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Abstract

The mechanisms of action and efficacy of four isolates (GS37, GS88, GA 102, and BIO126) of the yeast *Metschnikowia pulcherrima* against *Botrytis cinerea*, *Penicillium expansum*, *Alternaria* sp., and *Monilia* sp., all postharvest pathogens of apple fruits, were studied *in vitro* and on apples, in controlled and semi-commercial conditions.

An application of a cell suspension (10^8 cells ml^{-1}) of the antagonists in artificial wounds of apples permitted to reduce the growth of *B. cinerea* and *P. expansum* after storage at 23°C. A complete suppression of the pathogen was obtained against *Monilia* sp., storing at 23°C, and against *B. cinerea* and *P. expansum*, storing at 4°C. The results against *Alternaria* sp. were more variable. Applications of culture filtrates and autoclaved cells of the isolates were ineffective in reducing the diameter of the lesions on the fruits, supporting the hypothesis that living cells are necessary for biocontrol. In experiments of antagonism *in vitro*, on different solid substrates, a reduction of the micelial growth of the pathogens emerged, so that, at least *in vitro*, the antagonists could produce some diffusible toxic metabolites. Co-cultivating *in vitro* on a synthetic medium, *B. cinerea* spore (10^5 ml^{-1}) germination was completely inhibited by the presence of 10^8 cells of the antagonists, while culture filtrates and autoclaved suspensions were not able to reduce germination. Dipping boxes of apples cv Golden delicious in a suspension of 10^7 antagonist cells ml^{-1} and storing for 8 months in controlled atmosphere at 1°C, the isolates showed control capability against *B. cinerea* and *P. expansum* similar to thiabendazole.

Keywords: *Alternaria* sp.; Antagonism; Apple; Biocontrol; *Botrytis cinerea*; Integrated Pest Management, *Metschnikowia pulcherrima*; *Monilia* sp.; *Penicillium expansum*; Postharvest rot; Storage

1. Introduction

Apple Postharvest rots, caused by *Penicillium expansum*, *Botrytis cinerea*, and *Alternaria* sp., are particularly severe even in production areas where the most advanced storage technologies are available (Eckert and Ogawa, 1988). In Northern Italy they can cause serious losses, also due to the presence of populations of *B. cinerea* and *P. expansum* resistant to fungicides (Romano *et al.*, 1983). In this area during the last years, *Monilia* sp. has become an increasing problem (Trevisan *et al.*, 1992), also due to integrated pest management techniques, which avoid treatments at flowering.

Currently, fungicides, when admitted, are the major means to control postharvest diseases (Eckert and Ogawa, 1988). However, the growing public concern over the health and environmental hazards associated with pesticide use in orchards (Wisniewski and Wilson, 1992), the development of fungicide resistant strains of postharvest pathogens (Spotts and Cervantes, 1986) and the deregistration of some of the most effective fungicides (Gullino and Kuijpers, 1994) have generated interest in the development of alternative non chemical methods.

Biological control using microbial antagonists (Wilson and Wisniewski, 1994) has emerged as one of the most promising alternatives, either alone or as part of an integrated pest management to reduce pesticide use. Several biocontrol agents have been exploited and widely investigated against different postharvest fungal pathogens (*B. cinerea*, *Penicillium*, *Monilia*, and *Alternaria spp.*) and tested on apple fruits (Mc Laughlin, 1990; Roberts, 1990; Gullino *et al.*, 1994; Filonow *et al.*, 1996; Piano *et al.*, 1997; El-Ghaouth *et al.*, 1998; Janisiewicz, 1998). At present, three products containing *Pseudomonas syringae* Van Hall, active against *Botrytis*, *Penicillium*, *Mucor* and *Geotrichum* spp., named Bio-Save 100, Bio-Save 110 and Bio-Save 1000 and commercialized by EcoScience Corp. (Janisiewicz and Jeffers, 1997), and a product containing *Candida oleophila* Montrocher, effective against *Botrytis* and *Penicillium* spp., named Aspire and commercialized by Ecogen Inc. (Hofstein and Fridlender, 1994)**,** are available on the market for postharvest protection.

