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

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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. *Metschnikowia 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 the 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, including apple juice, amended with or without cell wall preparations (CWP) of *B. cinerea*. The study showed a 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, the 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 show 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 post-harvest biological control of *B. cinerea*.

Keywords *Botrytis cinerea* · Cell wall preparations · Chitinases · *Metschnikowia pulcherrima* · PCR · Post-harvest biocontrol

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Introduction

Post-harvest disease control is traditionally based on the application of synthetic fungicides (Eckert and Ogawa 1985). However, due to concerns related to fungicide toxicity, development of fungicide resistance by pathogens and potential harmful effects 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 post-harvest diseases (Wilson and Wisniewski 1994; Spadaro and Gullino 2004). Among the different biocontrol agents (BCAs), 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 BCA against post-harvest 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* has been reported as a BCA against post-harvest diseases (Calvente et al. 1999, 2001). When considering how to improve the performance of natural BCAs and hence to develop them as reliable commercial products, it is first necessary to characterise their mode of action.

Several possible biocontrol mechanisms have been suggested against post-harvest rots on fruit including competition for nutrients and space, antibiosis, parasitism or direct interaction with 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 post-harvest 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 hypothesised to be associated with the firm attachment of the yeast cells to fungal hyphae and the partial degradation of fungal mycelia. Although there is a sufficient amount of literature supporting the biocontrol efficacy of different species of *Metschnikowia* against post-harvest pathogens (Kurtzman and Droby 2001; Spadaro et al. 2002), there are no reports on the production of lytic enzymes and their role in the biological control of post-harvest 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 walls 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.

Materials and methods

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 *B. 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 granulated yeast 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 4°C.

Efficacy of antagonists against *B. cinerea* in vitro

The antagonistic yeast strains MACH1 and PW34 were inoculated into 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 h. Later, *B. cinerea* mycelial discs (8 mm diam) were placed into flasks containing antagonistic yeast strains and incubated under room temperature (25±2°C) for 10 days. The PDB inoculated with *B. cinerea* served as the control for the comparative studies. The culture filtrate was filtered through Whatman No.1 filter paper and the filtrates were observed for conidial production by *B. cinerea* under a 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 a hot air oven at 70°C for 10 days. Six replications were maintained for each treatment and the experiments were carried out three times.

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 2 weeks on PDA amended with 50 mg l^{-1} streptomycin. After 2 weeks of incubation at 25°C, spores from the three strains were collected and suspended in sterile Ringer's solution (Merck). After filtering through eight 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 with sodium hypochloride (NaClO, 1.0% as chlorine), rinsed under tap water, and when dry punctured with a sterile needle at the equatorial region (3 mm depth; three wounds per fruit). An antagonistic yeast suspension (30 µl: 2×10^8 cells ml^{-1}, adjusted using Burker chamber) was pipetted into the wound. Inoculated control fruits were pipetted, before pathogen inoculation, with 30 µl of distilled water. After 3 h, 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.

Antifungal activity of partially purified proteins

To test the effect of extracellular proteins produced by yeast biocontrol strains against *B. cinerea*, yeast cells of MACH1 and PW34 were inoculated into YPD broth and grown at 25°C; 4 l of 6 day-old cultures were used for the 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; approximately 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 dialysed (dialysis tubing cellulose membrane; Sigma Aldrich, Italy) three times against 5 l of distilled water at 4°C overnight. The protein solution was concentrated using lyophiliser and the protein concentration was determined according to Bradford (1976), with bovine serum albumin as the 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 sterilised by baking at 150°C for 2 h. Into three separate sterile 90 mm Petri dishes, 20 ml of PDA medium was poured, and three strips were placed equally spaced on the outside periphery of each dish. Ten micrograms of purified protein dissolved in sodium phosphate buffer pH 7.0 were added to the discs using a 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 the control. Fungal discs (5 mm) were punched from 5 day-old cultures of *B. cinerea* and placed in the middle of the Petri dish. The plates were incubated at room temperature (28±2°C) and observations were made after 5 days of incubation.

