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Efficacy of the antagonist *Aureobasidium pullulans* PL5 against postharvest pathogens of peach, apple and plum and its modes of action

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Abstract

The efficacy of *Aureobasidium pullulans* PL5 against different postharvest pathogens of fruits (*Monilinia laxa* on plums and peaches, *Botrytis cinerea* and *Penicillium expansum* on apples) were evaluated under storage conditions when applied at 10^8 cells ml^{-1} and their interactions were studied *in vitro* and *in vivo* to discover the possible modes of action. Under 1.2°C and 95% relative humidity (RH) for 28 days, the efficacy of PL5 against *M. laxa* on plums was 45%, reducing disease incidence from 78% to 43%. Under 1°C and 95% RH for 21 days, the efficacy against *M. laxa* on peaches was 63%, reducing disease incidence from 79% to 29%. Under 4°C and 95% RH for 45 days, the efficacy against *B. cinerea* and *P. expansum* on apples was 56% and 46%, respectively. In Lilly-Barnett minimal salt medium with the fungal cell walls of pathogens as sole carbon source, PL5 produced β-1,3-glucanase, exo-chitinase and endo-chitinase. Nutrient concentrations had significant effect on pathogen growth reduction by PL5. No attachment was observed in antagonist-pathogen interactions *in vitro* or *in vivo*. PL5 completely inhibited pathogen spore germination in PDB at 10^8 cells ml^{-1}, whereas at 10^6 cells ml^{-1} the efficacy was significantly decreased. However, inactivated cells and culture filtrate of PL5 had no effect on pathogen spore germination and germ tube elongation. Our results showed that *A. pullulans* PL5 could be introduced in commercial formulations to control postharvest pathogens on fruits and its activity was based on secretion of lytic enzymes and competition for nutrients.

***Keywords*:** apple, *Aureobasidium pullulans*, β-1,3-glucanase, biological control, *Botrytis cinerea*, chitinase, *Monilinia laxa*, peach, *Penicillium expansum*, plum.

1. Introduction

Fruit and vegetables are highly perishable products, especially during the postharvest phase and major losses are caused by postharvest pathogens (Ippolito and Nigro, 2000; Chan and Tian, 2005; Zhang et al., 2008). *Monilinia laxa, Botrytis cinerea* and *Penicillium expansum* are among the most important postharvest pathogens on fruit and vegetables (Snowdon, 1990). Among them, three species of *Monilinia* can cause severe losses on stone fruits (Karabulut et al., 2002; Pellegrino et al., 2009), but *M. laxa* is the most dangerous in European countries; *B. cinerea* could cause grey mold on pome and stone fruits, and *P. expansum* can cause blue mold decay, which is one of the most destructive disease of pear and apples and it is accompanied by the production of patulin, a mycotoxin with immunosuppressive effects on humans (Spotts and Chen, 1987; Spadaro et al., 2007). Chemical treatment is an important method for controlling postharvest diseases of fruits (Eckert and Ogawa, 1988). However, pathogen resistance to fungicides (Holmes and Eckert, 1999) and concern for public safety have resulted in the cancellation of some of the most effective fungicides in Europe (Directive 91/414 CE and Regulation) and the United States (Food Quality Protection Act). In addition, the use of synthetic fungicides to control postharvest diseases of peaches and plums is prohibited in European Union countries. Therefore, researches have been focused on the development of alternative control that should be both effective and economically feasible (El-Ghaouth et al., 1998). Biological control is an effective alternative to fungicidal treatment in controlling postharvest diseases of fruits (Jijakli and Lepoivre, 1998; Spadaro and Gullino, 2004). In 1995, the first commercial products were registered in the United States by the U.S. Enviromental Protection Agency (EPA) and are sold under the names BioSave 100 and 110 to control postharvest rots of pome and citrus fruit. In 2007, the biofungicide “Shemer” (based on a strain of *Metschnikowia fructicola* Kurtzman & Droby) was registered in Israel, and is commercially used for the control of sweet potato and carrot storage diseases (Blachinsky et al., 2007). In addition, a commercial

formulation of *Candida sake* was recently developed and registered for use on pome fruit in Spain under the name Candifruit® (Droby et al., 2009).

The comprehension of the modes of action of an antagonist is an important prerequisite both for enhancing their biocontrol activity and establishing screening criteria in search for new antagonists (Qin et al., 2003). Elucidation of the mechanisms of action is often hampered by the complex interaction among host-pathogen-antagonist (Jijakli and Lepoivre, 1998). The mode of action of antagonists generally involves antibiotics (Bull et al., 1998), nutrient competition and site exclusion (Bencheqroun et al., 2007), induced host resistance (El-Ghaouth et al., 1998), and direct interactions between the antagonist and the pathogen (Castoria et al., 1997). Additional modes of action including the production of lytic enzymes *viz.,* β-1,3-glucanase and chitinase were also reported (Ippolito et al., 2000; Saligkarias et al., 2002; Yu et al., 2008).