Information on the mechanisms of action of most of the antagonists is still incomplete, but it is essential to develop appropriate formulation and methods of application, to obtain registration and to select new effective microorganisms. The main mode of action of yeast biocontrol agents is believed to be competition for nutrients and space (Droby and Chalutz, 1994). Moreover, yeasts are able to colonize the carposphere for a long period in low humidity conditions, grow rapidly and are

generally poorly sensitive to fungicides (Janisiewicz, 1991). Yeasts deserve particular attention, as their activity does not generally depend on the production of toxic metabolites, which could have a negative environmental or animal toxicological impact (Smilanick, 1994). Additional modes of action such as mycoparasitism, induced resistance and the production of lytic enzymes such as β-1,3-glucanase have been suggested (Wisniewski *et al.*, 1991; El Ghaouth *et al.*, 1998; Jijakli and Lepoivre, 1998). It is likely that there are multiple interactions between antagonist, fruit, pathogen, and other components of the natural epicarpic microflora (Droby and Chalutz, 1994).

Recently we have isolated and selected four strains of the yeast *Metschnikowia pulcherrima*, named BIO126, GS 88, GA102 and GS37, which proved to be effective in containing *Botrytis* and *Penicillium* spp. rots in apple. Other strains of *M. pulcherrima*, coded 2.33 and 4.4, had already proved to be highly effective in the control of Botrytis rot of apple (Gullino *et al.*, 1991; 1994; Migheli *et al.*, 1997; Piano *et al.*, 1997). The aim of this work was to determine the mechanism of action of the antagonistic isolates and to evaluate their efficacy under controlled and semi-commercial conditions with low temperature and controlled atmosphere.

2. Materials and methods

2.1. Microorganisms and culture conditions

Metschnikowia pulcherrima (Pitt) M. W. Miller isolates BIO126, GS88, GA102, e GS37 were isolated from the carposphere of apple cv Golden delicious, harvested in unsprayed orchards located in Piedmont and Aosta Valley (GA102), Northern Italy. Cultures were stored at –20°C in cell suspension with 65% V/V of glycerol and 35% V/V of a solution $MgSO_4$ 100 mM and Tris (pH 8.0) 25mM. Yeasts were grown on Yeast Peptone Dextrose (YPD: 10 g l^{-1} of Extract of Yeast Granulated Merck; 20 g l^{-1} of Triptone-Peptone of Casein Difco; 20 g l^{-1} of D(+)-Glucose Monohydrate Merck). Inocula of the antagonists for all the experiments were prepared by subculturing in 250 ml Erlenmeyer flasks containing 75 ml of Yeast Peptone Dextrose (YPD) and incubating on a rotary shaker (100 rpm) at 25°C for 48 hours. Yeast cells were collected by centrifugation at 2500 x *g* for 5 minutes, washed and resuspended in sterilised Ringer solution (pH 6.9$\pm$0.1; Merck), and brought to a standard concentration of 10^8 cells ml^{-1}, unless otherwise stated, by direct counting with a haemacytometer.

Three strains of *Alternaria* sp., isolated from rotted apples belonging to the cvs Golden delicious and Red delicious and selected for their virulence by inoculation in artificially wounded apples, were used as a mixture throughout this work, to ensure a higher level of disease. The same

operations were accomplished for three strains of *Botrytis cinerea*, three of *Monilia* sp., and three of *Penicillium expansum*. Each strain was stored in tube with Potato Dextrose Agar (PDA; Merck) and 50 mg l^{-1} of streptomycin Merck at 4°C and was routinely inoculated and re-isolated from apple to maintain pathogenicity. Spore suspensions were prepared by growing the pathogens on Petri dishes for two weeks with Potato Dextrose Agar (PDA; Merck) and 50 mg l^{-1} of streptomycin Merck (strains of *Alternaria* sp. and *P. expansum*) or with Potato Glucose Malt (35 g l^{-1} of Potato Dextrose Agar Merck, 7 g l^{-1} of D(+)-Glucose Monohydrate Merck and 3 g l^{-1} of Malt Extract Merck) and 50 mg l^{-1} of streptomycin Merck (strains of *B. cinerea* and *Monilia* sp.). After two weeks incubation at 25°C, spores from the three strains of each pathogen were collected and suspended in sterile Ringer's solution (Merck). After filtering through 8 layers of sterile cheese-cloth, spores were counted and brought to a final concentration of 10^5 ml^{-1}.

