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21 **Potential biocontrol activity of a strain of *Pichia guilliermondii* against grey mould of**
22 **apples and its possible modes of action**

23

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36 **Abstract**

37 The efficacy of *Pichia guilliermondii* strain M8 against *Botrytis cinerea* on apples was
38 evaluated under storage conditions, and its possible modes of action were investigated both *in*
39 *vitro* and *in vivo* experiments. After storage at 1°C for 120 days, M8 reduced grey mould
40 incidence from 45.3% (control) to 20.0%. In apple juice medium (AJM) and in
41 wound-inoculated apples, M8 at 10^9 and 10^8 cells ml⁻¹ inhibited the spore germination of *B.*
42 *cinerea* and the grey mould development. When co-culturing *B. cinerea in vitro* or *in vivo* in
43 presence of the yeast, neither inactivated cells nor culture filtrate of the yeast had any effect
44 on spore germination or germ tube elongation. In AJM, the spore germination was
45 significantly recovered by the addition of 1% glucose, sucrose and fructose, or 0.5% and 1%
46 of (NH₄)₂SO₄, phenylalanine and asparagine. When the pathogen and the yeast were
47 co-incubated in apple wounds with addition of the same nutrients, the inhibition of rots was
48 significantly reduced by the supplemental nutrients. Light microscopy revealed that the yeast
49 strongly adhered to the hyphae and spores of *B. cinerea*. M8 produced hydrolytic enzymes,
50 including β-1,3-glucanase and chitinases in minimal salt media with different carbon sources.
51 Pretreatment with M8 at 10^8 cells ml⁻¹ followed by washing, significantly reduced grey
52 mould lesions, suggesting an induction of defence responses. Direct attachment, competition
53 for nitrogen and carbon sources, secretion of hydrolytic enzymes and induction of host
54 resistance play a role in the biocontrol mechanism of *P. guilliermondii* M8 against *B. cinerea*.

55

56 **Key words:** Biological control, *Botrytis cinerea*, Hydrolytic enzymes, Induced resistance,
57 Mechanism of action

59 **1. Introduction**

60 Fruits are highly perishable products, especially during the postharvest phase and major losses
61 are caused by postharvest pathogens (Spadaro and Gullino, 2004). *Botrytis cinerea* is among
62 the most important postharvest pathogens on fruit and vegetables (Snowdon, 1990). When
63 permitted, chemical treatment is a primary method for controlling postharvest diseases of fruits
64 (Janisiewicz and Korsten, 2002). However, pathogen resistance to fungicides (Holmes and
65 Eckert, 1999; Gabriolotto et al., 2009) and concern for public safety have resulted in the
66 cancellation of some of the most effective fungicides in Europe (Regulation 1107/2009 and
67 Directive 2009/128) and the United States (Food Quality Protection Act). Therefore, researches
68 have been focused on the development of alternative control that should be both effective and
69 economically feasible (Lopez-Reyes et al., 2010). The use of microbial antagonists to control
70 postharvest diseases of fruits and vegetables has shown during the last thirty years to be one
71 of the most promising alternatives to fungicides (Wisniewski et al., 1991; Wilson et al., 1993;
72 Droby et al., 2009). Some bacteria, actinomycetes and yeasts showed effectiveness against
73 postharvest diseases of fruit and vegetables (Smilanick et al., 1993; Karabulut et al., 2003;
74 Macagan et al., 2008; Spadaro et al., 2008; Zhang et al., 2010a). Among these microbial
75 antagonists, yeasts that naturally occur on fruits and vegetables have attracted the attention of
76 several researchers as potential antagonists of postharvest diseases due to the fast colonization
77 on fruit surfaces (Droby and Chalutz, 1994; Droby et al., 2009). Some yeasts have been
78 developed, but currently there are only a few commercial products available in the market for
79 postharvest use: BoniProtect® (*Aureobasidium pullulans* de Bary) registered in Germany and

80 used mostly for the control of postharvest diseases in apples (Weiss et al., 2006), Shemer
81 (*Metschnikowia fructicola* Kurtzman & Droby) registered in Israel and commercially used for
82 postharvest diseases of sweet potatoes, table grapes, strawberries, peppers, and carrots
83 (Kurtzman and Droby, 2001), and CandiFruit (Sipcam Inagri, SA Valencia), based on
84 *Candida sake* and registered in Europe, which has been recently commercialized in Spain
85 (Sanzani et al., 2009).

86 *Pichia guilliermondii* Wick has been successfully applied to control postharvest pathogens on
87 a number of fruits and vegetables, such as *P. digitatum* on grapefruits, *P. expansum* on apples,
88 *Rhizopus nigricans* on tomato fruit (Droby et al., 1997; Tian et al., 2002; Scherm et al., 2003;
89 Zhao et al., 2008). However, information about the application of *P. guilliermondii* in
90 controlling grey mould on apples is limited. In addition, the modes of action of *P.*
91 *guilliermondii* against pathogens have not been fully elucidated.

92 A better understanding of the modes of action is essential for developing appropriate
93 commercial production and formulation protocols (Spadaro et al., 2010a; 2010b) and for
94 maximizing the potential use of microbial biocontrol agents. The main mode of action of the
95 yeast biocontrol agents is believed to be competition for nutrients and space (Chan and Tian,
96 2005; Bencheqroun et al., 2007). In particular, competition for amino acids, sugars and Fe^{3+}
97 plays an important role in the mechanism of competition for nutrients of some antagonists
98 (Sipiczki, 2006; Bencheqroun et al., 2007; Saravanakumar et al., 2008). Additional modes of
99 action such as mycoparasitism, induced resistance and the production of lytic enzymes *viz.*,
100 β -1,3-glucanase and chitinase (Ippolito et al., 2000; Saligkarias et al., 2002; Yu et al., 2008)
101 were also proposed.

