**Use of AFLP for differentiation of Metschnikowia pulcherrima strains for postharvest disease biological control**

# *Metschnikowia pulcherrima*: a promising species isolated from different food matrices for biological control of postharvest diseases in apple

Davide Spadaro[1]*, Wilma Sabetta[1], Alberto Acquadro[2], Ezio Portis[2], Angelo Garibaldi[1] and Maria Lodovica Gullino[1]

[1]Centre of Competence for the Innovation in the Agro-environmental Sector, Università di Torino, via L. da Vinci 44, I-10095 Grugliasco (TO), Italy. [2]Di.Va.P.R.A. – Plant Genetics and Breeding, Università di Torino, via L. da Vinci 44, I-10095 Grugliasco (TO), Italy.

*Corresponding author. Phone: +39-011-6708942; fax: +39-011-6709307; e-mail address: davide.spadaro@unito.it

## Abstract

Seven strains of the yeast *Metschnikowia pulcherrima*, isolated from the carposphere of apples cv. Golden delicious, showed biocontrol capability against *B. cinerea* and *P. expansum*. The efficacy of these strains was compared with that of nineteen *M. pulcherrima* strains, isolated from different sources in different geographical regions. On an average, the strains were more effective in the control of *B. cinerea* than of *P. expansum*, after storage for 28 days at 4°C, with a mean reduction of the pathogen growth respectively to 30.0% and 49.0% of the control. Antagonistic properties could be owned to microorganisms of different origin. Strain 3043 isolated from grape must offered the best control of both diseases. To assess the genetic diversity of *M. pulcherrima*, the AFLP technique was used. With six AFLP primer pairs 729

polymorphic bands were scored. The genetic similarity coefficients obtained with AFLP technique were used to construct a dendrogram using UPGMA through SHAN routine. The similarity matrix generated by some AFLP primer pairs, such as McaEaa or McgEat, was sufficient to describe the genetic distance among the strains. A relationship between efficacy and genetic origin was not observed, because strains isolated in different locations, characterized by high genetic diversity, showed similar biocontrol potential.

*Keywords***:** amplified fragment length polymorphism, biocontrol agent, *Botrytis cinerea*, molecular characterization, *Penicillium expansum*, yeast.

## 1. Introduction

Fungal pathogens cause severe losses on apples during postharvest storage and commercialisation (Snowdon, 1990). Biological control, using microbial antagonists, has emerged as an effective strategy to combat major postharvest decays of fruit (Janisiewicz and Korsten, 2002; Spadaro and Gullino, 2004). During the last decade several yeast strains have been selected for their antagonistic properties on postharvest biological control of fruit (Gullino et al., 1991; 1994). Recently three strains of the yeast *Metschnikowia pulcherrima*, named BIO126, GS 88 and GS37, which proved to be effective in containing *Botrytis* and *Penicillium* spp. rots in apple, especially at low storage temperatures, were selected and studied (Spadaro et al., 2002; 2004). A strain of *M. pulcherrima*, coded 4.4, proved to be highly effective in the control of Botrytis rot of apple (Piano et al., 1997).

*Metschnikowia pulcherrima* is involved in the first step of the fermentation process of apples for cider-making (Beech, 1993). As the ethanol level raises (2 to 4% V/V),

these initial fermenters begin to die out and *Saccharomyces cerevisiae* takes over. In grape must and during the early phase of fermentation, apiculate yeasts belonging to the species *Kloeckera apiculata* are dominant and, to a lesser extent, isolates of *M. pulcherrima*, can also be detected (Fleet and Heard, 1993).

*Metschnikowia pulcherrima* occurs naturally on fruits, buds and floral parts of certain apple trees (Boekhout and Robert, 2002) and has been reported to be a yeast species effective as 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; 2004). *Metschnikowia pulcherrima* normally acts by consuming the nutrients on fruit and vegetable skins that allow rot-causing fungi to develop (Piano et al., 1997; Janisiewicz et al., 2001).

Random amplified polymorphic DNA (RAPD) and arbitrarily primed-PCR (AP-PCR) techniques were useful methods to identify and evaluate the survival rate of some fungi (*Aureobasidium pullulans*) and yeasts as agents for postharvest biological control (Schena et al., 1999; 2000). Specific fingerprints using amplified fragment length polymorphism (AFLP) technique have also been applied to monitor the population of *Rhodotorula glutinis*, *Cryptococcus laurentii* and *Aureobasidium pullulans* in both the field and cold storage (Lima et al., 2003).

