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Additional Information

1 **IMPROVING FUNCTION OF BIOCONTROL AGENTS INCORPORATED IN**

2 **ANTIFUGAL FRUIT COATINGS. A REVIEW.**

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6 **ABSTRACT**

7 The in-field performance of microbial biocontrol agents against fungal pathogens in

8 fruit is subject to considerable variability due to their sensitivity to both adverse

9 environmental conditions and their fluctuations. Therefore, to achieve an adequate

10 development and implementation of biological agent-based products, it is necessary to

11 improve their resistance and ability to control fungal diseases under a wide range of

12 conditions. In this review, an overview of the latest strategies for the enhancement of

13 the action of biocontrol agents is given. The combination of the antagonists with edible

14 polymers able to form coatings is one of the approaches with the greatest potential and

15 it is analysed in depth. This formulation approach of biocontrol products, including

16 adequate microbial protectants, can yield stable products with high microbial viability,

17 ready for field applications, with improved adherence and survival of the biocontrol

18 agent once applied in plant. The most recent studies into this field are reviewed and

19 summarized.

20 **Key words**: antagonists, biological control, biocontrol products, edible coatings,

21 postharvest decay

1. INTRODUCTION

Fruit losses caused by fungal diseases both in the field, during storage and under commercial conditions can reach more than 25% of the total production in industrialized countries, and over 50% in developing countries (Nunes, 2012; Spadaro & Gullino, 2004). Fungal diseases can be somewhat controlled by using non-chemical methods or non-selective fungicides, such as sodium carbonate, sodium bicarbonate, active chlorine and sorbic acid, although synthetic fungicides, applied both in orchard and post-harvest, represent the most widely-used method to control fungal diseases, with several shortcomings.

Firstly, synthetic pesticides are a source of environmental contamination and have a long degradation period (Tripathi & Dubey, 2004). Secondly, the use of these chemicals may lead to the presence of residues in food, which represent a toxicological hazard to human health. This is of particular importance in the case of fruit, since nowadays there is a rising consumer awareness of the need to follow a healthier diet, in which the role of fruit is essential. Ultimately, the continued use of chemical fungicides has generated the occurrence of resistance in the pathogen populations and, consequently, some of them have become ineffective against such strains (Panebianco et al., 2015; Tripathi & Dubey, 2004; Vitale, Panebianco & Polizzi, 2016). Consumer awareness in this regard has motivated an increasing demand for a reduction in the use of potentially harmful chemicals in order to obtain fruit free of pesticide residues (Liu, Sui, Wisniewski, Droby & Liu, 2013). Additionally, the authorities have developed stricter regulatory policies that require the search for eco-friendly strategies as an alternative to the chemical control of fungal decay.

45   In the past thirty years, the use of biocontrol agents (BCAs) or biological control has

46   been considered as one of the approaches with the greatest potential against fungal

47   pathogens, either alone or as part of integrated systems for pest management (Spadaro

48   & Gullino, 2004). Consequently, extensive research has been devoted to exploring and

49   developing this field, as recently reported by Spadaro & Droby (2016).

50   Fungi, yeasts and bacteria are potential microorganisms to be used as antagonists for

51   controlling the post-harvest diseases of fruits and vegetables. An ideal BCA should

52   meet a number of requirements, as reported by several authors (Abano & Sam-Amoah

53   2012; Droby, Wisniewski, Macarisin & Wilson, 2009; Sharma et al., 2009). The

54   characteristics of an ideal antagonist are that it must be: genetically stable, effective at

55   low concentrations, undemanding in terms of its nutrient requirements, capable of

56   surviving under adverse environmental conditions, effective against a wide range of

57   pathogens in different commodities, amenable to production on inexpensive growth

58   media, amenable to formulation with a long shelf-life, easy to dispense, resistant to

59   chemicals used in the post-harvest environment, not detrimental to human health,

60   compatible with other chemical and physical treatments and not detrimental to the

61   quality of the fruits and vegetables it preserves.

62   An extensive body of research has been devoted to the understanding of the

63   mechanisms by which BCAs exert their action against pathogens. Nonetheless, in many

64   cases, the suggested modes of action whereby antagonists wield their biocontrol effect

65   are not totally elucidated, especially due to the fact that several mechanisms frequently

66   take place at the same time since and successful BCAs are generally equipped with

67   several attributes which often work in concert and may be crucial for controlling disease

68   development (Droby et al., 2009; Jamalizadeh et al., 2011; Janisiewicz & Korsten,

69   2002). Despite the difficulties, insight into the action modes involved will permit an

70   improvement in both the biocontrol performance and the development of appropriate

71   formulations and methods of application. Competition for nutrients and space between

72   the pathogen and the antagonist is considered to be the major mode of action, but other

73   mechanisms such as parasitism, the production of secondary metabolites or the

74   induction of host defences, have also been reported, as shown in Table 1.

75   The potential BCAs often show some significant limitations, such as their sensitivity to

76   both adverse environmental conditions and their fluctuations, and their narrow range of

77   activity because BCAs act on specific hosts against well-defined pathogens (Spadaro &

78   Gullino, 2004). For these reasons, the performance of biological-based control strategies

79   in the field is subject to significant variability which constitutes a significant constraint

80   to their practical implementation (Droby et al., 2009; Wisniewski et al., 2007). In these

81   sense, different approaches have been reported to make the BCAs more efficient: the

82   use of mixed cultures (Conway, Janisiewicz, Leverentz, Saftner & Camp, 2007;

83   Panebianco, Vitale, Polizzi, Scala & Cirvilleri, 2016), their physiological manipulation

84   (Usall et al., 2009; Wang, He, Xia, Yu & Zheng, 2014) and their combination with

85   different types of substances (Guo et al., 2014; Qin et al., 2015; Zhou et al., 2016). The

86   application of BCAs in combination with coating materials has been reported to

87   enhance the BCA effectiveness at inhibiting the growth of plant pathogens, as discussed

88   in the next section.