2.2. Antagonism in apple artificial wounds

Apples (*Malus domestica* Borkh, cv Golden delicious), disinfected in sodium hypochloride (NaClO, 1.0 % as chlorine) and rinsed under tap water, when dry were punctured with a sterile needle at the equatorial region (3 mm depth; 3 wounds per fruit). An antagonistic yeast cell suspension (30 μl) was pipetted into wound. Autoclaved cells of *M. pulcherrima* and culture filtrates, prepared by centrifuging cultures of the antagonists and then filtering the supernatant through a 0.2 μm nitro-cellulose filter, were applied into wounds in order to evaluate their efficacy in reducing the incidence of the pathogens on apple fruit. Inoculated control fruits were pipetted, before pathogen inoculation, with 30 μl of Yeast Peptone Dextrose. After 3 hours, 30 μl of the spore suspension of the pathogen strains were pipetted in the wound. When dry, apples from different treatments were randomly packed in commercial plastic trays and either stored at 23°C for 6 days (*B. cinerea* and *P. expansum*), 12 days (*Monilia* sp.) or 18 days (*Alternaria* sp.) or kept at 4°C for 21 days (*B.cinerea*) or 28 days (*P. expansum*). Three fruits per treatment were used (9 inoculation sites) and each experiment was repeated three times.

2.3. Antagonism in vitro

The growth rate of the pathogens was tested in different solid synthetic mediums: Potato Dextrose Agar (PDA, Merck), NYDA (as in Droby *et al.*, 1989), Yeast Potato Dextrose-Agar (YPD with 20 g l^{-1} of Agar-agar Merck), CZAPEK-Agar (1 g l^{-1} of Potassium Phosphate Merck, 2 g l^{-1} of Sodium Nitrate Merck, 0.5 g l^{-1} of Magnesium Sulphate Merck, 0.5 g l^{-1} Potassium Chloride Merck, 0.01 g

l^{-1} of Ferrous Sulphate Merck, 30 g of Saccharose Merck e 15 g of Agar-Agar Merck), Malt Extract Agar (Merck) and Apple-Agar (80 % v/v of apple homogenised filtered through Whatman no. 1 filter paper and 20 g l^{-1} of Agar-Agar Merck; pH: 5.5). A drop of the yeast cell suspension was striped on the substrate in 90 mm diameter Petri dishes, 20 mm from the border. A 6 mm mycelium disk of the pathogen was put 32 mm from the border and 32 mm from the strip of the antagonist. The radial growth of the mycelium towards the yeast strip was measured when the pathogen reached 32 mm of diameter towards the dish border, after storage at 23°C and in the dark. If the mycelium did not reach the border in 28 days, the substrate was considered not optimal for that pathogen growth. Three Petri plates per treatment were used and the experiment was repeated twice.

2.4. Effect on B. cinerea *spores germination*

The effect of the isolates of *M. pulcherrima* on spore germination of *B. cinerea* isolate Gao1 was assessed in potato dextrose broth (PDB, Difco). Aliquots (100 µl) of spore suspension ($5x10^6$ spores ml^{-1}) of the pathogen in Ringer's solution were transferred to 10 ml plastic tubes containing 5 ml PDB. Living cells of each antagonistic yeast (100 µl of a suspension containing $5x10^7$, $5x10^8$, or $5x10^9$ cells ml^{-1}) or cells killed by autoclaving (100 µl of a suspension containing $5x10^8$ cells ml^{-1}) were added to each tube. As a control, the pathogen was added to 5 ml of a mixture (1:1) of PDB and of culture filtrates obtained as described from 48 hours old cultures of the 4 isolates of *M. pulcherrima* in PDB. After 12 h incubation of the 45° sloping tubes at 25°C on a rotary shaker (100 rpm), 100 spores per replicate were observed microscopically and their germination was evaluated. The treatments were replicated three times and the experiment repeated twice.

2.5. Experimental trials under semi-commercial conditions: storage in controlled atmosphere

An experimental trial was carried out in Aosta (Aosta Valley, Northern Italy) during the period November 1998 – July 1999 in cooperation with the Institut Agricole Regional on artificially infected apples of the cv Golden delicious. Apples were harvested in orchards conducted with integrated pest management. Four boxes were used in each treatment (100 fruits per box). Ten apples per box, to reproduce the most probable conditions after harvesting, were artificially wounded at the equatorial region (3 mm depth; 3 wounds per fruit). All fruits were artificially inoculated by dipping for 60 seconds in 100 l tanks containing a conidial suspension (10^5 spores ml^{-1} per pathogen) of *B. cinerea*, *P. expansum*, and *Alternaria* sp.. After 3 hours, biocontrol isolates were applied at 10^7 cells ml^{-1} completely dipping the boxes of fruits for 60 sec in 100 l tanks

containing cell suspensions prepared as described. The treatments included the four isolates of *M. pulcherrima* (BIO126, GS37, GS88 and GA102) and a chemical control (thiabendazole, Tecto 20 S, Elf Atochem Agri Italy, 19,7 % a.i., 30 g a.i. 100 l^{-1}). The inoculated, with the pathogens suspension, and uninoculated controls were represented by four boxes with 100 fruits per box, ten of them artificially wounded. When dry, apples were incubated at 23°C for 24 h and then stored at 1°C for 8 months in controlled atmosphere (2% O_2 and 3% CO_2). After 4 and 8 months storage, the rot incidence was evaluated and the relative importance of the different postharvest pathogens determined visually or through isolation on potato dextrose agar (PDA, Merck).

