Detection of enzymatic activity and partial sequence of a chitinase gene in Metschnikowia pulcherrima strain MACH1 used as post-harvest biocontrol agent

(Article begins on next page)

Detection of enzymatic activity and partial sequence of a chitinase in *Metschnikowia pulcherrima* strain MACH1 used as postharvest biocontrol agent

Duraisamy Saravanakumar[1]**, Davide Spadaro**[2]**, Angelo Garibaldi**[1] **and M. Lodovica Gullino**[1]

[1]Centre of Competence for the Innovation in the Agro-environmental Sector, Università degli Studi di Torino, via L. da Vinci 44, I-10095 Grugliasco (TO), Italy; [2]DiVaPRA – Plant Pathology, Università degli Studi di Torino, via L. da Vinci 44, I-10095 Grugliasco (TO), Italy.

Abstract

Two antagonistic yeast strains *Metschnikowia pulcherrima* MACH1 and *Rhodotorula sp.* PW34 were tested for their efficacy against *Botrytis cinerea in vitro* and *in vivo* on apples. *M. pulcherrima* strain MACH1 showed higher inhibition of *B. cinerea* compared to the strain PW34 *in vitro* on potato dextrose broth. Further, yeast strain MACH1 showed higher efficacy in reducing grey mould on apples compared to PW34 and untreated control. In addition, partially purified extracellular proteins from strain MACH1 showed an inhibition to *B. cinerea in vitro*. The antagonistic yeast strains were tested for their efficacy to produce chitinases in different liquid media, among which apple juice, amended with or without cell wall preparations (CWP) of *B. cinerea*. The study showed the higher production of chitinases from *M. pulcherrima* strain MACH1 when compared to PW34. Interestingly, the strain MACH1 secreted higher chitinases in the presence of cell wall fractions of *B. cinerea*. For this reason, chitinase gene of strain MACH1 was amplified using PCR reactions and the nucleotide sequence data showed high homology to chitinases of other yeast strains. The results of the current study permit to conclude that *M. pulcherrima* strain MACH1 has the ability to secrete chitinases in different liquid media including apple juice and the enzyme could be involved in the biological control of *B. cinerea* in postharvest.

Keywords: *Botrytis cinerea*, Cell wall preparations, Chitinases, *Metschnikowia pulcherrima*, PCR, Postharvest biocontrol

1. Introduction:

Postharvest disease control is traditionally based on the application of synthetic fungicides (Eckert and Ogawa 1985). However, due to the concern related to fungicide toxicity, development of fungicide resistance by pathogens and potential harmful effect on the environment and human health, alternatives to synthetic chemicals have been proposed (Eckert et al. 1994; Janisiewicz and Korsten 2002). Among the proposed alternatives, the development of antagonistic microorganisms has been the most studied and has made substantial progress in the management of postharvest diseases (Wilson and Wisniewski 1994; Spadaro and Gullino, 2004). Among the different biocontrol agents, yeasts attract particular attention due to the non-production of toxic metabolites which could have a negative environmental or toxicological impact. Recently, *Metschnikowia pulcherrima* has been reported as an effective biocontrol agent against postharvest decay of apple, table grape, grapefruit and cherry tomato (Piano et al. 1997; Schena et al. 2000; Janisiewicz et al. 2001; Spadaro et al. 2002) as well as against some food-borne pathogens (Leverentz et al. 2006). Similarly, the yeast species of *Rhodotorula* have been reported as biological control agents against postharvest diseases (Calvente et al. 1999; 2001). When considering how to improve the performance of natural biocontrol agents and hence to develop them as reliable commercial products, it is first necessary to characterize their mechanisms of action.

Several possible biocontrol mechanisms have been suggested against postharvest rots on fruit including competition for nutrients and space, antibiosis, parasitism or direct interaction with the pathogens and induction of resistance in the host tissue (Smilanick 1994). Competition for nutrients and space is believed to be the major mode of action of antagonistic yeasts (El-Ghaouth et al. 1998; Spadaro et al. 2002). Recently, we demonstrated that *M. pulcherrima* strain MACH1 could reduce the postharvest pathogens of apple through competition for iron nutrient (Saravanakumar et al.

2008). However, there is growing evidence to support the possible involvement of cell wall degrading enzymes in the action of yeast antagonists. Wisniewski et al. (1991) demonstrated that the yeast *Pichia guilliermondii* secreted high levels of exo-ß-1,3-glucanase and chitinase when cultured on various liquid media or on the cell walls of fungal pathogens. The ability of the yeast to produce lytic enzymes was hypothesized to be associated with the firm attachment of the yeast cells to fungal hyphae and the partial degradation of fungal mycelia. Though there are sufficient amount of literature supporting the biocontrol efficacy of different species of *Metschnikowia* against postharvest pathogens (Kurtzman and Droby 2001; Spadaro et al. 2002), there is no report about the production of lytic enzymes and their role in the biological control of postharvest diseases.

Therefore, the objectives of the study were: (1) to evaluate the level of efficacy of two yeast strains (*M. pulcherrima* MACH1 and *Rhodotorula* sp. PW34), previously selected at AGROINNOVA – University of Torino, on apples against *Botrytis cinerea*; (2) to study the antifungal activity of extracellular proteins secreted by yeast strains (3) to study the production of chitinases by both yeast strains on synthetic liquid media and on apple juice, in the presence and absence of cell wall of *B. cinerea*; (4) to detect the chitinase gene in order to develop a molecular method to screen the enzymatic activity of *M. pulcherrima* strains potentially useful for biocontrol.

