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Control of *Penicillium expansum* and *Botrytis cinerea* on apple combining a biocontrol agent with hot water dipping and acibenzolar-S-methyl, baking soda, or ethanol application

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ABSTRACT

The application of a cell suspension of the BIO126 strain of *Metschnikowia pulcherrima* proved to be highly effective in the control of blue and grey mould, two of the most severe postharvest diseases on apple fruit. The possibilities to integrate the application of the antagonist with chemicals, such as acibenzolar-S-methyl (ASM), ethanol, or sodium bicarbonate, and a heat treatment were investigated in this work. The fruits were stored at 23°C for 5 days and at 4°C for 20 days. The antagonist, applied at 10^8 cells ml^{-1}, proved to be the key element for the control of both pathogens, resulting more efficient after cold storage, with a reduction of 56.6% and 97.2% of the lesion diameter of blue and grey mould. Ethanol and acibenzolar-S-methyl permitted a partial reduction of grey mould severity. Heat treatment and sodium bicarbonate significantly improved the efficacy of the biocontrol agent against blue mould with storage at 23°C.

To study the possibility of a single application of the biocontrol agent (10^7 cells ml^{-1}) with reduced

dosages of sodium bicarbonate or ethanol, the viability of the microorganism with these chemicals was studied and a new set of experiments was established. Against both pathogens, the higher reduction of the lesion diameter was obtained treating simply with the biocontrol agent. Significant results on blue mould were provided by the application of 20% ethanol or 5% sodium bicarbonate before the biocontrol agent and by the application of BIO126 in 0.1% sodium bicarbonate.

The application of the cell suspension of BIO126 *M. pulcherrima*, preceded or not by a pre-treatment with sodium bicarbonate or ethanol, could become a successful alternative to fungicide usage in postharvest disease control of pome fruit. The fungistatic effect of ethanol or sodium bicarbonate could be associated to the effect of the biocontrol agent, persistent on the fruit for long periods.

Keywords: benzothiadiazole, biocontrol agent, blue mould, ethanol, grey mould, heat treatment, sodium bicarbonate.

1. Introduction

Fungal pathogens are the main cause of postharvest losses of apples. These losses may reach as much as 50% during the shelf life of the fruits (Eckert and Ogawa, 1988). Blue mould, caused by *Penicillium expansum* Link, and grey mould, caused by *Botrytis cinerea* Pers.:Fr., are severe diseases worldwide on pome fruit, even in production areas where the most advanced storage technologies are available, such as Northern Italy (Romano et al., 1983). Currently, the most used and effective management strategy is the application of synthetic fungicides, but alternative methods are needed because of growing public concerns over the human health and environmental risks, the development of fungicide resistant strains of both pathogens (Spotts and Cervantes, 1986), and the problems encountered in the reregistration process by some of the most effective fungicides, especially for minor uses (Gullino and Kuijpers, 1994).

Biological control with microbial antagonists has emerged as a promising alternative, with lower

environmental impact, either alone or as part of an integrated pest management to reduce synthetic fungicides usage (Wilson and Wisniewski, 1994). At present, a class of products containing *Pseudomonas syringae* Van Hall (Janisiewicz and Jeffers, 1997), a product containing *Candida oleophila* Montrocher (Hofstein and Fridlender, 1994), and a biofungicide based on *Cryptococcus albidus* (De Koch, 1998), active against *Botrytis* spp., *Penicillium* spp., and other fungal pathogens, are available for postharvest protection in a few countries, but other biological products are under development (Spadaro and Gullino, 2004).

Recently, different strains of the yeast *Metschnikowia pulcherrima* were isolated in our Department and studied for their efficacy and mode of action (Piano et al., 1997; Spadaro et al., 2002). All of them are effective against *P. expansum* and *B. cinerea* on apples with a varying degree of control and act through competition for nutrients and/or space, without producing toxic metabolites *in vivo*. The strain BIO126 was chosen for further studies. Different physical and chemical control methods could be used together with the application of antagonists to obtain more consistent results.

Pre-storage hot water dips of fruits at temperatures superior to 40°C are effective in controlling storage decays, not only by reducing the pathogen inoculum but also by enhancing the resistance of the fruit tissue, influencing host metabolism and ripening (Barkai-Golan and Philips, 1991). Postharvest dips are applied for a few minutes at high temperatures, because fungal spores and latent infections are either on the surface or in the first few cell layers under the peel of the fruit (Lurie, 1998). Hot water treatment may eliminate incipient infections, by removing spores from wounds and acting directly on their viability, and induce fruit defence mechanisms in the outer layers of epicarp which inhibit pathogen growth (Schirra et al., 2000). Moreover, generally regarded as safe (GRAS) compounds have been applied in hot water to improve the efficiency of their antifungal action (Smilanick et al., 1995).

