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Biocontrol activity of an alkaline serine protease from *Aureobasidium pullulans* expressed in *Pichia pastoris* against four postharvest pathogens on apple

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Abstract

The yeast-like fungus *Aureobasidium pullulans* PL5 is a microbial antagonist against postharvest pathogens of fruits. The strain is able to produce hydrolases, including glucanases, chitinases and proteases. The alkaline serine protease gene *ALP5* from *A. pullulans* was cloned, inserted into the vector pPIC9 to construct pPIC9/PL5, and then expressed in *Pichia pastoris* strain KM71. *ALP5* had a molecular mass of 42.9 kDa after 5 days growth with 1% methanol induction at 28 °C. The recombinant protease expressed in *P. pastoris* showed its highest activity under alkaline conditions (at pH 10) and temperature of 50 °C. The antifungal activity of the recombinant protease was investigated against *Penicillium expansum, Botrytis cinerea, Monilinia fructicola* and *Alternaria alternata in vitro* and on apple. The recombinant protease reduced significantly the spore germination and the germ tube length of the tested pathogens in PDB medium. The highest level of protease efficacy was observed against *M. fructicola* and *B. cinerea*, whereas a lower efficacy was observed against *P. expansum* and *A. alternata* indicating a possible effect of the pathogen cell wall composition on the proteolytic activity of the recombinant protease. The presence of protease was able to cause swelling of the hyphae of *B. cinerea*, under optical microscope. The recombinant protease expressed in the *P. pastoris* was more active against the pathogens *in vitro* than the same enzyme expressed in *E. coli* in previous studies. The efficacy of *ALP5* was also evaluated against the pathogens *in vivo* on apples cv Golden delicious. The protease was more efficient in controlling *M. fructicola*, *B. cinerea* and *P. expansum* than *A. alternata*. However, the extent of the activity was dependent on the enzyme concentration and the length of fruit storage. This study demonstrated the capacity of the alkaline serine protease to keep its enzymatic activity for some days in the unfavourable environment of the fruit wounds. The alkaline serine protease could be developed as a postharvest treatment with antimicrobial activity for fruit undergoing a short shelf life.

Keywords: Alternaria alternata, Botrytis cinerea, Monilinia fructicola, Penicillium expansum, postharvest, recombinant expression.

1. Introduction

Penicillium expansum, *Botrytis cinerea*, *Monilinia fructicola* and *Alternaria alternata* are among the most severe postharvest pathogens on apples in production areas where the most advanced storage technologies are available (Khamis et al., 2012; Martini et al., 2013; Snowdon, 1990). To control postharvest diseases of fruits, few synthetic fungicides are admitted (Singh and Sharma, 2007; Zhu, 2006). However, pathogen resistance to fungicides (Holmes and Eckert, 1999), and the willingness to use safer and eco-friendly treatments, have generated interest in the development of alternative non-chemical methods to reduce postharvest losses (Lopez-Reyes et al., 2010; Nunes et al., 2012). Biological control using microbial antagonists has emerged as one of the most promising alternatives to fungicides, either alone or as part of an integrated pest management (Janisiewicz and Korsten, 2002). A clear understanding about the mode of action of biocontrol agents is important for a successful implementation of postharvest biocontrol technology (Droby et al., 2009; Zhang et al. 2011).

Among the different biocontrol agents, yeasts are promising and gaining popularity (Jamalizadeh et al., 2011; Janisiewicz et al., 2010; Spadaro et al., 2008). In particular, the yeast-like fungus *Aureobasidium pullulans* De Bary (Arnaud), showed to be effective against *B. cinerea*, *P. expansum* and *Rhizopus stolonifer* on various fruit, including apple, grapes, sweet cherry, strawberry and peach (Bencheqroun et al., 2007; Ippolito et al., 2000; Lima et al., 1997; Schena et al., 2003). Moreover, the strain PL5 of *A. pullulans* showed high efficacy in the control of *B. cinerea* and *P. expansum* on apples, in addition to *Monilinia laxa* on plums and peaches (Zhang et al., 2010a).

Several mechanisms have been reported to play a significant role in the biocontrol activity of *A. pullulans* strains, including induction of defence responses (Ippolito et al., 2000) and competition for nutrients (Bencheqroun et al., 2007). Recently, it was demonstrated that the strain PL5 secretes

β-1,3-glucanase, exo-chitinase and endo-chitinase, in addition to the secretion of alkaline serine protease (Zhang et al., 2010a; 2012).

