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

10

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16

17 **Abstract**

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

33

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

36

37 **1. Introduction:**

38 Postharvest disease control is traditionally based on the application of synthetic fungicides (Eckert
39 and Ogawa 1985). However, due to the concern related to fungicide toxicity, development of
40 fungicide resistance by pathogens and potential harmful effect on the environment and human
41 health, alternatives to synthetic chemicals have been proposed (Eckert et al. 1994; Janisiewicz and
42 Korsten 2002). Among the proposed alternatives, the development of antagonistic microorganisms
43 has been the most studied and has made substantial progress in the management of postharvest
44 diseases (Wilson and Wisniewski 1994; Spadaro and Gullino, 2004). Among the different
45 biocontrol agents, yeasts attract particular attention due to the non-production of toxic metabolites
46 which could have a negative environmental or toxicological impact. Recently, *Metschnikowia*
47 *pulcherrima* has been reported as an effective biocontrol agent against postharvest decay of apple,
48 table grape, grapefruit and cherry tomato (Piano et al. 1997; Schena et al. 2000; Janisiewicz et al.
49 2001; Spadaro et al. 2002) as well as against some food-borne pathogens (Leverentz et al. 2006).
50 Similarly, the yeast species of *Rhodotorula* have been reported as biological control agents against
51 postharvest diseases (Calvente et al. 1999; 2001). When considering how to improve the
52 performance of natural biocontrol agents and hence to develop them as reliable commercial
53 products, it is first necessary to characterize their mechanisms of action.

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

60 2008). However, there is growing evidence to support the possible involvement of cell wall
61 degrading enzymes in the action of yeast antagonists. Wisniewski et al. (1991) demonstrated that
62 the yeast *Pichia guilliermondii* secreted high levels of exo- β -1,3-glucanase and chitinase when
63 cultured on various liquid media or on the cell walls of fungal pathogens. The ability of the yeast to
64 produce lytic enzymes was hypothesized to be associated with the firm attachment of the yeast cells
65 to fungal hyphae and the partial degradation of fungal mycelia. Though there are sufficient amount
66 of literature supporting the biocontrol efficacy of different species of *Metschnikowia* against
67 postharvest pathogens (Kurtzman and Droby 2001; Spadaro et al. 2002), there is no report about the
68 production of lytic enzymes and their role in the biological control of postharvest diseases.
69 Therefore, the objectives of the study were: (1) to evaluate the level of efficacy of two yeast strains
70 (*M. pulcherrima* MACH1 and *Rhodotorula* sp. PW34), previously selected at AGROINNOVA –
71 University of Torino, on apples against *Botrytis cinerea*; (2) to study the antifungal activity of
72 extracellular proteins secreted by yeast strains (3) to study the production of chitinases by both
73 yeast strains on synthetic liquid media and on apple juice, in the presence and absence of cell wall
74 of *B. cinerea*; (4) to detect the chitinase gene in order to develop a molecular method to screen the
75 enzymatic activity of *M. pulcherrima* strains potentially useful for biocontrol.

76

77 **2. Materials and methods:**

78 **2.1. Yeast and fungal cultures**

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

85 Casein Difco; 20 g l⁻¹ of D(+)-Glucose Monohydrate, Merck) and Potato Dextrose Agar (PDA;
86 Merck, Germany) respectively at 4°C.

87

88 **2.2. Efficacy of antagonists against *B. cinerea* in vitro**

89 The antagonistic yeast strains MACH1 and PW34 were inoculated onto 250 ml Erlenmeyer flasks
90 containing 75 ml of Potato Dextrose Broth (PDB, Sigma, Steinheim, Germany) and incubated on a
91 rotary shaker (100 rpm) at 25°C for 24 hours. Later, *B. cinerea* mycelial discs (8 mm diameter)
92 were placed onto flasks containing antagonistic yeast strains and incubated under room temperature
93 (25±2°C) for 10 days. The PDB inoculated with *B. cinerea* served as control for the comparative
94 studies. The culture filtrate was filtered through Whatman No.1 filter paper and the filtrates were
95 observed for the conidial production by *B. cinerea* under Burkner chamber. The mycelial wet weight
96 was recorded from each treatment and the mycelial dry weight was taken after removing the water
97 content by incubating in hot air oven at 70°C for 10 days. Six replications were maintained for each
98 treatment and the experiments were carried out three times.

