for nutrients and space between the two microorganisms was presumed, although other mechanisms of action have to be considered such as the production of volatile organic compounds previously elucidated by Di Francesco et al. (2015) in L1 and L8 strains.

A. pullulans produces antibiotics showing a fungicide activity against a variety of fungi including *Aspergillus* spp. (Takesako et al. 1993), yeast (*Saccharomyces cerevisiae*) (Endo et al. 1997) and some important human yeast-like pathogenic fungi (Zhong et al. 2000). In particular, aureobasidin A, an antifungal cyclic depsipeptide antibiotic, produced by *A. pullulans* R106, proved active against some postharvest pathogens like *B. cinerea*, *P. digitatum* and *M. fructicola*, although the latter appeared more resistant than the others to antibiotic, showing a reduction of conidia germination of less than 30% at 16 μg ml^{-1} of aureobasidin A (Liu et al. 2007). The cell-filtrates of both BCAs exert their antifungal activity affecting various aspects of fungal development such as reducing spore germination rate (Fig. 2), slowing the germ tube elongation (Fig. 3), disrupting polarized growth of the germ tube and excessive branching near the tip (data not shown). These data are consistent with the results obtained by other researchers on *A. nidulans* (Endo et al. 1997).

Among the modes of action of BCAs, several papers have focused on the production of extracellular glucanase and chitinase enzymes by different antagonists such as *A. pullulans* (Castoria et al. 2001), *Pichia membranefacines* and *Candida guilliermondii* (Fan et al. 2002), *Pichia anomala* (Jijakli and Lepoivre 1998). These enzymes are able to act as depolymerases on fungal pathogen cell walls, causing cell lysis and death (Castoria et al. 1997; Tseng et al. 2009). In particular, the glucanase and chitinases activities have been addressed as key elements for the antagonistic activity against pathogens (Jijakli and Lepoivre 1998; Chan and Tian, 2006). In this paper we demonstrated the activities of endo and exo-chitinases, and of β-1,3-glucanase enzymes in cell filtrates of *A. pullulans* L1 and L8 strains. The two strains exhibited a different timing in extracellular enzyme production, revealing substantial differences between L1

and L8, as already observed in previous trials (Mari et al. 2012a, b). The L8 strain displayed a peak in chitinase and β-1,3-glucanase activities after 48 h of incubation, in agreement with previous results obtained by Zhang et al. (2010) with the *A. pullulans* strain PL5 cultured in LBMS medium and *M. laxa* cell walls as sole carbon sources. Similarly, L1 showed the greatest β-1,3-glucanase activity after 48 h of incubation, while with respect to the tested incubation times, the endochitinase activity reached the highest level after 96 h of incubation. To our knowledge, there are no studies of enzymatic detection in culture filtrate in which no pathogen cell wall components were added. Our data reported values of β-1,3-glucanase, eso and endochitinase activities in culture filtrates produced by *A. pullulans* L1 and L8 strains at lower levels with respect to those mentioned above by other authors. We can explain our data with the absence of pathogen cell wall. In fact, it is know that cell wall highly stimulates enzymatic production of antagonists (Lopes et al. 2012). The reduction of pathogen conidia germination and germ tube length obtained with culture filtrates may also be due to the presence of other effective metabolites which cannot be excluded. In fact, the diversity of enzyme secretion even within the same species, as reported here for L1 and L8 strains, was observed by others (*Trichoderma* spp.) (Lopes et al. 2012). These present findings could be explained by the high complexity of cell wall degrading enzyme gene regulation, with different rates of evolution and adaptation caused by different environmental pressure. As far as we know, the mechanisms regulating the production of β-1,3-glucanase and chitinase enzymes by *A. pullulans* are still poorly understood, and it is therefore necessary to carry out more molecular studies on the regulation and function of the genes related to the enzyme production (Zhang et al. 2010).

The real time quantitative PCR (qPCR) assays confirmed the expression of chitinase and glucanase genes in the L1 and L8 yeasts; similarly, qPCR was used to evaluate and quantify the expression of specific genes from fungal pathogens and antagonists (Schena et al. 2004) and the effect of their interactions. In this study, qPCR experiments were aimed at quantifying the expression of L1 and L8 *chitinases* and *glucanase* gene in a specific lapse of time and possibly to correlate it with their antifungal activity. In most cases, the studies effectively demonstrated the expression of *endochitinase, N-β-acetylglucosaminidase* or *β-1,3-glucanase* genes in the presence of cell wall pathogens. Our results suggested that the chitinase and glucanase expressions were induced in yeasts at an early time of incubation, implying that these enzymes have a possible role at the initial stage of pathogen attack (Zeilinger et al. 1999). *N-β-acetylglucosaminidase* strongly decreased its expression from 0 h onward. Since yeast strains were removed from the solid medium (NYDA) after 48 h of growth and then introduced into liquid medium, it is possible that solid medium stimulates the expression of this gene with respect to liquid culture media.

Above all, it must be considered that the evaluated chitinase and glucanase activities are possibly due to the concurrent activities of several types of these enzymes displaying similar function. On the contrary, gene expression quantification is based on the used specific primers that, even if designed in conserved region of this enzyme class, might not be totally associated with the measured lytic activity in yeast filtrates. Biochemical characterization of the proteins associated with the lytic enzyme activity is needed in order to identify the actual target genes encoding for such activity.

References

Ashwell G (1957) Colorimetric analysis of sugars. *Methods Enzymol* **3:** 73–105.

Bautista-Rosales PU, Calderon-Santoya M, Servin-Villegas R, Ochoa-Alvarez NA, Ragazzo-Sanchez JA (2013) Action mechanisms of the yeast *Meyerozyma caribbica* for the control of the phytopathogen *Colletotrichum gloesporioides* in mangoes. Biol Control 65**:** 293-301.

Bencheqroun SK, Bajji M, Massart S, Labhilili M, El Jaafari S, Jijakli H (2007) *In vitro* and *in situ* study of postharvest apple blue mold biocontrol by *Aureobasidium pullulans*: Evidence for the involvement of competition for nutrients. Postharvest Biol Tec 46**:** 128–135.

Byrde RJW, Willetts HJ (1977) The brown rot fungi of fruit: their biology and control. Pergamon Press, Oxford.

Bradford A. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72**:** 248–254.

Castoria R, De Curtis F, Lima G, De Cicco V (1997) β–1,3–glucanase activity of two saprophytic yeasts and possible mode of action as biocontrol agents against postharvest diseases. Postharvest Biol Tec 12**:** 293–300.

Castoria R, De Curtis F, Lima G, Caputo L, Pacifico S, De Cicco V (2001) *Aureobasidium pullulans* (LS-30) an antagonist of postharvest pathogens of fruits: study on its modes of action. Postharvest Biol Technol 22**:**7-17.

Chan Z, Tian S (2006) Induction of H_2O_2-metabolizing enzymes and total protein synthesis by antagonistic yeast and salicylic acid in harvested sweet cherry fruit. Postharvest Biol Tec 39**:** 314–320.

Dake MS, Beniwal N (2014) Antibacterial activity of *Aureobasidium pullulans.* Int J Pharma Bio Sci 5: 1026-1048.

Di Francesco A, Ugolini L, Lazzeri L, Mari M (2015) Production of volatile organic compounds by *Aureobasidium pullulans* as a potential mechanism of action against postharvest fruit pathogens. Biol Control 81**:** 8-14.

Endo M, Takesako K, Kato I, Yamaguchi H. (1997) Fungicidal action of aureobasidin A, a cyclic depsipeptide antifungal antibiotic, against *Saccharomyces cerevisiae.* Antimicrob Agents Chemother 41**:** 672–676.

Fan Q, Tian SP, Liu HB, Xu Y (2002) Production of *β*-1, 3-glucanase and chitinase of two biocontrol agents and their possible modes of action. Chin Sci Bull 47**:** 292–296.

Gonzalez V, Tello ML (2011) The endophytic mycota associated with *Vitis vinifera* in central Spain. Fungal Divers 47**:** 29–42.

Ippolito A, El Ghaouth A, Wilson CL, Wisniewski M (2000) Control of postharvest decay of apple fruit by *Aureobasidium pullulans* and induction of defense responses. Postharvest Biol Tec 19**:** 265–272.

Janisiewicz WJ, Kurtzman CP, Buyer JS (2010) Yeasts associated with nectarines and their potential for biological control of brown rot. Yeast 27: 389–398.

Jijakli MH, Lepoivre P (1998) Characterization of an exo–1,3-glucanase produced by *Pichia anomala* strain K, antagonist of *Botrytis cinerea* on apples. Phytopathology 88**:** 335–343

Kauffmann S, Legrand M, Geoffroy P, Fritig B (1987). Biological function of `pathogenesis-related proteins: four PR proteins of tobacco have 1,3-β-glucanase activity. EMBO J, 6**:** 3209–3212.

Kohl J, Fokkema NJ (1994) Fungal interactions on living and necrotic leaves (Blakeman JP & Williamson B, eds.). Ecology of Plant Pathogens. CABI, Oxon 321.

Liu X, Wang J, Gou P, Mao C, Zhu ZR, Li H (2007) *In vitro* inhibition of postharvest pathogens of fruit and control of gray mold of strawberry and green mold of citrus by aureobasidin A. Int J Food Microbiol 119**:** 223-229.

Long CA., Deng BX, Deng XX (2007).Commercial testing of *Kloeckera apiculata*, isolate 34–9, for biological control of postharvest diseases of citrus fruit. Ann Microbiol 57**:** 203–207.

Lopes FAC, Steindorff AS, Geraldine AM, Brandao RS, MonteiroVN, Junior ML, Coelho ASG, Ulhoa CJ, Silva RN (2012) Biochemical and metabolic profiles of *Trichoderma* strains isolated from common bean crops in the Brazilian Cerrado, and potential antagonism against *Sclerotinia sclerotiorum*. Fungal Biol 116**:** 815-824.

Mari M, Martini C, Guidarelli M, Neri F (2012a) Postharvest biocontrol of *Monilinia laxa*, *Monilinia fructicola* and *Monilinia fructigena* on stone fruit by two *Aureobasidium pullulans* strains. Biol Control 60**:** 132–140.

Mari M, Martini C, Spadoni A, Rouissi W, Bertolini P. (2012b) Biocontrol of apple postharvest decay by *Aureobasidium pullulans.* Postharvest Biol Tec 73**:** 56–62.

Martini C, Mari M (2014) *Monilinia fructicola*, *Monilinia laxa* (*Monilinia* Rot, Brown Rot). (S. Bautista-Baňos, ed.), pp. 233-258. Postharvest decay. Control strategies. Eastbourne, UK, Academic Press, Elsevier.

McCormack PJ, Wildman HG, Jeffries P (1994). Production of antibacterial compounds by phylloplane-inhabiting yeasts and yeast-like fungi. Appl Environ Microbiol 60**:** 927–931

Miessner S, Stammler G (2010) *Monilinia laxa, M. fructigena* and *M. fructicolar* risk estimation of resistance to QoI fungicides and identification of species with cytochrome *b* gene sequences. J Plant Dis Protect 117**:** 162–167.

Morales H, Sanchis V, Usall J, Ramos AJ., Marín S (2008) Effect of biocontrol agents *Candida sake* and *Pantoea agglomerans* on *Penicillium expansum* growth and patulin accumulation in apples. Int J Food Microbiol 122**:** 61–67.

Schena L, Nigro F, Ippolito A, Gallitelli D (2004) Real time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. Eur J Plant Pathol 110**:** 893-908.

Takesako K, Kuroda H, Inoue T, Haruna F, Yoshikawa Y, Kato I, Uchida K, Hiratani T, Yamaguchi H (1993) Biological properties of aureobasidin A, a cyclic depsipeptide antifungal antibiotic. *J Antibiot* 46**:** 414–420.

Tronsmo A, Harman GE (1993) Detection and Quantification of *N*-Acetyl-β-D-glucosaminidase, Chitobiosidase, and Endochitinase in Solutions and on Gels. Anal Biochem 1**:** 74-79.

Tseng T, Tyler BM, Setubal JC (2009) Protein secretion systems in bacterial-host associations, and their description in the gene ontology. BMC Microbiol 9**:** S2.

Zeilinger S, Galhaup C, Payer K, Woo SL, Mach RL, Fekete C, Lorito M, Kubicek C (1999) Chitinase Gene Expression during Mycoparasitic Interaction of *Trichoderma harzianum* with its host. Fungal Genet Biol 26**:** 131-140.

Zhang D, Spadaro D, Garibaldi A, Gullino ML, (2010) Efficacy of the antagonist *Aureobasidium pullulans* PL5 against postharvest pathogens of peach, apple and plum and its modes of action. Biol Control 54: 172–180.

Zhong W, Jeffries MW, Georgopapadakou NH (2000) Inhibition of inositol phosphorylceramide synthase by aureobasidin A in *Candida* and *Aspergillus* species. Antimicrob Agents Chemother 44**:** 651–653.

Table 1 Genes and primers used in qRT-PCR.

Gene	Gene Id*	Forward and reverse primer
Tubulin beta chain	Aurpu2p4_003900	F:GTCGAGAACTCCGACGAGAC
		R:CGGCAGAGACGAGGTAGTTC
Endochitinase 2	Aurup2p4_002661	F:TTCATGCTGCTCCAAGTACG
		R:TTGGAAGAGCTGGAGGAAGA
N-acetylglucosaminidase GH18	Aurpu2p4_008454	F:CAACTCTTTGCCTGGAGAGC
		R:GGTCTCTCAACCCCTCTTCC
Glucan 1,3-beta glucosidase	Aurpu2p4_000459	F:AGCTGCAATGGCTGAAGTTT
		R:TTGAATGGCACTCGGATACA

The relative temperature is 58°C for all genes

*According to www.genome.fungalgenomics.com

Figure 1.

Population dynamics of *Aureobasidium pullulans* (L1 and L8 strains) cultured in Minimal Medium Broth at 25 °C for 144 h. Each point represents the mean of the number of colony forming units (CFUs) from three replicates (flask) + error bars, each plated in triplicate at each sampling time.
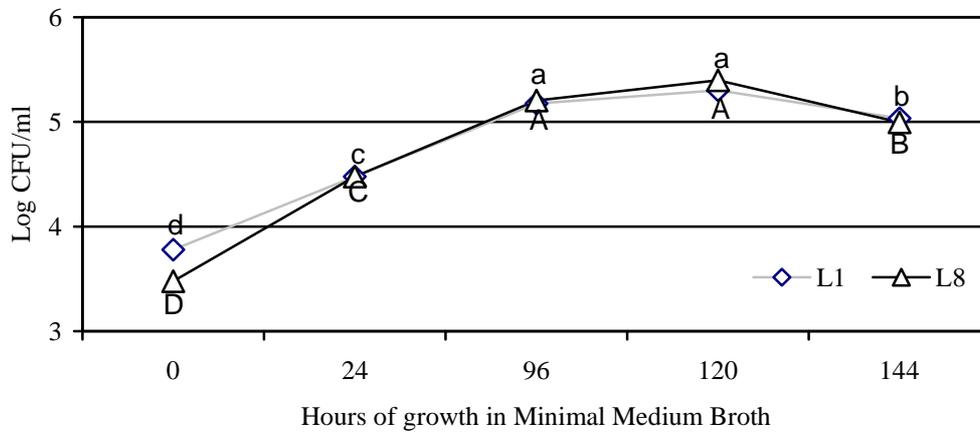
Figure 2.

