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17 **Effect of culture age, protectants and initial cell concentration on viability of freeze-**
18 **dried cells of *Metschnikowia pulcherrima***

19

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34

35 **Running title**

36 Freeze-drying of *Metschnikowia pulcherrima*

37

38 **ABSTRACT**

39

40 The effect of freeze-drying using different lyoprotectants at different concentrations on the
41 viability and biocontrol efficacy of *Metschnikowia pulcherrima* was evaluated. The effects of
42 initial yeast cell concentration and culture age on the viability were also considered. Yeast
43 cells grown for 36 h were more resistant to freeze-drying than 48 h-old cells. An initial
44 concentration of 10^8 cells mL⁻¹ favoured the highest survival after freeze-drying. When
45 maltose (25% w v⁻¹) was used as protectant, a high cell viability was obtained (64.2%). Cells
46 kept a high viability after 6 months of storage at 4°C. The biocontrol efficacy of freeze-dried
47 cells was similar to the activity of fresh cells on ‘Gala’ apples, and slightly lower on ‘Golden
48 Delicious’ apples. After optimizing the freeze-drying conditions, the viability of *M.*
49 *pulcherrima* cells were similar to that obtained in other studies. The results constitute a first
50 step towards the commercial development of *M. pulcherrima* as biocontrol agent.

51

52 **Key words**

53 Apple fruit, biological control, freeze-drying, postharvest, yeast.

54

55 INTRODUCTION

56

57 Several microorganisms, particularly yeast naturally occurring on the surface of fruit and
58 vegetables, have been widely studied for the control of postharvest diseases (Spadaro and
59 Gullino 2004). The acceptance of biocontrol agents (BCAs) depends on their effectiveness
60 and compatibility with current handling and storage practices used in the agrochemical
61 marketplace (Wraight et al. 2001). Formulation is necessary in order to present the product in
62 a usable form and to optimize its efficacy, stability, safety and ease of application. The
63 formulation must therefore provide conditions which retain viability during preparation,
64 storage and application, and favour survival of the agent, in the environment (Rhodes 1993).

65 Among the microorganisms under development, there are some antagonistic yeast strains
66 belonging to the species *Metschnikowia pulcherrima* (Pitt) M.W. Miller effective against
67 postharvest decay of apple, table grape, grapefruit, cherry tomato, and peach (Schena et al.
68 2000; Janisiewicz et al. 2001; Spadaro et al. 2002, 2008; Zhang et al. 2010). *M. pulcherrima*
69 normally acts as competitor for scarce nutrients, such as iron (Saravanakumar et al. 2008), or
70 by producing hydrolases, such as chitinases, able to degrade the cell wall of pathogenic fungi
71 (Saravanakumar et al. 2009).

72 Biocontrol of postharvest pathogens using competitive yeast is only effective when applying
73 high (at least 10^7 - 10^8 cells mL^{-1}) concentrations of microorganisms (Hofstein et al. 1994), and
74 its effectiveness increases with the concentration. The need of storing and preserving the
75 microbial pesticides requires an increase in the shelf-life, obtained by stabilizing the viability
76 of the microorganisms (Burgess 1998). This can be achieved i) by refrigeration in liquid state;
77 ii) by freezing in presence of cryoprotectant substances; iii) by dehydrating the product
78 (Montesinos, 2003).

79 Freeze-drying is the most convenient and successful method of preserving bacteria,
80 sporulating fungi and yeast (Berny and Hennebert 1991). Freeze-drying, compared to other
81 dehydration techniques (To and Etzel 1997), has the advantages of protecting from
82 contamination during storage, long viability and ease of distribution (Miyamoto-Shinohara et
83 al. 2006). Formation of a maximally freeze-concentrated matrix with entrapped microbial
84 cells is essential in freezing prior to freeze-drying. Freeze-drying must retain a solid
85 amorphous state of protectant matrices. Freeze-dried matrices contain cells entrapped in the
86 protective matrices in the freezing process. The retention of viability during storage seems to
87 be controlled by water plasticization of the protectant matrix and possibly interactions of
88 water with the dehydrated cells. Highest cell viability has been obtained in glassy protective
89 media (Pehkonen et al. 2008). Some investigators have already studied the effect of freeze-
90 drying on survival and efficacy of yeast biocontrol agents, such as *Candida sake* (Abadias et
91 al. 2001), *Cryptococcus laurentii* (Li and Tian 2007) and *Pichia anomala* (Melin et al. 2007).
92 The aim of the present research was to evaluate the effect of freeze-drying on the survival of
93 *M. pulcherrima*. Furthermore, the effect of different lyoprotectants used at different
94 concentrations was studied on the viability of yeast cells after freeze-drying. Moreover, the
95 initial yeast cell concentration and culture age on the viability of freeze-dried yeast cells were
96 taken into consideration. Finally, the biocontrol efficacy of frozen, freeze-dried and fresh cells
97 were compared.