Aureobasidium pullulans De Bary (Arnaud) is widely distributed in different environments. Different strains of *A. pullulans* can produce amylase, proteinase, lipase, cellulase, xylanase, mannanase, transferases, pullulan, siderophore, and single-cell protein. Therefore it is a biotechnologically important yeast that can be used in different fields (Chi et al., 2009). Moreover, different strains of *A. pullulans* showed wide efficacy against *B. cinerea*, *P. expansum* and *Rhizopus stolonifer* on apple, sweet cherry, grapes, strawberry and peach (Lima et al., 1997; Ippolito et al., 2000; Schena et al., 2003; Bencheqroun et al., 2007). In particular, the strain PL5 of *A. pullulans* showed a high efficacy in the control of postharvest diseases of peaches (Zhang et al., 2010). Strains of *A. pullulans* have been reported to act against fungal pathogens through competition for nutrients (Bencheqroun et al., 2007), secretion of exochitinase and β-1,3-glucanase (Castoria et al., 2001), or induction of defence responses (Ippolito et al., 2000). Understanding the modes of action is essential for developing appropriate commercial formulations and application methods to maximize the potential use of microbial biocontrol agents.

The aim of this research was to evaluate the efficacy of *A. pullulans* PL5 against *B. cinerea*, *P. expansum*, and *M. laxa* on postharvest fruits under storage conditions. Generally, biocontrol agents are selected and optimized for their efficacy just against one pathogen on one fruit. In our experiment, the antagonist was tested agiants three pathogens on three fruit species under standard storage conditions. A second aim was to evaluate the production of hydrolytic enzymes by *A. pullulans* PL5 *in vitro*, by studying the ß-1,3-glucanase (EC 3.2.1.39), exochitinase or *N*-acetyl-β-glucosaminidase (EC 3.2.1.52) and endochitinase (EC 3.2.1.14) activities. A third aim was to investigate the effects of different nutrient concentrations on the interactions with three postharvest pathogens (*M. laxa*, *B. cinerea* and *P. expansum*) *in vitro* and *in vivo* to reveal their possible modes of action.

2. Materials and methods

2.1 Microorganisms and fruit

The yeast-like fungus *Aureobasidium pullulans* De Bary (Arnaud) PL5 was isolated from a plum cv. Angeleno produced in Piemonte (Northern Italy) and maintained on potato dextrose agar plates (PDA; 39 g l^{-1}, Merck) at 4°C for further studies. The antagonist, selected for its efficacy (Zhang et al., 2009), was identified through microscopic observation of cell and colony morphology, and by sequencing the internal transcribed spacer 1 (ITS1), 5.8S ribosomal RNA gene, and internal transcribed spacer 2 (ITS2) according to White et al. (1990). The sequence was deposited in GenBank (FJ919775). The strain was grown at 25±1°C in 300 ml YPD (10 g l^{-1} of yeast extract; 20 g l^{-1} of triptone-peptone of casein and 20 g l^{-1} of D(+)-glucose) on a rotary shaker (250 rpm) for 48 h. Cells were harvested after centrifugation at 5000×g for 10 min, resuspended in sterile Ringer's solution (pH 6.9±0.1; Merck) and adjusted to the desired cell concentration (10^8 cells ml^{-1}) with a Bürker chamber.

The pathogens *Monilinia laxa* (Aderhold & Ruhland) Honey, *Botrytis cinerea* (de Bary) Whetzel, and *Penicillium expansum* Link were isolated from infected fruit, and pure cultures were maintained on PDA plates at 4°C. The spore suspensions of the pathogens were prepared from 7 days old mycelia by scraping the conidia on PDA plates with a sterile Ringer's solution and adjusting to the desired conidial concentration with a Bürker chamber. Fruit used throughout the experiments were apples (*Malus* × *domestica* Borkh.) cv. Golden delicious, plums (*Prunus domestica* L.) cv. Angeleno, and peaches [*Prunus persica* (L.) Batsch] cv. Redhaven harvested at commercial maturity and kept at 1°C until use. Fruit were disinfected with 2% sodium hypochlorite for 2 min, washed with tap water and air dried prior to wounding.

2.2 Efficacy of the antagonist PL5 against M. laxa *on plums and peaches under storage conditions*

The cells of the antagonist PL5 was harvested by centrifugation at 5000 ×g for 10 min after being grown in 300 ml YPD medium in 1000-ml Erlenmeyer flasks for 48 hours at 25°C on a rotary shaker at 250 rpm, and were diluted with 30 L tap water in 50 L tank into a final concentration of 1×10^{8} cells ml^{-1}. Thirty L of tebuconazole solution (2.5 mL/L of Folicur, Bayer Crop Science; 25.0 % a. i.) was prepared according to the manufacturer. The plums and peaches were surface sterilized with 1% commercial sodium hypochlorite solution for 1 min followed by rinsing with tap water. After 2 h air-drying at 25°C, fruits were treated with the antagonist suspension by dipping in tank for 1 min. Fruit surfaces were then air dried at 25°C for 2 h and 1 ml of a conidial suspension of *M. laxa* (5×10^{4} spores ml^{-1}) was sprayed universally onto each fruit. The fruits treated with tebuconazole constituted the chemical control, while the fruits simply inoculated with the pathogen served as inoculated control. Three replicates of 25 fruits were prepared for each treatment. Two hours after inoculation of the pathogen on fruit surface, the plums were stored at 1.2°C and 95% RH and the peach