Effect of *Aureobasidium pullulans* (L1 and L8) cell-filtrates on conidia germination of *Monilinia laxa*. Antagonists were grown in Nutrient Yeast Dextrose Broth at 25 °C for 144 h. Data are the means of germinated conidia of 30 conidia observed for each sample. For each antagonist, different letters (lowercase – L1; uppercase– L8) indicate significant differences according to LSD test, *P* < 0.05.
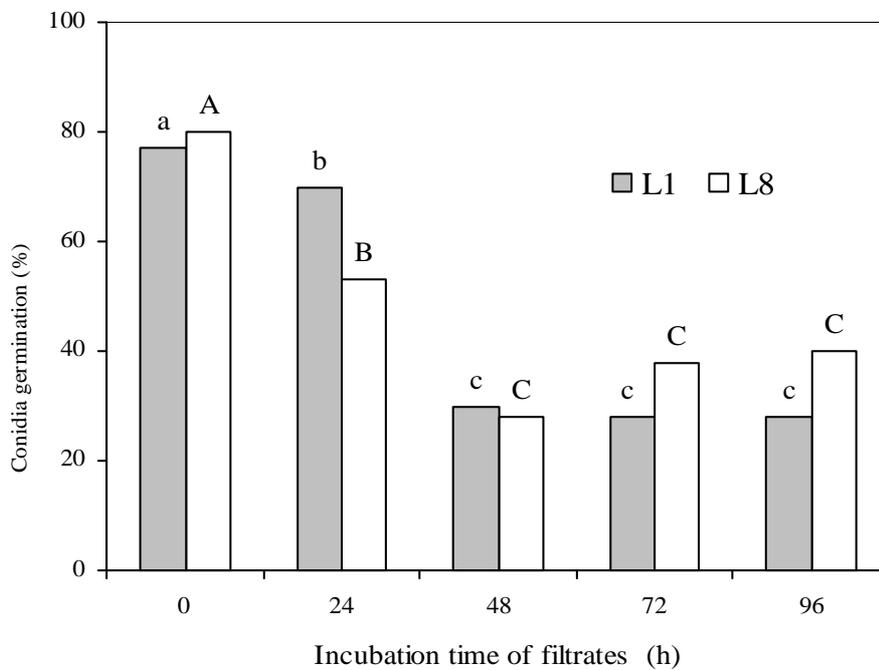
Figure 3.

Effect of *Aureobasidium pullulans* (L1 and L8) cell-filtrates on germ tube elongation of *Monilinia laxa* conidia. Antagonists were grown in Nutrient Yeast Dextrose Broth at 25 °C for 144 hours. Data are the means of 30 germ tube length of germinated conidia observed for each sample. For each antagonist, different letters (lowercase – L1; uppercase – L8) indicate significant differences according to LSD test, $P < 0.05$.
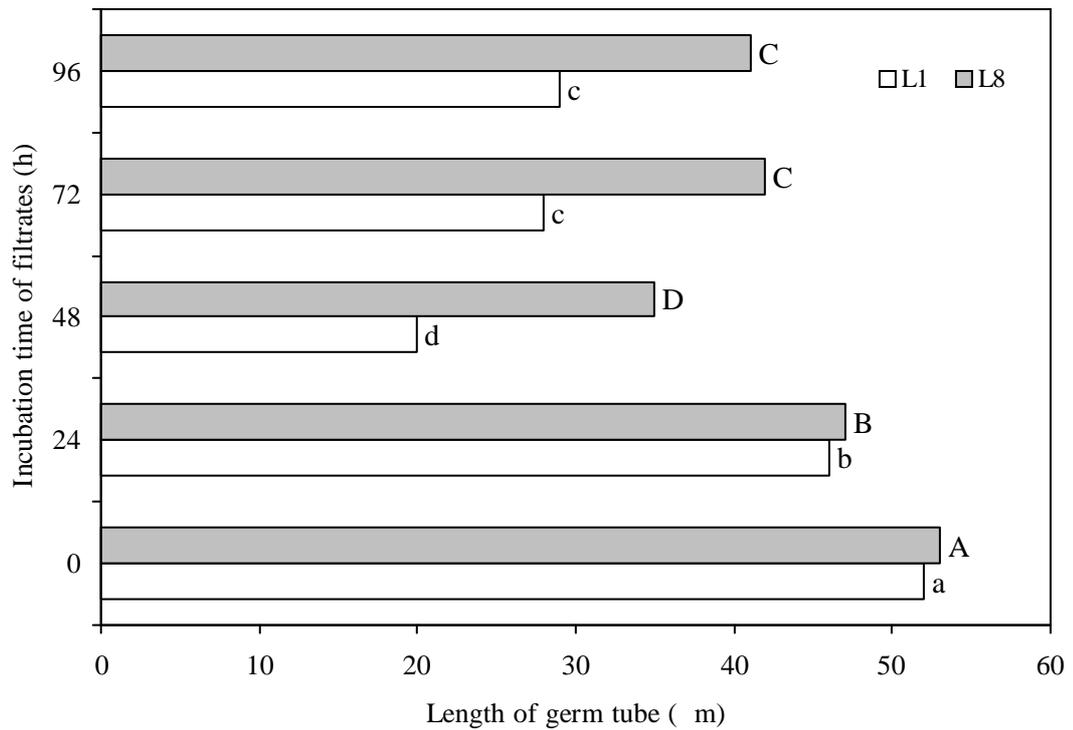
Figure 4.

Effect of *Aureobasidium pullulans* (L1 and L8) cell-filtrates on lesion diameter (mm) of *Monilinia laxa* on peach fruit artificially inoculated. Fruit wounds were first treated by 20 µl of cell-filtrates derived by liquid culture of antagonists grown in Nutrient Yeast Dextrose Broth at 25 °C for 144 hours; after air drying they were inoculated with 20 µl of *M. laxa* conidia suspension (10^5 conidia ml^{-1}). Fruits were kept at 20°C for 7 days. Data are the means of five fruits for each incubation time and each antagonist. For each antagonist (lowercase – L1; uppercase – L8), different letters indicate significant differences according to LSD test, $P < 0.05$.
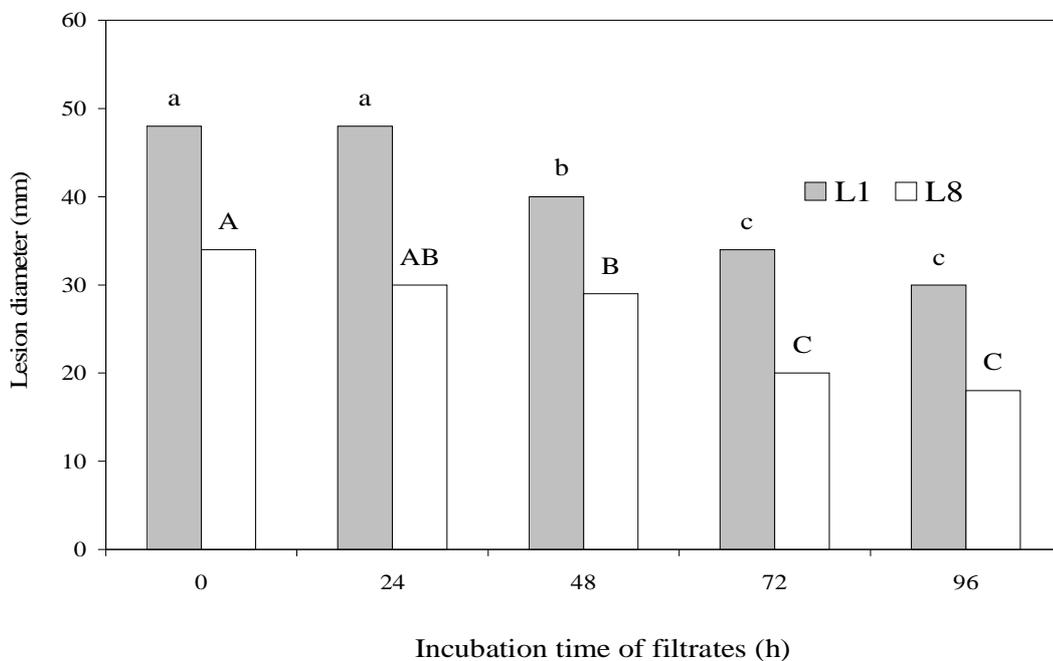
Figure 5.

N-β-acetylglucosaminidase (a), Endochitinase (b) and β-1,3-glucanase (c) activities of *Aureobasidium pullulans* (L1 and L8) cell-filtrates detected by spectrophotometer assays. Each point is the mean of three replicates $\pm$ standard deviations. For each antagonist (lowercase – L1; uppercase – L8), different letters indicate a significant difference, according to LSD test, $P < 0.05$.

a)

N-β-acetylglucosaminidase

b)

Endochitinase

c)
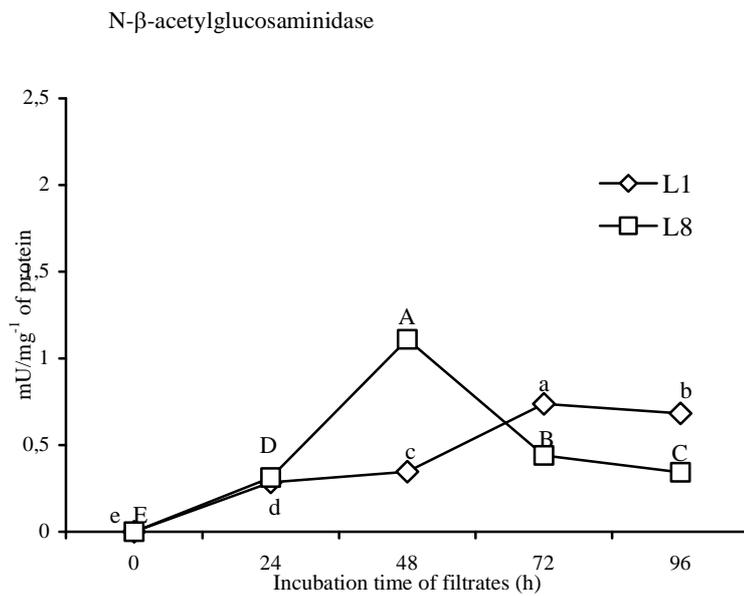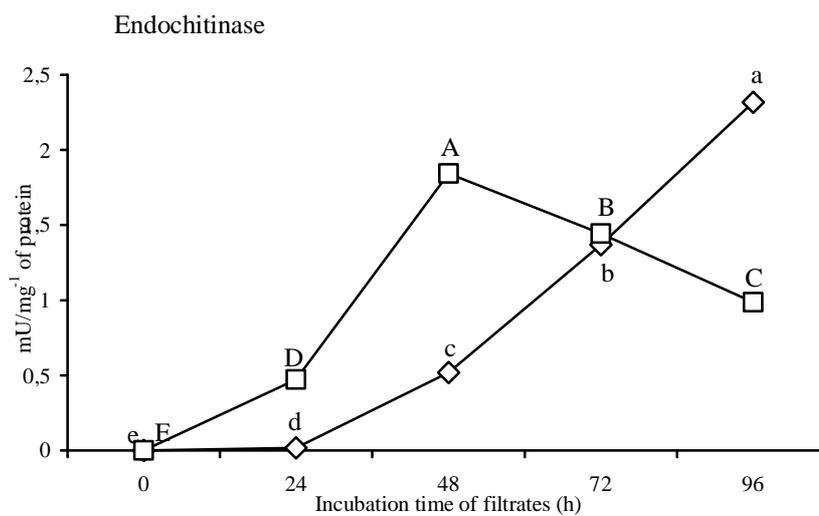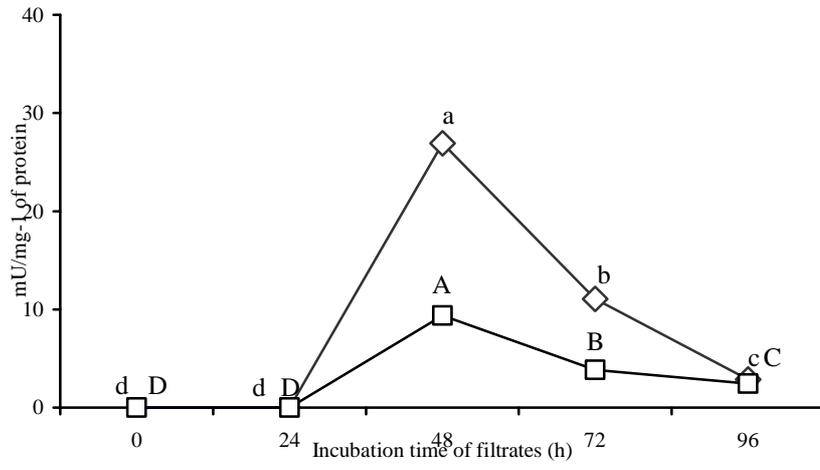
β-1,3-glucanase

Figure 6.

Relative expression levels of *N-acetylgllucosaminidase* (a), *Endochitinase* 2 (b) and *Glucan beta 1-3 glucosidase* (c) genes in *Aureobasidium pullulans* (L1 and L8) cell. Results in each histogram are the mean of three replicates ± standard deviations. For each antagonist (lowercase – L1; uppercase – L8), different letters indicate a significant difference, according to LSD test, *P* < 0.05.

a)

N-acetylglucosaminidase GH18

b)

Endochitinase

c)

Glucan β 1-3 glucosidase
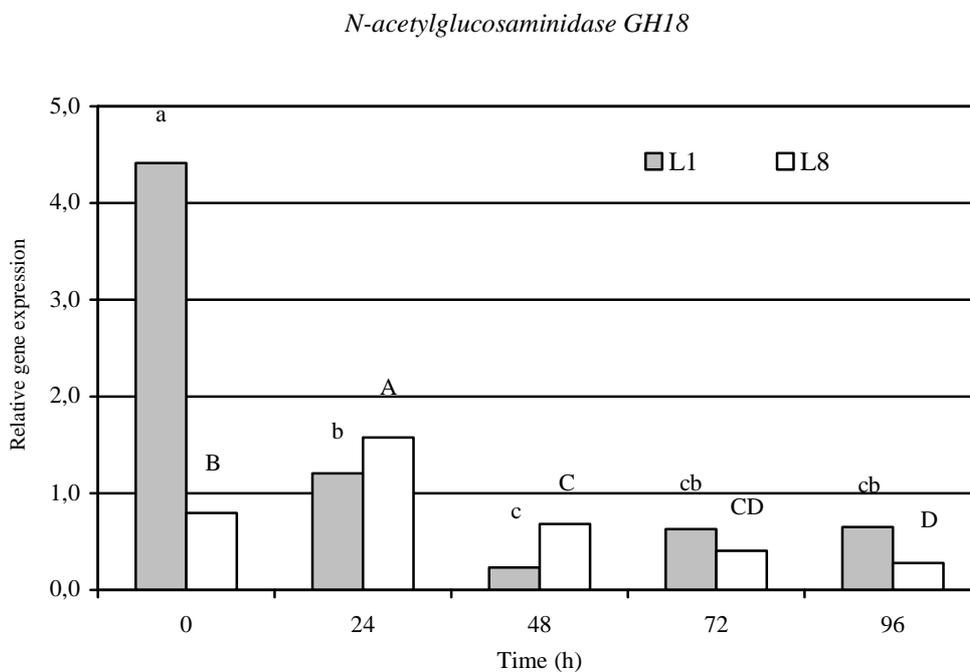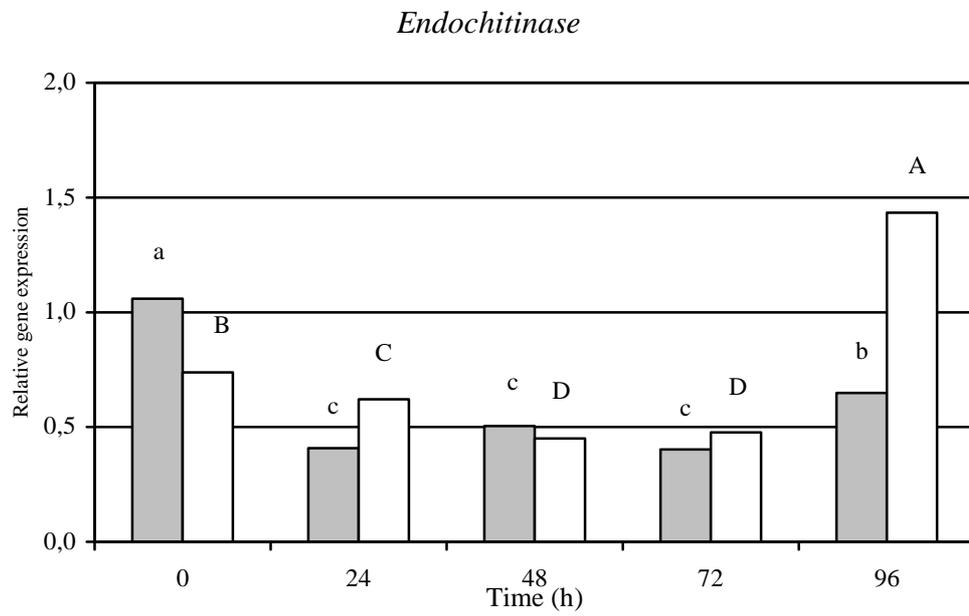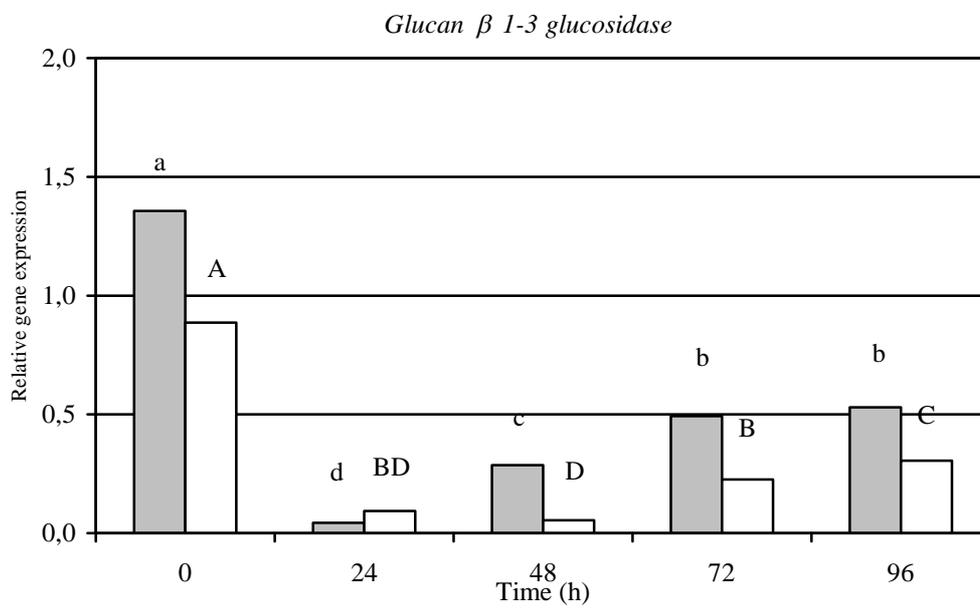
__Chapter III__