99

100 **2.3. Efficacy of antagonists against *B. cinerea* in vivo**

101 The biocontrol efficacy was tested on apples treated with the biocontrol strains MACH1 and PW34
102 and inoculated with *B. cinerea*. Antagonistic yeasts MACH1 and PW34 were grown on YPD and
103 inocula of the antagonists were prepared as described in Spadaro et al., (2002). Spore suspensions
104 were prepared by growing the pathogens on Petri dishes for two weeks on PDA added with 50 mg l⁻¹
105 of streptomycin. After two weeks of incubation at 25°C, spores from the three strains were
106 collected and suspended in sterile Ringer's solution (Merck). After filtering through 8 layers of
107 sterile cheese-cloth, spores were counted and brought to a final concentration of 10⁵ ml⁻¹. Apples
108 (cv Golden delicious) were disinfected in sodium hypochloride (NaClO, 1.0 % as chlorine) and
109 rinsed under tap water and when dry punctured with a sterile needle at the equatorial region (3 mm
110 depth; 3 wounds per fruit). An antagonistic yeast suspension (30 µl: 2 × 10⁸ cells ml⁻¹ were adjusted

111 using Burker chamber) was pipetted into wound. Inoculated control fruits were pipetted, before
112 pathogen inoculation, with 30 µl of distilled water. After 3 hours, 30 µl of the spore suspension of
113 the *B. cinerea* were pipetted in the wound. Fifteen fruits per treatment were used (45 inoculation
114 sites). Eight days after inoculation, rotten area, fruit weight and percent infected wounds were
115 recorded. The experiments were carried out twice.

116

117 **2.4. Antifungal activity of partially purified proteins**

118 To test the effect of extracellular proteins produced yeast biocontrol strains against *B. cinerea*, yeast
119 cells of MACH1 and PW34 were inoculated onto YPD broth and grown at 25°C and 4 l of 6-day-
120 old culture was used for partial purification of proteins. Following filtration through a Millipore
121 membrane (0.2 µm) (Sigma Aldrich, Italy), proteins in the supernatant fluid were precipitated with
122 (NH₄)₂SO₄ (Sigma Aldrich, Italy) (approx. 80% saturation) on ice. The precipitate was recovered by
123 centrifugation at 16,260 g for 30 min, dissolved in 50 ml 0.1 M phosphate buffer (pH 7.2) and
124 dialyzed (Dialysis Tubing Cellulose Membrane; Sigma Aldrich, Italy) three times against 5 l of
125 distilled water at 4°C overnight. The protein solution was concentrated using lyophilizer and the
126 protein concentration was determined according to Bradford (1976), with bovine serum albumin as
127 standard. The antifungal activity of the partially purified protein was studied based on the mycelial
128 growth inhibition of *B. cinerea*. Whatman No.1 filter paper strips of 25 mm² were cut and sterilized
129 by baking at 150°C for 2 h. In sterile 90-mm Petri plates of three numbers, 20 mL of PDA medium
130 was poured separately, and three strips were placed equally spaced on the outside periphery of each
131 plate. Ten micrograms of purified protein dissolved in sodium phosphate buffer pH 7.0 were added
132 to the discs using sterile pipette. Cell free culture filtrates (30 µl for each paper disc) of the two
133 yeast strains were also tested for their antifungal activity. Sterile distilled water served as control.
134 Fungal discs (5 mm) were punched from 5-day old culture *Botrytis cinerea* and placed in the middle
135 of the Petri plate. The plates were incubated at room temperature (28 ± 2°C) and observations were
136 made after 5 days of incubation.

