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Potential biocontrol activity of a strain of *Pichia guilliermondii* against grey mould of apples and its possible modes of action

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Abstract

The efficacy of *Pichia guilliermondii* strain M8 against *Botrytis cinerea* on apples was evaluated under storage conditions, and its possible modes of action were investigated both *in vitro* and *in vivo* experiments. After storage at 1°C for 120 days, M8 reduced grey mould incidence from 45.3% (control) to 20.0%. In apple juice medium (AJM) and in wound-inoculated apples, M8 at 10^9 and 10^8 cells ml^{-1} inhibited the spore germination of *B. cinerea* and the grey mould development. When co-culturing *B. cinerea in vitro* or *in vivo* in presence of the yeast, neither inactivated cells nor culture filtrate of the yeast had any effect on spore germination or germ tube elongation. In AJM, the spore germination was significantly recovered by the addition of 1% glucose, sucrose and fructose, or 0.5% and 1% of $(NH_4)_2SO_4$, phenylalanine and asparagine. When the pathogen and the yeast were co-incubated in apple wounds with addition of the same nutrients, the inhibition of rots was significantly reduced by the supplemental nutrients. Light microscopy revealed that the yeast strongly adhered to the hyphae and spores of *B. cinerea*. M8 produced hydrolytic enzymes, including β-1,3-glucanase and chitinases in minimal salt media with different carbon sources. Pretreatment with M8 at 10^8 cells ml^{-1} followed by washing, significantly reduced grey mould lesions, suggesting an induction of defence responses. Direct attachment, competition for nitrogen and carbon sources, secretion of hydrolytic enzymes and induction of host resistance play a role in the biocontrol mechanism of *P. guilliermondii* M8 against *B. cinerea*.

***Key words*:** Biological control, *Botrytis cinerea*, Hydrolytic enzymes, Induced resistance, Mechanism of action

1. Introduction

Fruits are highly perishable products, especially during the postharvest phase and major losses are caused by postharvest pathogens (Spadaro and Gullino, 2004). *Botrytis cinerea* is among the most important postharvest pathogens on fruit and vegetables (Snowdon, 1990). When permitted, chemical treatment is a primary method for controlling postharvest diseases of fruits (Janisiewicz and Korsten, 2002). However, pathogen resistance to fungicides (Holmes and Eckert, 1999; Gabriolotto et al., 2009) and concern for public safety have resulted in the cancellation of some of the most effective fungicides in Europe (Regulation 1107/2009 and Directive 2009/128) and the United States (Food Quality Protection Act). Therefore, researches have been focused on the development of alternative control that should be both effective and economically feasible (Lopez-Reyes et al., 2010). The use of microbial antagonists to control postharvest diseases of fruits and vegetables has shown during the last thirty years to be one of the most promising alternatives to fungicides (Wisniewski et al., 1991; Wilson et al., 1993; Droby et al., 2009). Some bacteria, actinomycetes and yeasts showed effectiveness against postharvest diseases of fruit and vegetables (Smilanick et al., 1993; Karabulut et al., 2003; Macagan et al., 2008; Spadaro et al., 2008; Zhang et al., 2010a). Among these microbial antagonists, yeasts that naturally occur on fruits and vegetables have attracted the attention of several researchers as potential antagonists of postharvest diseases due to the fast colonization on fruit surfaces (Droby and Chalutz, 1994; Droby et al., 2009). Some yeasts have been developed, but currently there are only a few commercial products available in the market for postharvest use: BoniProtect® (*Aureobasidium pullulans* de Bary) registered in Germany and

used mostly for the control of postharvest diseases in apples (Weiss et al., 2006), Shemer (*Metschnikowia fructicola* Kurtzman & Droby) registered in Israel and commercially used for postharvest diseases of sweet potatoes, table grapes, strawberries, peppers, and carrots (Kurtzman and Droby, 2001), and CandiFruit (Sipcam Inagri, SA Valencia), based on *Candida sake* and registered in Europe, which has been recently commercialized in Spain (Sanzani et al., 2009).

Pichia guilliermondii Wick has been successfully applied to control postharvest pathogens on a number of fruits and vegetables, such as *P. digitatum* on grapefruits, *P. expansum* on apples, *Rhizopus nigricans* on tomato fruit (Droby et al., 1997; Tian et al., 2002; Scherm et al., 2003; Zhao et al., 2008). However, information about the application of *P. guilliermondii* in controlling grey mould on apples is limited. In addition, the modes of action of *P. guilliermondii* against pathogens have not been fully elucidated.

A better understanding of the modes of action is essential for developing appropriate commercial production and formulation protocols (Spadaro et al., 2010a; 2010b) and for maximizing the potential use of microbial biocontrol agents. The main mode of action of the yeast biocontrol agents is believed to be competition for nutrients and space (Chan and Tian, 2005; Bencheqroun et al., 2007). In particular, competition for amino acids, sugars and Fe^{3+} plays an important role in the mechanism of competition for nutrients of some antagonists (Sipiczki, 2006; Bencheqroun et al., 2007; Saravanakumar et al., 2008). Additional modes of action such as mycoparasitism, induced resistance and the production of lytic enzymes *viz.*, β-1,3-glucanase and chitinase (Ippolito et al., 2000; Saligkarias et al., 2002; Yu et al., 2008) were also proposed.