Biological control of postharvest fungal pathogens by *Aureobasidium pullulans:* competition aspects

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Abstract

The yeast *Aureobasidium pullulans,* strains L1 and L8, were studied to evaluate their effectiveness against *Penicillium expansum* and *Monilinia laxa* in pome and stone fruit respectively and also to identify the possible mechanisms of action involved in the pathogen control. *A. pullulans* showed a great attitude to compete for nutrients (glucose, fructose and sucrose) and space (as observed by SEM micrographs). Both strains showed a high antagonistic potential *in vivo*, competing especially for sucrose and glucose in wounded apples and in presence of *P. expansum*. Electron microscopy also showed that the yeast colonized the fruit surface obstructing the pathogen access to fruit. Moreover *A. pullulans* showed a great ability to keep a higher pH (pH 6) in the medium in presence of pathogens than when it is alone (pH 3). This ability could contrast the pathogen action in fruit colonization and induce the rot development. *A. pullulans* seems to produce siderophores, competing for essential nutrients such as iron.

According to our results, the competition for nutrients and space represent an essential mechanism of action that could play an essential role in the antagonistic activity of two *A. pullulans* strains against *P. expansum* and *M. laxa.*

1. Introduction

The control of postharvest diseases by antagonistic yeasts involves several modes of action, including competition for nutrients and space. This mechanism is considered one of the major mechanisms of action because involves the nutritional requirements of both antagonists and pathogens (Elad and Chet 1987; Mekbib *et al.* 2011; Bautista-Rosales *et al.* 2013) and it is important mainly against wound pathogens that are typically dependent on exogenous nutrients for their development. In previous studies, *Aureobasidium pullulans* (Janisiewic*z et al.* 2000 and Bencheqroun *et al.* 2007), *Pichia caribbica* (Xu *et al.* 2013) and *P. guilliermondii* (Chanchaichaovivat *et al.* 2008) have showed respectively their antifungal activity against *P. expansum*, *Rhyzopus stolonifer* and *Colletotrichum capsici* by competition for nutrients and the addition of exogenous nutrients influenced the biocontrol showing different pathogen behaviours relating to their presence or absance (Nunes *et al.* 2001, Druvefors *et al.* 2005, Bencheqroun *et al.* 2006 and Liu *et al.* 2010).

Among competition for nutrients, competition for iron also may play a role in the biocontrol interactions (Raaska and Mattila-Sandholm 1995) by the production of siderophores, low molecular weight ferric chelating agents that may enhance the effectiveness of BCAs subtracting iron to pathogen development, inhibiting its growth and metabolic activity (Riquelme, 1996). It has been confirmed that yeasts produce only hydroxamate-type compound derived from amino acid ornithine (Riquelme, 1996).

Another interesting aspect, however few investigated is the indirect competition between an antagonist and a pathogen represented by the change of some environment conditions like pH that can have an influence on the pathogen development. In effect pathogens may enhance their virulence by locally modulating the host's ambient pH such as *Colletorichum* spp. in apple, tomato or avocado through host pH alkalinization (Prusky *et al.* 2001) or *M. fructicola* in peach fruit through host pH acidification. These initial attacks are accompanied by secretion of enzymes like endopolygalacturonases or pectin esterases (Hall, 1971 and Wade and Cruickshank, 1992) causing cell wall maceration and death of affected host cells.

The aims of this study were i) determine the ability of L1 and L8 to compete for sugars and space against *P. expansum* and *M. laxa* in apple and peach fruit respectively; ii) evaluate the potential of siderophores in the *A. pullulans* biocontrol activity and iv) by RT-qPCR analyze the gene expression of L-ornithine-N^5-monooxygenase gene, precursor of siderophore biosynthesis.

2. Materials and methods

2.1 Fruit

'Golden Delicious' apples (*Malus domestica* (L.) Borkh.) and 'Redhaven' peaches (*Prunus persica* (L.) Batsch) were harvested at commercial maturity in orchards located in Cadriano (Bologna, Italy). Fruits were stored at 0 °C and used within 5 days from harvest. Fruits were wounded by sterile nail (3x3x3 mm) on the equator (two wounds per fruit).

2.2 Pathogens

P. expansum was isolated from rotted stored apple, grown on potato dextrose agar (PDA, Oxoid, Hampshire, UK) (39 g in 1 L of distilled water) for 7 days at 20 °C. *M. laxa* strain (ML4) from our collection 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 both pathogens were collected and suspended in sterile distilled water containing Tween 80 0,05% (v/v). The concentration of each conidia suspension was quantified with a haematocytometer and adjusted with sterile distilled water relating to trials.

2.3 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}.

2.4 In vitro *antagonistic assay and pH determination*

Competition for sugars was evaluated using a liquid medium: Minimal Medium (MM) broth (2 g of NaNO$_3$, 1 g KH$_2$PO$_4$, 0.5 g MgSO$_4$x7H$_2$O, 0.5 g KCl, 0.2 mL of mineral solution in 1 L of distilled H$_2$O) adjusted to pH 7 using 1 mM NaOH and amended with sucrose, glucose and fructose, the concentrations of all sugars were 2%. Flasks with capacity of 50 mL containing 25 mL of MM broth amended with each sugar were

inoculated with 0.1 mL of conidia suspension (10^3 cell mL^{-1}) of *P. expansum* or *M. laxa* and subsequently with the same quantity of yeast cell suspension (10^8 cell mL^{-1}) and incubated at 25 °C for 7 days on a rotary shaker (250 rpm). The content of flasks was filtered using a Whatman filter number 1, preconditioned overnight at 80 °C and dried at 80 °C until reaching a constant weight to determine the mycelium dry weight. Flasks inoculated only with pathogens were used as the control. The sample unit was represented by three flasks (replicate) for each pathogen and sugar. The trials were performed twice. For each flask the pH values, before and after 7 days of incubation were determined.

2.5 In vivo *antagonistic assay*

The determination of the competition for nutrients was performed using the method described by Spadaro *et al.* (2002) and modified by Ragazzo-Sanchez *et al.* (2012). Ten apple and ten peach fruits were wounded and treated with 20 μL of each yeast suspension containing 10^7 cell mL^{-1}, after 1 hr at room temperature the fruit were inoculated with 20 μL of pathogen suspensions *P. expansum* or *M. laxa* (10^5 cell mL^{-1}) and 20 μL of a sugar solution (2%). Also ten apple and ten peach fruits were wounded and treated with 20 μL of each yeast suspension (10^7 cell mL^{-1}) and after 1 hr were inoculated with 20 μL of pathogen suspensions *P. expansum* or *M. laxa* (10^5 cell mL^{-1}). Control was represented by fruits treated with sterile distilled water instead of yeast cell suspension and inoculated with pathogen conidia and sugar solution as described above; in the case of fruit not inoculated with sugar solution, control was represented by fruits treated with sterile distilled water instead of yeast cell suspension. Fruits were stored at

20 °C for 7 days. The lesion diameters produced by each pathogen were recorded. The experiment was repeated twice.

2.6 Spectrophotometer siderophore assay

In order to determine the siderophore production by two BCAs and pathogens, one loop of L1 and L8 cells and one of *P. expansum* and *M. laxa* conidia was transferred in a siderophore solution contained sucrose (3%), 0.3% 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 at 200 rpm for 120 h. The culture was centrifuged at 10.000 rpm at 4 °C for 5 min, the supernatant (500 µL) were added to 2,5 mL of a solution contained 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. 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 pathogens was also determined.

2.7 Chromo-Azurol S assay

Chromo-Azurol S, CAS-blue agar was prepared according to Neilands and Schwyn (1987) using 60.5 mg CAS dissolved in distilled water (50 mL) and mixed with iron (III) solution (1mM $FeCl_3.6H_2O$, 10 mM HCl) (10 mL) The solution was slowly added to 72.9 mg HDTMA dissolved in 40 mL of distilled water. The resultant blue solution

was autoclaved at 121 °C for 15 min. A mixture of 750 mL distilled water, 15 g agar, 30.24 g Pipes was also autoclaved and a solution of NaOH was prepared to raise the pH of the solution to 6.8. Dishes were prepared with 30 mL of culturing medium for each antagonist and fungus: NYDA for L1 and L8, malt extract agar (MEA: 50 g of malt extract agar in 1 L of distilled water, Oxoid, UK) for *P. expansum* and PDA for *M. laxa*. Then the solidified growth medium was cut in two halves, one of which was replaced by CAS-blue agar. The dishes were inoculated placing near the borderline between the two medium, but in the culturing medium halve, a 6mm-plug of mycelium derived from a colony in active growth. The plates were incubated in the dark at 20 °C and 25 °C for *P. expansum* and *M. laxa* respectively for three weeks. The CAS reaction was determined by the advance of the color-change in the CAS-blue agar starting from the borderline between the two medium. 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 once.

2.8 Gene expression analysis

The gene expression analysis was performed only for *P. expansum*. *A. pullulans* L1 and L8 strains were grown with or without *P. expansum* conidia in siderophore medium at 28 °C on a rotary shaker (250 rpm) for 5 days. The total RNA was extracted from three biological replicates using the Spectrum Plant Total RNA Kit (Sigma). The extraction was performed according to the protocol and DNAse treatment was carried out using DNAseI (Sigma). RNA concentration and quality were assessed spectrophotometrically (260 nm) and by electrophoresis in 1% agarose gel. 300 ng of RNA were retrotranscribed with High-Capacity RNA-to-cDNA Kit (Applied Biosystem) in a final

volume of 25 µL. RT-qPCR was performed on Rotor-Gene 6000 (Corbett), using SYBR Select Master Mix (Applied Biosystem) in a final volume of 15 µL. The amplification cycle was 2 minutes at 95 °C, 15 second at 95 °C and 1 minute at 60 °C. Dissociation curve was performed from 60 °C to 95 °C.

Sequences for the target genes and the references were downloaded from the *A. pullulans* genome project (http://genome.fungalgenomics.ca/). The expression level of the two gene predictions annotated as *L*-ornithine-N^5-monooxygenase (Aurpu2p4_005796, Aurpu2p4_007237) was tested. Beta-tubulin (Aurpu2p4_003900) and glyceraldehyde-3-phosphate dehydrogenase (GADPH) (Aurpu2p4_004800) were chosen as reference genes. The primer sequences and the gene prediction IDs used in this study were reported in Table 2. Primers were purchased from PRIMM BIOTECH (Milan, Italy).

Real-time PCR efficiencies were calculated for each primer combination on a serial dilution of a pool of cDNA, according to the formula E =10^[–1/slope]. Ct were calculated with the Rotor-Gene 6000 Series Software 1.7 and a threshold manually set at 0.04. Data were analyzed according to the delta delta Ct formula, corrected for the real efficiency of primers as in Pfaffl (2001), by using the software REST 2009 (Qiagen) and L8 as control.

2.9 Scanning electron microscopy (SEM)

In order to investigate on the competition for the space between BCA and *M. laxa* some Scanning Electron Microscopy (SEM) observations were performed. Peach fruit were wounded once with sterile nail (2x2x2 mm), treated with 20 μL of L1 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 fruits, 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 tissue 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 fruits were examined during each evaluation; however only images from a single fruit are shown, because this sample most represented features seen in the others.

2.10 Statistical analysis

All data were analysed using one-way ANOVA and the least significant differences test (LSD) was used to separate differences among the means; statistical significance was considered as $P<0.05$. 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 In vitro *antagonistic assay and pH determination*

The antagonistic activity in terms of competition for nutrients exerted by L1 and L8 strains in a MM broth amended with 2% of glucose, sucrose or fructose was assayed towards *P. expansum* and *M. laxa.* Tested pathogens showed significant ($P<0.05$) decrease of the dry weight mycelium when grown with yeasts in presence or not (data

not shown) of sugars. In presence of glucose and sucrose the weight mycelium of *P. expansum* decrease more than 60% in both treatments; with fructose the reduction was less pronounced (41% and 32% respectively for L1 and L8) (Fig. 1a). Instead *M. laxa* weight mycelium was inhibited more than 80% in both treatments (L1 and L8) and with each sugar (Fig. 1b).

The greatest mycelium growth reduction was observed for both pathogens in presence of sucrose and L8 strain, 75% (*P. expansum)* and 88% (*M. laxa*) respectively.

The presence of L1 and L8 in the culture medium amended with sugars (2%) induced higher pH values (pH 7) than the control (pH 3) (Fig. 2, a and b) after 5 d of incubation for both pathogen. The treatments with glucose and sucrose showed the highest pH values especially for *M. laxa* samples.

3.2 In vivo *antagonistic assay*

After 7 days of incubation, diameters of the lesions caused by *P. expansum* on apples and *M. laxa* on peaches were measured (Fig. 3 a). Under *in vivo* condition L1 and L8 manifested different levels of competition for substrates.

In the case of apples*,* when sugars were added to fruit inoculated with the yeast and the pathogen, *e*specially whit fructose, there was an increase of the lesions (10 and 7 mm respectively for L1 and L8) respect to the treatment without sugar addition (0 mm for both strains). In peaches trials, L1 and L8 controlled totally the disease caused by *M. laxa* independently from the kind of sugar. In addition, when sugars were added to peaches inoculated with the yeast and the pathogen there was not an increase in the development of the disease in fact the addition of sugar seemed to slow down the

pathogen growth (data not shown). *A. pullulans* showed a good control on brown rot by the competition for sugars.

3.3. Spectrophotometer siderophore assay

L1 and L8 showed a considerable siderophore production activity which varied, even if only slightly, in presence of *P. expansum*.