In mycoparasitism, fungal proteases may be significantly involved in antagonistic activity, because they may play a significant role in fungal cell wall lysis, which is composed of chitin and glucan polymers embedded in, and covalently linked to a protein matrix (Wessels, 1986). The inner layer of fungal cell walls is primarily composed of glucans and chitin arranged as interwoven microfibrils, while the outer electron dense layer is mainly composed of covalently bound mannosylated proteins (Klis et al. 2002). Proteases catalyse the cleavage of peptide bonds in proteins. In recent years, there has been an increasing interest in the study of proteolytic enzymes, because they constitute one of the most important group of industrial enzymes due to their commercial value and potential application in several fields, including food science and technology, pharmaceutical industries and detergent manufactories (Feijoo-Siota and Villa, 2011).

The protease gene *ALP5* of *A. pullulans* strain PL5 was previously cloned and expressed in *Escherichia coli* BL21 (Zhang et al., 2012), showing a low enzymatic activity. Prokaryotic expression systems could present some drawbacks, including incorrect protein processing, folding and posttranslational modification, lower heterologous protein expression levels, and lower activity. *Pichia pastoris* has recently emerged as an important yeast host for heterologous protein expression (Cregg et al., 1993; Macauley et al., 2005). As an eukaryote, *P. pastoris* has many of the advantages of higher eukaryotic expression systems, such as protein processing and folding, and posttranslational modifications, while being as easy to manipulate as *Escherichia coli* (Balamurugan et al., 2007). In the yeast expression system, the secreted heterologous protein is the vast majority in the medium, and, if there are glycosylation sites, glycosylation may occur at these sites. For this reason, yeast genes could be better expressed in eukaryotic expression systems, such as *P. pastoris.*

Therefore, the objectives of this research were to clone the protease gene *ALP5* from *A. pullulans* strain PL5 and to express it in *P. pastoris* to evaluate its activity. A second objective was to

demonstrate the antifungal activity of the recombinant protease in controlling different postharvest pathogens *in vitro* and *in vivo* on fruits, and to prove its involvement in the biocontrol activity of the yeast-like fungus PL5.

2. Materials and Methods

2.1. Microorganisms, plasmids and molecular kits

Aureobasidium pullulans strain PL5 was isolated from the carposphere of plum cv Angeleno and selected for its efficacy (Zhang et al., 2010b). It was identified through microscopic observation of cell and colony morphology, and by sequencing of the ribosomal region ITS (Genbank accession number: FJ919775).

Strains of *Botrytis cinerea, Penicillium expansum, Monilinia fructicola* and *Alternaria alternata* were isolated from rotten apples or peaches (Pellegrino et al., 2009; Saravanakumar et al., 2008), then selected throughout this work for their virulence by inoculation in artificially wounded apples cv Golden delicious. Each strain was maintained at 4 °C on PDA (potato dextrose agar, Merck, Germany) slants.

The *Escherichia coli* strain DH5α used in this study as host for plasmids, was obtained from Invitrogen (Life Technologies, Carlsbad, USA). The oligonucleotides, pGEM-T vector and the *E. coli* strain JM109 were purchased from Promega (Madison, USA). *Pichia pastoris* KM71 strain (Invitrogen) was used as host for transformations with the plasmid pPIC9 (Invitrogen).

2.2. Total RNA isolation and first-strand cDNA synthesis

The strain PL5 was grown in liquid medium YPD (20 g D-glucose, 20 g peptone casein, and 10 g yeast extract per litre) at 25 °C. After 48h shaking at 200 rpm on a rotatory shaker (ASAL, Italy), the culture was centrifuged for 10 min at 5,000 g. RNA was extracted from the pellet with RNeasy® extraction kit (Qiagen, Hilden, Germany), then the first-strand cDNA was synthesized

using Reverse Transcript kit according to the manufacturer's instructions (Qiagen). RNA concentration and purity were checked by spectrophotometer (Nanodrop 2000, Thermo Scientific, Wilmington, USA).

2.3. Cloning of ALP5-Encoding cDNA gene

In order to amplify the cDNA of the *ALP5* gene encoding the alkaline serine protease by PCR, we designed the forward primer (Pf-*ALP5*) as 5′-ACTGAATTCATGTGGAAGAAGAGTGTTGC-3′ and reverse primer (Pr-*ALP5*) as 5′-AATGAATTCTAACGACCGCTGTTGTTGTAAAC-3′; (bases underlined encode *Eco*RI restriction site) according to the sequence of the protease gene obtained from the genomic DNA (GenBank accession number HQ113460.1). PCR conditions were as follows: an initial step at 94 °C for 3 min, and 35 cycles at 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 90 s. The purified PCR amplicons were ligated into pGEM-T-Easy cloning vector, then transformed into chemically competent cells of *E. coli* strain DH5α.

