Novel film-forming formulations of the biocontrol agent *Candida sake* CPA-1: biocontrol efficacy and performance at field conditions in organic wine grapes

Running title: Use of *C. sake* film-forming formulations

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ps.5200

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Abstract

BACKGROUND: The biocontrol agent (BCA) *Candida sake* CPA-1 has previously reduced effectively *Botrytis* bunch rot (BBR) and it was also suggested as a promising strategy to control sour rot in grapes under field conditions. However, biocontrol efficacy of solid formulations of CPA-1 has never been tested in field trials. The present study aims to confirm the efficacy against BBR and sour rot in grapes under field conditions of two novel formulations recently developed by the addition of biodegradable coatings using a fluidised-bed spray-drying system.

RESULTS: Novel film-forming formulations of the BCA *C. sake* CPA-1 controlled *B. cinerea* as well as liquid formulation. Sour rot control resulted better in the second season and severity reductions were more satisfactory than incidence control. Visual and cryoSEM observations revealed that film-forming treatments were uniformly distributed on plant surface. CPA-1 coating could be observed on grapes at harvest time.

CONCLUSION: The results of this work suggest that solid formulations would be a competitive alternative to conventional fungicides because they were easy to package and transport, and cells viability could be maintained for a long period of time.

Keywords: *Botrytis cinerea*, sour rot, fluidised-bed spray-drying, grapes, coating, solid formulation

1 INTRODUCTION

One of the major fruit rot diseases on grapes is caused by the filamentous fungus *Botrytis cinerea* that is the responsible for *Botrytis* bunch rot (BBR) or grey mould, which causes heavy economic losses and reductions in wine quality worldwide.[1] Sour rot is also becoming especially frequent in regions with hot summer season conditions.[2] However, despite of the significant damage of BBR, its control is still constrained. Indeed many fungicides have failed controlling the necrotrophic pathogen *B. cinerea* because of its resistance to synthetic fungicides[3], mainly due to its genetic plasticity.[4] For sour rot, management options are scarce as this disease involves a complex of bacteria and yeasts[5]. Moreover, pre-harvest fungicide applications are ineffective against sour rot.[6] Furthermore, currently there is a bid drive for innovative research for sustainable pest management aimed to reduce the negative impact of synthetic pesticides.[7]

Such novel biocontrol strategies have been tested under field conditions against *B. cinerea* and sour rot with good results. Despite of the unfavourable factors such as temperature, relative humidity and UV radiation occurred during summer season in western Catalonia (Spain), Cañamás *et al.*[8] demonstrated the potential of *Candida sake* CPA-1 for biocontrol of BBR of grapevine with heat-adapted cells and especially with the addition of a fatty acid-based additive

called Fungicover® (FC) as an edible coating. Later, biological control efficacy of CPA-1 with FC was confirmed in an organic vineyard under both Mediterranean[9] and Atlantic climatic conditions.[10] Recently, the efficacy of *C. sake* CPA-1 plus FC has been demonstrated in commercial conditions and their compatibility with the phytosanitary products commonly used in viticulture has been studied.[11] Regarding sour rot, *C. sake* CPA-1 was described as a promising strategy to control this disease due to the severity reductions obtained in an organic vineyard.[2] However, although the exceptional results obtained with CPA-1, the role of FC was essential under field conditions because this commercial coating could protect *C. sake* from environmental stresses[8]. Moreover, FC alone also reduced BBR incidence and severity at harvest.[9]

Nevertheless, this fatty acid-based additive is too expensive to use as coating for commercial applications. Another burden is the requirement of blending with the BCA just before the application. Thus alternative formulations had to be developed to reduce the costs and to improve the handling of the products.