98

99 **MATERIALS AND METHODS**

100

101 **Microorganisms**

102 *Metschnikowia pulcherrima* (Pitt) M.W. Miller strain BIO126 was isolated from the
103 carposphere of an apple 'Golden Delicious' harvested in an unsprayed orchard located in

104 Piedmont, Northern Italy (Spadaro et al. 2002). The strain was stored at -80°C in a cell
105 suspension with 20% v v⁻¹ of glycerol.

106 For the biocontrol assays, five strains of *Botrytis cinerea* were isolated from rotten apples and
107 selected for their high virulence. Each strain was stored on a slant of Potato Dextrose Agar
108 (Merck, Darmstadt, Germany) with 50 mg L⁻¹ streptomycin Merck at 4°C. Spore suspensions
109 were prepared by growing the fungal strains on Petri dishes on PDA amended with 50 mg L⁻¹
110 of streptomycin for 10 days. Spores from the five strains were collected, resuspended in
111 sterile Ringer's solution (Merck, Darmstadt, Germany), filtered through 8 layers of sterile
112 cheese-cloth and brought to a final concentration of 10⁵ spores mL⁻¹ per strain.

113

114 **Yeast growth**

115 Before use, the yeast was subcultured in Petri dishes with nutrient yeast dextrose agar
116 (NYDA, Droby et al. 1989). The growth medium was YEMS (yeast extract 30 g L⁻¹, D-
117 mannitol 5 g L⁻¹ and L-sorbose 5 g L⁻¹), which favoured a high yeast biomass (Spadaro et al.
118 2010). Well-controlled fermentations of 4.0 L working volume (nominal volume, 5 L) were
119 carried out in Applikon BioConsole ADI 1025 glass stirred tank vessels (Applikon™
120 Biotechnology, The Netherlands), integrated with the software Bioexpert Lite for data
121 acquisition. Yeast cultures were inoculated to 5×10⁵ cfu mL⁻¹. Operating conditions included
122 temperature controlled at 25± 0.2°C, floating dissolved oxygen monitored using a
123 polarographic probe, agitation with two equally spaced Rushton impellers controlled at a
124 constant speed of 450 rpm, and air sparging through a submerged ring sparger controlled at
125 4.0 L min⁻¹.

126

127 **Sample preparation**

128 Cells were collected at the beginning of the stationary phase by centrifugation (7500 rpm for

129 10 min at 4°C) in a J21-2 centrifuge (Beckman, Brea, USA) and resuspended in Ringer
130 solution. The concentration of the resuspended cells was determined by Bürker chamber and
131 adjusted to the desired concentrations. An aliquote (500 µl) of the yeast cells suspensions
132 were then deposited into vials.

133 The protective agents tested were two monosaccharide sugars (D-glucose and D-fructose),
134 four disaccharide sugars (lactose, maltose, sucrose and trehalose), a sugar alcohol (L-sorbitol)
135 and a complex matrix (yeast extract). Each protectant was dissolved in deionised water,
136 autoclaved and added to the yeast cell suspension to obtain the final concentration ($w v^{-1}$).
137 Throughout the experiments, different concentrations (1%, 5%, 10%, 15%, 20%) of
138 protectants were tested and also the effect of the initial cell concentration was evaluated. 4.5
139 ml of each protectant suspension were added into each vial containing the yeast cell
140 suspension (500 µl), to get the desired protectant concentration. For each protectant and/or
141 initial concentration tested, tenfold dilutions of the suspensions were made in Ringer solution,
142 and 100 µl aliquots of the suspensions were spread plated in triplicate on Petri dishes
143 containing NYDA in order to determine the initial fresh cell concentration. Dishes were
144 incubated at $25\pm 1^\circ\text{C}$ for 48 h, and the initial number of colony forming units per millilitre
145 (cfu mL^{-1}) was determined.