2. Materials and methods:

2.1. Yeast and fungal cultures

Metschnikowia pulcherrima strain MACH1 (Saravanakumar et al. 2008) and *Rhodotorula* sp. PW34 (Spadaro et al. 2005) were isolated from the carposphere of apple cv Golden delicious, harvested in organic orchards located in Piedmont, Northern Italy. Three strains of *Botrytis cinerea* were isolated from rotted apples and selected for their virulence by inoculation in artificially wounded apples. The antagonistic and pathogenic fungal strains were maintained in Yeast Peptone Dextrose (YPD: 10 g l^{-1} of Extract of Yeast Granulated Merck; 20 g l^{-1} of Triptone-Peptone of

Casein Difco; 20 g l^{-1} of D(+)-Glucose Monohydrate, Merck) and Potato Dextrose Agar (PDA; Merck, Germany) respectively at 4ºC.

2.2. Efficacy of antagonists against *B. cinerea in vitro*

The antagonistic yeast strains MACH1 and PW34 were inoculated onto 250 ml Erlenmeyer flasks containing 75 ml of Potato Dextrose Broth (PDB, Sigma, Steinheim, Germany) and incubated on a rotary shaker (100 rpm) at 25°C for 24 hours. Later, *B. cinerea* mycelial discs (8 mm diameter) were placed onto flasks containing antagonistic yeast strains and incubated under room temperature (25±2ºC) for 10 days. The PDB inoculated with *B. cinerea* served as control for the comparative studies. The culture filtrate was filtered through Whatman No.1 filter paper and the filtrates were observed for the conidial production by *B. cinerea* under Burker chamber. The mycelial wet weight was recorded from each treatment and the mycelial dry weight was taken after removing the water content by incubating in hot air oven at 70ºC for 10 days. Six replications were maintained for each treatment and the experiments were carried out three times.

2.3. Efficacy of antagonists against *B. cinerea in vivo*

The biocontrol efficacy was tested on apples treated with the biocontrol strains MACH1 and PW34 and inoculated with *B. cinerea*. Antagonistic yeasts MACHI and PW34 were grown on YPD and inocula of the antagonists were prepared as described in Spadaro et al., (2002). Spore suspensions were prepared by growing the pathogens on Petri dishes for two weeks on PDA added with 50 mg l^{-1} of streptomycin. After two weeks of incubation at 25°C, spores from the three strains were collected and suspended in sterile Ringer's solution (Merck). After filtering through 8 layers of sterile cheese-cloth, spores were counted and brought to a final concentration of 10^5 ml^{-1}. Apples (cv Golden delicious) were disinfected in sodium hypochloride (NaClO, 1.0 % as chlorine) and rinsed under tap water and when dry punctured with a sterile needle at the equatorial region (3 mm depth; 3 wounds per fruit). An antagonistic yeast suspension (30 μl: 2×10^8 cells ml^{-1} were adjusted using Burker chamber) was pipetted into wound. Inoculated control fruits were pipetted, before pathogen inoculation, with 30 µl of distilled water. After 3 hours, 30 µl of the spore suspension of the *B. cinerea* were pipetted in the wound. Fifteen fruits per treatment were used (45 inoculation sites). Eight days after inoculation, rotten area, fruit weight and percent infected wounds were recorded. The experiments were carried out twice.

2.4. Antifungal activity of partially purified proteins

To test the effect of extracellular proteins produced yeast biocontrol strains against *B. cinerea*, yeast cells of MACH1 and PW34 were inoculated onto YPD broth and grown at 25ºC and 4 l of 6-day-old culture was used for partial purification of proteins. Following filtration through a Millipore membrane (0.2 µm) (Sigma Aldrich, Italy), proteins in the supernatant fluid were precipitated with $(NH_4)_2SO_4$ (Sigma Aldrich, Italy) (approx. 80% saturation) on ice. The precipitate was recovered by centrifugation at 16,260 g for 30 min, dissolved in 50 ml 0.1 M phosphate buffer (pH 7.2) and dialyzed (Dialysis Tubing Cellulose Membrane; Sigma Aldrich, Italy) three times against 5 l of distilled water at 4ºC overnight. The protein solution was concentrated using lyophilizer and the protein concentration was determined according to Bradford (1976), with bovine serum albumin as standard. The antifungal activity of the partially purified protein was studied based on the mycelial growth inhibition of *B. cinerea.* Whatman No.1 filter paper strips of 25 mm^2 were cut and sterilized by baking at 150ºC for 2 h. In sterile 90-mm Petri plates of three numbers, 20 mL of PDA medium was poured separately, and three strips were placed equally spaced on the outside periphery of each plate. Ten micrograms of purified protein dissolved in sodium phosphate buffer pH 7.0 were added to the discs using sterile pipette. Cell free culture filtrates (30 µl for each paper disc) of the two yeast strains were also tested for their antifungal activity. Sterile distilled water served as control. Fungal discs (5 mm) were punched from 5-day old culture *Botrytis cinerea* and placed in the middle of the Petri plate. The plates were incubated at room temperature (28 ± 2ºC) and observations were made after 5 days of incubation.

