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Additional Information

1 **Dehydration of *Ampelomyces quisqualis* CPA-9 conidia by adding biodegradable**
2 **coatings: biocontrol activity against powdery mildew and physical characterization**
3 **of the formulated product**

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28 **Abstract**

29 *Ampelomyces quisqualis* CPA-9 was reported as a promising biocontrol fungus against powdery
30 mildew on cucurbits, although its formulation had not been developed. The present work aimed
31 to develop a fluidised-bed spray-dried formulation of CPA-9 that included film-forming
32 compounds, which could improve the behaviour of the microorganism under practical conditions.
33 Film forming compounds, efficacy, shelf life, physical stability as a function of a_w , and solubility
34 in water were determined. A film-forming formulation based on native potato starch and
35 pregelatinised potato starch as carrier and binder was obtained, and sucrose plus skimmed milk
36 were used as protective compounds. Dehydrated conidia maintained the efficacy of fresh cells
37 against *Podosphaera xanthii* on zucchini leaves, and powdery mildew was significantly reduced
38 compared with control treatment. Despite of results obtained for the glassy state guaranteed the
39 stability of the powder against sticking and caking processes, the shelf life of the product was
40 limited to 4 months of storage at 4 °C. On the other hand, the formulation was mainly dispersible,
41 due to the high concentration of native potato starch which reduces the solubility of the product.
42 Results obtained in his work, such as the high efficacy of the developed product, the low cost of
43 the employed substances, the film-forming ability of the rehydrated powder, and finally the
44 effectiveness of the dehydration process, confirmed the potential of the product. Further studies
45 to verify the improvement of CPA-9 behaviour under practical conditions or to improve the
46 storage conditions to enlarge the shelf life of the BCA should be done to consider the developed
47 product as a biocontrol-based product for cucurbits against powdery mildew.

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50 **Keywords:** fluidised-bed spray-drying; *P. xanthii*; film-forming; formulation; biological control;
51 zucchini.

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56 **1. Introduction**

57 Nowadays, commercial bioproducts are being part of integrated crop protection systems to reduce
58 the use of chemical fungicides, which are prejudicial for the environment and for human health
59 (Droby et al., 2009). However, the use of these products for preharvest applications is still limited
60 due to their narrow range of activity (Droby et al., 2016), and extending their use to preharvest
61 diseases should be necessary (Usall et al., 2016).

62 The obligate biotrophic fungus *Podosphaera xanthii* is considered the main widespread disease
63 of cucurbits in many countries (Pérez-García et al., 2009). The control of this disease is mainly
64 focused on the application of fungicides and the use of resistant cultivars (Caffi et al., 2013;
65 Gilardi et al., 2012; Kiss et al., 2004). Nevertheless, biocontrol fungi of genus *Ampelomyces* had
66 showed efficacy to control powdery mildew infections (Sucharzewska et al., 2012).

67 *Ampelomyces quisqualis* was commercialised as a water dispersible granules under the name of
68 AQ10 Biofungicide® (Sztejnberg, 1991, 1993). Although its biocontrol efficacy was questioned
69 in several studies due to the unsatisfactory levels of control (Dik and Verhaar, 1998; Kiss, 2003;
70 Shishkoff and McGrath, 2002), related to the high humidity required for its activation against
71 diseases (Romero et al., 2007). For that, there is still interest in finding more effective *A.*
72 *quisqualis* strains (Angeli et al., 2012), which can control effectively powdery mildew even under
73 unfavourable conditions. Additionally, a formulation process using biodegradable coatings could
74 be an approach to extend and improve the use of biocontrol agents to preharvest applications
75 (Usall et al., 2016). On this point, Carbó et al. (2017) developed film-forming formulations of
76 *Candida sake* CPA-1 that showed biocontrol efficacy and improved the survival under
77 environmental conditions.

78 During the development of a film-forming formulation of microbial biocontrol agents, the
79 selection of carriers-protectants of the BCAs is a key step for the shelf-life and stability of
80 formulations (Kinay and Yildiz, 2008). Carrier composition also defines sorption and
81 plasticization water, which have a great influence on the physical stability and microbial features.
82 Likewise, it also impacts on the powder dispersion capability in water, another key factor for their
83 practical application. Marín et al. (2017) demonstrated that *C. sake* CPA-1 formulations with low

84 water activity (a_w) were more stable at cold and room temperature; and that the use of
85 maltodextrins as carrier allowed for a better solubility in water.

86 There are several processes to dry microorganisms, as freeze-drying, vacuum-drying, or spray-
87 drying. Among these processes the fluidised-bed spray-drying system appeared to be more
88 advantageous due to permits to dry the cells at low temperature; the process is highly automatic
89 and it do not require any additional manipulation. Moreover, it is possible to obtain a high amount
90 of product and the methodology could be scaled-up easily (Carbó et al., 2017). Furthermore, the
91 process time is short and operating costs are low compared with other drying methodologies such
92 as a freeze-drying (Srivastava and Mishra, 2010). Despite this system has been recently used to
93 dry the yeast *C. sake* CPA-1 (Carbó et al., 2017) and the bacterium *Bacillus amyloliquefaciens*
94 CPA-8 (Gotor-Vila et al., 2017).

95 The present study was focused on obtaining a fluidised-bed spray-dried formulation of
96 *A. quisqualis* CPA-9 together with biodegradable coating-forming compounds to improve its
97 adherence and efficacy after field application. The specific objectives were as follows: (i) to study
98 the effect of protective substances on the survival of CPA-9 conidia; (ii) to adjust the carrier
99 compounds to achieve a suitable formulation; (iii) to evaluate the shelf life of the dried
100 formulation at 4 °C; (iv) to check the efficacy of the optimised formulation against
101 *P. xanthii* on zucchini leaves; and (v) to analyse its physical stability as a function of both a_w and
102 solubility in water.