158 fruits were stored at 1°C and 95% relative humidity (RH) under storage conditions. After 21
159 and 28 days of storage, incidence of the rotten peaches and plums were measured,
160 respectively. The experiments were repeated twice.
161
162 *2.3 Efficacy of the antagonist PL5 against* B. cinerea *and* P. expansum *on apples under*
163 *storage conditions*
164 To evaluate the efficacy of the antagonist PL5 against *B. cinerea* and *P. expansum* on
165 apples, the trials were prepared in a similar way, as described above. Briefly, the cells of
166 antagonist *A. pullulans* PL5 were diluted with 30 L tap water in 50 L tank into a final
167 concentration of 1×10^8 cells ml^{-1}. The apples were surface sterilized with 1% commercial
168 sodium hypochlorite solution for 1 min followed by rinsing with tap water. After 2 h of air-
169 dry at 25°C, fruits were treated with the antagonist suspension (1×10^8 cells ml^{-1}) by dipping in
170 tank for 1 min. Fruit surfaces were air dried at 25°C for 2 h and 1 ml of a conidial suspension
171 of *B. cinerea* (5×10^4 spores ml^{-1}) or *P. expansum* (5×10^4 spores ml^{-1}) were sprayed onto each
172 fruit according to the different trials. The fruits treated with 2.5 mL/L of Folicur (Bayer Crop
173 Science; tebuconazole: 25.0 %) played as chemical controls, while the fruits only inoculated
174 with the pathogen served as inoculated controls. Three replicates of 25 fruits were prepared
175 for each treatment. Two hours after inoculation of the pathogen on fruit surface, the apples
176 were stored at 4°C and 95% RH under storage conditions. After 45 days of storage, the rotten
177 apples were counted and the infected percentage of the apples was recorded. The experiment
178 was repeated twice.
179
180 *2.4 Pathogen cell wall preparation*
181 Cell wall preparations (CWP) of each pathogen were prepared as described by
182 Saligkarias et al. (2002) with small modifications. Briefly, the pathogens were grown in
183 potato dextrose broth media (Sigma-Aldrich, USA) and the mycelium was collected with

four-fold cotton gauzes and washed twice with deionized water followed by two washes through Whatman No.1 filter paper with deionized water and centrifuging (Centrifuger 6K15, Sigma, Germany) at 480×g for 2 min. After discarding the supernatant, the fungal mycelia were sonicated with a probe type sonicator (USC6000, VWR, Malaysia) for 20 min and centrifuged at 480×g for 5 min. The supernatant was discarded and the pellet was resuspended in deionized water. The same procedure was performed for six times. Then the crushed mycelia were resuspended into an equal volume of Tris/HCl buffer (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.

2.5 Preparation of colloidal chitin

Colloidal chitin was prepared according to the method described by Roberts and Selitrennikoff (1988) from shrimp shell chitin. Five grams of chitin powder (Sigma, USA) was added slowly into 100 ml of concentrated HCl and left overnight at 4°C 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 precipitate was collected by centrifugation at 3000×g for 20 min at 4°C and the precipitate was 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.

2.6 Culture conditions for enzyme production by the antagonist

The antagonist *A. pullulans* PL5 was cultured in modified Lilly-Barnett minimal salt medium (LBMS; Lilly and Barnett, 1951) containing 2 mg ml^{-1} CWP of each pathogen as sole carbon source. A 30 ml of culture media in 100-ml flask was incubated at 25°C on a rotary shaker at 150 rpm for 0, 24, 72, and 96 h. Culture filtrates from each individual culture

flask were collected by centrifuging at 7000×g for 8 min, and the supernatant was used for enzyme assays.

2.7 Determination of ß-1,3-glucanase activity produced by A. pullulans *PL5*

β-1,3-glucanase activity assays were carried out by measuring the amount of reducing sugars released from laminarin (Sigma, USA), using glucose as a standard (Masih and Paul, 2002). A reaction mixture was prepared by adding 250 µl of 0.005M 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 a water bath (Melsungen, Germany). Then 0.5 ml of dinitrosalicylic acid reagent was added and boiled at 100°C for 5 min. After cooling, 2 ml of deionized water were added directly and measured spectrophotometrically at 595 nm. Background levels of reducing sugars were determined with a time 0 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 (Sigma, USA) as a standard. The specific activity was expressed as micromoles of glucose per milligram protein per hour (µmol Glucose Released/mg Protein/h; Fan et al., 2002). Each treatment had three replications and the experiments were repeated twice.