The strain M8 of *P. guilliermondii*, previously isolated from the rhizosphere of maize cultivated in northern Italy and identified through morphological and molecular tools, showed high biocontrol activity against grey mould on apples. Therefore, the present work aimed at (1) evaluating the efficacy of *P. guilliermondii* strain M8 against *B. cinerea* of apples under controlled conditions; (2) investigating the effects of nutrient sources and Fe^{3+} on the efficacy of *P. guilliermondii* strain M8 against *B. cinerea in vitro* and *in vivo*; (3) studying the dynamics of β-1,3-glucanase and chitinase activities of the yeast *in vitro*; (4) observing the attachment of hyphae of *B. cinerea* by the yeast; and (5) checking the induction of defence responses to *B. cinerea*.

2. Materials and methods

2.1 Fruits, microorganisms and culture conditions

The antagonistic yeast *P. guilliermondii* strain M8 was isolated from the rhizosphere of maize cultivated in northern Italy and identified by sequencing the internal transcribed spacer 1 (ITS1), 5.8S ribosomal RNA gene, and internal transcribed spacer 2 (ITS2) (GenBank Accession n° GU478315) according to Cai et al. (1996) and the D1/D2 domain at the 5' end of the LSU rRNA gene (GenBank Accession n° GU478320) according to Kurtzman and Robnett (1998). The strain was maintained on nutrient yeast dextrose agar (NYDA) slants (nutrient broth 8 g l^{-1}, yeast extract 5 g l^{-1}, glucose 10 g l^{-1} and agar 20 g l^{-1}) in the culture collection centre at AGROINNOVA. The yeast was grown in YPD medium (10 g l^{-1} yeast extract, 20 g l^{-1} peptone casein, 20 g l^{-1} dextrose) at 25°C on a rotary shaker (ASAL, Italy;

200 rpm) for 48 h.

Five strains of *Botrytis cinerea* (De Bary) Whetzel were isolated from rotted peaches and selected for their virulence by inoculation in artificially wounded apples. They were used as a mixture (each strain accounted for 1/5 of the total final concentrations) throughout this work, to ensure a high level of disease. Each strain was maintained on PDA (potato dextrose agar) slants at 4°C and the spores were harvested after the pathogen was incubated on PDA in Petri dishes at 25°C for 7 days. The required concentrations of pathogen conidia were determined by a Bürker chamber (Knittel, Germany).

Apples (cv. Golden Delicious, at a maturity suitable for marketing) for all the experiments were bought from supermarkets.

2.2 Efficacy against grey mould incidence on apples under storage conditions

The cells of antagonist *P. guilliermondii* M8 were diluted in 50 L tank into a final concentration of 10^8 cells ml^{-1}. Apples were treated with the antagonist suspension (10^8 cells ml^{-1}) by dipping in tank for 1 min. Fruit surfaces were allowed to air dry at 25°C for 3 h. Fruits treated with a fungicide solution were used as a chemical control: 30 L of thiabendazole (Tecto 20 S, Elf Atochem Agri Italy, 19,7 % a.i., 30 g a.i. 100 l^{-1}) were used for dipping the fruits. Fruits treated with distilled water served as uninoculated controls. Three replicates of 50 fruits were prepared for each treatment. Apples were then stored at 1°C and 95% relative humidity (RH) in storage chambers. After 120 days of storage, the percentage of rotten apples was recorded. The experiment was repeated twice.

2.3 Effect on spore germination of *B. cinerea*

The effect of *P. guilliermondii* M8 on spore germination of *B. cinerea* was assessed in apple juice medium (AJM) as reported by Zhang et al. (2010b) with some modifications. AJM was prepared by homogenizing the apples with a grinding machine (HR1821-PHILIPS, China) followed by filtering with a Whatman No. 1 filter paper. Yeast cells were harvested by centrifugation at 5000×g for 10 min and then resuspended in sterile ringer solution (Merck, Germany) after growing in 300 ml YPD at 25°C on a rotary shaker (ASAL; 200 rpm) for 48 h. The culture medium was filtered through a 0.22 µm nitro-cellulose filter (Millipore, Billerica, MA, United States) for further use. Living cells of the antagonist (100 µL of a suspension containing 5×10^{7}, 5×10^{8}, 5×10^{9} or 5×10^{10} cells ml^{-1}) or cells (100 µl of a suspension containing 5×10^{9} cells ml^{-1}) inactivated by irradiation (Zhang et al., 2010b) for 30' with a germicidal lamp (General Electric, G15T8) that predominantly emitted UV light of a wavelength of 254 nm at fluence of 1.5 W/m^{2} posed at 5 cm from the cell suspension layer (2 mm thick), were added to tubes containing 4.8 ml AJM. The final living cell concentrations were 1×10^{6}, 1×10^{7}, and 1×10^{8} cells ml^{-1}, respectively. For the culture filtrate treatment, 100 µL of culture filtrate were added to 4.8 mL AJM. Aliquots (100 µl) of *B. cinerea* spore suspension (5×10^{6} spores ml^{-1}) in Ringer solution were transferred to each tube. As a control, 100 µl of *B. cinerea* spore suspension were added to tubes containing 4.9 ml AJM. After 20 h incubation of the 45° sloping tubes at 25°C on a rotary shaker (200 rpm), 100 spores per replicate were observed under microscope and their germination rate and germ tube length were measured. Three replications were prepared for each treatment and the experiments were performed three times.