2.4. Construction of the expression vector pPIC9-ALP5

The product was subsequently digested with *EcoRI* and ligated into the pPIC9 vector, which was previously digested with the same restriction enzyme. The consequent plasmid pPIC9-*ALP5* was transformed into *E. coli* JM109 (Promega). The recombinant plasmid was isolated from the positive transformants using Qiaprep Spin Miniprep Kit (Qiagen). The presence and correct orientation of the insert sequence was confirmed by DNA sequencing at BMR Genomics (Padova, Italy).

2.5. Transformation of P. pastoris

The pPIC9-*ALP5* construct was linearized by *Stu I* enzyme for efficient integration into the *P. pastoris* genome, then it was transformed into *P. pastoris* strain KM71. The empty vector (pPIC9) was also transformed in *P. pastoris* for negative control tests following the manufacturer’s

instructions. After transformation with plasmid pPIC9-*ALP5*, His+ transformants of *P. pastoris* KM71 were purified on minimal medium plates without histidine to ensure pure clonal isolates, then the genomic DNA of the transformants were isolated and PCR amplification and sequencing were done to confirm whether the protease cDNA was integrated into the genomic DNA of *P. pastoris*. Primers used for PCR, 5'AOX1 (5'-GACTGGTTCCAATTGACAGC-3') and 3'AOX1 (5'-GCAAATGGCATTCTGACATCC-3') were provided by the manufacturer. The PCR screening of the positive recombinants produced a 1.7 kb fragment, while the control yeast transformed with pPIC9 produced a 492 bp product (data not shown), which confirmed the integration of the insert into *P. pastoris* genome.

2.6. Expression and purification of A. pullulans *strain PL5 protease*

Transformed *P. pastoris* isolates were cultured in 100 mL of BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5} % biotin and 1% glycerol) for approximately 24 h at 28 °C with constant shaking till OD 600 nm reached about 2-6. Cells were centrifuged and the cell mass was resuspended in 20 ml of BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5} % biotin and 0.5% methanol) to induce expression of the recombinant proteins. The culture was supplemented daily with 100% methanol to a final concentration of 1% to maintain induction. Supernatants were then harvested by centrifugation at 3,000 g at 4 °C. The production of the protease in the supernatant of the culture medium was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis SDS-PAGE (Laemmli, 1970) (Amersham ECL Gel 10%, GE Healthcare Life science, Uppsala, Sweden) of aliquots taken at different times (0, 24, 48,72, 96, 120 and 144h). Large-scale production was performed under similar conditions using the isolate that rendered the best yield in the small-scale experiments. To purify the recombinant protease, the supernatant was collected and protein content was precipitated by adding ammonium sulphate (Sigma Aldrich, Milan, Italy, approximately 80% saturation). After centrifugation at 8,000 g for 15 min, the protein precipitate

was dissolved in 50 mM Tris-HCl (pH 8.0) and dialyzed overnight against three changes of the same buffer at 4 °C. The dialyzed sample was clarified by centrifugation and then applied to an anion-exchange column of DEAE Sepharose 1×20 cm (GE Healthcare, United Kingdom), previously equilibrated with 50 mM Tris-HCl (pH 8.0). To elute the bound proteins, 200 mL equilibration buffer were used with a linear gradient from 0 to 0.3 M NaCl at a flow rate of 40 mL/h. Purity of the recombinant protease was determined by SDS-PAGE.

2.7. Protease assay

The recombinant protease activity was assayed against azocasein according to Inamura et al. (1985) with minor modifications. Concisely, a linear rate of increase in activity was obtained up to an absorbance of 0.2 at 440 nm. One unit of the enzyme activity was defined as the quantity of protease which gave an absorbance of 0.001 at 440 nm. The protein concentration was determined by using the Bradford assay (Bradford, 1976). Bovine serum albumin (Sigma-Aldrich, Milan, Italy) was used as a standard. All the experiments and measurements were performed at least in triplicate.

2.8. The effect of temperature and pH on the activity and stability on the recombinant protease

To check the effect of temperature on the activity of the recombinant alkaline serine protease, the enzyme was incubated between 20 °C and 60 °C using the standard assay conditions for the determination of the protease activity. Similarly, the effect of pH on the enzyme activity was studied by incubating the protease at different pH values (ranging from 4.0 to 12.0). The recombinant *ALP5* activity obtained at temperature of 50 °C and pH of 10 was used as a reference (relative activity was 100%), in order to calculate the relative activity at different pH values and temperatures. The experiment was performed three times.