102 The strain M8 of *P. guilliermondii*, previously isolated from the rhizosphere of maize
103 cultivated in northern Italy and identified through morphological and molecular tools, showed
104 high biocontrol activity against grey mould on apples. Therefore, the present work aimed at
105 (1) evaluating the efficacy of *P. guilliermondii* strain M8 against *B. cinerea* of apples under
106 controlled conditions; (2) investigating the effects of nutrient sources and Fe³⁺ on the efficacy
107 of *P. guilliermondii* strain M8 against *B. cinerea* *in vitro* and *in vivo*; (3) studying the
108 dynamics of β -1,3-glucanase and chitinase activities of the yeast *in vitro*; (4) observing the
109 attachment of hyphae of *B. cinerea* by the yeast; and (5) checking the induction of defence
110 responses to *B. cinerea*.

111

112 **2. Materials and methods**

113

114 **2.1 Fruits, microorganisms and culture conditions**

115 The antagonistic yeast *P. guilliermondii* strain M8 was isolated from the rhizosphere of
116 maize cultivated in northern Italy and identified by sequencing the internal transcribed spacer
117 1 (ITS1), 5.8S ribosomal RNA gene, and internal transcribed spacer 2 (ITS2) (GenBank
118 Accession n° GU478315) according to Cai et al. (1996) and the D1/D2 domain at the 5' end
119 of the LSU rRNA gene (GenBank Accession n° GU478320) according to Kurtzman and
120 Robnett (1998). The strain was maintained on nutrient yeast dextrose agar (NYDA) slants
121 (nutrient broth 8 g l⁻¹, yeast extract 5 g l⁻¹, glucose 10 g l⁻¹ and agar 20 g l⁻¹) in the culture
122 collection centre at AGROINNOVA. The yeast was grown in YPD medium (10 g l⁻¹ yeast
123 extract, 20 g l⁻¹ peptone casein, 20 g l⁻¹ dextrose) at 25°C on a rotary shaker (ASAL, Italy;

124 200 rpm) for 48 h.

125 Five strains of *Botrytis cinerea* (De Bary) Whetzel were isolated from rotted peaches and
126 selected for their virulence by inoculation in artificially wounded apples. They were used as a
127 mixture (each strain accounted for 1/5 of the total final concentrations) throughout this work,
128 to ensure a high level of disease. Each strain was maintained on PDA (potato dextrose agar)
129 slants at 4°C and the spores were harvested after the pathogen was incubated on PDA in Petri
130 dishes at 25°C for 7 days. The required concentrations of pathogen conidia were determined
131 by a Bürker chamber (Knittel, Germany).

132 Apples (cv. Golden Delicious, at a maturity suitable for marketing) for all the
133 experiments were bought from supermarkets.

134

135 **2.2 Efficacy against grey mould incidence on apples under storage conditions**

136 The cells of antagonist *P. guilliermondii* M8 were diluted in 50 L tank into a final
137 concentration of 10^8 cells ml^{-1} . Apples were treated with the antagonist suspension (10^8 cells
138 ml^{-1}) by dipping in tank for 1 min. Fruit surfaces were allowed to air dry at 25°C for 3 h.
139 Fruits treated with a fungicide solution were used as a chemical control: 30 L of
140 thiabendazole (Tecto 20 S, Elf Atochem Agri Italy, 19,7 % a.i., 30 g a.i. 100 l^{-1}) were used for
141 dipping the fruits. Fruits treated with distilled water served as uninoculated controls. Three
142 replicates of 50 fruits were prepared for each treatment. Apples were then stored at 1°C and
143 95% relative humidity (RH) in storage chambers. After 120 days of storage, the percentage of
144 rotten apples was recorded. The experiment was repeated twice.

145

146 **2.3 Effect on spore germination of *B. cinerea***

147 The effect of *P. guilliermondii* M8 on spore germination of *B. cinerea* was assessed in
148 apple juice medium (AJM) as reported by Zhang et al. (2010b) with some modifications.
149 AJM was prepared by homogenizing the apples with a grinding machine (HR1821-PHILIPS,
150 China) followed by filtering with a Whatman No. 1 filter paper. Yeast cells were harvested by
151 centrifugation at 5000×g for 10 min and then resuspended in sterile Ringer solution (Merck,
152 Germany) after growing in 300 ml YPD at 25°C on a rotary shaker (ASAL; 200 rpm) for 48 h.
153 The culture medium was filtered through a 0.22 µm nitro-cellulose filter (Millipore, Billerica,
154 MA, United States) for further use. Living cells of the antagonist (100 µL of a suspension
155 containing 5×10^7 , 5×10^8 , 5×10^9 or 5×10^{10} cells ml⁻¹) or cells (100 µl of a suspension
156 containing 5×10^9 cells ml⁻¹) inactivated by irradiation (Zhang et al., 2010b) for 30' with a
157 germicidal lamp (General Electric, G15T8) that predominantly emitted UV light of a
158 wavelength of 254 nm at fluence of 1.5 W/m² posed at 5 cm from the cell suspension layer (2
159 mm thick), were added to tubes containing 4.8 ml AJM. The final living cell concentrations
160 were 1×10^6 , 1×10^7 , and 1×10^8 cells ml⁻¹, respectively. For the culture filtrate treatment, 100
161 µL of culture filtrate were added to 4.8 mL AJM. Aliquots (100 µl) of *B. cinerea* spore
162 suspension (5×10^6 spores ml⁻¹) in Ringer solution were transferred to each tube. As a control,
163 100 µl of *B. cinerea* spore suspension were added to tubes containing 4.9 ml AJM. After 20 h
164 incubation of the 45° sloping tubes at 25°C on a rotary shaker (200 rpm), 100 spores per
165 replicate were observed under microscope and their germination rate and germ tube length
166 were measured. Three replications were prepared for each treatment and the experiments
167 were performed three times.

