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**Mechanisms of action and efficacy of four isolates of the yeast *Metschnikowia pulcherrima* active against postharvest pathogens on apples**

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## UNIVERSITÀ DEGLI STUDI DI TORINO

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23 **Mechanisms of action and efficacy of four isolates of the yeast *Metschnikowia***  
24 ***pulcherrima* active against postharvest pathogens on apples**

25  
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36  
37 **Abstract**

38 The mechanisms of action and efficacy of four isolates (GS37, GS88, GA 102, and BIO126) of the  
39 yeast *Metschnikowia pulcherrima* against *Botrytis cinerea*, *Penicillium expansum*, *Alternaria* sp.,  
40 and *Monilia* sp., all postharvest pathogens of apple fruits, were studied *in vitro* and on apples, in  
41 controlled and semi-commercial conditions.

42 An application of a cell suspension ( $10^8$  cells ml<sup>-1</sup>) of the antagonists in artificial wounds of apples  
43 permitted to reduce the growth of *B. cinerea* and *P. expansum* after storage at 23°C. A complete  
44 suppression of the pathogen was obtained against *Monilia* sp., storing at 23°C, and against *B.*  
45 *cinerea* and *P. expansum*, storing at 4°C. The results against *Alternaria* sp. were more variable.  
46 Applications of culture filtrates and autoclaved cells of the isolates were ineffective in reducing the  
47 diameter of the lesions on the fruits, supporting the hypothesis that living cells are necessary for  
48 biocontrol. In experiments of antagonism *in vitro*, on different solid substrates, a reduction of the  
49 micelial growth of the pathogens emerged, so that, at least *in vitro*, the antagonists could produce  
50 some diffusible toxic metabolites. Co-cultivating *in vitro* on a synthetic medium, *B. cinerea* spore  
51 ( $10^5$  ml<sup>-1</sup>) germination was completely inhibited by the presence of  $10^8$  cells of the antagonists,  
52 while culture filtrates and autoclaved suspensions were not able to reduce germination. Dipping  
53 boxes of apples cv Golden delicious in a suspension of  $10^7$  antagonist cells ml<sup>-1</sup> and storing for 8  
54 months in controlled atmosphere at 1°C, the isolates showed control capability against *B. cinerea*  
55 and *P. expansum* similar to thiabendazole.

57 **Keywords:** *Alternaria* sp.; Antagonism; Apple; Biocontrol; *Botrytis cinerea*; Integrated Pest  
58 Management, *Metschnikowia pulcherrima*; *Monilia* sp.; *Penicillium expansum*; Postharvest rot;  
59 Storage

60

## 61 **1. Introduction**

62

63 Apple Postharvest rots, caused by *Penicillium expansum*, *Botrytis cinerea*, and *Alternaria* sp., are  
64 particularly severe even in production areas where the most advanced storage technologies are  
65 available (Eckert and Ogawa, 1988). In Northern Italy they can cause serious losses, also due to the  
66 presence of populations of *B. cinerea* and *P. expansum* resistant to fungicides (Romano *et al.*,  
67 1983). In this area during the last years, *Monilia* sp. has become an increasing problem (Trevisan *et al.*,  
68 1992), also due to integrated pest management techniques, which avoid treatments at flowering.  
69 Currently, fungicides, when admitted, are the major means to control postharvest diseases (Eckert  
70 and Ogawa, 1988). However, the growing public concern over the health and environmental  
71 hazards associated with pesticide use in orchards (Wisniewski and Wilson, 1992), the development  
72 of fungicide resistant strains of postharvest pathogens (Spotts and Cervantes, 1986) and the  
73 deregistration of some of the most effective fungicides (Gullino and Kuijpers, 1994) have generated  
74 interest in the development of alternative non chemical methods.

75 Biological control using microbial antagonists (Wilson and Wisniewski, 1994) has emerged as one  
76 of the most promising alternatives, either alone or as part of an integrated pest management to  
77 reduce pesticide use. Several biocontrol agents have been exploited and widely investigated against  
78 different postharvest fungal pathogens (*B. cinerea*, *Penicillium*, *Monilia*, and *Alternaria spp.*) and  
79 tested on apple fruits (Mc Laughlin, 1990; Roberts, 1990; Gullino *et al.*, 1994; Filonow *et al.*, 1996;  
80 Piano *et al.*, 1997; El-Ghaouth *et al.*, 1998; Janisiewicz, 1998). At present, three products  
81 containing *Pseudomonas syringae* Van Hall, active against *Botrytis*, *Penicillium*, *Mucor* and  
82 *Geotrichum* spp., named Bio-Save 100, Bio-Save 110 and Bio-Save 1000 and commercialized by  
83 EcoScience Corp. (Janisiewicz and Jeffers, 1997), and a product containing *Candida oleophila*  
84 Montrocher, effective against *Botrytis* and *Penicillium* spp., named Aspire and commercialized by  
85 Ecogen Inc. (Hofstein and Fridlender, 1994), are available on the market for postharvest protection.  
86 Information on the mechanisms of action of most of the antagonists is still incomplete, but it is  
87 essential to develop appropriate formulation and methods of application, to obtain registration and  
88 to select new effective microorganisms. The main mode of action of yeast biocontrol agents is  
89 believed to be competition for nutrients and space (Droby and Chalutz, 1994). Moreover, yeasts are  
90 able to colonize the carposphere for a long period in low humidity conditions, grow rapidly and are