The chemical products chosen for studying the possibility of integration with the biocontrol agent are two natural compounds, ethanol and sodium bicarbonate, and an elicitor of systemic acquired resistance in the host tissue, acibenzolar-S-methyl (ASM).

Ethanol occurs naturally in fruit and many other food products and the toxic effects of the alcohol on spores of fungal pathogens have been reported (Eckert and Ogawa, 1988). The alcohol has been tested for control of brown rot, also associated with hot water treatment (Margosan et al., 1997), with varying degrees of success: the flesh of the fruit treated with the alcohol was significantly firmer and injury to the fruit did not occur.

Baking soda ($NaHCO_3$), a carbonic acid salt, is a common food additive for pH-adjustment, taste, texture modification and spoilage control, permitted unrestrictedly for many applications by European and North American regulations. Moreover, it is listed as an approved ingredient on organic products (Mazzini, 2002). Sodium bicarbonate showed an antimicrobial activity against *Penicillium digitatum* on citrus fruit (Smilanick et al., 1999). Sodium bicarbonate is a poor eradicant that does not kill spores and its inhibitory action is not very persistent. Biocontrol agents, which can persist for long periods, may provide protection of the fruit from reinfection after treatment (Teixidó et al., 2001).

Acibenzolar-s-methyl (or benzothiadiazole) is a chemical plant activator of the systemic acquired resistance (SAR) for crop protection (Kessmann et al., 1996). It is commercialised in some countries, including Italy, where it can be used on tomatoes, tobacco, cucurbits, pear, and hazelnut trees (Friedrich et al., 1996, Benhamou and Belanger, 1998). Acibenzolar-S-methyl has been tested on strawberry against *B. cinerea*: sprayed several times it delayed the development of grey mould on harvested fruits by about two days, increasing their shelf-life (Terry and Joyce, 2000). One preharvest spray of the plant activator on melon leaves decreased the incidence and extent of postharvest diseases (Huang et al., 2000).

The aim of this study was to determine if the attacks of blue mould and grey rot on apple were reduced by a combination of the biocontrol agent *M. pulcherrima* strain BIO126 with a chemical elicitor of resistance, sodium bicarbonate, or ethanol and hot water treatment. The experiments were devoted to develop an integrated strategy to control postharvest decay on apple fruit caused by *B. cinerea* and *P. expansum*, as effective as the traditional chemical control. A specific objective was

the evaluation of positive or negative interactions between the three alternative methods of disease control. The possibility of a single application of the biocontrol agent together with a chemical compound – ethanol or sodium bicarbonate – was also considered.

2. Materials and methods

2.1 Antagonist and pathogens preparation

Metschnikowia pulcherrima (Pitt) M.W. Miller strain BIO126, was isolated from the carposphere of an apple cv Golden delicious harvested in an unsprayed orchard located in Piedmont, Northern Italy, and it was studied for its efficacy and mechanism of action (Spadaro et al., 2002). The microorganism culture was 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) 25 mM. The strain was grown in Nutrient Yeast Dextrose Broth (NYDB), as described by Droby et al., 1989. Inocula of the antagonist for all experiments were prepared by subculturing in 250 ml Erlenmeyer flasks containing 75 ml of NYDB and incubating on a rotary shaker (100 rpm) at 25°C for 48 h. Yeast cells were collected by centrifugation at 1500 x *g* for 10 min, washed and resuspended in sterilized Ringer solution (pH 6.9$\pm$0.1; Merck), and brought to a standard concentration of 10^8 cells ml^{-1} by direct counting with a haemacytometer.

Two isolates of *B. cinerea* and two isolates of *P. expansum*, obtained from rotted apples cv Golden delicious and selected for their virulence, were used as a mixture during the experiments to ensure a high level of disease. Each strain was stored in tubes with Potato Dextrose Agar (PDA; Merck) and 50 mg l^{-1} of Streptomycin Merck at 4°C. Spore suspensions used for fruit inoculation were prepared as described in Spadaro et al. (2002).