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, homogenised for 2 min and centrifuged for 2 min at 480×*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 six times. The crushed mycelium was then resuspended in an equal volume of Tris HCl buffer (50 mmol l^{-1} and pH 7.2), centrifuged for 10 min at 1,920×*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, lyophilised and stored at −20°C.

Preparation of enzyme extract

Antagonistic yeasts were inoculated into 250 ml Erlenmeyer flasks containing 75 ml of YPD broth, PDB and apple juice extracts (commercial juices from supermarkets, Italy; 15 lbs, 121°C for 15 min: autoclaved). Similarly, into 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 flask 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 used for each treatment and the experiment was repeated twice.

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 nine 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.

DNA extraction from yeast strain MACH1

Two ml of YPD culture of the strain MACH1 was centrifuged at 2,500×*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 1× 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 visualised 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^{-1} 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.

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

To confirm the species of strain MACH1 at the molecular level, an ITS region was amplified using universal primers ITS5 (5′-GGAAGTAAAAGTCG-TAACAAGG-3′) and ITS2 (5′-GCTGCGTT CTTCATCGATGC-3′; White et al. 1990). PCR reactions were carried out in 20 µl reaction mixture containing 10× 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: 95°C for 1 min, 52°C for 1 min and 72°C 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.

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

The chitinase gene of *M. pulcherrima* was amplified with degenerate primers CHI forward (5′-ATCATGR-TITAYTGGGGICARAA-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 10× 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.

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 the 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.

Statistical analysis

Data from all the experiments were analysed 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).

Results

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 the presence of *M. pulcherrima* strain MACH1 (115.8, 21.5 mg) than *Rhodotorula* sp. strain PW34 (385, 35.2 mg). *Botrytis cinerea* did not produce conidia co-cultured in media with the antagonistic yeast whereas a higher conidial (9.3×10^4 ml^{-1}) concentration was noticed in the *B. cinerea* inoculation alone treatment (Table 1). Apples treated with the antagonist had lower *B. cinerea* infection compared to the untreated

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}	1,564.0 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.

control. Furthermore, apples treated with *M. pulcherrima* strain MACH1 had a 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).

Antifungal activity of extracellular proteins of MACH1

The 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*.

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 the pathogen. However, less activity of chitinase was noticed in PW34 inoculated into YPD broth amended with or without CWP of *B. cinerea* (Fig. 2a).

Filtrates from MACH1 culture grown in PDB + CWP had a higher activity of chitinase than from cultures 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 a higher activity of chitinase than PW34 in apple juice and in apple juice amended with CWP of *B. cinerea* compared to the 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).

PCR amplification, cloning and sequencing of the ITS region of MACH1

Based on the superior biocontrol performance shown 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; the internal transcribed spacer 2; and partial sequence of 26S ribosomal RNA gene. The amplified rDNA fragments of strain MACH1 were cloned into the 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

