Verifying the biocontrol activity of novel film-forming formulations of *Candida sake* CPA-1: resilience in relation to environmental factors, rainfall episodes, and control of *Botrytis cinerea* on different hosts

Short running title (less than 80 characters): Verifying the potential of novel film-forming formulations of *C. sake* CPA-1

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Abstract:

BACKGROUND:

The efficacy of *C. sake* CPA-1 as a biocontrol agent against several diseases has been studied since it was isolated twenty years ago. However, it was only recently that two suitable and effective film-forming formulations based on potato starch and maltodextrins were developed by the fluidised-bed spray-drying system. The present work aimed to confirm the capability of both novel formulations by testing their resilience on grapes under different temperatures (0 ºC, 22 ºC and 30 ºC), relative humidities (40% and 85%) and simulated rainfall. Another objective was to examine control of *Botrytis cinerea* in different hosts.

RESULTS:

CPA-1 cells from both dried formulations survived better than the liquid formulation on grapes stored at 0 ºC and 22 ºC regardless of the relative humidity. After simulated rainfall, potato starch formulation achieved significantly higher populations than maltodextrin formulation, although the highest reduction was -1.6 Log N N_0^{-1}. A positive effect of cell establishment prior to the simulated rainfall was shown, and recovered cells from the potato starch formulation were significantly higher after 72 h of cell establishment. Finally, both formulations reduced the incidence and severity of *B. cinerea* on pears, apples and tomatoes.

CONCLUSION:

The potential of these novel film-forming formulations of *C. sake* CPA-1 was verified. The resilience of formulated *C. sake* was better than the commercialised liquid formulation, the adherence of the formulations on the grapes improved after an establishment period prior to rain exposure, and the control of *B. cinerea* was verified in wider range of hosts.

Keywords: temperature, relative humidity, rainfall, *B. cinerea*, efficacy, fluidised-bed spray-drying

1. Introduction

Twenty years ago, *Candida sake* CPA-1 was isolated from the surface of apples,[1] and since then, it has been thoroughly studied. Growth conditions of CPA-1 were optimised[2] and several formulations were tested,[3–7] although only a liquid formulation called Candifruit™ was commercialised.

The potential of *C. sake* CPA-1 as a biocontrol agent (BCA) is well-known and recent studies, together with technological advances, have allowed for the optimisation of two fluidised-bed spray-dried formulations with biodegradable coatings on their composition.[8]

However, the commercialisation of a biocontrol product is the most difficult stage in its development,[9] and an effort to anticipate any possible obstacles to commercialisation could be important to simplify the process.[10] The maintenance of cell viability[9] and abiotic stress tolerance[11] are crucial attributes for the commercialisation of yeasts used as BCAs, because of this, packaging and storage conditions for both film-forming formulations of CPA-1 have been optimised to maintain cell viability after 21 months (Carbó *et al.*, unpublished data). The spectrum of activity of BCAs is usually criticised[12], and in general, the spectrum might be quite specific for few pathogens and no systematically more generic, or even some BCAs could be efficient against one pathogen but they improved the development of other[13]. In this sense, it could be easier to extend the use of the BCA products to other hosts instead to other pathogens. Abiotic stress tolerance after application and the efficacy of these novel formulations on different hosts are still unknown.

The efficacy of fresh *C. sake* CPA-1 cells was demonstrated against the major postharvest fungal pathogens on pome fruits, such as *Penicillium expansum, Rhizopus stolonifer* and *Botrytis cinerea.*[1] The efficacy of liquid formulations of CPA-1 was also shown against *B. cinerea* on grapes in laboratory assays[14] and under field conditions with different strategies,[14–17] including the addition of biodegradable coatings. Liquid formulations of *C. sake* CPA-1 also significantly reduced sour rot severity under field conditions.[18] The efficacy of both novel film-forming formulations of CPA-1 was demonstrated against *B. cinerea* on grapes in a laboratory-based assay.[8] In addition, grey mould incidence and severity together with sour rot severity were

significantly reduced under field conditions during two growing seasons[19]. *B. cinerea* produces significant losses in more than 200 crops worldwide[20] and over 1400 plant species as possible hosts[21]; grey mould of grapes, berries, fruits, and tomatoes is one of the most common diseases produced by *B. cinerea*[22]. Therefore, it would be beneficial if biocontrol products could be effective against *B. cinerea* on different hosts in order to increase their potential for commercialisation.

Abiotic stress tolerance of BCAs is usually a weakness of biocontrol products, and ambient temperature is one of the major stresses to be confronted by yeasts.[23] *C. sake* CPA-1 was able to grow slowly at 1 ºC on apples,[24] and it shows a very wide tolerance from an ecological point of view.[25] Consequently, *C. sake* CPA-1 could be effective in pre- and postharvest applications, and this extended range of application is an advantage over other BCAs which can only be applied under controlled conditions.[26]

Rainfall events are probably most responsible for treatment wash-off after preharvest applications of BCAs, but there is a dearth of studies about this subject. Fortunately, the effect of simulated rainfall on *C. sake* CPA-1 blended with a commercial additive called Fungicover® was evaluated with positive results after an establishment period.[27] Fungicover® was also added to the liquid formulation of *C. sake* CPA-1[14–16] and other BCAs as *Bacillus ginsengihum*[28] to improve cell survival under field conditions. However, this additive is expensive and mixture before application was inconvenient. Therefore, solid formulations that included biodegradable coatings were optimised[8] with the aim of achieving high survival and the establishment of CPA-1 cells under field conditions without the further use of any additives.