103 **2. Materials and methods**

104 **2.1. Microorganisms**

105 *A. quisqualis* strain CPA-9 used in the present study was originally isolated from pumpkin leaves
106 at IRTA (Lleida, Catalonia, Spain), deposited at the Colección Española de Cultivos Tipo (CECT-
107 20749) at the University of Valencia (Burjassot, Spain) and it was patented by LAINCO, S.A.
108 (Rubí, Spain) (Garriga et al., 2014). The fungus was maintained in Glycerol 20% and Criobilles
109 (Deltalab 409113/6, Cryoinstant) at -80 °C for long term storage. When required, CPA-9 was
110 transferred to malt extract agar medium (MEA: malt extract, 30 g L⁻¹; peptone, 5 g L⁻¹; and agar

111 15 g L⁻¹) plates under a daily 12-h photoperiod (black light UV/dark at 25/18 °C) and periodically
112 sub-cultured at most three times, approximately every 10-15 days.

113 Sub-cultured colonies were mixed with sterile water and grinded with a sterile porcelain mortar
114 and pestle to obtain the suspension used as starter inoculum for conidia production. Conidia were
115 produced in 500-mL Erlenmeyer flasks with 75 mL of potato dextrose broth (PDB: potato
116 peptone, 4 g L⁻¹; and glucose, 20 g L⁻¹) modified with 2.5% (w/v) of glycerol with an initial
117 concentration of 10⁵ conidia mL⁻¹. Erlenmeyer flasks were incubated in the dark without agitation,
118 at 25 °C for 11 days (Carbó et al., 2020).

119 *P. xanthii* was originally isolated in IRTA (Lleida, Catalonia, Spain) from cucurbit leaves and it
120 was maintained by growing on zucchini cotyledons (variety “Black Beauty”) as follows: first,
121 cotyledons were disinfected thrice for two minutes with HgCl₂ (0.1% w/v); once dried at room
122 temperature, cotyledons were introduced in plates with agar (CTM: sucrose, 40 g L⁻¹;
123 benzimidazol, 40 mL
124 L⁻¹; and agar 2.4 g L⁻¹) by embedding their petioles in the medium; finally, a pinch of *P. xanthii*
125 was spread on the surface of the cotyledons with an eyelash to avoid injuring the leaves. Infected
126 cotyledons were incubated under a daily 12-h photoperiod (white light/dark at 21/18 °C).
127 *P. xanthii* was sub-cultured on new cotyledons approximately every 15 days.

128 **2.2. Fluidised-bed spray-drying process**

129 Fluidised-bed spray-drying system (Hüttlin GmbH, Bosch Packaging Technology, Stuttgart,
130 Germany) was used to dry *A. quisqualis* CPA-9 together with biodegradable coatings. The process
131 was carried out as described Carbó et al. (2017) with modifications.

132 Briefly, a suspension of 2-3 × 10⁸ conidia mL⁻¹ was prepared from 15-16 Erlenmeyer flasks. This
133 suspension was grinded with an homogeniser-disperser Micra D-9 (Micra GmbH, Heitersheim,
134 Germany) for 5 min at 21000 rpm and allowed to at rest for 5 min to facilitate the rupture of the
135 pycnidial wall and release the conidia. This process was repeated two times. Concentration of
136 conidia was determined in a Thoma-Zeiss counting chamber. For each formulated product, 150 g
137 of conidia suspension plus 3.5 g of binder were homogenised with the corresponding protective
138 substances using an Ultra-Turrax (IKA Ultra-turrax® T25 Digital, IKA®-Werke GmbH & Co.

139 KG, Munich, Germany) for 1 min at 10000 rpm and then shaken for 1 h before atomisation with
140 the fluidised-bed spray-drying system. The solution was sprayed onto 300 g of suspended carrier,
141 and the product was dried at 55 °C.

142 Three replicates of 0.5 g of the formulated products were rehydrated with 5 mL of sterile water,
143 which were then shaken for 1 min and allowed to rehydrate for 9 min. The total amount of conidia
144 was counted in a Thoma-Zeiss counting chamber and the percentage of conidia germination was
145 measured as described Carbó et al. (2020) after 40 h because dried conidia required more time to
146 germinate.

147 Cell survival was expressed as $\text{Log}(N/N_0)$, where N_0 represents the total conidia in the suspension
148 before being formulated, and N the total CPA-9 conidia obtained after the drying process. N_0 was
149 calculated as the concentration of the suspension (conidia mL^{-1}) \times amount of suspension (mL),
150 whereas N was calculated as the concentration of the formulated powder (conidia g^{-1}) \times amount
151 of powder (g).

152 **2.3. Optimisation of protective and carrier compounds**

153 Different combinations of skimmed milk and sucrose at different concentrations (wt/wt) were
154 tested as protective compounds during the fluidised-bed spray-dried process: (i) 10% skimmed
155 milk (SKM10); (ii) 10% skimmed milk plus 10% sucrose (SKM10+SUC10); (iii) 10% skimmed
156 milk plus 20% sucrose (SKM10+SUC20); (iv) 10% sucrose (SUC10); and (v) 20% sucrose
157 (SUC20). A formulation without any protective compound was used as control (NP).

158 The optimisation of protective compounds was carried out with a carrier based on potato starch
159 substances. Specifically, formulation was composed of pregelatinised potato starch (2/3) and
160 native potato starch (1/3) as carriers, and pregelatinised potato starch as binder.