89   **2.   EDIBLE COATING FORMULATIONS FOR ANTIFUNGAL CONTROL**

90   **ON FRUIT**

91   The application of commercial coatings is a common practice for many fruits. These

92   coatings are generically known as waxes, since their composition is based on paraffin

93   wax or a combination of various other waxes, such as beeswax or carnauba. They are

94   anionic microemulsions that may also contain synthetic components, such as

95   polyethylene and petroleum waxes, ammonia or morpholine, which are applied to

96   reduce fruit weight loss and shrinkage, while improving their appearance and physical

97   resistance. Commercial waxes are often amended with synthetic fungicides in order to

98   control post-harvest diseases (Palou et al., 2015).

99   However, due to the potential hazards of synthetic coatings, such as the presence of

100 potentially toxic substances on the fruit surface, the use of edible coatings (ECs) as a

101 replacement for these currently-used commercial waxes has been widely studied. Then,

102 the use of edible coatings (ECs) to protect fruits from fungal decay at postharvest

103 conditions cannot be considered as a new approach anymore, since there are a great

104 number of studies dealing this topic, in which different matrices and active compounds

105 are used, such as EOs and food preservatives (Table 2). However, the use of ECs to

106 carry antagonistic microorganisms, to be used at both pre and post-harvest conditions, is

107 an area that has been less widely explored.

108 Coating formation on the surface of a product implies the application of a film-forming

109 solution or dispersion of a polymeric material with filmogenic capacity (Campos,

110 Gerschenson & Flores, 2011). Those coatings and films obtained with food-grade

111 polymers/ingredients can be eaten as part of the whole product and their use is

112 interesting for fruits and vegetables which can be directly consumed. Therefore, the

113 composition of ECs and films must conform to the regulation that applies to the food

114 product concerned (Guilbert, Gontard & Cuq, 1995).

115 Their basic components are typically hydrocolloids (polysaccharides and proteins) and

116 lipids, and these can either be used individually or in combination, in order to obtain

117 composite or blend coatings. The composite coatings take advantage of the specific

118 functional characteristics of each group, reducing their drawbacks (González-Martínez

119 et al., 2011). Other components, such as plasticizers and emulsifiers (or surfactants),

120 may be added to the matrices as a means of improving the flexibility, extensibility

121 and/or the stability of the structure (Palou et al., 2015). Moreover, formulations can act

122 as carriers of a very wide range of other minor compounds, such as antioxidants,

123 antimicrobials, certain nutrients like vitamins, volatile precursors, flavours, firming

124 agents or colorants (González-Martínez et al., 2011). Multilayer coatings applied by the

125 "layer by layer" technology have also described as effective enhancers of fruit quality

126 during storage, optimizing the coating functionality through the complementary

127 properties of different hydrocolloids (Poverenov et al., 2014). Additionally, ECs may be

128 used as carrier matrices of bioactive compounds to enhance the safety and the quality of

129 fruit (Quirós-Sauceda, Ayala-Zavala, Olivas & González-Aguilar, 2014). Bioactive

130 compounds can be carried by the ECs to the fruit skin by diffusion release, which is

131 controlled by their solubility and permeability in the polymer matrix. Table 2 shows

132 some examples of different coatings applied to fruits and vegetables to improve their

133 quality preservation, using different polysaccharide or protein matrices.

134 Polysaccharides are the most commonly used components in fruit ECs, probably due to

135 their better microbial and physical stability over time in comparison with protein-based

136 coatings, especially in high relative humidity environments (González-Martínez et al.,

137 2011). Other compounds which are commonly used in fruit ECs are lipids, which have

138 low water vapour permeability and are very useful for controlling their desiccation

139 (Vargas et al., 2008). In fact, TAL-Prolong and Semperfresh are two commercially

140 available composite coating formulations based on carboxymethylcellulose, sucrose

141 fatty acid ester, sodium salt and an emulsifier, used for the shelf-life extension of

142 bananas and other fruits (Nisperos-Carriedo, Baldwin & Shaw, 1992; Tharanathan,

143 2003).

144 Intensive research has been devoted to the application of ECs as a means of improving

145 the quality and shelf-life of fruit. For instance, Fakhouri, Martelli, Caon, Velasco & Mei

146 (2015) studied the effect of ECs based on native and/or waxy corn starch and gelatine

147 on the quality of grapes; Nadim, Ahmadi, Sarikhani & Amiri Chayjan (2015) applied

148 methylcellulose-based coatings to strawberries for the purposes of studying their quality

149 throughout storage; and Muangdech (2016) developed ECs based on aloe vera gel,

150 chitosan and carnauba wax to study the post-harvest storage life of mango. These are

151 only some recent examples of the numerous studies published on this topic.

152 As far as the prevention of microbial decay is concerned, especially that caused by fungi

153 in fruit, ECs and films based on the biopolymer components (with the exception of CH)

154 are not capable of accomplishing this task. Hence, in order to obtain ECs with

155 antifungal properties, food-grade antimicrobial agents have to be incorporated into the

156 formulations (Liu, 2009; Palou et al., 2015). In this sense, the use of ECs containing

157 antimicrobial substances may be more efficient than the direct application of

158 antimicrobial agents, given that active compounds may selectively and gradually

159 migrate from the coating onto the surface of the fruit, helping to maintain a high

160 concentration of bioactive compounds where needed (Elsabee & Abdou, 2013; Quirós-

161 Sauceda et al., 2014).

162 According to Palou et al., (2015), the antifungal compounds that can be incorporated

163 into ECs might be classified in the following categories: (a) synthetic food preservatives

164 or GRAS compounds with antimicrobial activity, which include some organic and

165 inorganic acids and their salts (benzoates, carbonates, propionates or sorbates) and

166 parabens (ethyl and methyl parabens) and their salts, among others; (b) natural

167 compounds, such as EOs or other natural plant extracts (capsicum, carvacrol, cinnamon,

168 cinnamaldehyde, citral, eugenol, grape seed extracts, lemongrass, propolis extract,

169 oregano, rosemary, thyme oil, vanilla, vanillin, etc.); (c) antimicrobial antagonists, such

170 as BCAs. Several studies have been summarized in Table 2.

