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

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

Duraisamy Saravanakumar, Annalisa Ciavorella, Davide Spadaro, Angelo Garibaldi, M. Lodovica Gullino

Centre of Competence for the Innovation in the Agro-environmental Sector, Università degli Studi di Torino, via L. da Vinci 44, I-10095 Grugliasco (TO), Italy; DiVaPRA – Plant Pathology, Università degli Studi di Torino, via L. da Vinci 44, I-10095 Grugliasco (TO), Italy.

*Corresponding author. Tel.: +39-011-6708942; fax: +39-011-6709307.

E-mail address: davide.spadaro@unito.it (D. Spadaro)

Proofs should be sent to: Davide Spadaro, Centre of Competence for the Innovation in the Agro-environmental Sector, Università di Torino, via L. da Vinci 44, I-10095 Grugliasco (TO), Italy.

Abstract

A new strain of *Metschnikowia pulcherrima* (MACH1) was studied for its efficacy as biocontrol agent against *Botrytis cinerea*, *Penicillium expansum* and *Alternaria alternata* on apples stored for 8 months at 1°C. The results of two semi-commercial trials permitted to observe an interesting efficacy of the biocontrol strain MACH1. In order to understand the mechanism of action involved, the yeast strain was investigated for its competitive ability for iron against postharvest pathogens of apple. *M. pulcherrima* strain MACH1 was cultivated on PDA with different concentrations of iron (supplemented as $FeCl_3$) against *A. alternata* and *B. cinerea*. The yeast strain MACH1 produced a wider pigmented inhibition zone against both pathogens in low iron amendments while less 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

integrated pest management to reduce pesticide use. During the past 30 years, several biocontrol agents have been exploited and widely investigated against different postharvest fungal pathogens (Lima et al, 1997; Zahavi et al., 2000; Vivekananthan et al., 2004; Calvo et al., 2007). Among the different biocontrol agents, yeasts deserve particular attention, as their activity does not generally depend on the production of toxic metabolites, which could have a negative environmental or toxicological impact. Recently, *Metschnikowia pulcherrima* has been reported as an effective biocontrol agent against postharvest decay of apple, table grape, grapefruit and cherry tomato (Schena et al., 2000; Janisiewicz et al., 2001; Spadaro et al., 2002) as well as against some food-borne pathogens (Leverentz et al., 2006).

The strains of the yeast species mostly investigated generally act by consuming nutrients present on fruit and vegetable skins that allow rot-causing fungi to develop (Piano et al., 1997; Janisiewicz et al., 2001). A good understanding of the mode of action is essential before developing appropriate commercial formulations and application methods. Several possible biocontrol mechanisms have been suggested to be effective against postharvest rots on fruit including competition for nutrients and space, antibiosis, parasitism or direct interaction with the pathogens and induction of resistance in the host tissue (Smilanick, 1994). Competition for nutrients and space is considered to be a primary mode of action against postharvest fungal pathogens. In particular, competition for iron is believed to play a significant role in the biocontrol interactions (Raaska and Mattila-Sandholm, 1995). *M. pulcherrima* was found to produce a red pigment, pulcherrimin, that accumulates in the cells and is also secreted around the colonies (Miller and Phaff, 1998; Kurtzman and Droby, 2001).

The aim of this research was to assess the efficacy of a new strain of *M. pulcherrima* against *B. cinerea*, *P. expansum* and *A. alternata* on apples in semi-commercial conditions. A second objective was to study the competitive ability of *M. pulcherrima* strain MACH1 for iron sequestration against postharvest pathogens of apples.

2. Materials and methods

100 **2.1 Antagonist and pathogens preparation**

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

105 Yeast strain MACH1 was grown and cells were prepared as described in Spadaro et al. (2002). Two
106 strains each of *Alternaria alternata*, *Botrytis cinerea* and *Penicillium expansum* were isolated from
107 rotted apples and selected for their virulence by inoculation in artificially wounded apples. They
108 were used as a mixture throughout this work, to ensure high level of disease and the presence of rots
109 caused by multiple pathogens. Each strain was stored in slant on Potato Dextrose Agar (PDA;
110 Merck) with 50 mg l^{-1} of streptomycin Merck at 4°C. Spore suspensions were prepared by growing
111 the pathogens on Petri dishes for two weeks on PDA added with 50 mg l^{-1} of streptomycin. After
112 two weeks of incubation at 25°C, spores from the two strains of each pathogen species were
113 collected and suspended in sterile Ringer's solution (Merck, Darmstadt, Germany). After filtering
114 through 8 layers of sterile cheese-cloth, spores were counted and brought to a final concentration of
115 10^5 spores ml^{-1} per each pathogen.

