A new strain of Metschnikowia fructicola for postharvest control of Penicillium expansum and patulin accumulation on four cultivars of apple

A new strain of *Metschnikowia fructicola* for postharvest control of *Penicillium expansum* and patulin accumulation on four cultivars of apple

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ABSTRACT

The efficacy of three antagonistic yeasts – *Metschnikowia pulcherrima* strain MACH1, *M. pulcherrima* strain GS9, and *Metschnikowia fructicola* strain AL27 – against *Penicillium expansum* and patulin accumulation was evaluated on apples stored at room (22±1°C for 7 days) and cold temperatures (1±1°C for 56 days). To increase the potential range of application of the biocontrol agents (BCAs), their efficacy was evaluated on four cultivars of apple, i.e. Golden Delicious, Granny Smith, Red Chief and Royal Gala. AL27 was more effective than MACH1 and GS9 in the control of blue mold rot and in the reduction of patulin accumulation. The efficacy of AL27 was in most cases similar to the chemical control used, making the antagonist as competitive as chemical fungicides. Also *in vitro* experiments showed that AL27 reduced the conidial germination and germ tube length of *P. expansum* more than the other strains. The three BCAs were more effective in the control of blue mold rot on apples cv Golden Delicious than on the other tested cultivars.

Keywords:

Apple, Biological control, *Metschnikowia fructicola*, Mycotoxin, *Penicillium expansum*, Yeast

1. Introduction

Postharvest losses on fruit and vegetables are mainly due to attacks of pathogens during harvest, storage, transport and marketing (Snowdon, 1990). Some species of *Penicillium* are important plant pathogens causing decays on various fruit and vegetables, through their antioxidant proteins and hydrolytic enzymes (Bertolini et al., 1996; Qin et al., 2007). Particularly, *Penicillium expansum* can cause blue molds and blue rots on several plant species (Stange et al., 2002).

Besides its pathogenic activity, *P. expansum* is able to produce patulin, a highly reactive unsaturated lacton, that may cause acute and chronic toxicity, including carcinogenic, mutagenic, and teratogenic effects (Beretta et al., 2000; Hasan, 2000; McCallum et al., 2002). The mycotoxin causes impairment of kidney functions, oxidative damage, and weakness to the immune system. It also has a negative impact on reproduction in males via interaction with hormone production (Fuchs et al., 2008; Selmanoglu and Kockaya, 2004). Patulin can be found in several typologies of fruit-derived food, including apple, pear, peach and apricot juices and nectars (Spadaro et al., 2007; 2008a). The Joint FAO/WHO Expert Committee on Food Additives (JEFCA) established a provisional maximum tolerable daily intake (PMTDI) of 0.4 μg kg^{-1} body weight (bw) day^{-1}, based on a no observable effect level of 43 μg kg^{-1} $bwday^{-1}$ and a safety factor of 100 (World Health Organization, 1995). Based on this PMTDI, patulin is regulated in the European Union at levels of 50 mg kg^{-1} in fruit juices and fruit nectars, 25 mg kg^{-1} in solid apple products, and 10 mg kg^{-1} in apple-based products for infants and young children (European Commission, 2006).

The use of chemical fungicides is an important strategy for controlling *P. expansum* in harvested commodities (Eckert and Ogawa, 1990; Janisiewicz and Korsten, 2002; Zhou et al., 2002). However, during the last decades, some fungicides lost their efficacy due to the development of resistant strains. Several studies demonstrated resistance of *P. expansum* to the most common fungicides used in postharvest (Errampalli et al., 2006; Sholberg et al., 2005). Moreover, concern for public safety has resulted in the cancellation of some of the most effective fungicides in Europe (European Parliament, 2009) and the United States (United States Congress, 1996) (Dayan et al.,

2009). Therefore, research focused on the development of alternative control that should be both effective and economically feasible. The use of microbial antagonists to control postharvest diseases of fruit and vegetables is one of the most promising alternatives to fungicides (Droby et al., 2009; Qin et al., 2004). Some components of the microbial community present on the surface of fruit and vegetables, such as bacteria and yeasts, showed to have significant antagonistic activity against *P. expansum* (Janisiewicz and Korsten, 2002; Usall et al., 2001).

Different yeasts are also able to reduce the patulin level *in vitro* (Coelho et al., 2008; Reddy et al., 2011). Fermentative yeasts reduce patulin contamination during production of cider from apple juice (Harwig et al., 1973). Moss and Long (2002) showed that *Saccharomyces cerevisiae* metabolizes patulin to the less toxic E-ascladiol, whereas there are few studies on the effect of biological control yeasts on patulin accumulation in stored pome fruit (Castoria et al., 2005; Lima et al., 2011; Morales et al., 2008a).

Several studies revealed that fruit cultivars may differ in their susceptibility to blue mold rots and to patulin accumulation (Konstantinou et al., 2011; Neri et al., 2010). Therefore, the apple cultivar should be considered an essential factor influencing the biocontrol of *P.expansum* and its patulin accumulation on fruit. Morales et al. (2008b) found that the pH value of the apple varieties was a determinant factor in the patulin accumulation only under cold storage: apples cv Golden Delicious, characterized by a lower pH, were more prone to patulin accumulation at 1°C. At room temperatures, varieties of apple with higher amounts of organic acids, such as apples cv Golden Delicious and cv Fuji, accumulated more patulin. Another study showed that patulin accumulation was significantly higher in apples cv Golden Delicious and cv Red Delicious than in cv Granny Smith and cv Fuji, due to the lower acidity of the fruit (Konstantinou et al., 2011).