161 Conidia viability of the formulation was improved with protective compounds in the previous
162 experiment. Then, other two ratios of pregelatinised potato starch (PPS) and native potato starch
163 (NPS) were tested to use as carriers: (i) 1/3 of PPS; and (ii) 1/6 PPS. Once the carrier composition
164 was optimised, the effect of protective compounds that not showed significant differences with
165 the selected one, were tested again.

166 **2.4. Efficacy of the formulation**

167 The efficacy of the fluidised-bed spray-dried formulation was tested against *P. xanthii* strain
168 04/05 on detached leaves of zucchini plants (variety “Black Beauty”). Double Petri plates were
169 used to incubate the detached zucchini leaves by immersing their petioles in Hoagland’s solution
170 diluted to 50%. *P. xanthii* was spread on the nervation of detached leaves with an eyelash
171 previously sterilised with alcohol, then double Petri plates with infected zucchini leaves were
172 incubated in a plant growth chamber for 5 days. At that moment, low powdery mildew infection
173 was visible and the treatments were applied. Non-formulated conidia and the optimised
174 formulation of *A. quisqualis* CPA-9 were applied at 10^6 conidia mL⁻¹ by spraying each leaf for
175 10 s using an air brush, and water was used as control. Each replicate consisted of five leaves per
176 treatment.

177 Efficacy was estimated after 6, 10, and 13 days of the treatments and calculated as the incidence
178 disease index (IDI): $[(a \times 0) + (b \times 1) + (c \times 2) + (d \times 3)] / 3n \times 100$, where numbers designate
179 the relative surface of the leaf covered with powdery mildew (0: no powdery mildew; 1: only few
180 spots; 2: a half of leaf or more; 3: all surface practically covered); letters indicated the amount of
181 leaves assigned at each group; and n was the total amount of infected leaves.

182 **2.5. Shelf life of the formulation**

183 Shelf life of the optimised formulation was preliminary tested to evaluate the viability of conidia
184 under refrigerated conditions. Approximately, 250 g of each optimised formulation was stored in
185 plastic bags at 4 °C for 5 months. Triplicate samples of 0.5 g were rehydrated with sterile water
186 after 1, 2, 3, 4, and 5 months to check the viability of *A. quisqualis* conidia as it has been described
187 above. Total amount of conidia was counted in a Thoma-Zeiss counting chamber and viable
188 conidia was determined with the percentage of germination.

189 **2.6. Physical stability and solubility of the formulation**

190 Water sorption isotherm of the optimised formulation was obtained via a static gravimetric
191 method at 20 °C. Three replicates of the sample were weighed using an analytical balance
192 (ME235P-SD, SARTORIUS AG, Germany) and placed in hermetic recipients containing
193 oversaturated solutions of different salts providing known equilibrium relative humidity (RH)
194 from 11 to 98% (Greenspan, 1977). Samples were periodically weighed until constant weight was

195 reached. Initial moisture content of the sample was determined by drying for 24 h at 60 °C in a
196 vacuum oven and subsequent conditioning in a desiccator containing P₂O₅. Experimental data
197 were fitted to the Guggenheim–Anderson–de Boer (GAB) model (Equation 1) over the entire a_w
198 range.

$$199 \quad W_e = \frac{W_0 \cdot C \cdot a_w}{(1 - K \cdot a_w) \cdot (1 + (C - 1) \cdot K \cdot a_w)} \quad (1)$$

200 where W_e is the equilibrium moisture content on dry basis; W_0 , the monolayer moisture content;
201 C constant related to the heat sorption of multilayer; and K factor correcting properties of the
202 multilayer molecules.

203 Water plasticisation was analysed by determining the glass transition temperature (T_g) of the
204 formulation as a function of their a_w by means of differential scanning calorimetry (DSC) using
205 a DSC TA Instruments, model DSC1 STAR System (Mettler Toledo, Switzerland) as described
206 by Marín et al. (2017). Briefly, triplicate samples of 9 mg samples each were weighed and sealed
207 in aluminium pans. Three cycles of scanning (heating–cooling–heating) between 0 °C and 160
208 °C were performed at 10 °C min⁻¹, using a 20 mL min⁻¹ nitrogen flow. T_g was determined as the
209 midpoint temperature of the glass transition in the second heating scan. Measurements were taken
210 by duplicate. The relationship between T_g and water content at various a_w were modelled by using
211 the Gordon and Taylor equation (Equation 2).

$$212 \quad T_g = \frac{(1 - x_w) \cdot T_{gs} + k \cdot x_w \cdot T_{g(w)}}{(1 - x_w) + k \cdot x_w} \quad (2)$$

213 where x_w is the moisture content; $T_{g(s)}$ is the T_g value of the anhydrous solids; $T_{g(w)}$ is the
214 T_g value of the amorphous water; and k is a model parameter.

215 Water solubility of the formulate was measured at 5°C, 15°C and 25°C for different contact times
216 (5 to 50 min) under mild agitation conditions (200 rpm), with a modification of the method
217 described by Cano-Chauca et al., (2005). Samples of 0.25 g were dispersed in 25 mL of deionised
218 water and stirred for each time and temperature. Samples were centrifuged at 3000 g for 5 min
219 and 6.25 mL of the supernatants were transferred into pre-weighed glass Petri dishes. Dishes were
220 then oven-dried for 5 h at 105 °C to determine the content in soluble solids. Solubility was

221 expressed as percentage of dissolved solids with respect to the initial mass of dry formulation.
222 Determinations were carried out in triplicate. Peleg model was used to fit experimental data
223 (Equation 3).

$$224 \quad S = S_0 + \frac{t}{K_1 + K_2 \cdot t} \quad (3)$$

225 Where, S is the percentage of solubilised solids at time t; S₀ is the instantaneous solubility; t is
226 time (min); and K₁ and K₂ are the Peleg rate (min) and Peleg capacity constant (%⁻¹), respectively.