One goal of this study was to compare the biocontrol capability against *B. cinerea* and *P. expansum* of the seven *M. pulcherrima* strains, all of them isolated from the carposphere of apples, with the same capability of other strains of the same species coming from different sources. Another goal was the assessment of the genetic diversity of *M. pulcherrima* strains having a different origin and the evaluation of the relationship between biocontrol capability and genetic distance.

## 2. Materials and methods

102

103 *2.1. Microorganisms*

104 Seven yeast strains, isolated in Northern Italy from the carposphere of apples
105 organically grown (BIO114, BIO126, BIO131, GS9, GS37, GS88 and 4.4) were
106 included. They were identified by PCR-RFLP (restriction fragment length
107 polymorphism) analysis on the ITS region of the 5.8S rRNA gene and the two
108 ribosomal internal transcribed spacers as *Metschnikowia pulcherrima* (Pitt) M. W.
109 Miller (Esteve-Zarzoso et al., 1999) and identification was confirmed by morphological
110 and physiological methods by the Department of Plant Biology of the University of
111 Perugia, Italy. Three strains, 311, 291 and 320, were kindly given by the University of
112 Bari (Schena et al., 2000; see Table 1). Sixteen strains were purchased from the
113 Industrial Yeast Collection DBVPG, University of Perugia, Italy (see Table 1).

114 All the strains were stored at –80°C in a cell suspension with 20% V/V of glycerol
115 in the antagonist culture collection of the Centre of Competence for the Innovation in
116 the Agro-environmental Sector of the University of Torino (Italy). Yeasts were grown
117 on Yeast Peptone Dextrose (YPD): 10 g $l^{-1}$ of granulated yeast extract (Merck,
118 Darmstadt, Germany); 20 g $l^{-1}$ of triptone-peptone of casein (Difco, Detroit, MI, USA);
119 20 g $l^{-1}$ of D(+)-glucose monohydrate (Merck). Antagonists were prepared by
120 subculturing in YPD on a rotary shaker (100 rpm) at 25°C for 48 h. Yeast cells were
121 collected by centrifugation at 2500 x *g* for 5 minutes, washed and resuspended in
122 sterilised Ringer solution (pH 6.9±0.1; Merck), and brought to a standard concentration
123 of $10^8$ cells $ml^{-1}$ by direct counting with a haemacytometer.

124 Three strains of *Botrytis cinerea* Pers. : Fr. and three of *Penicillium expansum* Link,
125 isolated from rotten apples produced in Piedmont (Nothern Italy) and selected for their
126 different degree of virulence, were used as a single mixture in the experiments, to
127 ensure a consistent level of disease. Each strain was stored in tube with Potato Dextrose

Agar (PDA; Merck) and 50 mg $l^{-1}$ of streptomycin (Merck) at 4°C in the pathogen culture collection of the Centre of Competence for the Innovation in the Agro-environmental Sector of the University of Torino (Italy). Conidia suspensions were prepared by growing the pathogens on Petri dishes with PDA and 50 mg $l^{-1}$ of streptomycin (Merck) for 10 days at room temperature. Conidia were suspended in sterile Ringer's solution (Merck), filtered through 8 layers of sterile cheesecloth and brought to a final concentration of $10^5$ $ml^{-1}$.

*2.2. Biocontrol trials*

The experiments of biocontrol efficacy against *B. cinerea* and *P. expansum* were carried out in apples (*Malus domestica* Borkh, cv Golden delicious). The fruits, sanitized in sodium hypochlorite (NaClO, 1.0 % in water) and rinsed under tap water, when dry, were punctured with a sterile needle at the equatorial region (3 mm depth; 3 wounds per fruit). The cell suspensions ($10^8$ cells $ml^{-1}$) of the 26 *M. pulcherrima* strains were pipetted (30 µl) into the wounds. Control fruits were inoculated, before pathogen inoculation, with 30 µl of YPD. Also thiabendazole (Tecto 20S, Elf Atochem Agri Italy) was employed as standard chemical (0.3 mg $ml^{-1}$ of active ingredient in water suspension). After 3 h, 30 µl of the *B. cinerea* or *P. expansum* suspension ($10^5$ conidia $ml^{-1}$) were pipetted into the wound. When dry, apples were randomly packed in commercial plastic trays and stored at 4°C for 28 days. Five fruits per treatment were used (15 inoculation sites) and the biocontrol trials were repeated twice.