2.9. Effect on pathogen mycelium growth inhibition

219 The activity of the recombinant protease against postharvest pathogens was assayed in Petri dishes
220 containing PDA for *B. cinerea*, *P. expansum*, *M. fructicola* and *A. alternata* according to Zhang et
221 al. (2012) with some modifications. In brief, the mycelial plugs (5 mm in diameter) of the
222 pathogens were corked from a PDA culture and fixed in Petri dish. Ten µL of protease was streaked
223 into PDA at 30 mm from the pathogen plug, and after 4, 8, and 16 days of pathogens growing at 25
224 °C, direct interaction *in vitro* was observed. The inhibitory effect of the protease on the pathogens
225 mycelial growth was calculated by following the formula:

226 $$\text{Percentage of mycelium growth inhibition } (\%) = \left[\frac{DC - DP}{DC}\right]$$

227 DC and DP refers to the average diameters of fungal mycelia of control and protease respectively.
228 The experiment was performed three times.

229

230 *2.10. Effect on pathogen spore germination and germ tube elongation*

231 The effects of the protease on spores germination and germs tube elongation of *B. cinerea*, *P.*
232 *expansum*, *M. fructicola* and *A. alternata* were assayed in potato dextrose broth (PDB, Merck), by
233 using the method of Zhang et al. (2012). Briefly, 300 µL of 1×10^6 conidia/mL Ringer solution
234 (Merck) of each pathogen were transferred to a glass tube containing 2.4 mL PDB. Then 300 µL of
235 purified recombinant protease (62,5 ng/µl) was added to 45° sloping tubes and they were incubated
236 on a rotary shaker (200 rpm) at 25 °C for 9 h and 18 h. In addition, the control was incubated in
237 similar conditions, and it contained a PDB medium inoculated with the pathogens and treated with
238 300 µL inactivated enzymes boiled at 100 °C for 10 min. After 9h and 18 h of incubation, the
239 germination rate and germ tube length were measured on 100 conidia per replication by optical
240 microscopy (Eclipse 55i, Nikon, Tokyo, Japan). For each treatment and pathogen, three replications
241 of three tubes were prepared, and the experiment was performed twice.

242

243 *2.11. Antifungal activity of recombinant protease against postharvest pathogens on apple*

244 The antifungal activity of recombinant protease against *B. cinerea*, *P. expansum*, *M. fructicola* and
245 *A. alternata* was assessed following the method of Yan et al. (2008) with modifications. The
246 conidial suspension of each pathogen was prepared by flooding 14 day-old Petri dish cultures
247 incubated at 25 °C, and adjusted to 10^5 conidia/mL with sterile Ringer solution using a
248 haemocytometer (Zhang et al., 2011). Freshly harvested apples (cv Golden delicious) were surface-
249 sterilized with 1% sodium hypochlorite for 1 min, rinsed with tap water, air-dried, and punctured
250 with a sterile needle at the equatorial region (3 mm depth; three wounds per fruit).
251 In order to evaluate the effect of the protease concentration on the control of postharvest pathogens,
252 two concentrations of the protease PL5 were used: 62.5 ng/µL and 6.25 ng/µL, where 20 µL aliquot
253 of crude protease was pipetted into each wound. Heat inactivated crude protease and water served
254 as a control. Two hours later, 20 µL of conidial suspension (10^5 conidia/mL) of each pathogen was
255 applied into each wound. In order to compare the biocontrol activity of *A. pullulans* PL5 with the
256 efficacy of the protease against the pathogens, the antagonist PL5 was grown in YPD medium for
257 48 h at 25 °C on a rotary shaker at 250 rpm, then the cells were harvested by centrifugation at
258 5000×g for 10 min and adjusted to final concentration of 10^8 cells/mL, from which 20 µl was
259 pipetted into each wound. Two hours later, 20 µL of the pathogen suspension was inoculated into
260 each wound. The treated fruits were incubated at 23 °C, and the rot diameter was measured 4 and 7
261 days after inoculation (DAI) for *B. cinerea* and *P. expansum*, at 8 and 12 DAI for *M. fructicola*, and
262 at 12 and 21 DAI for *A. alternata*. Each treatment contained three replicates with ten fruits per
263 replicate and the experiment was performed three times.
264
265 *2.12. Data analysis*
266 All statistical analyses were performed with SPSS software (SPSS Inc., version 20.0, Chicago, IL,
267 USA). Data from all the experiments were analysed using analysis of variance (ANOVA), and the
268 treatment means were separated at 5% significance level by using Duncan's multiple range tests.
269

3. Results

3.1. Expression and purification of recombinant protease

The expression of purified recombinant protease from a transformed isolate of *P. pastoris* was analysed through SDS-PAGE (Figure 1). After 120 h induction, the protease band was observed in some isolates at 42.9 kDa, which corresponds to the same molecular weight of the *ALP5* protease (Zhang et al., 2012), while no band was present in the negative control (non-insert control: lane number 0). After small-scale production, the best producer isolates (colonies 4 and 5, Fig. 1) were selected for large-scale expression. The recombinant protein was easily purified with DEAE-Sepharose column protein purification system.

