Metschnikowia pulcherrima strain MACH1 outcompetes Botrytis cinerea, Alternaria alternata and Penicillium expansum in apples through iron depletion

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24 **and _Penicillium expansum_ in apples through iron depletion**

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38

39 **Abstract**

40 A new strain of _Metschnikowia pulcherrima_ (MACH1) was studied for its efficacy as biocontrol

41 agent against _Botrytis cinerea_, _Penicillium expansum_ and _Alternaria alternata_ on apples stored for

42 8 months at 1°C. The results of two semi-commercial trials permitted to observe an interesting

43 efficacy of the biocontrol strain MACH1. In order to understand the mechanism of action involved,

44 the yeast strain was investigated for its competitive ability for iron against postharvest pathogens of

45 apple. _M. pulcherrima_ strain MACH1 was cultivated on PDA with different concentrations of iron

46 (supplemented as $FeCl_3$) against _A. alternata_ and _B. cinerea_. The yeast strain MACH1 produced a

47 wider pigmented inhibition zone against both pathogens in low iron amendments while less

48 inhibition was measured in increased iron concentrations. At the coloured inhibition zone, _B._

cinerea and *A. alternata* conidia did not germinate and mycelial degeneration was observed. In addition, a high reduction in both pathogens infection was recorded in apples treated with *M. pulcherrima* strain MACH1 supplemented with less iron amendments compared to higher iron concentrations. The same experiments were carried out *in vivo* and *in vitro* against *P. expansum. M. pulcherrima* strain MACH1 amended with low iron concentration (5 μg ml^{-1} FeCl$_3$) showed modest lesion diameter reduction and it was not effective against *P. expansum* under increased iron and without iron amendments. This study illustrated that iron depletion by the yeast strain MACH1 under low iron conditions could reduce the growth of some postharvest pathogens *in vitro* and *in vivo*. Although, iron depletion seems to be a primary mode of action against the postharvest pathogens studied, other mechanisms of action cannot be excluded in the biocontrol employed by *M. pulcherrima* strain MACH1.

Keywords : Biocontrol; Competition; Iron depletion; Pulcherrimin; Postharvest diseases; Yeast

1. Introduction

Apple postharvest rots, caused by *Penicillium expansum* Link (blue mould), *Botrytis cinerea* Pers., (grey mould) and *Alternaria* sp. (Fr.) Keissl. (Alternaria rot) are particularly severe even in production areas where the most advanced storage technologies are available (Snowdon, 2003). When permitted, synthetic fungicides are the primary means to control postharvest diseases (Eckert and Ogawa, 1985). However, the growing public concern over the health and environmental hazards associated with pesticide use in orchards (Wilson and Wisniewski, 1994), the development of fungicide resistant strains of postharvest pathogens (Spotts and Cervantes, 1986) and the deregistration of some of the most effective fungicides (Ragsdale, 2000) have generated interest in the development of alternative non chemical methods.

Biological control using microbial antagonists (Janisiewicz and Korsten, 2002; Spadaro and Gullino, 2004) has emerged as one of the most promising alternatives, either alone or as part of an

75   integrated pest management to reduce pesticide use. During the past 30 years, several biocontrol

76   agents have been exploited and widely investigated against different postharvest fungal pathogens

77   (Lima et al, 1997; Zahavi et al., 2000; Vivekananthan et al., 2004; Calvo et al., 2007). Among the

78   different biocontrol agents, yeasts deserve particular attention, as their activity does not generally

79   depend on the production of toxic metabolites, which could have a negative environmental or

80   toxicological impact. Recently, *Metschnikowia pulcherrima* has been reported as an effective

81   biocontrol agent against postharvest decay of apple, table grape, grapefruit and cherry tomato

82   (Schena et al., 2000; Janisiewicz et al., 2001; Spadaro et al., 2002) as well as against some food-

83   borne pathogens (Leverentz et al., 2006).