2.2 Efficacy of the combination of biological, chemical and heat treatments

Apples (*Malus domestica*, cv Golden delicious), harvested in an Italian orchard conducted according to integrated pest management practices, were disinfected in sodium hypochloride

154 (NaClO, 1.0 % as chlorine) and rinsed under tap water, dried at room temperature and punctured
155 with a sterile needle at the equatorial region (3 mm depth; 3-4 mm wide; 3 wounds per fruit). Heat-
156 treated fruit were dipped in deionised water at 50°C for 3 and 10 minutes, and left to dry for 1 hour.
157 Benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester, kindly provided by Syngenta (50 % a.
158 i. in wettable powder; Bion; Syngenta), was applied at 1.0 mg ml^{-1}, commercial-grade ethanol at
159 10.0% wt vol^{-1}, and sodium bicarbonate (pH 8.3 to 8.6; Sigma-Aldrich) at 3.0 % wt vol^{-1}. Also a
160 standard chemical (thiabendazole, Tecto 20S, Elf Atochem Agri Italy, 19.7% a. i.) was employed at
161 0.3 mg a. i. ml^{-1}. The chemical compounds tested were applied diluted in sterile distilled water.
162 Apples were dipped for 10 seconds in beakers containing 500 ml of the chemical suspension.
163 After 3 hours, fruits exposed to treatments alternative to fungicides were treated with 30 µl of the
164 cell suspension (10^8 ml^{-1}) of *M. pulcherrima* strain BIO126 per wound. After 24 hours at room
165 temperature, 30 µl of the spore suspension of *B. cinerea* or *P.expansum* (10^5 ml^{-1}) were pipetted
166 into the apple wounds. When dry, fruits were randomly packed in commercial plastic trays and
167 stored at 23°C for 5 days and at 4°C for 20 days. Three fruits per treatment were used (9 inoculation
168 sites). The severity of the diseases was determined measuring the mean lesion diameter of the rotted
169 apples. The experiments were carried out three times.
170
171 **2.3 Antagonist survival in co-culture with ethanol and sodium bicarbonate**
172 Erlenmeyer flasks containing 30 ml of NYDB were prepared and ethanol or sodium bicarbonate
173 added at different concentrations. Commercial-grade ethanol was employed at the final
174 concentration of 20%, 10%, 5%, and 2% wt vol^{-1} in the first trial and 5%, 4%, 3%, 2%, and 1% wt
175 vol^{-1} in the second one. Sodium bicarbonate (pH 8.3 to 8.6; Sigma-Aldrich) was used at the final
176 concentration of 5%, 3%, 1%, and 0.5% wt vol^{-1} in the first experiment and 0.5%, 0.1%, 0.05%, and
177 0.01% wt vol^{-1} in the second one. After subculturing the antagonist in NYDB 48 hours and counting
178 with the haemacytometer as previously described, 30 µl of BIO126 cell suspension (10^8 or 10^7 cells
179 ml^{-1}) were added to the Erlenmeyer flasks containing the different suspensions of the two chemicals

(final concentration: respectively 10^5 or 10^4 cells ml^{-1}). The flasks were incubated on a rotary shaker (100 rpm) at 25°C for 36 hours. The viability of the cells was evaluated through direct observation and the cell suspension was counted by haemacytometer.

2.4 Efficacy of different combinations of the biological and chemical treatments

Apples cv Golden delicious, harvested in an Italian orchard conducted by following integrated pest management, were disinfected, dried and punctured as previously described. Some fruits were double-treated, by immersion in an ethanol or sodium bicarbonate suspension and successive inoculation with the antagonist. Other fruits were exposed to one single treatment, by immersion in a combination of the antagonist and lower concentrations of ethanol or sodium bicarbonate. The fruits treated twice were dipped for 60 seconds in a commercial-grade ethanol suspension (10 or 20% wt vol^{-1}) or in a sodium bicarbonate (pH 8.3 to 8.6; Sigma-Aldrich) suspension (3 or 5% wt vol^{-1}), left to dry for 3 hours and then treated with 30 µl of BIO126 cell (10^7 ml^{-1}) suspension. Fruits exposed to single treatment were dipped for 60 seconds in a water suspension containing 10^7 cells ml^{-1} of the antagonist and commercial-grade ethanol (1 or 2% wt vol^{-1}) or sodium bicarbonate (0.1% wt vol^{-1}). After 24 hours at room temperature, 30 µl of the spore suspension of *B. cinerea* or *P. expansum* (10^5 ml^{-1}) were pipetted in the wounds of each fruit. When dry, the apples treated differently were randomly packed in commercial plastic trays and stored at 23°C for 5 days. Five fruits per treatment were used (15 inoculation sites). The severity of the diseases was determined by the mean lesion diameter in mm of the rotted apples. The experiments were carried out twice.