2.4 Effect on grey mould rot severity on apples

Apples were surface-sterilized with 1% commercial sodium hypochlorite for 1 min followed by rinsing with tap water. Three artificial wounds (3 mm wide x 3 mm deep) along the equatorial zone of the apple were made. The four concentrations of the yeast, inactivated cells and culture filtrate were prepared as in section 2.3. Aliquots of 30 µl of each suspension were pipetted into each wound site for each treatment. After 2 hours of incubation at 25°C, the wounds were inoculated with 30 µl of *B. cinerea* spore suspension at 10^5 spore ml^{-1}. The treatments were designed as described in section 2.3. Three replicates of ten apples were used for each treatment. The wound-inoculated fruits were stored at 20°C and 95% RH. After 5 days, the diameters of the rotten lesions were recorded and the experiments were performed three times.

2.5 Interactions between pathogen hyphae and yeast in apple juice and on apple tissue

The possible interactions of the yeast and pathogen hyphae were evaluated in tubes containing AJM. The yeast at 10^8 cells ml^{-1} and the pathogen at 10^5 spores ml^{-1} were co-incubated in tubes at 25°C for 20 h on a rotary shaker at 200 rpm. Tubes inoculated only with the pathogen served as control. The interactions of the yeast and pathogen were directly observed under light microscope (Axioskop 40, Germany). Each treatment had three replications and the experiments were repeated twice.

To assess the interaction between yeast and *B. cinerea in vivo*, the experiment was prepared as described in section 2.3. Fruits were stored at 20°C and 95% RH for 7 days.

Wounded tissue (2–4 mm^2) was excised with a sterilized surgical blade (Swann Morton, England) from the treated fruit and immediately immersed into FAA fixing solution (89 ml 50% ethanol, 6 ml acetic acid and 5 ml formaldehyde) for 24 h. Samples were later dehydrated in a graded ethanol series and critical-point dried with CO_2. The treated tissues were then subjected to light microscope (Nikon, Eclipse 55i, Japan) and the interactions of the yeast and pathogen were directly observed.

2.6 *In vitro* competition for nitrogen sources, sugars and Fe^{3+} ions

To examine the effects of different nitrogen sources (asparagine, phenylalanine and $(NH_4)_2SO_4$), sugars (glucose, sucrose and fructose) and Fe^{3+} ion on biocontrol activity of *P. guilliermondii* strain M8 against *B. cinerea*, AJM was used throughout the experiment. AJM was prepared as described in section 2.3. The yeast (100 µl containing 5×10^9 cells ml^{-1}) and pathogen spore suspensions (100 µl containing 5×10^6 conidia ml^{-1}) were added to the test tubes (18×150 mm) containing 4.8 ml of AJM supplemented or not with nitrogen sources, sugars and Fe^{3+} ion. The nitrogen and sugars were used at final concentrations of 0.5 and 1.0%, and Fe^{3+} ion was supplemented as $FeCl_3.7H_2O$ at final concentrations of 0.1 mM and 0.5 mM. The inoculated tubes were incubated at 25°C for 20 h on a rotary shaker at 200 rpm. Then, 100 spores were randomly selected out from each tube and their germination was evaluated. Three replications were included for each treatment and the experiment was repeated twice.

2.7 *In vivo* competition for nitrogen sources, sugars and Fe^{3+} ions

To test the effect of different nitrogen sources, sugars and Fe^{3+} ion on the antagonist efficacy *in vivo*, the apples were prepared as described in section 2.3. Three artificial wounds along the equatorial zone of the each were made. Aliquots of 30 µl of each nitrogen source or each sugar at the final concentration of 0.5% and 1%, and Fe^{3+} ion at the final concentrations of 0.1 mM and 0.5 mM were pipetted into each wound site. After 3 hours incubation at 25°C, the wounds were treated with 30 µl of the yeast cell suspension containing 10^8 cells ml^{-1}, followed 3 h later by inoculation of 30 µl of a *B. cinerea* suspension (10^5 spores ml^{-1}). The treatments were designed as follows: the wounds treated with 30 µl of distilled water before treatment with the yeast and inoculated with the pathogen served as positive control and the apples inoculated only with pathogen spore suspension served as negative control. Three replicates of ten apples were used for each treatment. Apples were stored at 20°C for 6 days and the diameter of the rotten lesions was measured and the experiments were repeated twice.

2.8 Preparation of cell wall and colloidal chitin for enzymatic activity studies

Cell wall preparations (CWP) of the pathogen were prepared as described by Saligkarias et al. (2002) with small modifications. Briefly, the pathogen mycelium was harvested after growing in the potato dextrose broth media with four-fold cotton gauzes and then the mycelium was washed twice with deionised water through Whatman No.1 filter paper and centrifuged (Centrifuger: 6K15, Sigma, Germany) at 500×g for 2 min. After removing the supernatant, the mycelial mat was sonicated with a probe type sonicator (USC6000, Malaysia) for 20 min and centrifuged at 500×g for 5 min. The supernatant was removed and the pellet was resuspended in deionised water. Then the crushed mycelium was resuspended into an

equal volume of Tris/HCl buffer (50 mmol per liter and pH 7.2), centrifuged at 1900×g for 15 min, and the supernatant was discarded. The pellet was subjected to three successive cycles of centrifugation and resuspension. The final pellet was frozen with liquid N_2, lyophilized and stored at -20°C for further studies. Colloidal chitin was prepared according to the method provided by Roberts and Selitrennikoff (1988) from shrimp shell chitin. Then, 5 g of chitin powder (C9752, Sigma, USA) was added slowly into 100 ml of concentrated HCl and left at 4°C overnight with vigorous stirring. The mixture was added to 2 l of ice-cold 95% ethanol with rapid stirring and kept overnight at 25°C. The pellet was collected by centrifugation at 3000×g for 20 min at 4°C and washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0). Colloidal chitin solution (5 mg ml^{-1}) was prepared and stored at 4°C for further studies.