3.2. Effect of temperature and pH on protease activity

The effect of temperature and pH on the enzyme activity is shown in table 1**.** The recombinant protease had its maximal activity at 50 °C, however the activity started decreasing when the enzyme reaction temperature exceeded 50 °C.

Results on the effect of pH on the enzyme activity showed that the maximum activity of the protease was observed at pH 10, then it was reduced at higher pH values.

3.3 Effect on pathogen mycelium growth inhibition

After several days of pathogen growth in PDA plates streaked with the protease, the effect of the recombinant enzyme on pathogen mycelium growth was assessed. *M. fructicola* (A) *and B. cinerea* (B) mycelial growth were significantly inhibited by the presence of the recombinant protease (fig. 2a). After 4 days of incubation, the mean inhibition of mycelia growth of *M. fructicola* and *B. cinerea* were 43.3 % and 33.7 % respectively. The mycelial growth inhibition was progressively reduced with the incubation time and it decreased up to 37.7 % (A) and 12.2 % (B), respectively, after 16 days of incubation (Fig. 2b). On the opposite, the recombinant protease did not show any

296 inhibition of the mycelial growth of *P. expansum* (Fig. 2a (C)) and *A. alternata* (Fig. 2a (D)),
297 starting from the first assessment.
298
299 *3.3.Effect on pathogen spore germination and germ tube elongation*
300 The effect of protease on spore germination and germ tube elongation of *B. cinerea*, *P. expansum*,
301 *M. fructicola*, and *A. alternata* is shown in Figures 3 and 4. The data shows that after 9 hours of co-
302 culturing (pathogen+enzyme) in liquid medium (PDB), the protease completely inhibited spore
303 germination of *B. cinerea* (Fig 3a) and *M. fructicola,* (Fig 3b). After 18h of incubation, the
304 germination percentages of *B. cinerea* (Fig 3a) and *M. fructicola* (Fig 3b) were significantly
305 reduced in presence of the recombinant protease from 98% to 54%, and from 98% to 37%,
306 respectively. No significant differences in germination percentage comparing to the control (heat
307 inactivated crude protease) were observed on *P. expansum* (Fig 3c) and *A. alternata* (Fig 3d) treated
308 with the recombinant protease either at 9h or 18h of incubation.
309 In addition, the average germ tube lengths of *B. cinerea* (Fig 4a) and *M. fructicola* (Fig 4b) conidia
310 were 88 and 57 µm after 18 h of incubation, respectively, while the average germ tube lengths of
311 their untreated controls were 372 and 206 µm, respectively. At 9 h and 18 h of incubation, no
312 significant differences in germ tube length were observed on *P. expansum* and *A. alternata* treated
313 with the recombinant protease or with heat inactivated crude protease.
314
315 *3.4. Efficacy of recombinant protease against postharvest pathogens on apple*
316 The antifungal activity of the recombinant protease was investigated on apple (Table 2). Two
317 protease concentrations, 62.5 ng/µL and 6.25 ng/µL were applied in order to study the effect of the
318 enzyme concentration on the biocontrol of the postharvest pathogens.
319 After 4 days of inoculation of *B. cinerea* conidia (Fig. 5), the protease was highly efficient in
320 controlling the pathogen compared to the controls (heat inactivated enzyme and water: 24.0 mm and
321 23.4 mm). The highest protease concentration significantly controlled the lesion diameter on apple

(2.8 mm), similarly to the whole antagonistic cells of *A. pullulans* PL5 (0.0 mm). However, the lowest protease concentration was statistically less effective (10.8 mm) than the highest one or the whole antagonist *A. pullulans.* At 7 DAI, the lesion diameters of grey mould rot increased to 28.3 mm and 37.3 mm, respectively, on apples treated with the highest and the lowest protease concentration, compared to 15.0 mm on *A. pullulans* PL5 treated fruits, but the protease was still significantly more efficient than the controls, which showed 59.3 mm (heat inactivated protease) and 61.2 mm (water) rot diameter.