The specific *P. expansum* strain may be another important factor in its pathogenicity and in its ability to synthesize patulin in the fruit (Neri et al., 2010). Sommer et al. (1974) found that different *P. expansum* strains produced differing patulin levels, and the levels were not related to the virulence of the *P. expansum* strains (Neri et al., 2010; Reddy et al 2010). Beretta et al. (2000)

similarly found that the patulin content in apples was not always related to the diameter of the rotten areas, since very high levels were sometimes detected in fruit with small rots.

The aims of the present study were to evaluate the efficacy of three antagonistic yeasts *Metschnikowia pulcherrima* strain MACH1 (Saravanakumar et al., 2008), *M. pulcherrima* strain GS9 (Spadaro et al., 2008b), and *Metschnikowia fructicola* strain AL27, in the control of *P. expansum* and patulin accumulation in apples stored at room and cold temperatures. To increase the potential range of application of the biocontrol agents (BCAs), their efficacy was evaluated on four cultivars of apple, i.e. Golden Delicious, Granny Smith, Red Chief and Royal Gala.

2. Materials and methods

2.1 Microorganisms

M. pulcherrima strain MACH1 (Saravanakumar et al., 2008), *M. pulcherrima* strain GS9 (Spadaro et al., 2008b) and *M. fructicola* strain AL27 were isolated from the carposphere of apples cv Golden Delicious harvested in unsprayed orchards located in Northern Italy. The microorganism culture was stored at −20°C in cell suspension with 65% (v/v) glycerol and 35% (v/v) of a solution of 100 mM $MgSO_4$ and 25 mM Tris (pH 8.0). The strain AL27 was deposited within the Industrial Yeasts Collection (DBVPG) on March 29, 2011 with deposit designation 30P and its use were patented with the Italian patent application TO2011A000534, deposited on June 20, 2011. The strains were grown in YEMS (30 g L^{-1} yeast extract, 5 g L^{-1} D-mannitol, 5 g L^{-1} L-sorbose; Spadaro et al., 2010).

Inocula of the antagonists for all experiments were prepared by subculturing in 250 ml Erlenmeyer flasks containing 75 ml of YEMS and incubated on a rotary shaker (100 rpm) at 22°C for 48 h. Yeast cells were collected by centrifugation at 1,500 rpm for 10 min, washed and resuspended in sterilized Ringer solution (pH 6.9+0.1; Merck, Darmstadt, Germany) and brought to a standard concentration of 10^8 cells ml^{-1} by direct counting with a haemacytometer.

Four isolates of *P. expansum* (PEX06, PEX12, PEX25 and PEX27), each obtained from rotted apples harvested in Piedmont, Northern Italy, and selected for their virulence (Reddy et al., 2010), were used as a mixture during the experiments to ensure a high level of disease. Each strain belongs to AGROINNOVA collection and it was stored in tubes with potato dextrose agar (PDA; Merck) and 50 mg l^{-1} of streptomycin (Merck) at 4°C. Conidial suspensions used for fruit inoculation were prepared by growing the pathogens on Petri dishes on PDA containing 50 mg l^{-1} of streptomycin. After a week incubation at 22°C, conidia from the four strains were collected and resuspended in sterile Ringer's solution. After filtering through eight layers of sterile cheese-cloth, conidia were counted and brought to a final concentration of 10^5 ml^{-1}. The resultant suspensions were shaken using a vortex mixer for 30 s before inoculation.

2.2 Molecular and morphological identification

The yeast antagonist *Metschnikowia fructicola* strain AL27 was 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 was: 95°C, 3 min; 34 cycles: 94°C, 15 s; 55°C, 45 s; 72°C, 55 s; 72°C, 7 min; 4°C. PCR 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 microscope observation of the cell and colony morphology was complementary to the molecular analysis. *M. pulcherrima* strain MACH1 and *M. pulcherrima* strain GS9 were previously identified (Saravanakumar et al., 2008; Spadaro et al., 2008b).

2.3 Antagonism *in vitro*

The effect of the isolates of *Metschnikowia* spp. on conidial germination and on germ tube length of *P. expansum* was assessed in 5 ml of potato dextrose broth (PDB, Merck). A conidial suspension (100 µl; 5×10^6 conidia per ml) of *P. expansum* strain PEX06 was added to a 10 ml test tube. Living cells of each antagonistic yeast (100 µl of a suspension containing 5×10^7, 5×10^8, or 5×10^9 cells per ml), were added to the test tube. As control, 100 µl of the conidial suspension (5×10^6 conidia per ml) of the pathogen in Ringer's solution were added to 5 ml of PDB. After 12 h incubation of the 45° sloping tubes at 22±1°C on a rotary shaker (100 rpm), 100 conidia per replicate were observed microscopically and their germination was evaluated. The treatments were replicated three times. The experiment was carried out twice.

183 **2.4 Efficacy on four cultivars of apples**

184 Apples (*Malus* x *domestica*), 'Golden Delicious', 'Granny Smith', 'Red Chief' and 'Royal Gala',
185 harvested in an Italian orchard grown according to integrated pest management practices, were
186 disinfected in sodium hypochlorite (NaClO, 1.0% as chlorine) and rinsed under tap water, dried at
187 room temperature and punctured with a sterile needle at the equatorial region (3mm depth; 3–4mm
188 wide; 3 wounds per fruit). Fruit were exposed to treatments with 10 µl of the cell suspension (10^8
189 ml^{-1}) of *M. pulcherrima* strain MACH1, strain GS9 or *M. fructicola* strain AL27 per wound.
190 Chemical treatment consisted in the application into each inoculated wound (10 µl) of a suspension
191 (1.25 ml l^{-1} water) of imazalil and pyrimethanil (Philabuster 400SC®, Decco Italia srl, Belpasso,
192 Italy; imazalil 17.2% a.i.; pyrimethanil 17.2% a.i.). An inoculated control was also performed: after
193 3 h at room temperature, 10 µl of the conidial suspension mixture of *P. expansum* (10^5 ml^{-1}) were
194 pipetted into the apple wounds. Apples were randomly packed in commercial plastic trays and
195 stored either at 22±1°C for 7 days or at 1±1°C for 56 days.

