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Abstract

During the growing seasons 2007 and 2008, 210 isolates of yeasts or yeast-like fungi were obtained from the carposphere of temperate fruit collected from organic orchards in Northern Italy. Through six rounds of *in vivo* screening, three isolates showing the highest biocontrol efficacy against *Monilinia laxa* on peaches were selected. By using molecular and morphological tools, the strain AP6 was identified as *Pseudozyma fusiformata*, the strain AP47 as *Metschnikowia* sp., and the strain PL5 as *Aureobasidium pullulans*. This research represents the first evidence about the potential use of *P. fusiformata* to control postharvest diseases of fruit. By co-culturing *in vitro M. laxa* in the presence of the three antagonists, neither the inactivated cells nor the culture filtrate of the three isolates had any significant effect on spore germination or germ tube elongation, permitting to exclude the production of secreted toxic metabolites. The antagonistic activity of *A.*

pullulans PL5 and *P. fusiformata* AP6 was dependent on the cell concentration. *Metschnikowia* sp. AP47 significantly inhibited the spore germination at the three concentrations tested (10^6, 10^7, and 10^8 cells/mL). The efficacy of the three strains was tested on peaches stored at three different temperatures, and their effectiveness was higher at 1°C than at 8°C or 20°C. In trials carried out in semi-commercial conditions with peaches inoculated by spraying 10^5 spores/mL of *M. laxa* and stored for 21 days at 1°C and 96 % RH, a cell concentration effect on the control of brown rot incidence was observed. In such experiment, AP6 and PL5 showed no significant differences in the efficacy when applied at 1×10^8 cells/mL or at 1×10^7 cells/mL, indicating that they could be used at a lower concentration in potential biofungicide formulations. Finally, in an experiment in semi-commercial conditions on fruits not inoculated with the pathogen with 21 days storage at 1°C and 96 % RH, the evaluation of postharvest quality parameters, including firmness, total soluble solids, ascorbic acid content, and titratable acidity, showed that no one of the three screened antagonists impaired peach quality, when applied before storage. The present study permitted to obtain three antagonistic microorganisms with potential exploitation as active ingredients for the development of products for postharvest control of brown rot on peaches.

Key words: *Aureobasidium pullulans*; biological control; *Metschnikowia* sp.; *Monilinia laxa*; *Pseudozyma fusiformata.*

1. Introduction

Brown rot caused by *Monilinia* spp. is the most important postharvest disease of commercially grown stone fruit (Byrde and Willetts, 1977). In European countries brown rot of peaches is caused by two fungi, *M. laxa* and *M. fructigena* (De Cal and Melgarejo, 1999). The most common species isolated from rotten peaches and nectarines in Europe, South Africa, Chile, and Iraq is *M. laxa* (Tian and Bertolini, 1999; Larena et al., 2005). *M. fructicola* is commonly present in Asia, North America, and Australia and it is a quarantined pathogen in Europe, but in 2008 its presence was detected in

Italian orchards (Pellegrino et al., 2009).

The use of synthetic fungicides in preharvest represents the primary method to control post-harvest diseases on stone fruit (Eckert and Ogawa, 1988): a fungicide application is recommended during the bloom and pre-harvest phases if conditions are favourable to disease development and cultivars are susceptible to *Monilinia* spp.. Postharvest treatments are not performed. Control programs are often inefficient and significant levels of brown rot may occur during storage, transport, and marketing. Consumers are demanding less pesticide residues in foodstuffs (Spadaro and Gullino, 2004) and many fungi are developing resistance to the commonly used fungicides (Spotts and Cervantes, 1986; Lima et al., 2006). Moreover, 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 has emerged as one of the most promising alternatives, either alone or as part of an integrated pest management to reduce pesticide use (Janisiewicz and Korsten, 2002). Some filamentous fungi (Melgarejo et al., 1986; Hong et al., 1998), yeasts (Spotts et al., 2002; Karabulut and Baykal, 2003; Fiori et al., 2008), and bacteria (Pusey and Wilson, 1984; Smilanick et al., 1993; Bonaterra et al., 2003) have been identified as postharvest biocontrol agents of brown rot on stone fruit. Previous research focused in particular on the orchard application of filamentous fungi, such as *Epicoccum nigrum* or *Penicillium frequentans* (Larena et al., 2005; Guijarro et al., 2006; De Cal et al., 2009), but few studies were related to the potential postharvest use of yeast of yeast-like fungi. Despite a flourishing research on postharvest biocontrol, few biofungicides are available on the market, and none of them is effective against brown rot on stone fruit.

Therefore, more efforts are needed to screen and develop effective microbial antagonists against *Monilinia* spp. on stone fruit. Among the antagonistic microorganisms, yeasts and yeast-like microorganisms 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

