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

26

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37

38 **ABSTRACT**

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

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

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

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

60

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

63

64 **1. Introduction**

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

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

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

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

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

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

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

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

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

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

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

131

132 **2. Materials and methods**

133 **2.1 Antagonist and pathogens preparation**

134 *Metschnikowia pulcherrima* (Pitt) M.W. Miller strain BIO126, was isolated from the carposphere of
135 an apple cv Golden delicious harvested in an unsprayed orchard located in Piedmont, Northern
136 Italy, and it was studied for its efficacy and mechanism of action (Spadaro et al., 2002). The
137 microorganism culture was stored at -20°C in cell suspension with 65 % v/v of glycerol and 35 %
138 v/v of a solution MgSO₄ 100 mM and Tris (pH 8.0) 25 mM. The strain was grown in Nutrient Yeast
139 Dextrose Broth (NYDB), as described by Droby et al., 1989. Inocula of the antagonist for all
140 experiments were prepared by subculturing in 250 ml Erlenmeyer flasks containing 75 ml of NYDB
141 and incubating on a rotary shaker (100 rpm) at 25°C for 48 h. Yeast cells were collected by
142 centrifugation at 1500 x g for 10 min, washed and resuspended in sterilized Ringer solution (pH
143 6.9±0.1; Merck), and brought to a standard concentration of 10⁸ cells ml⁻¹ by direct counting with a
144 haemocytometer.

145 Two isolates of *B. cinerea* and two isolates of *P. expansum*, obtained from rotted apples cv Golden
146 delicious and selected for their virulence, were used as a mixture during the experiments to ensure a
147 high level of disease. Each strain was stored in tubes with Potato Dextrose Agar (PDA; Merck) and
148 50 mg l⁻¹ of Streptomycin Merck at 4°C. Spore suspensions used for fruit inoculation were prepared
149 as described in Spadaro et al. (2002).

150

151 **2.2 Efficacy of the combination of biological, chemical and heat treatments**

152 Apples (*Malus domestica*, cv Golden delicious), harvested in an Italian orchard conducted
153 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 (10⁸ 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* (10⁵ 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 haemocytometer as previously described, 30 µl of BIO126 cell suspension (10⁸ or 10⁷ cells
179 ml⁻¹) were added to the Erlenmeyer flasks containing the different suspensions of the two chemicals

180 (final concentration: respectively 10^5 or 10^4 cells ml^{-1}). The flasks were incubated on a rotary shaker
181 (100 rpm) at 25°C for 36 hours. The viability of the cells was evaluated through direct observation
182 and the cell suspension was counted by haemocytometer.

183

184 **2.4 Efficacy of different combinations of the biological and chemical treatments**

185 Apples cv Golden delicious, harvested in an Italian orchard conducted by following integrated pest
186 management, were disinfected, dried and punctured as previously described. Some fruits were
187 double-treated, by immersion in an ethanol or sodium bicarbonate suspension and successive
188 inoculation with the antagonist. Other fruits were exposed to one single treatment, by immersion in
189 a combination of the antagonist and lower concentrations of ethanol or sodium bicarbonate. The
190 fruits treated twice were dipped for 60 seconds in a commercial-grade ethanol suspension (10 or
191 20% wt vol^{-1}) or in a sodium bicarbonate (pH 8.3 to 8.6; Sigma-Aldrich) suspension (3 or 5% wt
192 vol^{-1}), left to dry for 3 hours and then treated with 30 μl of BIO126 cell (10^7 ml^{-1}) suspension. Fruits
193 exposed to single treatment were dipped for 60 seconds in a water suspension containing 10^7 cells
194 ml^{-1} of the antagonist and commercial-grade ethanol (1 or 2% wt vol^{-1}) or sodium bicarbonate
195 (0.1% wt vol^{-1}). After 24 hours at room temperature, 30 μl of the spore suspension of *B. cinerea* or
196 *P. expansum* (10^5 ml^{-1}) were pipetted in the wounds of each fruit. When dry, the apples treated
197 differently were randomly packed in commercial plastic trays and stored at 23°C for 5 days. Five
198 fruits per treatment were used (15 inoculation sites). The severity of the diseases was determined by
199 the mean lesion diameter in mm of the rotted apples. The experiments were carried out twice.

200

201 **2.5 Statistical analysis**

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

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

207

208 **3. Results**

209

210 **3.1 Combinations of biological, chemical and heat treatments**

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

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

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

225

226 **3.2 Efficacy of the combination of biological and chemical treatments**

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

231 pathogen growth significantly (respectively 29.7% and 14.8% of the lesion diameter). Storing at
232 4°C, ethanol and acibenzolar-S-methyl resulted in a higher protection of the fruit from grey mould,
233 although not significantly different from the control, with a reduction of the pathogen severity of
234 26.8% and 21.5%. Thiabendazole, the chemical product commercially used, resulted completely
235 ineffective against the strains of grey mould used.

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

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

252

253 **3.3 Efficacy of the combination of biological and heat treatments**

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

257 stored at room temperature, the best results were shown by the biocontrol agent applied alone (5.2%
258 of pathogen severity with respect to the control) or with heat treatment (4.8% and 4.5%). Ten
259 minutes of hot water treatment led to a significant reduction of the lesion diameter (12.9%) in
260 apples stored at 23°C, but the same treatment followed by storage at 4°C was ineffective. Three
261 minutes of hot water immersion brought a significant result only in the case of apples stored at 4°C
262 (12.9% of reduction of the lesion diameter).