146

147 **Freeze-drying**

148 The suspension was frozen at -20°C for 4 h and at -40°C for 12 h and subsequently
149 lyophilized in a Heto FD 1.0 Freeze-Dryer (Waltham, Massachusetts, USA) operating at a
150 chamber pressure of 1 Pa and at -45°C for 24 h. After freeze-drying, the vials were sealed
151 under vacuum. Experiments were replicated three times for each protectant and concentration.

152

153 **Reyhdration and yeast cell viability**

154 For each protectant and/or initial concentration tested, three replicates of freeze-dried vials
155 were resuspended in autoclaved Ringer solution to the original volume (5 mL) and left on a
156 rotary shaker for 15 min. Tenfold dilutions of the suspensions were made in Ringer solution,
157 and 100 μ l aliquots of the suspensions were spread plated in triplicate on Petri dishes
158 containing NYDA. Dishes were incubated at $25\pm 1^\circ\text{C}$ for 48 h, and the number of cfu mL^{-1}
159 was determined. Viability percentage for each protectant was assessed as ratio between
160 freeze-dried and fresh cells concentration. For the last viability assays about initial cell
161 concentration with high protectant, the vials were stored at 4°C for 2, 4, and 6 months before
162 rehydration and viability assessment. Experiments were repeated three times.

163

164 **Biocontrol assays**

165 To evaluate the efficacy of freeze-dried cells of *M. pulcherrima* strain BIO126 against *B.*
166 *cinerea* on apple, freeze-dried cells (with 25% w v^{-1} maltose or 25% w v^{-1} fructose) were
167 rehydrated as described above and compared with frozen and fresh yeast cells. Freeze-dried
168 and frozen cell concentration was adjusted at 10^8 cfu mL^{-1} with Ringer solution. Fresh yeast
169 cells were obtained by growing them in YEMS for 36 h, by centrifuging (7500 rpm for 10
170 min at 4°C) and resuspending them in Ringer solution to 10^8 cfu mL^{-1} . For a chemical control,
171 fruit were treated with thiabendazole (Tecto 20S, Syngenta Crop Protection, Milan, Italy, 20%
172 a.i., 200 $\mu\text{g mL}^{-1}$).

173 Two assays were carried out on two apple cultivars, 'Golden Delicious' and 'Gala'. Apples
174 were artificially wounded at the equatorial region (3 mm diameter; 6 mm depth; 3 wounds per
175 fruit). A 30 μ l yeast suspension was applied to each wound and, after 3 h, wounds were
176 inoculated with a 30 μ l of *B. cinerea* suspension (10^5 spores mL^{-1}). Five apples per replicate
177 and three replicates per treatment were used. Treated apples were stored at $20\pm 1^\circ\text{C}$ for 7 days,
178 after which the grey mould lesion diameters were measured. The assays were repeated twice.

179

180 **Data analysis**

181 Each experiment was repeated at least twice. No significant differences were found among
182 corresponding experiments so that the trials were pooled and statistical analysis was
183 performed by using the SPSS-WIN software. Statistical significance was judged at the level
184 of $P < 0.05$. When the analysis of variance was statistically significant, Duncan's multiple
185 range test was used for the separation of means.

186

187 **RESULTS AND DISCUSSION**

188

189 **Viability assays: culture age**

190 In a first set of experiments, the cells of *M. pulcherrima* strain BIO126 grown for 36 and 48 h
191 in YEMS were freeze-dried starting from an initial concentration of 1×10^9 cfu mL⁻¹ and
192 adding different protectant agents at 1% w v⁻¹ (Table 1). The formulations after freeze-drying
193 were porous cakes. Highest cell viability was obtained in glassy protective media, such as
194 sugars. In general, the addition of any of the eight protectants tested provided a survival rate
195 statistically higher than Ringer solution. No significant difference was found in the viability
196 of yeast cells after freezing for the protectants tested. For 36 h old cultures, a statistically
197 higher viability was obtained with maltose (8.81%) and fructose (9.94%). A noticeable result
198 was obtained also by the addition of yeast extract (7.52%). For 48 h old cultures in YEMS,
199 the viability was always lower than for 36 h old cultures, the protectant being equal. The
200 highest viability was obtained by using maltose (3.21%), followed by trehalose (2.12%) and
201 yeast extract (2.00%). Without protectants, viability after freeze-drying was extremely low:
202 0.11% for 36 h old cultures and 0.22% for 48 h old cultures. Cultures grown for 36 h in
203 YEMS were chosen to continue the experiments.