91 generally poorly sensitive to fungicides (Janisiewicz, 1991). Yeasts deserve particular attention, as  
92 their activity does not generally depend on the production of toxic metabolites, which could have a  
93 negative environmental or animal toxicological impact (Smilanick, 1994). Additional modes of  
94 action such as mycoparasitism, induced resistance and the production of lytic enzymes such as  $\beta$ -  
95 1,3-glucanase have been suggested (Wisniewski *et al.*, 1991; El Ghaouth *et al.*, 1998; Jijakli and  
96 Lepoivre, 1998). It is likely that there are multiple interactions between antagonist, fruit, pathogen,  
97 and other components of the natural epicarpic microflora (Droby and Chalutz, 1994).  
98 Recently we have isolated and selected four strains of the yeast *Metschnikowia pulcherrima*, named  
99 BIO126, GS 88, GA102 and GS37, which proved to be effective in containing *Botrytis* and  
100 *Penicillium* spp. rots in apple. Other strains of *M. pulcherrima*, coded 2.33 and 4.4, had already  
101 proved to be highly effective in the control of Botrytis rot of apple (Gullino *et al.*, 1991; 1994;  
102 Migheli *et al.*, 1997; Piano *et al.*, 1997). The aim of this work was to determine the mechanism of  
103 action of the antagonistic isolates and to evaluate their efficacy under controlled and semi-  
104 commercial conditions with low temperature and controlled atmosphere.

105

## 106 **2. Materials and methods**

107

### 108 *2.1. Microorganisms and culture conditions*

109

110 *Metschnikowia pulcherrima* (Pitt) M. W. Miller isolates BIO126, GS88, GA102, e GS37 were  
111 isolated from the carposphere of apple cv Golden delicious, harvested in unsprayed orchards located  
112 in Piedmont and Aosta Valley (GA102), Northern Italy. Cultures were stored at  $-20^{\circ}\text{C}$  in cell  
113 suspension with 65% V/V of glycerol and 35% V/V of a solution  $\text{MgSO}_4$  100 mM and Tris ( pH  
114 8.0) 25mM. Yeasts were grown on Yeast Peptone Dextrose (YPD: 10 g  $\text{l}^{-1}$  of Extract of Yeast  
115 Granulated Merck; 20 g  $\text{l}^{-1}$  of Triptone-Peptone of Casein Difco; 20 g  $\text{l}^{-1}$  of D(+)-Glucose  
116 Monohydrate Merck). Inocula of the antagonists for all the experiments were prepared by  
117 subculturing in 250 ml Erlenmeyer flasks containing 75 ml of Yeast Peptone Dextrose (YPD) and  
118 incubating on a rotary shaker (100 rpm) at  $25^{\circ}\text{C}$  for 48 hours. Yeast cells were collected by  
119 centrifugation at  $2500 \times g$  for 5 minutes, washed and resuspended in sterilised Ringer solution (pH  
120  $6.9 \pm 0.1$ ; Merck), and brought to a standard concentration of  $10^8$  cells  $\text{ml}^{-1}$ , unless otherwise stated,  
121 by direct counting with a haemocytometer.

122 Three strains of *Alternaria* sp., isolated from rotted apples belonging to the cvs Golden delicious  
123 and Red delicious and selected for their virulence by inoculation in artificially wounded apples,  
124 were used as a mixture throughout this work, to ensure a higher level of disease. The same

125 operations were accomplished for three strains of *Botrytis cinerea*, three of *Monilia* sp., and three of  
126 *Penicillium expansum*. Each strain was stored in tube with Potato Dextrose Agar (PDA; Merck) and  
127 50 mg l<sup>-1</sup> of streptomycin Merck at 4°C and was routinely inoculated and re-isolated from apple to  
128 maintain pathogenicity. Spore suspensions were prepared by growing the pathogens on Petri dishes  
129 for two weeks with Potato Dextrose Agar (PDA; Merck) and 50 mg l<sup>-1</sup> of streptomycin Merck  
130 (strains of *Alternaria* sp. and *P. expansum*) or with Potato Glucose Malt (35 g l<sup>-1</sup> of Potato Dextrose  
131 Agar Merck, 7 g l<sup>-1</sup> of D(+)-Glucose Monohydrate Merck and 3 g l<sup>-1</sup> of Malt Extract Merck) and 50  
132 mg l<sup>-1</sup> of streptomycin Merck (strains of *B. cinerea* and *Monilia* sp.). After two weeks incubation at  
133 25°C, spores from the three strains of each pathogen were collected and suspended in sterile  
134 Ringer's solution (Merck). After filtering through 8 layers of sterile cheese-cloth, spores were  
135 counted and brought to a final concentration of 10<sup>5</sup> ml<sup>-1</sup>.