3. Results

3.1. Antagonism in apple artificial wounds

M. pulcherrima BIO126, GS88, GA102, and GS37 cell suspension, applied at 10^8 cells ml^{-1}, on apple artificial wounds stored at 23°C generally reduced the lesion diameter of Alternaria rot but the results were not homogeneous (Table 1). The major reduction to 0.9 % was due to the application of the isolate GS37. Culture filtrates and autoclaved cell suspensions also caused reduction of the lesions but, even in the same treatment, there were differences among single fruits.

Culture filtrates and autoclaved cell suspensions of the four isolates were ineffective against *Monilia* sp. at 23°C, *B. cinerea* and *P. expansum* at 23° and 4°C (Tables 1 and 2).

Cell suspension (10^8 cells ml^{-1}) of the four isolates significantly reduced Botrytis rot on apples, but the best results were obtained storing the fruits at 4°C (Table 2). BIO126 and GS37 cell suspensions permitted to obtain a remarkable reduction of the lesions also at 23°C, respectively to 25.7 and 26.8 %. Addition of the cell suspensions of any of the four antagonistic strains completely inhibited the growth of *Monilia* sp. after 12 days of storage at 23°C (Table 1). The cell suspensions of the four strains applied on apple wounds resulted highly effective against *P. expansum* at 4°C after 28 days of storage (Table 2). Reduction of lesions was lower, ranging between 35.1 and 60.9 %, but anyway significantly different from the control, storing fruits at 23°C (Table 1).

In this experiment the effect of all the yeast isolated was studied on disease severity (lesion diameter) and not incidence (percent infection), evaluated in the trials under semi-commercial conditions.

3.2. Antagonism in vitro

The co-culture on different solid substrate antagonists and pathogens, permitted to study antagonism *in vitro*. As shown in Table 3-A and 3-C, on some media, even after 28 days, *Alternaria* and *Monilia* spp. did not grow sufficiently to be influenced by the presence of the biocontrol agent, so that it was not possible to measure the inhibition.

Alternaria sp. mycelium growth (Table 3-A) was significantly inhibited by the presence of the antagonist strip on APPLE (Figure 1) and CZAPEK. *B. cinerea* (Table 3-B) growth was reduced by the four strains on YPD and NYDA and by GA102 on PDA. *Monilia* sp. (Table 3-C) was partially inhibited by all the potential antagonists on APPLE. *P. expansum* (Table 3-D) radial growth was significantly inhibited by the four isolates on NYDA and partially on YPD and APPLE.

3.3. Effect on B. cinerea *spore germination*

By co-culturing on potato dextrose broth (PDB, Difco), the effect of *M. pulcherrima* BIO126, GS88, GA102, and GS37 on spore germination of *B. cinerea* was evaluated (Table 4).

A complete inhibition of the spore germination emerged in presence of 10^8 cells ml^{-1} of the four strains of *M. pulcherrima*. With 10^7 cells ml^{-1} there was a partial inhibition: the percentage of conidia germinated compared with the control varied from 25.7 % of BIO126 to 73.0 % of GA102. In the presence of 10^6 cells ml^{-1} of antagonist only a negligible and not significant reduction in the germination was observed. A culture filtrate and killed cell suspension permitted the full germination of the spores.

During the experiment, a strong attitude by the antagonist living cells to concentrate and adhere to non germinated spores of *B. cinerea* was observed. Adhesion was not observed with autoclaved cells.

3.4. Experimental trials under semi-commercial conditions: storage in controlled atmosphere

Trial carried out dipping boxes of apples cv Golden delicious in a cell suspension of the antagonist was followed by an 8 month storage in controlled atmosphere at 1°C. First survey, after 4 months, showed a reduction in the incidence of rotted apples for all biological treatments (Table 5).