L1 and L8 were found to produce 1.4 and 1.2 mg mL^{-1} of siderophore respectively. *P. expansum* and *M. laxa* produced both 0.64 mg mL^{-1} of siderophore respectively.

In presence of *M. laxa* siderophore quantity seems to remain almost the same with respect to the yeasts grown alone (1.4 and 1.25 mg mL^{-1} for L1 and L8 respectively); with *P. expansum* siderophore quantity changed significantly but slightly (1.3 and 1.15 mg mL^{-1} for L1 and L8 respectively) (Fig. 4).

3.4. Chromo - Azurol S assay

The quality results distinct in terms of color change of CAS agar from blue to shades of red were analyzed after 15th day of incubation when all the tested microorganisms had covered the plate halve containing the culture growth medium. (Fig. 5). Only L1 and L8 induced a color change of CAS-blue agar from blue to dark orange.

Table 1 reports the days required to change blu (CAS agar colour) to red shades and the mm of advance of color change in the CAS blue agar.

On the other hand *M. laxa* produced a stromata barrage between PDA and CAS-blue agar without colour change of the indicative substrate. *P. expansum* on the contrary grown also on CAS-blue agar but didn't change colour.

3.5 Gene expression analysis

Relative expression levels of *A. pullulans* L-ornithine-N^5-monooxygenase genes (Aurpu2p4_005796 and Aurpu2p4_007237) in presence or absence of the pathogen *P. expansum* were determined by RT-qPCR (Table 3).

In both strains L-ornithine-N^5-monooxygenase genes were constitutively expressed, even though in L1 Aurpu2p4_007237 was significantly less expressed than in L8. Moreover, data showed that L-ornithine-N^5-monooxygenase gene Aurpu2p4_005796 was down regulated in strain L8 in presence of the pathogen. In L1 with *P. expansum* both relative expression genes were not determined because the results showed too high Ct values (> 30).

3.6 Scanning electron microscopy (SEM)

Scannig 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, *M. laxa* conidia were germinated at 8 h, 16 h and 32 h but remain on the surface of the fruit. The yeast colonized the fruit surface and the fruit wound (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 cell and only above it there were pathogens hyphae.

4. Discussion

A. pullulans showed an effective antagonist action against *P. expansum* and *M. laxa* through the competition for sugars, space and the capability to create critical condition for pathogen growth by the environment pH change. Competition for nutrient was especially observed on lesion diameter caused by *P. expansum* inoculation and BCA with sugar addition compared whit the control, particularly for sucrose and fructose (Fig. 3). As observed by Bautista – Rosales *et al.* (2013), this result could derive by an environment containing an excess of carbohydrates with sufficient amounts of nutrients for free development of both: the pathogen and the yeast. On peaches the severity of disease caused by *M. laxa* was not increased by the sugars addition (data not shown), probably the pathogen competed for others nutrients or by other conditions like pH alteration.

P.expansum in presence of sugars but in the absence of the antagonist, produce the wider lesions *in vivo* trials confirming that it is nutrient-dependent and, as the necrotrophic pathogens, requires sufficient nutrients for its development. In the presence of the antagonist, however, the development of the pathogen was partially inhibited. Competition for nutrients was also involved in *Pichia guilliermondii* against *P. digitatum*, cocultivated on synthetic media (Droby *et al.* 1989): the addition of exogenous nutrients resulted in a reduced efficacy of BCA since the antagonist was able to compete with pathogen when nutrients were scarce.

L1 and L8 can subtract sugars to *M. laxa* more than *P. expansum,* in fact controlled totally brown rot disease regardless of the type of sugar. In presence of sucrose and fructose *P. expansum* seems to be less controlled by BCA strains (Fig. 3). These results

are partially confirmed also by *in vitro* test where L1 and L8 less inhibited *P. expansum* in presence of fructose.

Therefore we can presume that glucose and sucrose are the principal fruit sugars involved in pathogen and BCA competition; Bautista-Rosales *et al.* (2013) showed as *Colletotrichum gloesporioides* in mangoes increased its pathogenicity after addition of sucrose and fructose. Other authors (Filonow, 1998; Sharma *et al.* 2009 and Spadaro *et al.* 2010) reported as the yeasts generally have the ability to successfully assimilate a wide variety of mono- and di- saccharides, such as fructose and sucrose, making these nutrients unavailable to pathogens and allowing it to rapidly proliferate.

Several studies suggested that the direct penetration of fruit tissue by fungi required the lytic enzymes like cutinases (Wang *et al.* 2000 and Wang *et al.* 2002) and that their over expression increased fungal virulence (Lee e*t al.* 2010). Effectively fungi secrete cell wall degrading enzymes (endopolygalacturonases and pectin esterases) and low molecular weight metabolites associated with tissue degradation (Byrde and Willetts 1977 and Pring *et al.* 1981) modulating pH ambient. Postharvest pathogens can alkalinize the environment of growth or acidify it. *Colletotrichum* spp. and *Alternaria* spp. were described as pathogens that alkalinize the tissue during decay development in several subtropical fruit (Prusky and Yakoby 2003; Alkan *et al.* 2008), whereas *Penicillium* spp., *Sclerotinia* spp*., Phomopsis* spp*., Monilinia fructicola* (De Cal *et al.* 2013) and *Botrytis* spp. have been described as acidifiers of the decayed tissue.

Our results showed that L1 and L8 with sugars kept pH environment quite high, from 7 to 6, with *M. laxa* and glucose, to pH 5 with *P. expansum* and each tested sugar.

Gluconic acid was the main factor in acidification of the host by *Monilinia* spp and *P. expansum* (Prusky e*t al.* 2004; Barad *et al.* 2012) and was a key factor contributing to

the virulence of both pathogen, activating fungal transcription of polygalacturonases (Magro *et al.* 1984; Prusky and Yakoby 2003). The ability of *A. pullulans* to maintain a high pH can inhibit the fruit colonization by pathogen inducing a reduction in rot development. De Cal *et al.* (2013) showed that the acidification of the environment caused by pathogen enhanced the expression of pectolytic enzymes that could facilitate pathogen development on fruit. Therefore our data suggest that the capability of L1 and L8 to modulate the pH values in presence of sugars could be considered a part of antagonistic mechanisms of action.

Yeasts, fungi and bacteria are also strictly tied to iron, an essential nutrient for all forms of life (Wang *et al.* 2009), difficult to obtain for its low solubility often in alkaline environments. Riquelme (1996) found that yeasts produce only hydroxamate-type compound derived from the amino acid ornithine and classified into four structural families: fusarines, coprogens, ferrichromes and rhodotorulic acid (Johnson 2008). The production of siderophore, low molecular weight and iron chelating ligands, are often required for iron sequestration.

Competition for nutrients, in particular for iron is believed to play a significant role in biocontrol interaction (Raaska and Mattila-Sandholm 1995). Other studies have showed that *A. pullulans* produced siderophore, hydroxamate type, and under optimal conditions could produced 1.1 mg mL^{-1} (Wang *et al.* 2009). In our study, L1 and L8 were evaluated for their ability to produce siderophore by CAS assay, based on competition for iron between ferric complex of CAS and siderophore produced by *A. pullulans* strains. The assay described by Neilands and Shwyn (1997) was used for the detection of siderophores produced by different microorganisms (Milagres *et al.* 1999). The siderophores compounds were excreted by microorganisms inducing a color change of

CAS agar from blue to orange, red or purple. Our results confirmed that both *A. pullulans* strains produced siderophore (agar colour change, from blue to red) while *M. laxa* and *P. expansum* were not capable to produce siderophore in solid medium. These result were confirmed by siderophores analysis by spectrophotometer; L1 and L8 were found to produce high levels of cheleting molecules (1.4 and 1.2 mg mL^{-1} respectively) while the pathogens mentioned above resulted poor siderophore producers (0.64 mg mL^{-1} both). When yeast and pathogen grown together, the siderophore production seemed remain unchanged respect to the yeast growth alone. According to these results, we can suppose that the competition for iron is low in *M. laxa* and *P. expansum* and BCAs interactions.

The RT-qPCR assays confirmed that *L*-ornithine-N^5-monooxygenase gene expression was slightly influenced by the *P. expansum* presence. In accordance with the spectrophotometric test, these results showed that *A. pullulans* siderophore production is a constitutive characteristic of the yeast, and not clearly correlated or induced by the presence of the pathogen. On the contrary, the expression of Aurpu2p4_005796 seemed to be slightly, but significantly repressed in the presence of *P. expansum*. Further research is necessary to better understand the functional differences of the two gene predictions annotated as L-ornithine-N^5-monooxygenase, and the siderophore role in biocontrol agent activities, maybe using gene knockout techniques.

Scanning electron microscopy (SEM) revealed that the yeast was able to colonize the fruit surface also in presence of a pathogen, in this case *M. laxa*. In this way the yeast showed its better capability to compete for space respect to the pathogen. To compete successfully with pathogen the microbial antagonist should be better adapted to various environmental and nutritional conditions than the pathogen (Barkai-Golan 2001 and El-

Ghaouth *et al.* 2004) and scanning electron microscopy revealed that *A. pullulans* was really able to colonize the environment preventing the pathogen penetration in each time of inoculation.

In contrast with other BCAs as *Pichia guillermondi* that parasitized *B. cinerea* in apple (Wisniewski *et al.* 1991) or *Meyerozyma caribbica* against *C. gloesporioides* of mangoes (Bautista-Rosales *et al.* 2013), preliminary data from SEM observations revealed that *A. pullulans* was not able to adhere to the hyphae of *M. laxa* but occupied the peach surface and wounds making critical the pathogen penetration.

The results of this study clearly showed that *A. pullulans* L1 and L8 strains can compete with pathogens as *P. expansum* and *M. laxa* for nutrients (sugars, iron) and space, in addition modifying the environment pH can inhibit pathogen development. This important information can help to increase their activity, however further researches are required to obtain a commercial formulation.

References

Alkan N., Fluhr R., Sherman A., Prusky D. Role of ammonia secretion and pH modulation on pathogenicity of *Colletotrichum coccodes* on tomato fruit, 2008. Molecular Plant-Microbe Interaction 21: 1058-1066.

Barad S., Brown Horowitz S., Kobiler I., Sherman A., Prusky D. Accumulation of the mycotoxin patulin in the presence of gluconic acid contributes to pathogenicity of *Penicillium expansum,* 2012. Molecular Plant-Microbe Interaction, 27: 66-77.

Barkai-Golan R. Postharvest diseases of fruit and vegetables: development and control, 2001. Elsevier Sciences, Amsterdam, The Netherlands.

Bautista-Rosales P.U., Calderon-Santoya M., Servin-Villegas R., Ochoa-Alvarez N.A., Ragazzo-Sanchez J.A. Action mechanisms of the yeast *Meyerozyma caribbica* for the control of the phytopathogen *Colletotrichum gloesporioides* in mangoes, 2013. Biological Control 65: 293-301.

Bencheqroun S.K., Bajji M., Massart S., Bentata F., Labhilili M., Achbani H., El Jaafari S., Jijakli M.H. Biocontrol of blue mold on apple fruits by *Aureobasidium pullulans* (strain Ach 1–1): *in vitro* and *in situ* evidence for the possible involvement of competition for nutrients, 2006. Agricultural Application and Biological Science, 71: 1151–1157.

Bencheqroun S.K., Bajji M., Massart S., EI-Jaafari S., Jijakli M.H. *In vitro* and *in situ* study of postharvest apple blue mould biocontrol. by *Aureobasidium pullulans*: evidence for the involvement of competition for nutrients, 2007. Postharvest Biology and Technology, 46: 28–135.

Byrde R.J.W., Willetts H.J. The brown rot fungi of fruit. Their biology and control, 1977. The brown rot fungi of fruit. Their biology and control, pp. 171.

Chanchaichaovivat A., Panijpan B., Ruenwongsa P. Putative modes of action of *Pichia guilliermondii* strain R13 in controlling chilli anthracnose after harvest, 2008. Biological Control, 47: 207-215.

Chi Z.M, Wang F, Chi Z, Yue LX, Liu GL, Zhang T. Bioproducts from *Aureobasidium pullulans,* a biotechnologically important yeast, 2009. Applied Microbiology Biotechnology, 82: 793–804.

De Cal A., Martinez F., Egüen B., Chien-Ming C., Lee M.H., Melgarejo P., Prusky D. Role of gluconic acid and pH modulation in virulence of *Monilinia fructicola* on peach fruit, 2013. Postharvest Biology and Technology, 86: 418-423.

Droby S., Chalutz E., Wilson C.L., Wisniewski M.E. Characterization of the biocontrol activity of *Debaryomyces hansenii* in the control of *Penicillium digitatum* on grapefruit, 1989. Canadian Journal of Microbiology, 35: 794–800.

Druvefors U.A., Passoth V., Schnurer J. Nutrient effects on biocontrol of *Penicillium roqueforti* by *Pichiaanomala* J121 during airtight storage of wheat, 2005. Applied Environmental Microbiology, 71: 1865-1869.

Elad Y., Chet I. The role of competitionfor nutrients in biocontrol of *Pythium* damping off by bacteria, 1987. Phytopathology, 77: 190-195.

El Ghaouth A., Droby S., Wilson C.L., Wisniewski M., Smilanick J., Korsten L. Biological control of postharvest diseases of fruits and vegetables, 2004. In: Khachatourians, G.G. and Arora, D.K. (eds). Applied Mycology and Biotechnology: Agriculture and Food Production. Elsevier Science, Amsterdam, The Netherlands, pp. 11–27.

Filonow A.B. Role of competition for sugars by yeasts in the biocontrol of gray mold of apple, 1998. *Biocontrol Science and Technology,* 8: 243-256.

Guédez C., Castillo C., Cañizalez L., Olivar R. Control biológico: Una herramienta para el desarrollo sustentable y sostenible, 2009. Academia, 7: 50–74.

Hall R. Pathogenicity of *Monilinia fructicola*: I. Hydrolytic enzymes, 1971. Phytopathologische Zeitschrift – Journal of Phytopathology, 72: 245–254.

Janisiewicz W.J, Tworkoski T.J., Sharer C. Characterizing the mechanism of biological control of postharvest diseases on fruits with a simple method to study competition for nutrients, 2000. Phytopathology, 90: 1196–1200.

Janisiewicz W.J, Korsten L. Biological control of postharvest diseases of fruits, 2002. Annual Review of Phytopathology, 40: 411–441.

Johnson L. Iron and siderophores in fungal-host interactions. 2008. Mycological Research, 112: 170–183.

Lee M.E., Chiu C.M., Roubtsova T., Chou C.M., Bostock R.M. Overexpression of a redox-regulated cutinase gene, *MfCUT1*, Increases virulence of the brown rot pathogen *Monilinia fructicola* on *Prunus* spp, 2010. Molecular Plant Microbe Interaction, 23: 176-186.

Liu X., Fang W., Liu L., Yu T., Lou B, Zheng X. Biological control of postharvest sour rot of citrus by two antagonistic yeasts, 2010. Letters of Applicated Microbiology, 51: 30–35.

Magro P., Marciano P., Di Lenna P. Oxalic acid production and its role in pathogenesis of *Sclerotinia sclerotiorum,* 1984. FEMS Microbiology Letters, 24: 9–12.

Mari M., Martini C., Guidarelli M., Neri F. Postharvest biocontrol of *Monilinia laxa*, *Monilinia fructicola* and *Monilinia fructigena* on stone fruit by two *Aureobasidium pullulans* strains, 2012. Biological Control, 60: 132-140.

Mekbib S.B., Regnier T.J.C, Korsten L. Efficacy and mode of action of yeast antagonists for control of *Penicillium digitatum* in oranges, 2011. Tropical Plant Pathology, 36: 233–240.

Milagres A.M.F., Machuca A., Napoleão D. Detection of siderophore production from several fungi and bacteria by a modification of chrome azurol S (CAS) agar plate assay, 1999. Journal of Microbiological Methods, 37: 1-6.

Neilands J.B., Schwyn B. Universal chemical assay for the detection and determination of siderophores, 1987. Analysis Biochemestry, 160: 47-56.