Carbó *et al.*[12] optimised two novel film-forming formulations for *C. sake* CPA-1 using fluidised-bed spray-drying system to improve the survival of the BCA under unfavourable environmental conditions. Therefore, to enhance their efficacy under field conditions without the addition of any commercial coating prior field application. Efficacy of these novel formulations was successfully tested against *B. cinerea* on grapes under laboratory conditions. Additionally, the impact of environmental conditions forecasted under climate change scenarios on the resilience of the solid film-forming formulations of CPA-1 was examined for projecting the efficacy and resilience of these BCA formulations under expected environmental conditions.[13] Because of the enormous challenge presented by real "on-field" application of biological control strategies, microbe-plant interaction, and the inherent variability of the field

environment[1], there is a need for more scientific data to further confirm the efficacy of these novel film-forming formulations.

Thus, the main objective of the present study was to test the efficacy of four *C. sake* CPA-1 treatments against *Botrytis* bunch rot and sour rot under field conditions during two growing seasons (2015 and 2016). Strategies tested to compare the effect of coatings or film-forming substances in the BCA efficacy were: (i) a liquid formulation of CPA-1; (ii) the liquid formulation blended with Foodcoat® (commercial coating); (iii) and two solid film-forming formulations recently developed. Moreover, a wide study of the formulations was done in 2015: (1) to evaluate CPA-1 populations under these field conditions; (2) to observe the microstructural and visual appearances of different formulations on vine leaves after the applications; and (iii) to visually check the appearance of the novel film-forming formulations on grape bunches at the end of the season.

2 MATERIALS AND METHODS

2.1 The biocontrol agent

All the experiments were carried out with the yeast strain CPA-1 of *C. sake* which was isolated from the surface of apples. This strain belongs to the Collection of Postharvest Pathology Group of IRTA (Lleida, Catalonia) and it was deposited in the Colección Española de Cultivos Tipo (CECT-10817) at the University of Valencia, Burjassot, Spain. CPA-1 stock cultures were stored at 4 ºC 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}). When required, yeast cells were sub-cultured on NYDA plates at 25 ºC for 48 h.

For biomass production, a starter inoculum was prepared by transferring sub-cultured cells to 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). Cells were produced in a liquid fermentation system with 5 L

working volume (BIOSTAT-A modular fermenter, Braun Biotech International, Germany) using an initial concentration of 10^6 CFU mL^{-1} and 40 h of fermentation as described by Abadias *et al.*[14]

2.2 Experimental field site (2015 and 2016)

In both growing seasons, the study was conducted in a commercial organic vineyard in Catalonia, in the North-East of Spain. The vineyard was located in the Designation of Origin Costers del Segre, subzone Vall del Riu Corb (Vallbona de les Monges, Lleida). The grape cultivar used was Macabeo (or Macabeu), a white variety for white wine production which is susceptible to *B. cinerea* contamination due to their large and compact clusters and thick-skinned berries.[15]

2.3 Experimental design and field treatments

A completely randomized block design was used to distribute the plots and four replicates per treatment were done. Each plot consisted of seven to ten vines, depending on their size; the first and last vines of each plot were considered as buffer vines. In 2015, the second and the third vines were used to monitor BCA population dynamics and the others were used to check the efficacy of the treatments against *B. cinerea* and sour rot. In 2016, only buffer vines were discarded to evaluate CPA-1 efficacy.

Four CPA-1 treatments were applied in the both growing seasons: (i) a liquid formulation with trehalose 5% (CS)[16]; (ii) the liquid formulation blended with 35 g L^{-1} of the commercial edible coating Foodcoat® (Domca S.L., Granada, Spain), which is a more concentrated formulation of Fungicover® (CS+FC); (iii) a solid formulation based on potato starch (PS); and (iv) a solid formulation based on maltodextrin (MAL). Both solid formulations were dried using fluidised-bed spray-drying system by adding biodegradable coatings to enhance the BCA survival under stress conditions as described[12]. Untreated vines were used as control.

All treatments were applied at 2.5×10^7 CFU mL^{-1} at two early-season key phenological stages (80 % flowering and pre-bunch closure);[9] additionally, a late-season application from veraison to commercial harvest was applied (see dates on Table 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.