Treatments with cell suspensions differ significantly from the chemical control, while they did not one from the other. BIO126 cell suspension offered a control (9.0 % of rotted apples) similar to thiabendazole (8,7 %). Analyzing pathogens separately, it was possible to point out in all theses a major incidence of *B. cinerea* rots. *Alternaria* sp. rots were absent or at a very low level (1.4 %). Biological treatments showed a control of *P. expansum* (average incidence of 2.9 %) similar to thiabendazole (3.2 %), but a lower efficacy towards *B. cinerea* (average incidence of 9.7 %) in comparison with the chemical control (4.1 %).

After 8 months' storage (Table 5), biological treatments offered a control statistically not different from thiabendazole, but significantly different from the inoculated control, with a reduction of the incidence of rotted fruits, compared to first survey.

Alternaria rot incidence increased prolonging storage, but remained a minority of the total rotted fruits. In comparison with the first survey, the incidence of *B. cinerea* was lower and that of *P. expansum* was similar.

Summing up the percentages of rotted apples in the two surveys, the best result was offered by BIO126 (17.1 %) and the worst by GA102 (23.1 %). Compared with the chemical product (13.7 %), biocontrol agents showed an efficacy slightly lower, but the incidence of rotted fruits was significantly reduced with respect to the inoculated control (50.4 %).

4. Discussion

4.1. Mechanisms of action

Generally the activity of antagonist yeasts is not based on the production of antibiotics or other secondary toxic metabolites (Droby and Chalutz, 1994); the results of this study show that *M. pulcherrima* BIO126, GS88, GA102, and GS37 principally act for the competition for space and / or nutrients.

From the antagonism in apple artificial wounds, a substantial incapability emerged to antagonize all pathogens tested either by culture filtrate (without yeast cells) or by autoclaved cell suspension (killed cells). Living cells of the antagonists are necessary to guarantee the fungal control. The nutritional environment of the apple wound could be favourable to *M. pulcherrima*, that would colonize fruit tissues rapidly competing with pathogens for nutrients.

Also in *in vitro* experiments on *B. cinerea* spore germination, neither the culture filtrate nor autoclaved cells of the four isolates had any effect on the germination. The antagonistic activity of

M. pulcherrima was dependent on the concentration of the antagonist: when applied at 10^6 cells ml^{-1} no yeast provided a satisfactory level of control. During the study a tenacious adhesion of living yeast cells to *B. cinerea* spores and hyphae was observed, in a manner similar to that described by Wisniewski *et al.* (1991). Attachment to pathogen conidia was not observed after incubation of autoclaved antagonistic cells. This permits to suppose a direct interaction between antagonist and pathogen.

Often microbial antagonists provide different results in *in vitro* or *in vivo* conditions (Gullino, 1994). Co-culture experiments of antagonists and pathogens on different solid substrates bring to suppose that, at least in *in vitro* conditions, antagonistic yeasts could produce some metabolites toxic for the pathogens, differently from what results from the application of culture filtrate *in vivo*. Because inhibition of mycelial growth is only present on some substrates, it is probable that the nutritional environment influences the production of secondary metabolites. The CZAPEK substrate, for instance, is poor in simple and complex sugars, therefore it could not favor the radial growth of the pathogen; furthermore it is rich in nitrates, that remarkably reduce antagonistic capability of the isolates, as already observed by Piano *et al.*, 1997. APPLE substrate could easily simulate the nutritional conditions of the wound; for many of the pathogens tested it was possible to point out some inhibition. On MALT, PDA, and NYDA, around the yeast strip, a pink halo was also visible, indicative of the metabolism of some compounds present on the substrate.

4.2. Efficacy

To be commercially acceptable, antagonists must be effective under semi-commercial conditions. Since previous experiments, carried out measuring naturally developed rots, were ineffective because of the low incidence of the disease in the control, the trial of storage at 1°C in controlled atmosphere was carried out on partially wounded apples and inoculated with spore suspensions of *Alternaria* sp., *Botrytis cinerea*, and *Penicillium expansum*. This step permitted to obtain, after 8 months of storage, 50.4 % of rotted apples in the inoculated control, greatly increasing the probability to observe significant differences. *P. expansum* and *B. cinerea* were the pathogens more frequently isolated from the fruits. The low incidence of Alternaria rots gave no significant results. All biocontrol agents were effective in the reduction of the total rots. The origin of *M. pulcherrima* BIO126, GS88, GA102, and GS37, isolated from apple surface of “Golden delicious”, could have influenced positively the experiments since the isolates are naturally able to colonize the carposphere of the fruit.