116

117 **2.2 Efficacy trials in semi-commercial conditions**

118 Two experimental trials were carried out in Aosta (Aosta Valley, Northern Italy) in cooperation
119 with the Institut Agricole Regional on artificially infected apples of the cv Golden delicious. Apples
120 were harvested in orchards conducted by following integrated pest management. Four boxes were
121 used for each treatment (100 fruits per box). Ten apples per box, to reproduce the most probable
122 conditions after harvesting, were artificially wounded at the equatorial region (3 mm diameter; 6
123 mm depth; 3 wounds per fruit). All fruits were artificially inoculated by dipping for 60 seconds in
124 100 l tanks containing a conidial suspension (10^5 spores ml^{-1} per pathogen) of *B. cinerea*, *P.*
125 *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 (10^7cells 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 ($3x10^5$ 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 ($3x10^5$ 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; $2x10^8$ 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 ($2x10^5$ spores ml^{-1})
174 of *B. cinerea, A. alternata* and *P. expansum* were inoculated into wounds and apples were incubated
175 at 25ºC. 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

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.

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

parameters (11.5 mm, 3.0 g) in apples treated with the strain of *M. pulcherrima* and 5 μg ml^{-1} $FeCl_3$. This was comparable with that of chemical fungicide. Apples inoculated with *B. cinerea* alone recorded the highest rotten area (71.3 mm) and rotten fruit weight (54.0 g) (Fig. 3).

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

In the experiments against *A. alternata* in artificial wounds (Fig. 4), 11.3% infection was observed in *M. pulcherrima* strain MACH1 treatment compared to 34.3% in untreated control. Interestingly, *M. pulcherrima* strain MACH1 supplemented with the lowest concentrations (5, 10 μg ml^{-1} of $FeCl_3$) of iron reduced more the number of rotten fruits compared to the highest concentrations (20, 30 μg ml^{-1} of $FeCl_3$) of iron amendment. Similarly, the lesion diameter (16.0 mm) and the rotten fruit weight (5.9 g) provided by *M. pulcherrima* strain MACH1+5 μg ml^{-1} iron were significantly lower than the other treatments with increased iron amendments and the untreated control (lesion diameter: 52.7 mm; rotten fruit weight: 27.5 g).

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

The results of the *in vivo* experiments against *P. expansum* (Fig. 5) reproduced the same situation of the *in vitro* studies and *M. pulcherrima* strain MACH1 alone was not very effective in reducing *P. expansum* infections in apple fruits. On the other hand, *M. pulcherrima* strain MACH1 amended with 5 μg ml^{-1} $FeCl_3$ significantly increased the efficacy of the strain MACH1 compared to the other concentrations of iron. Also the diameter of the rotten area (45.5 mm) and the rotten fruit weight (23.9 g) were lower in treatment supplemented with 5 μg ml^{-1} $FeCl_3$, while high concentrations of iron showed lower effectiveness compared to the control. Among all the treatments, untreated control showed the highest infection rate by *P. expansum*.

4. Discussion

In the present study, *M. pulcherrima* strain MACH1, recently isolated from the carposphere of apple cv Golden delicious and previously selected for its efficacy under controlled conditions, was partially effective in controlling postharvest diseases caused by *B. cinerea, A. alternata* and *P. expansum* under semi-commercial conditions. The experiments in semi-commercial conditions were carried out to test the efficacy of the biocontrol agent under cold storage and controlled atmosphere used by the packinghouses. Actually, some biocontrol strains perform well under controlled conditions in laboratory, where normally the fruits used have the same level of maturity, but may not do the same under controlled atmosphere cold storage in the packinghouses, where it is not possible to control the physiological conditions of the fruits tested. Zheng et al. (2005) reported that the efficacy of the biocontrol strains at room temperature and under cold storage conditions was determined by the nutritional environment at the wound site which may favour or not the growth and the colonization.