100 (Spadaro and Gullino, 2004). Moreover, yeasts do not produce allergic spores or mycotoxins as
101 many filamentous fungi do (Fan and Tian, 2000). Finally yeasts are easy to be cultivated with
102 simple nutritional requests and are easy to be produced on a large scale (Droby and Chalutz, 1994).
103 The aim of the present research was the isolation of effective biocontrol agents against brown rot
104 caused by *M. laxa* on stone fruit. Three potential biocontrol agents were isolated and identified as
105 *Pseudozyma fusiformata*, *Metschnikowia* sp., and *Aureobasidium pullulans*. A second goal was the
106 assessment of the capability of the three microorganisms to control *M. laxa in vitro* and *in vivo*,
107 under controlled and semi-commercial conditions. Moreover, we wanted to evaluate the effect of
108 the temperature of storage and of the antagonist concentration on the biocontrol efficacy. Finally,
109 the effect of the biocontrol agent application on the fruit quality was assessed.
110
111 **2. Materials and Methods**
112
113 **2.1 Microorganisms and fruit**
114 Five strains of *Monilinia laxa* (Aderhold & Ruhland) Honey were isolated from rotted peaches and
115 selected for their virulence by inoculation in artificially wounded apples. They were used as a
116 mixture (each strain accounted for 1/5 of the total final concentrations) throughout this work, to
117 ensure a high level of disease. Each strain was stored in slant on Potato Dextrose Agar (39 g/L;
118 PDA; Merck, Darmstadt, Germany) with 50 mg/L of streptomycin (Merck) at 4°C. Spore
119 suspensions were prepared by growing the isolates on Petri dishes at 25°C for 7 days on Peach Agar
120 [PA; 500 mL/L peach juice + 20 g/L agar (Merck); pH 7.0] medium. *M. laxa* spores were collected
121 and suspended in sterile Ringer solution (pH 6.9±0.1; Merck). After filtering through 8 layers of
122 sterile cheese-cloth, spores were quantified with a Bürker chamber and brought to a final
123 concentration of 10^5 spores/mL.
124 The isolated antagonists were grown on yeast peptone dextrose [YPD: 10 g/L of granulated yeast
125 (Merck); 20 g/L of tryptone-peptone of casein (Difco, Detroit, United States); 20 g/L of D(+)-

126 glucose monohydrate (Merck)]. Inocula of the antagonists for all the experiments were prepared by
127 subculturing in YPD and incubating on a rotary shaker (200 rpm) at 25°C for 48 h. Antagonist cells
128 were collected by centrifugation at 5000 × *g* for 10 min, washed, resuspended in sterilized Ringer
129 solution, quantified with a Bürker chamber and brought to a standard concentration of 10^8 cells/mL,
130 unless otherwise stated.
131 Fruits used throughout the biocontrol experiments were peaches [*Prunus persica* (L.) Batsch] cv.
132 Redhaven harvested at commercial maturity. They were disinfected in sodium hypochlorite (NaClO,
133 1.0 % as chlorine), rinsed under tap water, and when dry punctured with a sterile needle at the
134 equatorial region (3 mm depth; 3 wounds/fruit).
135
136 **2.2 Antagonist isolation and screening against *M. laxa***
137 Epiphytic microorganisms were isolated during the growing seasons 2007 and 2008 from fruit
138 (apples, pears, peaches, plums, and apricots) collected from different organic orchards in Piedmont
139 (Northern Italy) according to Wilson et al. (1993) with slight modifications. Fruits were put into
140 beakers containing sterile Ringer solution and left on a rotary shaker (150 rpm) for 10 min. The
141 suspension was diluted in serial with Ringer solution and the diluted suspensions were transferred
142 in Petri dishes containing PDA with 50 mg/L of streptomycin sulphate (Merck; PDA+S). Petri
143 dishes were incubated at 25°C for 48 hours. After morphological selection under light microscope,
144 single colonies of yeasts and yeast-like fungi with different characteristics were picked up and
145 streaked onto NYDA (nutrient yeast dextrose agar; Droby et al., 1989).
146 To select potential biocontrol agents, the isolates were tested directly on wounded peaches
147 according to Lima et al. (1999). Aliquots of 30 µL of antagonist suspension (1×10^8 cells/mL) were
148 pipetted into each wound site. After 2 hours of incubation at room temperature, the wounds were
149 inoculated with 30 µL of *M. laxa* suspension at the concentration of 10^5 spores/mL. The control
150 fruits (water+pathogen) were rinsed with 30 µl distilled water, before pathogen inoculation. A
151 chemical control, treated with 2.5 mL/L of Folicur (Bayer Crop Science, Monheim, Germany;

tebuconazole: 25.0 %) was included. When dry, peaches from different treatments were randomly packed in commercial plastic trays and stored at 20°C for 5 days, when the diameters of the rotten lesions were measured. Three replicates of five peaches (15 fruit; 45 inoculation sites) were used for each treatment. Experiments were repeated six times.

2.3 Molecular and morphological identification

The three antagonists, selected for their efficacy, were identified by sequencing the internal transcribed spacer 1 (ITS1), 5.8S ribosomal RNA gene, and internal transcribed spacer 2 (ITS2) according to White et al. (1990) and the D1/D2 domain at the 5' end of the LSU rRNA gene according to Kurtzman and Robnett (1998). The DNA, coming from antagonist cell suspensions grown in YPD for 48 h, was extracted using NucleoMag 96 Plant Kit (Macherey Nagel, Oensingen, Switzerland) and Kingfisher magnetic particle processor (Thermo Labsystems, Basingstoke, United Kingdom) following the manufacturers' protocols. The ITS regions were amplified using genomic DNA as a template and universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The D1/D2 domains were amplified using the primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') on the genomic DNA. PCRs were performed using a TGradient thermal cycler (Biometra, Göttingen, Germany). Each 20 µL PCR contained 1 µL of DNA template (50 ng), 200 mM of each deoxynucleotide triphosphate, 2 µL of 10 X buffer (Taq DNA Polymerase, Qiagen, Chatsworth, CA, USA), 0.7 mM each primer, and 1.0 U Taq DNA Polymerase (Qiagen). PCR program for ITS regions followed: 95°C, 3 min; 34 cycles: 94°C, 15 s; 55°C, 45 s; 72°C, 55 s; 72°C, 7 min; 4°C. The program for D1/D2 domain was: 95°C, 10 min; 30 cycles: 94°C, 30 s; 55°C 30 s; 72°C, 45 s; 72°C, 7 min; 4°C. A 10 µL aliquot of PCR products from each reaction was electrophoresed in 2.0 % agarose gel in TBE buffer and then stained with SYBR SAFE (Invitrogen, Eugene, OR, USA). Gel images were acquired with a Gel Doc 1000 System (Bio-Rad Laboratories, Hercules, CA, USA). PCR amplification products were cloned into the PCR4 TOPO vector (Invitrogen) using