Nunes C., Usall J., Teixidó N., Miró M., Viñas I. Nutritional enhancement of biocontrol activity of *Candida sake* (CPA-1) against *Penicillium expansum* on apples and pears, 2001. European Journal of Plant Pathology, 107: 543–551.

Pfaffl M.W. A new mathematical model for relative quantification in real-time RT-PCR, 2001. Nucleic Acids Research**,** 29: 2002-2007.

Pring R.J., Byrde R.J.W., Willetts H.J. An ultrastructural study of infection on pear fruit by *Monilinia fructigena,* 1981. Physiological Plant Pathology, 19: 1-6.

Prusky D., McEvoy J.L, Leverentz B., Conway W.S. Local modulation of host pH by *Colletotrichum* species as a mechanism to increase virulence, 2001. Molecular Plant–Microbe Interactions, 14: 1105–1113.

Prusky D., McEvoy J.L., Saftner R., Conway W.S., Jones R. Relationship Between Host acidification and virulence of *Penicillium* spp. on apple and citrus fruit, 2004. Phytopathology, 94: 44-51.

Prusky D., Yakoby N. Pathogenic fungi: leading or led by ambient pH?, 2003. Molecular Plant Pathology, 4: 509–516.

Raaska L., Mattila-Sandholm T. Effects of iron level on the antagonistic action of siderophores from non-pathogenic *Staphylococcus* spp., 1995. Journal of Microbiology and Biotechnology, 15: 480-485.

Ragazzo-Sánchez J.A., Magallón-Andalón C.G., Luna-Solano G., Calderón-Santoyo M. Parasitism and substrate competitions effect of antagonistic yeasts for biocontrol of *Colletotrichum gloeosporioides* in papaya (*Carica papaya* L.) var Maradol, 2012. Mexican Journal of Science Research, 1: 2–9.

Riquelme M. Fungal siderophores in plant-microbe interactions, 1996. Microbiologia Sem, 12: 537–546.

Schirra M., D'Aquino S., Cabras P., Angioni A. Control of postharvest diseases of fruit by heat and fungicides: efficacy, residue levels, and residue persistence. A review, 2011. Journal of Agricultural Food Chemestry, 59: 8531–8542.

Sharma R.R., Singh D., Singh R. Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A review. 2009. Biological Control, 50: 205-221.

Spadaro D., Vola R., Piano S., Gullino M.L. Mechanisms of action and efficacy of four isolates of the yeast *Metschnikowia pulcherrima* active against postharvest pathogens on apples, 2002. Postharvest Biology and Technology, 24: 123-134.

Spadaro D., Ciavorella A., Zhang D., Garibaldi A., Gullino M.L. Effect of culture media and pH on the biomass production and biocontrol efficacy of a *Metschnikowia pulcherrima* strain to be used a fungicide for postharvest disease control, 2010. Canadian Journal of Microbiology, 56: 128–137.

Van Lenteren J.C., Loomans A.J., Babendreier D., Bigler F. Harmonia axyridis: an environmental risk assessment for Northwest Europe, 2008. Biological Control, 53: 37-54.

Wade G.C., Cruickshank R.H. Rapid development of resistance of wounds on immature apricot fruit to infection with *Monilinia fructicola,* 1992. Phytopathologische Zeitschrift – Journal of Phytopathology, 136: 89–94.

Wang W., Chi Z., Liu G., Buzdar M.A., Chi Z., Gu Q. Chemical and biological characterization of siderophore produced by the marine-derived *Aureobasidium pullulans* HN6.2 and its antibacterial activity, 2009. Biometals, 22: 965–972.

Wang C., Chin C.K, Gianfagna T. Relationship between cutin monomers and tomato resistance to powdery mildew infection, 2000. Physiologiacal and Molecular Plant Pathology, 57: 55–61.

Wang, Y., Georgi L.L., Reighard G.L., Scorza R., Abbott. A.G. Genetic mapping of the evergrowing gene in peach *Prunus persica* (L.) Batsch, 2002. Journal of Heredity, 93: 352-358.

Wisniewski M., Biles C., Droby S., McLauchlin R., Wilson C., Chalutz E. Mode of action of the postharvest biocontrol yeast, *Pichia guilliermondii*. Characterization of attachment to *Botrytis cinerea,* 1991. Physiology and Molecular Plant Pathology, 39: 245–25

Xu B., Zhang H., Chen K., Xu Q., Yao Y., Gao H. Biocontrol of postharvest *Rhizopus* decay of peaches with *Pichia caribbica,* 2013. Current Microbiology, 67: 255-261.

Figure 1.

Influence of L1 and L8 strains and sugars (glucose, sucrose and fructose) (2%) on *Penicillium expansum* (A) and *Monilinia laxa* (B) mycelium dry weight (MDW) (mg) determined after 7 days of incubation at 20 °C. Data represent the means of three replicates ± standard errors. Within the same sugar, different letters indicate significant difference according to LSD test (*P*<0.05).

A)
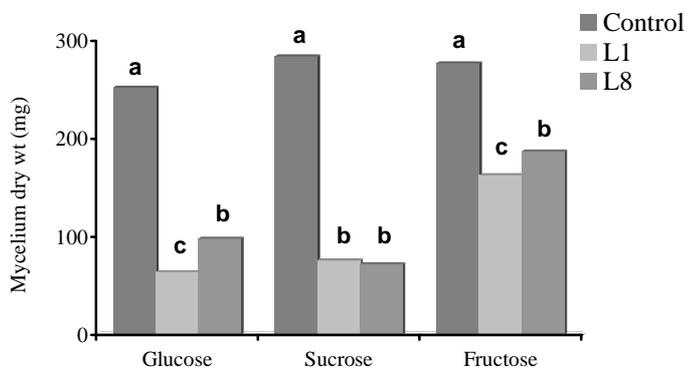
B)
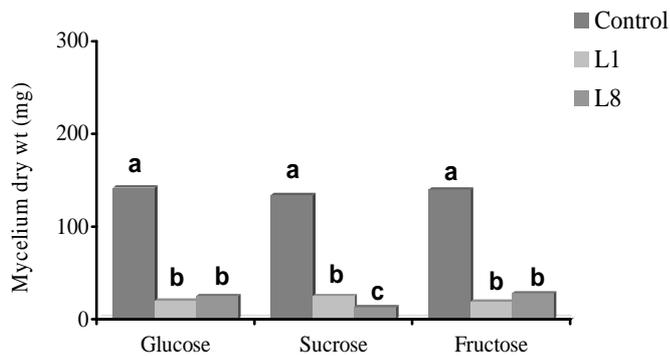
Figure 2.

Influence of L1 and L8 strains and sugars (glucose, sucrose and fructose) (2%) on pH values of liquid medium growth of *Penicillium expansum* (A) and *Monilinia laxa* (B).

Data of pH values represent the means of three replicates ± standard errors. Within the same sugar, different letters represent a significant difference according to LSD test ($P<0.05$) for each sugar.

A)
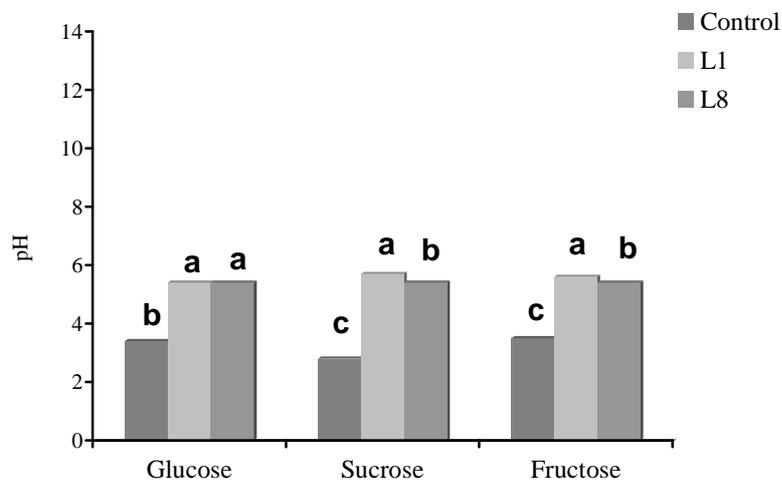
B)
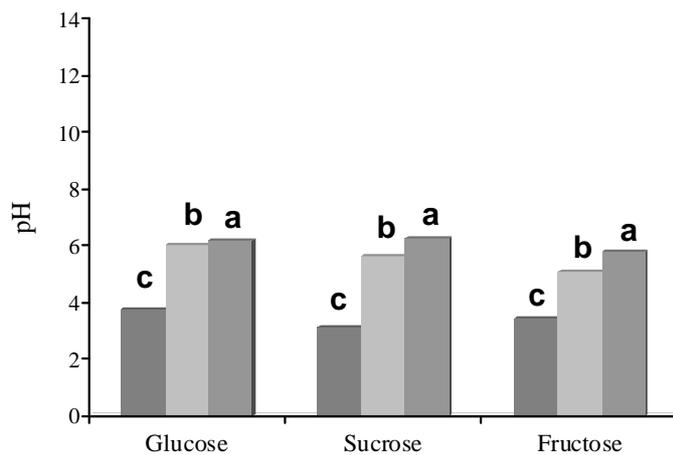
Figure 3.

Influence of L1 and L8 strains and sugars (glucose, sucrose and fructose) (2%) on severity (lesion diameter) of *Penicillium expansum* in artificially inoculated apple. Fruit were inoculated with pathogen conidia suspension and stored for 7 days at 20°C.

Data are the mean of 30 fruits each with two wounds (60 wounds). Different letters means significantly difference according to LSD ($P<0.05$).
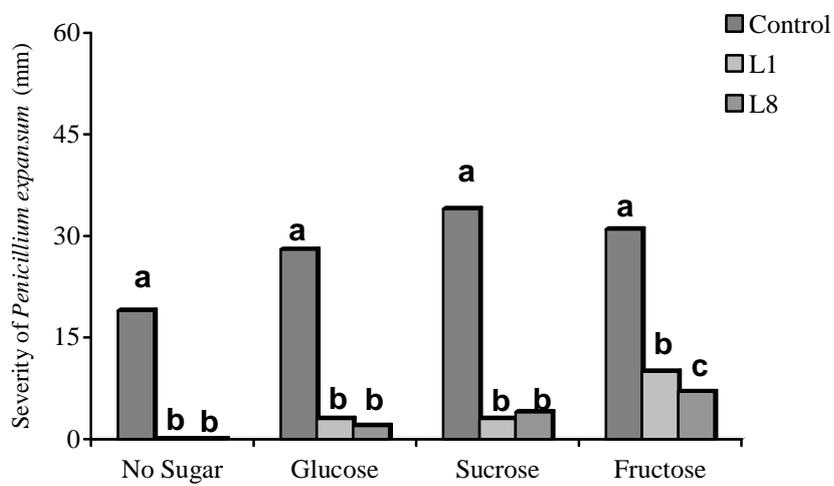
Figure 4.

Quantitative (mg mL^{-1}) determination of siderophore at 440 nm by spectrophotometer, on deferoxamine mesylate standard curve, of *Aureobasidium pullulans* L1 and L8 strains co-cultured with *Penicillium expansum* (P) and *Monilinia laxa* (M). Each value is the mean of three replicates ± standard deviations. For each antagonist, different letters indicate a significant difference, according to LSD test, $P < 0.05$.
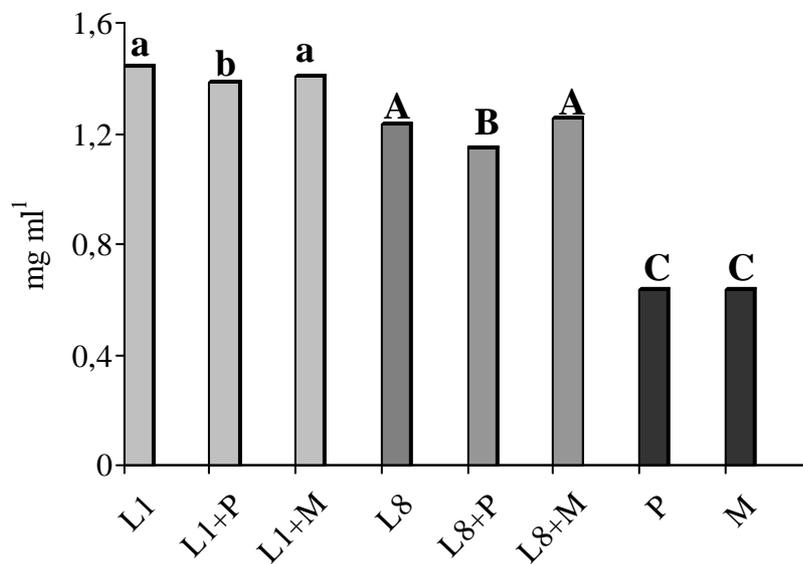
Figure 5.

CAS assay performed with *Aureobasidium pullulans* L1 strain (1), L8 strain (2), *Penicillium expansum* (3) and *Monilinia laxa* (4).
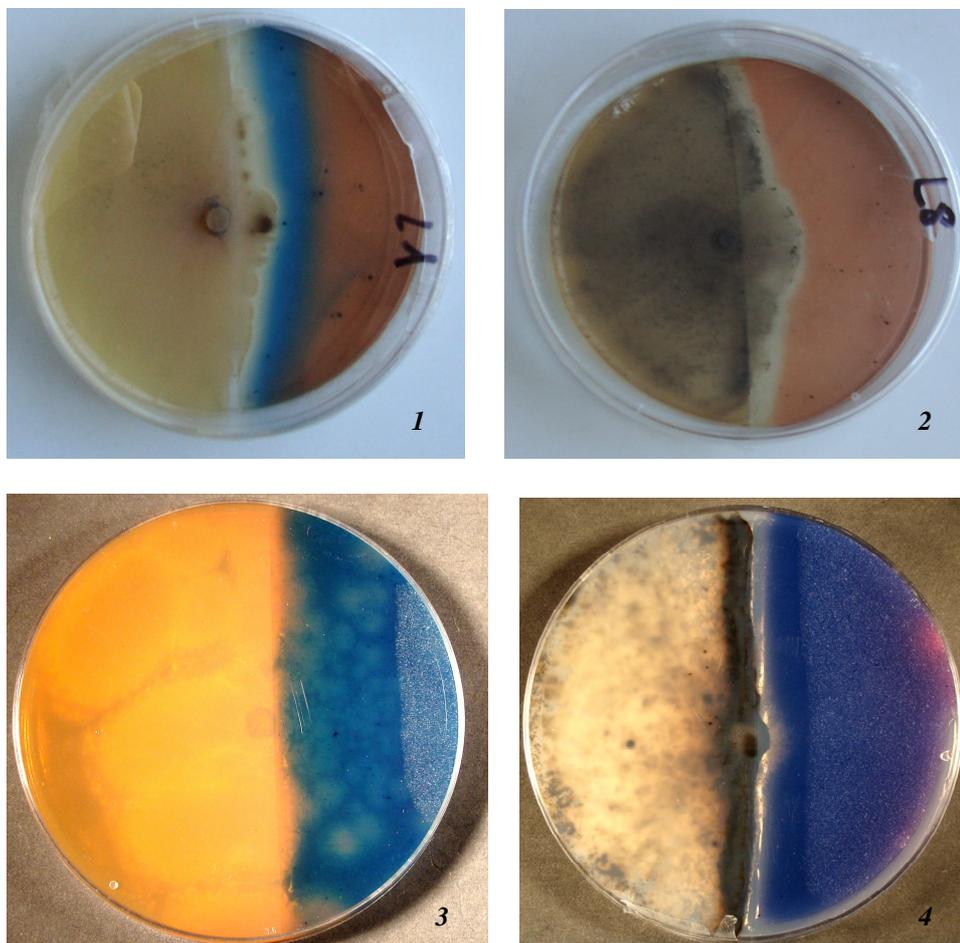

Table 1.