*2.3. DNA extraction*

Two ml of YPD culture of the yeast isolates were centrifuged at 2500 x *g* for 3 min. The pellets were suspended in 280 µl of EDTA 50 mM (pH 8-8.5) with 400 µg of lyticase (Sigma, St Louis, MO, USA) and incubated at 37°C for 45 min. After 3 min

centrifugation, the pellets were treated with the Wizard Genomic DNA Purification kit (Promega Corp., Madison, WI, USA). Genomic DNA was controlled by electrophoresis (30 min at 100 V/cm) on 1% SeaKem LE agarose gel (FMC BioProducts, Rockland, ME, USA) in 1X TAE buffer (40 mM Tris, 40 mM acetate, 2 mM EDTA, pH 8.0; Maniatis et al., 1982); the gel was stained with ethidium bromide and visualized through UV light. Gel images were acquired with a Gel Doc 1000 System (Bio-Rad Laboratories, Hercules, CA, USA). A 1 kb DNA ladder (Gibco BRL, Rockville, MD, USA) was used as a molecular weight marker for an approximate quantification of the genomic DNA. A precise quantification in ng/µl was obtained by a BioPhotometer (Eppendorf, Hamburg, Germany). Purified DNA was stored in TE buffer (10 mM Tris-HCl; 0.1 mM EDTA; pH 8) at 4°C.

*2.4. AFLP analysis*

The AFLP protocol was similar to that described by Vos et al. (1995). Each genomic DNA was diluted to 100 ng/µl with TE buffer. DNA digestion was carried out using *Eco*RI and *Mse*I (BioLabs, Beverly, MA, USA); 2 µl of genomic DNA were added to a reaction mixture containing 1X NEB buffer2, 1 µg/µl BSA, 10 U *Eco*RI, 10 U *Mse*I and water to a final volume of 20 µl. After 3 h incubation at 37°C, 20 µl containing 100 pmol/µg *Mse*I-adapter and 10 pmol/µg *Eco*RI-adapter, 4 U T4-DNA-Lygase (BioLabs) and 1X T4-buffer were added to the restriction mixture; the ligation was carried out for 16 h at 16°C. Two primers, Mc and Ea (Table 2), both with one selective base in the 3' position, were used for the pre-amplification reaction. 5 µl of DNA template 10-fold diluted with TE buffer were amplified in a final volume of 20 µl of a reaction mixture containing 1X PCR buffer (10 mM Tris-HCl; 50 mM KCl; pH 8.3), 1.5 mM $MgCl_2$, 200 µM dNTP, 50 ng each primer and 1 U Taq-polymerase

179 (Promega). The PCR reaction was performed with the following programme: 1 min at
180 94°C; 35 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C; 10 min at 72°C. The
181 presence of the pre-amplified products was verified through electrophoresis on a 2%
182 agarose gel in 1X TAE buffer.

183 The selective amplifications were carried out using couples of primers both with a
184 selective extension; the primer combinations indicated in Table 2 were used.

185 PCR mixture was the same as the pre-amplification reaction, except for the primers
186 (2 ng/µl each). The amplification program was: one cycle at 94°C for 1 min; 13 cycles
187 of 30 s at 94°C, 30 s ramping from 65°C to 56.6°C (-0.7°C per cycle) and 1 min at
188 72°C; 23 cycles of 30 s at 94°C, 30 s at 56°C and 1 min at 72°C; one cycle at 72°C for
189 10 min. 4 µl of amplification product were added to 15 µl loading buffer (98%
190 formamide; 10 mM EDTA pH 8.0; 0.01% bromophenol blue; 0.01% xylene cyanol),
191 denaturated at 95°C for 5 min and kept at 0°C before loading in a denaturing (7M urea)
192 5 % polyacrylamide (19:1) gel. A 5 ng/µl marker mixture, formed by 10 bp DNA ladder
193 (Sigma), 100 bp DNA ladder (Sigma) and formamide blue (5:5:40), was used.
194 Electrophoresis was carried out at 80 W for 2.5 h. Polyacrylamide gels were fixed for
195 30 min in a 10% acetic acid solution. Silver staining was carried out as described by
196 Bassam et al. (1991). Gel profiles were scanned and visualised by a Gel-Documentation
197 System (Quantity One Programme, Bio-Rad Labs). Every AFLP analysis was repeated
198 twice for each couple of primers.

