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IMPROVING FUNCTION OF BIOCONTROL AGENTS INCORPORATED IN ANTIFUGAL FRUIT COATINGS. A REVIEW.

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ABSTRACT

The in-field performance of microbial biocontrol agents against fungal pathogens in fruit is subject to considerable variability due to their sensitivity to both adverse environmental conditions and their fluctuations. Therefore, to achieve an adequate development and implementation of biological agent-based products, it is necessary to improve their resistance and ability to control fungal diseases under a wide range of conditions. In this review, an overview of the latest strategies for the enhancement of the action of biocontrol agents is given. The combination of the antagonists with edible polymers able to form coatings is one of the approaches with the greatest potential and it is analysed in depth. This formulation approach of biocontrol products, including adequate microbial protectants, can yield stable products with high microbial viability, ready for field applications, with improved adherence and survival of the biocontrol agent once applied in plant. The most recent studies into this field are reviewed and summarized.

Key words: antagonists, biological control, biocontrol products, edible coatings, 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.

In the past thirty years, the use of biocontrol agents (BCAs) or biological control has been considered as one of the approaches with the greatest potential against fungal pathogens, either alone or as part of integrated systems for pest management (Spadaro & Gullino, 2004). Consequently, extensive research has been devoted to exploring and developing this field, as recently reported by Spadaro & Droby (2016).

Fungi, yeasts and bacteria are potential microorganisms to be used as antagonists for controlling the post-harvest diseases of fruits and vegetables. An ideal BCA should meet a number of requirements, as reported by several authors (Abano & Sam-Amoah 2012; Droby, Wisniewski, Macarisin & Wilson, 2009; Sharma et al., 2009). The characteristics of an ideal antagonist are that it must be: genetically stable, effective at low concentrations, undemanding in terms of its nutrient requirements, capable of surviving under adverse environmental conditions, effective against a wide range of pathogens in different commodities, amenable to production on inexpensive growth media, amenable to formulation with a long shelf-life, easy to dispense, resistant to chemicals used in the post-harvest environment, not detrimental to human health, compatible with other chemical and physical treatments and not detrimental to the quality of the fruits and vegetables it preserves.

An extensive body of research has been devoted to the understanding of the mechanisms by which BCAs exert their action against pathogens. Nonetheless, in many cases, the suggested modes of action whereby antagonists wield their biocontrol effect are not totally elucidated, especially due to the fact that several mechanisms frequently take place at the same time since and successful BCAs are generally equipped with several attributes which often work in concert and may be crucial for controlling disease development (Droby et al., 2009; Jamalizadeh et al., 2011; Janisiewicz & Korsten, 2002). Despite the difficulties, insight into the action modes involved will permit an

improvement in both the biocontrol performance and the development of appropriate formulations and methods of application. Competition for nutrients and space between the pathogen and the antagonist is considered to be the major mode of action, but other mechanisms such as parasitism, the production of secondary metabolites or the induction of host defences, have also been reported, as shown in Table 1.

The potential BCAs often show some significant limitations, such as their sensitivity to both adverse environmental conditions and their fluctuations, and their narrow range of activity because BCAs act on specific hosts against well-defined pathogens (Spadaro & Gullino, 2004). For these reasons, the performance of biological-based control strategies in the field is subject to significant variability which constitutes a significant constraint to their practical implementation (Droby et al., 2009; Wisniewski et al., 2007). In these sense, different approaches have been reported to make the BCAs more efficient: the use of mixed cultures (Conway, Janisiewicz, Leverentz, Saftner & Camp, 2007; Panebianco, Vitale, Polizzi, Scala & Cirvilleri, 2016), their physiological manipulation (Usall et al., 2009; Wang, He, Xia, Yu & Zheng, 2014) and their combination with different types of substances (Guo et al., 2014; Qin et al., 2015; Zhou et al., 2016). The application of BCAs in combination with coating materials has been reported to enhance the BCA effectiveness at inhibiting the growth of plant pathogens, as discussed in the next section.