2.5. Preparation of *B. cinerea* cell walls

Cell wall preparations (CWP) of *B. cinerea* were prepared as reported by Saligkarias et al. (2002) with some modifications. Briefly, the mycelium collected by Whatman No.1 filter paper was washed four times with deionised water, homogenized for 2 min and centrifuged 2 min at 480 x g. After removing the supernatant, the fungal material was sonicated with a sonicator for 10 min and centrifuged for 5 min at 600 rpm. The supernatant was discarded and the pellet was resuspended in water. The samples were subjected to sonication and centrifugation as above for a total of six times. Then the crushed mycelium was resuspended in an equal volume of Tris HCl buffer (50 mmol l^{-1} and pH 7.2), centrifuged for 10 min at 1920 x g, and the supernatant was discarded. The pellet was subject to three successive cycles of centrifugation and resuspension. The final pellet was frozen in liquid N_2, lyophilized and stored at −20ºC.

2.6. Preparation of enzyme extract

Antagonistic yeasts were inoculated into 250 ml Erlenmeyer flask containing 75 ml of YPD broth, PDB and apple juice extracts (Commercial juices from supermarkets, Italy) (15 lbs, 121ºC for 15 min : autoclaved). Similarly, onto YPD broth, PDB and apple juice extracts, 2 mg ml^{-1} CWP of *B. cinerea* were added and yeast strains were inoculated. The cultures were incubated on a rotary shaker (200 rpm) at 25°C. Aliquots of 5 ml of each culture were withdrawn aseptically from each flasks after 24, 48, 72, 96, 144 and 196 h of inoculation and centrifuged at 9600 rpm for 10 min at 4°C to obtain the cell free culture filtrates. The supernatants were used for the enzyme assay. Five replications were maintained for each treatment and the experiment was repeated twice.

2.7. Assay of chitinase activity

The spectrophotometric assay of chitinase was carried out according to the procedure developed by Boller and Mauch (1988). The reaction mixture consisted of 10 µl of 0.1 M sodium acetate buffer (pH 4.0), 0.4 ml enzyme solution and 0.1 ml colloidal chitin. After incubation for 2 h at 37°C, the reaction was stopped by centrifugation at 3,000 g for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl of 1 M potassium phosphate buffer (pH 7.0) and incubated with 20 µl of 3% (w/v) snail gut enzyme (Sigma, Steinheim, Germany) for 1 h. After 1 h, the reaction mixture was brought to pH 8.9 by the addition of 70 µl of 0.1 M sodium borate buffer (pH 9.8). The mixture was incubated in a boiling water bath for 3 min and then rapidly cooled in an ice-water bath. After addition of 2 ml of DMAB (para-dimethylaminobenzaldehyde reagent was prepared by mixing 8 g of DMAB in 70 ml of glacial acetic acid along with 10 ml of concentrated HCl; one volume of stock solution was mixed with 9 volumes of glacial acetic acid and used for the reactions), the mixture was incubated for 20 min at 37°C and the absorbance was measured at 585 nm.

2.8. DNA extraction from yeast strain MACH1

Two ml of YPD culture of the strain MACH1 was centrifuged at 2500 x g for 3 min. The pellets were suspended in 280 µl of EDTA 50 mM (pH 8-8.5) with 400 µg of lyticase (Sigma, Steinhem, Germany) and incubated at 37°C for 45 min. After 3 min centrifugation, the pellets were treated with the Wizard Genomic DNA Purification kit (Promega Corp., Madison, WI, USA). Genomic DNA was controlled by electrophoresis (30 min at 100 V/cm) on 1% SeaKem LE agarose gel (FMC BioProducts, Rockland, ME, USA) in 1X TAE buffer (40 mM Tris, 40 mM acetate, 2 mM EDTA, pH 8.0; Maniatis et al. 1982); the gel was stained with SYBR-safe (Invitrogen, USA) and visualized through UV light. Gel images were acquired with a Gel Doc 1000 System (Bio-Rad Laboratories, Hercules, CA, USA). A 1 kb DNA ladder (Gibco BRL, Rockville, MD, USA) was used as a molecular weight marker for an approximate quantification of the genomic DNA. A precise quantification in ng/µl was obtained by a BioPhotometer (Eppendorf, Hamburg, Germany). Purified DNA was stored in TE buffer (10 mM Tris-HCl; 0.1 mM EDTA; pH 8) at 4°C for further reactions.

2.9. PCR amplification of ITS region of *M. pulcherrima* MACH1

To confirm the species of strain MACH1 at molecular level, an ITS region was amplified using universal primers ITS5 (5’-GGAAGTAAAAGTCGTAACAAGG-3’) and ITS2 (5’-GCTGCGTTCTTCATCGATGC-3’) (White et al. 1990). PCR reactions were carried out in 20 µl reaction mixture containing 10x buffer (with 2.5 mmol l^{-1} $MgCl_2$), 2 µl; 2 mmol l^{-1} dNTP mixture, 2 µl; 2 mol l^{-1} primer, 5 µl; Taq DNA polymerase, 3 U; H_2O, 8 µl and 50 ng of template. DNA samples were amplified on a DNA thermalcycler (Biometric, USA) using the PCR conditions: 95ºC for 1 min, 52ºC for 1 min and 72ºC for 2 min. The total number of cycles was 35 with a final extension time of 10 min. The PCR products were resolved on 2% agarose at 50 V stained with SYBR-safe (Invitrogen, USA) and photographed using a Gel Doc 1000 system.