3. EDIBLE COATINGS CONTAINING BIOCONTROL AGENTS

172 BCA agents can also be incorporated to the coating-forming formulations to obtain

173 coatings or films loaded with the antagonist cells, with the ability to maintain their

174 viability and allowing for cell distribution on the coated product. In this sense, the

175 coating-forming formulations should contain components which not only allow for

176 coating formation, but also be compatible with the cells and provide them an adequate

177 substrate for nutrition and growth. An ideal formulation for BCA-coating product must

178 be: 1) water soluble or dispersible, without organic solvents toxic for cells, 2) able to

179 maintain or increase cell population when applied in the product, 3) able to impart gloss

180 and modulate plant respiration and 4) contain safe ingredients for the final consumers.

181 Likewise, in the case of formulated BCAs with coating-forming agents, these should

182 provide adequate properties to maintain microbial and physical stability of the

183 formulation during storage throughout the commercialization period. The latter aspect is

184 highly dependent on the final format of the product (liquid or dried products).

185 In comparison with the large number of studies dealing with the incorporation of

186 antifungal compounds into ECs for fruit applications, there is little information about

187 coatings including antifungal microbial antagonists for the purposes of controlling fruit

188 pathogens. Some studies were published in the 1990s, but there has been little recent

189 research. This approach, consisting of the combination of BCA and coatings as a means

190 of preserving fruit from fungal decay has proven to be effective. This effectiveness is

191 attributed to the advantages of both strategies, which are summarized in Figure 1.

192 While BCA endows the coating with antifungal capacity, the coating provides good

193 adherence (binding element) and survival (potential nutrient) to the BCA, protecting

194 them against ultraviolet (UV) radiation, desiccation, and rain and temperature variations

195 in the field (Potjewijd, Nisperos, Burns, Parish, & Baldwin, 1995). Likewise, coating-

196 forming agents can improve the stability and dispersability of cell suspensions, which

197 could allow for a more homogeneous spatial distribution on the fruit surface. All these

198 aspects may extend the time available for the BCA to multiply and become established

199 (Cañamás et al., 2011). This is of vital importance in the case of antagonists whose

200 main mechanism of action is competition for nutrients and space, since to successfully

201 compete with pathogens, there has to be a sufficient quantity of cells at the correct time

202 and location (El-Ghaouth, Wilson & Wisniewski, 2004; Sharma et al., 2009).

203 ECs can also exert a direct effect against a pathogen both via their intrinsic antifungal

204 propertie or by acting as a mechanical barrier to protect fruit (Chien, Sheu & Lin, 2007;

205 Meng, Qin & Tian, 2010). When the EC exhibit antifungal properties (e.g. chitosan

206 based coatings) it could negatively influence the viability and performance of the BCA.

207 Therefore, in the design of the coating formulation aimed to carry microbial antagonists,

208 the study of their compatibility is required in order to optimize their ability and efficacy

209 in improving the performance of the microorganisms under practical conditions.

210 As regards the technique whereby the combined application of ECs and BCAs takes

211 place, some authors have reported the separated application of the EC and the BCA

212 suspension, where the microbial antagonists might be applied before or after coating

213 (Meng, Qin & Tian, 2010; Rahman, Mahmud, Kadir, Abdul Rahman & Begum, 2009).

214 Another option is the incorporation of the BCA directly into the coating-forming

215 dispersion and then their joint application in only one step (McGuire & Baldwin, 1994;

216 Aloui, Licciardello, Khwaldia, Hamdi & Restuccia, 2015; Marín et al., 2016)

217 Meng et al. (2010) applied the yeast *Cryptococcus laurentii* on grapes at pre-harvest and

218 then, the treated fruit was coated with CH solutions. The results revealed that the

219 combined treatments enhanced the control of the fungal decay of grapes. Rahman,

220 Mahmud et al. (2009) applied separated suspensions of the BCA *Burkholderia cepacia*

221 and CH on papaya at post-harvest, in combination with $CaCl_2$, as stimulant of the

222 antagonistic activity, showing that the combination of the different treatments resulted

223 in a more effective disease control. In these particular cases, the coating performed

224 more as an additional treatment than as a support for the BCA due to the antifungal

225 properties of the polysaccharide.

226 As concerns the joint application of coating agent and BCA in only one step, several

227 recent studies have been reported (Aloui et al., 2015; Marín et al., 2016; González-

228 Estrada, Carvajal-Millán, Ragazzo-Sánchez, Bautista-Rosales & Calderón-Santoyo,

229 2017). Aloui et al. (2015) incorporated the BCA *Wickerhamomyces anomalus* into

230 sodium alginate and locust bean gum coatings to control the growth of *Penicillium*

231 *digitatum* on oranges. These authors reported that the coatings maintained more than

232 85% of the initial BCA and that this combination completely inhibited the pathogen.

233 Similarly, Marín et al. (2016) applied on grapes several formulations of ECs, based on

234 polysaccharides or proteins, as support of the BCA *Candida sake* for the purposes of

235 controlling the pathogen *Botrytis cinerea*. The study showed that the adherence and

236 survival of the BCA was improved with the combined application and consequently a

237 better control of the fungal decay was observed. In the same way, González-Estrada

238 (2017) reported that the incorporation of the antagonist *Debaromyces hansenii* in a

239 coating matrix based on arabinoxylan allowed for a maintaince of more than 97% of the

240 initial yeast population. Moreover, they observed that the application of yeast entrapped

241 in the coating improved its efficacy against blue mold decay under storage of lime.