2. EDIBLE COATING FORMULATIONS FOR ANTIFUNGAL CONTROL ON FRUIT

The application of commercial coatings is a common practice for many fruits. These coatings are generically known as waxes, since their composition is based on paraffin wax or a combination of various other waxes, such as beeswax or carnauba. They are anionic microemulsions that may also contain synthetic components, such as

polyethylene and petroleum waxes, ammonia or morpholine, which are applied to reduce fruit weight loss and shrinkage, while improving their appearance and physical resistance. Commercial waxes are often amended with synthetic fungicides in order to control post-harvest diseases (Palou et al., 2015).

However, due to the potential hazards of synthetic coatings, such as the presence of potentially toxic substances on the fruit surface, the use of edible coatings (ECs) as a replacement for these currently-used commercial waxes has been widely studied. Then, the use of edible coatings (ECs) to protect fruits from fungal decay at postharvest conditions cannot be considered as a new approach anymore, since there are a great number of studies dealing this topic, in which different matrices and active compounds are used, such as EOs and food preservatives (Table 2). However, the use of ECs to carry antagonistic microorganisms, to be used at both pre and post-harvest conditions, is an area that has been less widely explored.

Coating formation on the surface of a product implies the application of a film-forming solution or dispersion of a polymeric material with filmogenic capacity (Campos, Gerschenson & Flores, 2011). Those coatings and films obtained with food-grade polymers/ingredients can be eaten as part of the whole product and their use is interesting for fruits and vegetables which can be directly consumed. Therefore, the composition of ECs and films must conform to the regulation that applies to the food product concerned (Guilbert, Gontard & Cuq, 1995).

Their basic components are typically hydrocolloids (polysaccharides and proteins) and lipids, and these can either be used individually or in combination, in order to obtain composite or blend coatings. The composite coatings take advantage of the specific functional characteristics of each group, reducing their drawbacks (González-Martínez et al., 2011). Other components, such as plasticizers and emulsifiers (or surfactants),

may be added to the matrices as a means of improving the flexibility, extensibility and/or the stability of the structure (Palou et al., 2015). Moreover, formulations can act as carriers of a very wide range of other minor compounds, such as antioxidants, antimicrobials, certain nutrients like vitamins, volatile precursors, flavours, firming agents or colorants (González-Martínez et al., 2011). Multilayer coatings applied by the "layer by layer" technology have also described as effective enhancers of fruit quality during storage, optimizing the coating functionality through the complementary properties of different hydrocolloids (Poverenov et al., 2014). Additionally, ECs may be used as carrier matrices of bioactive compounds to enhance the safety and the quality of fruit (Quirós-Sauceda, Ayala-Zavala, Olivas & González-Aguilar, 2014). Bioactive compounds can be carried by the ECs to the fruit skin by diffusion release, which is controlled by their solubility and permeability in the polymer matrix. Table 2 shows some examples of different coatings applied to fruits and vegetables to improve their quality preservation, using different polysaccharide or protein matrices.

Polysaccharides are the most commonly used components in fruit ECs, probably due to their better microbial and physical stability over time in comparison with protein-based coatings, especially in high relative humidity environments (González-Martínez et al., 2011). Other compounds which are commonly used in fruit ECs are lipids, which have low water vapour permeability and are very useful for controlling their desiccation (Vargas et al., 2008). In fact, TAL-Prolong and Semperfresh are two commercially available composite coating formulations based on carboxymethylcellulose, sucrose fatty acid ester, sodium salt and an emulsifier, used for the shelf-life extension of bananas and other fruits (Nisperos-Carriedo, Baldwin & Shaw, 1992; Tharanathan, 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,

oregano, rosemary, thyme oil, vanilla, vanillin, etc.); (c) antimicrobial antagonists, such as BCAs. Several studies have been summarized in Table 2.

3. EDIBLE COATINGS CONTAINING BIOCONTROL AGENTS

BCA agents can also be incorporated to the coating-forming formulations to obtain coatings or films loaded with the antagonist cells, with the ability to maintain their viability and allowing for cell distribution on the coated product. In this sense, the coating-forming formulations should contain components which not only allow for coating formation, but also be compatible with the cells and provide them an adequate substrate for nutrition and growth. An ideal formulation for BCA-coating product must be: 1) water soluble or dispersible, without organic solvents toxic for cells, 2) able to maintain or increase cell population when applied in the product, 3) able to impart gloss and modulate plant respiration and 4) contain safe ingredients for the final consumers. Likewise, in the case of formulated BCAs with coating-forming agents, these should provide adequate properties to maintain microbial and physical stability of the formulation during storage throughout the commercialization period. The latter aspect is highly dependent on the final format of the product (liquid or dried products).