196 Some quality parameters were assessed on healthy fruit of every cultivars. Firmness was measured
197 on each fruit at two opposite sites along the equatorial region with a FT327 – Fruit Pressure Tester
198 with an 11mm probe (EFFEGI, Alfonsine, Italy). The probe descended towards the sample at 1.0
199 mm/s and the maximum force (N) was defined as firmness. Total soluble solids (TSS) were
200 determined by measuring the refractive index of pressed juice (Larrigaudière et al., 2002) with a
201 digital refractometer (DBR95, Singapore) and the results were expressed as percentages (g/100 g
202 fruit weight). Acidity was measured by titration with 0.1N NaOH to pH 8.0: 5mL of pressed juice
203 diluted with 5mL of distilled water were evaluated. Titratable acidity was calculated as percent
204 malic acid (Wright and Kader, 1997).

205 Each treatment was replicated three times. Twenty fruit per replication were used (60 inoculation
206 sites). The severity of the diseases was determined by measuring the mean lesion diameter on the
207 rotted apples and the percentage of rot (fresh weight of rot/ fresh weight of fruit). The experiments
208 were carried out twice.

209

210 **2.5. Patulin analysis**

211 Patulin was extracted from rot caused by *P. expansum* on apples treated and stored at 22°C and at
212 1°C. The extraction procedure used was modified by AOAC Official Method 2000.02 Patulin in
213 Clear and Cloudy Apple Juices and Apple Puree. Twenty grams of sample were placed in a
214 centrifuge tube to which 20 drops of pectinase enzyme solution (Sigma Chemical Co., St Louis,
215 MO, USA; 5U/g of juice) and 10 ml of water were added. The mixture was left at 40°C for 2 hours
216 and then centrifuged at 4500 rpm for 5 min. Ten ml of clear juice were placed into 100 ml
217 separating funnel; patulin was extracted with 30 ml of ethyl acetate shaking for 1 min. The organic
218 layer was separated from the water layer. The procedure was repeated three times. The organic
219 phase was dehydrated with 25 g of sodium sulphate anhydrous and then evaporated to dryness
220 (Rotavapor Laborota 4000, Heidolph®, Schwaback, Germany). The residual was resumed with 2
221 ml of acidic water (pH 4.0) and transferred into a HPLC vial. The HPLC apparatus was an Agilent
222 1100 series equipped with G1379 degasser, G1313A autosampler, G1316A column thermostat set
223 at 30°C, G1315B UV diode array detector set at 276nm, G1311 quaternary pump and Agilent
224 Chemstation G2170AA Windows XP operating system (Agilent®, Waldbronn, Germany). A
225 stainless steel analytical column (250x4.6mm i.d., 4 µm, Synergy Hydro-RP C18; Phenomenex®,
226 Torrance, CA, USA) preceded by a guard column (4x3mm i.d.) with the same stationary phase was
227 used. The mobile phase, eluting at a flow rate of 0.800 ml/ min, consisted of an isocratic mixture of
228 water–acetonitrile–perchloric acid (95:4:1) for 20 min, followed by a washing step with an isocratic
229 mixture of water–acetonitrile (35:65). One hundred microliters of sample were injected onto the
230 HPLC column and the retention time of patulin was about 15 min. The amount of patulin in the
231 final solution was determined by using a calibration graph of concentration versus peak area and
232 expressed as ng/ml, achieved by injection onto the HPLC column of 100 µl of standard solutions of
233 patulin (Sigma Chemical Co., St Louis, MO, USA). The standard solutions had concentrations of
234 500 ng ml^{-1}, 400 ng ml^{-1}, 250 ng ml^{-1}, 100 ng ml^{-1} and 50 ng ml^{-1} of patulin. The recovery was

235 determined on a blank apple puree spiked at three concentrations of patulin (10, 50 and 100 ng g^{-1}).
236 Each test was performed three times and the mean recovery values were respectively 90.9%, 91.9%
237 and 100.9%. The repeatability ranged from 1.0% to 6.2% for duplicate analyses. The limit of
238 detection (LOD) and the limit of quantification (LOQ), based on the IUPAC definition (Thompson
239 et al., 2002), were respectively 1.04 and 1.57 ng g^{-1}. The high value of the regression coefficient (R^2
240 $\geq$0.99) obtained indicated a good linearity of the analytical response.
241
242 **2.6 Statistical analysis**
243 For the efficacy experiments, data from at least two experimental trials were pooled. For the
244 mycotoxin experiments, the analyses were carried out in triplicate and the values represented the
245 mean values. The statistical analysis was performed by one-way analysis of variance (ANOVA),
246 using SPSS-WIN software (17.0), and Duncan's multiple range test was employed; $p < 0.05$ was
247 considered significant.
248
249 **3. Results**
250
251 **3.1 Molecular and morphological identification**
252 The strain AL27 was identified by sequencing the ribosomal regions ITS1-5.8S-ITS2 with universal
253 primers ITS-1 and ITS-4 and sequencing the D1/D2 domain with the primers NL-1 and NL-4. The
254 sequences of the amplified regions were deposited in GenBank. The BLAST analysis of the ITS
255 sequence (accession number HQ682194.2; amplimer size: 251 bp) showed that the amplicon of
256 AL27 showed 99% (249/251) identity with the sequences of *Metschnikowia fructicola*. The analysis
257 of the D1/D2 domain (accession number HQ682195; amplimer size: 448 bp) confirmed that the
258 PCR product of AL27 had 99% (447/448) identity with the sequences of *Metschnikowia fructicola*,
259 while the identity with strains of *M. pulcherrima* was lower (98%; 437/444). The observation of the
260 morphological (colony morphology) and microscopic (cell shape and size) characteristics of AL27

confirmed the rDNA sequencing results. Colonies are milky white, cells are ovoid and they measure 1.66×3.30-2.54×7.21 µm.