168

169 **2.4 Effect on grey mould rot severity on apples**

170 Apples were surface-sterilized with 1% commercial sodium hypochlorite for 1 min
171 followed by rinsing with tap water. Three artificial wounds (3 mm wide x 3 mm deep) along
172 the equatorial zone of the apple were made. The four concentrations of the yeast, inactivated
173 cells and culture filtrate were prepared as in section 2.3. Aliquots of 30 μl of each suspension
174 were pipetted into each wound site for each treatment. After 2 hours of incubation at 25°C,
175 the wounds were inoculated with 30 μl of *B. cinerea* spore suspension at 10^5 spore ml^{-1} . The
176 treatments were designed as described in section 2.3. Three replicates of ten apples were used
177 for each treatment. The wound-inoculated fruits were stored at 20°C and 95% RH. After 5
178 days, the diameters of the rotten lesions were recorded and the experiments were performed
179 three times.

180

181 **2.5 Interactions between pathogen hyphae and yeast in apple juice and on apple tissue**

182 The possible interactions of the yeast and pathogen hyphae were evaluated in tubes
183 containing AJM. The yeast at 10^8 cells ml^{-1} and the pathogen at 10^5 spores ml^{-1} were
184 co-incubated in tubes at 25°C for 20 h on a rotary shaker at 200 rpm. Tubes inoculated only
185 with the pathogen served as control. The interactions of the yeast and pathogen were directly
186 observed under light microscope (Axioskop 40, Germany). Each treatment had three
187 replications and the experiments were repeated twice.

188 To assess the interaction between yeast and *B. cinerea* *in vivo*, the experiment was
189 prepared as described in section 2.3. Fruits were stored at 20°C and 95% RH for 7 days.

190 Wounded tissue (2–4 mm²) was excised with a sterilized surgical blade (Swann Morton,
191 England) from the treated fruit and immediately immersed into FAA fixing solution (89 ml
192 50% ethanol, 6 ml acetic acid and 5 ml formaldehyde) for 24 h. Samples were later
193 dehydrated in a graded ethanol series and critical-point dried with CO₂. The treated tissues
194 were then subjected to light microscope (Nikon, Eclipse 55i, Japan) and the interactions of
195 the yeast and pathogen were directly observed.

196

197 **2.6 *In vitro* competition for nitrogen sources, sugars and Fe³⁺ ions**

198 To examine the effects of different nitrogen sources (asparagine, phenylalanine and
199 (NH₄)₂SO₄), sugars (glucose, sucrose and fructose) and Fe³⁺ ion on biocontrol activity of *P.*
200 *guilliermondii* strain M8 against *B. cinerea*, AJM was used throughout the experiment. AJM
201 was prepared as described in section 2.3. The yeast (100 µl containing 5×10⁹ cells ml⁻¹) and
202 pathogen spore suspensions (100 µl containing 5×10⁶ conidia ml⁻¹) were added to the test
203 tubes (18×150 mm) containing 4.8 ml of AJM supplemented or not with nitrogen sources,
204 sugars and Fe³⁺ ion. The nitrogen and sugars were used at final concentrations of 0.5 and
205 1.0%, and Fe³⁺ ion was supplemented as FeCl₃·7H₂O at final concentrations of 0.1 mM and
206 0.5 mM. The inoculated tubes were incubated at 25°C for 20 h on a rotary shaker at 200 rpm.
207 Then, 100 spores were randomly selected out from each tube and their germination was
208 evaluated. Three replications were included for each treatment and the experiment was
209 repeated twice.

210

211 **2.7 *In vivo* competition for nitrogen sources, sugars and Fe³⁺ ions**

212 To test the effect of different nitrogen sources, sugars and Fe^{3+} ion on the antagonist
213 efficacy *in vivo*, the apples were prepared as described in section 2.3. Three artificial wounds
214 along the equatorial zone of the each were made. Aliquots of 30 μl of each nitrogen source or
215 each sugar at the final concentration of 0.5% and 1%, and Fe^{3+} ion at the final concentrations
216 of 0.1 mM and 0.5 mM were pipetted into each wound site. After 3 hours incubation at 25°C,
217 the wounds were treated with 30 μl of the yeast cell suspension containing 10^8 cells ml^{-1} ,
218 followed 3 h later by inoculation of 30 μl of a *B. cinerea* suspension (10^5 spores ml^{-1}). The
219 treatments were designed as follows: the wounds treated with 30 μl of distilled water before
220 treatment with the yeast and inoculated with the pathogen served as positive control and the
221 apples inoculated only with pathogen spore suspension served as negative control. Three
222 replicates of ten apples were used for each treatment. Apples were stored at 20°C for 6 days
223 and the diameter of the rotten lesions was measured and the experiments were repeated twice.

224

225 **2.8 Preparation of cell wall and colloidal chitin for enzymatic activity studies**

226 Cell wall preparations (CWP) of the pathogen were prepared as described by Saligkarias
227 et al. (2002) with small modifications. Briefly, the pathogen mycelium was harvested after
228 growing in the potato dextrose broth media with four-fold cotton gauzes and then the
229 mycelium was washed twice with deionised water through Whatman No.1 filter paper and
230 centrifuged (Centrifuger: 6K15, Sigma, Germany) at 500×g for 2 min. After removing the
231 supernatant, the mycelial mat was sonicated with a probe type sonicator (USC6000, Malaysia)
232 for 20 min and centrifuged at 500×g for 5 min. The supernatant was removed and the pellet
233 was resuspended in deionised water. Then the crushed mycelium was resuspended into an

234 equal volume of Tris/HCl buffer (50 mmol per liter and pH 7.2), centrifuged at 1900×g for 15
235 min, and the supernatant was discarded. The pellet was subjected to three successive cycles
236 of centrifugation and resuspension. The final pellet was frozen with liquid N₂, lyophilized and
237 stored at -20°C for further studies. Colloidal chitin was prepared according to the method
238 provided by Roberts and Selitrennikoff (1988) from shrimp shell chitin. Then, 5 g of chitin
239 powder (C9752, Sigma, USA) was added slowly into 100 ml of concentrated HCl and left at
240 4°C overnight with vigorous stirring. The mixture was added to 2 l of ice-cold 95% ethanol
241 with rapid stirring and kept overnight at 25°C. The pellet was collected by centrifugation at
242 3000×g for 20 min at 4°C and washed with sterile distilled water until the colloidal chitin
243 became neutral (pH 7.0). Colloidal chitin solution (5 mg ml⁻¹) was prepared and stored at 4°C
244 for further studies.