263 Combining biological and hot water treatments against *P. expansum*, the strain BIO126 of *M.*
264 *pulcherrima* provided a good control of the pathogen at 23°C (29.2% of reduction) and 4°C
265 (38.2%). The heat treatment improved the efficacy of the antagonist against blue mould after
266 storage at 23°C and 4°C, but the difference was statistically significant only at room temperature.
267 Immersion in hot water alone resulted in a significant control, with more effective result for the
268 longer treatment (41.3% of reduction), in the trial carried out at 23°C and in an inconsistent control
269 in the experiment conducted at 4°C.

270

271 **3.4 Antagonist survival in co-culture with ethanol and sodium bicarbonate**

272 When *M. pulcherrima* strain BIO126 was cultivated for 48 hours in NYDB with different
273 concentrations of ethanol (20%, 10%, 5%, and 2%) no cell growth and no viability was detectable
274 in all alcohol concentrations except at 2%. The experiment was repeated with co-culture in 5%, 4%,
275 3%, 2%, and 1% ethanol in the synthetic broth. At 1% and 2% ethanol, the total number of cells and
276 the viability were similar to the control. At 3% ethanol the total number of antagonistic cells were
277 0.5% compared to the control: the microorganism had a slowed growth but the cells were alive. At
278 4 and 5% ethanol all yeast cells were not viable and did not multiply.

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

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

284

285 **3.5 Efficacy of different combinations of the biological and chemical treatments**

286 All treatments were significantly different from the control in the trial against *B. cinerea* (Table 4).

287 Treatments where the biocontrol agent was applied alone (lesion diameter reduced to 5.7%) or after

288 the application of 10% ethanol (3.9%), 20% ethanol (5.2), 3% sodium bicarbonate (6.9%) or 5%

289 sodium bicarbonate (9.6%) were particularly effective. Fruits treated by immersion in a combination

290 of the antagonist at 10^7 ml⁻¹ and lower concentrations of ethanol or sodium bicarbonate, still

291 significantly different from the control, were more susceptible to *B. cinerea*: the BIO126 cell

292 suspension in 2% ethanol reduced the pathogen lesions by 55.4%, in 1% ethanol by 43.3% and in

293 0.1% sodium bicarbonate by 37.3%.

294 In the experiments carried out against *P. expansum* all treatments caused a significant reduction of

295 the lesion diameter compared to the control. The application of the cell suspension of BIO126

296 offered the higher level of control of the pathogen (14.7% of disease severity). When the

297 application of 20% ethanol or 5% sodium bicarbonate preceded the biological treatment, the lesion

298 diameter resulted greatly reduced (27.8 and 22.7%). Lower control resulted from the application of

299 10% ethanol or 3% sodium bicarbonate before the yeast cell suspension (42.0 and 50.3%). A single

300 application of the antagonist cell suspension in 1% and 2% ethanol resulted in a disease severity of

301 52.7 and 43.4%. A consistent efficacy was also showed by the application of the BIO126 cell

302 suspension in 0.1% sodium bicarbonate).

303 All fruits treated with 3% or 5% sodium bicarbonate, needed a final brushing or washing to

304 eliminate the residues of the salt. No sign of phytotoxicity was observed.

305

306 **4. Discussion**

307 The strain BIO126 of *Metschnikowia pulcherrima* proved its antagonistic potential in controlled and

308 semi-commercial trials reducing blue and grey mould on apples. When apples cv Golden delicious

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

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

321 For the experiments carried out, two temperatures of storage were chosen. Room temperature
322 (23°C) normally favours the growth of the pathogens (Snowdon, 1990), whereas 4°C is one of the
323 temperatures for commercial fruit storage and favours the yeast antagonist fitness (Spadaro et al.,
324 2002).

325 Heat treatment is effective in sanitizing the fruit and enhancing the wound curing process. It has the
326 added benefit of improving fruit colour but does not lead to softening, since it inhibits the synthesis
327 of cell wall hydrolytic enzymes in the apple fruit, and reduces ethylene production (Lurie, 1998).
328 Heat treatment could also damage the tissue of the fruit and, for this reason, some preliminary trials
329 were carried out to assess the optimum time-temperature regime (data not published). The lowest
330 times (1' or 2' at 50°C) were totally ineffective in controlling blue mould and grey rot and the
331 highest ones (30' at 50°C) caused damages to the apples, such as peel browning, as already noted
332 by Klein and Lurie (1992). Pasteurisation with hot water at 50°C showed an ET₅₀ of 1.5 minutes for
333 *B. cinerea* spore germination and an ET₅₀ of 0.9 minutes for the germ tube elongation of the same
334 pathogen (Fallik et al., 1996). Combining heat treatment with an antagonist, in some cases, could

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

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

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

356 Sodium bicarbonate is inexpensive, readily available and can be used with a minimal risk of injury
357 to the fruit. The inhibitory activity of sodium bicarbonate depends on the presence of salt residues
358 within the wound infection courts occupied by the fungus and on interactions between this residue
359 and constituents of the peel. In previous trials sodium bicarbonate was applied for control of *B.*
360 *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.

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

422

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429

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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

524 Table 2

525 Effect of a cell suspension of *M. pulcherrima* strain BIO126, combined or not with acibenzolar-S-

526 methyl, ethanol, and sodium bicarbonate, on *B. cinerea* and *P. expansum* growth on apples cv

527 Golden delicious. Storage at room temperature (23°C) for 5 days and at 4°C for 20 days.

528

Treatment	Grey mould severity (mm) ^a				Blue mould severity (mm) ^a			
	23°C storage	4°C storage	23°C storage	4°C storage	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

529 ^a Values in the same column followed by the same letter are not statistically different by Duncan's

530 Multiple Range Test ($P < 0,05$).

531 ^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)			
	<i>Botrytis cinerea</i>		<i>Penicillium expansum</i>	
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.