242 Different studies regarding the combined application of edible and commercial coatings

243 containing antagonistic microorganisms are summarized in Table 3. In the reported

244 studies the applied concentrations of the different BCA ranged from 10^7 to 10^9 CFU per

245 millilitre of coating suspension, depending on the antagonist, whereas greater

246 differences can be found in the concentration of solids in the coating dispersions (Table

247 3). In some of the studies, concentrations lower than 1% of coatings solids were applied

248 while percentages up to 20% were used in others cases. This is an important factor since

249 the amount of coating solids will affect not only to the final price if the commercial

250 application is intended, but also the efficacy of the BCA. Marín et al. (2016) observed

251 that a minimum value of the polymer:CFU ratio is required to observe significant

252 increase in the BCA population with respect to the control application (without coating)

253 in grapes. Similarly, Parafati et al. (2016) tested different concentrations of locust bean

254 gum dispersions incorporated with two yeasts and observe that the highest percentage of

255 coating solids enhanced to a greater extent their activity against blue mould decay of

256 mandarin.

257 In general, an enhancement of the BCA viability on the fruit surface and an increased

258 control of the pathogens can be achieved with combined applications of BCA and ECs,

259 even in applications carried out in the field (Cañamás et al., 2008b, Cañamás et al.

260 2011, Calvo-Garrido et al., 2013). Nevertheless the specific mechanisms whereby a

261 determined ECs influence the performance of a determined microbial antagonist, and

262 also their effects of pathogen, require further studies

4. FORMULATION OF BIOCONTROL AGENTS WITH COATING-

FORMING AGENTS

265 A biocontrol product (BCP) could be defined as a mixture of the active ingredient

266 (BCA), a carrier providing physical support for the microorganism, which can be liquid

267 (aqueous dispersion) or a dried powder, It is common practice to incorporate adjuvants

268 and/or protectants, which can be incorporated at different points, such as in the mass

269 production, formulation and storage steps or just before the application in the mixing

270 tanks (Cañamás et al., 2008a; 2008b). Additives can be used as stickers, diluents,

271 suppressants, dispersants, emulsifiers, wetters, gelants, humectants, brighteners,

272 spreaders, stabilizers, sunscreens, synergists, thickeners, nutrients, binders, or

273 protectants, depending on their function in the formulations. As previously described,

274 some of these functions can be accomplished by components of ECs.

275 The formulation of BCAs with EC agents can enhance their efficacy, extend the range

276 of conditions over which they are effective, increase their ability to withstand drastic

277 changes in the phyllosphere and improve their survival under unfavourable

278 microclimatic conditions (Cañamás et al., 2011). In this sense, the formulation process

279 is decisive and has a significant influence on the successful delivery of the antagonists,

280 the shelf-life and storage requirements of the BCP and on its cost (Janisiewicz &

281 Korsten, 2002; Spadaro & Gullino, 2004; Yánez-Mendizábal et al., 2012).

282 In comparison with the large number of effective antagonists under laboratory

283 conditions, the success of formulated BCA-based products has been limited and just a

284 few products have reached advanced stages of development and commercialization.

285 Generally, information on the specific composition and production of formulations of

286 commercial BCA is largely proprietary (Sztejnberg, 1993; Howard Davies, Ebbinghaus,

287 GÖRTZ & Carbonne, 2014; Brandi, Trainer & Westerhuis, 2016). Table 4 summarizes

288 some characteristics for different comercial BCP. As can be observed, the concentration

289 of BCA varies between 10^8-10^{10} CFU or conidia/g, depending on the product, and the

290 ratio of BCA:inert solids, also differs for the different products. For instance, Aspire^{TM}

291 a bioproduct based on *Candida oleophila*, contains 55% of the yeast isolate and 45% of

292 inert ingredients, while Bio-Save 10 LP, based on *Pseudomonas syringae* only contains

293 30% of the bacteria and 70% of other ingredients.

294 Some of the reasons for the limited success of BCA-based commercial products are the

295 inconsistency and variability of the efficacy under commercial conditions, the narrow

296 tolerance to fluctuating environmental conditions of the BCAs and the difficulties in

297 developing shelf-stable formulated products that retain a biocontrol activity similar to

298 that of the fresh cells (Janisiewicz & Jeffers, 1997; Usall et al., 2009). Another

299 drawback is the difficulty involved in the market penetration and perception of the

300 customers/industry and small-sized companies whose available resources are too low to

301 maintain development and commercialization (Spadaro & Droby, 2016).

302 According to Melin, Håkansson & Schnürer (2007) an ideal BCP should satisfy a set of

303 criteria. It should: be inexpensive to produce, be easy to distribute to the intended

304 environment, have a long shelf-life, preferentially also upon storage at ambient

305 temperature and be easily rehydrated (in the case of dry formulations). Finally, the

306 biocontrol activity must be maintained through all the formulation steps, long-term

307 storage and rehydration. Coating-forming agents can contribute to improve the

308 properties of formulations from different points of view, depending on kind of

309 formulation (physical state), as discussed below.

310 Liquid formulations are aqueous suspensions which consist of biomass suspensions in

311 water or oils, or combinations of both (emulsions) (Schisler, Slininger, Behle &

312 Jackson, 2004). They are the simplest way to stabilize the viability of microbial cells,

313 but require refrigeration (Droby, Wisniewski, Teixidó, Spadaro & Jijakli, 2016). In

314 aqueous formulations of BCA cells, different substances may be incorporated to adjust

315 the water activity (a_W) to obtain the same water chemical potential of the cells (isotonic

316 solutions).