CAS assay for analysis of siderophore production by *Aureobasidium pullulans* (L1 and L8), *Penicillium expansum* and *Monilinia laxa.*

Microorganism	Growth (days)*	Color change	CAS reaction **
L1	12	dark orange	20
L8	12	dark orange	27
Penicillium expansum	8	-	0
Monilinia laxa	10	-	0

* Days required for the fungal mycelium to cover the non-CAS half of the plate.
** mm of advance of color change front in the CAS blue agar after three weeks of incubation.

Table 2.

Primers used in RT-qPCR

| Name	5'-3' sequence	gene prediction
Ap_GAPDH_F	TTCGTCAAGCTTGTTTCGTG	Aurpu2p4_004800
Ap_GAPDH_R	TCGTCCTCTTTGCAGAGTCA	Aurpu2p4_004800
beta tub forward	GTCGAGAACTCCGACGAGAC	Aurpu2p4_003900
beta tub reverse	CGGCAGAGACGAGGTAGTTC	Aurpu2p4_003900
L-orn right	CACCTCTATCGCCCAAGAAA	Aurpu2p4_005796
L-orn left	TGGAGCAATGAAGTCGAGTG	Aurpu2p4_005796
L-orn F3	AAGAGACCACCACGAGCAGT	Aurpu2p4_007237
L-orn R2	TACATCCCTGCAACCAAACA	Aurpu2p4_007237

Table 3.

Relative expression levels of *Aurpu2p4_005796* and *Aurpu2p4_007237 L*-ornithine-N^5-monooxygenase gene predictions in *Aureobasidium pullulans* (L1 and L8) alone or co-cultivated with *P.expansum*. Results are obtained from three biological and three technical replicates. A star indicates a significant difference compare to the control L8, according to REST 2009 software ($P < 0.05$).

	Relative expression	
	Aurpu2p4_007237	*Aurpu2p4_005796*
L1	0.145*	0.029
L1 + *P. expansum*	N.D	N. D.
L8	1	1
L8 + *P. expansum*	1.812	0.317*

Figure 6.

Scanning electron micrographs (SEM) of "Redhaven" peach (surface and cross section) inoculated with *Monilinia laxa* (Control=C) or *M. laxa* and *Aureobasidium pullulans* (L1 strain) (Treatment=T) at different times from the inoculum (0, 4, 8, 16, 32 h).
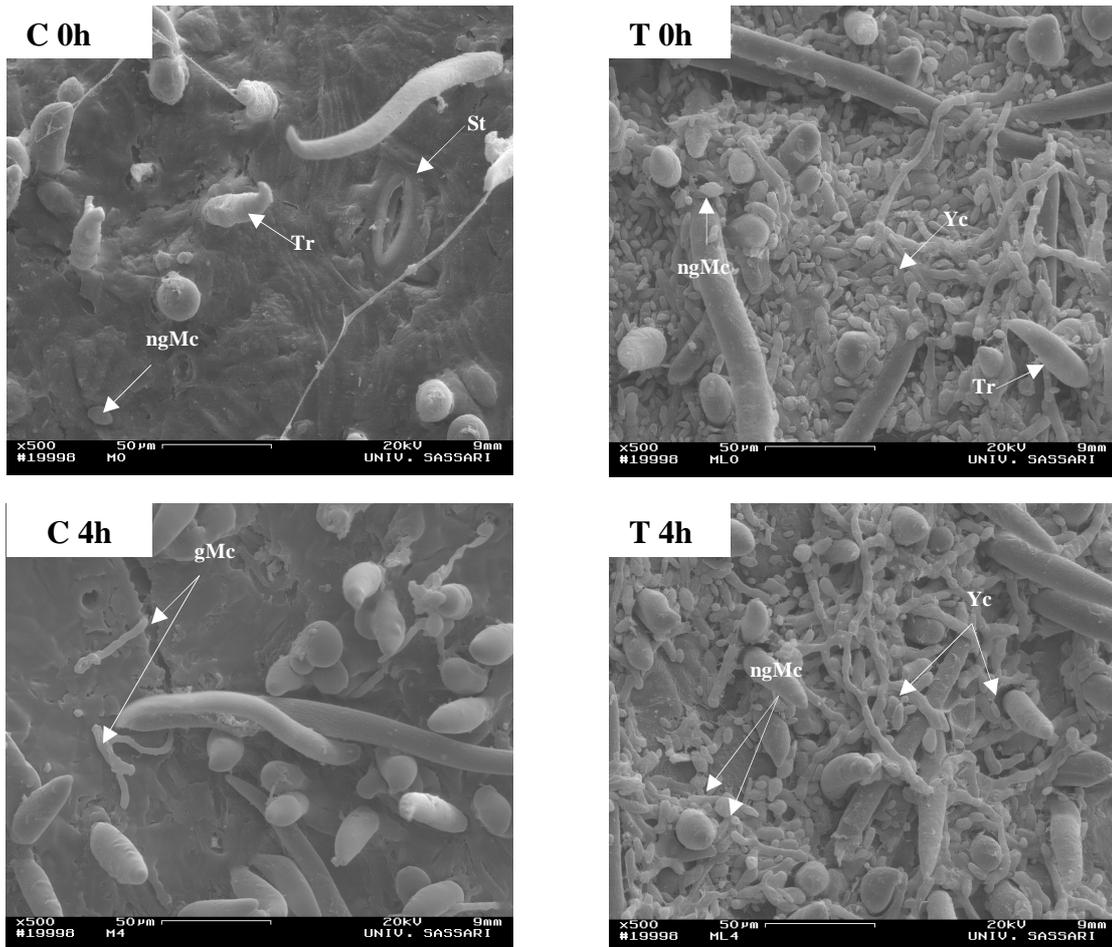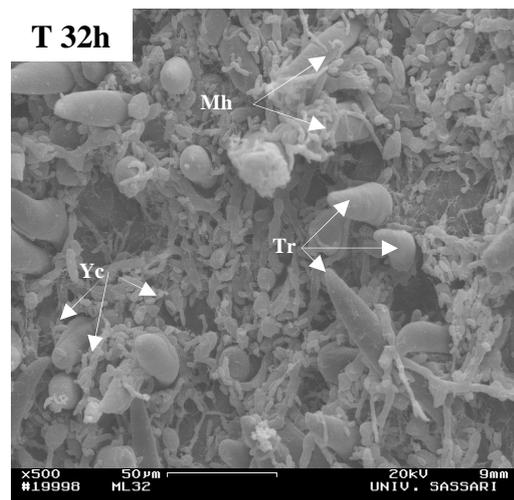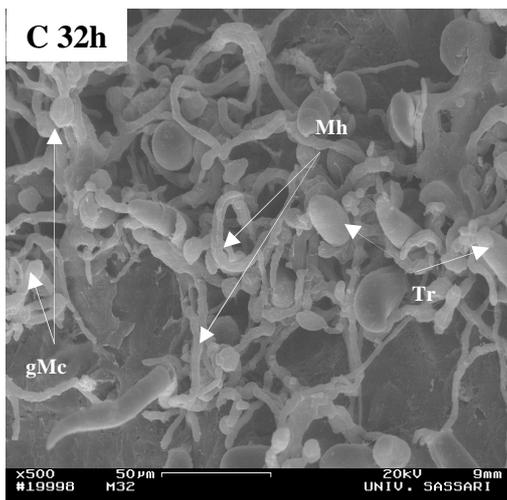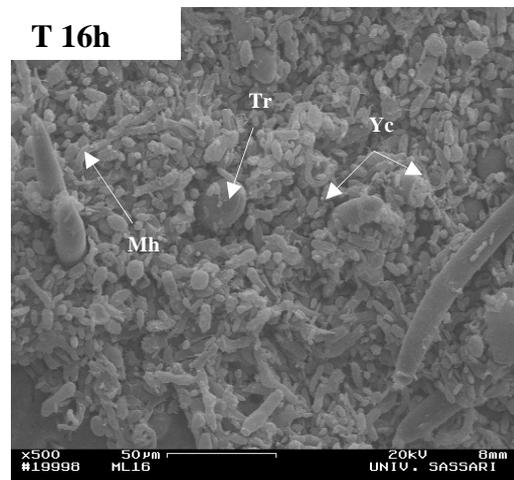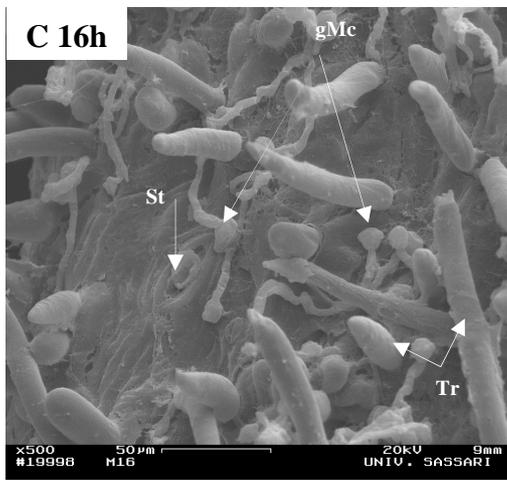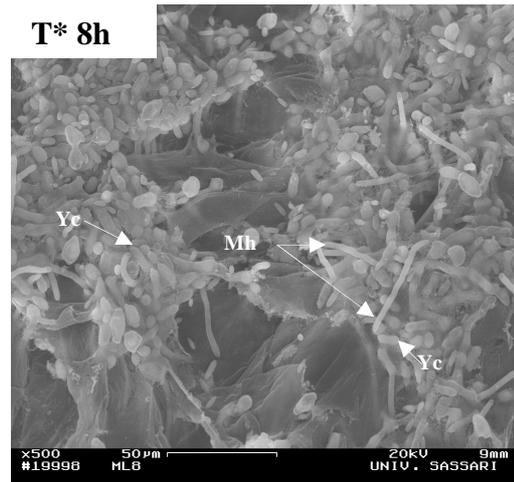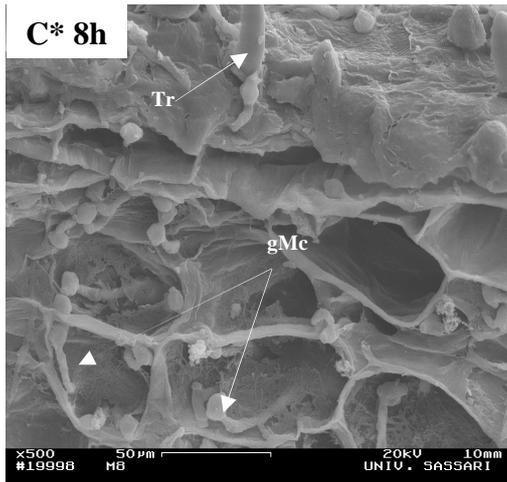
SEM showed, by magnification of 500 x in all images, peach stomata (St) and trichome (Tr), yeast cells (Yc), germinated and no germinated *M. laxa* conidia (gMc/ngMc), *M. laxa* hiphae (Mh).
* Cross section.

<u>Chapter IV</u>

Use of biocontrol agents in combination with physical and chemical treatments: efficacy assessment

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Purpose of review: This review discusses the combination of biological control agents with physical (heat, gamma or UV-C irradiation, controlled atmosphere) or chemical (salt additives, chitosan, natural plant products) treatments against the main postharvest fruit diseases.

Findings: The use of biocontrol agents (BCAs) to control postharvest fruit diseases is still constrained by the lack of high levels of disease control required in the postharvest phase (more than 95%). Their inconsistent activity is one of the main factors preventing their routine application on fruit after harvest. Therefore, to overcome this issue, integrated strategies were explored and continue to be one of the fields most investigated in postharvest fruit disease control, in order to achieve maximum effectiveness. The combination of BCAs with physical and chemical treatments, including fungicides at low doses, resulted in an increase of BCAs effectiveness against fungal diseases such as *Penicillium* spp., *Monilinia* spp., *Botrytis cinerea*, etc. Since the integration of different treatments could benefit from their additive or synergic effects and improve the efficacy of each single method.

Limitations: It is unrealistic to assume that BCAs have the same fungicidal activity as pesticides; the evaluation of their compatibility with other methods therefore seems to be the main topic that will be developed in the near future. Nevertheless, it is necessary to evaluate the possible detrimental effects of some treatments like gamma irradiation or essential oils on qualitative fruit parameters in view of their commercial application. In addition, the use of low doses of fungicides cannot be applied to organic production.

Directions for further research: Specific appropriate strategies have to be evaluated for each species and pathogen in order to tailor a complete integrated disease management. Moreover, these integrated applications have to be investigated at commercial levels, including during fruit handling, in order to ascertain their practical efficacy.

Keywords: physical treatments; salt additives; chitosan; fungicides

Introduction

The use of biocontrol agents (BCAs) to control postharvest fruit diseases is still constrained by the lack of high levels of disease control required in the postharvest phase, ie, more than 95% [1]. Their inconsistent activity is one of the main factors preventing their routine application on fruit after harvest, followed by the limited tolerance to fluctuating environmental conditions and the difficulties of developing shelf-stable formulated products with biocontrol activity similar to that of fresh cells [2]. Therefore, to overcome these issues, integrated strategies were explored and continue to be one of the fields most investigated in postharvest fruit disease control, in order to achieve maximum effectiveness. The use of BCAs in combination with physical and chemical methods has been widely proposed; physical methods including heat treatments, gamma or UV-C irradiation, and controlled atmosphere (CA) have been studied, achieving interesting results. As for chemical methods, the use of salt additives, chitosan and natural plant products could represent a valid alternative to only BCA-based treatments. The combination of low doses of fungicides and BCAs was also explored, appearing to be one of the reliable options for large-scale utilization of microbial antagonists in the control of postharvest fungal rots of fruit and vegetables [3**]. This review examines various combinations of treatments against the main postharvest fruit diseases.

BCAs in association with physical treatments

BCAs ,combined with physical treatments like hot water (HW), has been shown to be effective in reducing postharvest diseases of fruit. For example, strawberries dipped in water at 55°C for 30 s and treated with a suspension of *Cryptococcus laurentii* (10^8 cfu/mL) showed an incidence of *Rhizopus* rot lower than fruit treated with HW or BCA alone. Percentage of fruit infections was 43%, significantly reduced compared with 65% and 63% infections in fruit treated with HW and antagonist, respectively [4]. The combination of heat treatment with a biological control agent may have an additive effect on fruit such as berries since heat may partially disinfect the fruit surface, allowing the biological control agent to become established more quickly and completely [5].

The combination of two antagonists, *Candida guillermondii* or *Pichia membranaefaciens*, with HW was evaluated on tomato fruit against *Botrytis cinerea* [6]. Fruits were treated first by dipping in water at 42°C for 40 min and then treated with BCA. The application of HW did not affect the growth of two BCAs in tomato wounds, while the abiotic stress induced a significant increase in the activities of phenylalanine ammonia-lyase, chitinase and β-1,3 -glucanase in fruit. The mechanism by which HW enhanced the biocontrol efficacy of the antagonistic yeasts could be related to the elicitation of biochemical defense responses in tomato fruit.

When used alone, an isolate of *Aureobasidium pullulans* (PL5) reduced the *M. laxa* infections on peaches to 25.8%, compared with 74.2% for the control, but when applied together with HW (55°C per 50 s) the brown rot incidence decreased to 17.5%, while when HW was used alone the disease incidence was 30%. These results suggest that HW greatly increased the effectiveness of the antagonist [7]. A positive additive effect occurred with the combination of *Pantoea agglomerans* followed by a hot air treatment at 33°C for 6 h in controlling *Penicillium digitatum* of lemons [8*]. Heat treatment was also effective in reducing *M. fructicola* infections established in field but it did not provide protection for further infections after the treatment, before cool storage. Moreover, the combination of curing with BCA (*Bacillus subtilis*) showed the best brown rot control on 'Baby Gold' and 'Andros' peaches. Fruits were first cured (50°C for 2 h), then treated with BCA and finally inoculated with *M. fructicola* [9].

Use of biocontrol agents in combination with physical and chemical treatments: efficacy assessment

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Abstract

Purpose of review: This review discusses the combination of biological control agents with physical (heat, gamma or UV-C irradiation, controlled atmosphere) or chemical (salt additives, chitosan, natural plant products) treatments against the main postharvest fruit diseases.