2.4 Bunch rot assessment

Efficacy of the different CPA-1 formulations against BBR and sour rot were evaluated at the end of the season. In 2015, the evaluation was at commercial harvest time, whereas in 2016 grape bunches were over-ripe due to the low infection of BBR at commercial harvest time. Both BBR and sour rot were visually assessed on 50 bunches per replicate plot, 25 from each side of the row. Incidence was determined as the percentage of infected bunches and severity was measured as the percentage of rotted berries per bunch.

2.5 Population dynamics of *C. sake* CPA-1

Population dynamics of the different formulations were evaluated after each CPA-1 application (details in Table 1) during the first season (2015). Approximately, 8 bunches from each replicate were randomly sampled at flowering stage, whereas 20 bunches from each replicate were sampled at pre-bunch closure and veraison stages (details in Table 1).

C. sake populations were determined as described Cañamás et al. (2011); briefly, samples were weighted, transferred to Erlenmeyer flasks containing different volumes of phosphate buffer (details in Table 1), shaken in a rotary shaker at 150 rpm for 20 min and then sonicated for 10 min in an ultrasound bath (Selecta, Barcelona, Spain). Number of CFU ml^{-1} of the washings was determined by plating 100 µl of serial dilutions onto NYDA plates and incubating at 25 ºC for 48 h in the dark. Then, CPA-1 colonies were visually recognised based on their morphological characteristics. Data were expressed as CFU g^{-1}.

2.6 Meteorological data

Temperature, relative humidity and rainfall were logged at hourly intervals in both seasons using a weather station (Decagon Services Inc., Pullman, WA, USA) placed in the experimental vineyard. Mean, maximum and minimum daily values of temperature, relative humidity and accumulated rainfall were calculated later.

2.7 Microstructural analysis of *C. sake* CPA-1 treatments on vine leaves surface

Vine leaves samples were taken 24 h, 72 h and 7 days after the first application of the formulations to assess the distribution of the different component formulations on the plan tissue and the possible changes throughout time. The microstructural analysis of vine leaves surfaces was carried out by cryoSEM using a Scanning Electron Microscope (JEOL JSM-5410, Japan). Samples were cut into smalls pieces (approximately 0.5x0.5 cm), cryofixed in slush nitrogen and observed, after gold coating, using an accelerating voltage of 10 kV. Images of the vine leaves surface were obtained for each applied coating formulation and time.

2.8 Visual appearance of *C. sake* CPA-1 treatments on plant surface

Immediately after the CPA-1 applications, treatments were visually evaluated on the leaves to observe the distribution differences between film-forming and non-film-forming formulations. At harvest, the appearance of the novel film-forming formulations on grapes was also visually evaluated. In both cases, photographs were taken to check the differences.

2.9 Statistical analysis

For all the experiments, analysis of variance was performed using JMP8 software (SAS Institute Inc., Cary, NC, U.S.A). An arcsine-square root transformation was applied to severity and incidence values; also population dynamics counts (CFU g^{-1}) were log-transformed prior to

analysis of variance. Tukey's HSD test was used to separate means when significant effects were obtained after the analysis of variance ($P < 0.05$).

3 RESULTS

3.1 Efficacy of *C. sake* treatments against *Botrytis* bunch rot (2015 and 2016)

All *C. sake* treatments significantly ($P < 0.05$) reduced BBR incidence and severity during the two tested seasons and no differences were observed among the treatments (Fig. 1). BBR incidence in the untreated control was higher in 2015 than in 2016; specifically, in 2015 the incidence of BBR was 62% (Fig. 1a) whereas in 2016 it was lower than 20% (Fig. 1b). Moreover, in 2015 BBR incidence reductions ranged from 44% (PS) to 65% (CS+FC) and in 2016 the incidence was reduced from 77% (CS) to 100% (PS) compared to the untreated control. BBR severity in the untreated control was also higher in 2015 (6%) than in 2016 (1%) but severity reductions were very high in both seasons (from 68% to 100%).