In trials of antagonism in apple wounds, the isolates offered a satisfactory biocontrol efficacy

against *B. cinerea*, *Monilia*, sp. and *P. expansum*. Control of Botrytis and Penicillium rots by antagonist cell suspension was more homogeneous in the repetition, while efficacy towards Alternaria and Monilia rots resulted highly dependent on the fruit. An element of interference in the experiment was the difficulty to find fruits with the same degree of ripening; a high concentration in sugars, in fact, associated with senescence of the fruit, is a factor promoting the attack of certain pathogens, such as *P. expansum* and *B. cinerea* (Roberts, 1991). Comparing trials of antagonism carried out at 4 and at 23 °C, antagonists showed a major efficacy at 4°C and, in some cases, pathogens were completely inhibited.

Considering the results of the efficacy trials, a sharp difference in the biocontrol capability of the four antagonists does not exist: anyway BIO126 offered a higher control in semi-commercial conditions.

5. Conclusions

M. pulcherrima BIO126, GS88, GA102, and GS37 were tolerant to benomyl and thiabendazole (benzimidazoles), and to vinclozolin and procymidone (dicarboximides), all of them registered for postharvest use (data not shown), therefore it could be possible to employ the biocontrol agents together with reduced dosages of these fungicides, in an integrated control perspective.

The antagonists were tolerant to calcium chloride (data not shown). Between the strategies experimented during the last years in fruit protection, the association of biological agents with calcium chloride infiltration is to remember. The addition of this salt greatly enhances the action of the antagonist yeast (McLaughlin *et al.*, 1990; Piano *et al.*, 1998).

The biocontrol capability of the yeasts, during the experiment in semi-commercial conditions, was not affected by the low temperature of storage (1°C) of the fruits and by the controlled atmosphere. Antagonists are compatible with normal storage methods and with chemical products employed in post-harvest.

From growth at different temperatures (data not shown), it resulted that the tested isolates do not grow at 37°C, which is important from a toxicological point of view. Another point favourable for a future commercialization of the studied yeasts is the lack of production of antibiotics active against the tested pathogens *in vivo*. The main mechanism of action used by the biocontrol agents is competition with pathogens for space and nutrients, but a secondary mechanisms of action with a synergistic effect could be also a direct interaction, such as parasitism, and some production of toxic metabolites in particular nutritional conditions, not investigated in this work.

In future studies, to increase the knowledge on the mechanisms of action, it could be useful to

purify and characterize the substances released in the culture substrates. Enzymes surely involved in the process of antagonism, such as glucanases or chitinases, specific for the cell wall of the fungi, have already been isolated from the media where yeast antagonists were grown (Jijakli and Lepoivre, 1998; Wilson *et al.*, 1994). It could also be useful supply the microorganisms with different nutrients, sources of carbon or nitrogen, to understand which are involved in the mechanism of competition (Janisiewicz *et al.*, 1992; Piano *et al.*, 1998). Future studies will also concentrate on the potential of resistance induced in the host tissue (Arras, 1996; Wilson and El-Ghaouth, 1993).

It would be interesting to evaluate the curve of the antagonist population on the carposphere, but it was not possible to mark the biocontrol agents for the sensitivity to antibiotics to differentiate them from the indigenous population. Trials of sensitivity to seven antibiotics resulted in a similar level of tolerance, between the four isolates and the population of yeasts present on the fruit (data not shown).

The work is now continuing towards a molecular characterization of the isolates, either to evaluate the survival and dynamic of the population after application on the fruit or to study the environmental impact of a possible release in the open field. RAPD-PCR (Random Amplified Polymorphic DNA) and AP-PCR (Arbitrary Primed Polymerase Chain Reaction) techniques permit to distinguish efficiently also strictly correlated strains (Droby *et al.*, 1999; Schena *et al.*, 2000).

Finally, the formulation should constitute a fundamental field of the studies, to permit the commercialization of the product (Fravel *et al.*,1999).

Acknowledgements

This research was carried out with grants from the Institut Agricole Regional of Aosta, Italy. Particular thanks to R. Pramotton and C. Duverney, who cooperated in part of the experiments, and to the anonymous reviewers for their useful suggestions.

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Tables

Table 1

Effect of cell suspension, cell-free culture filtrate and autoclaved cells of *M. pulcherrima* isolates BIO126, GS88, GA102, and GS37, applied in artificial wounds of "Golden delicious" apple, on the growth of different post-harvest rots. Storage at 23°C for 6 (Botrytis and Penicillium rots), 12 (Monilia rot), and 18 days (Alternaria rot).