2.5 Statistical analysis

Data of the single experiments of the three combined treatments were analysed through a three-way variance analysis. The significant interactions were chosen and successively analysed through a Duncan's Multiple Range Test. The same test was employed for the analysis of the single

experiments of antagonist survival and efficacy of different combinations of biological and chemical treatments. The program SPSS-WIN was used.

3. Results

3.1 Combinations of biological, chemical and heat treatments

The experiments against *B. cinerea* and *P. expansum* were carried out three times and a three-way variance analysis was executed on each repetition (Table 1).

In the trials against *B. cinerea* and storage at 23°C, the interaction between the three variables was not significant (P=0.20) as the one between chemical and heat treatments (P=0.13), while the interactions between biological and chemical treatments (Table 2) and between biological and heat treatments (Table 3) were. In the experiment at 4°C against grey mould the analysis of variance gave in the four cases a P of 0.00. Although the interactions between chemical and heat treatments were significant, they were not considered because the study was focused on the identification of possible synergisms between the application of the biocontrol agent BIO126 and physical or chemical treatments alternative to the traditional fungicide.

In the experiments against *P. expansum*, after 5 days of storage at 23°C, the interaction between the three variables was significant, as were the interactions between biological and chemical treatments (Table 2) and between biological and heat treatments (Table 3). In the trial at 4°C against blue mould the analysis of variance gave in the four cases a P of 0.00.

3.2 Efficacy of the combination of biological and chemical treatments

The application of the cell suspension of BIO126 was effective against *B. cinerea*, providing an almost complete control of the disease, either alone or in combination with the chemical products, storing the apples at 23°C or 4°C (Table 2). Without biological treatment, the chemicals employed were less effective. After storage at 23°C, only ethanol and acibenzolar-S-methyl reduced the

pathogen growth significantly (respectively 29.7% and 14.8% of the lesion diameter). Storing at 4°C, ethanol and acibenzolar-S-methyl resulted in a higher protection of the fruit from grey mould, although not significantly different from the control, with a reduction of the pathogen severity of 26.8% and 21.5%. Thiabendazole, the chemical product commercially used, resulted completely ineffective against the strains of grey mould used.

From the analysis of the interactions between the biological and chemical treatments in the control of blue mould, after storage at room temperature (Table 2), the most consistent result was offered by the combined application of the antagonist and sodium bicarbonate (57.7% of control). The biocontrol agent employed alone was less effective (27.6%), than applied together with other chemical products, such as ethanol and acibenzolar-S-methyl (35.5% and 41.9%), although the difference was not significant. Only sodium bicarbonate improved significantly the efficacy of the application of BIO126 (56.7%). Also without biological treatment, sodium bicarbonate was effective (28.4%). Thiabendazole, applied alone, provided the more consistent efficacy with respect to the other chemicals (29.8%).

In the trial of efficacy against blue mould and storage at 4°C (Table 2), BIO126 acted significantly either alone (56.6% of control) or combined with acibenzolar-S-methyl (57.4%), sodium bicarbonate (56.2%) and ethanol (52.3%). In comparison with the experiment carried out at 23°C, the antagonist was much more efficient at the low temperatures of storage. The three chemicals and thiabendazole reduced significantly the pathogen attack with respect to the control, but the presence of the yeast resulted in a more consistent efficacy. Ethanol alone showed a disease severity of *P. expansum* (67.5%) similar to thiabendazole (65.1%).

3.3 Efficacy of the combination of biological and heat treatments

In Table 3 the effectiveness against *B. cinerea* of the application of two different hot water treatments with BIO126 is reported. The microorganism was effective in every treatment, especially after 20 days of storage of the fruit at 4°C, when the control was complete. When apples were

stored at room temperature, the best results were shown by the biocontrol agent applied alone (5.2% of pathogen severity with respect to the control) or with heat treatment (4.8% and 4.5%). Ten minutes of hot water treatment led to a significant reduction of the lesion diameter (12.9%) in apples stored at 23°C, but the same treatment followed by storage at 4°C was ineffective. Three minutes of hot water immersion brought a significant result only in the case of apples stored at 4°C (12.9% of reduction of the lesion diameter).