In comparison with the large number of studies dealing with the incorporation of antifungal compounds into ECs for fruit applications, there is little information about coatings including antifungal microbial antagonists for the purposes of controlling fruit pathogens. Some studies were published in the 1990s, but there has been little recent research. This approach, consisting of the combination of BCA and coatings as a means of preserving fruit from fungal decay has proven to be effective. This effectiveness is attributed to the advantages of both strategies, which are summarized in Figure 1.

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

combined treatments enhanced the control of the fungal decay of grapes. Rahman, Mahmud et al. (2009) applied separated suspensions of the BCA *Burkholderia cepacia* and CH on papaya at post-harvest, in combination with $CaCl_2$, as stimulant of the antagonistic activity, showing that the combination of the different treatments resulted in a more effective disease control. In these particular cases, the coating performed more as an additional treatment than as a support for the BCA due to the antifungal properties of the polysaccharide.

As concerns the joint application of coating agent and BCA in only one step, several recent studies have been reported (Aloui et al., 2015; Marín et al., 2016; González-Estrada, Carvajal-Millán, Ragazzo-Sánchez, Bautista-Rosales & Calderón-Santoyo, 2017). Aloui et al. (2015) incorporated the BCA *Wickerhamomyces anomalus* into sodium alginate and locust bean gum coatings to control the growth of *Penicillium digitatum* on oranges. These authors reported that the coatings maintained more than 85% of the initial BCA and that this combination completely inhibited the pathogen. Similarly, Marín et al. (2016) applied on grapes several formulations of ECs, based on polysaccharides or proteins, as support of the BCA *Candida sake* for the purposes of controlling the pathogen *Botrytis cinerea*. The study showed that the adherence and survival of the BCA was improved with the combined application and consequently a better control of the fungal decay was observed. In the same way, González-Estrada (2017) reported that the incorporation of the antagonist *Debaromyces hansenii* in a coating matrix based on arabinoxylan allowed for a maintaince of more than 97% of the initial yeast population. Moreover, they observed that the application of yeast entrapped in the coating improved its efficacy against blue mold decay under storage of lime.

Different studies regarding the combined application of edible and commercial coatings containing antagonistic microorganisms are summarized in Table 3. In the reported

studies the applied concentrations of the different BCA ranged from 10^7 to 10^9 CFU per millilitre of coating suspension, depending on the antagonist, whereas greater differences can be found in the concentration of solids in the coating dispersions (Table 3). In some of the studies, concentrations lower than 1% of coatings solids were applied while percentages up to 20% were used in others cases. This is an important factor since the amount of coating solids will affect not only to the final price if the commercial application is intended, but also the efficacy of the BCA. Marín et al. (2016) observed that a minimum value of the polymer:CFU ratio is required to observe significant increase in the BCA population with respect to the control application (without coating) in grapes. Similarly, Parafati et al. (2016) tested different concentrations of locust bean gum dispersions incorporated with two yeasts and observe that the highest percentage of coating solids enhanced to a greater extent their activity against blue mould decay of mandarin.

In general, an enhancement of the BCA viability on the fruit surface and an increased control of the pathogens can be achieved with combined applications of BCA and ECs, even in applications carried out in the field (Cañamás et al., 2008b, Cañamás et al. 2011, Calvo-Garrido et al., 2013). Nevertheless the specific mechanisms whereby a determined ECs influence the performance of a determined microbial antagonist, and also their effects of pathogen, require further studies

4. FORMULATION OF BIOCONTROL AGENTS WITH COATING-FORMING AGENTS

A biocontrol product (BCP) could be defined as a mixture of the active ingredient (BCA), a carrier providing physical support for the microorganism, which can be liquid (aqueous dispersion) or a dried powder, It is common practice to incorporate adjuvants and/or protectants, which can be incorporated at different points, such as in the mass

production, formulation and storage steps or just before the application in the mixing tanks (Cañamás et al., 2008a; 2008b). Additives can be used as stickers, diluents, suppressants, dispersants, emulsifiers, wetters, gelants, humectants, brighteners, spreaders, stabilizers, sunscreens, synergists, thickeners, nutrients, binders, or protectants, depending on their function in the formulations. As previously described, some of these functions can be accomplished by components of ECs.