137 **2.5. Preparation of *B. cinerea* cell walls**

138 Cell wall preparations (CWP) of *B. cinerea* were prepared as reported by Saligkarias et al. (2002)
139 with some modifications. Briefly, the mycelium collected by Whatman No.1 filter paper was
140 washed four times with deionised water, homogenized for 2 min and centrifuged 2 min at 480 x g.
141 After removing the supernatant, the fungal material was sonicated with a sonicator for 10 min and
142 centrifuged for 5 min at 600 rpm. The supernatant was discarded and the pellet was resuspended in
143 water. The samples were subjected to sonication and centrifugation as above for a total of six times.
144 Then the crushed mycelium was resuspended in an equal volume of Tris HCl buffer (50 mmol l⁻¹
145 and pH 7.2), centrifuged for 10 min at 1920 x g, and the supernatant was discarded. The pellet was
146 subject to three successive cycles of centrifugation and resuspension. The final pellet was frozen in
147 liquid N₂, lyophilized and stored at -20°C.

148

149 **2.6. Preparation of enzyme extract**

150 Antagonistic yeasts were inoculated into 250 ml Erlenmeyer flask containing 75 ml of YPD broth,
151 PDB and apple juice extracts (Commercial juices from supermarkets, Italy) (15 lbs, 121°C for 15
152 min : autoclaved). Similarly, onto YPD broth, PDB and apple juice extracts, 2 mg ml⁻¹ CWP of *B.*
153 *cinerea* were added and yeast strains were inoculated. The cultures were incubated on a rotary
154 shaker (200 rpm) at 25°C. Aliquots of 5 ml of each culture were withdrawn aseptically from each
155 flasks after 24, 48, 72, 96, 144 and 196 h of inoculation and centrifuged at 9600 rpm for 10 min at
156 4°C to obtain the cell free culture filtrates. The supernatants were used for the enzyme assay. Five
157 replications were maintained for each treatment and the experiment was repeated twice.

158

159 **2.7. Assay of chitinase activity**

160 The spectrophotometric assay of chitinase was carried out according to the procedure developed by
161 Boller and Mauch (1988). The reaction mixture consisted of 10 µl of 0.1 M sodium acetate buffer
162 (pH 4.0), 0.4 ml enzyme solution and 0.1 ml colloidal chitin. After incubation for 2 h at 37°C, the

163 reaction was stopped by centrifugation at 3,000 g for 3 min. An aliquot of the supernatant (0.3 ml)
164 was pipetted into a glass reagent tube containing 30 μ l of 1 M potassium phosphate buffer (pH 7.0)
165 and incubated with 20 μ l of 3% (w/v) snail gut enzyme (Sigma, Steinheim, Germany) for 1 h. After
166 1 h, the reaction mixture was brought to pH 8.9 by the addition of 70 μ l of 0.1 M sodium borate
167 buffer (pH 9.8). The mixture was incubated in a boiling water bath for 3 min and then rapidly
168 cooled in an ice-water bath. After addition of 2 ml of DMAB (para-dimethylaminobenzaldehyde
169 reagent was prepared by mixing 8 g of DMAB in 70 ml of glacial acetic acid along with 10 ml of
170 concentrated HCl; one volume of stock solution was mixed with 9 volumes of glacial acetic acid
171 and used for the reactions), the mixture was incubated for 20 min at 37°C and the absorbance was
172 measured at 585 nm.

173

174 **2.8. DNA extraction from yeast strain MACH1**

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

187

188 **2.9. PCR amplification of ITS region of *M. pulcherrima* MACH1**

189 To confirm the species of strain MACH1 at molecular level, an ITS region was amplified using
190 universal primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS2 (5'-
191 GCTGCGTTCTTCATCGATGC-3') (White et al. 1990). PCR reactions were carried out in 20 µl
192 reaction mixture containing 10x buffer (with 2.5 mmol l⁻¹ MgCl₂), 2 µl; 2 mmol l⁻¹ dNTP mixture, 2
193 µl; 2 mol l⁻¹ primer, 5 µl; Taq DNA polymerase, 3 U; H₂O, 8 µl and 50 ng of template. DNA
194 samples were amplified on a DNA thermalcycler (Biometric, USA) using the PCR conditions: 95°C
195 for 1 min, 52°C for 1 min and 72°C for 2 min. The total number of cycles was 35 with a final
196 extension time of 10 min. The PCR products were resolved on 2% agarose at 50 V stained with
197 SYBR-safe (Invitrogen, USA) and photographed using a Gel Doc 1000 system.