3.2 Antagonism *in vitro*

The effect of *M. fructicola* strain AL27, *M. pulcherrima* strain GS9 and *M. pulcherrima* strain MACH1 was evaluated on conidial germination and germ tube length of *P. expansum* (Table 1). In the control, 98.0% of the conidia germinated and the average germ tube length was 96.1 µm. The three microorganisms were able to significantly reduce the germination rate and the germ tube length of *P. expansum* at each concentration tested. Each microorganism showed a higher inhibition capability when co-cultivated at the highest concentration (10^8 cells ml^{-1}), than when applied at 10^7 or 10^6 cells ml^{-1}. AL27 was more effective than the other two microorganims in reducing the conidial germination at each of the three concentrations tested. In particular, when co-cultivated with AL27 at 10^8 and 10^7 cells ml^{-1}, the germination rates were only 5.0% and 8.7% respectively. The highest germinations, respectively 78.0% and 69.3%, were observed when co-cultivating with GS9 at 10^6 and 10^7 cells ml^{-1}. The smallest germ tube length (2.3 µm) was observed in presence of 10^8 cells ml^{-1} of AL27, followed by 10^8 cells ml^{-1} of MACH1 (11.2 µm). The germ tubes were longer when reducing the concentration of the yeast cells. Longer germ tubes where observed in presence of 10^6 cells ml^{-1} of AL27 (32.5 µm) and GS9 (31.0 µm).

3.3 Efficacy on four cultivars of apples

The efficacy of the antagonist yeasts was evaluated on apples ‘Golden Delicious’, ‘Granny Smith’, ‘Red Chief’, and ‘Royal Gala’, stored at room (22±1°C for 7 days) and low temperature (1±1°C for 56 days). Blue mold rot was evaluated as rot diameter (Fig. 1) and as percentage of rot weight (Fig. 2). The mixture of imazalil and pyrimethanil was chosen as a chemical control because it is registered in several European countries for use against postharvest rots on apple.

In the trials carried out at 22±1°C for 7 days, the three biocontrol agents (BCAs) were able to significantly reduce the blue mold rot diameter and weight compared to the control. AL27 was the most effective antagonist and provided an efficacy in reducing the rot diameter statistically similar to imazalil + pyrimethanil on 'Golden Delicious', 'Royal Gala' and 'Red Chief' (Fig. 1). On the cv Granny Smith, AL27 was as effective as the chemical in reducing the rot weight (Fig. 2).

When apples were stored at 1±1°C for 56 days, the three BCAs significantly reduced the blue mold lesion diameter, but AL27 was the most effective in reducing the rot diameter on all the apple cultivars (Fig. 1). Its efficacy was statistically similar to the chemical control and higher than the other two antagonists, GS9 and MACH1. By considering the reduction of the rot weight (Fig. 2), all the BCAs were effective against *P. expansum*. Again, AL27 reduced more than the other two BCAs the rot weight and its effect was similar to the application of imazalil + pyrimethanyl. The rot weight was only 0.8% on the cv Granny Smith, 1.0% on the cv Golden Delicious, 2.3% on the cv Red Chief and 3.9% on the cv Royal Gala.

Among the cultivars tested, AL27, MACH1 and GS9 showed a higher control of the rot lesion diameter on the apples cv Golden Delicious. The average values of some quality parameters have been measured on the apples before storage (Table 2). The values of firmness did not differ among the cultivars. On the other hand, total soluble solids and titratable acidity were significantly different among the cultivars. In particular, apples cv Golden Delicious showed a higher content in total soluble solids (14.5%), while the highest titratable acidity was observed on apples cv Granny Smith. The higher total soluble solids on the cv Golden Delicious could be related to a higher efficacy of the BCAs.

3.4 Patulin reduction

The patulin produced was significantly lower in the trials performed at low temperature compared to the experiments carried out at 22±1°C for 7 days, except for the cv Red Chief, where patulin content was significantly higher on the apples stored at 1±1°C for 56 days (Fig. 3). In general, the

three antagonists were able to significantly reduce the patulin content compared to the control. AL27 was the most effective BCA on all the apple cultivars, stored either at 1±1°C for 56 days or at 22±1°C for 7 days. The patulin level observed in the apples treated with AL27 was similar to the level of the chemical control on 'Golden Delicious', 'Granny Smith' and 'Royal Gala'. In particular, when the fruit where kept at 1±1°C for 56 days, the patulin level was lower on apples treated with AL27 (0.0 ng g^{-1} on cv Golden Delicious, 1.2 ng g^{-1} on cv Granny Smith, 24.0 ng g^{-1} on cv Royal Gala), than on apples treated with imazalil + pyrimethanil (0.7 ng g^{-1} on cv Golden Delicious, 4.2 ng g^{-1} on cv Granny Smith, 29.5 ng g^{-1} on cv Royal Gala). Only on cv Red Chief, the patulin level on the fruit treated with AL27 (78.0 ng g^{-1} at 22°C and 67.1 ng g^{-1} at 1°C) was higher than the level on the chemical control (56.4 ng g^{-1} at 22°C and 16.6 ng g^{-1} at 1°C). The highest concentrations of patulin were observed in the fruit treated with GS9, that was also the least effective antagonist.