2.8 Determination of chitinase activity produced by A. pullulans *PL5*

The exo-chitinase 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 the endo-chitinase assay, a reaction mixture was prepared by adding 0.1 ml of 3% (w/v) desalted snail gut enzyme cytohelicase (Sigma, USA) and 0.1 ml of 1 mol l^{-1} potassium phosphate buffer (pH

7.0) 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 same tube. The enzyme-substrate mixture was incubated for 2h at 37°C in the water bath with constant shaking. The supernatant was collected from the mixture by centrifuging at 7000×g for 8 min. In the following, 0.5 ml dinitrosalicylic acid reagent was added to the mixture and boiled at 100°C for 5 min. After cooling, 1.5 ml of deionized water was added directly and measured spectrophotometrically at 550 nm. Background levels of reducing sugars and the protein concentration of the enzyme solution was determined as described above. The specific activity was reported as micromoles of N-acetyl-D-glucosamine per milligram protein per hour (µmol GlcNAc Released/mg Protein/h) according to Reissig et al. (1955). Each treatment had three replications and the experiments were repeated twice.

2.9 Biocontrol activity of antagonist against postharvest pathogens in vivo

To assess the biocontrol activity of *A. pullulans* PL5 against *B. cinerea* and *P. expansum* on apples and against *M. laxa* on peaches, the fruits were surface sterilized with 1% commercial sodium hypochlorite solution for 1 min and then rinsed with tap water. Three artificial wounds (about 3mm wide× 3mm deep) along the equatorial zone of the each fruit were made. Aliquots of 30 µl of antagonist suspension containing 10^8cells ml^{-1} were pipetted into each wound. After 2 hours of incubation at 25±2°C, the wounds were inoculated with 30µl of pathogen conidial suspension containing 5×10^4 conidia ml^{-1}. The fruits inoculated with water served as control. Fifteen fruits with three wounds were used for each treatment. The fruits were stored at 25±2°C and after 7 days, the diameter of decay on fruits was measured. The experiments were repeated twice.

2.10 Testing of antagonist-pathogen direct interaction in vitro *and* in vivo

The interaction between the antagonist and the pathogen hyphae was assessed in Petri dishes (90 mm, containing 20 ml PDA for *B. cinerea* and *P. expansum* or 20 ml peach juice agar for *M. laxa*). The plates were divided into three sections, 30 mm wide each, along the diameter. Pathogen mycelial plugs (5mm in diameter) were corked from a 4 day old PDA culture plate and fixed upon one point on the agar surface. After 48h at 25±2°C, 50 µl aliquots of the antagonist cell suspension containing 10^8 cells ml^{-1} were streaked onto PDA and peach juice agar at 30 mm from the pathogen plug (Spadaro et al., 2002). The dual cultures were washed with deionized water for about 2 min after 48h co-culture at 25±2°C. The interactions of the antagonist and pathogen were directly observed under light microscope (Axioskop 40, Germany). The experiments were repeated twice.

To test the interaction between antagonist and pathogen *in vivo* the fruits were inoculated as described above. The fruits were stored at 25°C and the interaction of the antagonist and pathogens were directly observed under light-microscope 7 days after inoculation. Only the interactions between the antagonist and *B. cinerea* are presented in this paper.

2.11 Effect of nutrient concentration on antagonist-pathogen interaction in vitro

To characterize the nutrimental mechanism, different concentrations of PDA (39 g L^{-1}, 19.5 g L^{-1}, 9.7 g L^{-1}, and 3.9 g L^{-1}) for *B. cinerea* and *P. expansum* and peach juice agar (50%, 25%, 10%, and 5%) for *M. laxa* were used to examine the effect of nutrients on mycelial growth reduction by *A. pullulans* PL5. The co-inoculation of *A. pullulans* PL5 (10^8 cells ml^{-1}) and the pathogens was performed as described above (**2.9**). After 7 days of incubation at 25°C, the pathogen mycelial growth reduction was measured and recorded. Each treatment had three replications and the experiments were repeated twice.

2.12 Effect of antagonist on pathogen spore germination in vitro

The effect of the antagonist *A. pullulans* PL5 on *M. laxa*, *B. cinerea* and *P. expansum* spore germination was assessed in potato dextrose broth (PDB) according to the method described by Spadaro et al. (2002). Yeast cells grown at 25±2°C for 48h in YPD broth were harvested by centrifugation at 5000×g for 10 min, and then resuspended in sterile ringer solution. The culture media of YPD were filtered with 22 µm nitro-cellulose filter for further use. Living cells of the antagonist (100µl of a suspension containing 5×10^7, 5×10^8 and 5×10^9 cells ml^{-1}) or cells (100 µl of a suspension containing 5×10^9 cells ml^{-1}) inactivated by irradiation for 30' with a germicidal lamp (General Electric, G15T8) that emitted predominantly 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 5 ml PDB in 35 ml tubes. The final concentrations of living yeast cells were 1×10^6, 1×10^7, and 1×10^8 cells ml^{-1} respectively. One ml of culture filtrate was transferred into the 5 ml PDB tubes for the culture filtrate treatment. Pathogens were then inoculated into each tube containing 5×10^4 conidia ml^{-1}. The PDB tubes inoculated only with the pathogens served as control. Then the tubes were incubated at 25°C on a rotary shaker at 200 rpm for 20 h. One hundred spores were randomly selected from each treatment. The spore germination of each pathogen was assessed and the germ tube length was measured using light microscope. Three replications were maintained for each treatment and the experiments were repeated twice.