84   The strains of the yeast species mostly investigated generally act by consuming nutrients present on

85   fruit and vegetable skins that allow rot-causing fungi to develop (Piano et al., 1997; Janisiewicz et

86   al., 2001). A good understanding of the mode of action is essential before developing appropriate

87   commercial formulations and application methods. Several possible biocontrol mechanisms have

88   been suggested to be effective against postharvest rots on fruit including competition for nutrients

89   and space, antibiosis, parasitism or direct interaction with the pathogens and induction of resistance

90   in the host tissue (Smilanick, 1994). Competition for nutrients and space is considered to be a

91   primary mode of action against postharvest fungal pathogens. In particular, competition for iron is

92   believed to play a significant role in the biocontrol interactions (Raaska and Mattila-Sandholm,

93   1995). *M. pulcherrima* was found to produce a red pigment, pulcherrimin, that accumulates in the

94   cells and is also secreted around the colonies (Miller and Phaff, 1998; Kurtzman and Droby, 2001).

95   The aim of this research was to assess the efficacy of a new strain of *M. pulcherrima* against *B.*

96   *cinerea*, *P. expansum* and *A. alternata* on apples in semi-commercial conditions. A second

97   objective was to study the competitive ability of *M. pulcherrima* strain MACH1 for iron

98   sequestration against postharvest pathogens of apples.

99   **2. Materials and methods**

4

2.1 Antagonist and pathogens preparation

Metschnikowia pulcherrima (Pitt) M. W. Miller isolate MACH1 was isolated from the carposphere of apple cv Golden delicious, harvested in organic orchards located in Piedmont, Northern Italy. The strain was deposited within the American Type Culture Collection on June 19, 2007 with deposit designation PTA-8487.

Yeast strain MACH1 was grown and cells were prepared as described in Spadaro et al. (2002). Two strains each of *Alternaria alternata*, *Botrytis cinerea* and *Penicillium expansum* were isolated from rotted apples and selected for their virulence by inoculation in artificially wounded apples. They were used as a mixture throughout this work, to ensure high level of disease and the presence of rots caused by multiple pathogens. Each strain was stored in slant on Potato Dextrose Agar (PDA; Merck) with 50 mg l^{-1} of streptomycin Merck at 4°C. Spore suspensions were prepared by growing the pathogens on Petri dishes for two weeks on PDA added with 50 mg l^{-1} of streptomycin. After two weeks of incubation at 25°C, spores from the two strains of each pathogen species were collected and suspended in sterile Ringer's solution (Merck, Darmstadt, Germany). After filtering through 8 layers of sterile cheese-cloth, spores were counted and brought to a final concentration of 10^5 spores ml^{-1} per each pathogen.

2.2 Efficacy trials in semi-commercial conditions

Two experimental trials were carried out in Aosta (Aosta Valley, Northern Italy) in cooperation with the Institut Agricole Regional on artificially infected apples of the cv Golden delicious. Apples were harvested in orchards conducted by following integrated pest management. Four boxes were used for each treatment (100 fruits per box). Ten apples per box, to reproduce the most probable conditions after harvesting, were artificially wounded at the equatorial region (3 mm diameter; 6 mm depth; 3 wounds per fruit). All fruits were artificially inoculated by dipping for 60 seconds in 100 l tanks containing a conidial suspension (10^5 spores ml^{-1} per pathogen) of *B. cinerea*, *P. expansum*, and *A. alternata*. After 3 hours, biocontrol isolates were applied at 10^7 cells ml^{-1} by

126   completely dipping the boxes of fruits for 60 sec in 100 l tanks containing the cell suspensions

127   prepared as described. The treatments were a cell suspension of *M. pulcherrima* MACH1 and a

128   chemical control (thiabendazole, Tecto 20 S, Elf Atochem Agri Italy, 19,7 % a.i., 30 g a.i. 100 l^{-1}).

129   Moreover, an inoculated control was represented by four boxes with 100 fruits per box, ten of them

130   artificially wounded, dipped in the spore suspension of the three pathogens. When dry, apples were

131   incubated at 23°C for 24 h, then stored at 1°C for 8 months under controlled atmosphere (2% O_2

132   and 3% CO_2) and the same experiment was duplicated in another cold chamber storing the fruits at

133   1°C in 1% O_2 and 2% CO_2. After 5 and 8 month of storage, the total rot incidence and the relative

134   rot incidence (grey mould, blue mould, Alternaria rot) were determined.