Treatment	Percentage of control *							
	Alternaria		*B.cinerea*		*Monilia*		*P.expansum*	
Uninoculated Control	0.0	a	0.0	a	0.0	a	0.0	a
Inoculated control	100.0	f	100.0	d	100.0	b	100.0	ef
BIO 126 10^8 cells/ml	5.7	ab	25.7	b	0.7	a	35.1	b
BIO 126 culture filtrate	96.4	f	113.2	de	110.5	b	101.7	ef
BIO 126 autoclaved	52.1	cde	115.8	de	71.6	b	107.6	f
GS 88 10^8 cells/ml	51.8	cde	60.7	c	0.0	a	52.4	bc
GS 88 culture filtrate	69.9	def	103.6	de	75.1	b	81.0	de
GS 88 autoclaved	34.5	abcd	122.1	de	31.8	b	97.5	ef
GA 102 10^8 cells/ml	0.0	a	102.9	de	0.0	a	60.9	cd
GA 102 culture filtrate	42.3	bcde	113.9	de	105.2	b	102.5	ef
GA 102 autoclaved	81.8	ef	125.5	e	114.4	b	110.2	f
GS 37 10^8 cells/ml	0.9	ab	26.8	b	0.0	a	40.2	bc
GS 37 culture filtrate	28.0	abc	103.8	de	103.7	b	92.9	ef
GS 37 autoclaved	17.9	abc	109.4	de	97.4	b	107.4	f

*Calculated on the lesion diameter. Values in the same column followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P < 0,05$).

Table 2

Effect of cell suspension, cell-free culture filtrate and autoclaved cells of *M. pulcherrima* isolates BIO126, GS88, GA102, and GS37, applied in artificial wounds of "Golden delicious" apple, on the growth of different post-harvest rots. Storage at 4°C for 21 (Botrytis rot) and 28 days (Penicillium rot).

Treatment	Percentage of control*			
	B.cinerea		*P.expansum*	
Uninoculated Control	0.0	a	0.0	a
Inoculated control	100.0	cd	100.0	cd
BIO 126 10^8 cells/ml	57.1	b	0.0	a
BIO 126 culture filtrate	116.3	de	89.7	b
BIO 126 autoclaved	121.3	e	101.2	d
GS 88 10^8 cells/ml	5.3	a	0.0	a
GS 88 culture filtrate	103.6	cde	98.5	cd
GS 88 autoclaved	113.3	cde	98.2	cd
GA 102 10^8 cells/ml	9.5	a	0.0	a
GA 102 culture filtrate	95.6	c	85.5	b
GA 102 autoclaved	100.0	cd	93.1	bcd
GS 37 10^8 cells/ml	0.0	a	0.0	a
GS 37 culture filtrate	102.7	cd	88.5	b
GS 37 autoclaved	103.6	cde	92.4	bc

*See table 1.

Table 3

Inhibition of the mycelium growth of *Alternaria* sp. (3-A), *Botrytis cinerea* (3-B), *Monilia* sp. (3-C), *Penicillium expansum* (3-D) by *M. pulcherrima* isolates BIO126, GS88, GA102, and GS37 in dual culture on different media at room temperature in the dark.

3-A

Substrates	Days of co-culturing**	Mean inhibition (%) *			
		BIO 126	GS88	GA102	GS37
PDA	12	0.0 a	0.0 a	0.0 a	0.0 a
YPD	28	No growth on control plates			
NYDA	28	No growth on control plates			
CZAPEK	12	29.2 d	6.3 ab	10.4 bc	17.7 c
MALT	15	0.0 a	0.0 a	0.0 a	0.0 a
APPLE	12	24.0 b	25.0 b	24.0 b	31.3 b

3-B

Substrates	Days of co-culturing**	Mean inhibition (%) *			
		BIO 126	GS88	GA102	GS37
PDA	5	0.0 a	0.0 a	12.5 b	0.0 a
YPD	5	10.4 c	7.3 b	6.3 b	8.3 bc
NYDA	5	18.8 c	12.5 b	13.5 bc	14.6 bc
CZAPEK	5	0.0 a	0.0 a	0.0 a	0.0 a
MALT	5	0.0 a	0.0 a	0.0 a	0.0 a
APPLE	5	0.0 a	0.0 a	0.0 a	0.0 a

3-C

Substrates	Days of co-culturing**	Mean inhibition (%) *			
		BIO 126	GS88	GA102	GS37
PDA	20	11.5 b	4.2 a	4.2 a	1.0 a
YPD	28	No growth on control plates			
NYDA	28	No growth on control plates			
CZAPEK	28	No growth on control plates			
MALT	15	0.0 a	0.0 a	0.0 a	0.0 a
APPLE	10	6.3 a	20.8 b	15.6 b	15.6 b