317 Some examples of the liquid formulation of different BCA have been reported by

318 several authors: *Candida sake* CPA-1 formulated in isotonic solutions (Abadias et al.,

319   2003); heat-adapted *Candida sake* CPA-1 cells and combined with an EC (Cañamás et

320   al., 2011); *Cryptococcus laurentii* and *Pichia membranaefaciens* with sugar protectants

321   and antioxidants (Liu et al., 2009) and *Pichia anomala* with different substances (Melin,

322   Schnürer & Håkansson, 2011). For their part, Batta (2007) and Peeran, Nagendran,

323   Gandhi, Raguchander & Prabakar (2014) developed formulations of different BCAs

324   supported in emulsions. Coating forming agents could enhance the stability of the

325   emulsions without implying relevant changes in the a_w, because of their high molecular

326   weight, and could also play a nutrient and protectant role for the cells.

327   In general, dry formulations have a longer shelf life and exhibit a lower risk of

328   contamination than liquid ones, and allow for easier transport, distribution, storage and

329   manipulation (Fravel, 2005; Usall et al., 2009). However, they also present some

330   shortcomings, such as a marked loss of viability in the cells not only during dehydration

331   and storage but also during the subsequent rehydration process (Melin, Håkansson,

332   Eberhard & Schnürer, 2006).

333   Dry formulations of BCAs take several forms. Wettable powders consist of dry inactive

334   and active ingredients (BCA cells) intended to be applied as a suspension in liquid.

335   Dusts are powder-like and consist of dry inactive and active ingredients to be applied

336   dry, generally to seeds or foliage. Granules are described as free-flowing aggregated

337   products composed of dry inactive and active ingredients (Schisler et al., 2004). Dry

338   formulations can be applied directly to the target plant or, in the case of wettable

339   powders and water dispersible granules, mixed into water where the suspension of

340   biomass and inactive ingredients are applied as a spray.

341   The inactive ingredients of dry formulations act as carriers of the antagonists and may

342   be organic (grain flours, powders from plants, starches and their derivatives, etc.) or

343   mineral (peats, talc, diatomaceous earths, kaolin, clay, etc.) (Mokhtarnejada, Etebariana,

344   Fazelib & Jamalifarb, 2011; Schisler, Slininger & Olsen, 2016). The use of coating-

345   forming agents as carriers in the formulation of dry BCP can represent several

346   advantages. The EC compound would firstly act as support for the BCA cells during the

347   drying and storage steps and, when applied, the EC would provide the BCA with the

348   previously described benefits, such as an improved adherence and survival on the fruit

349   surface or as a source of nutrients. Wettable powders or water dispersible granules

350   would be the most adequate forms for dry BCA-EC formulations, since previous water

351   dispersion of coating-forming agents is necessary to form the ECs. Moreover, the

352   polymeric nature of the coating-forming agents confer them high values of the glass

353   transition temperature and water sorption capacity, which contributes to limit the

354   product a_w after drying, while they have a high value of the critical water content for

355   plasticization, which benefit the physical stability of the product.

356   There are few publications on the use of EC-forming agents as support for microbial

357   antagonist based formulations and, in most of the cases, different kinds of starch and

358   derivatives are the used ingredients. This is because the production cost is a key factor

359   that must be borne in mind and kept to a minimum (Melin et al., 2011), and starch is

360   low cost and readily available. Lewis, Fravel, Lumsden & Shasha (1995) obtained

361   granular formulations using pre-gelatinized starch and the BCA *Gliocladium virens* and

362   Mounir et al. (2007) used maize starch to produce a formulation of *A. pullulans*. More

363   recently, Soto-Muñoz et al. (2015) and Gotor-Vila et al. (2017) developed different dry

364   formulations of *Pantoea agglomerans* and *Bacillus amyloliquefaciens* respectively

365   using native potato starch as carrier and Marín, Atarés, Cháfer & Chiralt (2017)

366   characterized the most relevant properties of formulations of *Candida sake* based on

367   pre-gelatinized starch and maltodextrins.

368 Dehydration is a very critical step since not all microorganisms are amenable to drying

369 and many tend to lose viability during both the drying process and subsequent storage.

370 For that reason, many approaches have been developed in order to reduce the losses in

371 viability, such as adding protectants to growth media or directly to cells (Abadias,

372 Torres, Usall, Viñas & Magan, 2001; Schisler et al., 2016; Yánez-Mendizábal et al.,

373 2012). Of the protectant agents, skim milk and sugars, used either alone or in

374 combination, have been widely used because of their relatively low prices and

375 chemically innocuous nature (Costa, Usall, Teixidó, Torres & Viñas, 2002; Khem,

376 Woo, Small, Chen & May, 2015; Santivarangkna et al., 2008). Sugars, especially

377 disaccharides, are able to protect the cell membranes from dehydration (Leslie, Israeli,

378 Lighthart, Crowe & Crowe, 1995). The proteins present in milk provide a protective

379 coat for the cells and seem to restore injured cells during dehydration, avoiding osmotic

380 shock, disruption and the death of cells (Champagne, Gardner, Brochu & Beaulieu,

381 1991). Many coating-forming agents such as whey protein and maltodextrins has been

382 reported as excellent cell protectants during drying of different microorganisms,

383 especially probiotics (Eratte et al. 2015; Huang et al. 2017; Liao et al. 2017).

384 The classical dehydration processes applied to obtain BCPs are freeze-drying, spray-

385 drying and fluidized-bed drying. Although these methods present several differences,

386 the inclusion of cells in a carrier containing protectants or different adjuvants before the

387 drying step is common to all methodologies. In this sense, biopolymers used as coating-

388 forming agents could act as effective carriers together with other cell protectant agents.

389 **4.1 Stability of biocontrol formulations during storage**

390 The preservation of the cell viability during fermentation, drying, storage and

391 rehydration is one of the main goals of the formulation process (Schisler et al., 2004).

392 After the drying process, storage conditions have a great influence on the shelf-life of

393   the dry BCP. The final moisture content or, preferably, a_W of the products, and

394   temperature and relative humidity conditions during storage can profoundly affect the

395   survival of BCA in the formulations (Fravel, 2005). Therefore, all of these parameters

396   deserve careful research in order to maintain the formulation in optimal conditions for

397   its further applications (Torres et al., 2014).