199

200 *2.5. Data scoring and statistical analysis*

201 AFLP amplifications were repeated at least once in order to test their consistency.
202 Each PCR product was assumed to represent a single locus and only reproducible
203 polymorphic bands were manually scored as present (1) or absent (0). All fragments
204 were given equal weights. A binary matrix of isolates and markers for cluster analysis

was compiled using the NTSYS-pc version 1.80 package (Rohlf, 1993). Genetic similarity among accessions was calculated using the Dice Similarity Index (DSI) and the SIMQUAL routine. The similarity coefficients were used to construct a dendrogram using the UPGMA through the SHAN routine and a thousand bootstrap were performed over AFLP loci using PHYLIP software (Felsenstein, 1993; http://evolution.genetics.washington.edu/phylip.html). A co-phenetic matrix was produced using the hierarchal cluster system, by means of the COPH routine, and correlated with the original distance matrixes for AFLP data, in order to test for association between the cluster in the dendrogram and the DSI matrix.

Mantel tests (Mantel, 1967) were performed to check the correlation between the similarity matrixes generated by the single primer combinations and the total similarity matrix.

## 3. Results

### *3.1. Biocontrol trials*

Twenty-six *Metschnikowia pulcherrima* strains of different origin have been evaluated for their biocontrol efficacy in artificial wound of apple against *Penicillium expansum* and *Botrytis cinerea* (Table 3).

After 28 days of storage at 4°C the mean lesion diameter of blue mould (*Penicillium expansum*) was 49.3% of the control. Five strains (BIO114, GS9, 3008, 3435 and 4129) did not reduce significantly the growth of *P. expansum*. The other twenty-one strains reduced the pathogen growth ranging from 31.1 to 84.3% of the control. The most effective strains appeared to be BIO131, 3043, 4185 and 4292, one coming from apple surface, two from grape must and one from a winery surface.

Against *B. cinerea*, after storage for 28 days at 4°C, generally all strains were more

effective, with a mean reduction of the pathogen growth to 30.0% of the control. Two strains, BIO114 and 4354, did not significantly reduce the growth of grey mould on apple. The other strains reduced the lesion diameter ranging from 30.5% to the complete inhibition of the pathogen growth. GS37 and 3043 provided a complete inhibition of *B. cinerea*. Among the most effective strains, also 3835 (96.2% of reduction of the lesion diameter), 3041 (92.5%) and BIO126 (91.6%) showed a good capability of inhibiting *B. cinerea*. Eight strains were able to limit the pathogen growth to values inferior to 20% of the control: three were isolated from grape must, two from apple, one from quince, one from cherry and one from a snail.

*3.2. Molecular characterization*

The AFLP technique was chosen to assess the genetic variability among the twenty-six yeast strains. Preamplification reaction was carried out successfully using primer pair McEa. Six primer combinations of an initial 16 tested combinations were chosen for their ability to generate informative patterns rich of polymorphic bands. A representative result, obtained with primer pair MctEac, is given in Figure 1.

After scoring the AFLP profiles obtained with the six primer combinations selected, a total of 729 polymorphic bands (39% of the total amplified bands) were scored. The size of AFLP fragments was in a range from 40 bp to 1500 bp, but only fragments between 100 and 500 bp were taken into account to avoid scoring problems due to excess primer peaks near the front of the electrophoresed fragments and a decreasing signal for fragments longer than 500 bp.

The dendrogram, generated from the Dice distance matrix, using UPGMA clustering analysis, is shown in Figure 2. The co-phenetic correlation coefficient (r-value) between the data matrix and the co-phenetic matrix for AFLP data was 0.978, suggesting a very good fit between the dendrogram clusters and the similarity matrixes

from which they were derived.

Reproducibility of the groupings below each node of the dendrogram was verified by analysing 1000 multiple datasets from bootstrapping. Two major clusters could be distinguished that are supported by high bootstrap value (97): Cluster 1, including the seven strains isolated from the carposphere of apples, coming from orchard of Piedmont, in Northern Italy, and Cluster 2, including all the other strains, except for 311 and 291, that did not cluster to a specific group. Strains belonging to Cluster 1 showed a well-structured grouping. This was characterised by high bootstrap values.

Genetic similarities among the strains of *M. pulcherrima* ranged from 0.21 (between 291 and 311 and the other strains studied) and 0.94 (between 3435 and 3527).

Within Cluster 1, the strains BIO114 and BIO126 were the most similar strains (DSI of 0.91); quite high bootstrap values were obtained (96). BIO131 was the yeast more similar to them with a DSI of 0.80. GS37 was more similar to GS88 (DSI of 0.58) and GS9 to 4.4 (DSI of 0.59).