136

## 137 2.2. Antagonism in apple artificial wounds

138

139 Apples (*Malus domestica* Borkh, cv Golden delicious), disinfected in sodium hypochloride (NaClO,  
140 1.0 % as chlorine) and rinsed under tap water, when dry were punctured with a sterile needle at the  
141 equatorial region (3 mm depth; 3 wounds per fruit). An antagonistic yeast cell suspension (30 µl)  
142 was pipetted into wound. Autoclaved cells of *M. pulcherrima* and culture filtrates, prepared by  
143 centrifuging cultures of the antagonists and then filtering the supernatant through a 0.2 µm nitro-  
144 cellulose filter, were applied into wounds in order to evaluate their efficacy in reducing the  
145 incidence of the pathogens on apple fruit. Inoculated control fruits were pipetted, before pathogen  
146 inoculation, with 30 µl of Yeast Peptone Dextrose. After 3 hours, 30 µl of the spore suspension of  
147 the pathogen strains were pipetted in the wound. When dry, apples from different treatments were  
148 randomly packed in commercial plastic trays and either stored at 23°C for 6 days (*B. cinerea* and *P.*  
149 *expansum*), 12 days (*Monilia* sp.) or 18 days (*Alternaria* sp.) or kept at 4°C for 21 days (*B. cinerea*)  
150 or 28 days (*P. expansum*). Three fruits per treatment were used (9 inoculation sites) and each  
151 experiment was repeated three times.

152

## 153 2.3. Antagonism in vitro

154

155 The growth rate of the pathogens was tested in different solid synthetic mediums: Potato Dextrose  
156 Agar (PDA, Merck), NYDA (as in Droby *et al.*, 1989), Yeast Potato Dextrose-Agar (YPD with 20 g  
157 l<sup>-1</sup> of Agar-agar Merck), CZAPEK-Agar (1 g l<sup>-1</sup> of Potassium Phosphate Merck, 2 g l<sup>-1</sup> of Sodium  
158 Nitrate Merck, 0.5 g l<sup>-1</sup> of Magnesium Sulphate Merck, 0.5 g l<sup>-1</sup> Potassium Chloride Merck, 0.01 g

159  $l^{-1}$  of Ferrous Sulphate Merck, 30 g of Saccharose Merck e 15 g of Agar-Agar Merck), Malt Extract  
160 Agar (Merck) and Apple-Agar (80 % v/v of apple homogenised filtered through Whatman no. 1  
161 filter paper and 20 g  $l^{-1}$  of Agar-Agar Merck; pH: 5.5). A drop of the yeast cell suspension was  
162 striped on the substrate in 90 mm diameter Petri dishes, 20 mm from the border. A 6 mm mycelium  
163 disk of the pathogen was put 32 mm from the border and 32 mm from the strip of the antagonist.  
164 The radial growth of the mycelium towards the yeast strip was measured when the pathogen  
165 reached 32 mm of diameter towards the dish border, after storage at 23°C and in the dark. If the  
166 mycelium did not reach the border in 28 days, the substrate was considered not optimal for that  
167 pathogen growth. Three Petri plates per treatment were used and the experiment was repeated twice.

168

#### 169 2.4. *Effect on B. cinerea spores germination*

170

171 The effect of the isolates of *M. pulcherrima* on spore germination of *B. cinerea* isolate Gao1 was  
172 assessed in potato dextrose broth (PDB, Difco). Aliquots (100  $\mu$ l) of spore suspension ( $5 \times 10^6$   
173 spores  $ml^{-1}$ ) of the pathogen in Ringer's solution were transferred to 10 ml plastic tubes containing  
174 5 ml PDB. Living cells of each antagonistic yeast (100  $\mu$ l of a suspension containing  $5 \times 10^7$ ,  $5 \times 10^8$ ,  
175 or  $5 \times 10^9$  cells  $ml^{-1}$ ) or cells killed by autoclaving (100  $\mu$ l of a suspension containing  $5 \times 10^8$  cells  $ml^{-1}$ )  
176 were added to each tube. As a control, the pathogen was added to 5 ml of a mixture (1:1) of PDB  
177 and of culture filtrates obtained as described from 48 hours old cultures of the 4 isolates of *M.*  
178 *pulcherrima* in PDB. After 12 h incubation of the 45° sloping tubes at 25°C on a rotary shaker (100  
179 rpm), 100 spores per replicate were observed microscopically and their germination was evaluated.  
180 The treatments were replicated three times and the experiment repeated twice.

181

#### 182 2.5. *Experimental trials under semi-commercial conditions: storage in controlled atmosphere*

183

184 An experimental trial was carried out in Aosta (Aosta Valley, Northern Italy) during the period  
185 November 1998 – July 1999 in cooperation with the Institut Agricole Regional on artificially  
186 infected apples of the cv Golden delicious. Apples were harvested in orchards conducted with  
187 integrated pest management. Four boxes were used in each treatment (100 fruits per box). Ten  
188 apples per box, to reproduce the most probable conditions after harvesting, were artificially  
189 wounded at the equatorial region (3 mm depth; 3 wounds per fruit). All fruits were artificially  
190 inoculated by dipping for 60 seconds in 100 l tanks containing a conidial suspension ( $10^5$  spores  
191  $ml^{-1}$  per pathogen) of *B. cinerea*, *P. expansum*, and *Alternaria* sp.. After 3 hours, biocontrol isolates  
192 were applied at  $10^7$  cells  $ml^{-1}$  completely dipping the boxes of fruits for 60 sec in 100 l tanks