4. Discussion

Biocontrol agents can be applied as an alternative to fungicides to prevent and control postharvest diseases, and in particular *P. expansum*, of apples. Yeasts are suitable biocontrol agents against postharvest diseases, because they rapidly colonize and survive on fruit surfaces for long periods of time under different conditions, use available nutrients to proliferate rapidly, limit nutrient availability to the pathogen and generally are unaffected by fungicides used commercially (Droby et al., 2009). Previously, several isolates belonging to the yeast genus *Metschnikowia* were isolated from different sources and selected for their efficacy against postharvest diseases (Zhang et al., 2010). *M. pulcherrima*, in recent years, showed high efficacy as a BCA against postharvest decays of apples, grapes, grapefruit and tomatoes (Janisiewicz et al. 2001; Schena et al. 2000; Spadaro et al. 2002). Also *M. fructicola* effectively reduced the development of postharvest rots of grapes and strawberries (Karabulut et al., 2003, 2004; Kurtzman and Droby, 2001).

One strain of *M. pulcherrima*, named MACH1, was isolated from the surface of apple cv Golden Delicious, harvested in organic orchards located in Piedmont, and selected for its efficacy against *B. cinerea, A. alternata* and *P. expansum*. The strain showed a good efficacy against grey mold and alternaria rot, but its biocontrol capability was lower against blue mold rot (Saravanakumar et al., 2008). Its mechanism of action was mainly based on competition for nutrients and release of hydrolases, particularly chitinases (Saravanakumar et al., 2009). The same strain was evaluated for its capability to biodegrade patulin when grown *in vitro*: after 48 h growth of the yeast, patulin was not detected in the growth medium nor in the yeast cell wall, indicating that the mycotoxin was not absorbed but completely biodegraded (Reddy et al., 2011). Another strain of *M. pulcherrima*, named GS9, was previously isolated from an apple cv Golden Delicious and evaluated for its biocontrol against *B. cinerea* and *P. expansum* (Spadaro et al., 2008b) and its capacity to completely biodegrade patulin *in vitro* within 72 h (Reddy et al., 2011), showing lower efficacy compared to MACH1.

In the current research a new yeast strain, named AL27, isolated from the surface of apples cv Golden Delicious, was selected for its efficacy against *P. expansum* on four apple cultivars. Moreover, the capacity to reduce the patulin accumulation on apple was considered as an important feature for the antagonist selection. The yeast strain was identified as *M. fructicola* through its morphological characteristics and through sequencing of the ITS region and the D1/D2 domain. To our knowledge, this is the first report describing the efficacy of *M. pulcherrima* and *M. fructicola* in reducing the accumulation of patulin on apples.

The *in vitro* experiments showed that AL27 reduced the conidial germination and germ tube length of *P. expansum* more than the other strains. The yeast cell concentration was an important factor in determining the inhibition, and a higher inhibition was obtained in presence of higher concentrations of antagonist cells, as previously demonstrated for other antagonistic microorganisms (Hofstein et al. 1994). The results obtained *in vitro* were confirmed by the results of the trials on fruit, performed at 22±1°C for 7 days and at storage temperature. AL27 was more

363 effective than MACH1 and GS9 in the control of blue mold rot, either when the lesion diameter or
364 the rot weight were considered as parameters. These results are in agreement with previous studies,
365 where different strains of the same yeast species showed different biocontrol capabilities, due to
366 their genetic background (Spadaro et al., 2008b). The efficacy of AL27 was in most cases similar to
367 the chemical control used, which is a mixture of two active ingredients commercially available in
368 several European markets.
369 Generally, the efficacy of the three biocontrol agents was higher when the fruit were stored at 1°C
370 than at 22°C. In particular, the efficacy of *M. pulcherrima* MACH1 was significantly higher on
371 apples stored at 1±1°C for 56 days. Previous studies showed that low temperatures of storage
372 resulted in a higher efficacy of the antagonists, either yeast or bacteria (Morales et al., 2008a):
373 during shelf life, *P. expansum* may take advantage of the optimal conditions of growth and increase
374 the growth rate, resulting in a higher aggressiveness (Morales et al., 2010).
375 The three BCAs were more effective in the control of blue mold rot on apples cv Golden Delicious
376 than on the other cultivars. By considering the quality parameters of the fruit, apples cv Golden
377 Delicious showed a higher content in total soluble solids. The higher total soluble solids on the cv
378 Golden Delicious could be related to a higher efficacy of the BCAs, because one of the main
379 mechanisms of action exploited by yeast strains is competition for nutrients, and in particular for
380 carbon sources, such as sugars (Spadaro et al., 2010). A higher efficacy on apples cv Golden
381 Delicious could be also related to the source of isolation of the three BCAs, which is the surface of
382 apples cv Golden Delicious, so an environment where the antagonists were already able to grow.
383 Several BCAs showed good efficacy against *P. expansum*, but rarely the effect on patulin
384 accumulation was tested. There are recent studies about the effect of antagonists on patulin
385 accumulation on fruit (Castoria et al., 2005; Lima et al., 2011; Morales et al., 2008a). The studies of
386 Castoria et al. (2005) and Lima et al. (2011) were performed at room temperature, and not in cold
387 storage conditions. In our study, MACH1 was effective against postharvest pathogens but
388 inefficient in the reduction of patulin accumulation. In contrast, AL27 was effective both in the