2.10. PCR detection of chitinase gene of *M. pulcherrima* MACH1

The chitinase gene of *M. pulcherrima* was amplified with degenerate primers CHI forward (5’-ATCATGRTITAYTGGGGICARAA-3’) and CHI reverse (5’-GAGCARTARTARTTRTTR TA RAAYTG-3’), previously designed by McCreath et al. (1995) for *Candida albicans*. PCR reactions were carried out in 20 µl reaction mixture containing 10x buffer (with 2.5 mmol l^{-1} $MgCl_2$), 2 µl; 2 mmol l^{-1} dNTP mixture, 2 µl; 2 mol l^{-1} primer, 5 µl; Taq DNA polymerase, 3 U; H_2O, 8 µl and 50 ng of template. DNA samples were amplified on DNA thermalcycler (Biometric, USA) using the PCR conditions 94°C for 45 s, 56°C for 1 min and 72°C for 45 s. The total number of cycles was 35 with a final extension time of 10 min. The PCR products were resolved on 2% agarose at 50 V stained with SYBR-safe (Invitrogen, USA) and the images were obtained from Gel Doc 1000 system.

2.11. Cloning and sequencing of ITS region and chitinase gene of strain MACH1

Both amplified DNA regions were purified from agarose (1.2%, w/v) gel after electrophoresis. A small agarose slice containing the band of interest [observed under long-wavelength (312-nm) UV light] was excised from the gel and purified by using a QIAquick gel extraction kit (Qiagen Inc., Chatsworth, CA, USA) according to the supplier's instructions. This purification was performed to remove primer dimers and other residues from the PCR amplification. Fragments were cloned into the pCR 2.1-TOPO plasmid vector (Invitrogen, USA) and transformed into *Escherichia coli* strain DH5α according to the procedure recommended by the manufacturer. Transformants were selected on Luria Broth (LB) agar amended with ampicillin (75 mg ml^{-1}). Clones were randomly selected and used as templates in PCR to produce products of required sizes in agarose gel. The transformation of *E. coli* strains with the presence of the insert was confirmed by PCR using universal primer M13F (5'-CACGACGTTGTAAAACGAC-3') and M13R (5'-GGATAACAATTTCACACAGG-3') sequence. DNA sequencing was performed at BMR Genomics, DNA sequencing service centre, Padova, Italy. For sequence determination of the cloned PCR products, a generally applicable sequencing strategy was developed. The nucleotide sequences were submitted to the National Centre for Biotechnology Information (NCBI), GenBank, New York, NY, USA.

2.12. Statistical analysis

Data from all the experiments were analyzed using analysis of variance (ANOVA) and the SPSS version 12.0 (SPSS, 1989-2003). The treatment means were separated at the 5% significance level using Duncan's Multiple Range Test (DMRT).

3. Results

3.1. Biocontrol strain efficacy against *B. cinerea in vitro* and *in vivo*

The results of the *in vitro* study showed that *B. cinerea* produced less mycelium, considered as wet and dry matter, in PDB when cultured with both biocontrol strains than alone (1564, 150.3 mg). In particular, the mycelium produced was lower in presence of *M. pulcherrima* strain MACH1 (115.8, 21.5 mg) than of *Rhodotorula* sp. strain PW34 (385, 35.2 mg). *B. cinerea* did not produce conidia co-cultured in a media with the antagonistic yeast whereas higher conidial ($9.3x10^4$ ml^{-1}) concentration was noticed in the *B. cinerea* inoculation alone (Table 1). Apples treated with the antagonist had lower *B. cinerea* infection compared to untreated control. Further, apples treated with *M. pulcherrima* strain MACH1 had lower incidence of decay (8.47%). The diameter of the rotten areas and weight of the rotten tissue were lowest (2.13 cm, 6.33 g respectively) in apples treated with the strain of *M. pulcherrima*. Apples inoculated with *B. cinerea* alone had the highest decayed area (7.45 cm) and decayed tissue weight (51.78 g) (Table 1).

3.2. Antifungal activity of extracellular proteins of MACH1

Assay to test the antifungal activity of partially purified proteins from two different yeast strains indicated that proteins from *M. pulcherrima* strain MACH1 strongly inhibited the growth of *B. cinerea* mycelium *in vitro* (Fig 1). The culture filtrates of strain MACH1 did not show any inhibition to mycelial growth of *B. cinerea*. Both culture filtrates and partially purified proteins from strain PW34 did not show inhibition to mycelial growth of *B. cinerea*.

3.3. Enzymatic assay of the chitinase activity by both biocontrol agents

The assay of chitinase production in YPD broth inoculated with both yeast strains showed higher activity when inoculated with MACH1 than PW34. The highest chitinase activity was observed in *M. pulcherrima* MACH1 144 h after inoculation and later the activity started to decline. When YPD broth was amended with CWP of *B. cinerea*, the chitinase produced by strain MACH1 was higher than when the yeast was inoculated without pathogen preparation. However, less activity of chitinase was noticed in PW34 inoculated onto YPD broth amended with or without CWP of *B. cinerea* (Fig 2a).

Filtrates from MACH1 culture grown in PDB+CWP had higher activity of chitinase than from culture grown in PDB alone. The peak of chitinase activity occurred 6 days after inoculation (Fig 2b). In general the chitinase activity by both yeast strains was lower in PDB than in YPD broth.
The yeast strain MACH1 had higher activity of chitinase than PW34 in apple juice and in apple juice amended with CWP of *B. cinerea* compared to non-amended. The chitinase activity by *M. pulcherrima* strain MACH1 reached its maximum 6 days after inoculation and later it started to decline, whereas in PW34, throughout the assay period, lower activity was recorded (Fig 2c).

