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## State of art and future perspectives of biological control of postharvest fruit diseases

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23     **STATE OF ART AND FUTURE PERSPECTIVES OF BIOLOGICAL CONTROL OF**  
24                                     **POSTHARVEST FRUIT DISEASES**

25

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29

30     **Abstract**

31     Synthetic fungicides, when admitted, are the primary means to control postharvest diseases of  
32     fruits. Biological control using antagonists has emerged as one of the most promising  
33     alternatives to chemicals. During the last twenty years, several biocontrol agents have been  
34     widely investigated against different pathogens and fruit crops. Many biocontrol mechanisms  
35     have been suggested to operate on fruit including antibiosis, parasitism, induced resistance  
36     and competition. Trying to extend the use of the biofungicides, there have been deep studies  
37     on application of antagonists mixes, preharvest use, and integration with chemical and  
38     physical means of protection. The formulation and application methods are key issues for the  
39     efficacy and final success of the commercial product. The new molecular techniques are  
40     useful tools in the characterisation of the microorganisms and enhancement of their biocontrol  
41     capabilities, through genetic engineering. Although a huge number of scientific papers  
42     published on biological control, this method at the moment should be viewed as an important  
43     component of an integrated disease management scheme if a significant and permanent  
44     reduction of pesticide use is our goal. Anyway scientists, growers and consumers alike must  
45     accept the fact that BCA's are usually not as effective as pesticides.

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46

47 **Keywords:** antagonist, fruit, mechanism of action, postharvest disease.

48

## 49 **1. Introduction**

50

51 Fruits are an important part of the human diet, because they supply essential nutrients such as  
52 vitamins, minerals, and they are important to human health and well-being, for their contents  
53 in antioxidants and anticancer substances. An increasing awareness by consumers that diet  
54 and health are linked has resulted in a greater consumption of fruits. At the same time,  
55 consumers are also more concerned about the safety of the fruits they eat, and they ask for  
56 food free from pesticide residues, toxins and pathogens.

57 Losses due to pests and diseases attacks on fruit in field and during storage, transit, and  
58 commercialisation steps, before reaching the consumer, are not easily assessed, but can result  
59 in 25% of the total production in industrialised countries (Harvey, 1978). In developing  
60 countries damages are often higher, exceeding 50%, because of the lack of adequate storage  
61 structures (Eckert and Ogawa, 1985). The high water content of plant products, such as fruit,  
62 is one of the features that makes them more susceptible to pathogens attack, since they are in  
63 orchard (Harvey, 1978). Another factor favourable to pathogenic fungi, particularly to the  
64 necrotrophic ones, is the presence during storage on the plant organs of wounds, often  
65 produced during harvest and transport of fruit, which represent an ideal way of access for  
66 microorganisms.

67 Synthetic fungicides, when admitted, are the primary means to control postharvest diseases.  
68 However, several reasons, such as the growing public concern over the human health  
69 conditions and the environmental pollution associated with pesticide usage in orchards  
70 (Wilson and Wisniewski, 1994), the development of fungicide resistant strains of postharvest

71 pathogens (Romano et al., 1983; Spotts and Cervantes, 1986) and the lack of reregistration of  
72 some of the most effective fungicides (Gullino and Kuijpers, 1994) have encouraged the  
73 search of alternative approaches.

74 Biological control well fits with the concept of sustainable agriculture, because it mostly  
75 exploits natural cycles with zero or reduced environmental impact. Among the biological  
76 strategies adoptable in postharvest, the induction of resistance in the fruit, the use of plant or  
77 animal products with a fungicidal activity, and, above all, the application of antagonistic  
78 microorganisms can be considered. Biological control using antagonists (Wilson and  
79 Wisniewski, 1994) has emerged as one of the most promising alternatives, either alone or as  
80 part of an integrated pest management to reduce pesticide use.