The strains appeared to be less structured in Cluster 2. In fact lower bootstrap values were obtained here indicating a lower reliability of structure. Within Cluster 2, two sub-clusters could be identified: Cluster 2a, comprising strains 3041, 3042, 3043, 3435, 3527 and 3345, which separates from Cluster 2b, including 3835, 4130, 4185, 3938, 4064 and 4292, with quite high bootstrap values (72). Within Cluster 2a, the strains 3435 and 3527 were the most similar strains (DSI of 0.94) and were found in 100% of the bootstrap resamples. Also 3041, 3042 and 3043 were quite similar (DSI of 0.86) and were found in all the resamples. In the Cluster 2b, strains 4130 and 4185 are more similar (DSI of 0.91) and were found in 99% of the resamples.

In general, the bootstrap values that unite the strains are among the most highly supported: all bootstrap values are greater than 50 except in the case of cluster between 3348 and 4129, 3008, Cluster 2a and Cluster 2b (bootstrap value of 45).

283 In this study the choice of one of the six primer combinations did not have a large
284 influence on the amount of fragments recovered (ranging from 109 to 135) but did have
285 a strong effect on the correlation between the similarity matrixes generated by the single
286 primer combination and the total similarity matrix, ranging from 0,770 to 0.937 (data
287 not shown). To check this correlation, Mantel tests were performed. In general, the
288 similarity matrixes generated by every single primer pair showed few differences from
289 the total matrix.
290 Primer combinations McaEaa and McgEat resulted highly informative, because both
291 generated a similarity matrix closely related with the total similarity matrix (*r* of 0.937
292 and 0.931).
293 All the primer combinations permitted to obtain twenty-six unique electrophoretic
294 patterns, except for primer pair McaEat, that did not distinguish between strain 3041
295 and 3042, and primer pair MctEac, that was not able to separate strain BIO 126 from
296 BIO 131.
297
298 **4. Discussion**
299
300 *4.1. Biocontrol trials*
301 The twenty-six strains studied for their biocontrol activities were coming from
302 different sources: seven strains were isolated from the carposphere of apple, two from
303 the carposphere of other pome fruit (pear and quince), two from the carposphere of
304 stone fruit (cherry), eleven from different steps in the wine production chain (grape,
305 must, wine and winery), two from unusual origins (a snail and seawater) and for two of
306 them the origin is unknown.
307 The strains were tested for their efficacy in the control of *Botrytis cinerea* and
308 *Penicillium expansum*, causal agents of grey and blue mould on apple. Some strains

309 already (Spadaro et al., 2002) proved to be more effective in the control of these
310 diseases at the low temperatures of storage of the fruits than at room temperature,
311 probably because at 4°C the growth rate of the biocontrol agents is reduced less than the
312 growth rate of the pathogens. The main mode of action involved in the biocontrol is
313 competition for nutrients or space although a direct interaction can not be excluded
314 (Spadaro et al., 2002).

315 In the biocontrol activity experiments carried out, thiabendazole was used as
316 chemical control but the strains of *Penicillium expansum* and *Botrytis cinerea* used were
317 partially tolerant to benzimidazoles, as can be observed from the low efficacy of the
318 fungicide. In effect, this low sensitivity is also confirmed by some recent evaluations
319 carried out in Italy on postharvest pathogens of pome fruit (Bertetti et al., 2003).

320 In general the strains were more effective in the control of *B. cinerea* than of *P.*
321 *expansum*, after storage for 28 days at 4°C, with a mean reduction of the pathogen
322 growth respectively to 30.0% and 49.3% of the control. Five strains did not reduce
323 significantly the growth of *P. expansum* and only two the growth of *B. cinerea*. Only six
324 strains controlled better blue than grey mould. In general, *M. pulcherrima* is a yeast
325 species that possesses good antagonistic characteristics for biological control of
326 postharvest diseases of apple, and it is meanly more effective against *B. cinerea* than *P.*
327 *expansum*.