398   Low moisture content after the drying process and its maintenance at the same level

399   during storage has been reported as being critical to the preservation of cell viability.

400   Dunlap & Schisler (2010) obtained dried granules based on *Cryptococcus flavescens* in

401   an inert support with different moisture contents using a fluidized-bed dryer and

402   evaluated its storage stability at 4°C for up to one year. These authors reported that 4%

403   moisture content (the lowest tested) had the best long-term survival (1 year). Mokiou &

404   Magan (2008) found that a moisture content of >10% was best for the viability of

405   *Pichia anomala* formulations obtained by fluidized bed drying. Nevertheless, more than

406   moisture content, a_w is the most critical parameter at defining the cell viability

407   preservation during storage. Recently, Marín et al. (2017) reported that the viability of

408   *Candida sake* formulated in starch derivatives by fluidized bed drying was greatly

409   affected by the product a_w (or RH of equilibrium); the lower the a_w the higher the cell

410   viability preservation during storage. There are few reports dealing with the influence of

411   a_w during storage on BCA-formulations and the majority of the published studies have

412   been carried out using probiotics. Miao et al. (2008) observed that the retention of the

413   cell viability of *Lactobacillus paracasei* and *Lactobacillus rhamnosus* was greatest for

414   cells stored at a_w of 0.11 and compromised at higher a_w. Likewise, Poddar et al. (2014)

415   studied the viability of dried *Lactobacillus paracasei* during storage at 25°C under

416   different a_w. These authors reported that, at a_W of 0.11, cell viability loss was minimal,

417   while viability was lost in all powders within 22 days at a_w of 0.52. Recently, Agudelo,

418 Cano, González-Martínez & Chiralt (2017) reported that the lower the a_W of whey

419 protein-maltodextrin based formulations of *Lactobacillus rhamnosus* the better the cell

420 preservation during storage.

421 At high humidity conditions, water act as a plasticizer and increases the molecular

422 mobility of the components of the dry formulations (Poddar et al., 2014). This increase

423 results in caking and the crystallization of the amorphous structures and in an

424 instantaneous loss of microbial viability during storage. The glassy (non-crystalline)

425 state has been shown to enhance the storage life (Miao et al., 2008; Poddar et al., 2014).

426 In this sense, coating-forming agents increase the critical water content for the powder

427 plasticization, which contributes to enhance both physical and microbial stability.

428 Additionally, the shelf-life of dry products containing microorganisms is highly

429 dependent on the storage temperature. In general, as the storage temperature increases,

430 mortality also increases and storage time is reduced (Costa et al., 2002). This has been

431 attributed to the fact that temperatures between 4 and 10°C cause a slowing down of

432 both cell division and metabolic rate in microorganisms and, in this situation, cells are

433 capable of withstanding the depletion of nutrients and the accumulation of toxic

434 metabolites (Mejri, Gamalero & Souissi et al., 2013).

435 Several studies have reported the effect of the storage temperature on the viability of

436 different biological formulations. Abadias, Teixidó, Usall, Benabarre & Viñas (2001)

437 reported that storage at 4°C was required to maintain the viability of *Candida sake* cells

438 obtained by freeze-drying. Likewise, Torres et al. (2014) studied the viability of freeze-

439 dried *Pantoea agglomerans* cells, which was significantly higher at -20 and 5°C, as

440 opposed to at 25°C. Similar tendencies have been reported by other authors for different

441 microorganisms (Kinay & Yilniz, 2008; Melin et al., 2011; Spadaro, Ciavorella, Lopez-

442 Reyes, Garibaldi & Gullino, 2010).

443 **5.   FINAL REMARKS**

444 From a practical point of view, the obtaining of BCPs competitive in price and

445 effectiveness, with easy distribution in the market, offers many advantages both to the

446 production sector and to consumers. For producers, the BCA-based products might be a

447 way of reducing both the losses caused by fungal diseases and the presence of pesticide

448 residues on their fruit, thus being able to respond to the increasing consumer demand

449 for chemical-free products. For agrochemical companies, BCPs might represent a

450 viable alternative to gain access to both the organic fruit and vegetable markets and to

451 integrated production systems, which have shown huge potential in the last few years.

452 Dry formulations of BCA with edible coating-forming agents, including adequate

453 microbial protectants, can yield stable products with high microbial viability, ready for

454 field applications, with improved adherence and survival of the biocontrol agent once

455 applied in plant. Likewise, polymeric coating-forming agents exhibit high glass

456 transition temperatures and water sorption capacity, which contributes to limit the

457 product a_w after drying, while they have a high value of the critical water content for

458 plasticization, which benefits the product physical stability. On the other hand, the

459 control of the product a_w after drying and the storage conditions (temperature and water

460 impermeable packaging) are key factors to guarantee the stability and efficacy of BCP

461 in field applications. An ideal coating agent aimed to act as carrier of a BCA would be

462 one capable of supporting the antagonist cells when applied on fruit and during the

463 storage of the final product, both from a nutritional point of view but also regarding

464 their stability. Moreover, it should be adequate to participate in the formulation

465 processes and also inexpensive in order to ensure a competitive final price.

466   Therefore, more studies are necessary to elucidate the best polymer and protectant

467   components of the BCA formulation, the more adequate drying conditions and the

468   optimal storage conditions of the BCP in order to extend shelf life for crop applications.

469

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843   **FIGURE CAPTIONS**

844   **Figure 1**. Advantages of the joint application of biocontrol agents and edible coatings.