389 biocontrol of the pathogen and in the reduction of patulin accumulation. Yeast can be effective in
390 reducing the patulin accumulation on apples through their indirect effect on the reduction of *P.*
391 *expansum* growth and their direct effect in the patulin biodegradation (Coelho et al., 2007; Reddy et
392 al., 2011).The metabolization of patulin to E-ascladiol or Z-ascladiol by *Saccharomyces cerevisiae*
393 (Moss and Lang, 2002), or to desoxypatulinic acid by *Rhodosporidium kratochvilovae* (Castoria et
394 al., 2011) were previously reported.
395 This study considers the efficacy of a yeast biocontrol agent both against *P. expansum* and patulin
396 accumulation on more than one cultivar of apple. Previous studies were just performed on one
397 cultivar of apple, such as cv Golden Delicious (Lima et al., 2011; Morales et al., 2008a) or cv
398 Annurca (Castoria et al. 2005).
399 The analysis of the patulin content showed that its accumulation on apples was not always
400 correlated with the severity of the blue mold rots, since very high patulin levels could be associated
401 to small rots. BCAs or fungicides, though being able to limit the pathogen growth, could also
402 enhance the patulin accumulation in the fruit (Morales et al., 2007). Patulin, such as other
403 mycotoxins, is produced in response to a stress, and the biocontrol application or the chemical
404 application can be considered stress factors for the fungal pathogen (Bottalico and Logrieco, 1998;
405 Calvo et al., 2002).
406 On apples stored at cold temperature, the concentration of the mycotoxin was, generally, lower than
407 in apples stored at room temperature, confirming that the temperature may affect the activity of *P.*
408 *expansum*, including the mycotoxin production (Santos et al., 2002).
409 The patulin level was more markedly reduced by the three antagonistic yeasts on apples cv Golden
410 Delicious than on the other apple cultivars. On cv Granny Smith, the patulin accumulated was
411 lower than in the other apple cultivars, probably due to their high acidity (Konstantinou et al.,
412 2011). Finally, on apples cv Red Chief, a high patulin content on the control could be related to the
413 average low level of titratable acidity of the fruit (Morales et al., 2010).

414 Future prospects involve the study of the mechanisms of actions used by the antagonistic yeast
415 AL27 to control the development of *P. expansum* and to reduce the patulin accumulation on apple.
416 Moreover, semi-commercial and commercial trials will be performed to evaluate the efficacy of *M.*
417 *fructicola* AL27 on large scale applications. Further studies will involve the optimization of the
418 fermentation and stabilization processes, essential steps to be undertaken to develop a biofungicide
419 with commercial application.
420
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428
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589

590 **Table 1**

591 Conidial germination (%) and germ tube length (µm) of *Penicillium expansum* co-cultivated with a
592 cell suspension of antagonistic yeast in PDB at 22±1°C for 12 h.

593

	Penicillium expansum	
Treatment	Conidial germination (%) [a]	Germ tube length (µm) [a]
AL27 $1x10^6$ cfu ml^{-1}	16.7 b	32.5 f
AL27 $1x10^7$ cfu ml^{-1}	8.7 a	23.7 e
AL27 $1x10^8$ cfu ml^{-1}	5.0 a	2.3 a
GS9 $1x10^6$ cfu ml^{-1}	78.0 g	31.0 f
GS9 $1x10^7$ cfu ml^{-1}	69.3 f	25.0 e
GS9 $1x10^8$ cfu ml^{-1}	56.7 e	18.7 cd
MACH1 $1x10^6$ cfu ml^{-1}	58.7 e	20.9 d
MACH1 $1x10^7$ cfu ml^{-1}	40.7 d	16.9 c
MACH1 $1x10^8$ cfu ml^{-1}	29.3 c	11.2 b
Control	98.0 h	96.1 g

594

595 [a] Values in the same column followed by the same letter are not statistically different by Duncan's
596 Multiple Range Test ($p<0.05$).

597

598 **Table 2**

599 Average values of quality parameters on apples ‘Golden Delicious’, ‘Granny Smith’, ‘Red Chief’,
600 and ‘Royal Gala’ used during the trials of storage at 22±1°C for 7 days or at 1±1°C for 56 days.