2.9 Enzyme production and activity assay

The yeast strain M8 was cultured in modified Lilly-Barnett minimal salt medium (Lilly and Barnett, 1951) containing 2 mg ml^{-1} CWP as sole carbon source. A 30 ml of culture media in 100-ml flask (PYREX, England) was incubated at 25°C on a rotary shaker at 150 rpm for 0, 24, 72, 96 and 120 h. Culture filtrates from each individual flask were collected by centrifuging at 7,000×g for 8 min, and the supernatant was used for enzyme assays. β-1, 3-glucanase activity assay were carried out by measuring the amount of reducing sugars released from laminarin (L9634, Sigma, USA), using glucose as a standard (Masih and Paul, 2002). A reaction mixture was prepared by adding 250 µl of 50 mM potassium acetate buffer (pH 5.0) containing 2.5 mg of laminarin per ml into 250 µl of culture filtrate (Chan and Tian,

2005). The enzyme-substrate mixture was incubated for 2 h at 40°C in water bath (D-3508 Melsungen, Germany). Then 0.5 ml of dinitrosalicylic acid reagent was added, boiling at 100°C for 5 min. After cooling, 2 ml of deionised water was added directly and measured spectrophotometrically at 595 nm. Background levels of reducing sugars were determined with a time zero supernatant substrate just prior to boiling at 100°C for 5 min. The protein concentration of the enzyme solution was determined according to Bradford (1976) by using bovine serum albumin (A1933, Sigma, USA) as a standard. The specific activity was expressed as micromoles of glucose per milligram protein per hour (Fan et al., 2002). Each treatment had three replications and the experiments were performed three times.

The exo-chitinase assay was performed according to Abeles et al. (1970). A reaction mixture was prepared by adding 0.5 ml of 5 mg ml^{-1} colloidal chitin containing 1.2 µmol l^{-1} sodium azide and 56 µmol l^{-1} sodium acetate to 0.5 ml enzyme supernatant. For endo-chitinase assay, a reaction mixture was prepared by adding 0.1 ml of 3% (v/v) desalted snail gut enzyme cytohelicase (C8274, Sigma, USA) and 0.1 ml of 1 mol l^{-1} potassium phosphate buffer (pH 7.1) into 0.5 ml of 5 mg ml^{-1} colloidal chitin in a 2 ml tube and then 0.5 ml of enzyme supernatant was added to the tubes. The enzyme-substrate mixture was incubated for 2 h at 37°C in the water bath with constant shaking. The supernatant was collected from the mixture by centrifuging at 7,000×g for 8 min. In the following, 0.5 ml dinitrosalicylic acid reagent was added, boiled at 100°C for 5 min. After cooling, 1.5 ml deionised water was added directly and measured spectrophotometrically at 550 nm (Chan and Tian, 2005). Background levels of reducing sugars and the protein concentration of the enzyme solution was determined as described above. The specific activity was determined as

micromoles of N-acetyl-D-glucosamine per milligram protein per hour according to Reissig et al. (1955). Each treatment had three replications and the experiments were repeated twice.

2.10 Induction of apple resistance against *B. cinerea*

To assess the effect of different concentrations of the antagonist on elicitation of defence resistance in whole intact fruits was performed according to El-Ghaouth et al. (2003). Intact apples were dipped into the yeast suspension at 10^8, 10^7 and 10^6 cells ml^{-1} for 30 seconds. Apples treated with sterilized distilled water served as controls. The effect of inactivated antagonistic cells was also evaluated. Intact fruits were dipped into the inactivated cell suspension at 10^8 cells ml^{-1} for 30 seconds. After air dry, apples were kept at 20°C for 24 h. The fruits were then surface-sterilized by wiping extensively with pure ethanol to remove yeast cells from the surface and then fruits were gently wounded (2-3 mm deep) with a syringe needle at three different sites around the equatorial region and an aliquot (30 µl) of the pathogen suspension (10^5 spore ml^{-1}) was inoculated into each wound site. After 2 h air-dry, the fruits were stored at 20°C and 95% RH. The diameters of rotten lesions were determined 4 days after inoculation. Each treatment consisted of three replications of 10 fruits each. The experiments were performed three times.

2.11 Statistical analysis

Replications of all the experiments, when the means were similar, were pooled and analyzed together. Means and standard errors for each treatment were reported. Data analysis was performed by using the SPSS software (SPSS Inc., version 13.0, Chicago, IL, USA).

Statistical significance was judged at the level of *p*-value < 0.05. When the analysis of variance was statistically significant, Tukey's test was used to compare the means.

3. Results

3.1 Efficacy against grey mould incidence on apples under storage conditions

After storage at 1°C and 95% relative humidity for 120 days, *P. guilliermondii* M8 reduced grey mould incidence from 45.3% to 20.0% compared to the control. Correspondingly, the efficacy of *P. guilliermondii* strain M8 in controlling grey mould on apples was 55.8% (Table 1).