245

246 **2.9 Enzyme production and activity assay**

247 The yeast strain M8 was cultured in modified Lilly-Barnett minimal salt medium (Lilly
248 and Barnett, 1951) containing 2 mg ml⁻¹ CWP as sole carbon source. A 30 ml of culture
249 media in 100-ml flask (PYREX, England) was incubated at 25°C on a rotary shaker at 150
250 rpm for 0, 24, 72, 96 and 120 h. Culture filtrates from each individual flask were collected by
251 centrifuging at 7,000×g for 8 min, and the supernatant was used for enzyme assays. β-1,
252 3-glucanase activity assay were carried out by measuring the amount of reducing sugars
253 released from laminarin (L9634, Sigma, USA), using glucose as a standard (Masih and Paul,
254 2002). A reaction mixture was prepared by adding 250 μl of 50 mM potassium acetate buffer
255 (pH 5.0) containing 2.5 mg of laminarin per ml into 250 μl of culture filtrate (Chan and Tian,

256 2005). The enzyme-substrate mixture was incubated for 2 h at 40°C in water bath (D-3508
257 Melsungen, Germany). Then 0.5 ml of dinitrosalicylic acid reagent was added, boiling at
258 100°C for 5 min. After cooling, 2 ml of deionised water was added directly and measured
259 spectrophotometrically at 595 nm. Background levels of reducing sugars were determined
260 with a time zero supernatant substrate just prior to boiling at 100°C for 5 min. The protein
261 concentration of the enzyme solution was determined according to Bradford (1976) by using
262 bovine serum albumin (A1933, Sigma, USA) as a standard. The specific activity was
263 expressed as micromoles of glucose per milligram protein per hour (Fan et al., 2002). Each
264 treatment had three replications and the experiments were performed three times.

265 The exo-chitinase assay was performed according to Abeles et al. (1970). A reaction
266 mixture was prepared by adding 0.5 ml of 5 mg ml⁻¹ colloidal chitin containing 1.2 µmol l⁻¹
267 sodium azide and 56 µmol l⁻¹ sodium acetate to 0.5 ml enzyme supernatant. For
268 endo-chitinase assay, a reaction mixture was prepared by adding 0.1 ml of 3% (v/v) desalted
269 snail gut enzyme cytohellicase (C8274, Sigma, USA) and 0.1 ml of 1 mol l⁻¹ potassium
270 phosphate buffer (pH 7.1) into 0.5 ml of 5 mg ml⁻¹ colloidal chitin in a 2 ml tube and then 0.5
271 ml of enzyme supernatant was added to the tubes. The enzyme-substrate mixture was
272 incubated for 2 h at 37°C in the water bath with constant shaking. The supernatant was
273 collected from the mixture by centrifuging at 7,000×g for 8 min. In the following, 0.5 ml
274 dinitrosalicylic acid reagent was added, boiled at 100°C for 5 min. After cooling, 1.5 ml
275 deionised water was added directly and measured spectrophotometrically at 550 nm (Chan
276 and Tian, 2005). Background levels of reducing sugars and the protein concentration of the
277 enzyme solution was determined as described above. The specific activity was determined as

278 micromoles of N-acetyl-D-glucosamine per milligram protein per hour according to Reissig
279 et al. (1955). Each treatment had three replications and the experiments were repeated twice.

280

281 **2.10 Induction of apple resistance against *B. cinerea***

282 To assess the effect of different concentrations of the antagonist on elicitation of defence
283 resistance in whole intact fruits was performed according to El-Ghaouth et al. (2003). Intact
284 apples were dipped into the yeast suspension at 10^8 , 10^7 and 10^6 cells ml^{-1} for 30 seconds.
285 Apples treated with sterilized distilled water served as controls. The effect of inactivated
286 antagonistic cells was also evaluated. Intact fruits were dipped into the inactivated cell
287 suspension at 10^8 cells ml^{-1} for 30 seconds. After air dry, apples were kept at 20°C for 24 h.
288 The fruits were then surface-sterilized by wiping extensively with pure ethanol to remove
289 yeast cells from the surface and then fruits were gently wounded (2-3 mm deep) with a
290 syringe needle at three different sites around the equatorial region and an aliquot (30 μl) of
291 the pathogen suspension (10^5 spore ml^{-1}) was inoculated into each wound site. After 2 h
292 air-dry, the fruits were stored at 20°C and 95% RH. The diameters of rotten lesions were
293 determined 4 days after inoculation. Each treatment consisted of three replications of 10 fruits
294 each. The experiments were performed three times.

295

296 **2.11 Statistical analysis**

297 Replications of all the experiments, when the means were similar, were pooled and
298 analyzed together. Means and standard errors for each treatment were reported. Data analysis
299 was performed by using the SPSS software (SPSS Inc., version 13.0, Chicago, IL, USA).

300 Statistical significance was judged at the level of p -value < 0.05 . When the analysis of
301 variance was statistically significant, Tukey's test was used to compare the means.

302

303 **3. Results**

304 **3.1 Efficacy against grey mould incidence on apples under storage conditions**

305 After storage at 1°C and 95% relative humidity for 120 days, *P. guilliermondii* M8 reduced
306 grey mould incidence from 45.3% to 20.0% compared to the control. Correspondingly, the
307 efficacy of *P. guilliermondii* strain M8 in controlling grey mould on apples was 55.8% (Table
308 1).

309

310 **3.2 Effect on *B. cinerea* spore germination *in vitro* and on grey mould severity on apples** 311 ***in vivo***

312 By co-culturing in AJM, the effect of *P. guilliermondii* strain M8, applied as living cell
313 suspensions, inactivated cells or culture filtrate, on *B.cinerea* spore germination and germ
314 tube length were investigated (Table 2). *P. guilliermondii* strain M8, applied at 10^9 cells ml^{-1} ,
315 completely inhibited the pathogen spore germination in AJM. When co-cultured at 10^8 , 10^7
316 and 10^6 cells ml^{-1} , the spore germination were inhibited by 97.1%, 86.6% and 74.2%,
317 respectively, and in addition, the germ tube length was also reduced by 80.0%, 68.9% and
318 48.8%, respectively. However, no significant differences either on the spore germination rate
319 or on the germ tube elongation were observed compared to the control, when the pathogen
320 was co-cultured with inactivated cells or culture filtrate of the yeast.