Findings: The use of biocontrol agents (BCAs) to control postharvest fruit diseases is still constrained by the lack of high levels of disease control required in the postharvest phase (more than 95%). Their inconsistent activity is one of the main reasons preventing their routine application on fruit after harvest. Therefore, to overcome this issue, integrated strategies were explored and continue to be one of the fields most investigated in postharvest fruit disease control, in order to achieve maximum effectiveness. The combination of BCAs with physical and chemical treatments, including fungicides at low doses resulted in an increase of BCAs effectiveness against fungal diseases such as *Pencillium* spp., *Monilinia* spp., *Botrytis cinerea*, etc. Since the integration of different treatments could benefit from their additive or synergic effects and improve the efficacy of each single method.

Limitations: It is unrealistic to assume that BCAs have the same fungicidal activity as pesticides; the evaluation of their compatibility with other methods therefore seems to

be the main topic that will be developed in the near future. Nevertheless, it is necessary to evaluate the possible detrimental effects of some treatments like gamma irradiation or essential oils on qualitative fruit parameters in view of their commercial application. In addition, the use of low doses of fungicides can not be applied to organic production.

Directions for further research: Specific appropriate strategies have to be evaluated for each species and pathogen in order to tailor a complete integrated disease management. Moreover, these integrated applications have to be investigated at commercial levels, including during fruit handling, in order to ascertain their practical efficacy.

1. Introduction

The use of biocontrol agents (BCAs) to control postharvest fruit diseases is still constrained by the lack of high levels of disease control required in the postharvest phase, i.e. more than 95% (Chalutz E. and Droby S., 1997). Their inconsistent activity is one of the main reasons preventing their routine application on fruit after harvest, followed by the limited tolerance to fluctuating environmental conditions and the difficulties of developing shelf-stable formulated products with biocontrol activity similar to that of fresh cells (Janisiewicz W. *et al.* 1997). Therefore, to overcome these issues, integrated strategies were explored and continue to be one of the fields most investigated in postharvest fruit disease control, in order to achieve maximum effectiveness. The use of BCAs in combination with physical and chemical methods has been widely proposed; physical methods including heat treatments, gamma or UV-C irradiation, and controlled atmosphere (CA) have been studied, achieving interesting results. As for chemical methods, the use of salt additives, chitosan and natural plant

products could represent a valid alternative to only BCAs based treatments. The combination of low doses of fungicides and BCAs was also explored, appearing to be one of the reliable options for large-scale utilization of microbial antagonists in the control of postharvest fungal rots of fruit and vegetables (Droby S. *et al.* 2009). This review examines various combinations of treatments against the main postharvest fruit diseases.

2. Biocontrol agents in association with physical treatments

The use of BCAs combined with physical treatments like hot water (HW) has been shown to be effective in reducing postharvest diseases of fruit. For example, strawberries dipped in water at 55°C for 30 s and treated with a suspension of *Cryptococcus laurentii* (10^8 cfu per ml) showed an incidence of *Rhizopus* rot lower than fruit treated with HW or BCA alone. The percentage of fruit infections was 43%, significantly reduced compared to the 65% and 63% of infections in fruit treated with HW and antagonist, respectively (Zhang H. *et al* 2007). The combination of heat treatment with a biological control agent may have an additive effect on fruit such as berries since heat may partially disinfect the fruit surface, allowing the biological control agent to become established more quickly and completely (Wszelaki A. *et al.* 2003).

The combination of two antagonists, *Candida guillermondii* or *Pichia membranaefaciens,* with HW was evaluated on tomato fruit against *Botrytis cinerea* (Zhong Y. *et al.* 2010). Fruits were treated first by dipping in water at 42°C for 40 m and then treated with BCA. The application of HW did not affect the growth of two BCAs in tomato wounds, while the abiotic stress induced a significant increase in the

activities of phenylalanine ammonia-lyase, chitinase and β-1,3-glucanase in fruit. The mechanism by which HW enhanced the biocontrol efficacy of the antagonistic yeasts could be related to the elicitation of biochemical defense responses in tomato fruit.

When used alone, an isolate of *Aureobasidium pullulans* (PL5) reduced the *M. laxa* infections on peaches to 25.8%, compared with 74.2% for the control, but when applied together with HW (55°C per 50 s) the brown rot incidence decreased to 17.5%, while when HW was used alone the disease incidence was 30%. These results suggest that HW greatly increased the effectiveness of the antagonist (Zhang D. *et al.* 2010). A positive additive effect occurred with the combination of *Pantoea agglomerans* followed by a hot air treatment at 33°C for 6 h in controlling *Penicillium digitatum* of lemons (Torres R. *et al.* 2007). Heat treatment was also effective in reducing *M. fructicola* infections established in field but it did not provide protection for further infections after the treatment, before cool storage. Moreover, the combination of curing with BCA (*Bacillus subtilis*) showed the best brown rot control on 'Baby Gold' and 'Andros' peaches. Fruits were first cured (50°C for 2 h), then treated with BCA and finally inoculated with *M. fructicola* (Casals C. *et al.* 2012).

Some fruits, such as apple, pear, and kiwifruit, are stored for a long time (4-5 months) at low temperatures and in CA conditions in order to delay their senescence. CA storage has been found to be effective on the growth and development of various decay-causing fungi. For example, *P. expansum* is much more effectively inhibited during cold storage in CA than in air (Nilsson R. *et al.* 1956). However, once the fruits are removed from CA, there is no longer a protective effect, though not through any persistent inhibition on the pathogen. Effective BCAs have to be able to survive at refrigerated temperatures (0°-1°C) and low oxygen levels, as reported by Conway *et al.* 2007. For example, the

CA conditions had no adverse effect on the growth of two antagonists *Metschnikowia pulcherrima* or *C. laurentii* when compared to growth in air storage. Both antagonists rapidly colonized apple wounds, growing well at low temperatures and oxygen levels, proving useful for commercial application on apples designated for CA storage. Two *A. pullulans* isolates assayed against the *P. expansum* of apple exhibited greater inhibition on fruit stored at low oxygen levels (0.75% and 1.5%) than at the normal atmosphere (21%), showing an additive effect of low oxygen levels on their fungicidal activity (Mari M. *et al.* 2013).

The control of postharvest diseases can also be obtained applying gamma rays; however the complete elimination of pathogen depends upon dose that, moreover, may affect the fruit quality during storage. The combination of BCAs with gamma irradiation can increase the applied range of irradiation for postharvest control by decreasing the dose Mostafavi *et al.* 2011. The optimum dose of gamma irradiation in combination with *Pseudomonas fluorescens* for integrated management control of *P. expansum* was investigated on 'Golden Delicous' apple. Mostafavi *et al.* 2013 observed that *P. fluorescens* generally inhibited the growth of *P. expansum* similarly to irradiation at 200-400 Gy. On the other hand, irradiation at 600 Gy, the lethal dose for *P. expansum,* was negatively correlated with firmness and could accelerate softening of stored apples after 6 and 9 months storage. The combination of *P. fluorescens* and irradiation at 200-400 Gy could inhibit softening apple fruit during storage, controlling the green mould.

A possible synergistic effect between *D. hansenii*, an antagonist yeast, and UV-C irradiation in controlling brown rot incidence on both artificially and naturally infected peaches was observed by Stevens et al. (Stevens C. *et al.* 1997). The superiority of the combined treatment of UV-C and yeast was probably due to the ability of UV-C to

control deep-seated infections such as latent infections, whereas the yeast controlled only superficial infection originating in recent wounds. In addition, the control of latent infections by UV-C but not by an antagonist was achieved with the induction of fungitoxic substances within tissues of UV-C treated peaches (Wilson C.L. *et al.* 1994).

3. Biocontrol agents in association with salt additives, chitosan, natural plant products

Salt additives improve the efficacy of some BCAs in the postharvest sector (El Ghaouth A. *et al.* 2004). Sodium bicarbonate (SBC), commonly used as a food additive and considered a GRAS by the FDA of the United States, used at a concentration of 5%, showed a partial reduction (62.3%) of green mould in postharvest decay of citrus fruit. Similarly, in the same experiment, an antagonist, a marine yeast *Rhodosporidium paludigenum* (10^7 cells per ml), controlled the pathogen, reducing the incidence of disease by 37.4%. The combination of both, SBC and *R. paludigenum,* was as effective as the fungicide, reducing the infected fruit by 95.8% and showing an additive effect between the antagonist and SBC (Zu R.). Moreover, SBC led to a greater reduction of blue mould of apple when used with *M. pulcherrima* and *C. laurentii* during long storages than when used alone (Conway W.S. *et al.* 2007), and it appeared very effective in combination with *A. pullulans* against grey mould of sweet cherries (Ippolito A. *et al.* 2005) and against citrus fruit decay (Porat R. *et al.* 2003; Gamagae S.U. *et al.* 2004). The enhancement of biocontrol activity was also achieved in papaya using SBC and *C. oleophila*. The GRAS was incorporated in the wax coating to control anthracnose caused by *Colletotrichum gloeosporioides* during storage. The wax formulation acted as a medium for delivery of SBC, which may be considered as an

inoculum adjuvant to enhance yeast activity against the pathogen (Gamagae S.U. *et al.* 2004). Among other GRASs, mainly salt ammonium molybdate, despite the modest concentrations required, great reductions were achieved of gray mould in grape fruits or blue mould in peach fruit when combined respectively with *Hanseniaspora uvarum* (Liu H.M. *et al.* 2010) or *P. membranifaciens* (Cao S. *et al.* 2008). Significant effects were also shown when calcium chloride (CCH) was integrated with *C. laurentii* against gray mould of pear fruit (Zhang H. *et al.* 2005) or *A. pullulans* against brown rot of sweet cherries (Ippolito A. *et al.* 2005) or *P. membranifaciens* against antrachnose of loquat fruit (Cao S. *et al.* 2008). While CCH had no direct antifungal activity against postharvest pathogens like *P. expansum* and *B. cinerea* when applied alone to fruit wounds, its combination with the antagonist was more effective to control moulds of pear fruit than *C. laurentii* alone (Yu T. *et al.* 2012). The BCA could act as a primary defense line, inhibiting the initial attack by the pathogen, while CCH could induce resistance triggering defense responses in fruit tissue.

Chitosan has been proven to control numerous pre and postharvest diseases (Bautista-Banos S. *et al.* 2006). Treatments with chitosan revealed that it can especially affect the morphology of the pathogen hyphae, inducing defence reactions in plants correlated with enzymatic responses. Postharvest applications of chitosan were effective in reducing the production of pathogenic enzymes like polygalacturonases, pectinase and cellulose (El Ghaouth A. *et al.* 1997), showing a preventive action in delaying the onset of disease rather than offering an enduring resistance or a curative effectiveness (Fajardo J.E. *et al.* 1998). In the attempt to improve the antifungal activity of chitosan the combination with the yeast *C. laurentii* was evaluated against the *P. expansum* of pear (Yu T. *et al.* 2012). The results showed that the most effective concentration of

chitosan able to enhance blue mould control was 0.5% when combined with *C. laurentii*.

Infections of fruit by fungal pathogens often occur in the field, prior to harvest; pre-harvest treatment with BCAs would therefore be advantageous in order to reduce initial infection and to suppress pathogens in storage (Teixido N. *et al.* 1998). However, the combination of preharvest treatment with *C. laurentii* and chitosan coating of table grapes enhanced the control of fruit decay to a greater extent than the preharvest treatment alone (Meng X. *et al.* 2010).

The possibility to increase BCAs activity with natural substances was investigated for different combinations of substances and antagonists. Recently, a mixture of crude extract of *Eugenia caryophyllata* (15,000 mg/L) and *C. utilis* (10^8 cfu per mL) was established to be the best combination to attain a reduction of 90.3% of *P. digitatum* on citrus fruit (Sukorini H. *et al.* 2013). C. *laurentii* in combination with methyl jasmonate (MeJA) (naturally occurring plant regulator) inhibited *M. fructicola,* although MeJa had a slight inhibitory effect on the mycelial growth of pathogen; a concentration of 200 µmol per L increased the population of the antagonist and induced a stronger disease resistance in fruit than MeJa or yeast alone, resulting in a lower lesion diameter (Yao H.J. *et al.* 2005).

Essential oils (Eos) and their components are gaining increasing interest due to their volatility and relatively safe status; they have been widely investigated against postharvest fungal pathogens and previously reviewed (Mari M. *et al.* 2011). In addition, the monoterpene components of Eos increased the biofilm formation in gram-positive bacteria such as *B. amyloliquefaciens* (Sandasi M. *et al.* 2008)and it is known that the biofilm is important for the bacteria's ability to act as a biocontrol agent (Bais

H.P. *et al.* 2004). Therefore, the combination of thyme and lemongrass oils with *B. amyloliquefaciens* PPCB004 was tested against *B. cinerea* and *Rhizopus stolonifer* of peach. The biofilm formation of BCA was significantly higher in lemongrass oil than time oil and completely inhibited the mycelial growth of the pathogens (Arrebola E. *et al.* 2010).

4. Use of biocontrol agents in addition with low doses of fungicides

Considering an integrated control strategy to reduce postharvest fungal diseases, the resistance of BCAs to fungicides represents an important issue that still has to be investigated in depth. However, fungicides like imazalil and thiabendazole have been exceptionally effective in controlling postharvest diseases and it is quite difficult to find microbial antagonists able to perform as effectively as these (Sharma R.R. *et al.* 2009). The compatibility between a BCA and a synthetic fungicide could offer the option to use low dosages of fungicide and antagonist, thus helping to minimize the residue, the cost of biocontrol treatment and contributing also to management of fungicide resistance (Lima G. *et al.* 2011). Using this approach in pear, the combination of *C. laurentii* and pyrimethanil at low concentrations caused a greater inhibition of blue mould than the application of both at low concentrations (Yu C. *et al.* 2013). Lima *et al.* tested the compatibility of *Rhodosporidium kratochvilovae* and *C. laurentii* with boscalid, cyprodinil and fenhexamid to control blue mould on apples; the combination of low concentrations of BCAs and low doses of fungicides proved more efficient than thiabendazole treatment. *Trichoderma viride* (*T.v*-CIAH240) and low doses of fungicides were found to be effective against latent infections of *Alternaria alteranata* of over ripened ber fruit (*Ziziphus mauritiana*); the efficiency was more than 80% if the

biocontrol agent was used with fungicide at a concentration of 100 µg per g (Natallambi P. *et al* 2009). Mixing low doses of cypronidil and *P. syringae*, the development of *P. expansum* on apple and pear fruit in storage was reduced significantly (Errampalli D. *et al.* 2006; Sugar D. and Basile S.R. 2008).

5. Conclusion

Although the management of postharvest diseases by employing BCAs has been successfully evaluated on many species and against many pathogens, their efficacy could be increased by adopting an integrated strategy that combines antagonist and physico-chemical methods, including the use of fungicides at low doses. Since it is unrealistic to assume that BCAs have the same fungicidal activity as pesticides, the evaluation of their compatibility with other methods seems to be the main topic that will be developed in the near future. One approach that uses the integration of different treatments could benefit from their additive or synergic effects and improve the efficacy of each method. Moreover, specific appropriate strategies have to be evaluated for each species and pathogen in order to tailor a complete integrated disease management.

References

Arrebola E, Sivakumar D, Bacigalupo R and Korsten L. Combined application of antagonist *Bacillus amyloliquefaciens* and essential oils for the control of peach postharvest diseases. Crop Protection 2010: 29: 369-377.

Bais HP, Fall R and Vivanco JM. Biocontrol of *Bacillus subtilis* against infection of Arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiology 2004: 134: 307-319.

Bautista-Baňos S, Hernàndez-Lauzardo AN, Velàzquez-del Valle MG, Hernàndez-Lòpez M, Ait Barka E, Bosquez-Molina E and Wilson CL. Chitosan as a potential natural compound to control pre and postharvest diseases of horticultural commodities. Crop Protection 2006: 25: 108-118.

Cao S, Yuan Y, Hu Z and Zheng Y. Combination of *Pichia membranefaciens* and ammonium molybdate for controlling blue mould caused by *Penicillium expansum* in peach fruit. International Journal of Food Microbiology 2010: 141: 173-176.