135

136   **2.3 Effect of iron on pigment production and antagonism**

137   Cells of *M. pulcherrima* strain MACH1 (10^7 ml^{-1}) were streaked onto YPD and PDA plates (10

138   plates for each concentration of iron) with different concentrations of iron to test the pigment

139   production. Both media were amended with 5, 10, 15 and 20 μg ml^{-1} of $FeCl_3$ before autoclaving.

140   The widths of the reddish halos developing around the yeast colonies were measured after 5 days of

141   incubation at 25°C.

142   The antagonistic activity of the pigment produced by the yeast strain MACH1 was studied on PDA

143   plates supplemented with different concentrations of $FeCl_3$. The plates were flooded with 20 μl of a

144   conidial suspension ($3x10^5$ spores ml^{-1}) of the tested pathogens followed by yeast strain MACH1

145   (10^7 cells ml^{-1}) streaked onto the centre of each plate. The widths of the inhibition zones were

146   measured after 3 (*P. expansum*), 4 (*B. cinerea*) and 10 days (*A. alternata*) of incubation at 25°C.

147   The sensitivity of the test pathogens to iron depletion was tested with tropolone (2-

148   hydroxycyclohepta-2,4,6-trienone; T89702; Sigma-Aldrich Co.), a chelating agent with a strong

149   affinity for ferric ions (Diouf et al., 2002). An aqueous solution of the compound (50 μl; 0.5 mg

150   tropolone), was placed into wells (diameter, 5 mm) cut into PDA plates previously flooded with 20

151 µl of a conidial suspension (3×10^5 spores ml^{-1}) of the tested pathogens. The experiments were

152 repeated three times.

153

154 **2.4 Microscopic observations of the pathogen mycelium development**

155 To examine the effect of pigmented inhibition zone produced by MACH1 on the conidial

156 germination and hyphal growth of the tested pathogens, microscopic observations were carried out.

157 Conidial suspensions (3×10^5 spores ml^{-1}) were smeared (20 µl) onto PDA plates with and without

158 different concentrations of ferric chloride (5, 10, 15 and 20 µg ml^{-1}). Immediately after, MACH1

159 cell suspension was streaked onto the centre of each plate and the plates were incubated at 25°C.

160 After 36 h, 10 microscopic fields were randomly selected at the pigmented zone and they were

161 counted for conidial germination. After 48 h, hyphal germination in the coloured zone was observed

162 under light microscope (Nikon Eclipse 55i). The experiments were repeated twice.

163

164 **2.5 Study of iron competition in apples**

165 The strain MACH1 was inoculated into 250 ml Erlenmeyer flaks containing 100 ml of YPD broth

166 amended with 0, 5, 10, 15, 20, 30 µg ml^{-1} FeCl$_3$ and incubated on a rotary shaker (100 rpm) at 25°C

167 for 48 h. Apples, cv Golden delicious, disinfected in sodium hypochloride (NaOCl, 1.0% as

168 chlorine) for 30 sec and rinsed under tap water, when dry were punctured with a sterile needle at the

169 equatorial region (3 mm depth, 3 wounds per fruit). The broth containing the antagonistic yeast (30

170 µl; 2×10^8 cells ml^{-1}) was pipetted into wounds. Untreated controls were inoculated with YPD broth

171 and a chemical control applied at 30 µl per wound (thiabendazole, Tecto 20 S, Elf Atochem Agri

172 Italy, 19.7 % a.i., 200 µg a.i. ml^{-1}) was included in the experiment to have comparative studies with

173 the newly isolated yeast strain MACH1. After 24 h, 30 µl of conidial suspension (2×10^5 spores ml^{-1})

174 of *B. cinerea, A. alternata* and *P. expansum* were inoculated into wounds and apples were incubated

175 at 25°C. The diameter of the lesions, the weight of the rotten apple pulp and the percent infected

176 wounds were measured for each treatment. The measures were taken after 9 days for *B. cinerea*, 13

7

177 days for *P. expansum* and 21 days for *A. alternata.* Eight apples were used for each treatment (24

178 wounds) and the experiment was repeated twice.