227 **2.7. Statistical analysis**

228 The results of conidia mL⁻¹ were transformed to logarithmic values prior to one-way ANOVA
229 analysis and means separation were obtained. When the analysis was statistically significant,
230 Tukey's HSD test (P ≤ 0.05) was conducted for means comparison of survival of CPA-9 adding
231 different protective compounds to a potato starch carrier-based formulation. When comparisons
232 were conducted between two means of the optimised PPS ratio (SKM10+SUC10 and
233 SKM10+SUC20), Student's T-test (P ≤ 0.05). Data were analysed using JMP 13 software (SAS
234 Institute Inc., Cary, NC).

235 **3. Results**

236 **3.1. Fluidised-bed spray-drying process for *A. quisqualis* CPA-9**

237 **3.1.1. Optimisation of protective compounds**

238 The best survival of CPA-9 cells was obtained using skimmed milk 10% plus sucrose 20%
239 (SKM10 + SUC20) as protective compounds, despite of no significant differences were observed
240 with the combination of skimmed milk 10% plus sucrose 10% (SKM10 + SUC10) (Fig. 1).

241 Regarding other tested protective compounds, skimmed milk used alone as protective compound
242 reduced the survival of *A. quisqualis*, despite of survival reduction was not significant compared
243 with the control.

244 When sucrose was used as protectant substance, viability of cells was also improved, although
245 neither significant differences were observed compared with the control without protectant
246 compounds (NP) or compared with the use of sucrose 10% (SUC10) or sucrose 20% (SUC20).

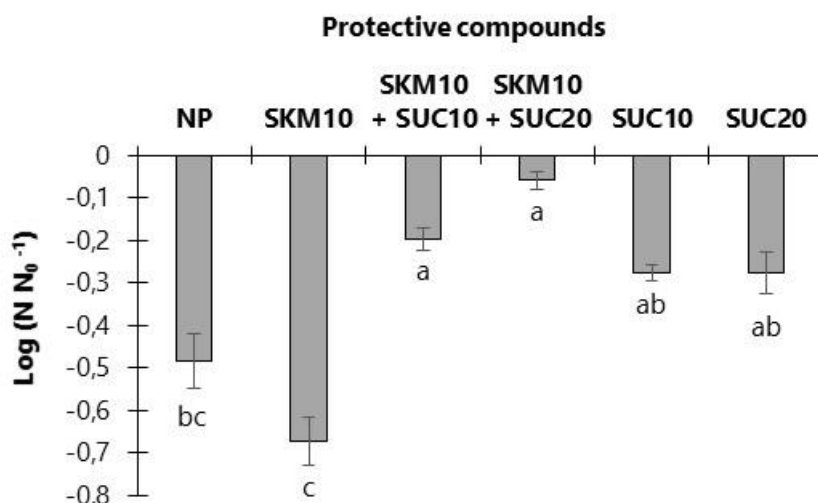


Fig. 1 Survival of *A. quisqualis* CPA-9 dried at 55 °C adding different protective compounds to a potato starch carrier-based formulation (□). Protective compounds were NP (control without protectant compounds); SKM10 (skimmed milk 10%); SKM10 + SUC10 (skimmed milk 10% and sucrose 10%); SKM10 + SUC20 (skimmed milk 10% and sucrose 20%); SUC10 (sucrose 10%); and SUC20 (sucrose 20%). Potato starch formulation was composed of pregelatinised potato starch (2/3) and native potato starch (1/3) as carriers and pregelatinised potato starch as binder. Mean values of three replicates are represented and columns with different letters indicate significant differences ($P < 0.05$) according to Tukey's test. Error bars represent the standard deviations of the means.

3.1.2. Adjustment of carrier compounds

Composition of carrier compounds was adjusted to increase the solubility of the product because the optimised potato starch formulation was not rehydratable at the required concentration of *A. quisqualis* CPA-9. Then, the amount of pregelatinised potato starch (PPS) had to be reduced from two-thirds to one-sixth to achieve a suitable product (Table 1).

Once PPS ratio was optimised, the best protective compounds obtained before were tested again. The combination of skimmed milk 10% plus sucrose 10% (SKM10 + SUC10) as protective compounds resulted to be significantly better than skimmed milk 10% plus sucrose 20% (SKM10 + SUC20) (Table 1). So, the optimised potato starch formulation was composed of one-sixth of PPS on its carrier and SKM10 + SUC10 were used as protectant substances during the drying process. Being the concentration of viable conidia 4.1×10^7 conidia g^{-1} . Also, one-third of PPS on the carrier was evaluated but suitability of the formulated product was not good.

Table 1 Viability of potato starch formulations with different ratio of PPS (pregelatinised potato starch) in the carrier. Optimised PPS ratio was studied with the best protective compounds (SKM10 + SUC10 and SKM10 + SUC20). Means with different letters indicate significant differences ($P < 0.05$) according to Student's *t* test. Native potato starch (NPS) completed the

274 total amount of carrier. Suitability problems were related to an excess of the viscosity of the
 275 rehydrated product.