193 containing cell suspensions prepared as described. The treatments included the four isolates of *M.*  
194 *pulcherrima* (BIO126, GS37, GS88 and GA102) and a chemical control (thiabendazole, Tecto 20 S,  
195 Elf Atochem Agri Italy, 19,7 % a.i., 30 g a.i. 100 l<sup>-1</sup>). The inoculated, with the pathogens  
196 suspension, and uninoculated controls were represented by four boxes with 100 fruits per box, ten  
197 of them artificially wounded. When dry, apples were incubated at 23°C for 24 h and then stored at  
198 1°C for 8 months in controlled atmosphere (2% O<sub>2</sub> and 3% CO<sub>2</sub>). After 4 and 8 months storage, the  
199 rot incidence was evaluated and the relative importance of the different postharvest pathogens  
200 determined visually or through isolation on potato dextrose agar (PDA, Merck).

201

### 202 **3. Results**

203

#### 204 *3.1. Antagonism in apple artificial wounds*

205

206 *M. pulcherrima* BIO126, GS88, GA102, and GS37 cell suspension, applied at 10<sup>8</sup> cells ml<sup>-1</sup>, on  
207 apple artificial wounds stored at 23°C generally reduced the lesion diameter of Alternaria rot but the  
208 results were not homogeneous (Table 1). The major reduction to 0.9 % was due to the application of  
209 the isolate GS37. Culture filtrates and autoclaved cell suspensions also caused reduction of the  
210 lesions but, even in the same treatment, there were differences among single fruits.

211 Culture filtrates and autoclaved cell suspensions of the four isolates were ineffective against  
212 *Monilia* sp. at 23°C, *B. cinerea* and *P. expansum* at 23° and 4°C (Tables 1 and 2).

213 Cell suspension (10<sup>8</sup> cells ml<sup>-1</sup>) of the four isolates significantly reduced Botrytis rot on apples, but  
214 the best results were obtained storing the fruits at 4°C (Table 2). BIO126 and GS37 cell suspensions  
215 permitted to obtain a remarkable reduction of the lesions also at 23°C, respectively to 25.7 and 26.8  
216 %. Addition of the cell suspensions of any of the four antagonistic strains completely inhibited the  
217 growth of *Monilia* sp. after 12 days of storage at 23°C (Table 1). The cell suspensions of the four  
218 strains applied on apple wounds resulted highly effective against *P. expansum* at 4°C after 28 days  
219 of storage (Table 2). Reduction of lesions was lower, ranging between 35.1 and 60.9 %, but anyway  
220 significantly different from the control, storing fruits at 23°C (Table 1).

221 In this experiment the effect of all the yeast isolated was studied on disease severity (lesion  
222 diameter) and not incidence (percent infection), evaluated in the trials under semi-commercial  
223 conditions.

224

### 225 3.2. Antagonism in vitro

226

227 The co-culture on different solid substrate antagonists and pathogens, permitted to study  
228 antagonism *in vitro*. As shown in Table 3-A and 3-C, on some media, even after 28 days, *Alternaria*  
229 and *Monilia* spp. did not grow sufficiently to be influenced by the presence of the biocontrol agent,  
230 so that it was not possible to measure the inhibition.

231 *Alternaria* sp. mycelium growth (Table 3-A) was significantly inhibited by the presence of the  
232 antagonist strip on APPLE (Figure 1) and CZAPEK. *B. cinerea* (Table 3-B) growth was reduced by  
233 the four strains on YPD and NYDA and by GA102 on PDA. *Monilia* sp. (Table 3-C) was partially  
234 inhibited by all the potential antagonists on APPLE. *P. expansum* (Table 3-D) radial growth was  
235 significantly inhibited by the four isolates on NYDA and partially on YPD and APPLE.

236

### 237 3.3. Effect on *B. cinerea* spore germination

238

239 By co-culturing on potato dextrose broth (PDB, Difco), the effect of *M. pulcherrima* BIO126,  
240 GS88, GA102, and GS37 on spore germination of *B. cinerea* was evaluated (Table 4).

241 A complete inhibition of the spore germination emerged in presence of  $10^8$  cells  $\text{ml}^{-1}$  of the four  
242 strains of *M. pulcherrima*. With  $10^7$  cells  $\text{ml}^{-1}$  there was a partial inhibition: the percentage of  
243 conidia germinated compared with the control varied from 25.7 % of BIO126 to 73.0 % of GA102.  
244 In the presence of  $10^6$  cells  $\text{ml}^{-1}$  of antagonist only a negligible and not significant reduction in the  
245 germination was observed. A culture filtrate and killed cell suspension permitted the full  
246 germination of the spores.

247 During the experiment, a strong attitude by the antagonist living cells to concentrate and adhere to  
248 non germinated spores of *B. cinerea* was observed. Adhesion was not observed with autoclaved  
249 cells.

250

### 251 3.4. Experimental trials under semi-commercial conditions: storage in controlled atmosphere

252

253 Trial carried out dipping boxes of apples cv Golden delicious in a cell suspension of the antagonist  
254 was followed by an 8 month storage in controlled atmosphere at 1°C. First survey, after 4 months,  
255 showed a reduction in the incidence of rotted apples for all biological treatments (Table 5).