179

180 **2.6 Statistical analysis**

181 The program SPSS-WIN was used for the statistical analysis to process the data and the Duncan's

182 Multiple Range Test was employed at $P<0.05$ to separate the values in the semi-commercial and

183 controlled condition experiments.

184

185 **3. Results**

186 **3.1 Efficacy of *M. pulcherrima* strain MACH1 against postharvest pathogens of apple at 1º C**

187 The semicommercial trials were conducted to test the efficacy of the strain MACH1 against

188 different postharvest pathogens of apple at 1°C, to assess the performance of the biocontrol agent

189 under storage conditions. The results (Table 1) showed that *M. pulcherrima* strain MACH1 after 5

190 and 8 months storage under $2\%O_2/3\%CO_2$ significantly reduced the percentage of fruits attacked by

191 postharvest pathogens (11.6%; 25.2%) compared to the control (19.4%; 41.3%). The treatment with

192 thiabendazole resulted significantly different from the control (13.5%) after 5 month storage, but

193 ineffective at the end of the trial (40.6%).

194 In the second trial with storage under $1\%O_2/2\%CO_2$ (Table 1), realized to assess the effectiveness

195 of the biocontrol agent under different semi-commercial conditions, the incidence of rotten fruits

196 between the treatment with strain MACH1 (12.9%) and the control (14.8%) was not significant

197 after 5 month storage. Also after 8 month storage, the biological treatment (29.0%) and the control

198 (34.8%) were not significantly different. After both surveys, an increase of the number of rotten

199 fruits compared to the control resulted after treatment with thiabendazole.

200 Analyzing pathogens separately, it was possible to point out in all theses a major incidence of *B.*

201 *cinerea* rots after 5 month storage and of *P. expansum* rots after 8 months storage. *Alternaria* sp.

202 rots were absent or at a very low level.

Comparing the two trials, the treatment that more effectively permitted to reduce the number of rotted fruits was the strain MACH1 application followed by storage at $2\%O_2/3\%CO_2$

3.2 Pigmented inhibition zone by _M. pulcherrima_ strain MACH1 against postharvest pathogens growth

M. pulcherrima strain MACH1 produced pale pink to dark red colonies under different iron conditions. The intensity of the colour increased with increasing iron concentrations (Table 2) while the strain produced white colonies without iron amendment in PDA. The antagonistic yeast produced wider pigmented halos in PDA without (7.5 mm) or with 5 µg ml^{-1} of FeCl$_3$ (5.0 mm). An increase in FeCl$_3$ concentration decreased the pigmented halo width and the narrowest halo was observed with a supplementation of 20 µg ml^{-1} of FeCl$_3$.

The strain showed an inhibition zone of 5.1 mm when cultured with _B. cinerea_, of 2.5 mm with _A. alternata_ and of 1.2 mm with _P. expansum_ in PDA without iron amendment. Similarly, when MACH1 was streaked onto the medium amended with 5 µg ml^{-1} FeCl$_3$, it produced wider pigmented inhibition zones against the tested pathogens when compared to increased concentrations (10, 15, 20 µg ml^{-1}) of iron salt (Fig. 1a). Compared to the effect on other pathogens, _M. pulcherrima_ did not show appreciable inhibition zone in the case of _P. expansum_, with or without iron amendment.

3.3 Tropolone activity

Tropolone was used to study the sensitivity of postharvest pathogens to iron chelation. A lack of germination of conidia of _B. cinerea_ and _A. alternata_ was observed in the zone near to wells treated with tropolone aqueous solution in PDA medium (Fig. 1b). Also, the mycelial growth of _B. cinerea_ and _A. alternata_ was restricted in the tropolone zone, whereas the fungi tend to grow freely in the medium outside the tropolone zone. Conspicuously, _P. expansum_ did not show restriction over its germination in the zone near to the wells treated with tropolone acquous solution.