204 In 5-liter fermenter experiments, the cultures of *M. pulcherrima* reached the stationary phase
205 after 32 h growth in YEMS (Spadaro et al. 2010), so 36 h old cultures corresponded to the
206 plain stationary phase, while 48 h old cultures corresponded to the beginning of the decline
207 phase, when viable count started to turn down. Indeed, preliminary experiments showed that
208 younger (grown for 24 or 30 h) or older cultures (grown for 42 or 48 h) had lower viability
209 after freeze-drying (data not shown). Similarly, freeze-drying of other biocontrol agents was
210 performed on cultures in plain stationary phase. In particular, the cultures of the bacterial
211 antagonist *Pantoea agglomerans* and the yeast BCA *Candida sake* were harvested
212 respectively after 24 h (Costa et al. 2000) and 38 h growth in liquid medium (Abadias et al.
213 2001).

214

215 **Viability assays: initial cell concentration with low protectant**

216 In a second set of experiments, the number of protectants tested was reduced to five, by
217 keeping one monosaccharide sugar (D-fructose), one sugar alcohol (L-sorbitol), one complex
218 matrix (yeast extract) and two disaccharide sugars (maltose and trehalose). The
219 microorganism cultures were freeze-dried after 36 h growth at three initial cell concentrations
220 (10^{11} , 10^{10} and 10^9 cfu mL⁻¹) and in presence of 5% w v⁻¹ of protectant (Figure 1). The lowest
221 initial concentration of microorganism (10^9 cfu mL⁻¹) provided the highest final viability. In
222 particular, starting with 10^{11} cfu mL⁻¹, fructose (0.69%) and sorbitol (0.63%) were the
223 protectants providing statistically higher viability. With an initial concentration of 10^{10} cfu
224 mL⁻¹, yeast extract (1.50%) and maltose (1.44%) resulted the most effective protectants.
225 Fructose (6.3%), followed by maltose (5.1%) and sorbitol (4.2%) provided the highest yeast
226 viabilities, when freeze-drying was realized on an initial concentration of 10^9 cfu mL⁻¹.
227 The viability of microorganisms, particularly of *M. pulcherrima*, was very poor after freeze-
228 drying without the use of any protective agent. Thus several protectants were tested at

229 different concentrations, throughout the experiments, without adding other chemical
230 compounds, to evaluate its single effect on viability and to keep the formulation as simple as
231 possible, as suggested by Nail et al. (2002). The experiments carried out showed that the type
232 and concentration of the protective agent used had a strong effect on the viability of the cells
233 of *M. pulcherrima*. Previously, Costa et al. (2000) found that maximum protection of cells of
234 *P. agglomerans* during freeze-drying was achieved with sugars, and in particular with
235 disaccharides. Sugars replace structural water in membranes after dehydration and prevent
236 unfolding and aggregation of proteins by hydrogen bonding with polar groups of proteins
237 (Patist and Zhoerb 2005).

238

239 **Viability assays: protectant concentration**

240 In the following series of experiments, cells with an initial concentration of 10^9 cfu mL⁻¹ were
241 freeze-dried in presence of four concentrations (5%, 10%, 15%, and 20% w v⁻¹) of four
242 protective agents (Figure 2). At 5% w v⁻¹ protectant, fructose and maltose provided a cell
243 survival respectively of 9.2% and 6.2% after freeze-drying, significantly higher than the other
244 two protectants. At 10% w v⁻¹ protectant, fructose (13.3%), followed by maltose (7.6%), was
245 the most effective protective agent tested. At 15% w v⁻¹, yet the most effective protectants
246 were fructose and maltose, able to guarantee respectively 11.8% and 10.7% viability. The
247 highest *M. pulcherrima* cell viability was achieved at high concentration (20%) of protective
248 agents: in particular, maltose (20.6%), followed by fructose (14.6%) and sorbitol (12.8%)
249 provided the best results.