PPS ratio	Protective compounds	Log N N ₀ ⁻¹	Suitability
2/3	SKM10 + SUC10	-0.20±0.048	No
1/3	SKM10 + SUC10	-0.44±0.042	No
1/6	SKM10 + SUC10	-0.22±0.033 A	Yes
1/6	SKM10 + SUC20	-0.41±0.021 B	Yes

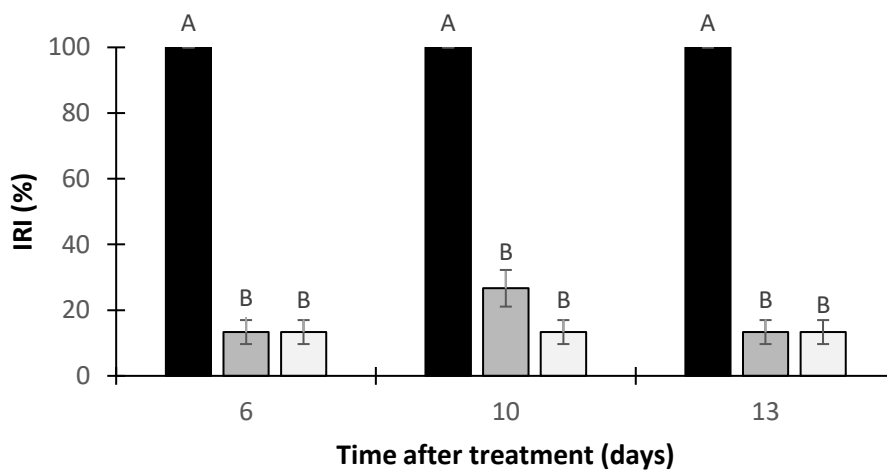
276

277 **3.2. Efficacy of the fluidised-bed spray-dried *A. quisqualis* CPA-9 against *P. xanthii***

278 The ability of formulated *A. quisqualis* CPA-9 to control powdery mildew (*P. xanthii*) is shown
 279 in Fig. 2a. The optimised formulation significantly reduced powdery mildew infection after 6, 10
 280 and 13 days of treatment and no significant differences were obtained among formulated and non-
 281 formulated products reducing powdery mildew infection.

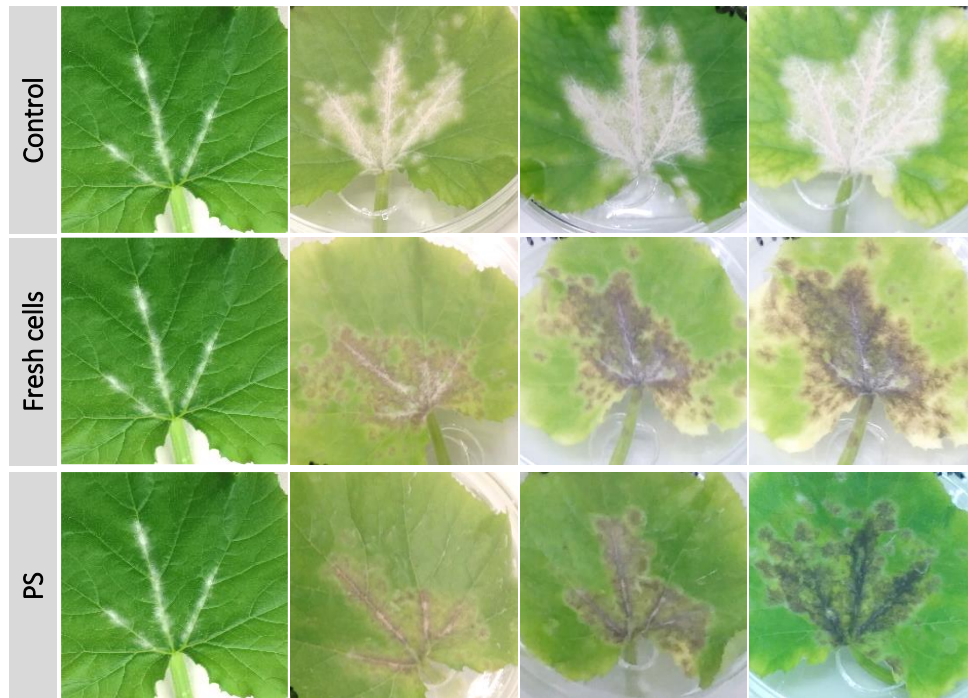
282 Visual observations (Fig. 2b) clearly revealed that both, formulated and non-formulated CPA-9
 283 completely inhibited mycelial growth of powdery mildew 13 days after treatment compared to
 284 the active colonization process of this pathogen observed in the untreated control. CPA-9 treated
 285 leaves became brownish and it was difficult to observe powdery mildew on their surface, whereas
 286 control leaves treated with water were covered with a whitish powdery mildew.

287



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 289
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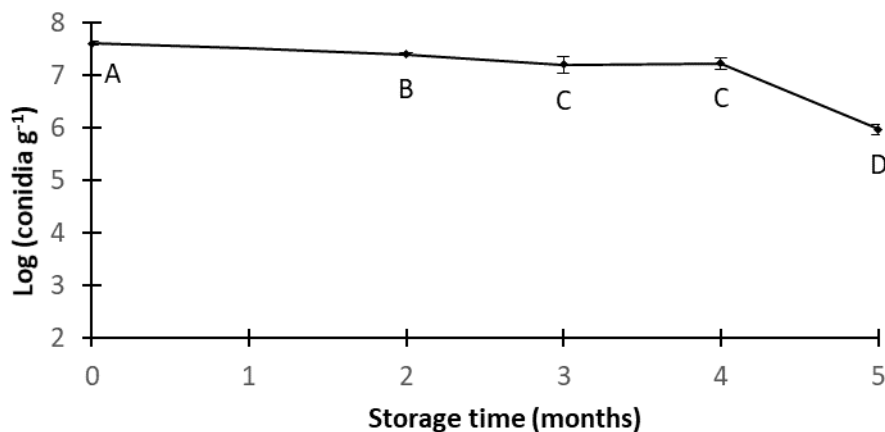