601

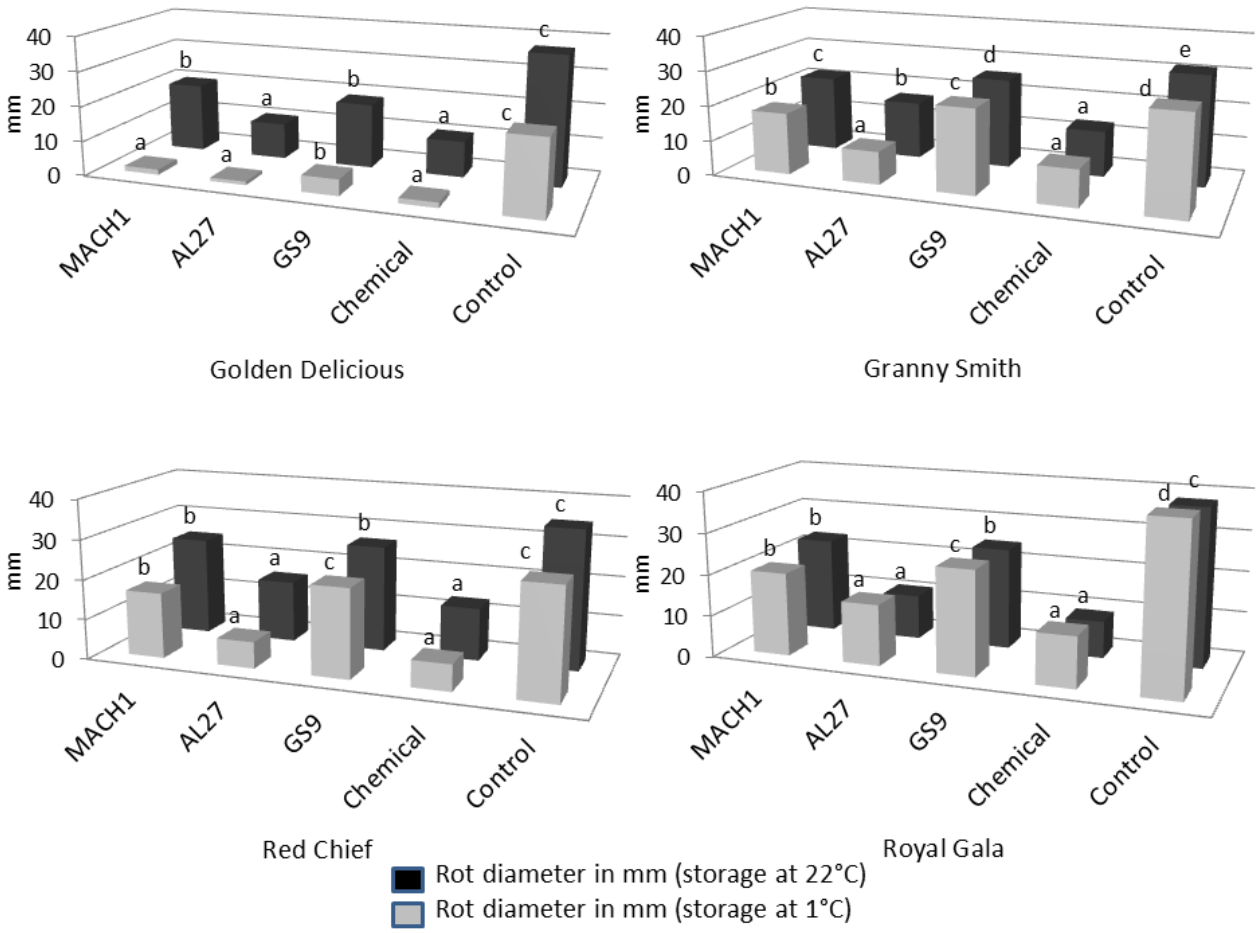
Apple cultivar	Total soluble solids (%)*	Firmness (N)*	Titratable acidity (g malic acid/100 mL*)
‘Golden Delicious’	14.5±0.8 a	70.6±8.4 a	0.509±0.027 b
‘Granny Smith’	10.8±0.6 c	74.5±9.1 a	0.811±0.034 a
‘Red Chief’	11.3±0.5 c	68.6±7.5 a	0.235±0.013 d
‘Royal Gala’	12.2±0.7 b	75.5±8.3 a	0.348±0.026 c

602

603 *The results are the means of two independent experiments. “±” stands for standard error of the
604 means. Values followed by the same letter are not statistically different by Duncan’s Multiple
605 Range Test (p<0.05).

606

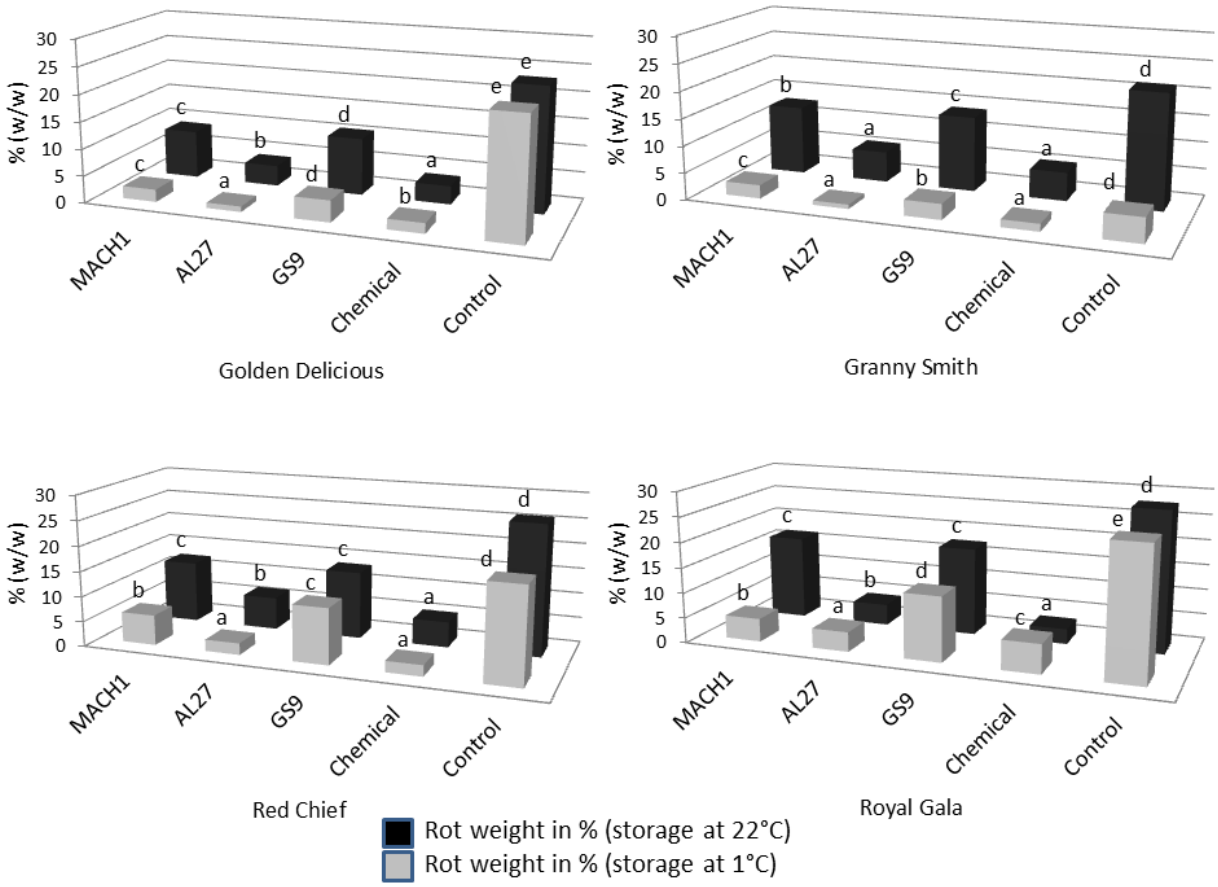
Figure 1. Blue mold rot diameter (mm) caused by *Penicillium expansum* on apples 'Golden Delicious', 'Granny Smith', 'Red Chief', and 'Royal Gala', treated with *Metschnikowia pulcherrima* strain MACH1, *M. pulcherrima* strain GS9 and *M. fructicola* strain AL27 and stored at 22±1°C for 7 days (dark grey) or at 1±1°C for 56 days (light grey).*



*Values of the same cultivar and the same storage trial, followed by the same letter, are not statistically different by Duncan's Multiple Range Test ($P<0.05$).