81 The postharvest environment represents a particular sector for the development of biological  
82 measures. Peculiar difficulties are present in the control of postharvest diseases (Chalutz and  
83 Droby, 1998): the disease control level required is extremely high (also 95-98%); the  
84 nutritional safety imposes special care to the direct use of living microorganisms on food  
85 products; the potential market to employ a biofungicide expressly developed for postharvest  
86 use is relatively small. On the other side, the possibilities of success for postharvest biological  
87 means can be numerous (Chalutz and Droby, 1998): the storage conditions partially  
88 controlled, such as temperature and humidity, can switch the host-pathogen-antagonist  
89 equilibrium towards the antagonist and the laboratory trials and results have a higher  
90 possibility to be transferred into practice; the application site of the antagonist, which is the  
91 fruit, is limited, permitting an increase of the biocontrol agent (BCA) efficacy and avoiding  
92 the presence of some interfering factors; finally the high value of fruit can justify a treatment  
93 with a product relatively expensive.

94 During the last 20 years, several biocontrol agents have been exploited and widely  
95 investigated against different postharvest fungal pathogens (*Alternaria*, *Botrytis*,

96 *Colletotrichum*, *Monilia*, *Penicillium*, *Rhizopus* spp.) on different host species. Many of the  
97 first studies were aimed at the study of the mode of action and the evaluation of the efficacy  
98 of some potential biocontrol bacteria, such as *Brevibacillus subtilis*, producer of antibiotics  
99 (Pusey et al., 1986). The consideration that the application of bacterial antagonists on the fruit  
100 was not commercially acceptable, at least in the short period, brought to switch the interest on  
101 antagonists using modes of action different from antibiosis. Wilson and Wisniewski (1994)  
102 indicated the characteristics of an ideal antagonist: genetic stability, efficacy at low  
103 concentrations and against a wide range of pathogens on various fruit products, simple  
104 nutritional requests, survival in adverse environmental conditions, growth on cheap substrates  
105 in fermenters, lack of pathogenicity for the host plant and lack of production of metabolites  
106 potentially toxic for humans, resistance to the most frequently used pesticides, compatibility  
107 with other chemical and physical treatments. Yeasts, between the potential BCAs, possess a  
108 good number of the mentioned features and, during the last years, research focused on the  
109 selection and study of these microorganisms.

110 At present, three products containing *Pseudomonas syringae* Van Hall, active against  
111 *Botrytis*, *Penicillium*, *Mucor* and *Geotrichum* spp., named Bio-Save 100, Bio-Save 110 and  
112 Bio-Save 1000 and commercialised by EcoScience Corp. (Janisiewicz and Jeffers, 1997), and  
113 a product containing *Candida oleophila* Montrocher, effective against *Botrytis* and  
114 *Penicillium* spp., named Aspire and commercialised by Ecogen Inc. (Hofstein et al., 1994),  
115 are available on the market for postharvest protection. Other yeast species have been  
116 extensively tested and could be registered in relatively short times. Among the  
117 microorganisms under development, there are antagonistic strains belonging to the species  
118 *Aureobasidium pullulans* (Ippolito et al., 2000), *Candida saitoana* (El-Ghaouth et al., 1998),  
119 *Candida sake* (Teixido et al., 1998) and *Metschnikowia pulcherrima* (Spadaro et al., 2001a).

120

## 121 **2. Mechanisms of action**

122

123 Information on the mechanisms of action for most of the antagonists investigated is still  
124 incomplete, because of the difficulties encountered during the study of the complex  
125 interactions between host, pathogen, antagonist and others microorganisms present in the site  
126 of interaction. However a good understanding of the mode of action is essential to develop  
127 appropriate formulation and methods of application, and to obtain registration.

128 Several possible biocontrol mechanisms have been suggested to operate against post-harvest  
129 rots on fruit including antibiosis, parasitism or direct interaction with the pathogen  
130 (extracellular hydrolases), induced resistance and competition for nutrients and space (Droby  
131 and Chalutz, 1994).