321 To determine the effects of *P. guilliermondii* strain M8 on grey mould decay, apples were

322 inoculated with the pathogen and treated with living cells, culture filtrate or inactivated cells
323 of the yeast. The living cells at 10^9 cells ml^{-1} completely controlled the development of grey
324 mould decay, while the living cells at 10^8 , 10^7 and 10^6 cells ml^{-1} reduced the diameter of grey
325 mould decay, to 66.7%, 47.9% and 35.2 %, respectively, compared to the control (Table 2).
326 When apples were treated with culture filtrate or inactivated cells, the mean diameter of grey
327 mould decays were not significantly different from that of inoculated control (Table 2).

328

329 **3.3 Interactions between pathogen hyphae and yeast in apple juice and on apple tissue**

330 To examine the attachment of the cells of the strain M8 to the hyphae of *B. cinerea*, the
331 pathogen and the yeast were co-cultured in AJM (Fig. 1a). After 20 h, yeast cells strongly
332 adhered to the pathogen conidia and hyphae of *B. cinerea* (Fig. 1b), and the yeast cells showed
333 to prefer the attachment to hyphal tips and conidia, inhibiting to some extent the germ tube
334 elongation. After 7 days of storage at 20°C and 95% RH, in apple fruit wounds, the yeast
335 cells adhered to the hyphae of the pathogen, apparently restricting the proliferation of *B.*
336 *cinerea in vivo* (Fig. 1d).

337

338 **3.4 *In vitro* and *in vivo* competition for nitrogen sources, sugars and Fe^{3+} ions**

339 To investigate the potential role of competition for nutrients in the interactions of the
340 strain M8 against *B. cinerea*, the effects of sugars, nitrogen sources and Fe^{3+} were studied on
341 spore germination in apple juice and on grey mould development on apples (Table 3). The *in*
342 *vitro* experiments showed that addition of glucose, sucrose and fructose reduced the spore
343 germination. Increasing concentrations of sugars, correspondingly increased the spore

344 germination rate in presence of M8. The addition of 1% $(\text{NH}_4)_2\text{SO}_4$, 0.5% and 1%
345 phenylalanine, 0.5% and 1% asparagine significantly increased the spore germination rate of
346 the pathogen in presence of M8. Compared to the control, the addition of these substrates also
347 increased the spore germination in absence of M8. However, addition of Fe^{3+} had no effect on
348 spore germination. Similar results were obtained both from *in vivo* and *in vivo* experiments,
349 with the addition of similar nutrients, the inhibition of rotten lesions was significantly
350 reduced by the addition of 1% glucose, 0.5% and 1% sucrose, 1% fructose, 0.5% and 1%
351 $(\text{NH}_4)_2\text{SO}_4$, phenylalanine and asparagine (Table 3). Summarizing the results *in vitro* and *in*
352 *vivo*, competition for sugars and nitrates played an important role in the interactions of the
353 strain M8 with *B. cinerea*.

354

355 **3.5 Production of β -1,3-glucanase and its activity**

356 The antagonistic yeast *P. guilliermondii* strain M8 produced extracellular
357 β -1,3-glucanase in culture media in presence of purified fungal cell walls, sucrose and
358 glucose used as sole carbon source. *P. guilliermondii* strain M8 produced more extracellular
359 β -1,3-glucanase at 24 h post-inoculation (hpi) in the three Lilly-Barnett minimal salt media,
360 with *B. cinerea* cell walls, sucrose and glucose as sole carbon sources. At 48 hpi, the activities
361 of extracellular β -1,3-glucanase were similar to those at 24 hpi. While at 72 hpi, the yeast
362 increasingly produced extracellular β -1,3-glucanase and its activity increased. At 96 hpi,
363 extracellular β -1,3-glucanase activity reached the maximum level and the activities in the
364 minimal salt medium with *B. cinerea* cell walls, sucrose and glucose, respectively, as sole
365 carbon sources, were 124.6, 132.7 and 125.8 U (μmol glucose released/mg protein/h),

366 respectively. However, at 120 hpi, the β -1,3-glucanase activity started to decrease (Fig. 2a).

367

368 **3.6 Production of chitinase and its activity**

369 The assay of exo-chitinase activity showed that *P. guilliermondii* strain M8, when
370 cultured in the three Lilly-Barnett minimal salt media with *B. cinerea* cell walls, sucrose and
371 glucose as sole carbon sources, demonstrated similar trends with different incubation periods
372 (hpi). The maximum level of exo-chitinase activity was detected between 48 hpi and 72 hpi.
373 At 96 hpi, the activities of exo-chitinase with *B. cinerea* cell walls, sucrose and glucose as
374 sole carbon sources were 2.36, 2.33 and 2.05 U ($\mu\text{mol GlcNAc released/mg protein/h}$),
375 respectively (Fig.2b).

376 The yeast also had the capability of producing endo-chitinase when cultured in
377 Lilly-Barnett minimal salt medium with *B. cinerea* cell walls, glucose and sucrose used as
378 carbon sources. The endo-chitinase activity reached the maximum level at 48 hpi and the
379 endo-chitinase activities were 8.6, 8.0 and 7.6 U, respectively for *B. cinerea* cell walls,
380 glucose and sucrose (Fig. 2c). No significant differences were experienced between the three
381 media tested.

382

383 **3.7 Induction of apple resistance against *B. cinerea***

384 After 4 days of storage at 20°C and 95% RH, the diameters of the rotten lesions of the
385 apples treated with the strain M8 at 10^8 , 10^7 and 10^6 cell ml^{-1} were 22, 26 and 27 mm,
386 respectively, and were lower than the control (29 mm (Fig. 3.)). The diameters of rotten
387 lesions of the fruits treated with the strain M8 at high concentration of 10^8 cell ml^{-1} were

388 significantly different from those of the control treatment, suggesting that at the highest cell
389 concentration *P. guilliermondii* strain M8 caused an induction of disease resistance to *B.*
390 *cinerea* on apples.