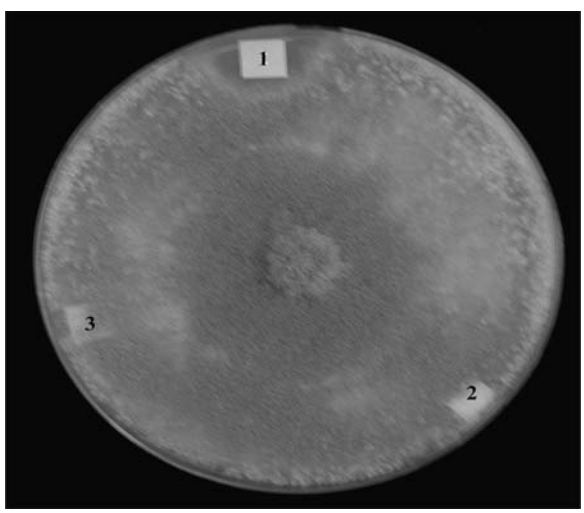
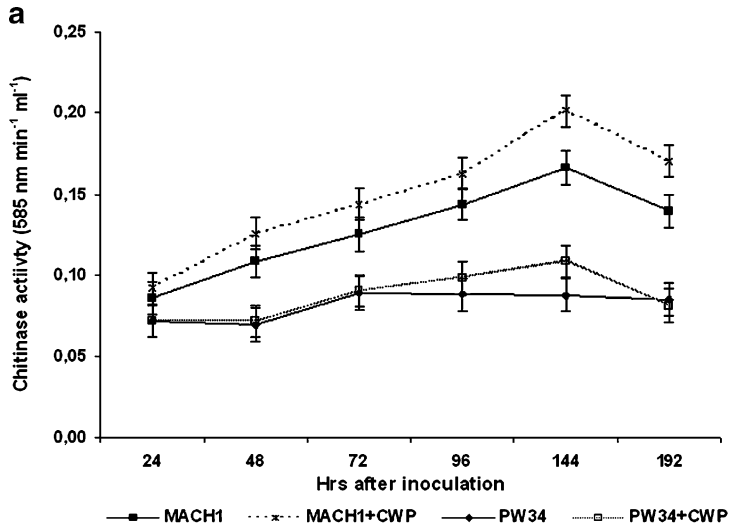


Fig. 1 Antifungal activity of partially purified proteins from *M. pulcherrima* strain MACH1 against *B. cinerea*. *1* Partially purified proteins of MACH1. *2* Cell-free culture filtrates of MACH1. *3* Sterile distilled water

Fig. 2 Chitinase secretion by antagonistic yeast strains in liquid media amended with or without CWP of *B. cinerea*. **a** Yeast peptone dextrose broth; *vertical bars* indicate standard deviations of five replications. **b** Potato dextrose broth; *vertical bars* indicate standard deviations of five replications. **c** Apple juice extracts; *vertical bars* indicate standard deviations of five replications


a
Chitinase activity (585 nm min-1 ml-1)
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Hrs after inoculation
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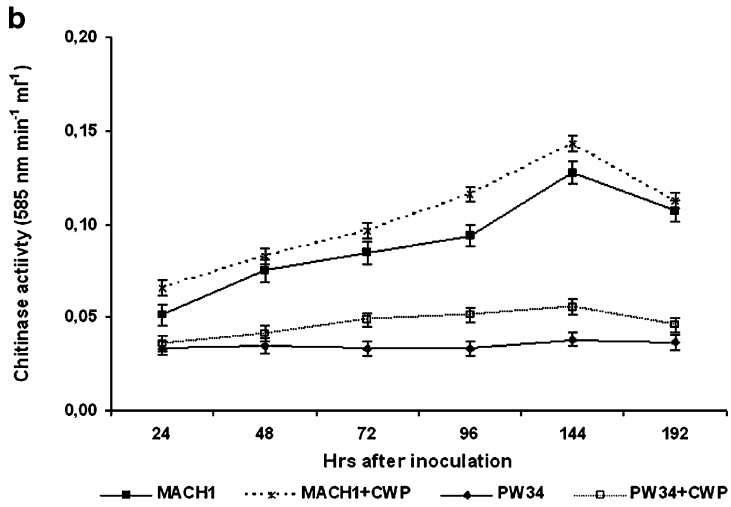
PW34
PW34+CWP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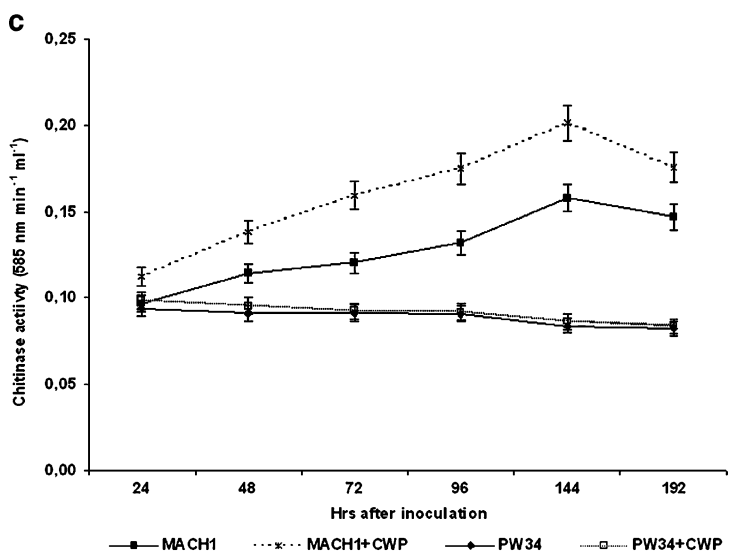
PW34+CWP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PW34+CWP