9

3.4 Conidial and mycelial inhibition

Microscopic observations at inhibition zone by *M. pulcherrima* in 5 μg ml^{-1} iron amendment showed interesting morphological deviations in the growth of *B. cinerea* and *A. alternata*. A count on the conidial germination at the coloured inhibition zone produced by strain MACH1 against *B. cinerea* revealed the lowest (4%) conidial germination in the medium amended with 5 μg ml^{-1} of FeCl$_3$. Similarly, *A. alternata* and *P. expansum* recorded 16% and 45% conidial germination at the inhibition zone produced by *M. pulcherrima* strain MACH1 in 5 μg ml^{-1} of FeCl$_3$. When the medium was added with increased concentrations of iron (10, 20 μg ml^{-1} of FeCl$_3$), the highest percent of conidial germination was observed in the case of *B. cinerea* and *A. alternata* (Table 3). The conidia in the inhibition zone did not germinate and even if germinating, malformation was observed for germination tube. *B. cinerea* growing at the tip of the colored inhibition zone exhibited mycelial degeneration (Fig. 2) and further growth was arrested. Similar to *B. cinerea*, *A. alternata* mycelial degeneration at the tip of the colored zone was observed. With regard to *P. expansum,* the conidial and mycelial inhibition was not observed as that of the other two pathogens used in this study. However, to some extend, *M. pulcherrima* strain MACH1 in 5 μg ml^{-1} iron concentration caused reduction in conidial germination of *P. expansum* (Table 3).

3.5 Iron competition in artificial wounds of apple against *B. cinerea*

Apples treated with the biocontrol agent amended with or without FeCl$_3$ showed lower *B. cinerea* disease incidence compared to the untreated control (Fig. 3). Further, apples treated with *M. pulcherrima* strain MACH1 recorded lower (6.4%) percentage of attack by *B. cinerea*. Similarly, apples treated with the strain MACH1 supplemented with 5 μg ml^{-1} FeCl$_3$ recorded the lowest (3.57%) percent infection by *B. cinerea* compared to all other treatments. The measurement of diameter of the rotten area and weight of the rotten fruits showed the lowest levels for both

254   parameters (11.5 mm, 3.0 g) in apples treated with the strain of *M. pulcherrima* and 5 μg ml^{-1} FeCl$_3$.

255   This was comparable with that of chemical fungicide. Apples inoculated with *B. cinerea* alone

256   recorded the highest rotten area (71.3 mm) and rotten fruit weight (54.0 g) (Fig. 3).

257

258   **3.6 Iron competition in artificial wounds of apple against *A. alternata***

259   In the experiments against *A. alternata* in artificial wounds (Fig. 4), 11.3% infection was observed

260   in *M. pulcherrima* strain MACH1 treatment compared to 34.3% in untreated control. Interestingly,

261   *M. pulcherrima* strain MACH1 supplemented with the lowest concentrations (5, 10 μg ml^{-1} of

262   FeCl$_3$) of iron reduced more the number of rotten fruits compared to the highest concentrations (20,

263   30 μg ml^{-1} of FeCl$_3$) of iron amendment. Similarly, the lesion diameter (16.0 mm) and the rotten

264   fruit weight (5.9 g) provided by *M. pulcherrima* strain MACH1+5 μg ml^{-1} iron were significantly

265   lower than the other treatments with increased iron amendments and the untreated control (lesion

266   diameter: 52.7 mm; rotten fruit weight: 27.5 g).

267

268   **3.7 Iron competition in artificial wounds of apple against *P. expansum***

269   The results of the *in vivo* experiments against *P. expansum* (Fig. 5) reproduced the same situation of

270   the *in vitro* studies and *M. pulcherrima* strain MACH1 alone was not very effective in reducing *P.*

271   *expansum* infections in apple fruits. On the other hand, *M. pulcherrima* strain MACH1 amended

272   with 5 μg ml^{-1} FeCl$_3$ significantly increased the efficacy of the strain MACH1 compared to the

273   other concentrations of iron. Also the diameter of the rotten area (45.5 mm) and the rotten fruit

274   weight (23.9 g) were lower in treatment supplemented with 5 μg ml^{-1} FeCl$_3$, while high

275   concentrations of iron showed lower effectiveness compared to the control. Among all the

276   treatments, untreated control showed the highest infection rate by *P. expansum*.