391

392 **4. Discussion**

393 *P. guilliermondii* strain M8 showed effective biocontrol capabilities, both in storage and
394 controlled conditions, in reducing the disease incidence and severity of grey mould of apples
395 caused by *B. cinerea*. Though some postharvest biocontrol agents are already on the market,
396 great potential exists for new biofungicide products, to be commercialized on a global scale
397 for the postharvest control of fungal diseases.

398 Multiple modes of actions were involved in the biocontrol of *B. cinerea* by the strain M8, but
399 most biocontrol agents use several modes of action against the pathogens (Janisiewicz and
400 Korsten, 2002; Ippolito et al., 2005). Extensive production of extracellular lytic enzymes by
401 the yeast antagonist, especially β -1,3-glucanase and chitinase, may play an important role by
402 enhancing either nutrient competition or other unknown mechanisms (Fan et al., 2002; Masih
403 and Paul, 2002; Chan and Tian, 2005). To our knowledge, this work represents the first
404 determination of the mechanisms used by the yeast *P. guilliermondii* to control *B. cinerea* on
405 apples.

406 In this research, the enzymes activities assay showed that *P. guilliermondii* strain M8
407 produced β -1,3-glucanase, exo-chitinase and endo-chitinase in Lilly-Barnett minimal salt
408 medium with *B. cinerea* cell walls, or glucose or sucrose as sole carbon source. The
409 β -1,3-glucanase activities of *P. guilliermondii* strain M8 with cell wall preparation (CWP)

410 were far higher than those of *P. guilliermondii* strain US7 (Castoria et al., 1997) and *P.*
411 *guilliermondii* strain R13 (Chanchaichaovivat et al., 2008), when grown on CWP. In addition,
412 the exo-chitinase and endo-chitinase activities of *P. guilliermondii* strain M8 were also higher
413 than those of the antagonistic yeasts *Metschnikowia pulcherrima* strain MACH1 and
414 *Rhodotorula* sp. PW34 (Saravanakumar et al., 2009), and *Pichia membranaefaciens* and
415 *Cryptococcus albidus* (Chan and Tian, 2005). Similar enzymatic activities from *P.*
416 *guilliermondii* strain M8 were detected on the three different carbon sources used. These
417 results suggest that *P. guilliermondii* strain M8 possesses a stronger capability of producing
418 enzymatic activities when compared with other antagonistic yeast strains under similar
419 conditions. Most phytopathogenic fungi have cell walls composed of complex polymers of
420 β -1,3- and β -1,6-glucans, mannoproteins, where chitin as a structural backbone is arranged in
421 regularly ordered layers, and β -1,3-glucan as a filling material is arranged in an amorphous
422 manner (Smits et al., 2001; Cheng et al., 2009). Thus, breakdown of the fungal cell wall
423 requires the participations of the different enzymes, especially β -1,3-glucanases and chitinase
424 (Marcello et al., 2010). Therefore, to further elucidate the roles of hydrolytic enzymes also *in*
425 *vivo*, the glucanase and chitinases genes from the antagonist yeast *P. guilliermondii* strain
426 M8 will be cloned and characterized.

427 Light microscope observations revealed that the fungal hyphae were strongly surrounded
428 attached by the antagonistic cells in AJM, and spores and germ tubes of the pathogen were
429 massively colonized by *P. guilliermondii* strain M8 in wounded tissue. Attachment of fungal
430 hyphae as an important mode of action in controlling pathogens has been discussed and
431 demonstrated in studies of other antagonistic yeasts including *Candida oleophila* strain I-182

432 colonizing the hyphal walls of *B. cinerea* (Saligkarias et al., 2002), and *P. membranefaciens*
433 and *C. albidus*, which attached the hyphal walls of *Monilinia fructicola*, *Penicillium*
434 *expansum* and *R. stolonifer* (Chan and Tian, 2005). Wisniewski et al. (1991) showed that the
435 attachment of the hyphal walls of *B. cinerea* by *P. guilliermondii* strain 87 could be blocked
436 by agents that alter protein integrity and certain sugars, and similar results were observed on
437 the fungal by *P. membranefaciens* and *C. albidus* cells (Chan and Tian, 2005), suggesting that
438 some functional proteins of the antagonists and pathogens were involved in the attachment
439 process. However, the attachment process and regulation mechanisms of the process are still
440 unknown.

441 Competition for nutrients and space is considered the main mode of action of yeast biocontrol
442 agents. Our research showed that competition for sugars and nitrates plays a key role in the
443 interactions of *P. guilliermondii* strain M8 with *B. cinerea*. The carbon, nitrogen and iron
444 sources tested are common sources generally used by microorganisms. Yeasts can
445 satisfactorily use a wide range of carbohydrates, which includes the disaccharide sucrose and
446 the monosaccharides glucose and fructose (Spadaro et al., 2010a). Based on the specific
447 growth rate in glucose containing media, nitrogen sources are classified as ‘good’ (‘preferred’)
448 or ‘poor’ (‘nonpreferred’) (Magasanik and Kaiser, 2002). Good nitrogen sources are generally
449 easily converted into glutamate and glutamine, with asparagine as an example (Hofman-Bang,
450 1999). Phenylalanine is considered to be an ‘average’ nitrogen sources (Magasanik and Kaiser,
451 2002). Ammonium sulphate $(\text{NH}_4)_2\text{SO}_4$ was tested to check the competition for ammonium
452 ion of the BCA and the pathogen (El-Mansi and Bryce, 1999). Finally, iron is essential for the
453 fungal growth and pathogenesis, and iron sequestration by non-pathogenic microbes could be

454 exploited in novel systems for biological control of postharvest pathogens (Saravanakumar et
455 al., 2008). Addition of increased concentrations of glucose, sucrose, fructose, phenylalanine,
456 asparagine and $(\text{NH}_4)_2\text{SO}_4$ significantly reduced the spore germination of pathogen in the
457 presence of the antagonist in AJM, which is in according to the result obtained *in vivo*,
458 suggesting that the antagonist is competing with *B. cinerea* for sugars and nitrogen sources.
459 Moreover, competition for nutrients plays a major role in the activities of the antagonist
460 against the postharvest pathogens and this is also supported by the fact that different
461 concentrations of the antagonist co-cultured with the pathogen in AJM influenced the spore
462 germination and germ tube elongation. When the concentrations of the antagonist were lower,
463 the spore germination rate significantly increased and the germ tubes were significantly
464 longer.