Cao S, Zheng Y, Tang S, Wang K. Improved control of anthracnose rot in loquat fruit by a combination treatment of *Pichia membranifaciens* with CaCl$_2$. International Journal of Food Microbiology 2008: 126: 216-220.

Casals C, Elmer P, Viňas I, Teixidò N, Sisquella M and Usall J. The combination of curing with either chitosan or *Bacillus subtilis* CPA-8 to control brown rot infections caused by *Monilinia fructicola*. Postharvest Biology and Technology: 2012: 64: 126-132.

Chalutz E and Droby S. Biological control of postharvest diseases. In: Plant microbe interaction and biological control. Edited by Boland GJ and Kuykendall LD (editors). New York, US: Dekker; 1997 pp. 157-170.

Conway WS, Janisiewicz WJ, Leverentz B, Saftener RA and Camp MJ. Control of blue mold of apple by combining controlled atmosphere, an antagonist mixture, and sodium bicarbonate. Postharvest Biology and Technology 2007: 45: 326-332.

Droby S, Wisniewski M, Macarisin D and Wilson C. Twenty years of postharvest biocontrol research: is it time for new paradigm? Postharvest Biology and Technology 2009: 52: 137-145.

El Ghaouth A, Arul J, Wilson C and Benhamou N. Biochemical and cytochemical aspects of the interactions of chitosan and *Botrytis cinerea* in bell pepper fruit. Postharvest Biology and Technology 1997: 12: 183-194.

El Ghaouth A, Droby S, Wilson CL, Wisniewski M, Smilanick J and Korsten L. Biological control of postharvest diseases of fruits and vegetables. In: Applied Mycology and Biotechnology. Agriculture and Food Production. Edited by Khachatourians GG and Arora DK (editors). Amsterdam, The Netherlands: Elsevier Science; 2004 pp. 11–27.

Errampalli D and Brubacher NR. Biological and integrated control of blue mold (*Penicillum expansum*) of apples by *Pseudomonas syringae* and cyprodinil. Biological Control 2006: 36: 49-56.

Fajardo JE, McCollum TG, McDonald RE and Mayer T. Differential induction of proteins in orange flavedo by biologically based elicitors and challenged by *Penicillium digitatum* Biological Control 1998: 13: 143-151.

Gamagae SU, Sivakumar D and Wijeratnam RLC. Evaluation of post-harvest application of sodium bicarbonate-incorporated wax formulation and *Candida oleophila* for the control of anthracnose of papaya. Crop Protection 2004: 23: 575–579.

Ippolito A, Schena L, Pentimone I and Nigro F. Control of postharvest rot of sweet cherries by pre- and postharvest applications of *Aureobasidium pullulans* in combination with calcium chloride or sodium bicarbonate. Postharvest Biology and Technology 2005: 36: 245-252.

Janisiewicz WJ and Jeffers SN. Efficacy of commercial formulation of two biofungicides for the control of blue mold and gray mold of apple in cold storage. Crop Protection 1997: 16 :629-633.

Lima G, Castoria R, De Curtis F, Raiola A, Ritieni A and De Cicco V. Integrated control of blue mould using new fungicides and biocontrol yeasts lowers levels

of fungicides residues and patulin contamination in apples. Postharvest Biology and Technology 2011: 60: 164-172.

Liu HM, Guo JH, Luo L, Liu P, Wang BQ, Cheng YJ, Deng BX and Long CA. Improvement of *Hanseniaspora uvarum* biocontrol activity against gray mold by the addition of ammonium molybdate and the possible mechanisms involved. Crop Protection 2010: 29: 277-282.

Mari M, Neri F and Bertolini P. Fruit postharvest disease control by plant bioactive compounds. In: Natural antimicrobials in food safety and quality. Edited by Rai M and Chikindas M (editors). Cambridge, USA: CABI; 2011 pp. 242-260.

Mari M, Rouissi W, Martini C and Spadoni A. Biocontrol of fruit postharvest diseases by *Aureobasidium pullulans*. IOBC-WPRS Bulletin 2013: 86: 49-54.

Mostafavi HA, Mirmajlessi H, Fathollahi H, Minassyan V and Mirjalili SM. Evaluation of gamma irradiation effect and *Pseudomonas fluorescens* against *Penicillium expansum*. African Journal of Biotechnology 2011: 10: 11290-11293.

Meng X, Qin G and Tian S. Influences of preharvest spraying *Cryptococcus laurentii* combined with postharvest chitosan coating on postharvest diseases and quality on table grapes in storage. LWT- Food Science and Technology 2010: 43: 596-601.

Mostafavi HA, Mirmajlessi SM, Fathollahi H, Shahbazi S and Mirjalili SM. Integrated effect of gamma radiation and biocontrol agent on quality parameters of apple fruit: An innovative commercial preservation method. Radiation Physics and Chemistry 2013: 91: 193-199.

Nallathambi P, Umamaheswari C, Thakore BBL and More TA. Post harvest management of ber (*Ziziphus mauritiana* Lambk) fruit rot (*Alternaria alternata* Fr. Keissler) using *Trichoderma* species, fungicides and their combinations. Crop Protection 2009: 28: 525-532.

Nilsson R, Nyhlen A, Nilsson R, Stoy V and Nilson T. Carbon dioxide storage of apples and pears 1951-1954. Journal Report Swedish Academic Agriculture 1956: 93: 318-647.

Obagwu J. and Korsten L. Integrated control of citrus green and blue molds using *Bacillus subtilis* in combination with sodium carbonate or hot water. Postharvest Biology and Technology 2003: 28: 187-194.

Porat R, Daus A, Weiss B, Cohen L and Droby S. Effects of combining hot water, sodium bicarbonate and biocontrol on postharvest decay of citrus fruit. The Journal of Horticulture Science and Biotechnology 2003: 77: 441-445.

Sandasi M, Leonard CM and Viljoen AM. The effect of five common essential oil components on *Listeria monocytogenes* biofilms. Food Control 2008: 19: 1070-1075.

Sharma RR, Singh D and Singh R. Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: a review. Biological Control 2009: 50: 205-221.

Stevens C, Khan VA, Lu JY, Wilson CL, Pusey LP, Igwegbe ECK, Kabwe K, Mafolo Y, Liu J, Chalutz E and Droby S. Integration of ultraviolet (UV-C) light with yeast treatment for control of postharvest storage rots of fruits and vegetables. Biological Control 1997: 10: 98-103.

Sugar D and Basile SR. Timing and sequence of postharvest fungicide and biocontrol agent applications for control of pear decay. Postharvest Biology and Technology 2008: 49: 107-112.

Sukorini H, Sangchote S and Khewkhom N. Control of postharvest green mold of citrus fruit with yeasts, medicinal plants, and their combination. Postharvest Biology and Technology 2013: 79: 24-31.

Teixido N, Vinas I, Usall J and Magan N. Control of blue mold of apples by preharvest application of *Candida sake* growth in media with different water activity. Phytopathology 1998: 88: 960–964.

Torres R, Nunes C, García JM, Abadias M, Vinas I, Manso T, Olmo M and Usall J. Application of *Pantoea agglomerans* CPA-2 in combination with heated sodium bicarbonate solutions to control the major postharvest diseases affecting citrus fruit at several Mediterranean locations. European Journal of Plant Pathology 2007: 118: 73-83.

Wilson CL, El Ghaouth A, Chalutz E, Droby S, Stevens C, Lu JY, Khan V and Arul J. Potential of induced resistance to control postharvest diseases of fruits and vegetables. Plant Disease 1994: 78: 837-844.

Wszelaki AL and Mitcham EJ. Effect of combinations of hot water dips, biological control and controlled atmospheres for control of gray mold on harvested strawberries. Postharvest Biology and Technology 2003: 27: 255-264.

Yao HJ and Tian SP. Effects of a biocontrol agent and methyl jasmonate on postharvest diseases of peach fruit and the possible mechanisms involved. Journal of Applied Microbiology 2005: 98: 941-950.

Yu C, Zhou T, Sheng K, Zeng L, Ye C, Yu T and Zheng X. Effect of pyrimethanil on *Criptococcus laurentii*, *Rhodosporidium paludigenum* and *Rhodotorula glutinis* biocontrol of *Penicillium expansum* infection in pear fruit. International Journal of Food Microbiology 2013: 164: 155-160.

Yu T, Yu C, Chen F, Sheng K, Zhou T, Zunun M, Abudu O, Yang S and Zheng X. Integrated control of blue mold of pear in fruit by combined application of chitosan, a biocontrol yeast and calcium chloride. Postharvest Biology and Technology 2012: 69: 49-53.

Zhang D, Spadaro D, Garibaldi A and Gullino ML. Efficacy of the antagonist *Aureobasidium pullulans* PL5 against postharvest pathogens of peach, apple and plum and its modes of action. Biological Control 2010: 54: 172-180.

Zhang H, Zheng X, Fu C and Xi Y. Postharvest biological control of gray mold rot of pear with *Cryptococcus laurentii*. Postharvest Biology and Technology 2005: 35: 79-86.

Zhang H, Zheng X, Wang L, Li S and Liu R. Effect of yeast antagonist in combination with hot water dips on postharvest *Rhyzopus* rot of strawberries. Journal of Food Engineering 2007: 78: 281-287.

Zhong Y, Liu J, Li B, Quin G and Tian S. Effects of yeast antagonists in combination with hot water treatment on postharvest diseases of tomato fruit. Biological Control 2010: 54: 316-321.

Zhu R, Yu T and Zheng X. Postharvest control of citrus green mold by the integration of sodium bicarbonate and *Rhodosporidium paludigenum*.[http://www.paper.edu.cn].

General conclusion

A great interest in the alternatives to the use of fungicides has produced an increasing regard about biocontrol agents such as *Aureobasidum pullulans.* Although the management of postharvest diseases by employing BCAs has been successfully evaluated on many species and against many pathogens, their efficacy could be increased by adopting an integrated strategy that combines antagonist and physico-chemical methods, including the use of fungicides at low doses. Since it is unrealistic to assume that BCAs have the same fungicidal activity as pesticides, the evaluation of their compatibility with other methods seems to be the main topic that will be developed in the near future. One approach that uses the integration of different treatments could benefit from their additive or synergic effects and improve the efficacy of each method. Moreover, specific appropriate strategies have to be evaluated for each species and pathogen in order to tailor a complete integrated disease management.

Postharvest disease management in effect needs the development of alternative strategies, like biofungicides. Although a large number of studies are focused on their modes of action, they need to be more developed to better understand the activity and the interaction between them and the environment, in particular postharvest conditions. The importance of each mode of action can vary relating to yeast-pathogen-host. The basic mode of action for yeast is competition for space and nutrients, while for bacteria it could be antibiotic production; however, several other modes of action can interact with each other, supporting the efficacy of BCAs in determining innovative solutions for sustainable fruit production.

In the last few years, the concept of biological control has considerably changed, involving not only a microorganism against its target pathogen but considering a wider notion of control of plant diseases that also takes into account the host and the environment, adopting integrated strategies in order to increase the efficacy level of the means proposed.

The present research involved new strategies to better understand specifically the mechanisms of action of L1 and L8 strains. The study approached several techniques for improving the knowledge of the mode of action of these biocontrol agents throught *in vitro* and *in vivo* trials, molecular techniques and electron microscopy. This reasearch reports the capability of L1 and L8 to control the principal pathogens disease through their mechanisms of action, sometime acting simultaneously, so it's difficult to establish the contribution of a single mechanism to a specific antifungal action, also it's all related to the specie of pathogen. However, L1 and L8 showed good antibiotic, enzymatic and competitiveness capabilities to control the main postharvest pathogens like *Penicillium* spp., *Monilinia* spp.

Studies for improving the knowledge of the host-pathogen-biocontrol agent interaction are also necessary to better understand these microorganisms action.

Next research objectives are i) obtain L1 and L8 strains bioformulation, ii) test the bioformulation effectiveness on the principal postharvest pathogens development and iii) assay their application in field. Also the search for new biocontrol strategies involving physical treatments, natural additives or low doses of fungicides to L1 and L8 action, could be a better control of pre- and postharvest disease to investigate.
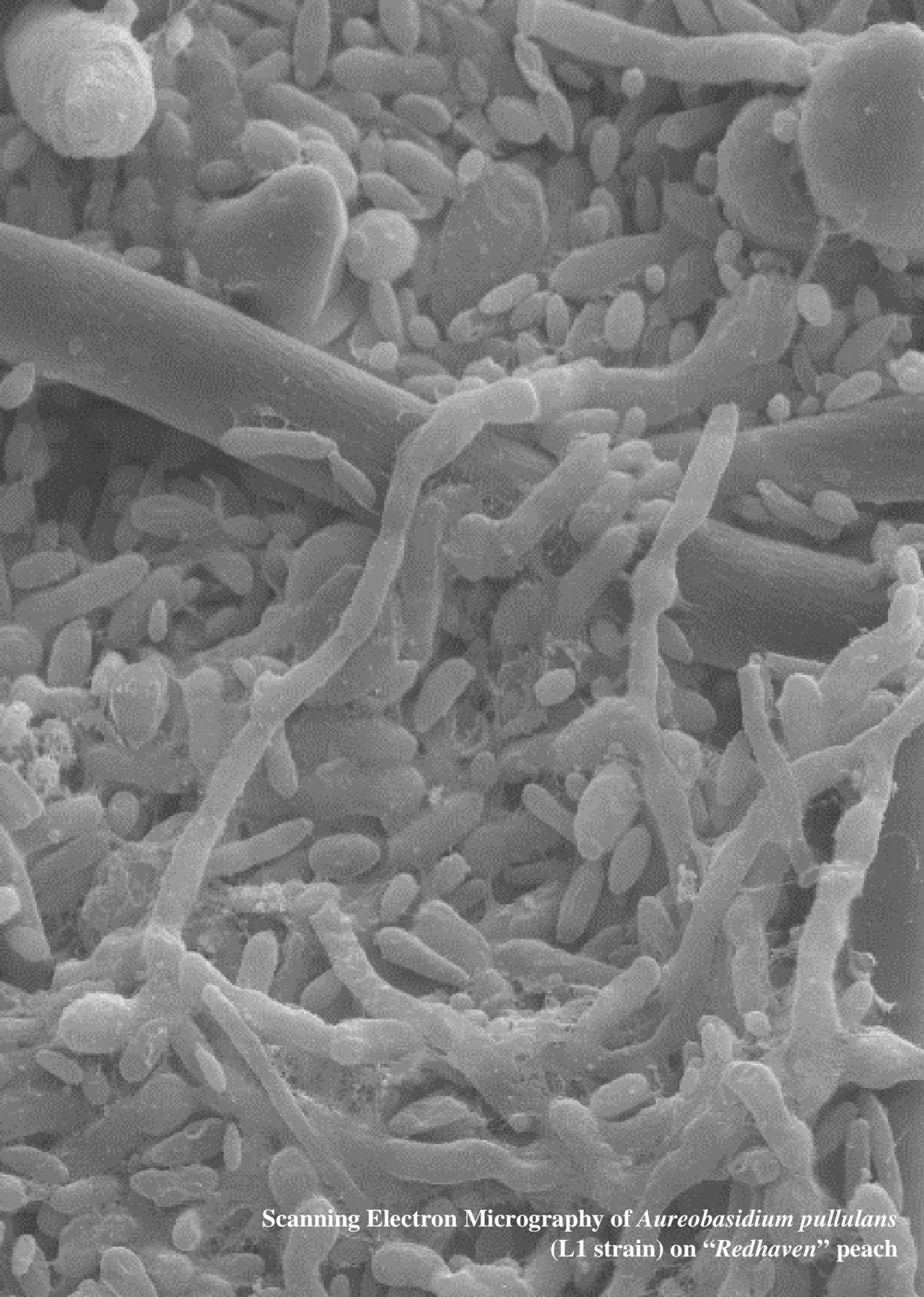
Scanning Electron Micrography of *Aureobasidium pullulans* (L1 strain) on "*Redhaven*" peach