256 Treatments with cell suspensions differ significantly from the chemical control, while they did not  
257 one from the other. BIO126 cell suspension offered a control (9.0 % of rotted apples) similar to  
258 thiabendazole (8,7 %). Analyzing pathogens separately, it was possible to point out in all theses a  
259 major incidence of *B. cinerea* rots. *Alternaria* sp. rots were absent or at a very low level (1.4 %).  
260 Biological treatments showed a control of *P. expansum* (average incidence of 2.9 %) similar to  
261 thiabendazole (3.2 %), but a lower efficacy towards *B. cinerea* (average incidence of 9.7 %) in  
262 comparison with the chemical control (4.1 %).

263 After 8 months' storage (Table 5), biological treatments offered a control statistically not different  
264 from thiabendazole, but significantly different from the inoculated control, with a reduction of the  
265 incidence of rotted fruits, compared to first survey.

266 *Alternaria* rot incidence increased prolonging storage, but remained a minority of the total rotted  
267 fruits. In comparison with the first survey, the incidence of *B. cinerea* was lower and that of *P.*  
268 *expansum* was similar.

269 Summing up the percentages of rotted apples in the two surveys, the best result was offered by  
270 BIO126 (17.1 %) and the worst by GA102 (23.1 %). Compared with the chemical product (13.7 %),  
271 biocontrol agents showed an efficacy slightly lower, but the incidence of rotted fruits was  
272 significantly reduced with respect to the inoculated control (50.4 %).

273

## 274 **4. Discussion**

275

### 276 *4.1. Mechanisms of action*

277

278 Generally the activity of antagonist yeasts is not based on the production of antibiotics or other  
279 secondary toxic metabolites (Droby and Chalutz, 1994); the results of this study show that *M.*  
280 *pulcherrima* BIO126, GS88, GA102, and GS37 principally act for the competition for space and /  
281 or nutrients.

282 From the antagonism in apple artificial wounds, a substantial incapability emerged to antagonize all  
283 pathogens tested either by culture filtrate (without yeast cells) or by autoclaved cell suspension  
284 (killed cells). Living cells of the antagonists are necessary to guarantee the fungal control. The  
285 nutritional environment of the apple wound could be favourable to *M. pulcherrima*, that would  
286 colonize fruit tissues rapidly competing with pathogens for nutrients.

287 Also in *in vitro* experiments on *B. cinerea* spore germination, neither the culture filtrate nor  
288 autoclaved cells of the four isolates had any effect on the germination. The antagonistic activity of

289 *M. pulcherrima* was dependent on the concentration of the antagonist: when applied at  $10^6$  cells  $\text{ml}^{-1}$   
290 no yeast provided a satisfactory level of control. During the study a tenacious adhesion of living  
291 yeast cells to *B. cinerea* spores and hyphae was observed, in a manner similar to that described by  
292 Wisniewski *et al.* (1991). Attachment to pathogen conidia was not observed after incubation of  
293 autoclaved antagonistic cells. This permits to suppose a direct interaction between antagonist and  
294 pathogen.

295 Often microbial antagonists provide different results in *in vitro* or *in vivo* conditions (Gullino,  
296 1994). Co-culture experiments of antagonists and pathogens on different solid substrates bring to  
297 suppose that, at least in *in vitro* conditions, antagonistic yeasts could produce some metabolites  
298 toxic for the pathogens, differently from what results from the application of culture filtrate *in vivo*.  
299 Because inhibition of mycelial growth is only present on some substrates, it is probable that the  
300 nutritional environment influences the production of secondary metabolites. The CZAPEK  
301 substrate, for instance, is poor in simple and complex sugars, therefore it could not favor the radial  
302 growth of the pathogen; furthermore it is rich in nitrates, that remarkably reduce antagonistic  
303 capability of the isolates, as already observed by Piano *et al.*, 1997. APPLE substrate could easily  
304 simulate the nutritional conditions of the wound; for many of the pathogens tested it was possible to  
305 point out some inhibition. On MALT, PDA, and NYDA, around the yeast strip, a pink halo was  
306 also visible, indicative of the metabolism of some compounds present on the substrate.

307

#### 308 4.2. Efficacy

309

310 To be commercially acceptable, antagonists must be effective under semi-commercial conditions.  
311 Since previous experiments, carried out measuring naturally developed rots, were ineffective  
312 because of the low incidence of the disease in the control, the trial of storage at  $1^{\circ}\text{C}$  in controlled  
313 atmosphere was carried out on partially wounded apples and inoculated with spore suspensions of  
314 *Alternaria* sp., *Botrytis cinerea*, and *Penicillium expansum*. This step permitted to obtain, after 8  
315 months of storage, 50.4 % of rotted apples in the inoculated control, greatly increasing the  
316 probability to observe significant differences. *P. expansum* and *B. cinerea* were the pathogens more  
317 frequently isolated from the fruits. The low incidence of *Alternaria* rots gave no significant results.  
318 All biocontrol agents were effective in the reduction of the total rots. The origin of *M. pulcherrima*  
319 BIO126, GS88, GA102, and GS37, isolated from apple surface of “Golden delicious”, could have  
320 influenced positively the experiments since the isolates are naturally able to colonize the  
321 carposphere of the fruit.