the TOPO TA cloning kit following the manufacturer protocol and sequenced by BMR Genomics (Padova, Italy) using an ABI PRISM 3730XL DNA Sequencer (AME Bioscience, Sharnbrook, United Kingdom). The sequences were analyzed by using the software BLASTn (Basic Local Alignment Search Tool; Altschul et al., 1990) for similarity. The microscopical observation of the cell and colony morphology was complementary to the molecular analysis.

2.4 Effect of antagonists on *M. laxa* spore germination *in vitro*

The effect of the three antagonists *P. fusiformata* AP6, *Metschnikowia* sp. AP47, and *A. pullulans* PL5 on *M. laxa* spores germination was assessed in PDB (Potato Dextrose Broth, Merck), as reported by Spadaro et al. (2002). Antagonist cells, grown at 25°C for 48 h in 300 mL YPD, were harvested by centrifugation and resuspended in sterile Ringer solution. The remaining cultural medium was filtered through a 22 µm nitrocellulose filter (Millipore, Billerica, MA, United States) for further use. Living cells of each antagonist (100 µL of a suspension containing 5×10^7, 5×10^8, or 5×10^9 cells/mL) or cells (100 µl of a suspension containing 5×10^9 cells ml^{-1}) inactivated by irradiation for 30' with a germicidal lamp (General Electric, G15T8) that emitted predominantly UV light of a wavelength of 254 nm at fluence of 1.5 W/m^2 posed at 5 cm from the cell suspension layer (2 mm thick), were added to tubes containing 4.8 mL PDB. The final living cell concentrations were 1×10^6 cells/mL, 1×10^7 cells/mL, and 1×10^8 cells/mL, respectively. For the culture filtrate treatment, 100 µL of culture filtrate were added to 4.8 mL PDB. Aliquots (100 µL) of *M. laxa* spore suspension (5×10^6 spores/mL) in Ringer solution were transferred to each tube. As a control (PDB+pathogen), 100 µL of *M. laxa* spore suspension were added to tubes containing 4.9 mL PDB. After 20 h incubation of the 45° sloping tubes at 25 °C on a rotary shaker (200 rpm), 100 spores/replicate were observed microscopically and their germination rate and germ tube length were measured. Three replications of three tubes were prepared for each treatment and the experiment was repeated twice.

204 **2.5 Effect of storage temperature on biocontrol efficacy**

205 To determinate the effect of storage temperatures on biocontrol efficacy, artificially wounded
206 peaches were treated with 30 µL of 1×10^8 cells/mL of each antagonist. After 2h of air drying, 30µL
207 of *M. laxa* (10^5 spores/mL) were inoculated as described above. Control fruits (water+pathogen)
208 were inoculated only with the pathogen spore suspension. Treated peaches were stored at 1°C, 8°C,
209 and 20°C, respectively for 21, 14, and 7 days. The severity was evaluated by measuring the
210 diameter of the brown rot lesions. Three replicates of 20 peaches (60 fruit; 180 inoculation sites)
211 were used for each treatment. The experiment was repeated twice.

212

213 **2.6 Effect of antagonist concentration on biocontrol efficacy under semi-commercial**
214 **conditions**

215 In order to evaluate the effect of the antagonist concentration on the biocontrol of *M. laxa*,
216 unwounded peaches were treated with three concentrations (1×10^6, 1×10^7, 1×10^8 cells/mL) of each
217 antagonist, by dipping in 100 L tanks for 60 s. After 2 h air drying, a suspension of *M. laxa* (10^5
218 spores/mL) was sprayed onto the surface of each peach. Peaches inoculated with the *M. laxa* spore
219 suspension acted as control (water+pathogen) and peaches treated with tebuconazole (2.5 mL/L of
220 Folicur, Bayer Crop Science; 25.0 % a. i.) performed as chemical control. After 2 h of air drying,
221 fruits were stored in boxes at 1°C and 96 % relative humidity (RH) in the dark. Fruits were kept in
222 cold chambers for 21 days before the disease incidence (number of rotten fruits) was measured.
223 Three replicates of 60 peaches were used for each treatment. The experiment was repeated twice.

224

225 **2.7 Biocontrol efficacy and evaluation of fruit quality parameters under semi-commercial**
226 **conditions**

227 Experimental trials under semi-commercial conditions were carried out to evaluate the effect of
228 antagonist application on biocontrol and fruit quality parameters. Peaches were treated with 1×10^8
229 cells/mL of each antagonist, by dipping in 100 L tanks for 60 s. Chemical control was represented

by fruit treated with a suspension containing 250 mL/100 L of Folicur (Bayer Crop Science; 25.0 % a. i.). Peaches treated only with tap water were included as uninoculated control. After 2 h air drying, the fruits were stored at 1°C and 96 % RH for 21 days. The brown rot incidence and the diameter of rotten lesions were measured.