198

199 **2.10. PCR detection of chitinase gene of *M. pulcherrima* MACH1**

200 The chitinase gene of *M. pulcherrima* was amplified with degenerate primers CHI forward
201 (5'-ATCATGRTITAYTGGGGICARAA-3') and CHI reverse (5'-GAGCARTARTARTTRTTR
202 TA RAAYTG-3'), previously designed by McCreath et al. (1995) for *Candida albicans*. PCR
203 reactions were carried out in 20 µl reaction mixture containing 10x buffer (with 2.5 mmol l⁻¹
204 MgCl₂), 2 µl; 2 mmol l⁻¹ dNTP mixture, 2 µl; 2 mol l⁻¹ primer, 5 µl; Taq DNA polymerase, 3 U;
205 H₂O, 8 µl and 50 ng of template. DNA samples were amplified on DNA thermalcycler (Biometric,
206 USA) using the PCR conditions 94°C for 45 s, 56°C for 1 min and 72°C for 45 s. The total number
207 of cycles was 35 with a final extension time of 10 min. The PCR products were resolved on 2%
208 agarose at 50 V stained with SYBR-safe (Invitrogen, USA) and the images were obtained from Gel
209 Doc 1000 system.

210

211 **2.11. Cloning and sequencing of ITS region and chitinase gene of strain MACH1**

212 Both amplified DNA regions were purified from agarose (1.2%, w/v) gel after electrophoresis. A
213 small agarose slice containing the band of interest [observed under long-wavelength (312-nm) UV
214 light] was excised from the gel and purified by using a QIAquick gel extraction kit (Qiagen Inc.,
215 Chatsworth, CA, USA) according to the supplier's instructions. This purification was performed to
216 remove primer dimers and other residues from the PCR amplification. Fragments were cloned into
217 the pCR 2.1-TOPO plasmid vector (Invitrogen, USA) and transformed into *Escherichia coli* strain
218 DH5 α according to the procedure recommended by the manufacturer. Transformants were selected
219 on Luria Broth (LB) agar amended with ampicillin (75 mg ml⁻¹). Clones were randomly selected
220 and used as templates in PCR to produce products of required sizes in agarose gel. The
221 transformation of *E. coli* strains with the presence of the insert was confirmed by PCR using
222 universal primer M13F (5'-CACGACGTTGTAAAACGAC-3') and M13R (5'-
223 GGATAACAATTCACACAGG-3') sequence. DNA sequencing was performed at BMR
224 Genomics, DNA sequencing service centre, Padova, Italy. For sequence determination of the cloned
225 PCR products, a generally applicable sequencing strategy was developed. The nucleotide sequences
226 were submitted to the National Centre for Biotechnology Information (NCBI), GenBank, New
227 York, NY, USA.

228

229 **2.12. Statistical analysis**

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

233

234 **3. Results**

235 **3.1. Biocontrol strain efficacy against *B. cinerea* *in vitro* and *in vivo***

236 The results of the *in vitro* study showed that *B. cinerea* produced less mycelium, considered as wet
237 and dry matter, in PDB when cultured with both biocontrol strains than alone (1564, 150.3 mg). In