277

278   **4. Discussion**

279 In the present study, *M. pulcherrima* strain MACH1, recently isolated from the carposphere of

280 apple cv Golden delicious and previously selected for its efficacy under controlled conditions, was

281 partially effective in controlling postharvest diseases caused by *B. cinerea, A. alternata* and *P.*

282 *expansum* under semi-commercial conditions. The experiments in semi-commercial conditions were

283 carried out to test the efficacy of the biocontrol agent under cold storage and controlled atmosphere

284 used by the packinghouses. Actually, some biocontrol strains perform well under controlled

285 conditions in laboratory, where normally the fruits used have the same level of maturity, but may

286 not do the same under controlled atmosphere cold storage in the packinghouses, where it is not

287 possible to control the physiological conditions of the fruits tested. Zheng et al. (2005) reported that

288 the efficacy of the biocontrol strains at room temperature and under cold storage conditions was

289 determined by the nutritional environment at the wound site which may favour or not the growth

290 and the colonization.

291 The only postharvest fungicide admitted in Italy and in many other countries is the benzimidazole

292 thiabendazole, whose efficacy is really poor due to the development of resistant populations of *P.*

293 *expansum* and *B. cinerea* (Baraldi et al., 2003; Bertetti et al., 2003). Also in our study,

294 thiabendazole was not working in the reduction of both postharvest pathogens on apple.

295 After testing the efficacy, we tried to elucidate the mechanism of action deployed by *M.*

296 *pulcherrima* strain MACH1 in the control of postharvest pathogens on apples. As reported by

297 Boekhout and Robert (2002), *M. pulcherrima* occurs naturally on fruits, buds and floral parts of

298 certain apple trees, is able to ferment glucose, assimilate various carbon compounds and produce

299 the pigment pulcherrimin (Miller and Phaff, 1998). Previous studies demonstrated the production of

300 pulcherrimin formed non enzymatically from pulcherriminic acid and ferric ions (Cook and Slater,

301 1956; MacDonald, 1965). Iron is essential for the fungal growth and pathogenesis, and iron

302 sequestration by non-pathogenic microbes could be exploited in novel systems for biological

303 control of postharvest pathogens (Calvente et al., 1999; Zhang et al., 2007).

304 The antagonistic strain MACH1 produced the red pigment pulcherrimin in presence of iron,

305 indicating the uptake of ferric ions from the surrounding substrate. Higher inhibition halos by the

306 antagonistic strain against *B. cinerea* and *A. alternata* in lower iron amendments indicated the

307 depletion of the micronutrient by the yeast strain under low iron conditions. On the opposite, a

308 reduced halo formed by MACH1 against *B. cinerea*, *A. alternata* and *P. expansum* in increased

309 concentrations of iron demonstrated the availability of a sufficient amount of iron closer to the yeast

310 cells. Iron depletion by the *M. pulcherrima* strain in the medium inhibited the growth of *B. cinerea*,

311 *A. alternata* and *P. expansum*. Our results are consistent with previous findings of Sipiczki (2006)

312 who demonstrated the iron competence between strains of *Metschnikowia pulcherrima* and

313 pathogenic fungi *in vitro*.

314 In order to assess the sensitivity of the pathogens to iron deprivation, tropolone was used in the

315 current study. Tropolone has strong affinity for ferric ions and is able to inhibit their reduction by

316 catecholates, lowering the redox potential of the iron couple. Diouf et al. (2002) reported that

317 tropolone inhibited the wood degradation by *Poria placenta* by chelating the iron present in wood.

318 In the currrent study, tropolone inhibited the growth of *B. cinerea* and *A. alternata*, demonstrating

319 their sensitivity to iron deprivation. On the other hand, the overgrowing of *P. expansum* onto the

320 zone produced by tropolone indicated its scarce sensitivity to iron chelation.

321 Similarly to the tropolone activity, the pigmented zone surrounding the streaks of the strain

322 MACH1 blocked the conidial germination and caused mycelial degeneration of *B. cinerea* and *A.

323 alternata*. As conidia require a large intake of iron for germination (Charlang et al., 1981; Calvente

324 et al., 1999), iron depletion by the *M. pulcherrima* strain delays or reduces conidial germination.

325 Moreover, the microscope observation that hyphae crack when entering the pigmented zones

326 around the *M. pulcherrima* streaks demonstrates that iron starvation elicits complex physiological

327 changes in the fungal cells. Similarly, Ippolito et al. (2005) reported that the ability of

328 *Aureobasidium pullulans* to out-compete the pathogens for nutrients and space might weaken the

329 pathogen cells.