2.13 Statistical analysis

Homogeneity of variances was tested by Levene's test ($P = 0.05$). When the variances were considered homogenous, data were subjected to analysis of variance (ANOVA) using SPSS (version 13.0) and statistical significance was assessed at the level of $P<0.05$. Duncan's multiple Range Test was used to separate the means.

3. Results

3.1 Efficacy of the antagonist PL5 against M. laxa *on plums and peaches under storage conditions*

After storage at 1.2°C and 95% RH for 28 days, brown rot disease incidence was recorded on plums (Table 1). Compared with that (78%) of inoculated control, the disease incidence caused by *M. laxa* of plums treated with *A. pullulans* PL5 was reduced to 43%. Correspondingly, the efficacy of PL5 against *M. laxa* on plums was 45% .

After the peach fruits were stored at 1°C and 95% RH for 21 days under storage conditions, the disease incidence was reduced from 79% (inoculated control) to 29% and the efficacy of PL5 against *M. laxa* on peaches was 63%. The biocontrol efficacy of tebuconazole to control brown rot on peaches and plums was 67% and 84%, respectively (Table 1).

3.2 Efficacy of the antagonist PL5 against B. cinerea *and* P. expansum *on apples under storage conditions*

After storage at 4°C and 95% RH under storage conditions for 45 days, the incidence of rotten apples caused by *B. cinerea* and *P. expansum* was recorded. The results are shown in Table 2. *A. pullulans* PL5 reduced gray mold incidence from 45% to 20% and reduced blue mold incidence from 47% to 25%. Correspondingly, the efficacy of *A. pullulans* PL5 against *B. cinerea* and *P. expansum* on apples was 56% and 46%, respectively.

3.3 Production of ß-1, 3-glucanase by A. pullulans *PL5 and its activity*

The antagonist *A. pullulans* PL5 produced extracellular β-1,3-glucanase in culture medium in presence of pathogen cell walls as sole carbon source. *A. pullulans* PL5 began to produce extracellular β-1,3-glucanase immediately after cultivation in LBMS medium with *B. cinerea* and *P. expansum* cell walls as sole carbon source. When *M. laxa* was supplied as sole carbon source, at 0 h incubation, no extracellular ß-1,3-glucanase was detected. At 48 h

incubation, the extracellular ß-1,3-glucanase activity reached the maximum level, and the ß-1,3-glucanase activity in the minimal salt medium with *M. laxa*, *B. cinerea* and *P. expansum* cell walls as sole carbon source were 46.9, 66.1 and 80.1 U (µmol Glucose Released/mg Protein/h), respectively. However, at 72 h the ß-1,3-glucanase activities began decreasing. At 96 h incubation, the ß-1,3-glucanase activity in the minimal salt medium with *M. laxa*, *B. cinerea* and *P. expansum* cell walls as sole carbon source were 32.9, 8.0, and 19.6 U (µmol Glucose Released/mg Protein/h), respectively (Fig. 1 a).

3.4 Production of chitinases by A. pullulans *PL5 and their activities*

The assay of chitinase activity showed that exo-chitinase activity of *A. pullulans* PL5, when cultured in minimal salt medium with the different fungal cell walls as sole carbon source, showed the different trends, according to different incubation periods and different pathogens. The maximum level of exo-chitinase activity in the medium with *M. laxa* and *P. expansum* as sole carbon source was detected after 48 h incubation. However, the maximum level of exo-chitinase activity in the medium with *B. cinerea* cell wall as sole carbon source appeared before 48 h incubation (Fig. 1 b). The maximum level of exo-chitinase activity in the medium with *P. expansum* as sole carbon source was 0.96 U (µmol GlcNAc Released/mg Protein/h) which was higher than the maximum levels of those in the medium with *M. laxa* and *B. cinerea* cell walls as sole carbon source (Fig. 1 b).

The endo-chitinase activity of *A. pullulans* PL5 cultured in LBMS medium with *P. expansum* and *M. laxa* cell walls as sole carbon sources reached the maximum level after 48 h incubation. However, when *A. pullulans* PL5 was cultured in the medium with *B. cinerea* cell walls as sole carbon source, the maximum level of endo-chitinase activity was detected at about 30 h incubation. Also the endo-chitinase activity assay showed that the maximum activity with *P. expansum* cell walls as sole carbon source was higher than the activity with *B. cinerea* or *M. laxa* cell walls (Fig. 1 c.).

3.5 Biocontrol activity of A. pullulans *PL5 and antagonist-pathogen direct interaction* in vitro *and* in vivo

After 7 days of co-incubation in PDA plates at 25±2°C, direct interaction *in vitro* was observed microscopically. *A. pullulans* PL5 significantly inhibited the hyphae elongation of the pathogens. However, no attachment of fungal hyphae was observed (Fig. 2) and the hyphae end close to the antagonist grew normally (Fig. 2b).