465 Iron (Fe^{3+}) is biologically important, being a constituent of cytochrome and other heme
466 or non-heme proteins and also a co-factor in various fungal enzymes (Meziane et al., 2005;
467 Macagan et al., 2008). Previous investigations have demonstrated that competition for Fe^{3+} is
468 the main modes of action of some antagonistic yeast against *B. cinerea* (Sipiczki, 2006;
469 Saravanakumar et al., 2008). However, both *in vitro* and *in vivo* experiments showed that the
470 addition of different concentrations of Fe^{3+} had no effect on the spore germination in the
471 presence of the antagonist, indicating that Fe^{3+} is not involved in the interaction of the
472 antagonist against *B. cinerea* in this study.

473 The study of the effects of *P. guilliermondii* strain M8 on *B. cinerea* *in vitro* and *in vivo*
474 shows that the strain is strongly concentration-dependent in controlling the pathogen. Culture
475 filtrate or inactivated cells of the yeast had no effect on the pathogen spore germination or

476 germ tube elongation or on the grey mould development, showing that antibiotic compounds
477 or killer toxin were not involved in the interactions of the antagonist and pathogen.

478 Induction of host plant resistance was also proposed as one of the modes of actions of
479 some antagonists in controlling pathogens (Castoria et al., 2003; Tian et al., 2007). *P.*
480 *guilliermondii* strain M8, when applied at high concentration, could induce the resistance of
481 host apples to *B. cinerea*, significantly inhibiting the grey mould decay. Resistance to
482 pathogens on fruits induced by other antagonistic yeasts has been documented: resistance to *B.*
483 *cinerea* on apples induced by *Aureobasidium pullulans* (Ippolito et al., 2000); resistance to *P.*
484 *digitatum* in grapefruits by *C. oleophila* (Droby et al., 2002); or resistance to postharvest
485 decays on apples by *C. saitoana* (EI-Ghaouth et al., 2003). The resistance induction is due to
486 the antagonist ability to elicit host plant defence responses. This process always involves
487 several chemical or biochemical reactions in the host tissue, including changes of tissue
488 structure and production of pathogenesis-related proteins, which can be expressed locally or
489 systemically (Kloepper et al., 1992; van Loon, 1997). Peroxidase, polyphenoloxidase,
490 catalase, phenylalanine ammonialyase and β -1,3-glucanase have been proved to be involved
491 in plant defence responses against fungal infection (Pieterse et al., 2002; van Loon, 2007).
492 Therefore, to further investigate the defence responses of apples induced by *P. guilliermondii*
493 strain M8, the specific activities of peroxidase, polyphenoloxidase, catalase, phenylalanine
494 ammonialyase, superoxide dismutase and β -1,3-glucanase in pericarp and flesh tissues of the
495 strain M8-treated fruits will be measured and confirmed with molecular tools.

496 Summarizing, it can be concluded that production of β -1,3-glucanase and chitinase,
497 direct attachment, competition for sugars and nitrogen sources, and indirect induction of

498 defence response as well as other undetermined mechanisms play a role in the interactions of
499 *P. guilliermondii* strain M8 with *B. cinerea*.

500

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506

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666 **Legends:**

667 **Figure 1**

668 **a)** *B. cinerea* in AJM (magnification 150 ×; bar = 40 μm); **b)** interaction of *P. guilliermondii*
669 strain M8 with *B. cinerea* in AJM (magnification 150 ×; bar = 40 μm); **c)** apple pulp tissue
670 (*Malus × domestica*; M) (magnification 150 ×; bar = 40 μm); **d)** interaction of *P.*
671 *guilliermondii* strain M8 with *B. cinerea* in apple pulp tissue (magnification 150 ×; bar = 40
672 μm).*

673

674 * **AT** – Apple tissue; **BH** - *B. cinerea* hyphae; **BC** - *B. cinerea* conidia; **PC** - *P. guilliermondii*
675 strain M8 cells

676

677 **Figure 2**

678 Hydrolytic enzyme activities of *P. guilliermondii* strain M8 grown in Lilly-Barnett minimal
679 salt medium supplemented with 2 mg ml⁻¹ CWP, glucose and sucrose as sole carbon source at
680 25°C for 120 h: **a)** β-1,3-glucanase (EC 3.2.1.39) activity; **b)** exo-chitinase (EC 3.2.1.52); **c)**
681 endo-chitinase (EC 3.2.1.14) activity. Bars represented standard deviations of the means.

682

683 **Figure 3**

684 Induction of apple resistance to *B. cinerea* by *P. guilliermondii* strain M8. Apples cv. Golden
685 Delicious were treated with the antagonist suspensions at 10⁸, 10⁷ and 10⁶ cells ml⁻¹, by
686 dipping for 30 seconds, respectively, and incubated at 20°C and 95% RH for 48 h, followed
687 by removing the cells from the fruits. The apples were then wound inoculated with *B. cinerea*

688 suspension at 10^5 spores ml^{-1} and after 4 days of storage at 20°C and 95% RH, the diameters
689 of the rotten lesions were recorded. Values of each column marked by different letters show
690 significant difference ($P<0.05$) according to analysis by Tukey's Test (SPSS 13.0). Bars
691 represented standard deviations of the mean.