Combining biological and hot water treatments against *P. expansum*, the strain BIO126 of *M. pulcherrima* provided a good control of the pathogen at 23°C (29.2% of reduction) and 4°C (38.2%). The heat treatment improved the efficacy of the antagonist against blue mould after storage at 23°C and 4°C, but the difference was statistically significant only at room temperature. Immersion in hot water alone resulted in a significant control, with more effective result for the longer treatment (41.3% of reduction), in the trial carried out at 23°C and in an inconsistent control in the experiment conducted at 4°C.

3.4 Antagonist survival in co-culture with ethanol and sodium bicarbonate

When *M. pulcherrima* strain BIO126 was cultivated for 48 hours in NYDB with different concentrations of ethanol (20%, 10%, 5%, and 2%) no cell growth and no viability was detectable in all alcohol concentrations except at 2%. The experiment was repeated with co-culture in 5%, 4%, 3%, 2%, and 1% ethanol in the synthetic broth. At 1% and 2% ethanol, the total number of cells and the viability were similar to the control. At 3% ethanol the total number of antagonistic cells were 0.5% compared to the control: the microorganism had a slowed growth but the cells were alive. At 4 and 5% ethanol all yeast cells were not viable and did not multiply.

Sodium bicarbonate was applied in the NYDB liquid substrate at 5%, 3%, 1%, and 0.5% in the first experiment. At all concentrations the antagonist could not survive and grow: the yeast cells were not viable. In the second trial the effects of 0.5%, 0.1%, 0.05%, and 0.01% sodium bicarbonate were tested on the viability of the strain BIO126. The antagonist had a slowed growth at 0.1% salt

concentration and the growth was similar to the control at 0.05% and 0.01% sodium bicarbonate.

3.5 Efficacy of different combinations of the biological and chemical treatments

All treatments were significantly different from the control in the trial against *B. cinerea* (Table 4). Treatments where the biocontrol agent was applied alone (lesion diameter reduced to 5.7%) or after the application of 10% ethanol (3.9%), 20% ethanol (5.2), 3% sodium bicarbonate (6.9%) or 5% sodium bicarbonate (9.6%)were particularly effective. Fruits treated by immersion in a combination of the antagonist at 10^7 ml^{-1} and lower concentrations of ethanol or sodium bicarbonate, still significantly different from the control, were more susceptible to *B. cinerea*: the BIO126 cell suspension in 2% ethanol reduced the pathogen lesions by 55.4%, in 1% ethanol by 43.3% and in 0.1% sodium bicarbonate by 37.3%.

In the experiments carried out against *P. expansum* all treatments caused a significant reduction of the lesion diameter compared to the control. The application of the cell suspension of BIO126 offered the higher level of control of the pathogen (14.7% of disease severity). When the application of 20% ethanol or 5% sodium bicarbonate preceded the biological treatment, the lesion diameter resulted greatly reduced (27.8 and 22.7%). Lower control resulted from the application of 10% ethanol or 3% sodium bicarbonate before the yeast cell suspension (42.0 and 50.3%). A single application of the antagonist cell suspension in 1% and 2% ethanol resulted in a disease severity of 52.7 and 43.4%. A consistent efficacy was also showed by the application of the BIO126 cell suspension in 0.1% sodium bicarbonate).

All fruits treated with 3% or 5% sodium bicarbonate, needed a final brushing or washing to eliminate the residues of the salt. No sign of phytotoxicity was observed.

4. Discussion

The strain BIO126 of *Metschnikowia pulcherrima* proved its antagonistic potential in controlled and semi-commercial trials reducing blue and grey mould on apples. When apples cv Golden delicious

were dipped in an antagonist cell suspension and stored at 1°C for 8 months, BIO126 showed postharvest rot control similar to benzimidazoles (Spadaro et al., 2002). The main mode of action involved in the biocontrol is competition for nutrients or space although a direct interaction can not be excluded (Spadaro et al., 2002). The biocontrol agent is very effective against *B. cinerea* but shows less consistent results towards *P. expansum*, and it is not as effective towards latent infections or previously established pathogens.

Since alternatives to chemical control do not possess generally a broad spectrum of activity and they are not as effective as fungicides, a combination of alternative methods could be more effective and consistent than one alternative alone. Hot water treatment, sodium bicarbonate and ethanol are non-curative treatments whose effects *in vivo* are primarily fungistatic and not very persistent. Acibenzolar-S-methyl is an elicitor of systemic acquired resistance in the host tissue, that could help in the defence of the fruit from the pathogens.