132

### 133 *2.1. Antibiosis*

134 Some of the most active bacteria are producer of antibiotics, whose action, at least partially,  
135 influences these BCA's effectiveness. *B. subtilis* was able to produce iturin, a powerful  
136 antifungal peptide (Gueldner et al., 1988) but also gramicidin S (Edwards and Seddon, 2001),  
137 *Pseudomonas cepacia* showed to synthesise pyrrolnitrin, that used alone can control *Botrytis*  
138 *cinerea* and *Penicillium expansum* attacks on pome fruit (Janisiewicz et al., 1991). Also from  
139 these observation, it was possible to develop phenylpyrroles fungicides (Gehmann et al.,  
140 1990). Often, over antibiosis, other mechanisms are present: *Penicillium digitatum* strains  
141 resistant to pyrrolnitrin are still inhibited by *P. cepacia* (Smilanick and Denis-Arrue, 1992).

142 The main concerns, related to the use of antibiotics in food products, regard the selection of  
143 human pathogens resistant to these chemicals and the likely rapid development of resistance  
144 in fruit pathogens. Even if antibiotic producers are able to control wound infections occurred  
145 before antagonist application or latent infections, at the moment there are not such antagonists

146 registered for use on food.

147

## 148 2.2 Competition

149 Others selected microorganisms, particularly yeasts, act mainly establishing with the pathogen  
150 a competition for space and/or utilisation of some nutrients (Piano et al., 1997; Filonow,  
151 1998; Spadaro et al., 2001a). Yeasts can successfully compete with the pathogen, inhibiting  
152 its growth but often leaving it alive. Also some bacteria act for competition, for example  
153 *Enterobacter cloacae* against *Rhizopus stolonifer* on peaches (Wisniewski et al., 1989). In the  
154 competition for space yeasts are helped by the formation of an extracellular polysaccharide  
155 capsule that can promote adhesion to fruit surface (Andrews et al., 1994). Competition for  
156 nutrients was demonstrated for *Pichia guilliermondii* against *P. digitatum* cocultivated on  
157 synthetic means (Droby et al., 1989): the addition of exogenous nutrients resulted in a reduced  
158 efficacy, because antagonists offered better results when nutrients were scarce. A rapid  
159 multiplication and colonisation by antagonist cells in the wound was elucidated in various  
160 interactions (Droby et al., 1989; Smilanick and Denis-Arrue, 1992; Piano et al., 1997). Recent  
161 studies on the repartition of radiolabelled glucose between the antagonist yeasts  
162 *Sporobolomyces roseus* or *Cryptococcus laurentii* and the pathogen *B. cinerea* point out a  
163 strong sugar assumption by the BCAs, that blocks fungus conidial germination for nutrients  
164 deprivation (Filonow, 1998). In fruit wounds, nutrients competition is likely extended to other  
165 nutrients, such as nitrogen compounds low concentrated. Janisiewicz et al. (2000) recently have  
166 developed a non-destructive method using tissue culture plates with cylinder inserts  
167 containing defusing membrane at one end to study competition for nutrients without  
168 competition for space.

169

## 170 2.3 Parasitism



171 Antagonist and pathogen can interact also through a direct parasitism. Wisniewski et al.  
172 (1991) observed a strong adhesion *in vitro* of *P. guilliermondii* antagonist cells to *B. cinerea*  
173 mycelium, perhaps due to a lectin link. Such adhesion is blocked by exposure to compounds  
174 able to alter the protein integrity and the respiration process. Moreover *P. guilliermondii*  
175 shows an high activity of  $\beta$ -1,3-glucanase enzyme, that could be associated to pathogen link  
176 and result in the degradation of the fungal cell walls (Jijakli and Lepoivre, 1998). Also  
177 *Aureobasidium pullulans* in apple wounds produces extracellular exochitinase and  $\beta$ -1,3-  
178 glucanase, that could play a role in the biocontrol activity (Castoria et al., 2001). El-Ghaouth  
179 et al. (1998) found, through ultrastructural and cytochemical studies, that *Candida saitoana*  
180 yeast cells, when cultivated together with *B. cinerea* mycelium, link fungal hyphae that show  
181 cytological damages, such as papillae and other protuberances in the cell wall, and  
182 degeneration of the cytoplasm. Finally some yeasts, such as *S. roseus* and *C. laurentii*, when  
183 applied, are able to reduce, on apple carposphere, the conidial adhesion and germination of *B.*  
184 *cinerea*, that is favoured by butyl acetate, volatile aroma produced by the fruit (Filonow,  
185 2001).