322 In trials of antagonism in apple wounds, the isolates offered a satisfactory biocontrol efficacy

323 against *B. cinerea*, *Monilia*, sp. and *P. expansum*. Control of Botrytis and Penicillium rots by  
324 antagonist cell suspension was more homogeneous in the repetition, while efficacy towards  
325 Alternaria and Monilia rots resulted highly dependent on the fruit. An element of interference in the  
326 experiment was the difficulty to find fruits with the same degree of ripening; a high concentration in  
327 sugars, in fact, associated with senescence of the fruit, is a factor promoting the attack of certain  
328 pathogens, such as *P. expansum* and *B. cinerea* (Roberts, 1991). Comparing trials of antagonism  
329 carried out at 4 and at 23 °C, antagonists showed a major efficacy at 4°C and, in some cases,  
330 pathogens were completely inhibited.

331 Considering the results of the efficacy trials, a sharp difference in the biocontrol capability of the  
332 four antagonists does not exist: anyway BIO126 offered a higher control in semi-commercial  
333 conditions.

334

## 335 **5. Conclusions**

336

337 *M. pulcherrima* BIO126, GS88, GA102, and GS37 were tolerant to benomyl and thiabendazole  
338 (benzimidazoles), and to vinclozolin and procymidone (dicarboximides), all of them registered for  
339 postharvest use (data not shown), therefore it could be possible to employ the biocontrol agents  
340 together with reduced dosages of these fungicides, in an integrated control perspective.

341 The antagonists were tolerant to calcium chloride (data not shown). Between the strategies  
342 experimented during the last years in fruit protection, the association of biological agents with  
343 calcium chloride infiltration is to remember. The addition of this salt greatly enhances the action of  
344 the antagonist yeast (McLaughlin *et al.*, 1990; Piano *et al.*, 1998).

345 The biocontrol capability of the yeasts, during the experiment in semi-commercial conditions, was  
346 not affected by the low temperature of storage (1°C) of the fruits and by the controlled atmosphere.  
347 Antagonists are compatible with normal storage methods and with chemical products employed in  
348 post-harvest.

349 From growth at different temperatures (data not shown), it resulted that the tested isolates do not  
350 grow at 37°C, which is important from a toxicological point of view. Another point favourable for a  
351 future commercialization of the studied yeasts is the lack of production of antibiotics active against  
352 the tested pathogens *in vivo*. The main mechanism of action used by the biocontrol agents is  
353 competition with pathogens for space and nutrients, but a secondary mechanisms of action with a  
354 synergistic effect could be also a direct interaction, such as parasitism, and some production of toxic  
355 metabolites in particular nutritional conditions, not investigated in this work.

356 In future studies, to increase the knowledge on the mechanisms of action, it could be useful to

357 purify and characterize the substances released in the culture substrates. Enzymes surely involved in  
358 the process of antagonism, such as glucanases or chitinases, specific for the cell wall of the fungi,  
359 have already been isolated from the media where yeast antagonists were grown (Jijakli and  
360 Lepoivre, 1998; Wilson *et al.*, 1994). It could also be useful supply the microorganisms with  
361 different nutrients, sources of carbon or nitrogen, to understand which are involved in the  
362 mechanism of competition (Janisiewicz *et al.*, 1992; Piano *et al.*, 1998). Future studies will also  
363 concentrate on the potential of resistance induced in the host tissue (Arras, 1996; Wilson and El-  
364 Ghaouth, 1993).

365 It would be interesting to evaluate the curve of the antagonist population on the carposphere, but  
366 it was not possible to mark the biocontrol agents for the sensitivity to antibiotics to differentiate  
367 them from the indigenous population. Trials of sensitivity to seven antibiotics resulted in a similar  
368 level of tolerance, between the four isolates and the population of yeasts present on the fruit (data  
369 not shown).

370 The work is now continuing towards a molecular characterization of the isolates, either to evaluate  
371 the survival and dynamic of the population after application on the fruit or to study the  
372 environmental impact of a possible release in the open field. RAPD-PCR (Random Amplified  
373 Polymorphic DNA) and AP-PCR (Arbitrary Primed Polymerase Chain Reaction) techniques permit  
374 to distinguish efficiently also strictly correlated strains (Droby *et al.*, 1999; Schena *et al.*, 2000).

375 Finally, the formulation should constitute a fundamental field of the studies, to permit the  
376 commercialization of the product (Fravel *et al.*, 1999).

377

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382

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478

479 **Tables**

480

481 Table 1

482 Effect of cell suspension, cell-free culture filtrate and autoclaved cells of *M. pulcherrima* isolates  
 483 BIO126, GS88, GA102, and GS37, applied in artificial wounds of “Golden delicious” apple, on the  
 484 growth of different post-harvest rots. Storage at 23°C for 6 (Botrytis and Penicillium rots), 12  
 485 (Monilia rot), and 18 days (Alternaria rot).