3.2 Effect on *B. cinerea* spore germination *in vitro* and on grey mould severity on apples *in vivo*

By co-culturing in AJM, the effect of *P. guilliermondii* strain M8, applied as living cell suspensions, inactivated cells or culture filtrate, on *B.cinerea* spore germination and germ tube length were investigated (Table 2). *P. guilliermondii* strain M8, applied at 10^9 cells ml^{-1}, completely inhibited the pathogen spore germination in AJM. When co-cultured at 10^8, 10^7 and 10^6 cells ml^{-1}, the spore germination were inhibited by 97.1%, 86.6% and 74.2%, respectively, and in addition, the germ tube length was also reduced by 80.0%, 68.9% and 48.8%, respectively. However, no significant differences either on the spore germination rate or on the germ tube elongation were observed compared to the control, when the pathogen was co-cultured with inactivated cells or culture filtrate of the yeast.

To determine the effects of *P. guilliermondii* strain M8 on grey mould decay, apples were

inoculated with the pathogen and treated with living cells, culture filtrate or inactivated cells of the yeast. The living cells at 10^9 cells ml^{-1} completely controlled the development of grey mould decay, while the living cells at 10^8, 10^7 and 10^6 cells ml^{-1} reduced the diameter of grey mould decay, to 66.7%, 47.9% and 35.2 %, respectively, compared to the control (Table 2). When apples were treated with culture filtrate or inactivated cells, the mean diameter of grey mould decays were not significantly different from that of inoculated control (Table 2).

3.3 Interactions between pathogen hyphae and yeast in apple juice and on apple tissue

To examine the attachment of the cells of the strain M8 to the hyphae of *B. cinerea*, the pathogen and the yeast were co-cultured in AJM (Fig. 1a). After 20 h, yeast cells strongly adhered to the pathogen conidia and hyhae of *B. cinerea* (Fig. 1b), and the yeast cells showed to prefer the attachment to hyphal tips and conidia, inhibiting to some extent the germ tube elongation. After 7 days of storage at 20°C and 95% RH, in apple fruit wounds, the yeast cells adhered to the hyphae of the pathogen, apparently restricting the proliferation of *B. cinerea in vivo* (Fig. 1d).

3.4 *In vitro* and *in vivo* competition for nitrogen sources, sugars and Fe^{3+} ions

To investigate the potential role of competition for nutrients in the interactions of the strain M8 against *B. cinerea*, the effects of sugars, nitrogen sources and Fe^{3+} were studied on spore germination in apple juice and on grey mould development on apples (Table 3). The *in vitro* experiments showed that addition of glucose, sucrose and fructose reduced the spore germination. Increasing concentrations of sugars, correspondingly increased the spore

germination rate in presence of M8. The addition of 1% $(NH_4)_2SO_4$, 0.5% and 1% phenylalanine, 0.5% and 1% asparagine significantly increased the spore germination rate of the pathogen in presence of M8. Compared to the control, the addition of these substrates also increased the spore germination in absence of M8. However, addition of Fe^{3+} had no effect on spore germination. Similar results were obtained both from *in vivo* and *in vivo* experiments, with the addition of similar nutrients, the inhibition of rotten lesions was significantly reduced by the addition of 1% glucose, 0.5% and 1% sucrose, 1% fructose, 0.5% and 1% $(NH_4)_2SO_4$, phenylalanine and asparagine (Table 3). Summarizing the results *in vitro* and *in vivo*, competition for sugars and nitrates played an important role in the interactions of the strain M8 with *B. cinerea*.

3.5 Production of β-1,3-glucanase and its activity

The antagonistic yeast *P. guilliermondii* strain M8 produced extracellular β-1,3-glucanase in culture media in presence of purified fungal cell walls, sucrose and glucose used as sole carbon source. *P. guilliermondii* strain M8 produced more extracellular β-1,3-glucanase at 24 h post-inoculation (hpi) in the three Lilly-Barnett minimal salt media, with *B. cinerea* cell walls, sucrose and glucose as sole carbon sources. At 48 hpi, the activities of extracellular β-1,3-glucanase were similar to those at 24 hpi. While at 72 hpi, the yeast increasingly produced extracellular β-1,3-glucanase and its activity increased. At 96 hpi, extracellular β-1,3-glucanase activity reached the maximum level and the activities in the minimal salt medium with *B. cinerea* cell walls, sucrose and glucose, respectively, as sole carbon sources, were 124.6, 132.7 and 125.8 U (μmol glucose released/mg protein/h),

respectively. However, at 120 hpi, the β-1,3-glucanase activity started to decrease (Fig. 2a).

3.6 Production of chitinase and its activity

The assay of exo-chitinase activity showed that *P. guilliermondii* strain M8, when cultured in the three Lilly-Barnett minimal salt media with *B. cinerea* cell walls, sucrose and glucose as sole carbon sources, demonstrated similar trends with different incubation periods (hpi). The maximum level of exo-chitinase activity was detected between 48 hpi and 72 hpi. At 96 hpi, the activities of exo-chitinase with *B. cinerea* cell walls, sucrose and glucose as sole carbon sources were 2.36, 2.33 and 2.05 U (µmol GlcNAc released/mg protein/h), respectively (Fig.2b).

The yeast also had the capability of producing endo-chitinase when cultured in Lilly-Barnett minimal salt medium with *B. cinerea* cell walls, glucose and sucrose used as carbon sources. The endo-chitinase activity reached the maximum level at 48 hpi and the endo-chitinase activities were 8.6, 8.0 and 7.6 U, respectively for *B. cinerea* cell walls, glucose and sucrose (Fig. 2c). No significant differences were experienced between the three media tested.