At the end of the optimisation of a biocontrol product, experts should be able to answer many questions about advantages over other products, efficacy, survival under abiotic conditions, or market size.[29] In connection therewith, the development process of both film-forming CPA-1 formulations was followed stepwise to provide satisfactory answers to these questions[8,17,19,24,30,31].

The present study aimed to verify the potential of two recently developed *C. sake* CPA-1 formulations using fluidised-bed spray-drying system [8], and the specific objectives were as follows: (i) to examine CPA-1 resilience on wine grape berries under controlled conditions of

temperature (0 ºC, 22 ºC and 30 ºC) and relative humidity (RH) (40% and 85%); (ii) to determine the effect of simulated rainfall with different rain intensities and rain volumes on CPA-1 formulations applied to wine grapes; (iii) to evaluate the effect of different periods of cell establishment prior to rain wash; and (iv) to test the efficacy of CPA-1 film-forming formulations to control *B. cinerea* on the surface of different hosts, such as apples, pears and tomatoes.

2. Materials and methods

2.1. The biocontrol agent

The assays conducted in the present study were carried out with the yeast strain CPA-1 of *C. sake*. CPA-1 belongs to the Collection of Postharvest Pathology Group of IRTA (Lleida, Catalonia, Spain) and was obtained originally from University of Lleida-IRTA. CPA-1 was deposited in the Colección Española de Cultivos Tipo (CECT-10817) at the University of Valencia, Burjassot, Spain.

C. sake CPA-1 stock cultures were stored in Criobilles tubes (Criobilles AEB 400100, AES Laboratory, Comburg, France) at -80 ºC for long term storage. When required, CPA-1 cells were sub-cultured on nutrient yeast dextrose agar plates (NYDA: nutrient broth, 8 g L^{-1}; yeast extract, 5 g L^{-1}; dextrose, 10 g L^{-1}; and agar, 15 g L^{-1}) at 25 ºC for 48 h. After growth, yeast cells were sub-cultured to use or to store at 4 ºC on NYDA plates for a short time.

Biomass production was conducted as described by Abadias *et al.*[2]. Briefly, cells were produced in a liquid fermentation system with a 5 L working volume (BIOSTAT-A modular fermenter, Braun Biotech International, Germany) at an initial concentration of 10^6 CFU mL^{-1} for 40 h. Starter inoculum was prepared by transferring sub-cultured cells to a potassium phosphate buffer (pH 6.5; KH_2PO_4 0.2 mol L^{-1}, 70 ml; K_2HPO_4 0.2 mol L^{-1}, 30 ml and deionized water, 300 mL).

2.2. *C. sake* CPA-1 formulations

The experiments were conducted with two solid and film-forming formulations of the biocontrol agent *C. sake* CPA-1. Both of the following formulations were optimised with the fluidised-bed spray-drying system described by Carbó *et al.*[8]: (i) the potato starch formulation (PS) and (ii) the maltodextrin formulation (MAL). When required, (iii) Candifruit™ (CS) was used as a liquid and non-film-forming formulation to compare with both solid and film-forming formulations.

Dried formulations were rehydrated with the necessary sterile water depending on the required concentration, and were then shaken for 1 min and allowed to rehydrate for 9 min.

2.3. Influence of abiotic factors on *C. sake* CPA-1 formulations

2.3.1. Plant material

The influence of abiotic factors on *C. sake* CPA-1 formulations was evaluated on wine grapes. Grapes were washed with tap water to remove possible residues and were left to dry at room temperature. Then, the grapes were cut into clusters leaving the pedicel attached, and five-berry clusters were used to evaluate the resilience of CPA-1 formulations under controlled conditions of temperature and RH, whereas twenty-berry clusters were exposed to simulated rainfall. Four clusters formed one replicate and each treatment consisted of four replicates.

2.3.2. Treatments application

Each treatment (four clusters × four replicates) was placed onto a grid and all CPA-1 treatments were sprayed using a motorised backpack sprayer (model WJR2225; Honda Motor Company Ltd, Frankfurt, Germany) with 1 mm nozzle and 15 bar pressure until run-off. After air drying, the grids were placed into trays and treated grapes were stored under controlled conditions of temperature and RH or exposed to simulated rainfall.

2.3.3. Resilience of *C. sake* CPA-1 formulations on grape berries under controlled conditions of temperature and RH

In this assay, wine grapes (cultivar "Tempranillo") were treated at 2.5×10^7 CFU ml^{-1} with both fluidised-bed spray-dried formulations and with Candifruit™. Treated grapes were exposed to six different scenarios: (i) 0 ºC and 40% RH; (ii) 0 ºC and 85% RH; (iii) 22 ºC and 40% RH; (iv) 22 ºC and 85% RH; (v) 30 ºC and 40% RH; and (vi) 30 ºC and 85% RH. Climatic chambers programmed at 0 ºC, 22 ºC or 30 ºC were used to control the temperature. For each RH value, treated grapes were placed inside a sealed plastic chamber with a dehumidifier (FDC32S, FRAL, Carmignano di BR., PD, Italy) to maintain the RH with ±10% variation. External data loggers (Testo 175H1, Testo Inc., Sparta Township, NJ, USA) were used to monitor temperature and RH during storage. The assay was carried out during 30 days at 0 ºC, 15 days at 22 ºC and only two days at 30 ºC due to the grapes' increased damage under elevated temperatures.

