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

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**Abstract**

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

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

## 1. Introduction

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

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

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

During the development of a film-forming formulation of microbial biocontrol agents, the selection of carriers-protectants of the BCAs is a key step for the shelf-life and stability of formulations (Kinay and Yildiz, 2008). Carrier composition also defines sorption and plasticization water, which have a great influence on the physical stability and microbial features. Likewise, it also impacts on the powder dispersion capability in water, another key factor for their practical application. Marín et al. (2017) demostrated 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 ºC; (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 ºC 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

15 g $L^{-1}$) plates under a daily 12-h photoperiod (black light UV/dark at 25/18 ºC) and periodically sub-cultured at most three times, approximately every 10-15 days.

Sub-cultured colonies were mixed with sterile water and grinded with a sterile porcelain mortar and pestle to obtain the suspension used as starter inoculum for conidia production. Conidia were produced in 500-mL Erlenmeyer flasks with 75 mL of potato dextrose broth (PDB: potato peptone, 4 g $L^{-1}$; and glucose, 20 g $L^{-1}$) modified with 2.5% (w/v) of glycerol with an initial concentration of $10^5$ conidia $mL^{-1}$. Erlenmeyer flasks were incubated in the dark without agitation, at 25 ºC for 11 days (Carbó et al., 2020).

*P. xanthii* was originally isolated in IRTA (Lleida, Catalonia, Spain) from cucurbit leaves and it was maintained by growing on zucchini cotyledons (variety "Black Beauty") as follows: first, cotyledons were disinfected thrice for two minutes with $HgCl_2$ (0.1% w/v); once dried at room temperature, cotyledons were introduced in plates with agar (CTM: sucrose, 40 g $L^{-1}$; benzimidazol, 40 mL $L^{-1}$; and agar 2.4 g $L^{-1}$) by embedding their petioles in the medium; finally, a pinch of *P. xanthii* was spread on the surface of the cotyledons with an eyelash to avoid injuring the leaves. Infected cotyledons were incubated under a daily 12-h photoperiod (white light/dark at 21/18 ºC). *P. xanthii* was sub-cultured on new cotyledons approximately every 15 days.

### 2.2. Fluidised-bed spray-drying process

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

Briefly, a suspension of 2-3 × $10^8$ conidia $mL^{-1}$ was prepared from 15-16 Erlenmeyer flasks. This suspension was grinded with an homogeniser-disperser Miccra D-9 (Miccra GmbH, Heitersheim, Germany) for 5 min at 21000 rpm and allowed to at rest for 5 min to facilitate the rupture of the pycnidial wall and release the conidia. This process was repeated two times. Concentration of conidia was determined in a Thoma-Zeiss counting chamber. For each formulated product, 150 g of conidia suspension plus 3.5 g of binder were homogenised with the corresponding protective substances using an Ultra-Turrax (IKA Ultra-turrax® T25 Digital, IKA®-Werke GmbH & Co.

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

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

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

### 2.3. Optimisation of protective and carrier compounds

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

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

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

### 2.4. Efficacy of the formulation

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

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

### 2.5. Shelf life of the formulation

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

### 2.6. Physical stability and solubility of the formulation

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

reached. Initial moisture content of the sample was determined by drying for 24 h at 60 °C in a vacuum oven and subsequent conditioning in a desiccator containing $P_2O_5$. Experimental data were fitted to the Guggenheim–Anderson–de Boer (GAB) model (Equation 1) over the entire $a_W$ range.

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

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

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

$$T_g = \frac{(1 - x_W) \cdot T_{gs} + k \cdot x_W \cdot T_{g\,(W)}}{(1 - x_W) + k \cdot x_W} \quad (2)$$

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

Water solubility of the formulate was measured at 5°C, 15°C and 25°C for different contact times (5 to 50 min) under mild agitation conditions (200 rpm), with a modification of the method described by Cano-Chauca et al., (2005). Samples of 0.25 g were dispersed in 25 mL of deionised water and stirred for each time and temperature. Samples were centrifuged at 3000 *g* for 5 min and 6.25 mL of the supernatants were transferred into pre-weighed glass Petri dishes. Dishes were 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 $$S = S_0 + \frac{t}{K_1 + K_2 \cdot t} \quad (3)$$

225 Where, S is the percentage of solubilised solids at time t; $S_0$ is the instantaneous solubility; t is
226 time (min); and $K_1$ and $K_2$ 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 ($P \leq 0.05$) 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 ($P \leq 0.05$). 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).