250

251 **Viability assays: initial cell concentration with high protectant**

252 The last set of experiments related to the viability of *M. pulcherrima* was carried out by
253 freeze-drying the yeast cells at three initial concentrations and in presence of two high

254 concentrations (20 and 25%) of maltose and fructose (Figure 3). At the highest initial
255 concentration (10^9 cells mL^{-1}) 25% of maltose permitted the highest yeast viability (47.0%).
256 When the initial cell concentration was 10^8 cells mL^{-1} , noticeable viabilities were obtained for
257 both protectants and both concentrations tested. In particular, 20% and 25% of maltose
258 provided a survival rate respectively of 62.5% and 64.2%. The viability in presence of 20%
259 and 25% fructose were respectively 21.4% and 43.4%. The viability when the initial cell
260 concentration was 10^7 cells mL^{-1} was lower compared to the other two initial concentrations,
261 and the best results was provided by 25% maltose (35.4%) or 25% fructose (35.6%).

262 When increasing the protectant concentration, a lower microorganism concentration (10^8 cells
263 mL^{-1}) guaranteed a higher viability. With 5% protectant, the highest viability was guaranteed
264 with 10^9 cells mL^{-1} of antagonistic yeast (Figure 1), but with 20% or 25% protectant, the
265 highest viability was obtained with 10^8 cells mL^{-1} (Figure 3). A high initial yeast concentration
266 (higher than 10^{10} cells mL^{-1}) was related to a drastic decrease in the viable count. Costa et al.
267 (2000) studied the effect of the initial cell concentration on the viability of *P. agglomerans*,
268 finding that the effect of the initial cell concentration was related to the protective medium
269 used. When sucrose was used as a protectant, the highest recovery was obtained at high
270 concentration (10^{10} cfu mL^{-1}). On the opposite, when protein based protectants were used, the
271 lowest viabilities were obtained at high bacterial concentration. Bozoglu et al. (1987)
272 suggested that the death of microorganisms is proportional to their area of contact with the
273 external medium. Pehkonen et al. (2008) showed that the differences in cell viabilities after
274 dehydration were depending on the protective agents used, and in particular on the glass
275 transition properties of the disaccharides used.

276 The protective effect provided by some disaccharides, such as maltose or trehalose, on living
277 organisms under desiccation could be explained by several mechanisms. In the water-
278 replacement hypothesis, the stabilization was attributed to the formation of hydrogen bonds

279 between sensitive components and disaccharide molecules when water is removed,
280 maintaining the structural integrity of membranes and proteins (Crowe et al. 1987). A second
281 hypothesis is related to the ability of disaccharides to form a glassy structure during drying
282 under suitable conditions where the sensitive components are embedded (Crowe et al. 1993).
283 Lodato et al. (1999) found that the presence of a certain amount of amorphous disaccharides
284 (such as maltose) during freeze-drying was critical for ensuring the cell viability of
285 *Saccharomyces cerevisiae*.

286 The shelf-life of a biological product refers to the period of time during which the
287 antagonistic cells remain viable and effective (Elzein et al. 2004). In our study, no significant
288 loss of viability on freeze-dried cells appeared after a storage at 4°C of the vials obtained in
289 the last experiments for 2, 4, and 6 months. In particular, when 10^8 cells mL⁻¹ of BIO126
290 were freeze-dried in presence of 20% or 25% maltose, the cell viabilities after 2, 4, and 6
291 months were 61.4%, 61.3%, and 59.7% with 20% maltose and 63.8%, 63.2% and 60.3% with
292 25% maltose. The low temperature helped to keep the metabolic activity at a low level, and
293 contributed to increase the storage stability and shelf life. Freeze-drying is probably the most
294 effective desiccation technique for microorganism long storage, and recent studies have
295 showed that it is suitable to keep alive not only gram-negative or gram-positive bacteria, but
296 also yeast for up to 20 years (Miyamoto-Shinohara et al. 2006).