Depending on the scenario, quantification of *C. sake* cells on berry surfaces was undertaken at different times as described by Calvo-Garrido *et al.*[27]. Briefly, at each timepoint, the grapes of each replicate were placed into Erlenmeyer flasks with 50 ml of buffer phosphate. Flasks were then shaken for 20 min at 150 rpm on a rotatory shaker and sonicated for 10 min in an ultrasonic bath (JP Selecta S.L., Abrera, Spain). The viability of the cells was checked by plating on NYDA with a serial ten-fold dilution of the washings, then colonies were counted after incubating at 25 ºC for 48 h. Data were expressed as CFU g^{-1}.

2.3.4. Adherence of *C. sake* CPA-1 formulations on berry surface under simulated rainfall

Two different assays were carried out to evaluate the wash-off caused by simulated rainfall on both fluidised-bed spray-dried formulations of *C. sake* CPA-1 applied at 5×10^7 CFU ml^{-1} on grapes. (i) In the first approach, the effect of three rain intensities (60, 100 and 150 mm h^{-1}) and three rain volumes (20, 60 and 120 mm) was evaluated on grapes (cultivar "Monastrell") treated as previously described. Treated grapes not exposed to simulated rainfall were used as a control. All wash-off resistance results were expressed in relation to the control. (ii) In the second approach, grapes (cultivar "Macabeu") treated with *C. sake* CPA-1 were incubated at 20 ºC and 85% RH during periods of 24, 48 and 72 h prior to being exposed to rainfall to evaluate the influence of an establishment period on the wash-off caused by simulated rainfall. Treated and incubated grapes which were not exposed to simulated rainfall were used as a control. The rainfall conditions which produced the highest *C. sake* CPA-1 wash-off in the first approach were used for this trial.

Rainfall was simulated as described by Calvo-Garrido *et al.*[27]. Briefly, a metallic box (100 × 50 × 20 cm) with a drop generator system at the bottom was used as rainfall simulator. Treated grapes were placed 1.5 m above with a moving fan located in front of the water curtain to avoid the continuous impact of drops on the same part of the cluster. Rain intensity was measured before and after each rain event and it was regulated by maintaining a constant water layer in the metallic box.

196 Quantification of *C. sake* cells on berry surfaces was done as described by Carbó *et al.*[31] with
197 minor modifications. For each replicate, five berries of each of the four twenty-berry clusters were
198 cut leaving the pedicel attached and introduced into sterile plastic filter bags (BagPage 400 mL,
199 Interscience BagSystem, ST Nom la Brètech, France) with 50 ml sterile water supplemented with
200 Tween 80 (one drop per litre). Bags were homogenised in a Stomacher blender (Masticator Basic
201 400 mL, IUL S.A., Torrent de l'Estadella, Barcelona, Spain) for 10 min. The viability of cells
202 was checked as described above.
203 The rate of reduction was calculated as Log $N\,N_0^{-1}$, were N_0 represents the total CFU g^{-1} recovered
204 from treated grapes which were not exposed to rainfall, and N was the amount recovered after a
205 simulated rainfall event.

206 **2.4. Efficacy of *C. sake* CPA-1 formulations against *B. cinerea* on apple, tomato and**
207 **pear**

208 The efficacy of both fluidised-bed spray-dried formulations of *C. sake* CPA-1 was evaluated
209 against *B. cinerea* on (i) apples (cultivar "Golden delicious"); (ii) pears (cultivar "Conference");
210 and (iii) tomatoes (cultivar "Marglobe") to test the efficacy of these novel formulations on
211 different hosts. The efficacy of freshly made formulations (PS0 and MAL0) and formulations
212 stored for 6 months (PS6 and MAL6) were tested together with Candifruit™ (CS) as a positive
213 control. Deionised water was used as a negative control (CK). Each treatment was replicated four
214 times with five fruits per replicate.
215 All the fruits were washed with tap water and left to dry, then they were wounded with a nail to
216 produce an injury (3 mm in diameter × 3 mm in depth). In each wound, 15 µL of each treatment
217 was applied preventively at 2.5×10^7 CFU mL^{-1}. Once the treatments dried, 15 µL of the pathogen
218 *B. cinerea* at 10^4 conidia mL^{-1} was added in each wound. Then, the fruits were allowed to dry
219 again at room temperature, and after that they were incubated at 20 ºC and 85% RH until the
220 determination of incidence and severity, which were measured as the number of infected wounds
221 and the rot lesion diameters, respectively. Apples were incubated for 3 days, whereas pears and
222 tomatoes were incubated for 4 days.
223

2.5. Statistical analysis

To evaluate the resilience of *C. sake* CPA-1 formulations under controlled conditions of temperature and relative humidity and the adherence of the yeast after the rainfall simulation, data were analysed using one-way ANOVA. Differences at $P < 0.05$ were considered to be significant and means separations were obtained by Tukey's test. The efficacy of CPA-1 treatment was analysed using a generalised linear model (GLIM). Incidence response was based on a binomial distribution and logit-link function and severity response was based on a normal distribution and identity link function. For GLIM analysis, means separations were obtained by orthogonal contrasts. Also differences at $P < 0.05$ were considered to be significant. Data analysis was performed using JMP 13 software (SAS Institute Inc., Cary, NC).