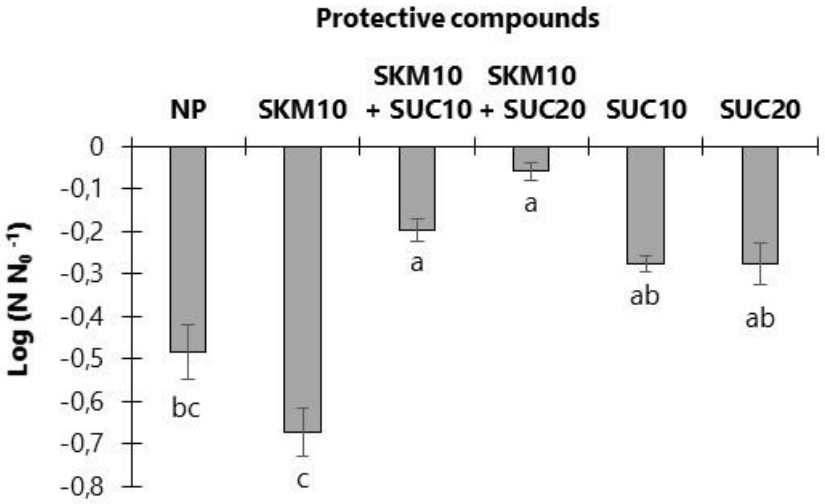



**Fig. 1** Survival of *A. quisqualis* CPA-9 dried at 55 °C adding different protective compounds to a potato starch carrier-based formulation (□). Protective compounds were NP (control without protectant compounds); SKM10 (skimmed milk 10%); SKM10 + SUC10 (skimmed milk 10% and sucrose 10%); SKM10 + 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 de 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 $4.1 \times 10^7$ 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

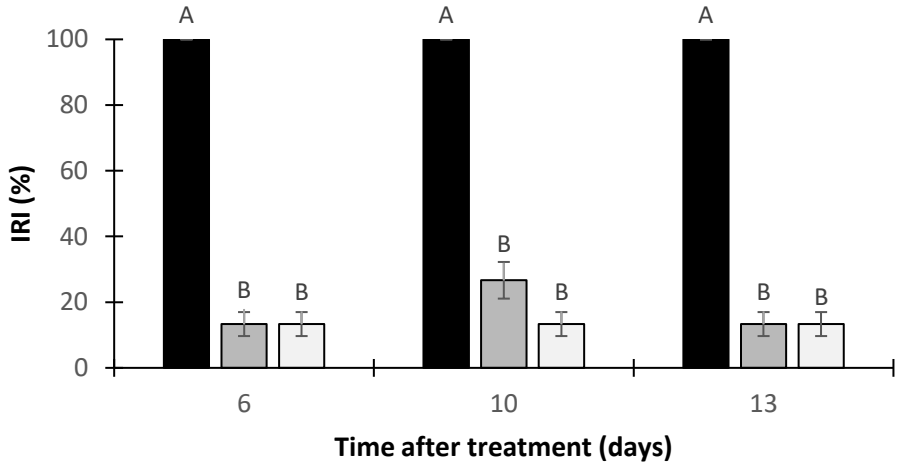
total amount of carrier. Suitability problems were related to an excess of the viscosity of the rehydrated product.