297

298 **Biocontrol assays**

299 To test the efficacy of the freeze-dried cells of *M. pulcherrima* strain BIO126 in controlling *B.*
300 *cinerea* on apple, two trials were carried out on the cultivars ‘Golden Delicious’ and ‘Gala’
301 (Figure 4). The fruit were stored at 20°C for 7 days. The biocontrol trials showed a significant
302 reduction of grey mould severity on both apple cultivars by fresh, frozen as well as freeze-
303 dried yeast cells. The biocontrol efficacy of the freeze-dried cells was similar on ‘Golden

304 Delicious' and 'Gala' apples, with a disease severity reduction of 45.3% and 48.1%
305 respectively, as compared to the control. On both cultivars, the antagonistic activity of fresh
306 and frozen cells amended with protective agents was similar. On 'Golden Delicious' apples,
307 freeze-dried yeast cells showed a slightly lower biocontrol efficacy, as compared to the fresh
308 and frozen cells, but on 'Gala' apples the efficacy of freeze-dried cells was not statistically
309 different from the biocontrol provided by the fresh cells.

310 The biocontrol efficacy trials confirmed that freeze-dried cells of *M. pulcherrima* after
311 rehydration not only were viable, but still kept a biocontrol activity against *B. cinerea* similar
312 to that shown by fresh cells of the same strain when applied at the same concentration.
313 Previous, Abadias et al. (2001) showed a lower efficacy of freeze-dried *Candida sake* cells
314 against *P. expansum* on apples compared to the fresh cells, partially due to the damage created
315 to the cells, which could not totally recover their function after the dehydration-rehydration
316 process. On the opposite, the biocontrol activity of freeze-dried cells of *Pichia anomala*
317 against moulds on moist cereal grains remained intact (Melin et al. 2007).

318 The present work permitted to set up a freeze-drying protocol for the cells of *M. pulcherrima*
319 characterized by a high viability and the preservation of the biocontrol efficacy against
320 postharvest pathogens. Previous studies showed that the cell viability could significantly
321 increase when a suitable rehydrating medium is used instead of Ringer solution. Future
322 research will focus on the effect of the rehydrating media, of longer storage times and of
323 storage temperatures on yeast cell viability.

324

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332

333 REFERENCES

334

335 Abadias, M., Teixido, N., Usall, J., Benabarre, A., and Vinas, I. 2001. Viability, efficacy, and
336 storage stability of freeze-dried biocontrol agent *Candida sake* using different protective and
337 rehydration media. *J. Food. Protect.* **64**(6): 856-861.

338

339 Berny, J.F., and Hennebert, G.L. 1991. Viability and Stability of Yeast-Cells and Filamentous
340 Fungus Spores during Freeze-Drying - Effects of Protectants and Cooling Rates. *Mycologia*
341 **83**(6): 805-815.

342

343 Bozoglu, T.F., Ozilgen, M., and Bakir, U. 1987. Survival kinetics of lactic acid starter cultures
344 during and after freeze-drying. *Enzyme Microb. Tech.* **9**: 531-537.

345

346 Burges, H.D. 1998. Formulation of Microbial Biopesticides: Beneficial microorganism,
347 nematodes and seed treatments. Kluwer Academic Publishers, Dordrecht, The Netherlands.

348

349 Costa, E., Usall, J., Teixido, N., Garcia, N., and Vinas, I. 2000. Effect of protective agents,
350 rehydration media and initial cell concentration on viability of *Pantoea agglomerans* strain
351 CPA-2 subjected to freeze-drying. *J. Appl. Microbiol.* **89**(5): 793-800.

352

353 Crowe, J.H., Crowe, L.M., and Carpenter, J.F. 1993. Preserving dry bio-materials: the water
354 replacement hypothesis. *Biopharmacology* **6**: 28-37.

355

356 Crowe, J.H., Crowe, L.M., Carpenter, J.F., and Aurell Wistrom, C. 1987. Stabilization of dry
357 phospholipid bilayers and proteins by sugars. *Biochem. J.* **242**(1): 1-10.

358

359 Droby, S., Chalutz, E., Wilson, C.L., and Wisniewski, M.E. 1989. Characterization of the
360 biocontrol activity of *Debaryomyces hansenii* in the control of *Penicillium digitatum* on
361 grapefruit. *Can. J. Microbiol.* **35**(8): 794-800.

362

363 Elzein, A., Kroschel, J., and Muller-Stover, D. 2004. Effects of inoculum type and propagule
364 concentration on shelf life of Pesta formulations containing *Fusarium oxysporum* Foxy 2, a
365 potential mycoherbicide agent for *Striga* spp. *Biol. Control.* **30**(2): 203-211.