3.7 Induction of apple resistance against *B. cinerea*

After 4 days of storage at 20°C and 95% RH, the diameters of the rotten lesions of the apples treated with the strain M8 at 10^8, 10^7 and 10^6 cell ml^{-1} were 22, 26 and 27 mm, respectively, and were lower than the control (29 mm (Fig. 3.). The diameters of rotten lesions of the fruits treated with the strain M8 at high concentration of 10^8 cell ml^{-1} were

significantly different from those of the control treatment, suggesting that at the highest cell concentration *P. guilliermondii* strain M8 caused an induction of disease resistance to *B. cinerea* on apples.

4. Discussion

P. guilliermondii strain M8 showed effective biocontrol capabilities, both in storage and controlled conditions, in reducing the disease incidence and severity of grey mould of apples caused by *B. cinerea*. Though some postharvest biocontrol agents are already on the market, great potential exists for new biofungicide products, to be commercialized on a global scale for the postharvest control of fungal diseases.

Multiple modes of actions were involved in the biocontrol of *B. cinerea* by the strain M8, but most biocontrol agents use several modes of action against the pathogens (Janisiewicz and Korsten, 2002; Ippolito et al., 2005). Extensive production of extracellular lytic enzymes by the yeast antagonist, especially β-1,3-glucanase and chitinase, may play an important role by enhancing either nutrient competition or other unknown mechanisms (Fan et al., 2002; Masih and Paul, 2002; Chan and Tian, 2005). To our knowledge, this work represents the first determination of the mechanisms used by the yeast *P. guilliermondii* to control *B. cinerea* on apples.

In this research, the enzymes activities assay showed that *P. guilliermondii* strain M8 produced β-1,3-glucanase, exo-chitinase and endo-chitinase in Lilly-Barnett minimal salt medium with *B. cinerea* cell walls, or glucose or sucrose as sole carbon source. The β-1,3-glucanase activities of *P. guilliermondii* strain M8 with cell wall preparation (CWP)

were far higher than those of *P. guilliermondii* strain US7 (Castoria et al., 1997) and *P. guilliermondii* strain R13 (Chanchaichaovivat et al., 2008), when grown on CWP. In addition, the exo-chitinase and endo-chitinase activities of *P. guilliermondii* strain M8 were also higher than those of the antagonistic yeasts *Metschnikowia pulcherrima* strain MACH1 and *Rhodotorula* sp. PW34 (Saravanakumar et al., 2009), and *Pichia membranaefaciens* and *Cryptococcus albidus* (Chan and Tian, 2005). Similar enzymatic activities from *P. guilliermondii* strain M8 were detected on the three different carbon sources used. These results suggest that *P. guilliermondii* strain M8 possesses a stronger capability of producing enzymatic activities when compared with other antagonistic yeast strains under similar conditions. Most phytopathogenic fungi have cell walls composed of complex polymers of β-1,3- and β-1,6-glucans, mannoproteins, where chitin as a structural backbone is arranged in regularly ordered layers, and β-1,3-glucan as a filling material is arranged in an amorphous manner (Smits et al., 2001; Cheng et al., 2009). Thus, breakdown of the fungal cell wall requires the participations of the different enzymes, especially β-1,3-glucanases and chitinase (Marcello et al., 2010). Therefore, to further elucidate the roles of hydrolytic enzymes also *in vivo*, the glucanase and chitinases genes from the antagonist yeast *P. guilliermondii* strain M8will be cloned and characterized.

Light microscope observations revealed that the fungal hyphae were strongly surrounded attached by the antagonistic cells in AJM, and spores and germ tubes of the pathogen were massively colonized by *P. guilliermondii* strain M8 in wounded tissue. Attachment of fungal hyphae as an important mode of action in controlling pathogens has been discussed and demonstrated in studies of other antagonistic yeasts including *Candida oleophila* strain I-182

colonizing the hyphal walls of *B. cinerea* (Saligkarias et al., 2002), and *P. membranefaciens* and *C. albidus,* which attached the hyphal walls of *Monilinia fructicola, Penicillium expansum* and *R. stolonifer* (Chan and Tian, 2005). Wisniewski et al. (1991) showed that the attachment of the hyphal walls of *B. cinerea* by *P. guilliermondii* strain 87 could be blocked by agents that alter protein integrity and certain sugars, and similar results were observed on the fungal by *P. membranefaciens* and *C. albidus* cells (Chan and Tian, 2005), suggesting that some functional proteins of the antagonists and pathogens were involved in the attachment process. However, the attachment process and regulation mechanisms of the process are still unknown.

Competition for nutrients and space is considered the main mode of action of yeast biocontrol agents. Our research showed that competition for sugars and nitrates plays a key role in the interactions of *P. guilliermondii* strain M8 with *B. cinerea*. The carbon, nitrogen and iron sources tested are common sources generally used by microorganisms. Yeasts can satisfactorily use a wide range of carbohydrates, which includes the disaccharide sucrose and the monosaccharides glucose and fructose (Spadaro et al., 2010a). Based on the specific growth rate in glucose containing media, nitrogen sources are classified as ‘good’(‘preferred’) or ‘poor’ (‘nonpreferred’) (Magasanik and Kaiser, 2002). Good nitrogen sources are generally easily converted into glutamate and glutamine, with asparagine as an example (Hofman-Bang, 1999). Phenylalanine is considered to be an ‘average’ nitrogen sources (Magasanik and Kaiser, 2002). Ammonium sulphate $(NH_4)_2SO_4$ was tested to check the competition for ammonium ion of the BCA and the pathogen (El-Mansi and Bryce, 1999). Finally, iron is essential for the fungal growth and pathogenesis, and iron sequestration by non-pathogenic microbes could be