The two protease concentrations similarly reduced *P. expansum* growth on apple at 4 DAI, with a rot diameter of 8.3 mm and 9.0 mm respectively, compared to 15.0 mm and 17.1 mm in the control fruits (heat inactivated protease and water treated) and no rot in PL5 treated fruit. At 7 DAI, the blue mould lesion diameter on apple fruit treated with both protease concentrations increased to become similar to the controls. The biocontrol agent PL5 showed more efficacy in controlling *P. expansum* compared to the other treatments.

At 8 DAI with *M. fructicola*, the rot diameter of fruits treated with the highest protease concentration (33.3 mm) was lower than the rots in apples treated with the lowest concentration (43.7 mm), with water or with the inactivated enzyme (50.6 mm and 49.8 mm). The best control against brown rot caused by *M. fructicola* was observed in *A. pullulans* PL5 treated fruits (20.8 mm). At 12 DAI, the rot diameters enlarged, and *A. pullulans* PL5 remained the best treatment with 41.0 mm rot, although the protease at the highest concentration controlled better *M. fructicola* (54.5 mm) than its lowest concentration (72.5 mm) or the control fruits (treated with heat inactivated protease or water: 73.2 mm and 75.8 mm).

Only *A. pullulans* PL5 significantly reduced the rot diameter caused by *A. alternata* on apple, and neither protease concentrations were efficient in controlling the pathogen at 12 or 21 DAI.

4. **Discussion**

348 *Aureobasidium pullulans* is a yeast-like fungus that resides in different environments, such as
349 woody tissues and leaves (Gonzalez and Tello, 2011), the surface of fruits from the early stages of
350 their development to maturity (Janisiewicz et al., 2010), and also human skin (Hawkes et al., 2005).
351 Different strains of *A. pullulans* have shown significant control of postharvest pathogens of fruits
352 (Bencheqroun et al., 2007, Ippolito et al., 2000; Zhang et al., 2010a). Recently, *Aureobasidium*
353 *pullulans* DSM 14940 and DSM 14941 have been registered as antimicrobial agents to control fire
354 blight *Erwinia amylovora* on pomefruit (EFSA, 2011). According to toxicological studies, *A.*
355 *pullulans* DSM 14940 and DSM 14941 are not acutely toxic, pathogenic or infective, and not able
356 to replicate within the human body. Anyway, some strains of this species have been recognized as
357 etiologic agents of unusual mycoses in immunosuppressed patients (Bolignano and Criseo, 2003; de
358 Oliveira et al., 2013; Huang et al., 2008).
359 In the present study, we cloned and expressed the protease gene *ALP5* of the antagonistic yeast-like
360 fungus *A. pullulans* in the methylotrophic yeast *P. pastoris* in order to verify its involvement in the
361 biocontrol activity of *A. pullulans* against four pathogens both *in vitro* and *in vivo*. Microbial
362 alkaline proteases are generally considered as safe (GRAS) substances and they dominate the world
363 enzyme market, because of their extensive use in the detergent and food industry, especially the
364 ones produced by some *Bacillus* species which (Schallmey et al., 2004).
365 The alkaline serine protease gene ALP5 was successfully expressed in *P. pastoris* and, as expected,
366 the proteolytic activity of the recombinant protease showed its highest activity under alkaline
367 conditions (at pH 10) and temperature of 50 °C, which was very similar to the activity of the
368 recombinant protease expressed in *E. coli* and to other alkaline serine proteases of bacterial (Gupta
369 et al., 2002) and fungal (Macchione et al., 2008) origin. These results are consistent with a previous
370 report indicating that the optimal pH and temperature of a related protein (alkaline α-amylase gene)
371 from alkaliphilic *Alkalimonas amylolytica* expressed both in *E. coli* and *P. pastoris* are similar
372 (Yang et al., 2012).