The only postharvest fungicide admitted in Italy and in many other countries is the benzimidazole thiabendazole, whose efficacy is really poor due to the development of resistant populations of *P. expansum* and *B. cinerea* (Baraldi et al., 2003; Bertetti et al., 2003). Also in our study, thiabendazole was not working in the reduction of both postharvest pathogens on apple.

After testing the efficacy, we tried to elucidate the mechanism of action deployed by *M. pulcherrima* strain MACH1 in the control of postharvest pathogens on apples. As reported by Boekhout and Robert (2002), *M. pulcherrima* occurs naturally on fruits, buds and floral parts of certain apple trees, is able to ferment glucose, assimilate various carbon compounds and produce the pigment pulcherrimin (Miller and Phaff, 1998). Previous studies demonstrated the production of pulcherrimin formed non enzymatically from pulcherriminic acid and ferric ions (Cook and Slater, 1956; MacDonald, 1965). Iron is essential for the fungal growth and pathogenesis, and iron sequestration by non-pathogenic microbes could be exploited in novel systems for biological 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.

In the study of iron competition in artificial wounds on apple, a reduced disease incidence by *B. cinerea, A. alternata* and *P. expansum* was observed in apples treated with the strain MACH1 when supplemented with lower concentrations of iron. The external supplementation of high concentrations of iron reduced the efficacy of the biocontrol yeast compared to non-iron and low iron concentrations. Our findings are similar to those of He et al. (2003), who supposed the involvement of iron in the mode of action of antagonistic yeasts against postharvest pathogens of apple. The same authors reported that the biocontrol activity of the microbial antagonists was concentration dependent and reversed by the addition of exogenous nutrients.

Some precursors from *M. pulcherrima* move further from the yeast colony before they are immobilized by iron, resulting in a increased efficacy of biocontrol. At higher concentrations of iron, the infection rate was higher because the precursor molecules did not diffuse far, because they bound sufficient amount of iron closer to the yeast cells, and the pathogens had an increased availability of free ferric irons for their growth. The pulcherrimic acid-ferric ion complex formed near to the yeast cells depletes the iron in the substrate and creates an environment unsuitable for fungal microbes requiring the micronutrients for their growth (Sipiczki, 2006; Sanson et al., 2005). Moreover, the increased efficacy by *M. pulcherrima* strain MACH1 under low iron conditions indicated the movement of precursors farther from the yeast to find sufficient amount of iron in the substrate.

The higher reduction in the incidence of rotten fruits caused by *B. cinerea*, *A. alternata* and *P. expansum*, when apples were treated with MACH1+5 $\mu g\ ml^{-1}$, indicated that small amounts of iron could elicit the production of pulcherrimic acid that depleted iron necessary for the pathogen growth.

From the above-mentioned results, this study clearly demonstrated that iron depletion from the substrate, used by *M. pulcherrima* strain MACH1 for the production of pulcherrimin, ultimately inhibited the growth of postharvest pathogens.

The measurement of the iron content in the fruits could become an interesting parameter to establish in advance the potential control of postharvest pathogens on fruits, before their application, and could possibly be used to establish if a low iron supplementation is needed to guarantee an effective level of pathogen control during postharvest.

Acknowledgements

The authors gratefully acknowledge Dr. Sandro Frati for helping in the experimental trials and Dr. Jeanne Griffin for the linguistic advice.

Duraisamy Saravanakumar acknowledges the Italian Ministry for University and Research for the PostDoc grant.

References

Baraldi, E., Mari, M., Chierici, E., Pondrelli, M., Bertolini, P., Pratella G., 2003. Studies on thiabendazole resistance of *Penicillium expansum* of pears: pathogenic fitness and genetic characterization. Plant Pathol. 52(3), 362-370.

Bertetti, D., Keji, S., Garibaldi, A., Gullino, M.L., 2003. Valutazione della sensibilità di alcuni agenti di marciume su pomacee in post-raccolta nei confronti di diversi fungicidi. Inf.tore fitopat. 53 (6), 57-60.