238 particular, the mycelium produced was lower in presence of *M. pulcherrima* strain MACH1 (115.8,
239 21.5 mg) than of *Rhodotorula* sp. strain PW34 (385, 35.2 mg). *B. cinerea* did not produce conidia
240 co-cultured in a media with the antagonistic yeast whereas higher conidial ($9.3 \times 10^4 \text{ ml}^{-1}$)
241 concentration was noticed in the *B. cinerea* inoculation alone (Table 1). Apples treated with the
242 antagonist had lower *B. cinerea* infection compared to untreated control. Further, apples treated
243 with *M. pulcherrima* strain MACH1 had lower incidence of decay (8.47%). The diameter of the
244 rotten areas and weight of the rotten tissue were lowest (2.13 cm, 6.33 g respectively) in apples
245 treated with the strain of *M. pulcherrima*. Apples inoculated with *B. cinerea* alone had the highest
246 decayed area (7.45 cm) and decayed tissue weight (51.78 g) (Table 1).

247

248 **3.2. Antifungal activity of extracellular proteins of MACH1**

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

254

255 **3.3. Enzymatic assay of the chitinase activity by both biocontrol agents**

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

263 Filtrates from MACH1 culture grown in PDB+CWP had higher activity of chitinase than from
264 culture grown in PDB alone. The peak of chitinase activity occurred 6 days after inoculation (Fig
265 2b). In general the chitinase activity by both yeast strains was lower in PDB than in YPD broth.
266 The yeast strain MACH1 had higher activity of chitinase than PW34 in apple juice and in apple
267 juice amended with CWP of *B. cinerea* compared to non-amended. The chitinase activity by *M.*
268 *pulcherrima* strain MACH1 reached its maximum 6 days after inoculation and later it started to
269 decline, whereas in PW34, throughout the assay period, lower activity was recorded (Fig 2c).

270

271 **3.4. PCR amplification, cloning and sequencing of ITS region of MACH1**

272 Based on the superior biocontrol performance provided by the yeast strain MACH1, a PCR was
273 carried out on the ribosomal DNA to identify the species, using ITS5 and ITS2 primers. The ITS
274 primers amplified a DNA fragment of 371 bp corresponding to the region including partial
275 sequence of the 18S ribosomal RNA gene; the internal transcribed spacer 1; the 5.8S ribosomal
276 RNA gene; and the internal transcribed spacer 2; and partial sequence of 26S ribosomal RNA gene.
277 The amplified rDNA fragments of strain MACH1 was cloned into pCR 2.1-TOPO plasmid vector
278 and transformed into *E. coli* strain DH5 α . Transformants on LB agar amended with ampicillin were
279 randomly selected and used as templates in PCR to verify products of 371 bp in agarose gel (Fig. 3).
280 The sequencing was performed using M13 universal primers and sequence of the ITS rDNA region
281 from MACH1 was submitted to the NCBI and given the Accession No. EU037994.

282

283 **3.5. PCR detection, cloning and sequencing of chitinase gene of MACH1**

284 A PCR performed on the genomic DNA of the antagonistic yeast MACH1 using degenerative
285 resulted in amplification of 566 bp product (Fig 3). The amplified product was cloned into pCR
286 2.1-TOPO plasmid vector and transformed into *E. coli* strain DH5 α . Transformants on LB agar
287 amended with ampicillin were randomly selected and used as templates in PCR to produce products
288 of the required size (566 bp). Sequencing was performed using M13 universal primers and

289 nucleotide sequences of chitinase gene from antagonistic yeast strain MACH1 was submitted to the
290 NCBI and the Accession No. EU153550 was assigned.

291

292 **4. Discussion**

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

308 An extensive review on literatures indicates that among different mechanisms of action displayed
309 by the biocontrol agents, one of the major mechanism is parasitism via degradation of the cell wall
310 (Vaidya et al. 2003; Joo 2005). Chitin, the unbranched homopolymer of N-acetyl glucosamine in a
311 β -1,4 linkage, is a structural component of cell walls in many fungi. Chitinases which hydrolyse this
312 polymer are produced by various organisms and have been implicated in the biocontrol process
313 (Castoria et al. 2001; Gohel et al. 2006). So far none of the *M. pulcherrima* isolates have been
314 studied for their chitinase activity. Our *in vitro* and *in vivo* experiments permitted to study the

