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

This is the author's manuscript

Original Citation:

Availability:
This version is available http://hdl.handle.net/2318/81402 since

Terms of use:
Open Access
Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.
This Accepted Author Manuscript (AAM) is copyrighted and published by Elsevier. It is posted here by agreement between Elsevier and the University of Turin. Changes resulting from the publishing process - such as editing, corrections, structural formatting, and other quality control mechanisms - may not be reflected in this version of the text. The definitive version of the text was subsequently published in [Zhang D., Spadaro D., Garibaldi A., Gullino M.L. (2010) - Efficacy of the antagonist Aureobasidium pullulans PL5 against postharvest pathogens of peach, apple and plum and its modes of action. Biological Control, 54, 172-180. DOI: 10.1016/j.biocontrol.2010.05.003].

You may download, copy and otherwise use the AAM for non-commercial purposes provided that your license is limited by the following restrictions:

(1) You may use this AAM for non-commercial purposes only under the terms of the CC-BY-NC-ND license.

(2) The integrity of the work and identification of the author, copyright owner, and publisher must be preserved in any copy.

(3) You must attribute this AAM in the following format: Creative Commons BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/deed.en), [10.1016/j.biocontrol.2010.05.003]
Efficacy of the antagonist *Aureobasidium pullulans* PL5 against postharvest pathogens of peach, apple and plum and its modes of action

Dianpeng Zhang¹, Davide Spadaro²*, Angelo Garibaldi¹ and Maria Lodovica Gullino¹

¹AGROINNOVA - Centre of Competence for the Innovation in the Agro-environmental Sector. University of Torino. Via L. da Vinci 44. 10095 Grugliasco (TO). Italy.

²DiVaPRA – Sector of Plant Pathology. University of Torino. Via L. da Vinci 44. 10095 Grugliasco (TO). Italy.

* Corresponding author. : DiVaPRA – Sector of Plant Pathology. University of Torino. Via L. da Vinci 44. 10095 Grugliasco (TO). Italy. E-mail: davide.spadaro@unito.it. Tel.: +39-011-6708942. Fax: +39-011-6709307.
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.

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. Environmental 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 against 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 β-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 (10⁸ 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
fruits were stored at 1°C and 95% relative humidity (RH) under storage conditions. After 21 and 28 days of storage, incidence of the rotten peaches and plums were measured, respectively. The experiments were repeated twice.

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

To evaluate the efficacy of the antagonist PL5 against B. cinerea and P. expansum on apples, the trials were prepared in a similar way, as described above. Briefly, the cells of antagonist A. pullulans PL5 were diluted with 30 L tap water in 50 L tank into a final concentration of 1×10^8 cells ml⁻¹. The apples were surface sterilized with 1% commercial sodium hypochlorite solution for 1 min followed by rinsing with tap water. After 2 h of air-dry at 25°C, fruits were treated with the antagonist suspension (1×10^8 cells ml⁻¹) by dipping in tank for 1 min. Fruit surfaces were air dried at 25°C for 2 h and 1 ml of a conidial suspension of B. cinerea (5×10⁴ spores ml⁻¹) or P. expansum (5×10⁴ spores ml⁻¹) were sprayed onto each fruit according to the different trials. The fruits treated with 2.5 mL/L of Folicur (Bayer Crop Science; tebuconazole: 25.0 %) played as chemical controls, while the fruits only inoculated with the pathogen served as inoculated controls. Three replicates of 25 fruits were prepared for each treatment. Two hours after inoculation of the pathogen on fruit surface, the apples were stored at 4°C and 95% RH under storage conditions. After 45 days of storage, the rotten apples were counted and the infected percentage of the apples was recorded. The experiment was repeated twice.

2.4 Pathogen cell wall preparation

Cell wall preparations (CWP) of each pathogen were prepared as described by Saligkarias et al. (2002) with small modifications. Briefly, the pathogens were grown in 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 (Centrifug 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 N₂, 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 β-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 dinitrosalicicylic 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 (\(\mu\text{mol GlcNAc Released/mg Protein/h}\)) 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 \textit{A. pullulans} PL5 against \textit{B. cinerea} and \textit{P. expansum} on apples and against \textit{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 \(10^8\) cells 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 \(5\times10^4\) 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 (5 mm in diameter) were corked from a 4 day old PDA culture plate and fixed upon one point on the agar surface. After 48 h 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 48 h 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 \textit{A. pullulans} PL5 on \textit{M. laxa}, \textit{B. cinerea} and \textit{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/m² 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 β-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 β-1,3-glucanase was detected. At 48 h
incubation, the extracellular β-1,3-glucanase activity reached the maximum level, and the β-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 β-1,3-glucanase activities began decreasing. At 96 h incubation, the β-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-
incubation at 25 ± 2°C on 9.7 g L⁻¹ and 3.9 g L⁻¹ PDA, the pathogen *P. expansum* mycelial growth reduction by the antagonist was 3.3 mm and 7.2 mm. However, on 39 g L⁻¹ and 19.5 g L⁻¹ PDA, no mycelial growth reduction of *P. expansum* was observed (Table 4).