366

367 Hofstein, R., Friedlender, B., Chalutz, E., and Droby, S. 1994. Large scale production and
368 pilot testing of biological control agents for post harvest diseases. *In* Biological control of
369 post-harvest diseases: theory and practice. *Edited by* C.L. Wilson and M.E. Wisniewsky. CRC
370 Press, Boca Raton, FL, USA. pp. 89-100.

371

372 Janisiewicz, W.J., Tworkoski, T.J., and Kurtzman, C.P. 2001. Biocontrol potential of
373 *Metchnikowia pulcherrima* strains against blue mold of apple. *Phytopathology* **91**(11): 1098-
374 1108.

375

376 Li, B.Q., and Tian, S.P. 2007. Effect of intracellular trehalose in *Cryptococcus laurentii* and
377 exogenous lyoprotectants on its viability and biocontrol efficacy on *Penicillium expansum* in
378 apple fruit. *Lett. Appl. Microbiol.* **44**(4): 437-442.
379

380 Lodato, P., de Huergo, M.S., and Buera, M.P. 1999. Viability and thermal stability of a strain
381 of *Saccharomyces cerevisiae* freeze-dried in different sugar and polymer matrices. *Appl.*
382 *Microbiol. Biotechnol.* **52**(2): 215-220.
383

384 Melin, P., Hakansson, S., and Schnurer, J. 2007. Optimisation and comparison of liquid and
385 dry formulations of the biocontrol yeast *Pichia anomala* J121. *Appl. Microbiol. Biotechnol.*
386 **73**(5): 1008-1016.
387

388 Miyamoto-Shinohara, Y., Sukenobe, J., Imaizumi, T., and Nakahara, T. 2006. Survival curves
389 for microbial species stored by freeze-drying. *Cryobiology* **52**(1): 27-32.
390

391 Montesinos, E. 2003. Development, registration and commercialization of microbial
392 pesticides for plant protection. *Int. Microbiol.* **6**(4): 245-252.
393

394 Nail, S.L., Jiang, S., Chongprasert, S., and Knopp, S.A. 2002. Fundamentals of freeze-drying.
395 *Pharm. Biotechnol.* **14**: 281-360.
396

397 Patist, A., and Zoerb, H. 2005. Preservation mechanisms of trehalose in food and biosystems.
398 *Coll. Surf. B: Biointerf.* **40**: 107–113.
399

400 Pehkonen, K.S., Roos, Y.H., Miao, S., Ross, R.P., and Stanton, C. 2008. State transitions and
401 physicochemical aspects of cryoprotection and stabilization in freeze-drying of *Lactobacillus*
402 *rhamnosus* GG (LGG). *J. Appl. Microbiol.* **104**: 1732–1743.

403

404 Rhodes, D.J. 1993. Formulation of biocontrol agents. *In* Exploitation of Microorganism.
405 *Edited by* D.G. Jones. Chapman and Hall, London, UK. pp. 411-439.

406

407 Saravanakumar, D., Ciavarella, A., Spadaro, D., Garibaldi, A., and Gullino, M.L. 2008.
408 *Metschnikowia pulcherrima* strain MACH1 outcompetes *Botrytis cinerea*, *Alternaria*
409 *alternata* and *Penicillium expansum* in apples through iron depletion. *Postharvest Biol.*
410 *Technol.* **49**(1): 121-128.

411

412 Saravanakumar, D., Spadaro, D., Garibaldi, A., and Gullino, M. 2009. Detection of enzymatic
413 activity and partial sequence of a chitinase gene in *Metschnikowia pulcherrima* strain
414 MACH1 used as post-harvest biocontrol agent. *Eur. J. Plant Pathol.* **123**(2): 183-193.

415

416 Schena, L., Ippolito, A., Zahavi, T., Cohen, L., and Droby, S. 2000. Molecular approaches to
417 assist the screening and monitoring of postharvest biocontrol yeasts. *Eur. J. Plant Pathol.*
418 **106**(7): 681-691.

419

420 Spadaro, D., and Gullino, M.L. 2004. State of the art and future prospects of the biological
421 control of postharvest fruit diseases. *Int. J. Food Microbiol.* **91**(2): 185-194.

422

423 Spadaro, D., Vola, R., Piano, S., and Gullino, M.L. 2002. Mechanisms of action and efficacy
424 of four isolates of the yeast *Metschnikowia pulcherrima* active against postharvest pathogens
425 on apples. *Postharvest Biol. Technol.* **24**(2): 123-134.