692 **Table 1.**

693 Efficacy of *P. guilliermondii* M8 against *B. cinerea* on apples cv. Golden Delicious under
694 storage conditions. Apples were dipped in the antagonist suspension (10^8 cells ml⁻¹) for 1 min,
695 air-dried at 25°C for 2 h, and then stored at 1°C and 95% RH. After 120 days of storage, the
696 incidence of rotten apples was counted. Fruits treated with the fungicide solution played as
697 chemical treatment, while fruits treated with distilled water served as uninoculated control.

698

| Treatments | <i>B. cinerea</i> disease incidence (%)* | |
|-----------------------------|--|---|
| <i>P. guilliermondii</i> M8 | 20.0 ± 4.0 | b |
| Thiabendazole* | 10.7 ± 2.3 | a |
| Uninoculated control | 45.3 ± 4.0 | c |

699

700 * The results are the mean of two independent experiments. “±” stands for standard error of
701 the means. Values of each column followed by different letters show significant difference
702 (P<0.05) according to analysis by Tukey’s Test (SPSS 13.0).

703 “±” stands for standard deviation of the means. Values followed by the same letter are not
704 statistically different

705 * Fruits were treated with thiabendazole (Tecto 20 S, Elf Atochem Agri Italy, 19,7 % a.i., 30 g
706 a.i. 100 l⁻¹).

707 **Table 2.**

708 Effect of *P. guilliermondii* strain M8 on *B. cinerea* spore germination and germ tube

709 elongation in AJM and decay development in wound inoculated apples cv. Golden Delicious.

710

| Treatments | <i>In vitro</i> | | <i>In vivo</i> |
|--|------------------------------------|------------------------------------|--|
| | Spore germination (%) [*] | Germ tube length (μm) [*] | Diameter of rotten lesions (mm) [*] |
| 10 ⁹ cells ml ⁻¹ | 0.0±0.0 a | 0.0±0.0 a | 0.0 ± 0.0 a |
| 10 ⁸ cells ml ⁻¹ | 2.7±0.6 a | 79.8±12.6 b | 11.2 ±1.2 b |
| 10 ⁷ cells ml ⁻¹ | 12.3±2.5 b | 112.6±12.6 b | 17.6 ±2.6 c |
| 10 ⁶ cells ml ⁻¹ | 23.7±3.2 c | 185.4±7.8 c | 21.9 ±2.1 d |
| Inactivated cells | 92.7±0.6 d | 369.1±14.6 d | 33.2 ±1.3 e |
| Culture filtrate | 91.7±1.5 d | 362.0±18.2 d | 32.9 ±1.0 e |
| Inoculated control | 91.7±2.9d | 362.4±23.7 d | 33.8 ±1.9 e |

711

712 * The results are the mean of two independent experiments. “±” stands for standard error of

713 the means. Values of each column followed by different letters show significant difference

714 (*P*<0.05) according to analysis by by Tukey’s Test (SPSS 13.0).

715 **Table 3.**

716 Effects of the yeast *P. guilliermondii* strain M8 along with sugars, nitrogen substrates and
 717 Fe³⁺ on control of *B. cinerea in vitro* on AJM and *in vivo* on apples cv. Golden Delicious. To
 718 assess the spore germination incidence, 100 spores were randomly selected from each
 719 replication and the germ tube length was measured microscopically.

720

| Treatments | <i>In vitro</i> | | <i>In vivo</i> | |
|--|------------------------------------|------------|---------------------------------------|------------------|
| | Spore germination (%) [*] | | Diameter of lesions (mm) [*] | |
| Control | 90 ±1.5 i | | 46.3 ± 3.5 g | |
| yeast | 2 ± 0.6 a | | 11.5 ± 4.4 a | |
| | Concentration | | | |
| | 0.5% | 1.0% | 0.5% | 1.0% |
| glucose | 91±2.3 i | 92 ±2.1 i | 46.4 ± 4.0 g | 49.5 ± 4.50 ghi |
| yeast+ glucose | 26 ±1.5 b | 42 ±2.0 d | 12.1 ± 3.5 a | 23.7 ± 4.85 c |
| sucrose | 90 ±3.2 i | 93 ±2.1 i | 46.9 ±4.5 g | 53.0 ± 3.40 ij |
| yeast + sucrose | 28 ±1.5 bc | 52 ±3.8 e | 19.9 ± 7.5 bc | 28.9 ± 5.31d |
| fructose | 91 ±2.1 i | 92 ±2.6 i | 46.4 ± 2.8 g | 52.1 ± 2.66 hij |
| yeast + fructose | 26 ±1.5 b | 34 ±2.0 c | 13.4 ± 4.8 a | 18.5 ± 4.49 b |
| (NH ₄) ₂ SO ₄ | 93 ±2.1 i | 96 ±1.0 i | 51.1 ± 2.8 jhij | 55.1 ± 2.50 j |
| yeast+ (NH ₄) ₂ SO ₄ | 66 ±2.1 f | 80 ±2.1 h | 29.3 ± 2.7 d | 37.7 ± 3.58 f |
| phenylalanine | 91 ±1.2 i | 94 ±1.0 i | 48.9 ± 3.9 ghi | 49.9 ± 3.38 ghi |
| yeast + phenylalanine | 53 ±4.2 e | 69 ±1.2 fg | 29.3 ± 3.6 d | 36.0 ± 3.02 ef |
| asparagine | 92 ±1.5 i | 93 ±1.5 i | 48.9 ± 3.1 ghi | 51.2 ± 1.61 ghij |
| yeast + asparagine | 64 ±4.0 f | 73 ±3.1 g | 32.3 ± 3.5 de | 33.4 ± 4.79 def |
| | 1 mM | 5 mM | 1 mM | 5 mM |
| Fe ³⁺ | 90 ±2.1 i | 90 ±2.0 i | 47.3 ± 3.87 gh | 48.8 ± 1.97 ghi |
| yeast + Fe ³⁺ | 2 ±0.0 a | 2 ±0.6 a | 14.1 ± 4.61 a | 12.9 ± 2.49 a |

721

722 * The results are the mean of two independent experiments. “±” stands for standard error of

723 the means. Values of each column followed by different letters show significant difference

724 (*P*<0.05) according to analysis by by Tukey’s Test (SPSS 13.0).