845 **Table 1**. Representative antagonistic fungi, bacteria and yeasts used as biocontrol agents and suggested mechanisms of action
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Biocontrol agent	Mechanism of action	Source	Pathogen	Application	Reference
Bacillus amyloliquefaciens	Competition for nutrients and space, production of secondary metabolites	Apple	*Botryosphaeria dothidea*	Apple	Li et al. (2013)
Bacillus subtilis	Competition for nutrients and space, production of secondary metabolites	Soil and stone fruit	*Monilinia fructicola*	Stone fruit	Yánez-Mendizábal et al. (2012)
Cryptococcus laurentii	Competition for nutrients	Pear	*Penicillium expansum*	Pear	Zeng et al. (2015)
Hanseniaspora uvarum	Competition for nutrients and space, induction of host defense	Strawberry	*Botrytis cinerea Rhizopus stolonifer*	Strawberry	Cai, Yang, Xiao, Qin & Si (2015)
Metschnikowia pulcherrima	Competition for nutrients, parasitism	Fig	*Botrytis cinerea Cladosporium cladosporioides Monilia laxa Penicillium expansum*	Apple and nectarine	Ruiz-Moyano et al. (2016)
Pantoea agglomerans	Competition for space and nutrients, attachment to pathogen, parasitism	Plum	*Monilinia fructicola*	Plum	Janisiewicz, Jurick, Vico, Peter & Buyer (2013)

Penicillium oxalicum	Induction of host defense	-	*Fusarium oxysporum*	Melon and watermelon	De Cal, Sztejnberg, Sabuquillo & Melgarejo (2009)
Pichia membranaefaciens	Competition for nutrients and space, attachment to pathogen, induction of host defense		*Colletotrichum gloeosporioides*	Citrus	Zhou, Zhang & Zeng (2016)
Pseudomonas aeruginosa	Induction of host defense	Grape	*Aspergillus* spp.	Grape	El-Shanshoury, Bazaid, El-Halmouchm & Ghafar (2013)
Trichoderma spp.	Competition for nutrients and space, induction of host defense, production of secondary metabolites, parasitism	Soil	*Fusarium oxysporum*	Melon	Gava & Pinto, 2016

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852 **Table 2**. Edible coatings carrying different antimicrobial agents with antifungal effects in different fruits. AG: Arabic gum; AV: aloe vera; CH:
853 chitosan; CMC: carboxymethylcellulose; EOs: essential oils; G: gelatin; HPMC: hydroxypropylmethylcellulose LBG: locust bean gum; MC:
854 methylcellulose; P: pectin; QP: quinoa protein; S: starch; SB: sodium benzoate; SP: soy protein; SEP: sodium ethyl paraben; SMP: sodium
855 methyl paraben; WP: whey protein
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Matrix	Antimicrobial agent	Application	Pathogen	Additional beneficial effects	Reference
CH	Citral, lemongrass EO	Lime and orange	*Penicillium digitatum* *Penicillium italicum*	-	El-Mohamedy, El-Gamal, & Bakeer (2015)
CH	Thyme or cinnamon EOs	Strawberry	*Botrytis cinerea*	-	Mohammadi, Hashemi & Hosseini (2015)
CH	AV	Blueberry	*Botrytis cinerea*	Slowing down of water and weight loss and preservation of pH values and total soluble solids	Vieira et al. (2016)
CH, AG	AV, Thyme EO,	Avocado	*Colletotrichum gloeosporioides*	Slowing down of weight loss and preservation of firmness and flesh colour	Bill, Sivakumar, Korsten & Thompson (2014)
CH, LBG	Citrus EOs	Date	*Aspergillus flavus*	-	Aloui et al. (2014)
CMC, MC, CH	-	Strawberry	Molds and yeasts	Slowing down of weight loss and preservation of total soluble solids, pH	Gol, Patel & Rao

				values, titratable acidity, ascorbic acid content, phenolic compounds and anthocyanins	(2013)
G, shellac	-	Banana	Molds and yeasts	Delay of ripening process	Soradech, Nunthanid, Limmatvapirat, & Luangtana-anan (2017)
HPMC, beeswax	$(NH_4)_2CO_3$, NH_4HCO_3, $NaHCO_3$	Plum	*Monilinia fructicola*	-	Karaca, Pérez-Gago, Taberner & Palou (2014)
HPMC, beeswax	SMP, SEP, SB	Cherry tomato	*Alternaria alternata*	Preservation of firmness and slowing down of respiration rate and weight loss	Fagundes, Palou, Monteiro & Pérez-Gago (2015)
QP, CH, sunflower oil	-	Blueberry	Molds and yeasts	Preservation of firmness	Abugoch *et al.*, (2016)
S	AV	Cherry tomato	*Fusarium oxysporum*	Slowing down of weight loss	Ortega-Toro, Collazo-Bigliardi, Roselló, Santamarina, Chiralt (2017)
S, gum	Ascorbic acid, $CaCl_2$, cinnamon	Fresh-cut apple	Molds, yeasts, aerobic mesophilic and	Preservation of firmness, delay of browning, respiration rate and CO_2 and	Pan, Chen & Lai (2013)

	oil		psychrophilic	ethylene production	
SP	Limonene	Lime	*Penicillium italicum*	Slowing down of water loss and preservation of colour	González-Estrada, Chalier, Ragazzo-Sánchez, Konuk, & Calderón-Santoyo (2017)
WP, P	-	Strawberry	Molds and yeasts	Slowing down of respiration rate	Valenzuela *et al*., (2015)
WP, P	-	Fresh-cut apple	Molds and yeasts	Slowing down of weight loss and preservation of firmness	Rossi Marquez *et al*., (2017)

Table 3. Edible coatings containing biocontrol agents and different coating forming agent concentration (CFA: wt. %) applied to preserve different fruits against target pathogens. A: arabinoxylan; C: cellulose; CMC-Na: carboxylmethylcellulose sodium; GlyCH: glycolchitosan; HPC: hydroxypropylcellulose; LBG: locust bean gum; MC: methylcellulose; NaAL: sodium alginate; NaCas: sodium caseinate; S: starch; PP: pea protein.