373 This study demonstrated that the recombinant protease gene *ALP5* expressed in *P. pastoris* is
374 effective in reducing spore germination and germ tube length of some postharvest pathogens. The
375 dual culture procedure was adopted to study the enzymatic activity of the alkaline serine protease,
376 showing that this *in vitro* method could be applied not only to hypothesize an antimicrobial activity
377 due to release of antibiotics, but also to evaluate the presence of active hydrolases. The presence of
378 protease was able to cause swelling of the hyphae of *B. cinerea*, under optical microscope.
379 Different levels of ALP5 efficacy were observed, indicating a possible effect of the pathogen cell
380 wall composition on the proteolytic activity of the recombinant protease. The highest levels of
381 protease efficacy were observed in *M. fructicola* and *B. cinerea*. Conversely, ALP5 protease was
382 less effective against *P. expansum* and *A. alternata*. These data suggest that the postharvest
383 pathogens display differential sensitivity to protease, as shown in previous studies which revealed
384 that despite having similar cell wall constituents (Bartnicki-Garcia, 1968), the filamentous fungi are
385 differentially sensitive to the cell wall degrading enzymes (chitinases and β-1,3-glucanases;
386 Schlumbaum et al., 1986; Saravanakumar et al., 2009). Proteins represent an important component
387 of the fungal cell wall. Many fungal cell wall proteins identified by tandem mass-spectroscopy have
388 putative carbohydrate-modifying functions involved in cell wall synthesis and remodeling, others
389 are classified as adhesins or heme-binding proteins involved in iron uptake (De Groot et al. 2005).
390 Proteins are the second major component of the cell wall prepared from *B. cinerea* cultures (Cantu
391 et al., 2009). In *Penicillium* spp. (Pessoni et al., 2005) the percentage of proteins is lower (24%),
392 and carbohydrate represent the major component (43%). In species of *Alternaria*, the protein
393 composition has not been characterized, but it is lower than in other fungal species, and melanin, a
394 dark pigment, plays a major role in strengthening the cell wall resistance to proteases (Kishore et
395 al., 2005). The different composition of the cell wall of different pathogens could justify the results
396 of the protease activity both *in vivo* and *in vitro*.
397 In addition, the current study confirms that the recombinant protease *ALP5* expressed in *P. pastoris*
398 exhibited higher proteolytic activity than the protease expressed in *E. coli*, since it is more efficient

399 in reducing spore germination of *M. fructicola* and *B. cinerea* than the protease expressed in the
400 prokaryotic expression system (Zhang et al., 2012). Similar results were obtained by Morton and
401 Potter (2000) which performed a comparative analysis between different model systems including
402 *E. coli* and *P. pastoris* for the expression of a carboxylesterase enzyme. Although the recombinant
403 carboxylesterase protein was observed in *E. coli*, little or no enzymatic activity was detected. In
404 contrast, active protein was produced in *P. pastoris*. These results demonstrate that *P. pastoris* is
405 more convenient than *E. coli* for an efficient expression of the protease from the antagonist *A.*
406 *pullulans* PL5.
407 As a prosecution of these studies, the biocontrol activity of the recombinant protease was evaluated
408 *in vivo* on apple wounds. The results demonstrated the capacity of the alkaline serine protease to
409 keep its enzymatic activity for some days in the unfavourable environment of the fruit wounds. The
410 recombinant protease reduced the activity of *M. fructicola, B. cinerea* and *P. expansum* for the first
411 days of the treatment, after which the pathogen lesion diameters increased. The protease reduced the
412 lesion diameter compared with the controls (the heat inactivated enzyme or water), but the extent
413 was dependent on the enzyme concentration and the temporal distance from the protease treatment.
414 This may be due to the loss of the proteolytic activity with increasing the number of storage days.
415 Our results are in accordance with the results obtained by Yan et al.(2008), which demonstrated that
416 the efficacy of recombinant rice chitinase expressed in *P. pastoris* against *B. cinerea* of loquat
417 fruits, is dependent on the concentration of the enzyme and the time of chitinase treatment and
418 pathogen inoculation.
419 Concerning *A. alternata*, the protease was totally ineffective, and only the biocontrol agent could
420 control the pathogen growth. The protease did not reduce the rot diameter caused by *A. alternata*,
421 which has a slower growth rate compared to the other pathogens (Kader, 2002); after 12 and 21
422 DAI the activity of the protease in degrading the cell wall of the pathogen was lost. Another reason
423 could be also the chemical composition of the cell wall (Kawamura et al., 1999; Wang et al., 1996).
424 The microorganism PL5 better reduced the rot diameter of the pathogens studied, compared to the

protease, probably because other mechanisms of action, and possibly hydrolases, are synergistically involved in pathogen control. Previous work reported the broad efficacy of *A. pullulans*, as the result of a multicomponent action, involving competition for nutrients (Bencheqroun et al., 2007), induction of defense responses (Ippolito et al., 2000), and production of lytic enzymes (Zhang et al., 2010; 2012). To our knowledge, this work provides the clarification of the protease role in the antagonistic activity of the biocontrol agent *A. pullulans* PL5.

In conclusion, either the use of microbials or enzymes as biocontrol approaches presents advantages and drawbacks. The biocontrol agent guarantees higher and longer activity, but it could imply human safety issues, while the enzyme is generally regarded as safe compound, also for the food industry, it has been deeply studied and characterized, but it can be used for limited storage periods. The alkaline serine protease could be developed as a postharvest treatment with antimicrobial activity for fruit undergoing a short shelf life.