For the experiments carried out, two temperatures of storage were chosen. Room temperature (23°C) normally favours the growth of the pathogens (Snowdon, 1990), whereas 4°C is one of the temperatures for commercial fruit storage and favours the yeast antagonist fitness (Spadaro et al., 2002).

Heat treatment is effective in sanitizing the fruit and enhancing the wound curing process. It has the added benefit of improving fruit colour but does not lead to softening, since it inhibits the synthesis of cell wall hydrolytic enzymes in the apple fruit, and reduces ethylene production (Lurie, 1998). Heat treatment could also damage the tissue of the fruit and, for this reason, some preliminary trials were carried out to assess the optimum time-temperature regime (data not published). The lowest times (1' or 2' at 50°C) were totally ineffective in controlling blue mould and grey rot and the highest ones (30' at 50°C) caused damages to the apples, such as peel browning, as already noted by Klein and Lurie (1992). Pasteurisation with hot water at 50°C showed an ET_{50} of 1.5 minutes for *B. cinerea* spore germination and an ET_{50} of 0.9 minutes for the germ tube elongation of the same pathogen (Fallik et al., 1996). Combining heat treatment with an antagonist, in some cases, could

complement the sanitary effect of the heat treatment with the residual protection of the biocontrol agent (Conway et al., 1999).

Mainly for its inability to survive at 50°C, the antagonist was applied after hot water treatment. The problem of applying the biocontrol agent before hot water treatment is that the microorganism must be heat-tolerant (Leverentz et al., 2000), but in this case problems of registration could rise. From growth at different temperatures (data not shown), it resulted that the BIO126 isolate does not grow at temperatures of 37°C or more, which is important from a toxicological point of view, especially in the case of contact with immunosuppressed patients (Mohl et al., 1998).

Ethanol can be effective in reducing postharvest decay immediately after harvest by disinfecting the fruits. The major target of ethanol stresses is the lipid membrane but it has many other effects, such as denaturation of proteins on fungal cells (Mishra, 1993). A 10% ethanol solution, concentration chosen for the experiments carried out, had previously shown to be effective in controlling *Monilinia fructicola* and *Rhizopus stolonifer* on peaches and nectarines (Margosan et al., 1997) and *Penicillium digitatum* on lemons (Smilanick et al., 1995). Injury to the fruit did not occur, no odours or residues (differently from sodium bicarbonate) from the fruit were detected and an increased firmness of the fruit was a benefit, permitting an extension of the shelf-life (Margosan et al., 1997). On the other side, ethanol vapours can induce concern about manipulation and storage, so that a vapour abatement system should be developed, with increased cost for equipment and energy to operate it and a delay in cooling fruit before storage. The loss of ethanol efficacy after prolonged storage periods is probably an indication that decays developing at this time are the result of latent or secondary infections, rather than of surface wounds infections (Lichter et al., 2002).

Sodium bicarbonate is inexpensive, readily available and can be used with a minimal risk of injury to the fruit. The inhibitory activity of sodium bicarbonate depends on the presence of salt residues within the wound infection courts occupied by the fungus and on interactions between this residue and constituents of the peel. In previous trials sodium bicarbonate was applied for control of *B. cinerea* on apple at 1% but it resulted ineffective (data not published). Oranges dipped for three

361 minutes at room temperature in water with 2 to 4 % of sodium bicarbonate reduced decay caused by
362 *Penicillium italicum* more than 50 % (Palou et al., 2001). For our experiments a concentration of
363 3% sodium bicarbonate was chosen. A disadvantage of sodium bicarbonate is that heating the
364 solution will cause carbon dioxide evolution into air with a concomitant increase in solution pH but
365 the addition of hypochlorite should permit the heating of the salt solution (Smilanick et al., 1999).
366 Another issue of the treatments with sodium bicarbonate, differently from ethanol, is that the salt
367 residues should be eliminated from the fruit skin before commercialisation.
368 Acibenzolar-S-methyl has been used until now before harvesting for the protection of fruit from
369 postharvest diseases. The chemical has an efficacy inferior to traditional fungicides and it needs a
370 relatively long period of time after its application, before pathogen infection, to provide positive
371 results (Kessmann et al., 1996). Moreover, to show positive results, it needs more than one
372 application. In these experiments, it has been used once in postharvest 48 hours before the pathogen
373 inoculation. In previous trials conducted in our laboratory, also acetylsalicylic acid was employed
374 but it resulted totally ineffective (data not shown).
375 In the experiments carried out, the strains of *B. cinerea* and *P. expansum* used were probably
376 resistant to benzimidazoles, as can be observed from the low efficacy of thiabendazole. This low
377 sensitivity is confirmed by recent evaluations on postharvest pathogens (Bertetti et al., 2003).
378 The yeast antagonist resulted really effective in the control of grey mould on apples stored at room
379 temperature or at 4°C. The experiments of combination with other physical or chemical treatments
380 resulted unnecessary, with no significant increase of the protection from the pathogen. Hot water
381 treatment alone showed inconsistent results against grey mould. Ethanol and acibenzolar-S-methyl
382 permitted a partial reduction of the disease severity of *B. cinerea* but at a level not commercially
383 acceptable.
384 The effect of the application of BIO126 on apples was less consistent against blue mould, a more
385 harmful disease also involved in the production of mycotoxins. After storage at 23°C, heat
386 treatment significantly improved the efficacy of the biocontrol agent, but not storing at low