3.7 Effect of antagonist on pathogen spore germination in vitro

By co-culturing in PDB, the effect of different concentrations of cell suspension, inactivated cells and culture filtrate of *A. pullulans* PL5 were investigated on *M. laxa*, *B. cinerea* and *P. expansum* spore germination and germ tube length (Table 5). After 20 h incubation at 25±2°C almost complete inhibition of the spore germination was observed in presence of 1×10⁸ cells ml⁻¹ of the antagonist. The percentage of spore germination of *M. laxa*, *B. cinerea*, and *P. expansum* were 1.7%, 1.7% and 2.7%, respectively. Correspondingly, at 1×10⁸ cells ml⁻¹, the antagonist inhibition efficacies were 98.1%, 98.2% and 96.9%, respectively. When co-cultured with 1×10⁷ cells ml⁻¹ of the antagonist, the percentage of spore germination of *M. laxa*, *B. cinerea* and *P. expansum* were 31.7%, 28.7% and 26.7%, respectively. Whereas at 1×10⁶ cells ml⁻¹ of the antagonist applied, the percentage of spore germination were reduced only into 60.0%, 61.7% and 62.7%, respectively. No significant difference of the percentage of spore germination was observed when the pathogens were co-cultured with inactivated cells or culture filtrate.

Germ tube elongation of *M. laxa*, *B. cinerea* and *P. expansum* in PDB was greatly controlled by the living cells of *A. pullulans* PL5. In the inoculated control, length of the germ tubes of *M. laxa* and *B. cinerea* were 123.1 µm and 148.4 µm. However, the length of germ tubes of *M. laxa* co-cultured with 1×10⁸, 1×10⁷, and 1×10⁶ cells ml⁻¹ of the antagonist were 4.2 µm, 37.7 µm and 58.1 µm, respectively, and the length of the germ tubes of *B. cinerea* co-cultured with 1×10⁸, 1×10⁷, and 1×10⁶ cells ml⁻¹ of the antagonist was reduced by 85.9%, 70.8% and 38.1%, respectively. The length of germ tubes of *P. expansum* in the presence of 1×10⁸, 1×10⁷ and 1×10⁶ cells ml⁻¹ of the antagonist was reduced from 117.5 µm to 13.4 µm,
20.1 \( \mu m \) and 65.3 \( \mu m \), respectively. When the pathogens were co-cultured with inactivated cells or culture filtrate of the antagonist, no significant difference in germ tube length was observed (Table 5).

4. Discussion

The efficacy of the antagonistic yeast-like fungus *A. pullulans* PL5 against three postharvest pathogens on three fruit species (*M. laxa* on plums and peaches, *B. cinerea* and *P. expansum* on apples) was evaluated under storage conditions.

Though much work has been done to develop biocontrol agents against postharvest pathogens of fruits, none of the commercialized biofungicides is effective against brown rot on stone fruit (Zhang et al., 2010). The antagonist *A. pullulans* PL5 was effective in controlling brown rot on peaches as well as on plums under storage conditions. Few antagonists have been selected for their biocontrol potential on different fruit species. A broader range of efficacy gives more opportunities for the commercial development of antagonists. Our research demonstrated that *A. pullulans* PL5 had also a high efficacy in controlling grey mould and blue mould on apples, beyond its strong biocontrol activity against brown rot on peaches and plums.

The lack of secretion of antibiotic compounds in the culture filtrate is an important and positive factor for potential registration of the biocontrol agents. In this research, the trials, carried out *in vitro* showed that neither inactivated cells nor culture filtrate of the antagonist had effect on the pathogens spore germination or germ tube elongation, suggesting that production of antibiotics were not involved in the modes of action of *A. pullulans* PL5 against the pathogens.

Mycoparasitism of antagonists is associated with the production of cell wall-degrading enzymes and induction of host defense. Concurrent induction of chitinase and glucosidase has been described in plant as a response to infection by microbial pathogen (Lorito et al., 1994),
and the two classes of enzymes exhibit synergistic activity against growth of several fungi (Qin et al., 2003). Peroxidase in combination with chitinase and β-1,3-glucanase has also shown to inhibit the growth of several pathogenic fungi in vitro (Schlumbaum et al., 1986). Lorito et al. (1994) anyway proved that a synergistic antifungal effect of either exo- or endo-chitinases in combination with glucosidase of T. harzianum was able to inhibit B. cinerea. The strain PL5 showed a strong antagonistic activity in controlling postharvest pathogens, such as M. laxa on peaches, B. cinerea and P. expansum on apples. A. pullulans PL5, when applied as a wound treatment, was effective in controlling postharvest decay of apple and peach. Moreover, when co-cultured with the pathogens in vitro or in vivo, it was also effective in inhibiting the pathogen mycelial growth.