Boekhout, T., Robert, V., 2002. Yeasts in Food – Beneficial and Detrimental Aspects. Hamburg. Behr's Verlag.

Calvente, V., Benuzzi, D., de Tosetti, M.I.S., 1999. Antagonistic action of siderophores from *Rhodotorula glutinis* upon the postharvest pathogen *Penicillium expansum*. Int. Biodeterior. Biodegrad. 43, 167-172.

Calvo, J., Calvente, V., de Orellano, M.E., Benuzzi, D., Tosetti, M.I.S., 2007. Biological control of postharvest spoilage caused by *Penicillium expansum* and *Botrytis cinerea* in apple by using the bacterium *Rahnella aquatilis*. Int. J. Food Microbiol. 113, 251-257.

Charlang, G., Bradford, N., Horowitz, N., Horowitz, R. 1981. Cellular and extracellular siderophores of *Aspergillus nidulans* and *Penicillium chrysogenum*. Mol. Cellular Biol. 1:94-100.

Cook, A. H., Slater, C. A., 1956. The structure of pulcherrimin. J. Chem. Soc. 4133-4135.

Diouf, P. N., Delbarre N., Perrin D., Gerardin P., Rapin C., Jacquot J.P., Gelhaye, E., 2002. Influence of tropolone on *Poria placenta* wood degradation. Appl. Environ. Microbiol. 68, 4377-4382.

Eckert, J.W., Ogawa, J.M., 1985. The chemical control of postharvest diseases: subtropical and tropical fruits. Annu. Rev. Phytopathol. 23, 421-454.

He, D., Zheng, X., Yin, Y., Sun, P., Zhang, H., 2003. Yeast application for controlling apple postharvest diseases associated with *Penicillium expansum*. Bot. Bull. Acad. Sin. 44, 211-216.

Ippolito, A., Schena L., Pentimone I., Nigro, F., 2005. Control of postharvest rots of sweet cherries by pre and postharvest applications of *Aureobasidium pullulans* in combination with calcium chloride or sodium bicarbonate. Postharvest Biol. Technol. 36, 245-252.

Janisiewicz, W.J., Tworkoski, T.J., Kurtzman, C.P., 2001. Biocontrol potential of *Metchnikowia pulcherrima* strains against blue mold of apple. Phytopathology 91, 1098-1108.

Janisiewicz W. J., Korsten, L., 2002. Biological control of postharvest diseases of fruits. Annu. Rev. Phytopathol. 40, 411-441.

Leverentz, B., Conway, W.S., Janisiewicz, W., Abadias, M., Kurtzman, C.P., Camp, M.J., 2006. Biocontrol of the food-borne pathogens *Listeria monocytogenes* and *Salmonella enterica* serovar Poona on fresh-cut apples with naturally occurring bacterial and yeast antagonists. Appl. Environ. Microbiol. 72, 1135-1140.

Kurtzman, C. P., Droby, S., 2001. *Metschnikowia fructicola*, a new ascosporic yeast with potential for biocontrol of postharvest fruit rots. Syst. Appl. Microbiol. 24, 395–399.