3. Results

3.1. Resilience of *C. sake* CPA-1 formulations on grape berries under controlled conditions of temperature and RH

Storage temperature and RH influenced the viability of different formulations of *C. sake* CPA-1 applied on grapes (Fig. 1). Viability of CPA-1 from both solid formulations followed a similar trend at 0 ºC regardless of the RH. In fact, no treatment showed significant differences due to RH (CS: $F_{1,41} = 0.0891$, $P = 0.7669$; MAL: $F_{1,45} = 0.0106$, $P = 0.9184$; and PS: $F_{1,44} = 0.2789$, $P = 0.6001$). Survival of dried *C. sake* cells from grape surfaces was always higher than for the liquid formulation cells without coatings (Fig. 1a, 1b), and both film-forming formulations achieved significantly higher populations after 30 days at 0 ºC. Under these conditions, the solid formulations nearly retained the initial viability of CPA-1, whereas the viability of the liquid formulation decreased by 3.2 Log CFU g^{-1} at 40% RH and 1.4 Log CFU g^{-1} at 85% RH.

Differences in CPA-1 survival between liquid and solid formulations were lower when the treated grapes were stored at 22 ºC, although significant differences were observed among treatments at the end of the assay (Fig. 1c, 1d). Regardless of the RH, recovered populations from solid film-forming formulations were always significantly higher after 15 days of storage at 22 ºC. Significant differences were also observed between 40% and 85% RH for all the treatments stored at 22 ºC (CS: $F_{1,42} = 27.4378$, $P < 0.0001$; MAL: $F_{1,46} = 49.7546$, $P < 0.0001$; and PS: $F_{1,44} =$

252 37.0540, $P < 0.0001$), and the greatest resilience of CPA-1 was obtained when the grapes were
253 stored at 85% RH. Under these favourable conditions all the formulations showed increased CPA-
254 1 concentration on the grape surface of 0.5 Log CFU g^{-1} (liquid formulation) to 1.3 Log CFU g^{-1}
255 (MAL solid formulation). After 15 days at 22 ºC and 40% RH, the viability of solid formulations
256 decreased by approximately 1 Log CFU g^{-1}, although the viability of the liquid formulation
257 decreased by 2.5 Log CFU g^{-1} (Fig. 1c).
258 The shelf life of detached grapes at high temperatures was very short and despite the CPA-1
259 viability showing almost no decrease, the grapes were greatly damaged after two days of storage
260 at 30 ºC (Fig. 1e, 1f). No significant differences were observed among treatments after two days
261 at 30 ºC, and differences between RH were only observed for the PS formulation, which showed
262 resilience significantly higher at 85% RH (CS: $F_{1,16} = 3.7172$, $P = 0.0718$; MAL: $F_{1,22} = 0.9014$,
263 $P = 0.3527$; and PS: $F_{1,22} = 13.1412$, $P = 0.0015$).

264 **3.2. Adherence of *C. sake* CPA-1 formulations on berry surface under simulated**
265 **rainfall**

266 **3.2.1. *C. sake* CPA-1 wash-off under different intensity and rain volume**

267 Performance of both fluidised-bed spray-dried formulations under the same conditions of
268 simulated rainfall was significantly different In general, the potato starch formulation achieved
269 significantly higher populations of CPA-1 cells than the maltodextrin formulation for all the tested
270 intensities and rain volumes with the exception of the most intensive wash-off (120 mm and 150
271 mm h^{-1}) (Fig. 2). However, differences in intensities at the same rain volume were not observed,
272 and significant differences in intensity among rain volumes were only observed for the potato
273 starch formulation (Fig. 2b).
274 Specifically, CPA-1 reductions for the potato starch formulation were from -0.5 Log N N_0^{-1} (20
275 mm and 60 mm h^{-1}) to -1.4 Log N N_0^{-1} (120 mm and 150 mm h^{-1}) (Fig. 2b). Wash-off of the potato
276 starch formulation significantly increased with the rain volume: after 20 mm of rain exposure, the
277 mean reduction of three intensities was -0.5 Log N N_0^{-1}; after 60 mm of rain, -0.9 Log N N_0^{-1}; and
278 after 120 mm of rain, -1.2 Log N N_0^{-1}. It is worth mentioning that *C. sake* adherence decreased

279 with the rain intensity after 120 mm of rain exposure, although there were not significant
280 differences among intensities within the same rain volume.
281 For the maltodextrin formulation, CPA-1 reductions due to wash-off caused by simulated rainfall
282 were from -1.2 Log N N_0^{-1} (20 mm and 150 mm h^{-1}) to -1.6 Log N N_0^{-1} (120 mm and 100 mm h^{-1}),
283 although no significant differences were observed among intensities or rain volumes for this
284 formulation (Fig. 2a).