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Tables

Table 1 - Determination of the effect of temperature (a) and pH (b) on the enzymatic reaction of the recombinant alkaline serine protease. The recombinant *ALP5* activity obtained at 50 °C and pH 10.0 was used as a reference in order to calculate the relative activity at different temperatures and pH values. Experiments were performed three times and the data are presented as mean ± standard error.

a)

Temperature (°C)	Relative activity (%)
20	42±2.4
30	51±1.8
40	77±2.2
50	100±3.1
60	62±3.9

b)

pH value	Relative activity (%)
5	33±2.7
6	42±2.3
7	67±4.6
8	79±3.9
9	90±4.5
10	100±4.8
11	83±5.1

606 **Table 2** Antifungal activity of the recombinant protease ALP5 in controlling the decay development of *B. cinerea, P. expansum, M. fructicola,* and
607 *A. alternata* in wound-inoculated apples cv Golden Delicious (20 µL of a suspension at 10^5 conidia/mL). The protease was applied at 62.5 ng/µL
608 and 6.25 ng/µL . The results are the mean of three independent experiments.
609

Treatment	**Rot lesion diameters (mm)$_a$**							
	Botrytis cinerea		***Penicillium expansum***		***Monilinia fructicola***		***Alternaria alternata***	
	4 days	**7 days**	**4 days**	**7 days**	**8 days**	**12 days**	**12 days**	**21 days**
Protease (62.5 ng/µL)	2.8±1.0a	28.3±3.2b	8.3±1.5b	31.0±4.6b	33.3±3.5b	54.5±4.1b	8.2±1.8b	48.5±4.9b
Protease (6.25 ng/µL)	10.8± 2.3b	37.3± 3.8b	9.0±1.3b	31.7±3.8b	43.7±4.7c	72.5±9.5c	9.5c1.3b	52.0±7.2b
***A. pullulans* PL5 10^8 cells/ml**	0.0±0.0a	15.0±2.3a	0.0±0.0a	8.2 l±1.4a	20.8±2.6a	41.0±4.6a	0.0±0.0a	35.2±3.8a
Control (inactivated protease)	24.0±2.6c	59.3±7.2c	15.0±3.6c	35.0±6.6b	49.8±6.1c	73.2±8.6c	11.8±2.6b	53.0±4.4b
Control (water)	23.4±3.0c	61.2±5.3c	17.1±2.9c	34.2±5.8b	50.6±6.4c	75.8±10.1c	12.1±3.2b	55.6±3.9b

610
611 '' ± '' stands for standard error of the means. Values of each column followed by different letters show significant difference ($P < 0.05$) according to
612 Duncan's multiple range Test (SPSS 20.0).

Figure captions

Figure 1 - Figure 1: SDS-PAGE analysis of the recombinant protease expressed in *P. pastoris.* Supernatants of the yeast culture were taken from different isolates after 120 h of induction. Lanes: M: molecular weight marker (Precision Plus Protein Dual Color Standards, BIO RAD); 0: *P. pastoris* KM71 isolate transformed with PPIC9 (Control); 1, 2, 3, 4 and 5: some transformed *P. pastoris* isolates with PPIC9-*ALP5.*

Figure 2 - Antifungal activity of the recombinant protease from *A. pullulans* PL5 expressed in *P. pastoris* against postharvest pathogens. (a): inhibition of pathogen growth *in vitro*: A) *M. fructicola;* B): *B. cinerea;* C) *P. expansum*, D) *A. alternata*, (b): Mycelial growth inhibition (%) of *B. cinerea , P. expansum*, *M. fructicola* and *A. alternata* after, respectively, 4, 4, 8, and 16 days of growth at 25 °C.

Figure 3 - Effect of the recombinant protease ALP5 on spore germination of *B. cinerea*, *P. expansum*, *M. fructicola* and *A. alternata* in potato dextrose broth medium. Treatments followed by different letters are statistically different following the Duncan's multiple range test ($p < 0.05$). Spore germination were measured microscopically after 9 h and 18 h of incubation at 25 °C.

Figure 4 - Effect of the recombinant protease ALP5 on germ tube length of *B. cinerea*, *P. expansum*, *M. fructicola* and *A. alternata* in potato dextrose broth medium. Treatments followed by different letters are statistically different following the Duncan's multiple range test ($p < 0.05$). Germ tube length were measured microscopically after 9h and 18 h of incubation at 25 °C.

Figure 5 – Grey mould on apple cv Golden Delicious after 4 days of *B. cinerea* (20 µL of a suspension at 10^5 conidia/mL) inoculation and treatment with (a): heat-inactivated protease as a control, (b): Protease at 6.25 ng/µL, (c): Protease at 62.5 ng/µL.