291
 292 **Fig. 2** Efficacy of the biocontrol agent *A. quisqualis* CPA-9 against *P. xanthii* on zucchini leaves
 293 (variety “Black Beauty”): (a) Efficacy represented as IDI (Incidence Disease Index); and (b)
 294 representative images of the infection control. Fluidised-bed spray-dried formulation was tested
 295 (□), and compared with non-formulated CPA-9 (▣). Control (■) was treated with water. Potato
 296 starch formulation was composed of pregelatinised potato starch (1/6) and native potato starch
 297 (5/6) as carriers, pregelatinised potato starch as binder, and 10% of skimmed milk plus 10% of
 298 sucrose as protective compounds. Fresh cells were produced in 250 mL Erlenmeyer flasks with
 299 50 mL of PDB with 2.5% (w/v) Glycerol. Zucchini leaves were artificially infected with *P. xanthii*
 300 5 days before the application of the treatments. *A. quisqualis* CPA-9 treatments were applied at
 301 10^6 conidia mL^{-1} and CPA-9 parasitization was evaluated 6, 10, and 13 days after the treatment.
 302 Error bars represent de standard deviations of the means.

303
 304

3.3. Shelf life of the fluidised-bed spray-dried formulation of CPA-9

305 The optimised product stored at 4 °C showed a decrease of viability of 1.64 Log after 5 months
 306 of storage, but first from 0 to 4 months of storage the viability is maintained (Fig. 3). Nevertheless,
 307 the highest reduction of viability was observed after 4 months of storage. CPA-9 viability only
 308 decreased 0.39 Log after 4 months.



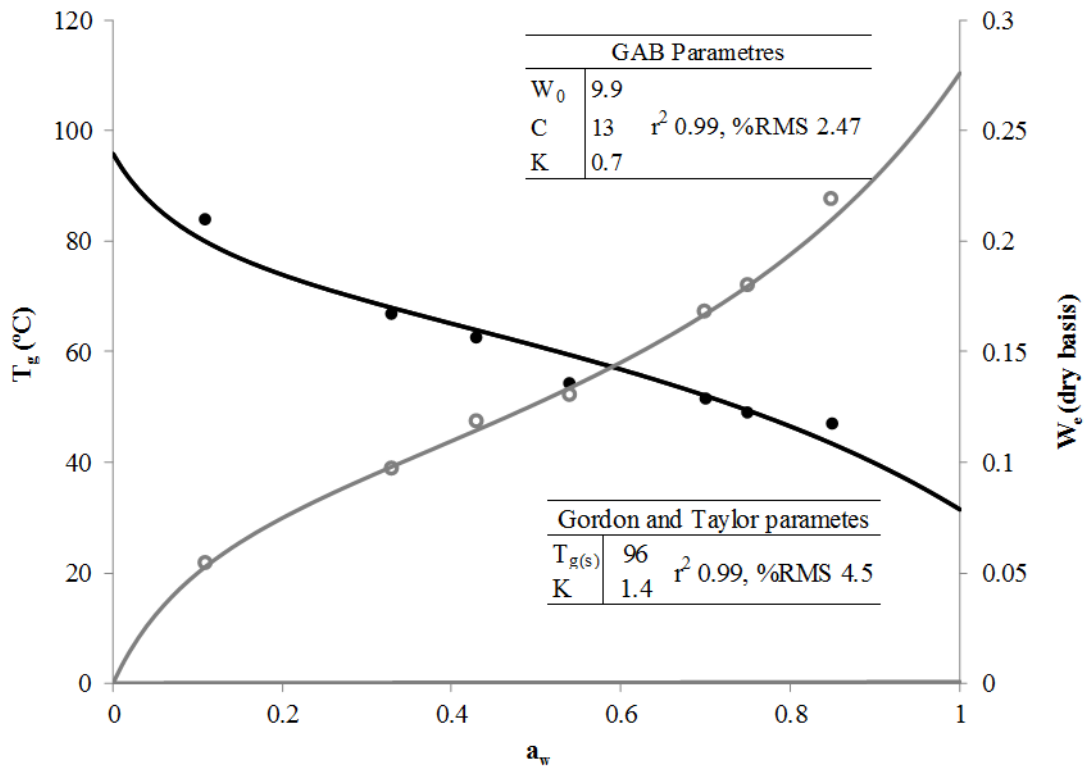
309 **Fig. 3** Shelf life of the fluidised-bed spray-dried formulation of *A. quisqualis* CPA-9 stored at
 310 4 °C for 5 months (●). Dried formulation was composed of pregelatinised potato starch (1/6) and
 311 native potato starch (5/6) as carriers, pregelatinised potato starch as binder, and 10% of skimmed
 312 milk plus 10% of sucrose as protective compounds. Mean values of three replicates are
 313 represented and vertical bars indicate standard deviation of the means. Where bars are not shown,
 314 they were smaller than symbol size and different letters indicate significant differences ($P < 0.05$)
 315 according to Tukey's test
 316

3.4. Physical stability and water solubility of the fluidised-bed spray-dried formulation of CPA-9

3.4.1. Water sorption and plasticisation

317
 318
 319
 320
 321 Figure 4 shows the initial moisture content of the formulation was 7.85 ± 0.05 g/100 g product
 322 and the equilibrium moisture content associated to the different values of a_w or relative humidity
 323 ($a_w = HR/100$). Also the fitted GAB model from which the monolayer moisture content of the
 324 powder could be deduced. This value (9.9 g/100 g dried product) was near the initial moisture
 325 content of the product, as usually occurs for spray dried materials. Then, the points in the isotherm
 326 for $a_w \geq 0.33$ corresponded to water adsorption while point below 0.33 a_w correspond to water
 327 desorption of the initial product. The values of the glass transition temperature (T_g) of the product
 328 equilibrated at different a_w values are also shown, together with the predicted values (Curve)
 329 obtained from the Gordon and Taylor and GAB fitted models. The water plasticisation analysis
 330 shows that the higher the moisture content the lower the T_g values (Roos & Karel, 1991). T_g values
 331 ranged between 47 and 84 °C in the 0.11 – 0.85 a_w range, and therefore no rubbery solid at 20 °C
 332 (sorption temperature) was obtained in this a_w range. In no case was the critical moisture content
 333 (CMC) exceeded at a relative humidity below 85% at 20 °C. Therefore, no stickiness or caking

334 was expected for the powder below this temperature, since powder will be at glassy state, as can
 335 be deduced from the plot.