328 The seven strains isolated from apple carposphere are the result of a selection for
329 biocontrol capabilities against *B. cinerea* and *P. expansum* on apple among about 400
330 strains (Gullino et al., 1994). The other strains were randomly chosen in the yeast
331 collection of DBVPG, University of Perugia, Italy, or (strains 291, 311 and 320) are the
332 result of a selection based on their biocontrol potential but on different host species. The
333 first group of microorganisms, in general, controlled better *B. cinerea* (23.1% compared
334 to the control) and *P. expansum* (40.3%) than the second group (29.8% and 50.8%). The

strains previously selected in our laboratory were among the more effective but not all of them. GS37 and 3043 provided a complete control of Botrytis rot. BIO131 and 3043 were the most effective against Penicillium rot. Strain 3043 offered the best control of both diseases and was randomly chosen among the isolates of DBVPG from grape must.

In this study we tried to clarify one question of biological control of postharvest diseases. Is the substratum of the antagonists so important in the determination of the biocontrol capability of the microorganisms? Normally it is believed that the fruit surface is an excellent source of naturally occurring microorganisms against postharvest rot agents (Wilson and Wisniewski, 1994; Droby et al., 1999). The carposphere or the phylloplane have provided the major source for antagonists and in a few cases microorganisms have been isolated from other matrices: one yeast collection has been screened (Filonow et al., 1996) and, in one example, starter cultures used in the food industry were used as possible sources of biocontrol agents (Wilson and Chalutz, 1991).

There has seldom been a comparison between the efficacy of microorganisms coming from the carposphere and the biocontrol capability of other microorganisms coming from other sources (Filonow et al., 1996). In this paper we showed that antagonistic properties for biological control in the carposphere can be possessed by microorganisms isolated from the same source where they will be applied as antagonists but also microorganisms of different origin can have biocontrol potential.

*4.2. Molecular characterization*

In the second part of the work, we wanted to assess the genetic diversity of *M. pulcherrima* strains of different origins and to discover if a relationship between biocontrol activity and genetic distance existed.

Genetic variability was assessed by RAPD (data not shown) and AFLP techniques.

AFLP technique permitted to obtain a superior number of polymorphisms and resulted more reproducible and than RAPD technique, though its analysis is more laborious and expensive (Jones et al., 1997; Blears et al., 1998).

The AFLP technique has been widely used to study plant genomes but rarely for yeast studies. Some applications in literature are for wine spoilage yeasts (Barros-Lopes et al., 1999) and recently for postharvest biological control (Lima et al., 2003). From the genetic analysis, co-phenetic correlation values showed that the genetic clusters accurately represented the estimates of genetic similarity. The high bootstrap values at each node indicate that the this tree is robust and reproducible. Isolates coming from the carposphere of apple were grouped in a cluster (Cluster 1) with a high bootstrap value (97). Within Cluster 1, the genetic closeness was supported by the similar origin of the strains.

The most effective strains in the control of *P. expansum* were BIO131, 3043, 4292 and 4185, the first one grouped in Cluster 1, the second in Cluster 2a and the last two in Cluster 2b. Against *B. cinerea*, after storage for 28 days at 4°C, the strains that provided better control of *B. cinerea* were BIO126 and GS37 (Cluster 1), 3041 and 3043 (Cluster 2a) and 3835 (Cluster 2b). We showed that there was not a relationship among biocontrol capability and origin of the microorganisms, but also that biocontrol efficacy and genetic distance among the strains were not related. Strains of the same species isolated from the same location (BIO114, BIO126 and BIO131) can be very similar from the genetic point of view but greatly differ for their biocontrol potential. On the contrary, strains isolated in different locations (GS37 and 3043), with a high genetic diversity, can have a similar biocontrol potential. The biocontrol potential is the result of a large quantity of genetic traits that contribute to provide the antagonist a fitness advantage towards the pathogen. This is especially true for microbial antagonists using competition as main mechanism of biocontrol. When other mechanisms are involved,

such as antibiosis or mycoparasitism, it is easier to identify key genetic traits, such as genes involved in the antibiotic biosynthetic pathway or genes for lytic enzymes. It is important also to point out that it is not known yet to which degree AFLP amplification patterns are representative for the overall genetic similarity among the strains. There are indications that AFLP bands preferably are amplified from repetitive DNA and therefore are a biased sample of heritable polymorphism (Potokina et al., 2002).

Many of the AFLP fragments only occurred in one or a few strains of *M. pulcherrima*. This pointed towards a high mutation rate and a low information content of a large fraction of the AFLP bands. Such rare polymorphisms would add random noise when AFLP patterns were analysed for overall similarity, and there is the possibility that this highly variable fraction changes quickly with time.

Assessing the correlation between the similarity matrixes generated by the single primer combination and the total similarity matrix we found that one primer pair, such as McaEaa or McgEat, resulted highly informative and sufficient to describe the genetic distance among the strains. AFLP patterns result highly informative (number of polymorphic bands generated) also using few primer combinations.