387 temperatures, where BIO126 was effective alone. Sodium bicarbonate significantly improved the
388 efficacy of the antagonistic microorganism when apples were stored at 23°C but any chemical did
389 not improve significantly the effect of BIO126 at 4°C.
390 In the experiments carried out to study the possibility of a single application of the biocontrol agent
391 with reduced dosages of sodium bicarbonate or ethanol, the strain of *M. pulcherrima* resulted
392 compatible with low concentrations of ethanol (1 to 2%), as results also from the fact that this
393 species of yeast is involved in the first step of the fermentation process of apples for cider-making
394 (Beech, 1993). As the ethanol level raises (2 to 4%), these initial fermenters die out and the
395 microbial succession is taken over by *Saccharomyces cerevisiae.*
396 BIO126 and other biocontrol agents are not in general compatible with high concentrations of
397 sodium bicarbonate, that reduces the growth and the viability of the microorganisms. Other
398 organisms, such as *Pantoea agglomerans*, are tolerant to 2% sodium bicarbonate at room
399 temperature, although the culturability of the bacterium is reduced by more than 1000-fold after 30
400 minutes in 2% sodium bicarbonate (Teixidó et al., 2001).
401 In the new set of experiments, BIO126 was applied at lower concentrations (10^7 cells ml^{-1} instead of
402 10^8 cells ml^{-1}) to assess possible synergistic effects with the two chemicals.
403 Against grey mould, the best results were obtained when the yeast was applied alone. Ethanol and
404 sodium bicarbonate, when applied before, were not necessary to improve the efficacy. When the
405 biocontrol agent was applied in a solution with 1% and 2% ethanol or 0.1% sodium bicarbonate, the
406 control was reduced, probably because the fitness of the microorganism was lower.
407 All the treatments against *P. expansum* showed a significant reduction of the disease severity. The
408 higher reduction of the lesion diameter was obtained simply treating with the biocontrol agent.
409 Significant results were provided also by the application of 20% ethanol or 5% sodium bicarbonate
410 before the biocontrol agent. Also the application of BIO126 in 0.1% sodium bicarbonate
411 significantly reduced the lesion diameter of the rots. *P. expansum* has a behaviour related to the
412 physiology of the fruit: smaller and less ripe apples are more resistant to the attack of the pathogen.

During the experiments, great effort was employed in the selection of uniform fruits.

In conclusion, it is possible to associate the fungistatic effect of ethanol or sodium bicarbonate to the effect of the biocontrol agent, persistent on the fruit for long periods. It is not useful, even if it could be more practical to apply the two treatments in one single step, because positive effects can not be revealed and the chemicals could inhibit the growth of the antagonist. An evaluation of the antagonist population survival in apple wound could clarify this question. Pre-treatment with sodium bicarbonate or ethanol and successive application of the cell suspension of BIO126 *M. pulcherrima* could become an alternative to fungicide usage in postharvest disease control of pome fruit, but registration and development studies to obtain a commercial product are necessary.

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517 **Tables**

518 Table 1

519 Summary of the significant effects (indicated as *P*>F) of antagonist (Ant), heat treatment (Heat),
520 and chemical compounds (Chem) on the lesion size of grey and blue mould on apples cv Golden
521 delicious after storage at 23°C for 5 days and at 4°C for 20 days.