426

427 Spadaro, D., Sabetta, W., Acquadro, A., Portis, E., Garibaldi, A., and Gullino, M.L. 2008.
428 Use of AFLP for differentiation of *Metschnikowia putcherrima* strains for postharvest disease
429 biological control. *Microbiol. Res.* **163**(5): 523-530.

430

431 Spadaro, D., Ciavorella, A., Zhang, D., Garibaldi, A., and Gullino, M.L. 2010. Effect of
432 culture media and pH on the biomass production and biocontrol efficacy of a *Metschnikowia*
433 *pulcherrima* strain to be used as a biofungicide for postharvest disease control. *Can. J.*
434 *Microbiol.*, **56**(2): 128-137.

435

436 To, B.C.S., and Etzel, M.R. 1997. Spray drying, freeze drying, or freezing of three different
437 lactic acid bacteria species. *J. Food Sci.* **62**(3): 576-578.

438

439 Wraight, S.P., Jackson, M.A., and de Kock, S.L. 2001. Production, stabilization and
440 formulation of fungal biocontrol agents. *In Fungi as biocontrol agents. Edited by T.M. Butt,*
441 *C. Jackson, N. Magan. CAB International. Wallingford, CT, USA. pp. 253-287.*

442

443 Zhang, D.P., Spadaro, D., Garibaldi, A., and Gullino, M.L. 2010. Selection and evaluation of
444 new antagonists for their efficacy against postharvest brown rot of peaches. *Postharvest Biol.*
445 *Technol.* **55**(3): 174-181.

446

447 **Figure captions**

448

449 **Figure 1** Viability of *Metschnikowia pulcherrima* cells (%) after 36h growth in YEMS and
450 freeze-drying, using different sugar solutions (5% w v⁻¹) as protectants and Ringer solution as
451 rehydration medium. Cells at three different initial concentrations were freeze-dried. Samples
452 were analyzed as soon as the freeze-drying process finished. Each bar represents the mean
453 value and standard deviation of 12 samples obtained from three independent experiments.
454 Bars of the same colour followed by the same letter are not statistically different by Duncan's
455 Multiple Range Test ($P < 0.05$).

456

457 **Figure 2** Viability of *Metschnikowia pulcherrima* cells (%) after 36h growth in YEMS and
458 freeze-drying, using different sugar solutions at different concentrations (5%, 10%, 15%, 20%
459 w v⁻¹) as protectants and Ringer solution as rehydration medium. Initial microorganism
460 concentration was 10⁹ cfu mL⁻¹. Samples were analyzed as soon as the freeze-drying process
461 finished. Each bar represents the mean value and standard deviation of 12 samples obtained
462 from three independent experiments. Bars followed by the same letter are not statistically
463 different by Duncan's Multiple Range Test ($P < 0.05$).

464

465 **Figure 3** Viability of *Metschnikowia pulcherrima* cells (%) after 36h growth in YEMS and
466 freeze-drying, using maltose and fructose, at two concentrations (20% and 25% w v⁻¹) as
467 protectants and Ringer solution as rehydration medium. Three different initial concentrations
468 were freeze-dried. Samples were analyzed as soon as the freeze-drying process finished. Each
469 bar represents the mean value and standard deviation of 12 samples obtained from three
470 independent experiments. Bars followed by the same letter are not statistically different by
471 Duncan's Multiple Range Test ($P < 0.05$).

472

473 **Figure 4** Efficacy of fresh, frozen or freeze-dried cells of *Metschnikowia pulcherrima* strain
474 BIO126 in reducing the development of *Botrytis cinerea* on apples ‘Golden Delicious’ and
475 ‘Gala’. Fruit were artificially wounded, treated with 5×10^8 antagonist cells mL⁻¹ and
476 inoculated after 3h with 5×10^5 conida mL⁻¹ of *Botrytis cinerea*. Thiabendazole (200 µg mL⁻¹;
477 p.a. 19.7%) was used as chemical control. Fruit were stored at 20±1°C for 7 days. Lesion
478 diameters (cm) were measured. Bars of the same colour followed by the same letter are not
479 statistically different by Duncan’s Multiple Range Test ($P < 0.05$).