3.2 Efficacy of *C. sake* treatments against sour rot (2015 and 2016)

Candida sake efficacy against sour rot (Fig. 2) was lower than against BBR and was season dependent. In 2015, despite of all treatments reduced the disease severity compared to the untreated control, only *C. sake* CPA-1 without biodegradable coatings (CS) reduced significantly sour rot incidence and severity (Fig. 2a). In 2016, all CPA-1 treatments reduced the incidence from 24% (CS) to 35% (PS) but only potato starch formulation (PS) achieved a significant reduction ($P < 0.05$) (Fig. 2b). Also, in 2016, the severity was significantly ($P < 0.05$) reduced for all the treatments by 56% (CS) to 84% (CS+FC) compared to the untreated control but no differences were observed among CPA-1 treatments.

3.3 Meteorological data

Temperature and relative humidity (RH) patterns were similar during the two seasons but with minor differences (Fig. 3). In 2016, the temperatures were slightly lower from the start of the assay until the beginning of September, whereas the 2015 season was cooler during the first half of September. Moreover, the 2015 season was characterized by weekly rainfall events before veraison, which increased occasionally the daily RH average. Indeed, accumulated rainfall in 2015 was raised to 56 mm, whereas in 2016 was 34 mm and it principally occurred at the end of the season. The major differences were observed between minimum RH values, mean of which was 44% in the first season and 39% in the second one. Nevertheless, pre-bunch closure period in 2015 was very dry, mainly due to the dearth of rain.

3.4 Population dynamics of *C. sake* CPA-1 on grapevine tissues exposed to field conditions

Population dynamics after each CPA-1 treatment decreased sharply after the applications at flowering and pre-bunch closure. However, there was a progressive decline of CPA-1 populations after the additional treatment, when berries were totally developed (Fig. 4).

The populations recovered ranged from 5.54 (CS+FC) to 5.13 (PS) Log CFU g^{-1} after flowering application; from 4.65 (PS) to 2.92 (CS) Log CFU g^{-1} when *C. sake* was sprayed at pre-bunch closure; and from 5.38 (CS) to 4.92 (CS+FC) Log CFU g^{-1} after the additional treatment.

At flowering, no significant differences among treatments were observed with the exception of the PS formulation, where CPA-1 populations were significantly higher ($P<0.05$) than with the liquid formulation without coatings (Table 2). At the other phenological stages, significant differences were observed among the treatments immediately after the applications. Specifically, during the two days after application (30/06/2015) at pre-bunch closure, the populations of the liquid formulation without coatings were significantly lower than the others,

whereas three days after the application (02/07/2015), CPA-1 formulations with film-forming compounds achieved higher populations than the liquid formulation without the commercial coating, but no significant differences were observed between CS and MAL.

The additional treatment was applied later in season, after a rainfall event on August 19th, and significant differences were observed among CPA-1 treatments during the first week after the applications. In general, along this phenological stage, *C. sake* populations recovered from formulations with biodegradable coatings were significantly higher than the recovered from the liquid formulation without any adjuvant. Moreover, during this period, solid formulations survived equal or greater compared with the liquid formulation plus Foodcoat®.

The high populations obtained two weeks after the last application were probably caused by minor rainfall events (4 mm of rain in 4 days) at the beginning of September (01/09/2015). Before harvest (10/09/2015), a noteworthy rainfall event (7 mm of rain in 2 hours) reduced the number of viable *C. sake* cells recovered from berries. After that, the populations ranged from 3.92 (CS+FC) to 2.41 (PS) Log CFU g^{-1}, but only the potato starch formulation was significantly different from the others.

3.5 Microstructural analysis of *C. sake* CPA-1 treatments on vine leaves surface after field applications

Representative cryoSEM images of the vine leaves surfaces after the application of the different treatments are shown on Fig. 5, where CS cells can be observed on the plant tissue. Likewise, the coating effect of the solids present in the film-forming formulations can be appreciated through the smoothing effect on the aspect of the plant cellular arrangement. Twenty four hours after the application, few differences were observed between CS and CS+FC treatments in terms of the cell presence, which appeared as cell aggregates, partially embedded in the coating in the case of CS+FC treatment. In the case of the maltodextrin formulation (MAL), *C. sake* cells