CFA	wt. %	Biocontrol agent	CFU/ml	Fruit	Pathogen	Reference
A	1	*Debaromyces hansenii*	10^8	Lime	*Penicillium italicum*	González-Estrada, Carvajal-Millán, Ragazzo-Sánchez, Bautista-Rosales & Calderón-Santoyo (2017)
CMC, HPC, MC	2	*Candida guilliermondii* *Debaromyces* spp.	$2.3 \cdot 10^8$ $1.7 \cdot 10^8$	Orange	*Geotrichum candidum* *Penicillium digitatum* *Penicillium italicum*	Potjewijd et al. (1995)
CMC-Na	0,3	*Rhodosporidium paludigenum*	10^8	Jujube	*Alternaria alternata*	Wang et al. (2011)
C, shellac, sucrose ester	-	*Candida oleophila* *Pseudomonas* spp.	$4 \cdot 10^8$ $8 \cdot 10^9$	Grapefruit	-	McGuire (2000)
Commercial EC	5	*Candida sake*	$5 \cdot 10^7$	Grape	*Botrytis cinerea*	Cañamás et al. (2011)*

(Fungicover®)						Calvo-Garrido et al. (2013)*
	5	*Pantoea agglomerans*	$2 \cdot 10^8$	Orange	*Penicillium digitatum*	Cañamás et al. (2008b)*
Commercial wax	20	*Pichia guilliermondii*	10^8	Orange	*Penicillium italicum*	Lahlali et al. (2014)
GlyCH	0,2	*Candida saitoana*	10^8	Apple, citrus	*Diplodia natalensis* *Penicillium digitatum* *Penicillium italicum* *Phomopsis citri*	El-Ghaouth, Smilanick & Wilson (2000)
HPC, MC	4, 2	*Candida oleophila* *Cryptococcus albidus* *Rhodotorula mucilaginosa*	$4 \cdot 10^8$	Grapefruit	-	McGuire & Baldwin, 1994
HPMC, S, NaCas, PP	2	*Candida sake*	$5 \cdot 10^7$	Grape	*Botrytis cinerea*	Marín et al. (2016)
LBG	0.5, 1	*Aureobasidium pullulans* *Metschnikowia pulcherrima* *Wickerhamomyces*	10^9	Mandarin	*Penicillium digitatum* *Penicillium italicum*	Parafati, Vitale, Restuccia & Cirvilleri, (2016)

		anomalus				
Shellac	-	*Candida oleophila*	$4 \cdot 10^8$	Grapefruit	*Penicillium italicum*	McGuire & Dimitroglou (1999)
NaAL	2	*Cryptococcus laurentii*	10^9	Strawberry	Non specified	Fan et al. (2009)
NaAL, LBG	2, 1	*Wickerhamomyces anomalus*	10^7	Orange	*Penicillium digitatum*	Aloui et al. (2015)

862   **Field application*

863 **Table 4**. Some characteristics of different commercial biocontrol products.

Product	Biocontrol agent	Concentration	Application	Crop	Physical state
AQ-10-biofungicide™ Fargro Ltd (West Sussex, UK)	*Ampelomyces quisqualis*	$5 \cdot 10^9$ spores/g	Pre-harvest	Apple, curcubits, grape, strawberry,	Solid
Aspire™** Ecogen, Inc., (Langhorne, PA, USA)	*Candida oleophila*	$2 \cdot 10^{10}$ CFU/g	Post-harvest	Apple, citrus, pear	Solid
Binab™ Binab USA, Inc. (Bridgeport, CT, USA)	*Trichoderma harzianum, T. polysporum*	10^5 spores/g	Pre-harvest	Strawberry	Solid
Bio-Save 10LP, 11LP, 110™ JET Harvest Solutions (Longwood, FL, USA)	*Pseudomonas syringae*	$9 \cdot 10^{10}$ CFU/g	Post-harvest	Apple, citrus, cherry, pear, potato	Solid
BlightBan A506™ Nufarm Americas Inc. (Burr Ridge, IL, USA)	*Pseudomonas fluorescens*	10^{10} CFU/g	Pre-harvest	Apple, pear, potato, strawberry	Solid

BoniProtect™ Bio-Protect Gmbh (Konstanz, GER)	*Aerobasidium pullulans*	$5 \cdot 10^9$ CFU/g	Pre-harvest	Apple	Solid
Botry-Zen™ Botry-Zen Ltd. (Dunedin, NZ)	*Ulocladium oudemansii*	$2 \cdot 10^8$ spores/g	Pre and post-harvest	Grape, kiwi	Solid
Candifruit™** Sipcam Iberia (Valencia, SP)	*Candida sake*	10^9 CFU/ml	Pre and post-harvest	Apple	Liquid
Nexy™ BioNext sprl (Gembloux, BE)	*Candida oleophila*	$8 \cdot 10^9$ CFU/g	Post-harvest	Banana, citrus, pome fruit	Solid
Serenade™ Bayer Crop Science (Leverkusen, GE)	*Bacillus subtilis*	$10^9 - 1010$ CFU/g	Pre-harvest	Apple, grape, pear, vegetables	Solid and liquid
Shemer™ Bayer Crop Science (Leverkusen, GE)	*Metschnikowia fructicola*	$1.6 \cdot 10^{10}$ CFU/g	Pre and post-harvest	Apricots, citrus, tropical fruits, grape, peach, strawberry	Solid and liquid
Trichodex™ Makhteshim-Agan (DeCeuster,	*Trichoderma harzianum*	10^9 spores/g	Pre-harvest	Grape	Solid

BE)

Yieldplus™**	*Cryptococcus albidus*	Not available	Pre and post-harvest	Pome fruit	Not available
Anchor Yeast (Cape Town, SA)					

*CFU/g (colony forming units per gram)
**Not currently commercialized