It has been suggested that extensive production of the extracellular lytic enzymes, especially β-1,3-glucanase and chitinase, by yeast or filamentous fungi may play an important role either by enhancing nutrient competition or by some other unknown mechanisms (Jijakli and Lepoivre, 1998; Fan et al., 2002; Masih and Paul, 2002; Chan and Tian, 2005). In our investigation, the in vitro assays showed that the antagonist A. pullulans PL5 produced high β-1,3-glucanase, exo-chitinase and endo-chitinase, especially when co-cultured in the LBMS medium supplied with P. expansum cell wall as sole carbon source. Our results in vitro are in accordance with previous researches of Castoria et al. (1997 and 2001) and Ippolito et al. (2000) that demonstrated the capacity of some strains of A. pullulans of producing β-1,3-glucanase and chitinase. However, the strain A. pullulans PL5 used in this research produced higher β-1,3-glucanase than the strain A. pullulans L-30 tested by Castoria et al. (2001), when the two strains were cultured in a medium with the same pathogen cell walls as sole carbon source. Anyway, the biocontrol efficacy cannot be compared among the two strains because Ippolito et al. (2000) worked on artificially wounded and inoculated apples, while we tested PL5 on intact fruits under standard storage conditions. Moreover, in this paper, the dynamics of time-course changes of enzyme activities produced by the antagonist was studied. The
production of β-1,3-glucanase and chitinase by A. pullulans PL5 and their activities demonstrated a time-curve trend of the enzyme activities. A. pullulans PL5 began to produce extracellular β-1,3-glucanase immediately after inducing the treatment in LBMS medium with B. cinerea and P. expansum cell walls used as sole carbon source, while when M. laxa was supplied as sole carbon source, no extracellular β-1,3-glucanase was detected at 0 h. At 24 h, the activity of β-1,3-glucanase began increasing and reached the maximum level at 48 h. However, at 72 h the β-1,3-glucanase activity began decreasing. Even if the exo- and endo-chitinase activities of A. pullulans PL5 showed a similar trend to that of β-1,3-glucanase activities, the maximum activities in LBMS medium with the three fungal cell walls as sole carbon source were different. Probably A. pullulans PL5 showed a different response to the presence of the cell walls of the three pathogens in producing chitinase. At present, the production pathways of β-1,3-glucanase and chitinase by A. pullulans PL5 are still poorly understood. Therefore, it is necessary to carry out more molecular research about the regulation and function of the genes related to the enzyme production.

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

Our results indicated that competition for nutrients played a significant effect on the biocontrol activities of A. pullulans PL5 against the three pathogens, in accordance to
previous studies of Lima et al. (1997). When co-cultured with the pathogens on culture medium with lower nutrient concentrations, the ability of \textit{A. pullulans} PL5 to inhibit the mycelial growth of pathogens was greatly improved. More consistent evidence was supplied by the co-culture of \textit{A. pullulans} PL5 with the pathogen \textit{P. expansum} on different concentrations of PDA. Also the co-culture experiment, where different concentrations of \textit{A. pullulans} PL5 had effects on the spore germination and germ tube elongation, supported a major role of the competition for nutrients in the activity of \textit{A. pullulans} PL5 against postharvest pathogens. When the concentrations of \textit{A. pullulans} PL5 were lowered, the spore germination incidence significantly increased and the germ tubes were longer. Competition for nutrients and space are considered among the main modes of action of yeast biocontrol agents (Spadaro et al., 2002). 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) and Saravanakumar et al. (2008) demonstrated that several strains of \textit{Metschnikowia pulcherrima} control \textit{B. cinerea} and \textit{P. expansum} in apples through competing for Fe$^{3+}$ with the pathogens. Bencheqroun et al. (2007) proposed that apple blue mould biocontrol by \textit{A. pullulans} was based on competition for amino acids. The main mode of action of \textit{Pichia guilliermondii} strain R13 in controlling anthracnose on sweet peppers after harvest was competition for nitrogen sources and sugars (Chanchaichaovivat et al., 2008). However, which nutrient sources are exactly involved in the competition of \textit{A. pullulans} PL5 with the pathogens is still under investigation. Summarizing the results above, production of \(\beta\)-1,3-glucanase and chitinase together with out-competition for nutrients and space as well as other undetermined mechanisms constitute important modes of action of \textit{A. pullulans} PL5 against postharvest pathogens of fruits.

\textbf{Acknowledgements}

This research was funded by the projects “CIPE – Production of stone fruit in Piedmont:
monitoring, prevention and control of pathogenic and mycotoxinogenic fungi to guarantee food safety” and “DRUMP – Drupacee minori in Piemonte: problemi fitopatologici e difesa post-raccolta” granted by the Piedmont Region.

References


Aureobasidium pullulans (LS-30) an antagonist of postharvest pathogens of fruits: study on its modes of action. Postharvest Biology and Technology 22, 7-17.


**Figure captions**

**Figure 1.**

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

**Figure 2.**

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