315 ability of *M. pulcherrima* to secrete chitinases. A higher production of chitinases by yeast strain
316 MACH1 in PDB and YPD in the presence of CWP indicated the induction of chitinases by
317 biocontrol yeast. Similarly, a superior production of chitinases by *M. pulcherrima* strain MACH1
318 occurred in apple juice extract amended with CWP indicated the possible involvement of the
319 chitinase production by yeast strain MACH1 in the control of *B. cinerea* on apples. The results are
320 similar to previous findings by Wisnieswski et al. (1991) who reported that *Pichia guilliermondii*
321 and *P. anomala* produce higher levels of lytic enzymes when grown in media supplemented with
322 fungal cell walls than when grown in media containing only glucose. Saligkarias et al. (2002)
323 reported the secretion of detectable amounts of β -1,3-exoglucanase and chitinase by *C.*
324 *guilliermondii* (strains 101 and US 7) and *C. oleophila* (strain I-182) grown in different carbon
325 sources.

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

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

346 The biochemical assays of chitinase demonstrated the secretion of enzyme by *M. pulcherrima* strain
347 MACH1. In addition, PCR amplification confirmed the presence of chitinase gene in the yeast
348 strain MACH1. Similarly, several chitinase genes have been amplified from different yeast and
349 bacterial strains using PCR techniques (McCreath et al. 1995; Chernin et al. 1997; Huang et al.
350 2005). The nucleotide data of chitinase gene from *M. pulcherrima* strain MACH1 showed high
351 homology to chitinase of *Pichia stipitis* CBS 6054, a yeast whose genome has been sequenced for
352 its industrial applications (GenBank Accession No. XM 001386570). The deduced amino acid
353 sequence of the chitinase gene from *M. pulcherrima* strain MACH1 showed higher homology
354 (>80%) to chitinases from *Pichia stipitis*, *Candida albicans* and *Pichia guilliermondii* (data from
355 GenBank, NCBI). Conspicuously, the chitinase protein code of strain MACH1 belongs to the group
356 of glycosyl hydrolases family 18, which generally contains enzymes that are involved in the lysis of
357 chitin molecules (Funkhouser and Aronson, 2007). This suggests that the chitinase gene amplified
358 from the biocontrol strain MACH1 could also have a role in the biological control of postharvest
359 pathogens. To our knowledge, this is the first report on the secretion and detection of chitinases
360 from biocontrol *M. pulcherrima* and the identification of a putative chitinase gene from a yeast used
361 as biocontrol agent. In addition, the current study suggested that the primers designed for chitinase
362 amplification from *C. albicans* by McCreath et al., (1995) could be used for the detection of
363 chitinases from other yeast biocontrol strains.

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

366 2004). Similar to this, the current study also documented the higher secretion of chitinases in the
367 presence of CWP of *B. cinerea*. Further, PCR amplification and sequencing of chitinase gene from
368 *M. pulcherrima* strain MACH1 has confirmed the presence of the gene at molecular level. With the
369 supportive evidence of the previous findings, it is assumed in the present study that secretion of
370 chitinases could be involved in biocontrol efficacy of *M. pulcherrima* strain MACH1 against
371 postharvest fungal pathogens. However, more in depth study is needed to elucidate the role of the
372 chitinases and other cell wall degrading enzymes in the antagonistic activity of *M. pulcherrima*
373 strain MACH1. Current work addresses direct role of lytic enzymes produced by strain MACH1 in
374 the biological control of postharvest pathogens.

375

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384

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521

522

523 **Figure legends:**

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

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

529

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

532

533 **Fig 2a. Yeast Peptone Dextrose Broth**

534 Vertical bars indicate standard deviations of five replications

535

536 **Fig 2b. Potato Dextrose Broth**

537 Vertical bars indicate standard deviations of five replications

538

539 **Fig 2c. Apple Juice Extracts**

540 Vertical bars indicate standard deviations of five replications

541

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

543

544

545

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

547

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

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