| 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 |

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

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

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



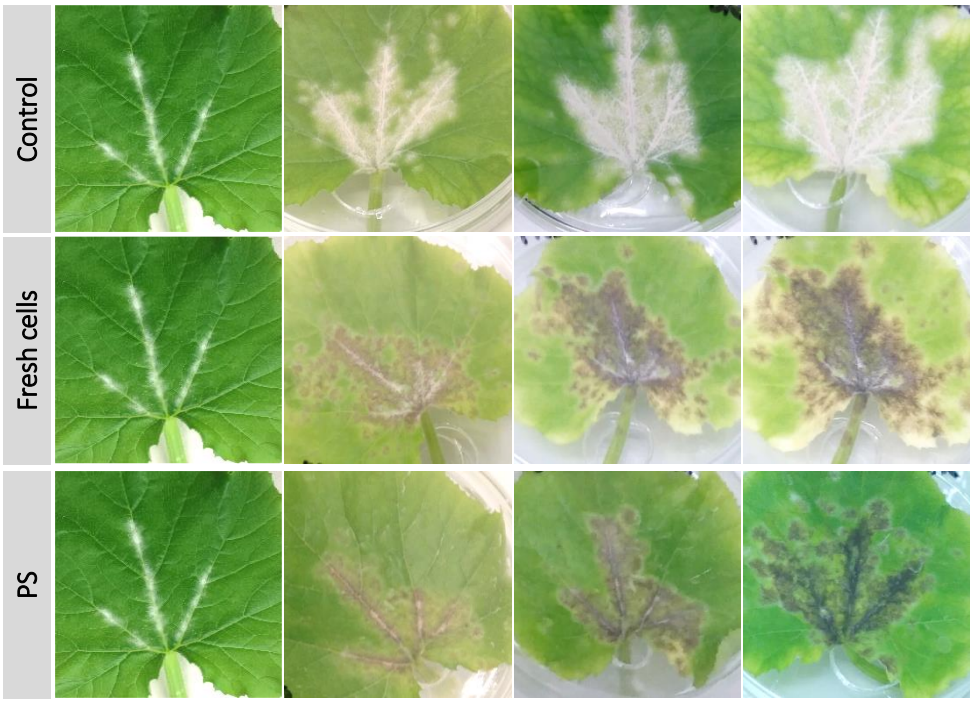




**Fig. 2** Efficacy of the biocontrol agent *A. quisqualis* CPA-9 against *P. xanthii* on zucchini leaves (variety "Black Beauty"): (a) Efficacy represented as IDI (Incidence Disease Index); and (b) representative images of the infection control. Fluidised-bed spray-dried formulation was tested (□),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

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

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

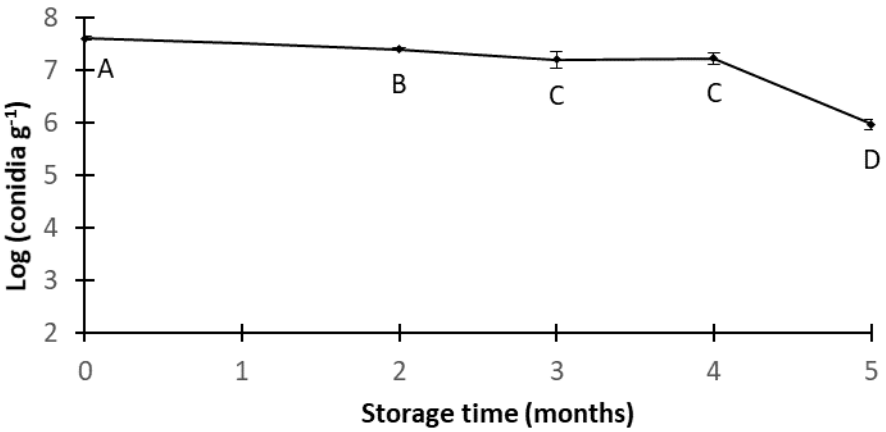



**Fig. 3** Shelf life of the fluidised-bed spray-dried formulation of *A. quisqualis* CPA-9 stored at 4 ºC for 5 months (●). Dried formulation was composed of pregelatinised potato starch (1/6) and native potato starch (5/6) as carriers, pregelatinised potato starch as binder, and 10% of skimmed milk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