486

Treatment	Percentage of control *							
	<i>Alternaria</i>		<i>B.cinerea</i>		<i>Monilia</i>		<i>P.expansum</i>	
Uninoculated Control	0.0	a	0.0	a	0.0	a	0.0	a
Inoculated control	100.0	f	100.0	d	100.0	b	100.0	ef
BIO 126 10 <sup>8</sup> cells/ml	5.7	ab	25.7	b	0.7	a	35.1	b
BIO 126 culture filtrate	96.4	f	113.2	de	110.5	b	101.7	ef
BIO 126 autoclaved	52.1	cde	115.8	de	71.6	b	107.6	f
GS 88 10 <sup>8</sup> cells/ml	51.8	cde	60.7	c	0.0	a	52.4	bc
GS 88 culture filtrate	69.9	def	103.6	de	75.1	b	81.0	de
GS 88 autoclaved	34.5	abcd	122.1	de	31.8	b	97.5	ef
GA 102 10 <sup>8</sup> cells/ml	0.0	a	102.9	de	0.0	a	60.9	cd
GA 102 culture filtrate	42.3	bcde	113.9	de	105.2	b	102.5	ef
GA 102 autoclaved	81.8	ef	125.5	e	114.4	b	110.2	f
GS 37 10 <sup>8</sup> cells/ml	0.9	ab	26.8	b	0.0	a	40.2	bc
GS 37 culture filtrate	28.0	abc	103.8	de	103.7	b	92.9	ef
GS 37 autoclaved	17.9	abc	109.4	de	97.4	b	107.4	f

487

488 \*Calculated on the lesion diameter. Values in the same column followed by the same letter are not  
 489 statistically different by Duncan’s Multiple Range Test ( $P < 0,05$ ).

490

491 Table 2  
 492 Effect of cell suspension, cell-free culture filtrate and autoclaved cells of *M. pulcherrima* isolates  
 493 BIO126, GS88, GA102, and GS37, applied in artificial wounds of “Golden delicious” apple, on the  
 494 growth of different post-harvest rots. Storage at 4°C for 21 (Botrytis rot) and 28 days (Penicillium  
 495 rot).  
 496

Treatment	Percentage of control*			
	<i>B.cinerea</i>		<i>P.expansum</i>	
Uninoculated Control	0.0	a	0.0	a
Inoculated control	100.0	cd	100.0	cd
BIO 126 10 <sup>8</sup> cells/ml	57.1	b	0.0	a
BIO 126 culture filtrate	116.3	de	89.7	b
BIO 126 autoclaved	121.3	e	101.2	d
GS 88 10 <sup>8</sup> cells/ml	5.3	a	0.0	a
GS 88 culture filtrate	103.6	cde	98.5	cd
GS 88 autoclaved	113.3	cde	98.2	cd
GA 102 10 <sup>8</sup> cells/ml	9.5	a	0.0	a
GA 102 culture filtrate	95.6	c	85.5	b
GA 102 autoclaved	100.0	cd	93.1	bcd
GS 37 10 <sup>8</sup> cells/ml	0.0	a	0.0	a
GS 37 culture filtrate	102.7	cd	88.5	b
GS 37 autoclaved	103.6	cde	92.4	bc

497  
 498 \*See table 1.  
 499

500 Table 3  
 501 Inhibition of the mycelium growth of *Alternaria* sp. (3-A), *Botrytis cinerea* (3-B), *Monilia* sp. (3-  
 502 C), *Penicillium expansum* (3-D) by *M. pulcherrima* isolates BIO126, GS88, GA102, and GS37 in  
 503 dual culture on different media at room temperature in the dark.

504 3-A

Substrates	Days of co-culturing**	Mean inhibition (%) *			
		BIO 126	GS88	GA102	GS37
PDA	12	0.0 a	0.0 a	0.0 a	0.0 a
YPD	28	No growth on control plates			
NYDA	28	No growth on control plates			
CZAPEK	12	29.2 d	6.3 ab	10.4 bc	17.7 c
MALT	15	0.0 a	0.0 a	0.0 a	0.0 a
APPLE	12	24.0 b	25.0 b	24.0 b	31.3 b

505 3-B

Substrates	Days of co-culturing**	Mean inhibition (%) *			
		BIO 126	GS88	GA102	GS37
PDA	5	0.0 a	0.0 a	12.5 b	0.0 a
YPD	5	10.4 c	7.3 b	6.3 b	8.3 bc
NYDA	5	18.8 c	12.5 b	13.5 bc	14.6 bc
CZAPEK	5	0.0 a	0.0 a	0.0 a	0.0 a
MALT	5	0.0 a	0.0 a	0.0 a	0.0 a
APPLE	5	0.0 a	0.0 a	0.0 a	0.0 a

506 3-C

Substrates	Days of co-culturing**	Mean inhibition (%) *			
		BIO 126	GS88	GA102	GS37
PDA	20	11.5 b	4.2 a	4.2 a	1.0 a
YPD	28	No growth on control plates			
NYDA	28	No growth on control plates			
CZAPEK	28	No growth on control plates			
MALT	15	0.0 a	0.0 a	0.0 a	0.0 a
APPLE	10	6.3 a	20.8 b	15.6 b	15.6 b

507 3-D

Substrates	Days of co-culturing**	Mean inhibition (%) *			
		BIO 126	GS88	GA102	GS37
PDA	5	14.6 b	12.5 b	7.3 ab	10.4 b
YPD	5	17.7 b	19.8 b	16.7 b	18.8 b
NYDA	5	12.5 b	20.8 b	16.7 b	18.8 b
CZAPEK	5	0.0 a	0.0 a	0.0 a	0.0 a
MALT	5	4.2 a	0.0 a	0.0 a	0.0 a
APPLE	5	17.7 b	18.8 b	29.2 c	17.7 b

508  
 509 \*Values in the same row followed by the same letter are not statistically different by Duncan's  
 510 Multiple Range Test ( $P < 0,05$ ). Control, always indicated with an a, is implied.