186

#### 187 *2.4 Induced resistance*

188 Some BCAs can interact with the host tissue, particularly the wounds, increasing the  
189 cicatrization processes (Droby and Chalutz, 1994). Several antagonistic yeasts are as much  
190 effective as applied before pathogen inoculation. This observation has brought to suppose that  
191 yeast cell application could induce resistance processes in the fruit skin. Some *Candida*  
192 strains are able to cause chemical and osmotic changes in apple tissues, favouring antagonist  
193 settlement (McLaughlin et al., 1990). A *P. guilliermondii* strain stimulates the production of  
194 ethylene, an hormone able in grapefruit to activate the phenylalaninammonium-lyase  
195 (Wisniewski et al., 1991), enzyme involved in the phenols, phytoalexins and lignins synthesis.

196 A phytoalexins accumulation (scoparon and scopoletin) was noted in citrus fruit treated with  
197 yeast cell (Rodov et al., 1994). In addition to control decays, *A. pullulans* can cause on apple  
198 fruit a transient increase in  $\beta$ -1,3-glucanase, chitinase, and peroxidase activities starting 24 h  
199 after treatment (Ippolito et al., 2000).

200

### 201 **3. Extension of use**

202

203 Potential biocontrol agents often have some significant limitations: they have a reduced action  
204 range, because they partially act on specific hosts against well defined pathogens on particular  
205 environmental conditions. A method to select antagonist with a broader spectrum of action,  
206 preferably for commercial development, is the carrying out of initial efficacy tests on various  
207 pathogens and fruit species (Wilson et al., 1993; Lima et al., 1999).

208

#### 209 *3.1 Antagonist mixtures*

210 In the enhancement of a biocontrol system, work could focuses on a promising approach,  
211 which is the development of antagonist mixtures. There is a superior probability to have an  
212 effective biological control with a mixture of several antagonists, complementary and non  
213 competitive, that with only one. Such mixture has some peculiar advantages (Janisiewicz,  
214 1998): over the broadening of the spectrum of action (different fruits, cultivars and ripening  
215 stages), it can increase the efficacy (less biomass necessary) and offer a higher reliability,  
216 permitting a reduction in the application times and treatment costs. Moreover, it permits the  
217 combination of different genetic traits, avoiding genetic engineering. The evaluation of casual  
218 combinations of antagonists isolated from apple fruits and leaves resulted in the selection of a  
219 mixture of *P. syringae* and *S. roseus*, superior to both BCAs applied alone in the control of  
220 the *Penicillium* rot (Janisiewicz and Bors, 1995).

221

### 222 3.2 Preharvest use

223 One of the major obstacles to the development of postharvest BCAs is their inability to  
224 control previously established infections, such as latent infections. Field application of the  
225 BCAs may enable early colonisation of the fruit surfaces, protecting from these infections  
226 (Ippolito and Nigro, 2000). To be successful in preharvest application, potential antagonists  
227 should be able to tolerate low-nutrient availability, UV rays, high temperature and dry  
228 conditions. Leibinger et al. (1997) applied in preharvest a mixture of a yeast *A. pullulans* and  
229 a bacterium (*B. subtilis*) obtaining a level of control of *P. expansum* and *B. cinerea* on apple  
230 similar to fungicides. Teixido et al. (1998) applied unmodified and low water activity tolerant  
231 cells of *Candida sake* before harvest to control blue mould of apples, obtaining similar  
232 disease control.