3.4. PCR amplification, cloning and sequencing of ITS region of MACH1

Based on the superior biocontrol performance provided by the yeast strain MACH1, a PCR was carried out on the ribosomal DNA to identify the species, using ITS5 and ITS2 primers. The ITS primers amplified a DNA fragment of 371 bp corresponding to the region including partial sequence of the 18S ribosomal RNA gene; the internal transcribed spacer 1; the 5.8S ribosomal RNA gene; and the internal transcribed spacer 2; and partial sequence of 26S ribosomal RNA gene. The amplified rDNA fragments of strain MACH1 was cloned into pCR 2.1-TOPO plasmid vector and transformed into *E. coli* strain DH5α. Transformants on LB agar amended with ampicillin were randomly selected and used as templates in PCR to verify products of 371 bp in agarose gel (Fig. 3). The sequencing was performed using M13 universal primers and sequence of the ITS rDNA region from MACH1 was submitted to the NCBI and given the Accession No. EU037994.

3.5. PCR detection, cloning and sequencing of chitinase gene of MACH1

A PCR performed on the genomic DNA of the antagonistic yeast MACH1 using degenerative resulted in amplification of 566 bp product (Fig 3). The amplified product was cloned into pCR 2.1-TOPO plasmid vector and transformed into *E. coli* strain DH5α. Transformants on LB agar amended with ampicillin were randomly selected and used as templates in PCR to produce products of the required size (566 bp). Sequencing was performed using M13 universal primers and nucleotide sequences of chitinase gene from antagonistic yeast strain MACH1 was submitted to the NCBI and the Accession No. EU153550 was assigned.

4. Discussion

Strains of *Metschnikowia pulcherrima* isolated from carposphere can be effective in protecting apples, peaches, and grapes against postharvest rots caused by *Botrytis cinerea* and other postharvest pathogens (De Curtis et al. 1996; Janisiewicz et al. 2001; Spadaro et al. 2002). In the present study, *M. pulcherrima* strain MACH1 showed higher inhibition of the mycelial growth of *B. cinerea* in liquid media compared to *Rhodotorula* sp. strain PW34. Further, the yeast strain MACH1 reduced more the spore production by *B. cinerea* under *in vitro* conditions. This indicates the possible role of extracellular proteins and metabolites in the arrest of the conidial production and mycelial growth of *B. cinerea*. To demonstrate this, the study was conducted to test the effect of extracellular proteins produced by yeast strain MACH1 and it revealed the antifungal activity against *B. cinerea in vitro*. Our findings are similar to Harish et al. (1998) who reported the reduced growth of *Fusarium udum* in the presence of extracellular proteins of culture filtrate of *Bacillus subtilis* AF1. The yeast strain MACH1 showed higher efficacy against *B. cinerea* on apples and there are several works performed in our Centre on the biocontrol efficacy of *M. pulcherrima* strains against postharvest diseases of pome fruits (Piano et al. 1997; Spadaro et al. 2004; Saravanakumar et al. 2008).

An extensive review on literatures indicates that among different mechanisms of action displayed by the biocontrol agents, one of the major mechanism is parasitism via degradation of the cell wall (Vaidya et al. 2003; Joo 2005). Chitin, the unbranched homopolymer of N-acetyl glucosamine in a β-1,4 linkage, is a structural component of cell walls in many fungi. Chitinases which hydrolyse this polymer are produced by various organisms and have been implicated in the biocontrol process (Castoria et al. 2001; Gohel et al. 2006). So far none of the *M. pulcherrima* isolates have been studied for their chitinase activity. Our *in vitro* and *in vivo* experiments permitted to study the ability of *M. pulcherrima* to secrete chitinases. A higher production of chitinases by yeast strain MACH1 in PDB and YPD in the presence of CWP indicated the induction of chitinases by biocontrol yeast. Similarly, a superior production of chitinases by *M. pulcherrima* strain MACH1 occurred in apple juice extract amended with CWP indicated the possible involvement of the chitinase production by yeast strain MACH1 in the control of *B. cinerea* on apples. The results are similar to previous findings by Wisnieswski et al. (1991) who reported that *Pichia guilliermondii* and *P. anomala* produce higher levels of lytic enzymes when grown in media supplemented with fungal cell walls than when grown in media containing only glucose. Saligkarias et al. (2002) reported the secretion of detectable amounts of β-1,3-exoglucanase and chitinase by *C. guilliermondii* (strains 101 and US 7) and *C. oleophila* (strain I-182) grown in different carbon sources.

Similarly, *P. guilliermondii* and *P. membranaefaciens* exhibited high levels of lytic enzymes activity, when cultured on various carbon sources or with cell walls of several fungi (Masih and Paul 2002). Cells of these yeasts were attached to the mycelium of *B. cinerea* and caused partial degradation of the cell wall. Similarly, *M. pulcherrima* strain MACH1 could attach to cell walls of *B. cinerea* and secrete chitinases. It is possible that CWP of *B. cinerea* might be utilised by *M. pulcherrima* strain MACH1 as carbon source and the yeast could produce higher levels of chitinase. Other works reported the higher lytic enzymes activity from *Trichoderma harzianum* and *Pichia anomala*, when grown on media supplemented with fungal cell walls than in media containing glucose (Elad et al. 1982; Jijakli and Lepoivre 1998). Similarly, it was demonstrated by several workers that the presence of glucose repressed the chitinase activities for a range of ectomycorrhizal and non-mycorrhizal fungi (El-Katatny et al. 2000; Bougoure and Cairney 2006) and for expression of some chitinase genes (Dana et al. 2001). Recently, Kaur et al. (2005) reported the higher production of chitinases by fungal biocontrol strains in the presence of sclerotia of *Sclerotinia sclerotiorum* which is similar to our current findings.