Moreover, some quality parameters were assessed, once discarded the rotten peaches, on the healthy fruit of every treatment. Firmness was measured for each fruit at two opposite sites along the equatorial region with a FT327 - Fruit Pressure Tester having an 11 mm probe (EFFEGI, Alfonsine, Italy). The probe descended toward the sample at 1.0 mm/s and the maximum force (*N*) was defined as firmness. Total soluble solids (TSS) were determined by measuring the refractive index of the pressed juice (Larrigaudière et al., 2002) with a digital refractometer (DBR95, Singapore) and the results were expressed as percentages (g/100 g fruit weight). The 2,6-dichloroindophenol titrimetric method (AOAC, 1995) was employed to determine the ascorbic acid content of pressed peach juice. Results were reported as mg ascorbic acid/100 g sample. Acidity was measured by titration with 0.1 N NaOH to pH 8.0: 5 mL of pressed juice diluted with 5 mL of distilled water were evaluated. Titratable acidity was calculated as percent malic acid (Wright and Kader, 1997).

Three replicates of 60 peaches were used for each treatment. The experiment was repeated twice.

2.8 Statistical analysis

Replications of all the experiments, when the means were similar, were pooled and analyzed together. Means and standard errors for each treatment were reported. Data analysis was performed by using the SPSS software (SPSS Inc., version 17.0, Chicago, IL, USA). Statistical significance was judged at the level of p-value < 0.05. When the analysis of variance was statistically significant, Tukey's test was used to compare the means.

3. Results

3.1 Antagonist isolation and screening against *M. laxa*

During the growing seasons 2007 and 2008, 210 isolates of yeasts or yeast-like fungi were obtained from apples, pears, peaches, plums, and apricots collected from organic orchards in Piedmont (Northern Italy). They were isolated on PDA containing streptomycin in order to avoid the bacterial isolates. After morphological selection under light microscope, only colonies of yeasts and yeast-like microorganisms were kept. The isolates were morphologically classified into four groups: 43 showing pink or red colonies; 80 with milky-white colonies; 46 with light-white colonies; and 41 showing butyrous colonies. By microscope observation of the cell shape, the isolates were arranged into three groups: 104 isolates with ovoid cells; 57 with spherical cells; and 49 with shuttle-like cells.

The selection process was carried out directly *in vivo* by treating wounds of peaches with cell suspensions of the isolates, inoculating after 2 hours with *M. laxa*, and storing the fruit at 20°C for 5 days. Through six rounds of screening, three isolates showing the highest biocontrol effectiveness in reducing the severity of brown rot caused by *M. laxa* were selected for continuing the studies (Table 1). Fruits treated with AP6, isolated from apple cv. Golden delicious, showed a brown rot diameter that was 31.4 % compared to the control (water+pathogen). The brown rot diameters on peaches treated with AP47, isolated from apple cv. Golden delicious, and with PL5, isolated from plums cv. Angeleno, were, respectively, 35.7 % and 44.4 % lower with respect to control. The chemical control, i.e. fruit treated with tebuconazole, showed the lowest severity of brown rot lesions caused by *M. laxa*. However, no significant difference was observed in the percentage of infected fruits between antagonist treatments and the control (water+pathogen) when fruits were stored at 20°C for 5 days (Table 1).

3.2 Molecular and morphological identification

The three strains selected for their biocontrol effectiveness against *M. laxa* were identified by sequencing the ribosomal regions ITS1-5.8S-ITS2 with universal primers ITS-1 and ITS-4 and

sequencing the D1/D2 domain with the primers NL-1 and NL-4. The sequences of the amplified regions were deposited in GenBank (accession numbers and amplimer sizes are indicated in Table 2). The BLAST analysis of the ITS sequences showed that the product of strain AP6 had 99 % (630/633) identity to the sequences of *Pseudozyma fusiformata* (Buhagiar) Boekhout, the PCR product of PL5 had 100 % (479/479) identity to the sequences of *Aureobasidium pullulans* De Bary (Arnaud), and the amplicon of AP47 showed 98 % (482/490) identity with the sequences of other species of the genus *Metschnikowia* Kamienski. The analysis of the D1/D2 domains confirmed that the PCR product of AP6 had 99 % (595/598) identity to the sequences of *P. fusiformata*, the product of PL5 had 99 % (564/569) identity to the sequences of *A. pullulans*, and the amplicon of AP47 showed 98 % (491/500) identity with the sequences of other species of the genus *Metschnikowia*. The observation of the morphological (colony morphology) and microscopic (cell shape and size) characteristics of AP6, AP47, and PL5 confirmed the rDNA sequencing results.