AFLP patterns could clearly distinguish the different strains of *M. pulcherrima* and, within the limits of the restricted samples, we have found some putative specific bands for single tag sequence (STS) conversion (data not shown). AFLP fingerprinting confirmed in other studies to be a useful method for the identification of specific DNA sequences suitable as a source of template for the production of STS markers (Shan et al., 1999). One of the major characteristics for an antagonist to be used in biological control is its precise identification and its traceability, to permit to follow its environmental fate in space (dispersion) and in time (survival) after release, and to assess the genetic stability and the effects on microbial communities of the introduced antagonists (Gullino et al., 1995). Suitable and reproducible strain authentication

methods are necessary in commercial procedures such as filling patents and product licensing. Moreover it is essential to develop a quality control system that allows to monitor the genetic stability over time of the biofungicide as a commercial product (Avis et al., 2001).

Research is in progress to develop STS isolate-specific markers for some of the antagonistic strains of *M. pulcherrima*.

**Acknowledgements**

The authors gratefully acknowledge Dr. Franco Nigro (University of Bari, Italy) and Dr. Ann Vaughan-Martini (University of Perugia, Italy) for having provided some of the strains studied in this work and Dr. Jeanne Griffin for the linguistic advice.

This research was carried out with a grant from the Italian Ministry for the Environment and Territory within the Framework Agreement “Crop Protection with Respect of the Environment”.

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**Figure captions**

Fig. 1. AFLP patterns of the twenty-six *M. pulcherrima* strains, using primer pair MctEac. 5 % polyacrylamide denaturing (7M urea) gel silver stained.

Fig. 2. Dendrogram describing the relationships among the twenty-six isolates of *M. pulcherrima*, based on the Dice similarity index of AFLP-PCR banding profiles. The analysis of grouping was undertaken by unweighted pair-group method using arithmetic averages (UPGMA). Numbers at the nodes represent the proportion of 1,000 bootstrap samples in which a particular clade was found.

538 **Tables**

539

540 Table 1. The twenty-six *Metschnikowia pulcherrima* strains studied in this paper

541

| Number | Code | Sustratum | Geographical origin | Source |
|---|---|---|---|---|
| 1 | BIO114 | apple | Northern Italy | University of Torino |
| 2 | BIO126 | apple | Northern Italy | University of Torino |
| 3 | BIO131 | apple | Northern Italy | University of Torino |
| 4 | GS37 | apple | Northern Italy | University of Torino |
| 5 | GS88 | apple | Northern Italy | University of Torino |
| 6 | GS9 | apple | Northern Italy | University of Torino |
| 7 | 4.4 | apple | Northern Italy | University of Torino |
| 8 | 291 | pear | Southern Italy | University of Bari |
| 9 | 311 | quince | Southern Italy | University of Bari |
| 10 | 320 | table grape | Southern Italy | University of Bari |
| 11 | 3008 | grape must | Central Italy | University of Perugia |
| 12 | 3041 | cherry | Italy | University of Perugia |
| 13 | 3042 | sour black cherry | Italy | University of Perugia |
| 14 | 3043 | grape must | Central Italy | University of Perugia |
| 15 | 3345 | wine | Northern Italy | University of Perugia |
| 16 | 3348 | * | Sicily | University of Perugia |
| 17 | 3435 | * | Sardinia | University of Perugia |
| 18 | 3527 | grape | Spain | University of Perugia |
| 19 | 3835 | *Helix pomatia* (snail) | * | University of Perugia |
| 20 | 3938 | grape must | * | University of Perugia |
| 21 | 4064 | grape must | Greece | University of Perugia |
| 22 | 4129 | grape | Greece | University of Perugia |
| 23 | 4130 | grape must | Slovenia | University of Perugia |
| 24 | 4185 | grape must | Greece | University of Perugia |
| 25 | 4292 | winery surface | Central Italy | University of Perugia |
| 26 | 4354 | seawater | Italy | University of Perugia |