exploited in novel systems for biological control of postharvest pathogens (Saravanakumar et al., 2008). Addition of increased concentrations of glucose, sucrose, fructose, phenylalanine, asparagine and $(NH_4)_2SO_4$ significantly reduced the spore germination of pathogen in the presence of the antagonist in AJM, which is in according to the result obtained *in vivo*, suggesting that the antagonist is competing with *B. cinerea* for sugars and nitrogen sources. Moreover, competition for nutrients plays a major role in the activities of the antagonist against the postharvest pathogens and this is also supported by the fact that different concentrations of the antagonist co-cultured with the pathogen in AJM influenced the spore germination and germ tube elongation. When the concentrations of the antagonist were lower, the spore germination rate significantly increased and the germ tubes were significantly longer.

Iron (Fe^{3+}) is biologically important, being a constituent of cytochrome and other heme or non-heme proteins and also a co-factor in various fungal enzymes (Meziane et al., 2005; Macagan et al., 2008). Previous investigations have demonstrated that competition for Fe^{3+} is the main modes of action of some antagonistic yeast against *B. cinerea* (Sipiczki, 2006; Saravanakumar et al., 2008). However, both *in vitro* and *in vivo* experiments showed that the addition of different concentrations of Fe^{3+} had no effect on the spore germination in the presence of the antagonist, indicating that Fe^{3+} is not involved in the interaction of the antagonist against *B. cinerea* in this study.

The study of the effects of *P. guilliermondii* strain M8 on *B. cinerea in vitro* and *in vivo* shows that the strain is strongly concentration-dependent in controlling the pathogen. Culture filtrate or inactivated cells of the yeast had no effect on the pathogen spore germination or

germ tube elongation or on the grey mould development, showing that antibiotic compounds or killer toxin were not involved in the interactions of the antagonist and pathogen.

Induction of host plant resistance was also proposed as one of the modes of actions of some antagonists in controlling pathogens (Castoria et al., 2003; Tian et al., 2007). *P. guilliermondii* strain M8, when applied at high concentration, could induce the resistance of host apples to *B. cinerea*, significantly inhibiting the grey mould decay. Resistance to pathogens on fruits induced by other antagonistic yeasts has been documented: resistance to *B. cinerea* on apples induced by *Aureobasidium pullulans* (Ippolito et al., 2000); resistance to *P. digitatum* in grapefruits by *C. oleophila* (Droby et al., 2002); or resistance to postharvest decays on apples by *C. saitoana* (EI-Ghaouth et al., 2003). The resistance induction is due to the antagonist ability to elicit host plant defence responses. This process always involves several chemical or biochemical reactions in the host tissue, including changes of tissue structure and production of pathogenesis-related proteins, which can be expressed locally or systemically (Kloepper et al., 1992; van Loon, 1997). Peroxidase, polyphenoloxidase, catalase, phenylalanine ammonialyase and β-1,3-glucanase have been proved to be involved in plant defence responses against fungal infection (Pieterse et al., 2002; van Loon, 2007). Therefore, to further investigate the defence responses of apples induced by *P. guilliermondii* strain M8, the specific activities of peroxidase, polyphenoloxidase, catalase, phenylalanine ammonialyase, superoxide dismutase and β-1,3-glucanase in pericarp and flesh tissues of the strain M8-treated fruits will be measured and confirmed with molecular tools.

Summarizing, it can be concluded that production of β-1,3-glucanase and chitinase, direct attachment, competition for sugars and nitrogen sources, and indirect induction of

defence response as well as other undetermined mechanisms play a role in the interactions of *P. guilliermondii* strain M8 with *B. cinerea*.

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Legends:

Figure 1

a) *B. cinerea* in AJM (magnification 150 ×; bar = 40 µm); **b)** interaction of *P. guilliermondii* strain M8 with *B. cinerea* in AJM (magnification 150 ×; bar = 40 µm); **c)** apple pulp tissue (*Malus* × *domestica*; M) (magnification 150 ×; bar = 40 µm); **d)** interaction of *P. guilliermondii* strain M8 with *B. cinerea* in apple pulp tissue (magnification 150 ×; bar = 40 µm)**.***

***** A**T** – Apple tissue; BH - *B. cinerea* hyphae; BC - *B. cinerea* conidia; PC - *P. guilliermondii* strain M8 cells

Figure 2

Hydrolytic enzyme activities of *P. guilliermondii* strain M8 grown in Lilly-Barnett minimal salt medium supplemented with 2 mg ml^{-1} CWP, glucose and sucrose as sole carbon source at 25°C for 120 h: **a)** β-1,3-glucanase (EC 3.2.1.39) activity; **b)** exo-chitinase (EC 3.2.1.52); **c)** endo-chitinase (EC 3.2.1.14) activity. Bars represented standard deviations of the means.

Figure 3

Induction of apple resistance to *B. cinerea* by *P. guilliermondii* strain M8. Apples cv. Golden Delicious were treated with the antagonist suspensions at 10^8, 10^7 and 10^6 cells ml^{-1}, by dipping for 30 seconds, respectively, and incubated at 20°C and 95% RH for 48 h, followed by removing the cells from the fruits. The apples were then wound inoculated with *B. cinerea*

suspension at 10^5 spores ml^{-1} and after 4 days of storage at 20°C and 95% RH, the diameters of the rotten lesions were recorded. Values of each column marked by different letters show significant difference ($P<0.05$) according to analysis by Tukey's Test (SPSS 13.0). Bars represented standard deviations of the mean.