233

### 234 3.3 Integrated use

235 Biological means of control can not at the moment solve all the problems of postharvest rots  
236 during fruit storage and they must be considered instruments to be used in combination with  
237 other methods of control in an integrated vision of disease management. For example,  
238 biocontrol agents can be combined with waxes and fungicides applied not only in post but  
239 also in pre-harvest (Pusey, 1994). In some laboratory and semi-commercial trials the efficacy  
240 of the BCA *P. guilliermondii* was consistently increased by the addition of small  
241 concentrations of imazalil or thiabendazole, reaching the levels of control of the fungicide  
242 alone (Droby et al., 1993). Antagonists, if employed together with fungicides, have to be  
243 tolerant or they request the development of resistant strains. Yeasts generally are tolerant to  
244 many of the fungicides used in postharvest: *Metschnikowia pulcherrima* (Spadaro et al.,  
245 2001a) is tolerant to relative high concentrations of benzimidazoles (benomyl and

246 thiabendazole) and dicarboximides (vinchlozolin and procymidone). Moreover  
247 microorganisms need to survive to thermal, hygrometry and atmospheric storage conditions.  
248 The efficacy of *M. pulcherrima* against *B.cinerea* and *P. expansum* is increased when fruits  
249 are stored at low temperatures optimal for host, respect to storage at 23°C, because the  
250 antagonist is more adaptable than the pathogens to cold temperatures (Spadaro et al., 2001a).  
251 Among the strategies evaluated during the last years, it is to remember the combination of  
252 biological treatments with other techniques alternative to chemicals: thermotherapy (Barkai-  
253 Golan and Phillips, 1991), ultraviolet rays (Chalutz et al., 1992), animal and plant natural  
254 products (Aharoni et al., 1993), calcium infiltrations (Janisiewicz et al.,1998), sodium  
255 bicarbonate (Teixido et al., 2001) or ethanol (Spadaro et al., 2001b).  
256 Calcium chloride infiltrations combined to antagonist application on apples increases control  
257 of *P. expansum* after 3 and 6 months of storage at 1°C, compared to simple biological  
258 treatment (Janisiewicz et al., 1998). A even more integrated approach, experienced on “Gala”  
259 apples (Conway et al., 1999) consists of a heat treatment (4 days at 38°C), followed by a  
260 calcium chloride (2%) infiltration and a *Pseudomonas syringae* cell suspension application.  
261 The result of the three treatments carried out together was a reduction of the Penicillium rots  
262 (91%) higher respect to calcium, antagonist, or heat treatments applied alone.  
263 The efficacy of *Pantoea agglomerans* for the control of green mould was improved when  
264 combined with sodium bicarbonate, or baking soda (Teixido et al., 2001), a common food  
265 additive, allowed with no restrictions for many application by European and North American  
266 regulations, and listed as approved ingredients on products labelled as “organic”. The  
267 combination of sodium bicarbonate, or ethanol, or acybenzolar-S-methyl with *M. pulcherrima*  
268 cell suspension and heat treatment was also studied by Spadaro et al. (2001b). Ethanol occurs  
269 naturally in fruit and many other food products, and its toxic effects on spores of fungal  
270 pathogens have been reported: the flesh treated with the alcohol was significantly firmer and

271 injury to the fruit did not occur.

272 It is possible to conclude that the possibilities to apply antagonists in the context of integrated  
273 control are very extensive.

274

#### 275 **4. Improvement of biocontrol agents**

276

##### 277 *4.1 Formulation and application*

278 Differently from soilborne or open field pathogens, where a disease control of 70-80% can be  
279 considered satisfying, postharvest disease control requests a higher level of efficacy and more  
280 consistent results. Even considering the most effective BCA's studied until now, they rarely  
281 reach the levels of efficacy of the fungicides. It is therefore necessary an increase in their  
282 antagonistic activity to achieve a practical use.