330 In the study of iron competition in artificial wounds on apple, a reduced disease incidence by *B.*

331 *cinerea, A. alternata* and *P. expansum* was observed in apples treated with the strain MACH1 when

332 supplemented with lower concentrations of iron. The external supplementation of high

333 concentrations of iron reduced the efficacy of the biocontrol yeast compared to non-iron and low

334 iron concentrations. Our findings are similar to those of He et al. (2003), who supposed the

335 involvement of iron in the mode of action of antagonistic yeasts against postharvest pathogens of

336 apple. The same authors reported that the biocontrol activity of the microbial antagonists was

337 concentration dependent and reversed by the addition of exogenous nutrients.

338 Some precursors from *M. pulcherrima* move further from the yeast colony before they are

339 immobilized by iron, resulting in a increased efficacy of biocontrol. At higher concentrations of

340 iron, the infection rate was higher because the precursor molecules did not diffuse far, because they

341 bound sufficient amount of iron closer to the yeast cells, and the pathogens had an increased

342 availability of free ferric irons for their growth. The pulcherrimic acid-ferric ion complex formed

343 near to the yeast cells depletes the iron in the substrate and creates an environment unsuitable for

344 fungal microbes requiring the micronutrients for their growth (Sipiczki, 2006; Sanson et al., 2005).

345 Moreover, the increased efficacy by *M. pulcherrima* strain MACH1 under low iron conditions

346 indicated the movement of precursors farther from the yeast to find sufficient amount of iron in the

347 substrate.

348 The higher reduction in the incidence of rotten fruits caused by *B. cinerea*, *A. alternata* and *P.*

349 *expansum*, when apples were treated with MACH1+5 μg ml^{-1}, indicated that small amounts of iron

350 could elicit the production of pulcherrimic acid that depleted iron necessary for the pathogen

351 growth.

352 From the above-mentioned results, this study clearly demonstrated that iron depletion from the

353 substrate, used by *M. pulcherrima* strain MACH1 for the production of pulcherrimin, ultimately

354 inhibited the growth of postharvest pathogens.

355 The measurement of the iron content in the fruits could become an interesting parameter to establish

356 in advance the potential control of postharvest pathogens on fruits, before their application, and

357 could possibly be used to establish if a low iron supplementation is needed to guarantee an effective

358 level of pathogen control during postharvest.

359

360 **Acknowledgements**

365

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453

454 **Legends:**

455 **Figure 1.**

456 **Fig. 1a.** A pigmented inhibition zones produced by strain MACH1 in PDA with different

457 concentrations of iron against *B. cinerea*

458 M - *M. pulcherrima* strain MACH1; B - *B. cinerea*

459 **Fig. 1b.** Tropolone activity against *B. cinerea*

460 T - Tropolone inhibition zone 5 mm; ** sporulation by *B. cinerea* at the point of entry onto

461 inhibition zone; Bc - *B. cinerea*; E - stimulated growth of *B. cinerea* in the tropolone diffused zone.

462

463 **Figure 2.**

464 An observation on *B. cinerea* mycelial distruption at pigmented inhibition zone produced by

465 MACH1

466 CIZ - coloured inhibition zone; MD - mycelial distruption; BC - *B. cinerea*

467

468 **Figure 3.**

469 Efficacy of yeast strain MACH1 with different iron concentrations on apples against *B. cinerea*

470 infection *in vivo*

471 Vertical bars indicated the standard error of mean. Same letter over bars represents that treatment

472 are not significantly different from each other

473

474 **Figure 4.**

475 Efficacy of yeast strain MACH1 with different iron concentrations on apples against *A. Alternata*

476 infection *in vivo*

477 Vertical bars indicated the standard error of mean. Same letter over bars represents that treatment

478 are not significantly different from each other

479

480

Figure 5.

Efficacy of yeast strain MACH1 with different iron concentrations on apples against *P. expansum*

infection *in vivo*

Vertical bars indicated the standard error of mean. Same letter over bars represents that treatment

are not significantly different from each other