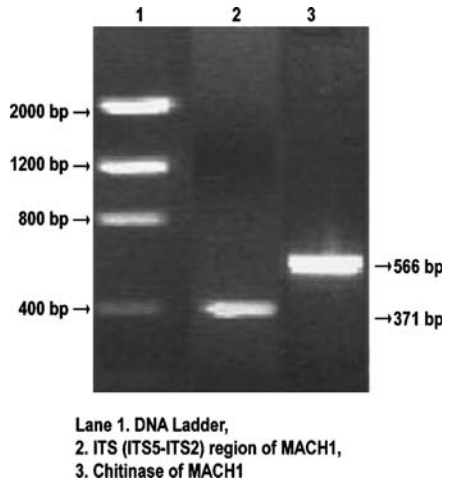

Fig. 3 PCR amplification of ITS region and chitinase gene of *M. pulcherrima* strain MACH1

primers and the sequence of the ITS rDNA region from MACH1 was submitted to the NCBI and given the Accession No. EU037994.

PCR detection, cloning and sequencing of chitinase gene of MACH1

A PCR performed on the genomic DNA of the antagonistic yeast MACH1 resulted in amplification of 566 bp product (Fig. 3). The amplified product was cloned into the 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 the nucleotide sequences of the chitinase gene from antagonistic yeast strain MACH1 were submitted to the NCBI and the accession no. EU153550 was assigned.

Discussion

Strains of *M. pulcherrima* isolated from the carposphere can be effective in protecting apples, peaches, and grapes against postharvest rots caused by *B. cinerea* and other post-harvest 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. Furthermore, the yeast strain MACH1 reduced 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, a 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 the culture filtrate of *Bacillus subtilis* AF1. The yeast strain MACH1 showed higher efficacy against *B. cinerea* on apples and there are several examples of work 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 of the literature indicates that among different modes of action displayed by the BCAs, one of the major modes 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 allowed us 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 and indicated the possible involvement of chitinase production by yeast strain MACH1 in the control of *B. cinerea* on apples. The results are similar to the previous findings of Wisniewski 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 enzyme 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 the 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 a carbon source and the yeast could produce higher levels of chitinase. Other work reported the higher lytic enzyme 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 the 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 agrees with our current findings.

Various molecular methods have been developed and used to identify various microorganisms at species level (White et al. 1990; Stafford et al. 2005). To date, many applications of molecular identification have focused on the 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 the ITS region has confirmed that strain MACH1 used in this study is *M. pulcherrima*.

The biochemical assays of chitinase demonstrated the secretion of the enzyme by *M. pulcherrima* strain MACH1. In addition, PCR amplification confirmed the presence of the 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 the chitinase gene from *M. pulcherrima* strain MACH1 showed high homology to the 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 post-harvest pathogens. To our knowledge, this is the first report of the secretion and detection of chitinases from the BCA *M. pulcherrima* and the identification of a putative chitinase gene from a yeast used as a BCA. In addition, the current study suggests 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 BCAs 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*. Furthermore, PCR amplification and sequencing of the chitinase gene from *M. pulcherrima* strain MACH1 has confirmed the presence of the gene at the 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 the biocontrol efficacy of *M. pulcherrima* strain MACH1 against post-harvest 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. The current work addresses the direct role of lytic enzymes produced by strain MACH1 in the biological control of post-harvest pathogens.

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