283 A rapid, efficient and cheap mass production of the antagonist, generally by fermentation, is a  
284 key issue. The efficacy of many antagonists of wound pathogens is directly related to the  
285 number of antagonist propagules inoculated (Hofstein et al., 1994), so that a really simple way  
286 to increase the effectiveness is the application of a higher number of cells. To scale-up a  
287 laboratory fermentation process to an industrial level, it is fundamental to find the nitrogen  
288 and carbon sources that provide maximum biomass production and minimum cost of media,  
289 whilst maintaining biocontrol efficacy. Costa et al. (2001) have studied yeast extract, dry beer  
290 yeast, sucrose, and molasses as possible substrate for the production of the biocontrol agent *P.*  
291 *agglomerans*.

292 A correct formulation can be decisive in the improvement of the efficacy and extension of the  
293 product shelf-life, facilitating the storage for periods of time commercially acceptable  
294 (Janisiewicz and Jeffers, 1997). The application of adjuvants can protect and stimulate the  
295 establishing of the antagonist on the host surface. The addition of calcium salts increases the

296 activity of several antagonistic yeasts (Janisiewicz et al., 1998). The addition of glycerol and  
297 trealose to the culture means augmented the osmotic tolerance and control capability of *C.*  
298 *sake* against *P. expansum* on apple (Janisiewicz, 1998). Sodium alginate,  
299 carboxymethylcellulose and chitosan, are adhesivants and can be added to yeast cell  
300 suspension, to increase the activity of the formulate. The three adhesivants have been added to  
301 a strain of *M. pulcherrima* (Piano et al., 1998) significantly increasing the efficacy against  
302 grey rot on apple. Chitosan has also a fungistatic activity demonstrated against the main  
303 postharvest pathogens of strawberry (El-Ghaouth et al., 1992). Recently, El-Ghaouth et al.  
304 (2000a) developed a biocontrol product called "bioactive coating" consisting of a unique  
305 combination of an antagonist with glycolchitosan, a chemically-modified chitosan. The  
306 bioactive coating made it possible to exploit the antifungal property of glycolchitosan and the  
307 biological activity of the antagonist. Moreover, when applied as a pretreatment, sodium  
308 carbonate enhanced the efficacy of the bioactive coating (El-Ghaouth et al., 2000b).

309 Also application systems have a fundamental role in the determination of the final results. The  
310 coatings most often applied to citrus fruit contain shellac, which is a purified product of the  
311 hardened resinous secretion of the scale insect *Kerria lacca*. Having developed coating  
312 formulations before biological control, it is important to test the suitability of such products  
313 for antagonistic applications. McGuire (2000) found that, after 3 or 4 months storage, *C.*  
314 *oleophila* survival was more efficacious when applied in the shellac than when applied by  
315 preliminary immersion and subsequent drying of fruit prior to shellacking. On the contrary,  
316 Chalutz and Droby (1998) had noted that the application of antagonistic yeasts in aqueous  
317 suspension before waxing results in an efficacy much more consistent respect to a unique  
318 application of antagonists and wax.

319 Another issue to face in the commercial production of biocontrol agents is the storage, that  
320 should be as long as possible. The BCA's should have a storability of at least 6 months and

321 preferably of 2 years (Pusey, 1994). Abadias et al. (2001a) found that freezing at  $-20^{\circ}\text{C}$  was  
322 the best method to preserve the viability of *C. sake* cells after freeze drying. Survival of the  
323 cells was higher using 10% skim milk as a protectant, and increased by using other  
324 appropriate protectants, such as lactose, glucose, fructose or sucrose. Skimmed milk was also  
325 the best rehydration medium with 1% peptone (Abadias et al., 2001b). However the efficacy  
326 of freeze-dried cells was significantly lower than that of fresh cells.