3.3 Effect of antagonists on *M. laxa* spore germination *in vitro*

By co-culturing in liquid medium (PDB), the effect of the three antagonists (AP6, AP47, and PL5), applied as living cell suspensions, inactivated cells, or culture filtrate, on *M. laxa* spore germination and germ tube length were investigated (Table 3). *Metschnikowia* sp. AP47 and *P. fusiformata* AP6, applied at 1×10^8 cells/mL, completely inhibited the pathogen spore germination in PDB and a strong inhibition of the spore germination was observed also in the presence of 1×10^8 cells/mL of *A. pullulans* PL5 (1.3 %). When co-cultured with 1×10^7 cells/mL of AP6 or PL5, the spore germination were 2.3 % and 29.7 %, respectively. However, when 1×10^6 cells/mL of both antagonists were applied, the spore germination increased to 29.3 % and 58.0 %, respectively. On the other side, even in presence of 1×10^7 cells/mL and 1×10^6 cells/mL of *Metschnikowia* sp. AP47, the pathogen germination rate was very low (0.7 % and 1.3 %, respectively). No significant difference on the spore germination rate, compared to the control, was observed when the pathogen was co-cultured with inactivated cells and culture filtrate of the three antagonists.

Germ tube elongation of *M. laxa* in PDB was greatly reduced by the presence of the living cells of the three antagonists (Table 3). Compared with the control, the length of germ tube of *M. laxa* co-cultured with 1×10^8 cells/mL, 1×10^7 cells/mL, or 1×10^6 cells/mL of the *P. fusiformata* AP6 was inhibited by 100.0 %, 95.5 %, and 61.2 %, respectively. The length of germ tube of *M. laxa* co-cultured with 1×10^8 cells/mL, 1×10^7 cells/mL, or 1×10^6 cells/mL of *Metschnikowia* sp. AP47 was 0.0 μm, 4.7 μm, and 7.4 μm, respectively. The length of germ tube of *M. laxa* co-cultured with 1×10^8 cells/mL, 1×10^7 cells/mL, or 1×10^6 cells/mL of *A. pullulans* PL5 was reduced by 92.3 %, 64.9 %, and 54.3 %, respectively. When the pathogen was co-cultured with the inactivated cells or culture filtrate of the antagonists, no significant differences in germ tube length were observed compared to the control. In the presence of the culture filtrate of AP6, the germ tube length was even higher than the control (127.6 μm).

3.4 Effect of storage temperature on biocontrol efficacy

To determine the effects of different storage temperatures on the biocontrol efficacy against brown rot, the peaches inoculated with the pathogen and treated with antagonists were stored at 20°C for 7 days, at 8°C for 14 days, and at 4°C for 21 days. After storage at 20°C (Fig.1), the antagonists AP6, AP47, and PL5 reduced the diameter of brown rot lesions, respectively, to 13.7 mm, 24.5 mm, and 24.6 mm, compared to the control (49.4 mm). When the peaches were stored at 8°C (Fig. 1-A), the antagonists more effectively reduced the diameters of brown rots: AP6, AP47, and PL5 reduced the severity of brown rot to 8.8 mm, 10.6 mm, and 13.1 mm, respectively, compared to 54.3 mm of the control fruit. After 21 days of storage at 1°C (Fig. 1-A), AP6, AP47, and PL5 provided the best efficacy, by reducing the lesion diameters from 47.4 mm (in the inoculated fruit) to 2.2 mm, 2.1 mm, and 9.1 mm, respectively.

3.5 Effect of antagonist concentration on biocontrol efficacy under semi-commercial conditions

To confirm *in vivo* the effect of the antagonist cell concentration on the pathogen *in vitro,* peaches were treated with the three antagonists at various concentrations (Fig. 1-B). At the concentration of 1×10^8 cells/mL, the three strains provided the highest biocontrol: the disease incidence was 29.3 %, 21.3 %, and 29.3 %, by treating the fruit with AP6, AP47, and PL5, respectively. The disease incidence on the peaches treated with *Metschnikowia* sp. AP47 was significantly lower than the other two. When the three antagonists were applied at the concentration of 1×10^7 cells/mL **,** the disease incidence on the treated peaches was similar (30.7 %, 30.0 %, and 30.7 %, respectively). When the antagonists were used at the concentration of 1×10^6 cells/mL, the reduction of disease incidence was lower, although the number of rotten fruit continued to be significantly lower compared to the control (water+pathogen) (78.7 %). The lowest disease incidence was obtained by treating the fruit with tebuconazole (13.5 %).

3.6 Biocontrol efficacy and evaluation of fruit quality parameters under semi-commercial conditions

In the experimental trials carried out under semi-commercial conditions, the effect on disease incidence and severity and fruit quality parameters were evaluated. After 21 days of storage at 1°C and 96 % RH (Table 4), the disease incidence on peaches treated with the three antagonists (AP6, AP47, and PL5) at the concentration of 1×10^8 cells/mL was significantly lower (18.3 %, 16.7 %, and 20.0 %, respectively) than that on the control (55.0 %). The lesion diameters on peaches treated with the three antagonists were 35.6 mm, 39.4 mm, and 32.5 mm, respectively, lower than that measured on the control (46.0 mm). Anyway, for the fruit treated with *Metschnikowia* sp. AP47 the severity was not significantly different from the control.

Considering the fruit quality parameters, after 21 days of storage at 1°C and 96 % RH, the three antagonists had no significant effect on fruit firmness, total soluble solids, ascorbic acid, or titratable acidity, compared with the control (Table 4).