Various molecular methods have been developed and used for identify various microorganisms at species level (White et al. 1990; Stafford et al. 2005). To date, many applications of molecular identification have focussed on differentiation of important yeast species used in the field of biological control (Daniel et al. 2001; Spadaro et al. 2008). In the present study, PCR amplification and sequencing of ITS region has confirmed that strain MACH1 used in this study is *Metschnikowia pulcherrima*.

The biochemical assays of chitinase demonstrated the secretion of enzyme by *M. pulcherrima* strain MACH1. In addition, PCR amplification confirmed the presence of chitinase gene in the yeast strain MACH1. Similarly, several chitinase genes have been amplified from different yeast and bacterial strains using PCR techniques (McCreath et al. 1995; Chernin et al. 1997; Huang et al. 2005). The nucleotide data of chitinase gene from *M. pulcherrima* strain MACH1 showed high homology to chitinase of *Pichia stipitis* CBS 6054, a yeast whose genome has been sequenced for its industrial applications (GenBank Accession No. XM 001386570). The deduced amino acid sequence of the chitinase gene from *M. pulcherrima* strain MACH1 showed higher homology (>80%) to chitinases from *Pichia stipitis*, *Candida albicans* and *Pichia guilliermondii* (data from GenBank, NCBI). Conspicuously, the chitinase protein code of strain MACH1 belongs to the group of glycosyl hydrolases family 18, which generally contains enzymes that are involved in the lysis of chitin molecules (Funkhouser and Aronson, 2007). This suggests that the chitinase gene amplified from the biocontrol strain MACH1 could also have a role in the biological control of postharvest pathogens. To our knowledge, this is the first report on the secretion and detection of chitinases from biocontrol *M. pulcherrima* and the identification of a putative chitinase gene from a yeast used as biocontrol agent. In addition, the current study suggested that the primers designed for chitinase amplification from *C. albicans* by McCreath et al., (1995) could be used for the detection of chitinases from other yeast biocontrol strains.

In general, other chitinase producing bacteria or filamentous fungi have been reported as biocontrol agents against different kinds of fungal diseases of plants (Kobayashi et al. 2002; Freeman et al.

2004). Similar to this, the current study also documented the higher secretion of chitinases in the presence of CWP of *B. cinerea*. Further, PCR amplification and sequencing of chitinase gene from *M. pulcherrima* strain MACH1 has confirmed the presence of the gene at molecular level. With the supportive evidence of the previous findings, it is assumed in the present study that secretion of chitinases could be involved in biocontrol efficacy of *M. pulcherrima* strain MACH1 against postharvest fungal pathogens. However, more in depth study is needed to elucidate the role of the chitinases and other cell wall degrading enzymes in the antagonistic activity of *M. pulcherrima* strain MACH1. Current work addresses direct role of lytic enzymes produced by strain MACH1 in the biological control of postharvest pathogens.

Acknowledgements

This research was carried out partly with a grant from the Piedmont Region for the project "Selection, study of the efficacy and of the mechanism of action, characterization and development of antagonistic yeast against post-harvest fruit pathogens" and partly with a grant from the Italian Ministry for the Environment and Land and Sea within the Framework Agreement "Sustainable Agriculture" to AGROINNOVA. Moreover, Duraisamy Saravanakumar acknowledges the Italian Ministry for University and Research and the Compagnia di San Paolo for the PostDoc grant "Bando per borse a favore di giovani ricercatori indiani".

References

Boller, T., & Mauch, F. (1988). Colorimetric assay for chitinase. Methods In Enzymology, 161, 430-435.

Bougoure, D.S., & Cairney, J.W.G. 2006. Chitinolytic activities of ericoid mycorrhizal and other root-associated fungi from *Epacris pulchella* (Ericaceae). Mycological Research, 110, 328-334.

Bradford, M.M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, 248-254.

Calvente, V., Benuzzi, D., & de Tosetti, M.I.S. (1999). Antagonistic action of siderophores from *Rhodotorula glutinis* upon the postharvest pathogn *Penicillium expansum*. International Biodeterioration & Degradation, 43, 167-172.

Calvente, V., de Orellano, M.E., Sansone, G., Benuzzi, D., & Tosetti, M.I.S. (2001). Effect of nitrogen source and pH on siderophore production by *Rhodotorula* strains and their application to biocontrol phytopathogenic moulds. Journal of Industrial Microbiology & Biotechnology, 26, 226-229.

Castoria, R., De Curtis, F., Lima, G., Caputo, L., Pacifico, S., & De Cicco, V. (2001). *Aureobasidium pullulans* (LS-30) an antagonist of postharvest pathogens of fruits: study on its modes of action. Postharvest Biology & Technology, 22, 7 – 17.

Chernin, L.S., De La Fuente, L., Sobolev, V., Haran, S., Vorgias, C.E., Oppenheim, A.B. et al. (1997). Molecular cloning, structural analysis, and expression in *Escherichia coli* of a chitinase gene from *Enterobacter agglomerans*. Applied Environmental Microbiology, 63(3), 834-839.

Dana, M.M., Limon, M.C., Mejıàs, R., Mach, R.L., Benıtez, T., Pintor-Toro, J.A., Kubicek, C.P. 2001. Regulation of chitinase 33 (chit33) gene expression in *Trichoderma harzianum*. Current Genetics, 38, 335-342.

Daniel, H.M., Sorrell, T.C. & Meyer, W. (2001). Partial sequence analysis of the actin gene and its potential for studying the phylogeny of *Candida* species and their teleomorphs. International Journal of Systematic and Evolutionary Microbiology, 51, 1593-1606.