285 **3.2.2.*C. sake* CPA-1 wash-off after cell establishment**

286 The establishment of CPA-1 cells prior exposure to rainfall (120 mm of rain with an intensity of
287 150 mm h^{-1}) affected the wash-off resistance of *C. sake* from grape surfaces (Fig. 3). In general,
288 population reductions were lower when the CPA-1 cells' establishment time increased.
289 Significant differences were observed for the potato starch formulation, whereas only a trend was
290 observed for the maltodextrin formulation.
291 Population reduction of CPA-1 in the potato starch formulation was significantly lower after 72
292 h of cell establishment. Specifically, after 72 h of establishment the population reduction was only
293 -0.4 Log N N_0^{-1}, whereas without cell establishment, the cell losses were approximately -0.9 Log
294 N N_0^{-1}. Therefore, an establishment time of 72 h was necessary when the potato starch formulation
295 was applied to grapes.
296 When both formulations were compared, the results showed that the potato starch formulation
297 losses were lower regardless of the establishment time, although the differences were only
298 significant after 72 h of establishment.

299 **3.3. Control of *B. cinerea* on different hosts using *C. sake* CPA-1 formulations**

300 Biocontrol efficacy of *C. sake* CPA-1 against *B. cinerea* was tested in pears, apples and tomatoes
301 and showed good reductions of the disease (Fig. 4). The best results were achieved with pears,
302 where all the treatments significantly reduced the incidence and severity of *B. cinerea.* No
303 significant differences in severity and incidence were observed among treatments in relation to
304 the control. The liquid formulation (CS) resulted in a 100% reduction in disease incidence, and
305 the solid formulations showed disease incidence reductions of 81% (MA0) to 94% (PS6), and
306 severity reductions of 85% (MA0) to 93% (PS6) (Fig. 4a).

Regarding apples, the liquid formulation (CS) and the potato starch formulation after 6 months of storage (PS6) also achieved a 100% reduction in disease incidence (Fig. 4b). The other tested CPA-1 treatments reduced *B. cinerea* incidence by 43% (MA0) to 79% (PS0). However, in spite of the high reduction, MA0 reduction was not significant ($P = 0.0545$) compared to the control. All the *C. sake* CPA-1 treatments significantly reduced *B. cinerea* severity on apples compared to the control, specifically, severity reductions ranged from 62% (MA0) to 100% (CS/PS6).

All the treatments also reduced *B. cinerea* incidence and severity on tomatoes (Fig. 4c). Incidence reductions ranged from 44% (MA6/PS6) to 81% (PS0) although no significant differences were obtained when MA6 ($P = 0.0506$) and PS6 ($P = 0.0577$) were compared to the control. The same occurred with severity reductions and despite the finding that all the treatments reduced *B. cinerea* severity, MA6 and PS6 reductions were not considered significant compared to the control.

4. Discussion

The present study provides relevant information on two recently optimised fluidised-bed spray-dried formulations of *C. sake* CPA-1. It focuses on the resilience of CPA-1 dried cells applied to wine grapes under different abiotic factors and on the matter of the spectrum of application of these novel formulations.

Both solid formulations (PS and MAL) maintained or even increased their CPA-1 populations on wine grapes incubated at 0 ºC or 22 ºC after 30 days and 15 days, respectively. The liquid formulation populations were always significantly lower at the end of the assays; therefore, the drying process did not affect the resilience of cells after rehydration. Unfortunately, wine grapes showed high damage at 30 ºC, and CPA-1 survival could only be evaluated up to 72 h. Regarding RH, no differences between 40% and 85% RH were observed at 0 ºC for any treatment, whereas all the treatments showed better resilience at 85% RH when they were stored at 22ºC. *C. sake* CPA-1 cells kept metabolic activity low under cold storage conditions and the effect of RH was insignificant. However, at 22 ºC cells were active and the effect of RH gained importance. In previous studies, different coating-forming dispersions also improved the viability of *C. sake* CPA-1 cells applied on grapes and incubated at 20 ºC for 24 h and 7 days.[32] Both solid formulations were applied previously on table grapes incubated at 35 ºC to evaluate the impact of

climate change on *C. sake* CPA-1 resilience, and in that case, table grapes could be incubated up to 96 h, although CPA-1 had a lower resilience at 35 ºC with the existing CO_2 concentration.[31] Calvo-Garrido *et al.*[15] also tested the viability of CPA-1 under limiting conditions of temperature and RH (40 ºC and 30% RH) and yeast populations decreased approximately 3 Log CFU g^{-1} after 72 h, whereas after the same time, the viability of cells remained stable at 21 ºC and 100% RH. Despite the low resilience of *C. sake* CPA-1 at temperatures higher than 30 ºC, the efficacy of fluidised-bed spray-dried formulations was demonstrated under field conditions on grapes, where maximum temperatures were higher than 35 ºC.[19] In contrast, *C. sake* CPA-1 was isolated from apples after several months in a storage atmosphere[1] and the present study showed that the PS and MAL formulations maintained their viability after 30 days at 0 ºC regardless of the RH. Therefore, it may be possible to consider both pre- and postharvest applications as registered uses for the products.