4. Discussion

Three new biocontrol agents active against *M. laxa*, agent of postharvest brown rot on peaches, have been isolated, selected and evaluated for their efficacy, showing potential for the development of at least one biofungicide for postharvest application. This research represents the first evidence about the potential use of *P. fusiformata* to control postharvest diseases of fruit and, to our knowledge, there are no other reports about *A. pullulans* against *M. laxa* on peaches. Moreover, it was the first time that the effects of *Metschnikowia* sp. on peach fruit quality was evaluated under semi-commercial conditions, providing the more reliable evidence that *Metschnikowia* sp. had the great potential to be commercialized as biocontrol agents against *M. laxa* on peach fruits. While different filamentous fungi, yeast and bacteria have been selected and studied against *Monilinia* spp. on peaches and nectarines (De Curtis et al., 1996; Ippolito et al., 2000; Karabulut et al., 2004), only a very few of them is commercially available. The main problems showed by the biocontrol agents studied are the lack of consistent results, or the production of antibiotics (Spadaro and Gullino, 2004). Yeasts and yeast-like microorganisms are promising because their activity does not generally depend on the production of toxic metabolites, which could have a negative environmental or toxicological impact.

Yeasts or yeast-like microorganisms isolated from the carposphere of pome fruit or stone fruit growing in temperate regions constituted the source to select the antagonists.

The screening was realized directly *in vivo* through six rounds of trials carried out in controlled conditions, although other researches first select the biocontrol agents *in vitro* (Janisiewicz and Korsten, 2002). *In vivo* screening was preferred because the results obtained are more reliable and transferable to the postharvest environment. It should be noticed that tebuconazole, used as chemical control in all the experiments, guaranteed an effectiveness superior to the biocontrol agents, but its use is not admitted for postharvest control of stone fruit diseases inside the European Union.

The strains selected were identified by molecular tools as *Pseudozyma fusiformata* AP6,

386 *Metschnikowia* sp. AP47, and *Aureobasidium pullulans* PL5. Initially, the increasing presence in
387 GenBank of sequences of the ribosomal regions ITS1-5.8S-ITS2 of fungal and yeast species (Cai et
388 al., 1996; James et al., 1996) induced to choose the sequence of the ITS regions as main diagnostic
389 technique. Then, to confirm the results, the D1/D2 domain sequencing was performed as a more
390 reliable and preferable tool for both ascomycetous (Kurtzman and Robnett, 1998) and
391 basidiomycetous (Fell et al. 2000) yeasts. Moreover, the sequencing of the D1/D2 domain was
392 already been used for identifying species of *Metschnikowia* (Kurtzman and Droby, 2001),
393 *Pseudozyma* (Sugita et al., 2003) and *Aureobasidium* (Sasahara and Izumori, 2005). The
394 morphological and microscopical observations confirmed the results of the molecular analysis.
395 Some strains of *P. fusiformata* showed antifungal activity due to the production of ustilagic acid, a
396 glycolipid active against different species of yeasts, yeast-like and filamentous fungi (Golubev et al.,
397 2001; Kulakovskaya et al., 2005). Recently, isolates of *P. fusiformata* have been tested as potential
398 biocontrol agents against *Podosphaera xanthii* on cucumber and *Blumeria graminis* f. sp. *tritici* on
399 wheat with scarce results (Clément-Mathieu et al., 2008). However, there are no previous reports
400 about the application of *P. fusiformata* to control postharvest diseases. This research represents the
401 first evidence about the potential use of *P. fusiformata* to control postharvest diseases of fruit, and in
402 particular *M. laxa* on peaches.
403 *Metschnikowia* species have been tested as biocontrol agents against postharvest diseases, mainly
404 *Botrytis cinerea* and *Penicillium* spp., on apple, table grape, grapefruit, and cherry tomato (De
405 Curtis et al., 1996; Schena et al., 2000; Janisiewicz et al., 2001; Spadaro et al., 2002; 2008).
406 *Metschnikowia* species normally acts against the pathogens by competing for scarce nutrients, such
407 as iron (Saravanakumar et al., 2008), or by producing hydrolases, such as chitinases, able to degrade
408 the cell wall of pathogenic fungi (Saravanakumar et al., 2009). In particular, *M. fructicola* has
409 recently been isolated from the surface of table grape berries, and effectively reduced the
410 development of postharvest rots of grapes and strawberry (Kurtzman and Droby, 2001; Karabulut et
411 al. 2003; Karabulut et al. 2004). Recently, a strain of *M. fructicola* has been applied as a post-

412 harvest treatment on peach and nectarine artificially inoculated with different pathogens including
413 *M. fructigena* (Ferrari et al., 2007), while there is a report about the use of a strain of *M.*
414 *pulcherrima* against brown rot of apricot (Grebenisan et al., 2008).
415 Different strains of *A. pullulans* showed wide efficacy against *B. cinerea, P. expansum*, and
416 *Rhizopus stolonifer* on apple, sweet cherry, grapes, and strawberry (Lima et al., 1997; Ippolito et al.,
417 2000; Schena et al., 2003; Bencheqroun et al., 2007). *A. pullulans* has been reported to act against
418 fungal pathogens through competition for nutrients (Bencheqroun et al., 2007), secretion of
419 exochitinase and β-1,3-glucanase (Castoria et al., 2001), or induction of defence responses (Ippolito
420 et al., 2000). Moreover, *in vitro* tests showed that aureobasidin A, an antifungal cyclic depsipeptide,
421 produced by *A. pullulans*, can inhibit the spore germination, germ tube elongation and hyphal
422 growth of *M. fructicola* (XiaoPing et al., 2007). Previous work reported the efficacy of *A. pullulans*
423 against *M. laxa* on sweet cherry (Schena et al., 2003) or *M. fructicola* on cherry blossom (Wittig et
424 al., 1997), but, to our knowledge, so far no strains of *A. pullulans* have been used as biocontrol
425 agent against *M. laxa* on peaches. Hong et al. (2000) were able to isolate different strains of *A.*
426 *pullulans* from the stone fruit mummies infected by *M. fructicola*. The strain PL5 was isolated from
427 plum fruit.
428 The *in vitro* studies, mainly carried out to confirm the *in vivo* results against the pathogens, were
429 also aiming at roughly elucidate the mechanism of action involved in the biocontrol. The results
430 showed that neither the inactivated cells nor the culture filtrate of the three isolates had any
431 significant effect on the germination, permitting to exclude the production of secreted toxic
432 metabolic compounds. Living cells of the antagonists are necessary to guarantee the fungal control.
433 The antagonistic activity of *A. pullulans* PL5 and *P. fusiformata* AP6 was dependent on the cell
434 concentration: when the antagonist concentrations increased, the spore germination rate of *M. laxa*
435 correspondingly decreased. These results suggest that competition for nutrients may play an
436 important role in controlling *M. laxa* by both antagonists. In a different way, *Metschnikowia* sp.
437 AP47 significantly inhibited the spore germination at the three concentrations tested. The inhibition