542 * data not available

543 Table 2. Adapter and primer sequences for the AFLP preamplification and selective
544 amplification
545

| | Restriction site | Primer | Sequence |
|---|---|---|---|
| Adapter | *Mse*I | | 5’-GACGATGAGTCCTGAG-3’ |
| Universal primer | | | 3’-TACTCAGGACTCAT-5’ |
| Preamplification | | Mc | 5’-GATGAGTCCTGAGTAA**C**-3’ |
| 1st selective amplification | | Mca | 5’-GATGAGTCCTGAGTAA**CA**-3’ |
| 2nd selective amplification | | Mct | 5’-GATGAGTCCTGAGTAA**CT**-3’ |
| 3rd selective amplification | | Mca | 5’-GATGAGTCCTGAGTAA**CA**-3’ |
| 4th selective amplification | | Mct | 5’-GATGAGTCCTGAGTAA**CT**-3’ |
| 5th selective amplification | | Mca | 5’-GATGAGTCCTGAGTAA**CA**-3’ |
| 6th selective amplification | | Mcg | 5’-GATGAGTCCTGAGTAA**CG**-3’ |
| Adapter | *Eco*RI | | 5’-CTCGTAGACTGCGTACC-3’ |
| Universal primer | | | 3’-CATCTGACGCATGGTTAA-5’ |
| Preamplification | | Ea | 5’-GACTGCGTACCAATTC**A**-3’ |
| 1st selective amplification | | Eaa | 5’-GACTGCGTACCAATTC**AA**-3’ |
| 2nd selective amplification | | Eac | 5’-GACTGCGTACCAATTC**AC**-3’ |
| 3rd selective amplification | | Eag | 5’-GACTGCGTACCAATTC**AG**-3’ |
| 4th selective amplification | | Eag | 5’-GACTGCGTACCAATTC**AG**-3’ |
| 5th selective amplification | | Eat | 5’-GACTGCGTACCAATTC**AT**-3’ |
| 6th selective amplification | | Eat | 5’-GACTGCGTACCAATTC**AT**-3’ |

546
547 Note: Abbreviations of the primers are given. Nucleotide extensions for
548 preamplification and selective amplification are indicated in bold.
549

550 Table 3. Effect of the cell suspensions of the twenty-six *M. pulcherrima* strains on
551 *Penicillium expansum* and *Botrytis cinerea* growth on apples cv Golden delicious.
552 Storage at 4°C for 28 days
553

| Treatment | *Penicillium expansum* severity (%) [a] | | *Botrytis cinerea* severity (%)[a] | |
|---|---|---|---|---|
| Inoculated control | *100.0 | g | **100.0 | h |
| Thiabendazole | 66.8 | e-f | 74.0 | f-h |
| BIO114 | 84.3 | f-g | 81.9 | g-h |
| BIO126 | 32.5 | b-d | 8.4 | a-c |
| BIO131 | 15.7 | a-b | 23.0 | a-e |
| GS37 | 32.7 | b-d | 0.0 | a |
| GS88 | 33.6 | b-d | 36.5 | c-e |
| GS9 | 81.8 | f-g | 36.0 | c-e |
| 4.4 | 31.8 | b-d | 24.3 | a-e |
| 291 | 53.9 | d-e | 33.5 | b-e |
| 311 | 36.6 | b-d | 18.6 | a-d |
| 320 | 68.2 | e-f | 39.0 | d-e |
| 3008 | 79.0 | f-g | 17.3 | a-d |
| 3041 | 68.9 | e-f | 7.5 | a-c |
| 3042 | 68.0 | e-f | 21.7 | a-e |
| 3043 | 16.6 | a-b | 0.0 | a |
| 3345 | 35.9 | b-d | 23.0 | a-e |
| 3348 | 36.9 | b-d | 69.5 | f-g |
| 3435 | 87.3 | f-g | 38.0 | c-e |
| 3527 | 34.3 | b-d | 51.4 | e-f |
| 3835 | 32.5 | b-d | 3.8 | a-b |
| 3938 | 66.6 | e-f | 30.7 | a-e |
| 4064 | 47.2 | c-e | 29.2 | a-e |
| 4129 | 88.9 | f-g | 38.8 | d-e |
| 4130 | 45.2 | c-d | 10.7 | a-d |
| 4185 | 26.5 | b-c | 25.0 | a-e |
| 4292 | 25.6 | b-c | 37.5 | c-e |
| 4354 | 50.0 | d-e | 74.4 | f-h |

554
555 [a]Calculated on the lesion diameter (0% is complete inhibition of the pathogen). Values
556 in the same column followed by the same letters are not statistically different by Tukey
557 b Test ($P < 0,05$).
558 [b] 300 µg a.i. $ml^{-1}$: used as chemical control.
559 Diameter of lesions in the control: *28.9 mm;**35.4 mm.