The potato starch formulation showed higher resistance to rain wash than the maltodextrin formulation after a simulated rainfall event of 20 or 60 mm, regardless of the intensity. Differences between the formulations' adherence on grapes decreased when the rain volume was increased to 120 mm and became no significant after the most intensive rain wash-off (120 mm and 150 mm h^{-1}). The physical properties of the formulation compounds could be the responsible for the wash-off differences. Solubility analysis of both fluidised-bed spray-dried CPA-1 formulations was carried out in previous studies and the maltodextrin formulation exhibited the fastest solubilisation.[33] In fact, maltodextrin is mainly used due to its high solubility in water,[34] whereas the solubilities of potato starch and pregelatinised potato starch are lower.[35] Therefore, the higher adherence of potato starch formulation coincides with its lower solubility. In this way, better results were obtained with a formulated product of *Bacillus amyloliquefaciens* CPA-8 based on potato starch than with another composed of maltodextrin substances, both of which were applied to nectarines and peaches.[36] Moreover, after 20 mm of rain exposure, the mean population reduction in the three rain intensities for the PS formulation was approximately 0.3 Log higher than that obtained in previous studies for the liquid formulations of *C. sake* CPA-1 blended with

362 Fungicover®.[27] In fact, it was reported that additives such as starch could provide shelter from UV
363 rays and reduce wash-off during rainfalls.[37]
364 The establishment of CPA-1 cells before exposure to simulated rainfall reduced losses due to rain
365 wash-off. The reduction of CPA-1 cells in the PS formulation was significantly lower after 72 h
366 of establishment. The benefits of an establishment period before exposure to limiting conditions[15]
367 or simulated rainfall[27] were also demonstrated for the liquid formulation of CPA-1 blended with
368 Fungicover®. Promoting an effective establishment of BCAs could be crucial, and several studies
369 also tested some protectants to enhance the viability of cells after application and to protect BCAs
370 against abiotic factors.[38–40]
371 These results highlight the importance of the abiotic factors when BCAs are applied under field
372 conditions. Despite the fact that *C. sake* CPA-1 develops in a wide range of conditions, rainfall
373 events after application could determine its biocontrol efficacy. Therefore, the timing of
374 treatments might be determined by rainfall episodes as suggested Calvo-Garrido *et al*.[27]. For
375 fungicides such as Mancozeb, which is applied to grapes, it is also recommended to repeat the
376 treatment when it rains the day after the application.[41]
377 Although formulations were developed and optimised against grey mould on grapes, the
378 possibility to control *B. cinerea* also on other hosts could increase the market size and the
379 company's interest. The formulated CPA-1 products reduced grey mould incidence and severity
380 on pears, apples and tomatoes. The most significant results were obtained on pears, with a
381 reduction of disease incidence of at least 81%. On apples and tomatoes, the reduction of disease
382 incidence was at least 43% and 44%, respectively. Severity reductions were also high in pears
383 and apples, by 62% to 100%, whereas severity decreased by 18% to 75% in tomatoes. The
384 reduction of *B. cinerea* on tomatoes was relevant because it is an economically important crop
385 which is especially affected by grey mould.[21] The efficacy of fresh cells or different formulations
386 of *C. sake* CPA-1 was previously tested against *Penicillium expansum*,[1,4,6,7] *B. cinerea*[1] and
387 *Rhizopus stolonifer*[1] on apples with good results. Previous studies also demonstrated the efficacy
388 of Candifruit (liquid formulation) against grey mould on tomatoes under semi-controlled

greenhouse conditions.[42] For future studies, it could be interesting to test the efficacy of the PS and MAL formulations against other pathogens.

5. Conclusions

The present study tested the resistance to rain wash of formulated CPA-1 cells applied on wine grapes, confirmed the possibility of both pre- and postharvest application of these novel formulations and increased their market size to control *B. cinerea* on a wider range of hosts. Therefore, these fluidised-bed spray-dried formulations could be potential biocontrol products for commercialisation. That said, it is still necessary to overcome extensive regulations to register these products.

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Conflict of interest

The authors declare that they have no conflict of interest.