522

	Grey mould (23°C)	Grey mould (4°C)	Blue mould (23°C)	Blue mould (4°C)
Ant x Chem x Heat	0.20	0.00	0.02	0.00
Ant x Chem	0.01	0.00	0.04	0.00
Ant x Heat	0.00	0.00	0.05	0.00
Chem x Heat	0.13	0.00	0.09	0.00

523

Table 2

Effect of a cell suspension of *M. pulcherrima* strain BIO126, combined or not with acibenzolar-S-methyl, ethanol, and sodium bicarbonate, on *B. cinerea* and *P. expansum* growth on apples cv Golden delicious. Storage at room temperature (23°C) for 5 days and at 4°C for 20 days.

Treatment	Grey mould severity (mm)[a]				Blue mould severity (mm) [a]			
	23°C storage		4°C storage		23°C storage		4°C storage	
Control	37.6	d	30.2	bc	28.7	d	25.8	d
Thiabendazole [b]	36.2	d	29.4	bc	20.1	bc	16.8	bc
Acibenzolar-S-methyl [c]	32.0	c	23.7	b	23.4	cd	20.1	c
Ethanol [d]	26.4	b	22.1	b	25.5	d	17.4	bc
$NaHCO_3$ [e]	35.3	d	34.0	c	20.5	bc	19.0	c
10^8 cells ml^{-1} BIO126	1.5	a	0.8	a	20.8	bc	11.2	a
Thiabendazole [b] + 10^8 cells ml^{-1} BIO126	1.7	a	1.1	a	15.9	ab	7.9	a
Acibenzolar-S-methyl [c] + 10^8 cells ml^{-1} BIO126	1.8	a	1.1	a	16.7	ab	11.0	a
Ethanol [d] + 10^8 cells ml^{-1} BIO126	1.5	a	0.3	a	18.5	abc	12.3	a
$NaHCO_3$ [e] + 10^8 cells ml^{-1} BIO126	1.1	a	1.9	a	12.1	a	11.3	a

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

[b] 300 µg a.i. ml^{-1}: used as chemical control; [c] 500 µg a.i. ml^{-1}; [d] 10.0% wt vol^{-1}; [e] 3.0% wt vol^{-1}.

532 Table 3

533 Effect of a cell suspension of *M. pulcherrima* strain BIO126, combined or not with two hot water
534 treatments (3' and 10' at 50°C), on *B. cinerea* and *P. expansum* growth on apples cv Golden
535 delicious. Storage at room temperature (23°C) for 5 days and at 4°C for 20 days.

536

Treatment	Grey mould severity (mm)[a]				Blue mould severity (mm) [a]			
	23°C storage		4°C storage		23°C storage		4°C storage	
Control	35.4	c	27.3	c	32.2	d	23.9	b
3' 50°C	34.7	c	23.8	b	25.2	c	21.2	b
10' 50°C	30.8	b	29.1	c	18.9	ab	23.3	b
10^8 cells/ml BIO126	1.8	a	0.0	a	22.8	bc	14.8	a
10^8 cells/ml BIO126 + 3' at 50°C	1.7	a	0.0	a	15.1	a	14.5	a
10^8 cells/ml BIO126 + 10' at 50°C	1.6	a	0.0	a	15.6	a	12.9	a

537 [a] See Table 2.

538

539 Table 4

540 Effect of a cell suspension of *M. pulcherrima* strain BIO126, applied alone, after or together with
541 different concentrations of ethanol or sodium bicarbonate, on *B. cinerea* and *P. expansum* growth
542 on apples cv Golden delicious. Storage at room temperature (23°C) for 5 days.

543

Treatment	Disease severity (mm)			
	Botrytis cinerea		*Penicillium expansum*	
Control	35.2	c	23.5	d
10^7 cells/ml BIO126	2.0	a	3.5	a
10^7 cells/ml BIO126 in 1% ethanol	20.0	b	12.4	c
10^7 cells/ml BIO126 in 2% ethanol	15.7	b	10.2	bc
10% ethanol and 10^7 cells/ml BIO126	1.4	a	9.9	bc
20% ethanol and 10^7 cells/ml BIO126	1.8	a	6.6	ab
10^7 cells/ml BIO126 in 0.1% $NaHCO_3$	21.9	b	6.5	ab
3% sodium bicarbonate and 10^7 cells/ml BIO126	2.4	a	11.8	c
5% sodium bicarbonate and 10^7 cells/ml BIO126	3.4	a	5.3	ab

544 [a] See Table 2.