After 7 days of co-incubation in artificial wounds of apples, the pathogen spore germination was almost completely inhibited by *A. pullulans* PL5 (Fig. 2d) and the population of *A. pullulans* PL5 greatly increased. However, no attachment to the pathogen spore surfaces was observed.

In artificial wound inoculation tests, *A. pullulans* PL5 was effective in controlling decays of fruits caused by *M. laxa*, *B. cinerea* and *P. expansum* (Table 3). After 7 days of storage at 25 ± 2°C, *A. pullulans* PL5 significantly reduced the decay of apples caused by *B. cinerea* and *P. expansum* from 61.9 mm and 35.3 mm to 9.5 mm and 7.1 mm, respectively. While the diameter of rotten lesions caused by *M. laxa* on peaches was significantly reduced from 49.4 mm to 24.6 mm.

3.6 Effect of nutrient concentration on antagonist-pathogen interaction in vitro

The effect of nutrient concentrations on the interaction of *A. pullulans* PL5 with the three pathogens (*M. laxa, B. cinerea* and *P. expansum*) was studied *in vitro*. After incubation at 25±2°C for 7 days *A. pullulans* PL5 co-cultured with *B. cinerea* on different concentrations of PDA (39 g L^{-1}, 19.5 g L^{-1}, 9.7 g L^{-1}, and 3.9 g L^{-1}) reduced the mycelial growth by 8.0 mm, 10.7 mm, 12.0 mm and 14.8 mm, respectively (Table 4). When co-cultured with *M. laxa* on 50%, 25%, 10%, and 5% peach juice agar, *A. pullulans* PL5 reduced the mycelial growth of *M. laxa* by 4.0 mm, 8.2 mm, 11.5 mm and 14.2 mm, respectively. After 7 days of co-

391 incubation at 25 ± 2°C on 9.7 g L^{-1} and 3.9 g L^{-1} PDA, the pathogen *P. expansum* mycelial
392 growth reduction by the antagonist was 3.3 mm and 7.2 mm. However, on 39 g L^{-1} and 19.5 g
393 L^{-1} PDA, no mycelial growth reduction of *P. expansum* was observed (Table 4).
394
395 *3.7 Effect of antagonist on pathogen spore germination* in vitro
396 By co-culturing in PDB, the effect of different concentrations of cell suspension,
397 inactivated cells and culture filtrate of *A. pullulans* PL5 were investigated on *M. laxa, B.*
398 *cinerea* and *P. expansum* spore germination and germ tube length (Table 5). After 20 h
399 incubation at 25±2°C almost complete inhibition of the spore germination was observed in
400 presence of 1×10^8 cells ml^{-1} of the antagonist. The percentage of spore germination of *M.*
401 *laxa, B. cinerea,* and *P. expansum* were 1.7%, 1.7% and 2.7%, respectively. Correspondingly,
402 at 1×10^8 cells ml^{-1}, the antagonist inhibition efficacies were 98.1%, 98.2% and 96.9%,
403 respectively. When co-cultured with 1×10^7 cells ml^{-1} of the antagonist, the percentage of
404 spore germination of *M. laxa, B. cinerea* and *P. expansum* were 31.7%, 28.7% and 26.7%,
405 respectively. Whereas at 1×10^6 cells ml^{-1} of the antagonist applied, the percentage of spore
406 germination were reduced only into 60.0%, 61.7% and 62.7%, respectively. No significant
407 difference of the percentage of spore germination was observed when the pathogens were co-
408 cultured with inactivated cells or culture filtrate.
409 Germ tube elongation of *M. laxa, B. cinerea* and *P. expansum* in PDB was greatly
410 controlled by the living cells of *A. pullulans* PL5. In the inoculated control, length of the germ
411 tubes of *M. laxa* and *B. cinerea* were 123.1 µm and 148.4 µm. However, the length of germ
412 tubes of *M. laxa* co-cultured with 1×10^8, 1×10^7, and 1×10^6 cells ml^{-1} of the antagonist were
413 4.2 µm, 37.7 µm and 58.1 µm, respectively, and the length of the germ tubes of *B. cinerea* co-
414 cultured with 1×10^8, 1×10^7, and 1×10^6 cells ml^{-1} of the antagonist was reduced by 85.9%,
415 70.8% and 38.1%, respectively. The length of germ tubes of *P. expansum* in the presence of
416 1×10^8, 1×10^7 and 1×10^6 cells ml^{-1} of the antagonist was reduced from 117.5 µm to 13.4 µm,