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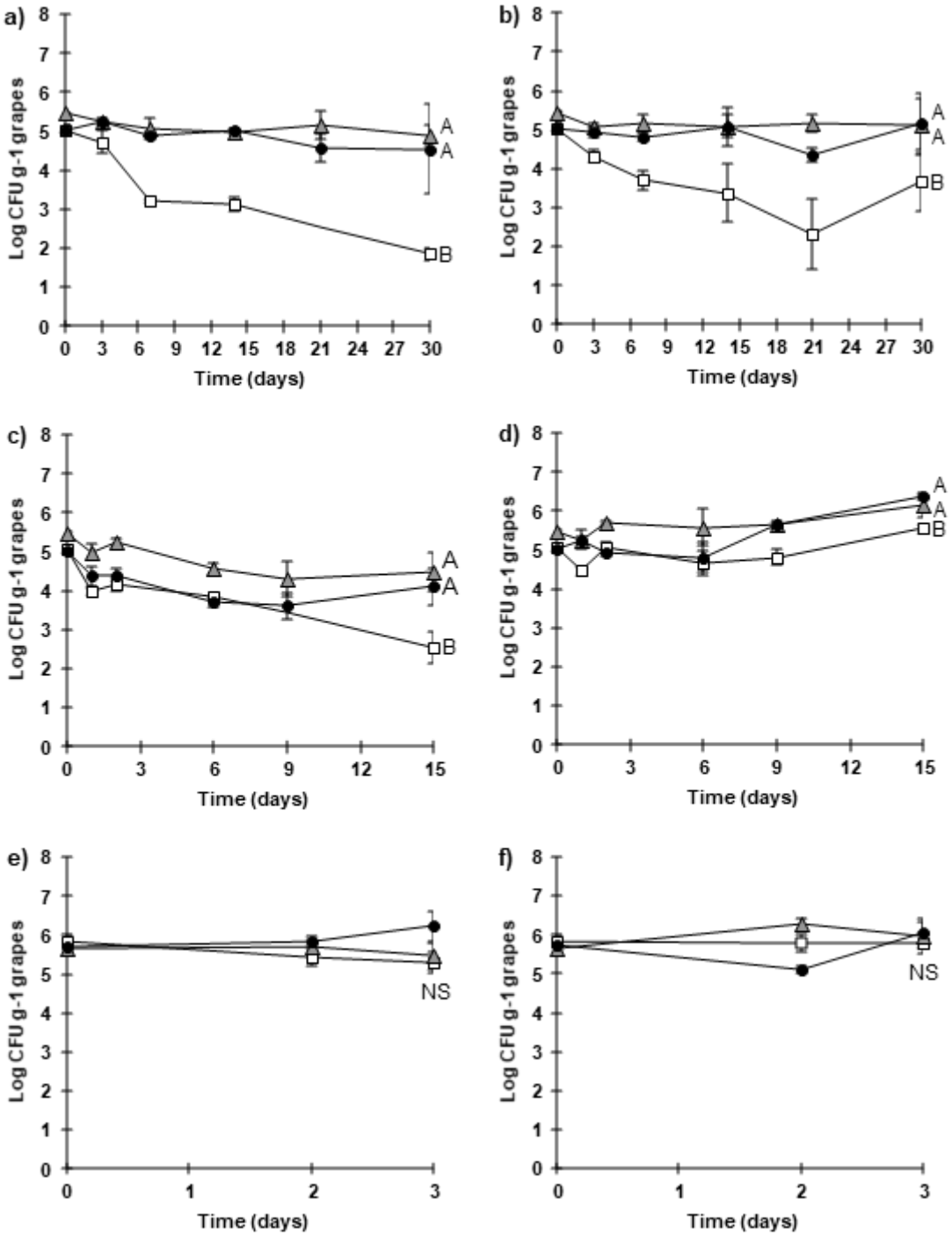

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557 **Figure 1.** Resilience of *C. sake* CPA-1 formulations on grape berries under controlled conditions
558 of temperature and RH: (a) 0 ºC and 40% RH; (b) 0 ºC and 85% RH; (c) 22 ºC and 40% RH; (d)
559 22 ºC and 85% RH; (e) 30 ºC and 40% RH; and (f) 30 ºC and 85% RH. Candifruit ( ), potato
560 starch formulation (▲) and maltodextrin formulation (■) are shown. Values are the mean of four
561 replicates and vertical bars indicate standard deviation of the means. Where bars are not shown,
562 they were smaller than the symbol size. Different letters indicate significant differences ($P < 0.05$)
563 between treatments at the end of the assay. Mean separations were obtained by Tukey's test.

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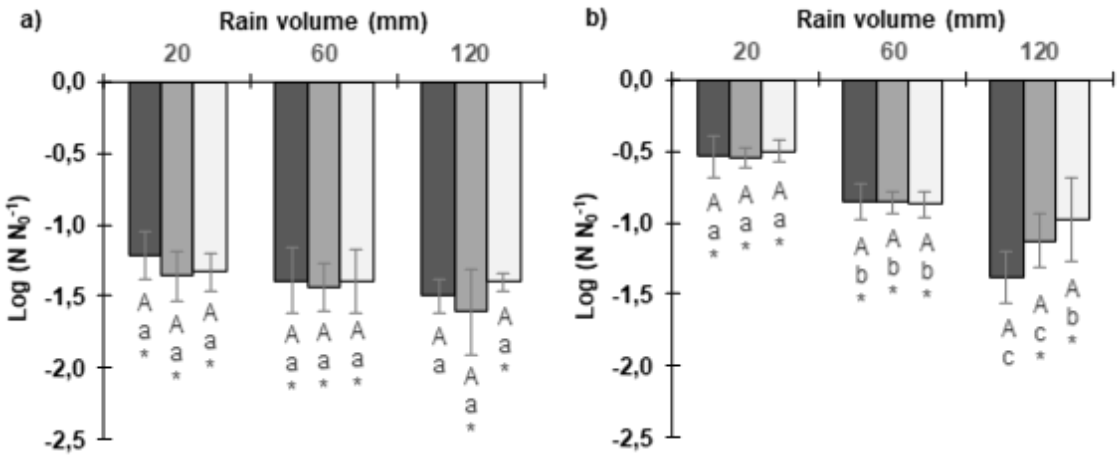

Figure 2. Adherence of *C. sake* CPA-1 fluidised-bed spray-dried formulations on berry surfaces after simulated rainfall at different rain volumes (mm) and intensities (mm h^{-1}). Three intensities are represented as follows: 150 mm h^{-1} (■); 100 mm h^{-1} (■); and 60 mm h^{-1} (■) for both formulations (a) maltodextrin formulation, and (b) potato starch formulation. Values are the mean of four replicates and vertical bars indicate standard deviation of the means. Uppercase letters indicate significant differences in intensities within rain volume; different lowercase letters indicate significant differences in rain volumes within intensities. Asterisks indicate significant differences between formulations. Means separations were obtained by Tukey's test and considered significant at $P < 0.05$.