327 The transition from fungicide use to employment of biological means will be as easier as  
328 more flexible are the biofungicides.

329

#### 330 4. 2 *The nutritional environment*

331 Temperature, humidity and gas composition in warehouse are optimised to guarantee a high  
332 fruit quality during storage and should not be changed but it is possible to manipulate the  
333 chemical environment to favour the antagonist. The addition of nutrients preferably  
334 metabolised by the antagonist and difficultly by the pathogen was suggested in several  
335 antagonist-pathogen interactions (Janisiewicz, 1998). The application of two nitrogen  
336 compounds, L-asparagine and L-proline (Janisiewicz et al., 1992), and an analogue of sugar,  
337 2-deoxy-D-glucose (Janisiewicz, 1994) showed a consistent increase of the control of *P.*  
338 *expansum* on apple. Both aminoacids stimulated *P. expansum* germination but slowed  
339 mycelial growth. Also L-glutamine added to *M. pulcherrima* showed a direct influence on the  
340 yeast, because its application with the antagonist contributed to reduce Botrytis rot, while  
341 without yeast, it was ineffective (Piano et al., 1998). The sugar analogue 2-deoxy-D-glucose  
342 could be a useful additive to antagonistic microorganisms, provided that it has a fungicidal  
343 action on the major postharvest pathogens of apple and peach fruit (El-Ghaouth et al., 1997)  
344 and that the antagonist is resistant to its toxic effects. Recently the sugar analogue was found  
345 to be compatible with the antagonistic yeast *C. saitoana* and effective against apple and citrus

346 fruit decay (El-Ghaouth et al., 2000c).

347 Nutritional composition can influence not only density and competitiveness of the population,  
348 but also the production of metabolites crucial in many control systems, such as antibiotics  
349 (Gueldner et al., 1988) and cell wall degrading enzymes (Wisniewski et al., 1991). The form  
350 and concentration of nitrogen and carbon sources can therefore be important factors in the  
351 synthesis and secretion of key compounds in the biocontrol mechanism.

352

#### 353 *4.3 Manipulation of antagonists*

354 It is possible to increase the ecological competence of microorganisms manipulating  
355 antagonists with techniques of mutagenesis (physical or chemical) or of sexual recombination,  
356 through protoplast fusion or continued culture.

357 Because nitrogen seems to be a limiting substance, it was noted that biocontrol increases  
358 adding some aminoacids. Therefore biocontrol strains with a superior capability of  
359 exploitation of nitrogen compounds present, or with a higher transport or metabolism rate of  
360 the limiting factor, could be developed (Janisiewicz, 1998). Some phenolic compounds,  
361 among them benzoic or chlorogenic acid, present at the wound sites, influence negatively the  
362 colonisation by yeasts; it is therefore conceivable trying to obtain strains resistant to these  
363 phenolic compounds (Bizeau et al., 1989). Mutants that use new substrates, not metabolised  
364 by the pathogen, to provide a nutritional advantage, could also be induced (Janisiewicz,  
365 1998).

366 With genetic engineering techniques, features for the exploitation of plant products or  
367 additives applied together with the antagonist, for fungicide resistance, for carposphere  
368 colonisation in storage conditions, for synthesis of compounds favouring antagonism  
369 (antibiotics or siderophores), could be transferred into the potential antagonistic  
370 microorganisms (Pusey, 1994). Because one mechanism involved in the biocontrol of



371 postharvest fruit pathogens is mycoparasitism, lytic enzymes such as chitinases (Chernin et  
372 al., 1997), proteases and glucanases (De la Cruz et al., 1995) produced by bacterial and fungal  
373 microorganisms, could be inserted in the potential antagonists to improve the degradation of  
374 the pathogen cell walls, resulting in death or inhibition of growth of the attacked fungus. For  
375 biocontrol of soilborne pathogens, for example, a chitinase gene, isolated from *Serratia*  
376 *marcescens*, was introduced into the endophytic bacterium *Pseudomonas fluorescens* to  
377 improve the control of *Rhizoctonia solani* on bean (Downing and Thomson, 2000).