De Curtis, E., Torriani, S., Rossi, E., & De Cicco, V. (1996). Selection and use of *Metschnikowia pulcherrima* as a biological control agent for postharvest rots of peaches and table grapes. Annals of Microbiol. Enzymology, 46, 45-55.

Eckert, J.W., & Ogawa, J.M. (1985). The chemical control of postharvest diseases: subtropical and tropical fruits. Annual Review of Phytopathology, 23, 421-454.

Elad, Y., Chet, I., & Henis, Y. (1982). Degradation of plant pathogenic fungi by *Trichoderma harzianum*. Can. J. Microbiol. 28, 719-725.

El-Ghaouth, A., Wilson, C.L., & Wisniewski, M. (1998). Ultrastructural and cytochemical aspects of the biological control of *Botrytis cinerea* by *Candida saitoana* in apple fruit. Phytopathology, 88, 282-291.

El-Katatny, M.H., Somitsch, W., Robra, K.H, El-Katatny, M.S., & Gu¨bitz, G.M. (2000). Production of chitinase and β-1,3-glucanase by *Trichoderma harzianum* for control of the phytopathogenic fungus *Sclerotium rolfsii*. Food Technology and Biotechnology, 38, 173-180.

Freeman, S., Minzm O., Kolesnik, I., Barbul, O., Zveibil, A., Maymon, M. et al. (2004). *Trichoderma* biocontrol of *Colletotrichum acutatum* and *Botrytis cinerea* and survival in strawberry. European Journal of Plant Pathology, 110, 361-370.

Funkhouser, J.D. & Aronson, N.N. (2007). Chitinase family GH18: evolutionary insights from the genomic history of a diverse protein family. BMC Evolutionary Biology, 7:96.

Gohel, V., Singh, A., Vimal, M., Ashwini, P., & Chhatpat, H.S. (2006). Bioprospecting and antifungal potential of chitinolytic microorganisms. African Journal of Biotechnology, 5(2), 54-72.

Harish, S., Manjula, K., & Podile, A.R. 1998. *Fusarium udum* is resistant to the mycolytic acitivity of a biocontrol strain of *Bacillus subtilis* AF1. FEMS Microbiology Ecology, 25, 385-390.

Huang, C., Wang, T., Chung, S., & Chen, C. 2005. Identification of an antifungal chitinase from a potential biocontrol agent, *Bacillus cereus* 28-9. Journal of Biochemistry and Molecular Biology, 38, 82-88.

Eckert, J.W., Sievert, J.R., & Ratnayake, M. (1994). Reduction of imazalil effectiveness against citrus green mold in California packinghouses by resistant biotypes of *Penicillium digitatum*. Plant Disease, 78, 971-974.

Janisiewicz, W.J., & Korsten, L. (2002). Biological control of postharvest diseases of fruits. Annual Review of Phytopathology, 40, 411-441.

Janisiewicz, W.J., Tworkoski, T.J., & Kurtzman, C.P. (2001). Biocontrol potential of *Metchnikowia pulcherrima* strains against blue mold of apple. Phytopathology 91, 1098-1108.

Jijakli, M.W., & Lepoivre, P. (1998). Characterization of an Exo-β-1,3-glucanase produced by *Pichia anomala* strain K, antagonist of *Botrytis cinerea* on apples. Phytophatology, 88, 335-343.

Joo, G. 2005. Purification and characterization of an extracellular chitinase from the antifungal biocontrol agent *Streptomyces halstedii*. Biotechnology Letters, 27, 1483-1486.

Kaur, J., Munshi, G.D., Singh, R.S., & Koch, E. (2005). Effect of carbon source on production of lytic enzymes by the sclerotial parasites *Trichoderma atroviride* and *Coniothyrium minitans*. Journal of Phytopathology, 153, 274-279.

Kobayashi, D.Y., Reedy, R.M., Bick, J.A., & Oudemans, P.V. (2002). Characterization of a chitinase gene from *Stenotrophomonas maltophilia* strain 34S1 and its involvement in biological control. Applied Environment and Microbiology, 68, 1047-1054.

Kurtzman, C.P., & Droby, S. (2001). *Metschnikowia fructicola*, a new ascosporic yeast with potential for biocontrol of postharvest fruit rots. Systematic and Applied Microbiology, 24, 395-399.

Leverentz, B., Conway, W.S., Janisiewicz, W., Abadias, M., Kurtzman, C.P., & Camp, M.J. (2006). Biocontrol of the food-borne pathogens *Listeria monocytogenes* and *Salmonella enterica* serovar Poona on fresh-cut apples with naturally occurring bacterial and yeast antagonists. Applied Environment Microbiology, 72, 1135-1140.

Maniatis, T.E., Fritsch, E.F., & Sambrook, J. (1982). Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor.

Masih, E.I., & Paul, B. (2002). Secretion of beta-1,3-glucanases by the yeast *Pichia membranifaciens* and its possible role in the biocontrol of *Botrytis cinerea* causing grey mould disease of the grapevine. Current Microbiology, 44, 391–395.

McCreath, K.J., Specht, C.A., & Robbins, P.W. (1995). Molecular cloning and characterization of chitinase genes from *Candida albicans*. Proceedings of National Academy of Science, 92, 2544-2548.

Piano, S., Neyrotti, V., Migheli, Q., & Gullino, M.L. (1997). Characterization of the biocontrol capability of *Metschnikowia pulcherrima* against *Botrytis* postharvest rot of apple. Postharvest Biology Technology, 11, 131-140.

