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

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ABSTRACT

The *Aureobasidium pullulans* L1 and L8 strains are known as efficient biocontrol agents against several postharvest fungal pathogens. In order to better understand the mechanism of action underneath the antifungal activity of L1 and L8 strains, yeast cell filtrates grown at different times were evaluated *in vivo* against *Monilinia laxa* on peach. Lesion diameters on peach fruit were reduced by L1 and L8 culture filtrates of 42.5% and 67% respectively. The ability of these filtrates to inhibit *M. laxa* conidia germination and germ tube elongation was studied by *in vitro* assays. The results showed a 70% reduction of conidia germination for both strains while for germ tube elongation, it was 52% and 41% for L1 and L8 culture filtrates respectively. Finally, the activity of cell wall hydrolytic enzymes such as chitinase and glucanase in cell filtrates was analysed and the expression of genes encoding these activities was quantified during yeast growth. From 24 h onward, both culture filtrates contained β,1-3,glucanase and. chitinase activities, the most pronounced of which was *N*-β-acetylglucosaminidase. Gene expression level encoding for these enzymes in L1 and L8 varied according to the strain. These results indicate that L1 and L8 strains culture filtrates retain the yeast antagonistic activity and suggest that the production of hydrolytic enzymes plays an important role in this activity.

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1. Introduction

Monilinia laxa (Aderhold and Ruhland) Honey, *Monilinia fructigena* (Aderhold and Ruhland) and *Monilinia fructicola* (Winter and Honey) are three important fungal pathogens of stone fruit (Byrde and Willetts, 1977). These fungi infect aerial parts of host plants with a variety of symptoms, including blighting of blossoms, cankers on woody tissues and fruit rotting, 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, such as *Kloeckera apiculate* (Long et al., 2007), *Meyerozyma caribbica* (Bautista-Rosales et al., 2013), *Pichia membranifaciens*, *Pichiaanomala* and *Debaryomyces hansenii* (Santos et al., 2004), *Aureobasidium pullulans* (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: dry and wet environments, controlled atmosphere and a wide range of temperatures (Kohl and Fokkema, 1994). Previous works revealed that competition for nutrients (Bencheqroun et al., 2007), induction of host defence (Ippolito et al., 2000), antibiosis, parasitism and production of lytic enzymes (exochitinase, endochitinase and β-1,3-glucanase) (Zhang et al., 2010) are the main mechanisms responsible for yeast efficacy. Promising results were also obtained with *A. pullulans* L1 and L8 strains, isolated from the surface of 'Redhaven' peaches, active against brown rot of stone fruit (Mari et al., 2012a); however, besides the recently reported production of volatile organic compounds (Di Francesco et al., 2015), little is known about the mechanisms of action involved in the biocontrol potential of these two *A. pullulans* strains.

Based on the above considerations, the main aim of this study was to evaluate the antifungal activity of *A. pullulans* L1 and L8

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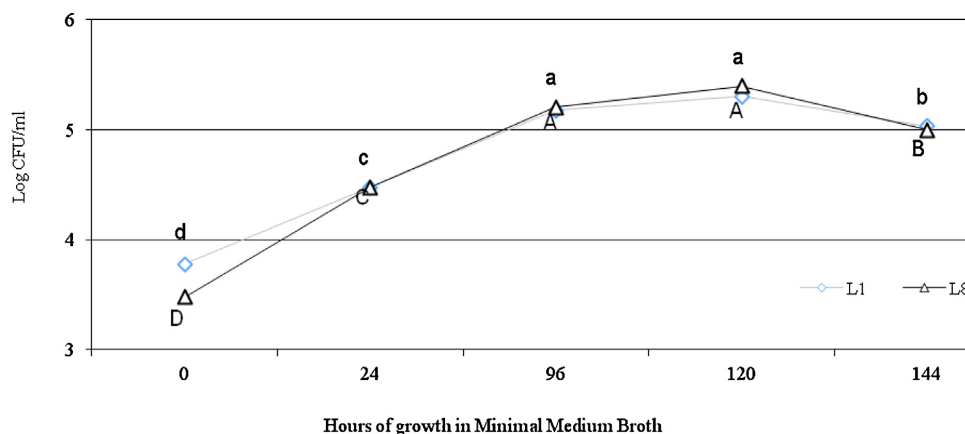

Fig. 1. Population dynamics of *Aureobasidium pullulans* (L1 and L8 strains) cultured in Minimal Medium Broth at 25 °C for 144 h. Each point represents the mean of the number of colony forming units (CFUs) from three replicates (flask) + error bars, each plated in triplicate at each sampling time.

culture filtrates against *M. laxa*. In particular, (i) the reduction of brown rot severity on artificially infected peaches; (ii) the inhibition of *M. laxa* conidia germination and germ-tube elongation, (iii) the display of chitinolytic (*N*-acetyl-β-glucosaminidase and endo-chitinase) and glucanolytic (β-1,3-glucanase) enzymatic activities and (iv) the expression of the genes encoding for these enzymes were investigated.

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. (2012a,b), 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, both antagonists were cultured in NYDB (NYDA without agar) at 25 °C in a rotary shaker (250 rpm) for 0 (1 h), 24, 48, 72 and 96 h. The cultures of each incubation time were centrifuged at 5000 × *g* for 20 min at 4 °C and, supernatants were sterilized with Millex-GV 0.22 μm syringe filters (Millipore, UK), concentrated and desalted with Ultrafree®-4Centrifugal Filter Unit (Millipore Corporation, USA). The ultrafiltrates (cell 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., 2012a). 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 × 3 × 3 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 days. 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 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.

2.6. 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 incu-

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