336

337 **Figure 4.** Moisture content-water activity-glass transition relationships of the fluidised-bed spray-
 338 dried formulation of CPA-9: water sorption isotherm (line: GAB fitted model) and T_g vs. a_w
 339 values (line: predicted values based on Gordon and Taylor and GAB fitted models). Model
 340 parameters are shown in the embedded tables within the plot, with the correlation coefficient (r^2)
 341 and the relative per cent root mean square (% RMS *) of the fitted models.
 342

343 * % RMS =
$$\left[\sqrt{\frac{\sum [(M^{exp} - M^{calc}) / M^{exp}]^2}{N}} \right] \cdot 100$$

344

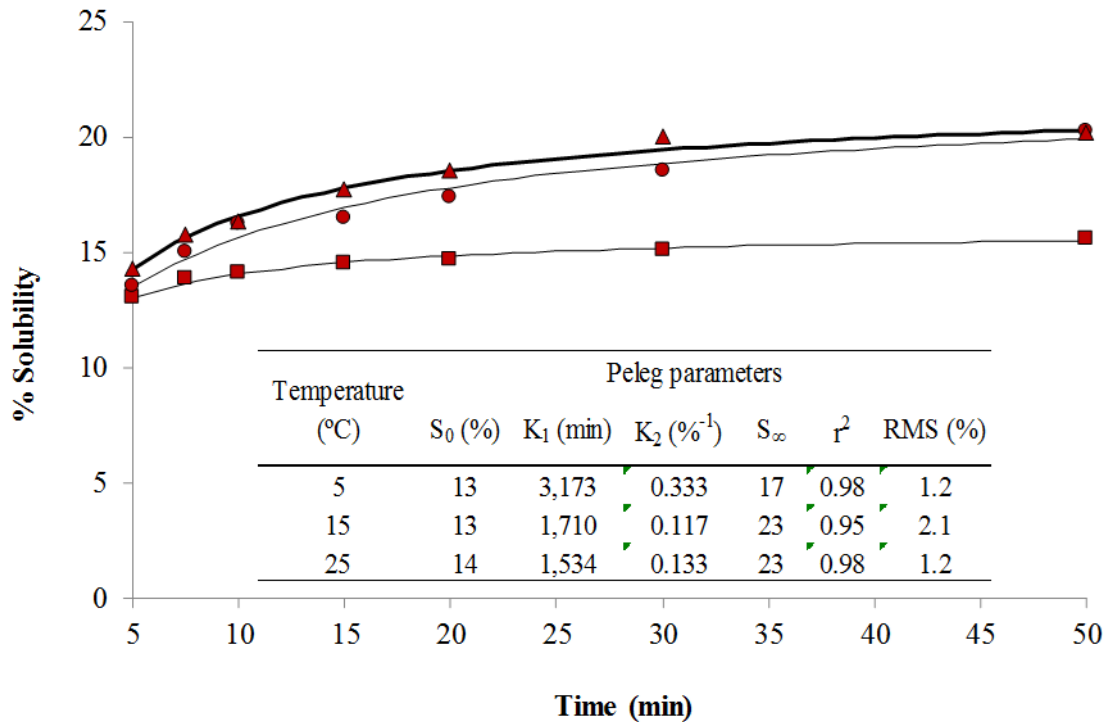
345

3.4.2. Water solubility

346 The water solubility (expressed as % of solubilised solid) of the powder product at 5, 15 and 25
 347 °C is shown in figure 5. Instantaneous solubility was about 13% regardless the temperature and
 348 the values progressively increased throughout up to 23 %. This indicates that the product was
 349 more dispersible than soluble, in agreement with the non-water soluble main component native
 350 starch. There were no notable differences in the solubility between 15 and 25 °C, being these
 351 slightly higher than at 5°C. The analysis of water solubilisation of the formulation at different
 352 temperatures base in the Peleg model was used to model water solubilisation of the formulation
 353 at different temperatures with good correlation coefficient ($r^2 > 0.953$) and model parameters are

354 shown in figure 5. K_1 is related to mass transfer rate while K_2 is associated with water absorption
 355 capacity.

356



357
 358

359 **Figure 5.** Percentage of solubility of the fluidised-bed spray-dried formulation of CPA-9 at 5
 360 (■), 15 (●) and 25 (▲) °C. Solid lines correspond to Peleg fitted model. The model
 361 parameters are shown in the embedded table, with the correlation coefficient (r^2) and the
 362 relative per cent root mean square (% RMS) of the fitted model.

363
 364

4. Discussion

365 The potential of the mycoparasite *A. quisqualis* against powdery mildew has been largely
 366 demonstrated (Angeli et al., 2013; Gautam and Avasthi, 2016; Gilardi et al., 2008, 2017). Although
 367 production and formulation design process for production and formulation of this fungus to
 368 achieve a suitable product is still limited this fungus to achieve a suitable biocontrol product is
 369 still limited (Angeli et al., 2017; Szejnberg, 1991, 1993; Szejnberg and Galper, 1989).