3-D

Substrates	Days of co-culturing**	Mean inhibition (%) *			
		BIO 126	GS88	GA102	GS37
PDA	5	14.6 b	12.5 b	7.3 ab	10.4 b
YPD	5	17.7 b	19.8 b	16.7 b	18.8 b
NYDA	5	12.5 b	20.8 b	16.7 b	18.8 b
CZAPEK	5	0.0 a	0.0 a	0.0 a	0.0 a
MALT	5	4.2 a	0.0 a	0.0 a	0.0 a
APPLE	5	17.7 b	18.8 b	29.2 c	17.7 b

*Values in the same row followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P < 0,05$). Control, always indicated with an a, is implied.

**Days needed by control mycelium to reach a radius of 32 mm.

Table 4

Effect of cell suspension, cell-free culture filtrate and autoclaved cells of *M. pulcherrima* isolates BIO126, GS88, GA102, and GS37, on spore germination of *Botrytis cinerea*, by co-culturing in PDB at 25°C for 12 hours.

		B. cinerea spore germination (%)*	
Control		99.0	e
BIO126	culture filtrate	98.7	e
	autoclaved	99.3	e
	10^8 cells ml^{-1}	0.0	a
	10^7 cells ml^{-1}	25.7	b
	10^6 cells ml^{-1}	98.7	e
GS88	culture filtrate	97.7	e
	autoclaved	98.7	e
	10^8 cells ml^{-1}	0.0	a
	10^7 cells ml^{-1}	47.7	c
	10^6 cells ml^{-1}	99.3	e
GA102	culture filtrate	98.3	e
	autoclaved	99.3	e
	10^8 cells ml^{-1}	0.0	a
	10^7 cells ml^{-1}	73.0	d
	10^6 cells ml^{-1}	97.0	e
GS37	culture filtrate	99.0	e
	autoclaved	99.7	e
	10^8 cells ml^{-1}	0.0	a
	10^7 cells ml^{-1}	51.7	c
	10^6 cells ml^{-1}	97.7	e

* Values in the same column followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P < 0,05$).

Table 5

Efficacy of *Metschnikowia pulcherrima* isolates BIO126, GS88, GA102, and GS37 against *Penicillium expansum*, *Alternaria* sp., and *Botrytis cinerea*, evaluated by dipping boxes of "Golden delicious" apples in a cell suspension of the antagonist and storing in controlled atmosphere at 1°C for 4 months (first survey) and 8 months (second survey).

First Survey Treatments	Rotted apples (%) *P. expansum*	*B. cinerea*	*Alternaria* sp.	Total
Uninoculated Control	2.3	4.9	0.0	7.2 a**
Inoculated control	7.8	15.2	0.3	23.3 b
Thiabendazole ($3*10^{-2}$ g l^{-1})*	3.2	4.1	1.4	8.7 a
Yeast BIO 126	0.9	8.1	0.0	9.0 a
Yeast GS 88	4.5	9.1	0.0	13.6 a
Yeast GA 102	1.7	12.2	0.0	13.9 a
Yeast GS 37	4.6	9.4	0.0	14.0 a

Second Survey Treatments	Rotted apples (%) *P. expansum*	*B. cinerea*	*Alternaria* sp.	Total
Uninoculated Control	2.4	5.1	1.1	8.7 a**
Inoculated control	3.1	21.7	2.3	27.1 b
Thiabendazole ($3*10^{-2}$ g l^{-1})*	1.5	1.2	2.3	5.0 a
Yeast BIO 126	2.1	5.7	0.3	8.1 a
Yeast GS 88	3.1	4.1	0.7	7.9 a
Yeast GA 102	4.7	2.4	2.0	9.2 a
Yeast GS 37	3.6	4.7	0.9	8.1 a

All isolates were applied at 10^7cells ml^{-1}.
*Apples were treated with 150 ml hl^{-1} of Tecto 20S (thiabendazole: 19,7 %)
**Values followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P < 0,05$).

Figure captions

Fig. 1. Dual culture of *Alternaria* sp. and *M. pulcherrima* BIO126 (left dish) and GS37 (right dish) on APPLE substrate in Petri dish for 12 days at room temperature in the dark. It is possible to notice the inhibition of the pathogen mycelium growth on the direction of the yeast strip side.

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