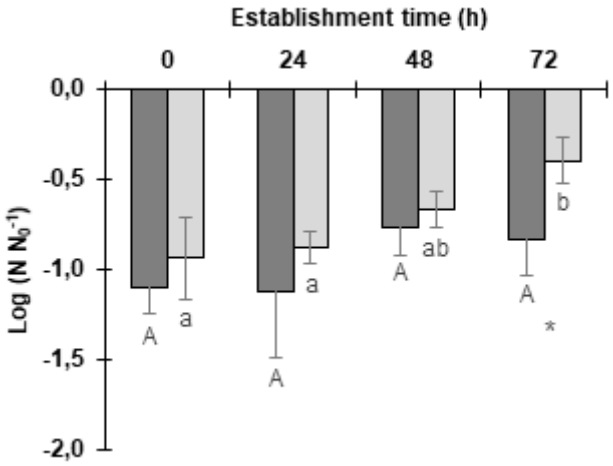

Figure 3. Adherence of *C. sake* CPA-1 fluidised-bed spray-dried formulations on berry surfaces after simulated rainfall at 150 mm h^{-1} and 120 mm of rain volume. *C. sake* was applied on grape clusters at $5 \cdot 10^7$ CFU ml^{-1} and then clusters were incubated for 0, 24, 48 and 72 h at 20 ºC and 85% RH, prior to exposure to simulated rainfall. Maltodextrin formulation (■) and potato starch formulation (■) are represented. Values are the mean of four replicates and vertical bars indicate standard deviation of the means. Columns with different letters indicate significant differences according Tukey's test ($P < 0.05$). Establishment times marked with an asterisk indicate significant differences between formulations.

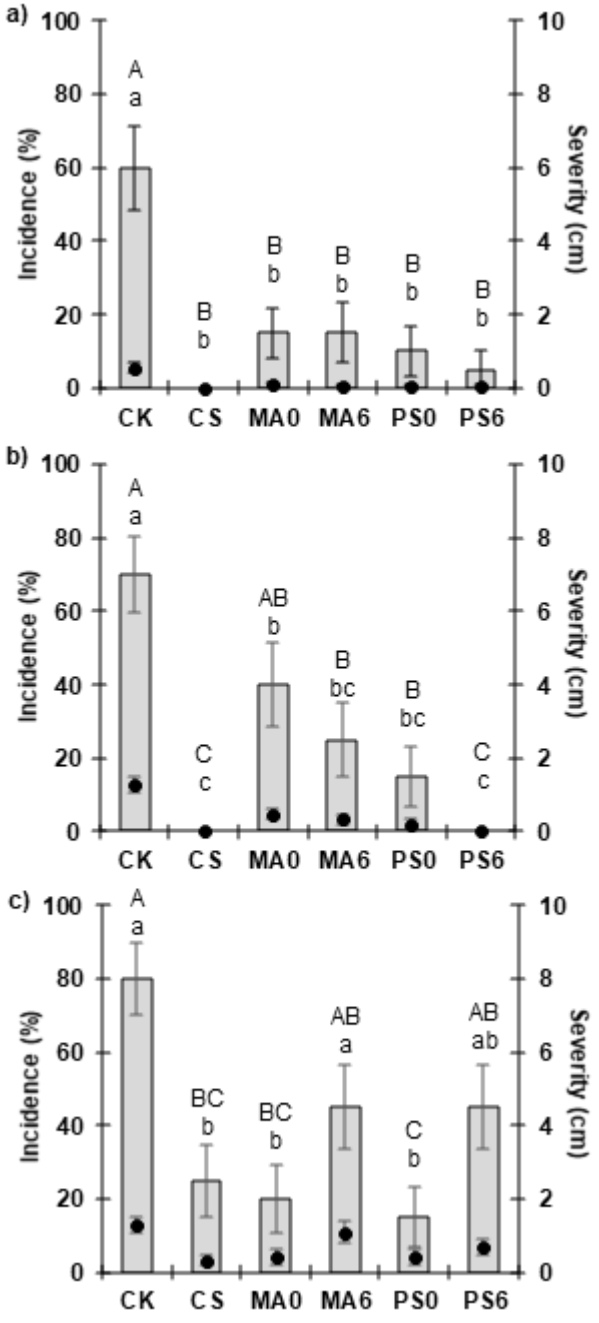

Figure 4. Efficacy of different formulations of *C. sake* CPA-1 against *B. cinerea* on (a) Conference pears; (b) Golden Delicious apples; and (c) Marglobe tomatoes. Incidence (columns) and severity (points) were evaluated after 3 days on pears and after 4 days on apples and tomatoes. *C. sake* formulations were: Candifruit (CS), maltodextrin formulation freshly made (MA0), maltodextrin formulation stored at 4 ºC for 6 months (MA6), potato starch formulation freshly made (PS0), and potato starch formulation stored at 4 ºC for 6 months (PS6). All the treatments were compared according to orthogonal contrasts analysis. Uppercase letters indicate significant differences ($P < 0.05$) in treatment incidence; different lowercase letters indicate significant differences ($P < 0.05$) in treatment severity. The values are means of 5 fruits × 4 replicates and error bars represent the standard errors of the means.