370 In the present study, the fluidised-bed spray-dried of *A. quisqualis* CPA-9 was tested using potato
 371 starch derivatives as carrier and binder compounds. Also different protective substances were
 372 evaluated to achieve a formulation which was able to control powdery mildew on cucurbits. After
 373 drying a film-forming formulation composed of biodegradable coatings to control *P. xanthii* on
 374 zucchini leaves was obtained based on pregelatinised potato starch (1/6) and native potato starch

375 (5/6) as carriers, pregelatinised potato starch as binder, and 10% of skimmed milk plus 10% of
376 sucrose as protective compounds. Moreover, the effectiveness of the dehydration system and the
377 low cost of the employed substances favour the scale-up of the formulation process.

378 The carrier of the optimised formulation was obtained using one-third of native potato starch
379 (NPS) and two-thirds of pregelatinised potato starch (PPS) as carriers, and binder. However,
380 sucrose alone also improved the viability of dehydrated cells, the reduction of PPS to one-sixth
381 and with skimmed milk 10% plus sucrose 10% enhanced the viability of dehydrated cells. In fact,
382 sugars and skimmed milk also prevented cellular damages during freeze-drying (Strasser et al.,
383 2009) and *C. sake* CPA-1 required skimmed milk and sucrose as protectors for a fluidised-bed
384 spray-dried maltodextrin-based product (Carbó et al., 2017).

385 The concentration of viable conidia of the formulated product was low compared to the
386 commercialised product AQ10 Biofungicide® (5×10^9 conidia g⁻¹). However, the viability of
387 CPA-9 only decreased 0.22 Log during the dried process. This fact suggests that the issue resides
388 in the difficulties to achieve a high concentration of conidia during the mass production;
389 unfortunately, conidia production of *A. quisqualis* in culture media is relatively poor (Angeli,
390 2013). So far, a maximum concentration of $2\text{-}3 \times 10^8$ conidia mL⁻¹ was achieved to continue with
391 the formulation process. However, regardless the concentration of conidia, the product was
392 formulated with cheap substances, so the requirement of a high amount of powder was balanced
393 out with the low cost of the compounds; additionally, the selected compounds were able to
394 develop a coating on the leave surface to improve the activity of CPA-9.

395 Regarding the product efficacy, the formulation of *A. quisqualis* CPA-9 controlled *P. xanthii* on
396 zucchini leaves with only one application at 10^6 conidia mL⁻¹ under controlled conditions. AQ10
397 Biofungicide® required repeated applications under field conditions suggesting that number of
398 applications for CPA-9 might also increase under unfavourable conditions (Gilardi et al., 2012;
399 Szejnberg, 1993). Other shortcoming of *A. quisqualis* is the requirement of high relative humidity
400 (RH) to be effective (Romero et al., 2007) In this sense, the RH during the efficacy trial conducted
401 in this study was higher than 85% and other experiments will be required to confirm the
402 germination of formulated CPA-9 at low RH values. In relation to the applied dose, despite of it

403 is also recommended to apply AQ10 at 10^6 conidia mL⁻¹ (Sztejnberg, 1991), although it was
404 applied at higher doses in several experiments (Caffi et al., 2013; Pertot et al., 2008). Surely, the
405 use of biodegradable coatings as carriers or binders during the drying process entailed important
406 advantages for preharvest applications of the optimised CPA-9 formulation. It has been
407 previously demonstrated that biodegradable coatings enhanced the distribution and adherence of
408 other biocontrol agents (Carbó et al., 2017); protected against environmental fluctuations (Lahlali
409 and Jijakli, 2009); and solar radiation (Lahlali et al., 2011). Therefore, biodegradable coatings
410 might avoid the necessity to increase the application dose in some conditions.

411 With respect to the conidia concentration and the shelf life of the optimised product, concentration
412 of viable conidia after 5 months of storage under refrigerated conditions was rather low to apply
413 the product at the desirable dose. From these results and until the package and the storage
414 conditions could be optimised, 4 months of shelf-life at 4 °C gave a commercially suitable
415 product.

416 In terms of physical stability, the powder formulate exhibited glassy state practically in the
417 complete range of relative humidity below 20 °C, which guarantees its stability against sticking
418 and caking processes which could compromise their usefulness. On the other hand, the powder
419 exhibited low water solubility due to the higher proportion of native starch, where the starch
420 polymers, amylose and amylopectin, are within structure of the granules (Mandala and Bayas,
421 2004; Marín et al., 2017). Then, the product is mainly dispersible, and this aspect must be taken
422 into account for practical field applications. In fact, in the first approach of the formulation
423 product, the proportion of native potato starch was lower, and it was not possible to rehydrate the
424 powder at the desired concentration due to the high stickiness of the solution. This characteristic
425 could be attributed to the presence of glycerol in the sprayed suspension, which came from the
426 production media. Commonly, glycerol is considered a plasticizer of starch substances (Dhall,
427 2013) to improve the flexibility and elasticity of the coating (Palou et al., 2015). However, an
428 excess of glycerol could produce too sticky coatings (Rodríguez et al., 2006). Moreover, the
429 rehydrated product required a high proportion of powder due to the low concentration of conidia
430 in the formulated powder, wherewith the solubilisation was more complicated.

431 In summary, the optimised formulation of *A. quisqualis* CPA-9 represents a promising biocontrol
432 product against powdery mildew on cucurbits, although the shelf-life of the product should be
433 improved. The drying process was carried out with the fluidised-bed spray-drying system, which
434 is an efficient, easy to use and scale-up technology. The obtained product was based on low-
435 priced coating forming substances that enhanced the survival and efficacy of the BCA under
436 practical conditions; and the product was effective against *P. xanthii* on zucchini leaves with just
437 one application at 10^6 conidia mL⁻¹. Moreover, the powder was physically stable against sticking
438 and caking. A challenge for these formulations will be to improve their shelf life with suitable
439 package and storage conditions, and to test their efficacy under field conditions.

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