406 Lima, G., Ippolito, A., Nigro, F., Salerno, M., 1997. Effectiveness of *Aureobasidium pullulans* and
407 *Candida oleophila* against postharvest strawberry rots. Postharvest Biol. Technol. 10, 169-
408 178.
409 MacDonald, J. C., 1965. Biosynthesis of pulcherriminic acid. Biochem. J. 96, 533-538.
410 Miller, M.W., Phaff, H.J., 1998. *Metschnikowia* Kamienski. In: Kurtzman, C.P., Fell, J.P. (Eds.),
411 The Yeasts. Elsevier, Amsterdam, pp. 256-267.
412 Piano, S., Neyrotti, V., Migheli, Q., Gullino, M.L., 1997. Characterization of the biocontrol
413 capability of *Metschnikowia pulcherrima* against *Botrytis* postharvest rot of apple.
414 Postharvest Biol. Technol. 11, 131-140.
415 Raaska, L., Mattila-Sandholm, T., 1995. Effects of iron level on the antagonistic action of
416 siderophores from non-pathogenic *Staphylococcus* spp. J. Ind. Microbiol. Biotechnol. 15,
417 480-485.
418 Ragsdale, N.N., 2000. The Impact of the Food Quality Protection Act on the Future of Plant
419 Disease Management. Annu. Rev. Phytopathol. 38, 577-596.
420 Sanson, G., Rezza, I., Calvente, V., Benuzzi, D., de Tosetti, M.I.S. 2005. Control of *Botrytis*
421 *cinerea* strains resistant to iprodione in apple with rhodotorulic acid and yeasts. Postharvest
422 Biol. Technol. 35, 245-251.
423 Schena, L., Ippolito, A., Zahavi, T., Cohen, L., Droby, S., 2000. Molecular approaches to assist the
424 screening and monitoring of postharvest biological yeasts. Eur. J. Plant Pathol. 106, 681-
425 691.
426 Sipiczki, M., 2006. *Metschnikowia* strains isolated from botrytized grapes antagonize fungal and
427 bacterial growth by iron depletion. Appl. Environ. Microbiol. 72, 6716-6724.
428 Smilanick, J.L., 1994. Strategies for the isolation and testing of biocontrol agents. In: Wilson, C.L.,
429 Wisniewski, M.E. (Eds.), Biological Control of Postharvest Diseases - Theory and Practice.
430 CRC Press, Boca Raton, pp. 25-42.

Snowdon, A.L., 2003. A Colour Atlas of Postharvest Diseases and Disorders of Fruits and Vegetables - Volume One: Fruits. Manson Publishing, London.

Spadaro, D., Vola, R., Piano, S., Gullino, M.L., 2002. Mechanisms of action, efficacy and possibility of integration with chemicals of four isolates of the yeast *Metschnikowia pulcherrima* active against postharvest pathogens on apples. Postharvest Biol. Technol. 24, 123-134.

Spadaro, D., Gullino, M.L., 2004. State of art and future perspectives of biological control of postharvest fruit diseases. Int. J. Food Microbiol. 91, 185-194.

Spotts, R.A., Cervantes, L.A., 1986. Population, pathogenicity, and benomyl resistance of *Botrytis* spp., *Penicillium* spp., and *Mucor piriformis* in packinghouses. Plant Dis. 70, 106-108.

Vivekananthan, R., Ravi, M., Saravanakumar, D., Kumar, N., Prakasam, V., Samiyappan., R., 2004. Microbially induced defense related proteins against post harvest anthracnose infection in mango. Crop Protection 23, 1061-1067.

Wilson, C.L., Wisniewski, M.E. (Eds.), 1994. Biological Control of Postharvest Diseases. Theory and Practice. CRC Press, Boca Raton.

Zhang, H., Zheng, X., Yu, T. 2007. Biological control of postharvest diseases of peach with *Cryptococcus laurentii*. Food control 18, 287-291.

Zheng, X.D., Zhanga, H.Y., Sun, P. 2005. Biological control of postharvest green mold decay of oranges by *Rhodotorula glutinins*. Eur. Food Res. Technol. 220, 353-357.

Zahavi, T., Cohen, L., Weiss, B., Schena, L., Daus, A., Kaplunov, Tl, Zutkhi, J., Ben-Arie, R., Droby, S., 2000. Biological control of *Botrytis*, *Aspergillus* and *Rhizopus* rots on table and wine grapes in Israel. Postharvest Biol., Technol. 20, 115-124.

Legends:

Figure 1.

Fig. 1a. A pigmented inhibition zones produced by strain MACH1 in PDA with different concentrations of iron against *B. cinerea*

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

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

T - Tropolone inhibition zone 5 mm; ** sporulation by *B. cinerea* at the point of entry onto inhibition zone; Bc - *B. cinerea*; E - stimulated growth of *B. cinerea* in the tropolone diffused zone.

Figure 2.

An observation on *B. cinerea* mycelial distruption at pigmented inhibition zone produced by MACH1

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

Figure 3.

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

Figure 4.

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

480

481 **Figure 5.**

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

483 infection *in vivo*

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

485 are not significantly different from each other