417 20.1 µm and 65.3 µm, respectively. When the pathogens were co-cultured with inactivated
418 cells or culture filtrate of the antagonist, no significant difference in germ tube length was
419 observed (Table 5).
420
421 **4. Discussion**
422 The efficacy of the antagonistic yeast-like fungus *A. pullulans* PL5 against three
423 postharvest pathogens on three fruit species (*M. laxa* on plums and peaches, *B. cinerea* and *P.*
424 *expansum* on apples) was evaluated under storage conditions.
425 Though much work has been done to develop biocontrol agents against postharvest
426 pathogens of fruits, none of the commercialized biofungicides is effective against brown rot
427 on stone fruit (Zhang et al., 2010). The antagonist *A. pullulans* PL5 was effective in
428 controlling brown rot on peaches as well as on plums under storage conditions. Few
429 antagonists have been selected for their biocontrol potential on different fruit species. A
430 broader range of efficacy gives more opportunities for the commercial development of
431 antagonists. Our research demonstrated that *A. pullulans* PL5 had also a high efficacy in
432 controlling grey mould and blue mould on apples, beyond its strong biocontrol activity
433 against brown rot on peaches and plums.
434 The lack of secretion of antibiotic compounds in the culture filtrate is an important and
435 positive factor for potential registration of the biocontrol agents. In this research, the trials,
436 carried out *in vitro* showed that neither inactivated cells nor culture filtrate of the antagonist
437 had effect on the pathogens spore germination or germ tube elongation, suggesting that
438 production of antibiotics were not involved in the modes of action of *A. pullulans* PL5 against
439 the pathogens.
440 Mycoparasitism of antagonists is associated with the production of cell wall-degrading
441 enzymes and induction of host defense. Concurrent induction of chitinase and glucosidase has
442 been described in plant as a response to infection by microbial pathogen (Lorito et al., 1994),

443 and the two classes of enzymes exhibit synergistic activity against growth of several fungi
444 (Qin et al., 2003). Peroxidase in combination with chitinase and β-1,3-glucanase has also
445 shown to inhibit the growth of several pathogenic fungi *in vitro* (Schlumbaum et al., 1986).
446 Lorito et al. (1994) anyway proved that a synergistic antifungal effect of either exo- or endo-
447 chitinases in combination with glucosidase of *T. harzianum* was able to inhibit *B. cinerea*.
448 The strain PL5 showed a strong antagonistic activity in controlling postharvest pathogens,
449 such as *M. laxa* on peaches, *B. cinerea* and *P. expansum* on apples. *A. pullulans* PL5, when
450 applied as a wound treatment, was effective in controlling postharvest decay of apple and
451 peach. Moreover, when co-cultured with the pathogens *in vitro* or *in vivo*, it was also effective
452 in inhibiting the pathogen mycelial growth.

453 It has been suggested that extensive production of the extracellular lytic enzymes,
454 especially β-1,3-glucanase and chitinase, by yeast or filamentous fungi may play an important
455 role either by enhancing nutrient competition or by some other unknown mechanisms (Jijakli
456 and Lepoivre, 1998; Fan et al., 2002; Masih and Paul, 2002; Chan and Tian, 2005). In our
457 investigation, the *in vitro* assays showed that the antagonist *A. pullulans* PL5 produced high
458 β-1,3-glucanase, exo-chitinase and endo-chitinase, especially when co-cultured in the LBMS
459 medium supplied with *P. expansum* cell wall as sole carbon source. Our results *in vitro* are in
460 accordance with previous researches of Castoria et al. (1997 and 2001) and Ippolito et al.
461 (2000) that demonstrated the capacity of some strains of *A. pullulans* of producing β-1,3-
462 glucanase and chitinase. However, the strain *A. pullulans* PL5 used in this research produced
463 higher β-1,3-glucanase than the strain *A. pullulans* L-30 tested by Castoria et al. (2001), when
464 the two strains were cultured in a medium with the same pathogen cell walls as sole carbon
465 source. Anyway, the biocontrol efficacy cannot be compared among the two strains because
466 Ippolito et al. (2000) worked on artificially wounded and inoculated apples, while we tested
467 PL5 on intact fruits under standard storage conditions. Moreover, in this paper, the dynamics
468 of time-course changes of enzyme activities produced by the antagonist was studied. The

469 production of β-1,3-glucanase and chitinase by *A. pullulans* PL5 and their activities
470 demonstrated a time-curve trend of the enzyme activities. *A. pullulans* PL5 began to produce
471 extracellular β-1,3-glucanase immediately after inducing the treatment in LBMS medium with
472 *B. cinerea* and *P. expansum* cell walls used as sole carbon source, while when *M. laxa* was
473 supplied as sole carbon source, no extracellular β-1,3-glucanase was detected at 0 h. At 24 h,
474 the activity of β-1,3-glucanase began increasing and reached the maximum level at 48 h.
475 However, at 72 h the β-1,3-glucanase activity began decreasing. Even if the exo- and endo-
476 chitinase activities of *A. pullulans* PL5 showed a similar trend to that of β-1,3-glucanase
477 activities, the maximum activities in LBMS medium with the three fungal cell walls as sole
478 carbon source were different. Probably *A. pullulans* PL5 showed a different response to the
479 presence of the cell walls of the three pathogens in producing chitinase. At present, the
480 production pathways of β-1,3-glucanase and chitinase by *A. pullulans* PL5 are still poorly
481 understood. Therefore, it is necessary to carry out more molecular research about the
482 regulation and function of the genes related to the enzyme production.