The formulation of BCAs with EC agents can enhance their efficacy, extend the range of conditions over which they are effective, increase their ability to withstand drastic changes in the phyllosphere and improve their survival under unfavourable microclimatic conditions (Cañamás et al., 2011). In this sense, the formulation process is decisive and has a significant influence on the successful delivery of the antagonists, the shelf-life and storage requirements of the BCP and on its cost (Janisiewicz & Korsten, 2002; Spadaro & Gullino, 2004; Yánez-Mendizábal et al., 2012).

In comparison with the large number of effective antagonists under laboratory conditions, the success of formulated BCA-based products has been limited and just a few products have reached advanced stages of development and commercialization. Generally, information on the specific composition and production of formulations of commercial BCA is largely proprietary (Sztejnberg, 1993; Howard Davies, Ebbinghaus, GÖRTZ & Carbonne, 2014; Brandi, Trainer & Westerhuis, 2016). Table 4 summarizes some characteristics for different comercial BCP. As can be observed, the concentration of BCA varies between 10^8-10^{10} CFU or conidia/g, depending on the product, and the ratio of BCA:inert solids, also differs for the different products. For instance, Aspire^{TM} a bioproduct based on *Candida oleophila*, contains 55% of the yeast isolate and 45% of inert ingredients, while Bio-Save 10 LP, based on *Pseudomonas syringae* only contains 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.,

2003); heat-adapted *Candida sake* CPA-1 cells and combined with an EC (Cañamás et al., 2011); *Cryptococcus laurentii* and *Pichia membranaefaciens* with sugar protectants and antioxidants (Liu et al., 2009) and *Pichia anomala* with different substances (Melin, Schnürer & Håkansson, 2011). For their part, Batta (2007) and Peeran, Nagendran, Gandhi, Raguchander & Prabakar (2014) developed formulations of different BCAs supported in emulsions. Coating forming agents could enhance the stability of the emulsions without implying relevant changes in the a_w, because of their high molecular weight, and could also play a nutrient and protectant role for the cells.

In general, dry formulations have a longer shelf life and exhibit a lower risk of contamination than liquid ones, and allow for easier transport, distribution, storage and manipulation (Fravel, 2005; Usall et al., 2009). However, they also present some shortcomings, such as a marked loss of viability in the cells not only during dehydration and storage but also during the subsequent rehydration process (Melin, Håkansson, Eberhard & Schnürer, 2006).

Dry formulations of BCAs take several forms. Wettable powders consist of dry inactive and active ingredients (BCA cells) intended to be applied as a suspension in liquid. Dusts are powder-like and consist of dry inactive and active ingredients to be applied dry, generally to seeds or foliage. Granules are described as free-flowing aggregated products composed of dry inactive and active ingredients (Schisler et al., 2004). Dry formulations can be applied directly to the target plant or, in the case of wettable powders and water dispersible granules, mixed into water where the suspension of biomass and inactive ingredients are applied as a spray.

The inactive ingredients of dry formulations act as carriers of the antagonists and may be organic (grain flours, powders from plants, starches and their derivatives, etc.) or mineral (peats, talc, diatomaceous earths, kaolin, clay, etc.) (Mokhtarnejada, Etebariana,

Fazelib & Jamalifarb, 2011; Schisler, Slininger & Olsen, 2016). The use of coating-forming agents as carriers in the formulation of dry BCP can represent several advantages. The EC compound would firstly act as support for the BCA cells during the drying and storage steps and, when applied, the EC would provide the BCA with the previously described benefits, such as an improved adherence and survival on the fruit surface or as a source of nutrients. Wettable powders or water dispersible granules would be the most adequate forms for dry BCA-EC formulations, since previous water dispersion of coating-forming agents is necessary to form the ECs. Moreover, the polymeric nature of the coating-forming agents confer them high values of the glass transition temperature and water sorption capacity, which contributes to limit the product a_w after drying, while they have a high value of the critical water content for plasticization, which benefit the physical stability of the product.