Table 1.

Efficacy of *P. guilliermondii* M8 against *B. cinerea* on apples cv. Golden Delicious under storage conditions. Apples were dipped in the antagonist suspension (10^8 cells ml^{-1}) for 1 min, air-dried at 25°C for 2 h, and then stored at 1°C and 95% RH. After 120 days of storage, the incidence of rotten apples was counted. Fruits treated with the fungicide solution played as chemical treatment, while fruits treated with distilled water served as uninoculated control.

Treatments	*B. cinerea* disease incidence (%)*	
P. guilliermondii M8	20.0 ± 4.0	b
Thiabendazole*	10.7 ± 2.3	a
Uninoculated control	45.3 ± 4.0	c

* The results are the mean of two independent experiments. "±" stands for standard error of the means. Values of each column followed by different letters show significant difference ($P<0.05$) according to analysis by Tukey's Test (SPSS 13.0).

"±" stands for standard deviation of the means. Values followed by the same letter are not statistically different

* Fruits were treated with thiabendazole (Tecto 20 S, Elf Atochem Agri Italy, 19,7 % a.i., 30 g a.i. 100 l^{-1}).

Table 2.

Effect of *P. guilliermondii* strain M8 on *B. cinerea* spore germination and germ tube elongation in AJM and decay development in wound inoculated apples cv. Golden Delicious.

Treatments	*In vitro*		*In vivo*
	Spore germination (%)*	Germ tube length (μm)*	Diameter of rotten lesions (mm)*
10^9 cells ml^{-1}	0.0±0.0 a	0.0±0.0 a	0.0 ± 0.0 a
10^8 cells ml^{-1}	2.7±0.6 a	79.8±12.6 b	11.2 ±1.2 b
10^7 cells ml^{-1}	12.3±2.5 b	112.6±12.6 b	17.6 ±2.6 c
10^6 cells ml^{-1}	23.7±3.2 c	185.4±7.8 c	21.9 ±2.1 d
Inactivated cells	92.7±0.6 d	369.1±14.6 d	33.2 ±1.3 e
Culture filtrate	91.7±1.5 d	362.0±18.2 d	32.9 ±1.0 e
Inoculated control	91.7±2.9d	362.4±23.7 d	33.8 ±1.9 e

* The results are the mean of two independent experiments. "±" stands for standard error of the means. Values of each column followed by different letters show significant difference (P<0.05) according to analysis by by Tukey's Test (SPSS 13.0).

Table 3.

Effects of the yeast *P. guilliermondii* strain M8 along with sugars, nitrogen substrates and Fe^{3+} on control of *B. cinerea in vitro* on AJM and *in vivo* on apples cv. Golden Delicious. To assess the spore germination incidence, 100 spores were randomly selected from each replication and the germ tube length was measured microscopically.

Treatments	*In vitro* Spore germination (%)*		*In vivo* Diameter of lesions (mm)*	
Control	90 ±1.5 i		46.3 ± 3.5 g	
yeast	2 ± 0.6 a		11.5 ± 4.4 a	
	Concentration			
	0.5%	1.0%	0.5%	1.0%
glucose	91±2.3 i	92 ±2.1 i	46.4 ± 4.0 g	49.5 ± 4.50 ghi
yeast+ glucose	26 ±1.5 b	42 ±2.0 d	12.1 ± 3.5 a	23.7 ± 4.85 c
sucrose	90 ±3.2 i	93 ±2.1 i	46.9 ±4.5 g	53.0 ± 3.40 ij
yeast + sucrose	28 ±1.5 bc	52 ±3.8 e	19.9 ± 7.5 bc	28.9 ± 5.31d
fructose	91 ±2.1 i	92 ±2.6 i	46.4 ± 2.8 g	52.1 ± 2.66 hij
yeast + fructose	26 ±1.5 b	34 ±2.0 c	13.4 ± 4.8 a	18.5 ± 4.49 b
$(NH_4)_2SO_4$	93 ±2.1 i	96 ±1.0 i	51.1 ± 2.8 jhij	55.1 ± 2.50 j
yeast+ $(NH_4)_2SO_4$	66 ±2.1 f	80 ±2.1 h	29.3 ± 2.7 d	37.7 ± 3.58 f
phenylalanine	91 ±1.2 i	94 ±1.0 i	48.9 ± 3.9 ghi	49.9 ± 3.38 ghi
yeast + phenylalanine	53 ±4.2 e	69 ±1.2 fg	29.3 ± 3.6 d	36.0 ± 3.02 ef
asparagine	92 ±1.5 i	93 ±1.5 i	48.9 ± 3.1 ghi	51.2 ± 1.61 ghij
yeast + asparagine	64 ±4.0 f	73 ±3.1 g	32.3 ± 3.5 de	33.4 ± 4.79 def
	1 mM	5 mM	1 mM	5 mM
Fe^{3+}	90 ±2.1 i	90 ±2.0 i	47.3 ± 3.87 gh	48.8 ± 1.97 ghi
yeast + Fe^{3+}	2 ±0.0 a	2 ±0.6 a	14.1 ± 4.61 a	12.9 ± 2.49 a

* The results are the mean of two independent experiments. "±" stands for standard error of the means. Values of each column followed by different letters show significant difference (P<0.05) according to analysis by by Tukey's Test (SPSS 13.0).