483 Attachment to fungal hyphae or mycoparasitism has also been proposed as a mode of
484 action of biocontrol agents against pathogens of fruits and vegetables (Wisniewski et al.,
485 1991; Arras, 1996; Wan and Tian, 2002). However, in our research, the observation of the
486 antagonist-pathogen interactions *in vitro* and *in vivo* indicated the lack of attachment to the
487 pathogen hyphae. Interaction test *in vitro* showed that the mycelia of the three pathogens were
488 greatly reduced by *A. pullulans* PL5, while no attachment of the antagonist to the pathogens
489 was observed on PDA, proving that inhibition of pathogen by *A. pullulans* PL5 was not based
490 on the mycoparasitism. Attachment of fungal hyphae by *A. pullulans* PL5 was not even
491 detected in the interaction tests performed *in vivo*.

492 Our results indicated that competition for nutrients played a significant effect on the
493 biocontrol activities of *A. pullulans* PL5 against the three pathogens, in accordance to

494 previous studies of Lima et al. (1997). When co-cultured with the pathogens on culture
495 medium with lower nutrient concentrations, the ability of *A. pullulans* PL5 to inhibit the
496 mycelial growth of pathogens was greatly improved. More consistent evidence was supplied
497 by the co-culture of *A. pullulans* PL5 with the pathogen *P. expansum* on different
498 concentrations of PDA. Also the co-culture experiment, where different concentrations of *A.*
499 *pullulans* PL5 had effects on the spore germination and germ tube elongation, supported a
500 major role of the competition for nutrients in the activity of *A. pullulans* PL5 against
501 postharvest pathogens. When the concentrations of *A. pullulans* PL5 were lowered, the spore
502 germination incidence significantly increased and the germ tubes were longer. Competition
503 for nutrients and space are considered among the main modes of action of yeast biocontrol
504 agents (Spadaro et al., 2002). In particular, competition for amino acids, sugars and Fe^{3+} plays
505 an important role in the mechanism of competition for nutrients of some antagonists. Sipiczki
506 (2006) and Saravanakumar et al. (2008) demonstrated that several strains of *Metschnikowia*
507 *pulcherrima* control *B. cinerea* and *P. expansum* in apples through competing for Fe^{3+} with
508 the pathogens. Bencheqroun et al. (2007) proposed that apple blue mould biocontrol by *A.*
509 *pullulans* was based on competition for amino acids. The main mode of action of *Pichia*
510 *guilliermondii* strain R13 in controlling anthracnose on sweet peppers after harvest was
511 competition for nitrogen sources and sugars (Chanchaichaovivat et al., 2008). However,
512 which nutrient sources are exactly involved in the competition of *A. pullulans* PL5 with the
513 pathogens is still under investigation. Summarizing the results above, production of β-1,3-
514 glucanase and chitinase together with out-competition for nutrients and space as well as other
515 undetermined mechanisms constitute important modes of action of *A. pullulans* PL5 against
516 postharvest pathogens of fruits.
517

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523

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642

643 **Figure captions**

644

645 **Figure 1.**

646 a) β-1,3-glucanase activity (EC 3.2.1.39) of *A. pullulans* PL5 grown in LBMS medium
647 supplemented with 2 mg ml^{-1} CWP (cell wall preparation of each pathogen: *M. laxa*, *B.*
648 *cinerea*, or *P. expansum*) as sole carbon source for 96 h at 25°C. Bars represented standard
649 deviations of the means; b) Exo-chitinase activity (EC 3.2.1.52) of *A. pullulans* PL5 grown in
650 LBMS medium supplemented with 2 mg ml^{-1} CWP (cell wall preparation of each pathogen:
651 *M. laxa*, *B. cinerea*, or *P. expansum*) as sole carbon source for 96 h at 25°C. Bars represented
652 standard deviations of the means; c) Endo-chitinase activity (EC 3.2.1.14) of *A. pullulans* PL5
653 grown in LBMS medium supplemented with 2 mg ml^{-1} CWP (cell wall preparation of each
654 pathogen: *M. laxa*, *B. cinerea*, or *P. expansum*) as sole carbon source for 96 h at 25°C. Bars
655 represented standard deviations of the means.

656

657 **Figure 2.**

658 a) Interaction of (A) and *B. cinerea* (B) *in vitro* (magnification 150 × ; bar = 40 µm); b) *B.*
659 *cinerea* hyphae (B) close to *A. pullulans* PL5 *in vitro* (magnification 600 × ; bar = 10 µm); c)
660 apple pulp tissue (*Malus* × *domestica*; M) as a control (magnification 150 × ; bar = 40 µm); d)
661 Interaction of *A. pullulans* PL5 (A), apple pulp (M) tissue and *B. cinerea* (B) *in vivo*
662 (magnification 150 × ; bar = 40 µm).