438 of the spore germination by AP47 did not significantly decrease with decreasing the living cell
439 concentration. Nevertheless, a cell concentration effect on the control of brown rot incidence was
440 observed in the trials carried out in semi-commercial conditions.
441 The efficacy of the three strains was tested on peaches stored at three different temperatures,
442 simulating the temperature normally used during cold storage (1°C; Snowdon, 1990), the room
443 temperature of the shelf life (20°C), and an intermediate temperature (8°C) typical of the fruit
444 handling areas of the fruit packinghouses. The storage temperature played an important role on the
445 biocontrol effectiveness of the three antagonists against brown rot decay caused by *M. laxa*. The
446 effectiveness was higher at 1°C than at 8°C, and higher at 8°C than at 20°C. Low temperatures
447 during storage and/or shipping can extend market up to 4 weeks, also thanks to the reduction of
448 losses for the higher efficacy of the biocontrol agents.
449 The experiments on the effects of the cell concentration on biocontrol showed that the efficacy of
450 the three antagonists increased when cell concentrations increased. *Metschnikowia* sp. AP47 was
451 more effective at 1×10^8 cells/mL than at 1×10^7 cells/mL. However, AP6 and PL5 showed no
452 significant differences in the efficacy when applied at 1×10^8 cells/mL or at 1×10^7 cells/mL,
453 indicating that they could be used at a lower concentration in a potential biofungicide formulation.
454 Most of the studies related to the use of microorganisms as biocontrol agents against postharvest
455 diseases focus on the efficacy, ignoring the effect of the microorganisms on the fruit quality. Not
456 impairing quality parameters of fruit is one of the characteristics of an ideal antagonist (Zhang et al.,
457 2008). In our research, the evaluation of postharvest quality parameters showed that no one of the
458 three screened antagonists reduced the peach quality, compared to the control, under cold storage
459 for 21 days.
460 In conclusion, the present study permitted to obtain three antagonistic microorganisms with
461 potential exploitation as active ingredients for the development of products for postharvest control
462 of brown rot on peaches. Future research will focus on the elucidation of the mechanisms of action
463 involved in biological control and on the adaptation of the microorganisms to the fermentation and

formulation conditions requested by the bioindustries to develop a formulated biofungicide with a potential market.

Acknowledgements

This research was funded by the projects "CIPE – Production of stone fruit in Piedmont: monitoring, prevention and control of pathogenic and mycotoxigenic fungi to guarantee food safety" and "DRUMP – Drupacee minori in Piemonte: problemi fitopatologici e difesa post-raccolta" granted by the Piedmont Region.

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630

631 **Tables**

632

633 **Table 1.** Biocontrol efficacy of the three selected antagonistic isolates (applied at 10^8 cells/mL) in
634 reducing the severity of rotten lesions caused by *M. laxa* on peaches cv. Redhaven. Fruits were
635 stored at 20°C for 5 days.

636

Treatment	Diameters of brown rot lesions (mm)** (p= 0.001)	Percentage of infected wounds (%) (p= 0.001)
AP6	17.7 ± 5.9 b	97 ± 0.9 b
AP47	20.1 ± 2.9 bc	97 ± 1.2 b
PL5	25.0 ± 4.6 c	98 ± 0.5 b
Tebuconazole*	2.7 ± 4.5 a	58 ± 2.4 a
Control (water+pathogen)	56.3 ± 3.5 d	100 ± 0.0 b

637 * Peaches were treated with 2.5 mL/L of Folicur (Bayer Crop Science; tebuconazole: 25.0 %).
638 ** The results are the mean of six independent experiments. "±" stands for standard deviation of the
639 means. Values followed by the same letter are not statistically different by Turkey's Test ($p < 0.05$).