511 \*\*Days needed by control mycelium to reach a radius of 32 mm.

512

513 Table 4  
 514 Effect of cell suspension, cell-free culture filtrate and autoclaved cells of *M. pulcherrima* isolates  
 515 BIO126, GS88, GA102, and GS37, on spore germination of *Botrytis cinerea*, by co-culturing in  
 516 PDB at 25°C for 12 hours.

<i>B. cinerea</i> spore germination (%)*			
Control		99.0	e
BIO126	culture filtrate	98.7	e
	autoclaved	99.3	e
	10 <sup>8</sup> cells ml <sup>-1</sup>	0.0	a
	10 <sup>7</sup> cells ml <sup>-1</sup>	25.7	b
	10 <sup>6</sup> cells ml <sup>-1</sup>	98.7	e
GS88	culture filtrate	97.7	e
	autoclaved	98.7	e
	10 <sup>8</sup> cells ml <sup>-1</sup>	0.0	a
	10 <sup>7</sup> cells ml <sup>-1</sup>	47.7	c
	10 <sup>6</sup> cells ml <sup>-1</sup>	99.3	e
GA102	culture filtrate	98.3	e
	autoclaved	99.3	e
	10 <sup>8</sup> cells ml <sup>-1</sup>	0.0	a
	10 <sup>7</sup> cells ml <sup>-1</sup>	73.0	d
	10 <sup>6</sup> cells ml <sup>-1</sup>	97.0	e
GS37	culture filtrate	99.0	e
	autoclaved	99.7	e
	10 <sup>8</sup> cells ml <sup>-1</sup>	0.0	a
	10 <sup>7</sup> cells ml <sup>-1</sup>	51.7	c
	10 <sup>6</sup> cells ml <sup>-1</sup>	97.7	e

517  
 518 \* Values in the same column followed by the same letter are not statistically different by Duncan's  
 519 Multiple Range Test ( $P < 0,05$ ).  
 520

521 Table 5

522 Efficacy of *Metschnikowia pulcherrima* isolates BIO126, GS88, GA102, and GS37 against  
 523 *Penicillium expansum*, *Alternaria* sp., and *Botrytis cinerea*, evaluated by dipping boxes of “Golden  
 524 delicious” apples in a cell suspension of the antagonist and storing in controlled atmosphere at 1°C  
 525 for 4 months (first survey) and 8 months (second survey).

First Survey Treatments	Rotted apples (%)			Total
	<i>P. expansum</i>	<i>B. cinerea</i>	<i>Alternaria</i> sp.	
Uninoculated Control	2.3	4.9	0.0	7.2 a**
Inoculated control	7.8	15.2	0.3	23.3 b
Thiabendazole ( $3 \cdot 10^{-2}$ g l <sup>-1</sup> )*	3.2	4.1	1.4	8.7 a
Yeast BIO 126	0.9	8.1	0.0	9.0 a
Yeast GS 88	4.5	9.1	0.0	13.6 a
Yeast GA 102	1.7	12.2	0.0	13.9 a
Yeast GS 37	4.6	9.4	0.0	14.0 a

  

Second Survey Treatments	Rotted apples (%)			Total
	<i>P. expansum</i>	<i>B. cinerea</i>	<i>Alternaria</i> sp.	
Uninoculated Control	2.4	5.1	1.1	8.7 a**
Inoculated control	3.1	21.7	2.3	27.1 b
Thiabendazole ( $3 \cdot 10^{-2}$ g l <sup>-1</sup> )*	1.5	1.2	2.3	5.0 a
Yeast BIO 126	2.1	5.7	0.3	8.1 a
Yeast GS 88	3.1	4.1	0.7	7.9 a
Yeast GA 102	4.7	2.4	2.0	9.2 a
Yeast GS 37	3.6	4.7	0.9	8.1 a

526

527

528 All isolates were applied at  $10^7$  cells ml<sup>-1</sup>.529 \*Apples were treated with 150 ml hl<sup>-1</sup> of Tecto 20S (thiabendazole: 19,7 %)530 \*\*Values followed by the same letter are not statistically different by Duncan's Multiple Range  
 531 Test ( $P < 0,05$ ).

532

533 **Figure captions**

534

535 Fig. 1. Dual culture of *Alternaria* sp. and *M. pulcherrima* BIO126 (left dish) and GS37 (right dish)  
536 on APPLE substrate in Petri dish for 12 days at room temperature in the dark. It is possible to notice  
537 the inhibition of the pathogen mycelium growth on the direction of the yeast strip side.

538

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