378 Another idea could be the insertion of the gene for amylase under constitutive promoter in  
379 some BCA's, because many fruits are rich in amid and antagonists could use effectively this  
380 carbon source having a consistent advantage. First experiments of transformation have been  
381 successful: *M. pulcherrima* was transformed with the green fluorescent protein gene (Nigro et  
382 al., 1999), *C. oleophila* was transformed with  $\beta$ -glucuronidase gene (Chand-Goyal et al.,  
383 1998), and histidine auxotrophs of *C. oleophila* were transformed with *HIS3*, *HIS4* and *HIS5*  
384 genes (Chand-Goyal et al., 1999). All these studies were accomplished only to obtain variants  
385 of the antagonistic strains with a genetically stable marker to expedite studies on the ecology  
386 of the yeasts on the fruit surface, but are really useful to put successive insertions of useful  
387 genes right. In all cases the transformed antagonists maintained the biocontrol capability.

388

## 389 **5. Molecular characterisation**

390

391 New molecular technologies improving the preliminary selection of biocontrol agents and the  
392 monitoring of field applied antagonists may greatly facilitate the selection and screening of  
393 yeast isolates having a superior antagonistic activity. Morphological and cultural  
394 characteristics alone are not sufficient to distinguish between strains of the same species and  
395 do not allow the exploitation of any possible naturally available genetic variability. Moreover

396 the effectiveness of the preharvest strategy depends to a large extent upon the survival of the  
397 antagonists in competition with other microorganisms on the surface of fruits. A study of the  
398 ecology of the BCA's is therefore necessary but often complicated by the existence of other  
399 microorganisms on the fruit that are morphologically similar to the antagonistic strain.  
400 Molecular approaches can help to differentiate a biocontrol agent from an epiphytic  
401 microorganism. Between the technologies already used to obtain DNA fingerprintings the  
402 random amplified polymorphic DNA (RAPD) and the arbitrarily primed PCR (AP-PCR) are  
403 to remember (Schena et al., 1999; 2000). Both these methods are based on the amplification  
404 of random genomic DNA fragments by arbitrarily selected PCR primers and have the major  
405 disadvantage that they are very sensitive to the reaction conditions, DNA quality and PCR  
406 temperature profiles, which limit their applications.

407 New techniques more robust and reliable, such as amplified fragment length polymorphism  
408 (Vos et al., 1995; De Barros et al., 1999), based on the selective PCR amplification of  
409 restriction fragments from a total digest of genomic DNA, and PCR amplification of the ITS  
410 regions of the ribosomal DNA (Masih et al., 2001) can be applied on potential biocontrol  
411 agents.

412

## 413 **6. Conclusions**

414

415 From this review, it appears that some significant progress has been made toward biological  
416 and integrated control of postharvest diseases on fruits. Some biofungicides are already on the  
417 market in a few countries, and will probably become more widely available as they are  
418 registered in more areas. Other BCA should reach the market soon. Postharvest conditions  
419 provide an ideal niche for BCAs since they are less subject to sudden climate changes, and are  
420 often equipped with a sophisticated system of climate control.

421 It is unrealistic to assume that perfect conditions for the development of BCA's will always  
422 prevail in the warehouse, and as a result, biofungicides will rarely stand alone as a complete  
423 measure of disease control under all conditions. For this reason, scientists, growers and  
424 consumers alike must accept the fact that BCA's are usually not as effective as pesticides. The  
425 success of biological control greatly depends in a change of mentality of the consumer, not  
426 anymore willing to have fruit with satisfying exterior aspects, but with an inner quality.  
427 Biological control should be viewed at the moment as an important if not essential component  
428 of an integrated disease management scheme if a significant and permanent reduction of  
429 pesticide use is our goal.

430

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432

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435

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