There are few publications on the use of EC-forming agents as support for microbial antagonist based formulations and, in most of the cases, different kinds of starch and derivatives are the used ingredients. This is because the production cost is a key factor that must be borne in mind and kept to a minimum (Melin et al., 2011), and starch is low cost and readily available. Lewis, Fravel, Lumsden & Shasha (1995) obtained granular formulations using pre-gelatinized starch and the BCA *Gliocladium virens* and Mounir et al. (2007) used maize starch to produce a formulation of *A. pullulans*. More recently, Soto-Muñoz et al. (2015) and Gotor-Vila et al. (2017) developed different dry formulations of *Pantoea agglomerans* and *Bacillus amyloliquefaciens* respectively using native potato starch as carrier and Marín, Atarés, Cháfer & Chiralt (2017) characterized the most relevant properties of formulations of *Candida sake* based on pre-gelatinized starch and maltodextrins.

Dehydration is a very critical step since not all microorganisms are amenable to drying and many tend to lose viability during both the drying process and subsequent storage. For that reason, many approaches have been developed in order to reduce the losses in viability, such as adding protectants to growth media or directly to cells (Abadias, Torres, Usall, Viñas & Magan, 2001; Schisler et al., 2016; Yánez-Mendizábal et al., 2012). Of the protectant agents, skim milk and sugars, used either alone or in combination, have been widely used because of their relatively low prices and chemically innocuous nature (Costa, Usall, Teixidó, Torres & Viñas, 2002; Khem, Woo, Small, Chen & May, 2015; Santivarangkna et al., 2008). Sugars, especially disaccharides, are able to protect the cell membranes from dehydration (Leslie, Israeli, Lighthart, Crowe & Crowe, 1995). The proteins present in milk provide a protective coat for the cells and seem to restore injured cells during dehydration, avoiding osmotic shock, disruption and the death of cells (Champagne, Gardner, Brochu & Beaulieu, 1991). Many coating-forming agents such as whey protein and maltodextrins has been reported as excellent cell protectants during drying of different microorganisms, especially probiotics (Eratte et al. 2015; Huang et al. 2017; Liao et al. 2017).

The classical dehydration processes applied to obtain BCPs are freeze-drying, spray-drying and fluidized-bed drying. Although these methods present several differences, the inclusion of cells in a carrier containing protectants or different adjuvants before the drying step is common to all methodologies. In this sense, biopolymers used as coating-forming agents could act as effective carriers together with other cell protectant agents.

4.1 Stability of biocontrol formulations during storage

The preservation of the cell viability during fermentation, drying, storage and rehydration is one of the main goals of the formulation process (Schisler et al., 2004). 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,

Cano, González-Martínez & Chiralt (2017) reported that the lower the a_W of whey protein-maltodextrin based formulations of *Lactobacillus rhamnosus* the better the cell preservation during storage.

At high humidity conditions, water act as a plasticizer and increases the molecular mobility of the components of the dry formulations (Poddar et al., 2014). This increase results in caking and the crystallization of the amorphous structures and in an instantaneous loss of microbial viability during storage. The glassy (non-crystalline) state has been shown to enhance the storage life (Miao et al., 2008; Poddar et al., 2014). In this sense, coating-forming agents increase the critical water content for the powder plasticization, which contributes to enhance both physical and microbial stability.

Additionally, the shelf-life of dry products containing microorganisms is highly dependent on the storage temperature. In general, as the storage temperature increases, mortality also increases and storage time is reduced (Costa et al., 2002). This has been attributed to the fact that temperatures between 4 and 10°C cause a slowing down of both cell division and metabolic rate in microorganisms and, in this situation, cells are capable of withstanding the depletion of nutrients and the accumulation of toxic metabolites (Mejri, Gamalero & Souissi et al., 2013).

Several studies have reported the effect of the storage temperature on the viability of different biological formulations. Abadias, Teixidó, Usall, Benabarre & Viñas (2001) reported that storage at 4°C was required to maintain the viability of *Candida sake* cells obtained by freeze-drying. Likewise, Torres et al. (2014) studied the viability of freeze-dried *Pantoea agglomerans* cells, which was significantly higher at -20 and 5°C, as 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.

Therefore, more studies are necessary to elucidate the best polymer and protectant components of the BCA formulation, the more adequate drying conditions and the optimal storage conditions of the BCP in order to extend shelf life for crop applications.

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FIGURE CAPTIONS

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

**CFU/g (colony forming units per gram)*

***Not currently commercialized*