640

641 **Table 2.** Morphological and molecular characteristics of the three antagonistic isolates used as
642 biocontrol agents.
643

Isolate	Source	Colony	Cell shape and size	GenBank accession n° (Amplimer size)		Species
				ITS	D1/D2	
AP6	Apple	Milky white	spherical (2.60-4.50 µm)	FJ919774 (676 bp)	GQ281760 (600 bp)	*Pseudozyma fusiformata*
AP47	Apple	Milky white	ovoid (1.70×3.37-2.46×7.02 µm)	FJ919773 (488 bp)	GQ281759 (508 bp)	*Metschnikowia* sp.
PL5	Plum	Butyrous	shuttle-like (3.11×5.76-5.52×7.92 µm)	FJ919775 (479 bp)	GQ281758 (569 bp)	*Aureobasidium pullulans*

644
645

646 **Table 3.** Effect of the three antagonists on *M. laxa* (10^5/mL) spore germination and germ tube
647 elongation by co-culturing in PDB at 25°C for 20 h **.

Treatments	**Spore germination (%)** (p= 0.001)	**Germ tube length (µm)** (p= 0.001)
AP6 10^8 cells/mL	0.0 ± 0.0 a	0.0 ± 0.0 a
AP6 10^7 cells/mL	2.3 ± 0.6 a	5.5 ± 1.1 a
AP6 10^6 cells/mL	29.3 ± 3.1 b	47.2 ± 3.1 b
AP6 inactivated cells	90.7 ± 2.1 d	118.8 ± 4.5 c
AP6 culture filtrate	91.0 ± 2.0 d	127.6 ± 4.4 c
AP47 10^8 cells/mL	0.0 ± 0.0 a	0.0 ± 0.0 a
AP47 10^7 cells/mL	0.7 ± 0.6 a	4.7 ± 4.1 a
AP47 10^6 cells/mL	1.3 ± 0.6 a	7.4 ± 1.6 a
AP47 inactivated cells	90.3 ± 1.5 d	115.8 ± 8.7 c
AP47 culture filtrate	90.0 ± 2.6 d	116.9 ± 4.3 c
PL5 10^8 cells/mL	1.3 ± 0.6 a	9.4 ± 0.8 a
PL5 10^7 cells/mL	29.7 ± 3.8 b	42.7 ± 3.6 b
PL5 10^6 cells/mL	58.0 ± 2.0 c	55.5 ± 6.4 b
PL5 inactivated cells	91.0 ± 2.6 d	117.9 ± 5.0 c
PL5 culture filtrate	90.3 ± 2.5 d	115.1 ± 6.1 c
Control (PDB+pathogen)	92.0 ± 1.7 d	121.5 ± 9.9 c

648

649 ** The results are the mean of two independent experiments. "±" stands for standard error of the
650 means. Values followed by the same letter are not statistically different by Tukey's Test ($p < 0.05$).

Table 4. Biocontrol efficacy against brown rot and effect on postharvest quality parameters of three antagonists applied at 10^8 cells/mL on peaches cv. Redhaven stored at 1°C and 96 % relative humidity for 21 days**.

Treatments	**Disease incidence (%)** (p= 0.001)	**Lesion diameter (mm)** (p= 0.001)	**Firmness (*N*)** (p= 0.484)	**Total soluble solids (%)** (p= 0.605)	**Ascorbic acid (mg/100g)** (p= 0.536)	**Titratable acidity (% malic acid)** (p= 0.500)
P. fusiformata AP6	18.3 ±5.8 a	35.6 ±5.1 ab	1.22 ±0.53 a	9.8 ±1.1 a	2.29 ±0.11 a	0.268 ±0.027 a
Metschnikowia sp. AP47	16.7 ±2.9 a	39.4 ±4.2 ab	1.48 ±0.43 a	10.5 ±0.3 a	2.13 ±0.18 a	0.246 ±0.034 a
A. pullulans PL5	20.0 ±0.0 a	32.5 ±5.4 ab	1.43 ±0.53 a	10.2 ±0.4 a	2.16 ±0.20 a	0.282 ±0.013 a
Tebuconazole*	11.7 ±2.9 a	30.3 ±4.6 a	1.26 ±0.35 a	10.4 ±0.4 a	2.34 ±0.19 a	0.268 ±0.013 a
Uninoculated control	55.0 ±5.0 b	46.0 ±4.1 b	1.37 ±0.64 a	10.1 ±0.3 a	2.28 ±0.14 a	0.264 ±0.028 a

* Peaches were dipped in a suspension containing 250 mL/100 L of Folicur (Bayer Crop Science; tebuconazole: 25.0 %).

** See Table 3.

The results are the mean of two independent experiments. "±" stands for standard error of the means. Values followed by the same letter are not statistically different by Tukey's Test ($p < 0.05$).

Figure captions

Fig. 1. Biocontrol efficacy of the three selected antagonistic isolates (applied at 10^8 cells/mL) in reducing the severity (measured as diameter of rotten lesions in mm) of *M. laxa* on peaches cv. Redhaven. Fruits were stored at 20°C for 7 days, at 8°C for 14 days, and at 1°C for 21 days*(**A**) or biocontrol efficacy of the three selected antagonistic isolates applied at 10^6, 10^7, and 10^8 cells/mL in reducing the incidence of brown rot caused by *M. laxa* on peaches cv. Redhaven stored stored at 1°C in 96 % RH for 21 days*. Fruits were inoculated by spraying a suspension (10^5 spores/mL) of *M. laxa*.(**B**).

*The results are the mean of two independent experiments. Standard error bars of the means are included. Values followed by the same letter are not statistically different by Tukey's Test ($p < 0.05$): Fig.1-A. ($p= 0.001$) and Fig.1-B ($p= 0.001$)

** Peaches were treated with 2.5 mL/L of Folicur (Bayer Crop Science; tebuconazole: 25.0 %).