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

#### 3.4.1. Water sorption and plasticisation

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

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

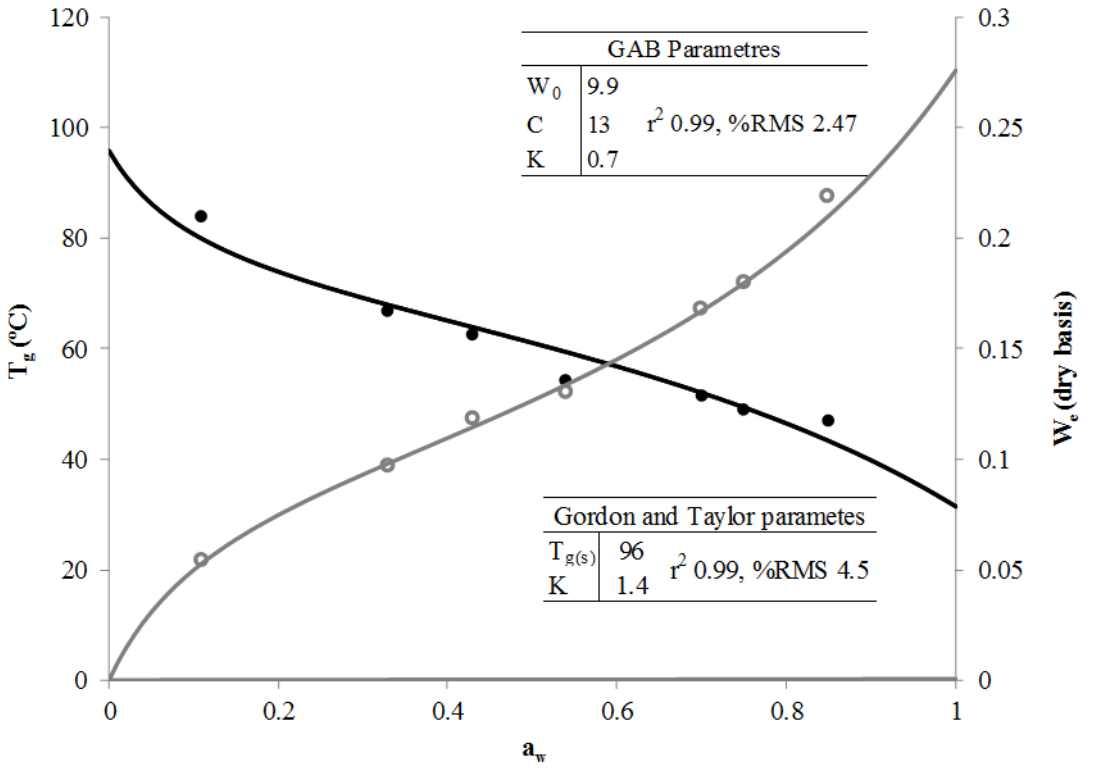


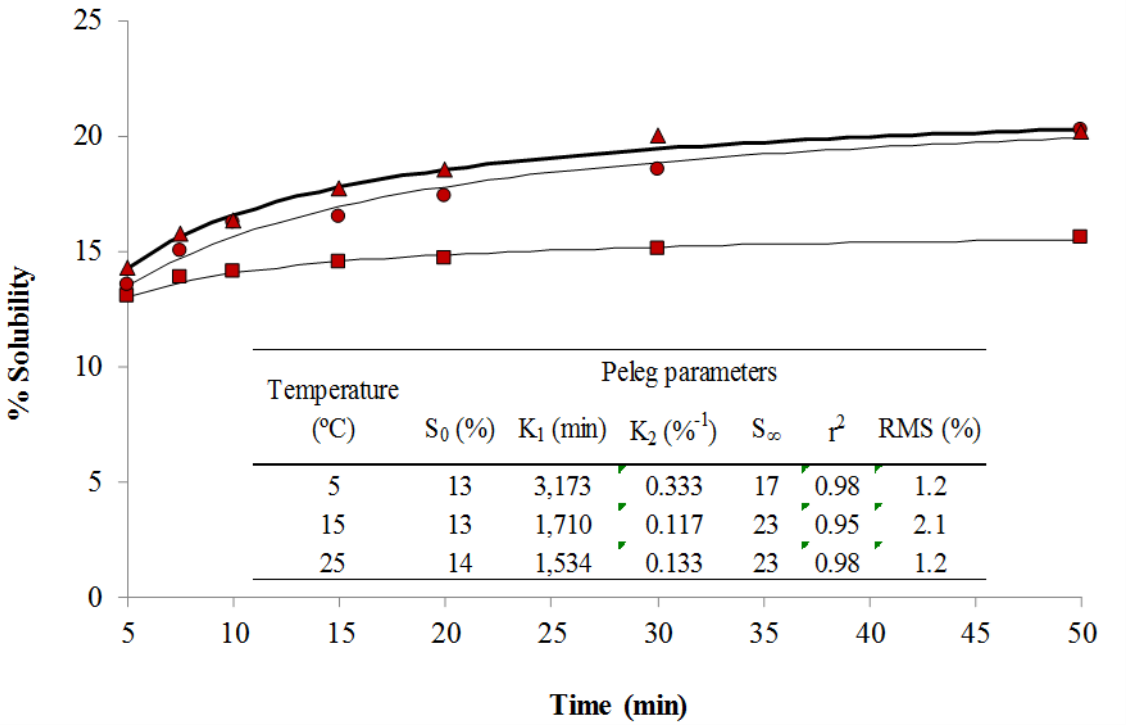

**Figure 4**. Moisture content-water activity-glass transition relationships of the fluidised-bed spray-dried formulation of CPA-9: water sorption isotherm (line: GAB fitted model) and $T_g$ vs. $a_w$ values (line: predicted values based on Gordon and Taylor and GAB fitted models). Model parameters are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

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

### 3.4.2. Water solubility

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

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




| Temperature (ºC) | Peleg parameters $S_0$ (%) | $K_1$ (min) | $K_2$ (%$^{-1}$) | $S_\infty$ | $r^2$ | RMS (%) |
|---|---|---|---|---|---|---|
| 5 | 13 | 3,173 | 0.333 | 17 | 0.98 | 1.2 |
| 15 | 13 | 1,710 | 0.117 | 23 | 0.95 | 2.1 |
| 25 | 14 | 1,534 | 0.133 | 23 | 0.98 | 1.2 |

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

## 4. Discussion

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

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

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

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

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

Regarding the product efficacy, the formulation of *A. quisqualis* CPA-9 controlled *P. xanthii* on zucchini leaves with only one application at $10^6$ conidia $mL^{-1}$under controlled conditions. AQ10 Biofungicide® required repeated applications under field conditions suggesting that number of applications for CPA-9 might also increase under unfavourable conditions (Gilardi et al., 2012; Sztejnberg, 1993). Other shortcoming of *A. quisqualis* is the requirement of high relative humidity (RH) to be effective (Romero et al., 2007) In this sense, the RH during the efficacy trial conducted in this study was higher than 85% and other experiments will be required to confirm the 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 ºC 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 ºC, 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.

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

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