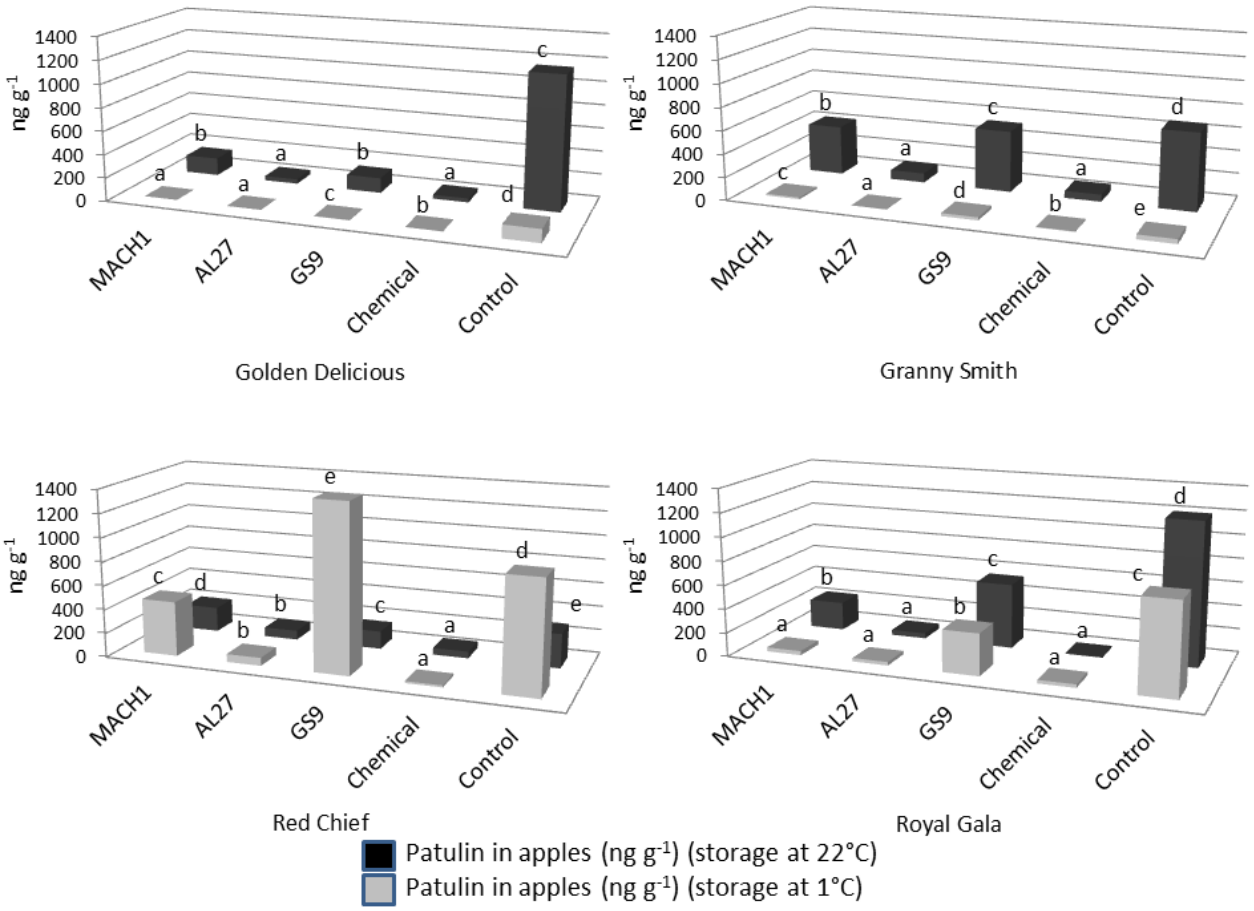
Chemical treatment consisted in the application into each inoculated wound (10 µl) of a suspension (1.25 ml l^{-1} water) of imazalil and pyrimethanil (Philabuster 400SC®, Decco Italia srl, Belpasso, Italy; imazalil 17.2% a.i.; pyrimethanil 17.2% a.i.).

Figure 2. Blue mold rot percentage (fresh weight) caused by *Penicillium expansum* on apples 'Golden Delicious', 'Granny Smith', 'Red Chief', and 'Royal Gala', treated with *Metschnikowia pulcherrima* strain MACH1, *M. pulcherrima* strain GS9 and *M. fructicola* strain AL27 and stored at 22±1°C for 7 days (dark grey) or at 1±1°C for 56 days (light grey).*****



*See Fig. 1.

Figure 3. Patulin concentration (ng g^{-1}) produced by *Penicillium expansum* on apples 'Golden Delicious', 'Granny Smith', 'Red Chief', and 'Royal Gala', treated with *Metschnikowia pulcherrima* strain MACH1, *M. pulcherrima* strain GS9 and *M. fructicola* strain AL27 and stored at 22±1°C for 7 days (dark grey) or at 1±1°C for 56 days (light grey).*



*See Fig. 1.