Saligkarias, I.D., Gravanis, F.T., & Eptona, H.A.S. (2002). Biological control of *Botrytis cinerea* on tomato plants by the use of epiphytic yeasts *Candida guilliermondii* strains 101 and US 7 and *Candida oleophila* strain I-182: II. a study on mode of action. Biological Control, 25, 151-161.

Saravanakumar, D., Ciavorella, A., Spadaro, D., Garibaldi, A., & Gullino, M. (2008). *Metschnikowia pulcherrima* strain MACH1 outcompetes *Botrytis cinerea*, *Alternaria alternata* and *Penicillium expansum* in apples through iron depletion. Postharvest Biology and Technology, 49, 121-128.

Schena, L., Ippolito, A., Zahavi, T., Cohen, L., & Droby, S. (2000). Molecular approaches to assist the screening and monitoring of postharvest biological yeasts. European Journal of Plant Pathology, 106, 681-691.

Smilanick, J.L. (1994). Strategies for the isolation and testing of biocontrol agents. In: Wilson, C.L., Wisniewski, M.E. (Eds.), Biological Control of Postharvest Diseases - Theory and Practice. CRC Press, Boca Raton, pp. 25-42.

Spadaro, D., Vola, R., Piano, S., & Gullino, M.L. (2002). Mechanisms of action, efficacy and possibility of integration with chemicals of four isolates of the yeast *Metschnikowia pulcherrima* active against postharvest pathogens on apples. Postharvest Biology and Technology, 24, 123-134.

Spadaro D., & Gullino M.L. (2004). State of art and future perspectives of biological control of postharvest fruit diseases. International Journal of Food Microbiology, 91, 185-194.

Spadaro, D., Garibaldi, A., & Gullino, M.L. (2004). Control of *Penicillium expansum* and *Botrytis cinerea* on apple combining a biocontrol agent with hot water dipping and acibenzolar-*S*-methyl, baking soda, or ethanol application. Postharvest Biology and Technology, 33, 141-151.

Spadaro, D., Ciavorella, A., Garibaldi, A., & Gullino, M.L. (2005). Ampliamento dello spettro d'azione di lieviti antagonisti per la lotta biologica in post-raccolta su pomacee. Informatore fitopatologico - La Difesa delle Piante, 55 (10), 56-59.

Spadaro D., Sabetta W., Acquadro A., Portis E., Garibaldi A., Gullino M.L. (2008). Use of AFLP for differentiation of *Metschnikowia pulcherrima* strains for postharvest disease biological control. Microbiological Research, (doi:10.1016/j.micres.2007.01.004).

Stafford, W.H.L., Baker, G.C., Brown, S.A., Burton, S.G., & Cowan, D.A. (2005). Bacterial diversity in the rhizosphere of proteaceae species. Environmental Microbiology, 11, 1175-1768.

Vaidya, R., Roy, S., Macmil, S., Gandhi, S., Vyas, P., & Chhatpar, H.S. (2003). Purification and characterization of chitinase from *Alcaligenes xylosoxydans*. Biotechnology Letters, 25, 715-717.

White, T.J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Shinsky JJ, White TJ, editors. PCR Protocols: A Guide to Methods and Applications, 315–322. Academic Press, San Diego.

Wilson, C.L., & Wisniewski, M.E. (1994). Biological Control of Postharvest Diseases - Theory and Practice. CRC Press, Boca Raton.

Wisniewski, M.E., Biles, C., Droby, S., Mclaughlin, R., Wilson, C.L., & Chalutz, E. (1991). Mode of action of the postharvest biocontrol yeast, *Pichia guilliermondii*. I. Characterization of attachment to *Botrytis cinerea*. Physiology and Molecular Plant Pathology, 39, 245-258.

Figure legends:

Fig 1. Antifungal activity of partially purified proteins from *M. pulcherrima* strain MACH1 against *B. cinerea*.

1. Partially purified proteins of MACH1
2. Cell free culture filtrates of MACH1
3. Sterile distilled water

Fig 2. Chitinase secretion by antagonistic yeast strains in liquid media amended with or without CWP of *B. cinerea*

Fig 2a. Yeast Peptone Dextrose Broth

Vertical bars indicate standard deviations of five replications

Fig 2b. Potato Dextrose Broth

Vertical bars indicate standard deviations of five replications

Fig 2c. Apple Juice Extracts

Vertical bars indicate standard deviations of five replications

Fig 3. PCR amplification of ITS region and chitinase gene of *M. pulcherrima* strain MACH1

Table 1. Effect of yeast antagonistic strains against *B. cinerea in vitro* and *in vivo*

Treatments	***In vitro***			***In vivo* experiment on apples**		
	Conidial concentration of *B. cinerea*	***Botrytis* wet mycelial weight (mg)**	***Botrytis* dry mycelial weight (mg)**	**Diameter of rotten area (cm)**	**Weight of rotten fruit (g)**	**Percent of infected wounds**
M. *pulcherrima* MACH1	No conidia	115.8 c	21.5 c	2.13 a	6.33 a	8.47 a
Rhodotorula spp. PW34	No conidia	385.0 b	35.2 b	5.68 b	40.15 b	40.91 b
Control (*B. cinerea*)	9.3×10^4 ml^{-1}	1564.0 a	150.3 a	7.45 c	51.78 c	77.11 c

In a column, mean values followed by a common letter are not significantly different (P =0.05) by DMRT.