appeared much more dispersed and isolated on the leaves surface. This was also observed for the potato starch formulation (PS), although in this case the footprint of some cells can be appreciated on the leaves' surface, suggesting that the cells could become detached from the plant surface. Lower number of cells was observed throughout time, except for the maltodextrin formulation. This showed a visual increase of cell aggregates 72 h after the application and maintained a higher number of cells than in the other cases 7 days after the application. The cell aggregates observed 72 h after the application, in contrast with the individualized initial cells after 24 h, suggest the effective cell growth when the maltodextrin formulation was applied. This effect was not appreciated for the other formulations, where the number on cells on the plant surface decreased after 24 h.

3.6 Visual appearance of *C. sake* CPA-1 treatments

3.6.1 Appearance of treatments on vine leaves after field applications

The appearance of the CPA-1 treatments on leaves immediately after the applications was different depending on the formulation (Fig. 6). The main difference among the formulations was observed on the CS treatment, this liquid formulation without the commercial coating Foodcoat® was distributed on droplets on the leaves and the treatment did not wet the entire plant surface. In contrast, the same liquid formulation plus Foodcoat® and the solid formulations that included coating compounds were uniformly distributed on the leaves. Nevertheless, the distribution of the potato starch solid formulation was a combination of both situations, because despite of it was possible to see some droplets on the leaves, the treatment also wet the leaves completely.

3.6.2 Appearance of film-forming formulations on grapes at harvest

Both tested film-forming formulations included coating substances on their composition that were visible on grapes at harvest stage (Fig. 7), three weeks after the last application.

Conversely, the liquid formulation was not perceptible at harvest, neither with the commercial coating nor without it. In fact, the liquid formulation showed the same appearance than untreated grapes.

Visual differences were perceived when comparing the maltodextrin formulation and the potato starch formulation. Specifically, the grapes surface treated with maltodextrin formulation appeared as a uniform and homogeneous layer coating (Fig. 7A, 7B and 7C). In contrast, the grapes surface coated with potato starch formulation exhibited a partially broken film after the fruit grew (Fig. 7D, 7E and 7F) thus making more evident the presence of the film over the grapes. Nevertheless, the coating was observed in both novel film-forming formulations verifying their coating capacity under field applications.

4 DISCUSSION

Biocontrol agents have been mainly applied at postharvest conditions due to their narrow range of activity; however, the BCAs application at preharvest conditions could improve their commercial success.[17] In this way, some coating additives as Fungicover® were required to improve *C. sake* CPA-1 efficacy under environmental stress conditions.[8–11] Fortunately, a high compatibility with phytosanitary products commonly used in viticulture has been also confirmed for *C. sake* CPA-1.[11]

Nevertheless, the commercialization of a competitive solid formulation easy to manage and transport would be an important way to promote the use of CPA-1. Unfortunately, most of the previously optimised solid formulations of CPA-1 resulted in a lack of efficacy or low cell viability after the dehydration process.[18–20] Fortunately, a fluidised-bed dried CPA-1 formulation[21] and two fluidised-bed spray-dried formulations with biodegradable coatings in their composition[12] achieved good survival (shelf life) and efficacy results under controlled conditions. Therefore, to the best of our knowledge, the present work represents the first

published study that attempt to test the efficacy of solid formulations of *C. sake* CPA-1 under field conditions. Notwithstanding, the efficacy of some dried BCAs has been demonstrated under controlled conditions; for example, fluidised-bed spray-dried *Bacillus amyloliquefaciens* was effective against *Monilinia spp.* On stone fruits,[22] spray-dried *Bacillus megaterium* reduced rice sheath blight disease in the laboratory and greenhouse[23] or freeze-dried *Pseudomonas* spp. was as effective as fresh cells in two different plant-pathogen systems.[24]

The efficacy of CPA-1 solid formulations against BBR in small-scale field trials has been demonstrated. Both tested solid formulations significantly reduced the disease at least as well as the liquid formulation of the BCA compared with the control. Despite of no significant differences were observed among treatments, BBR reductions in 2016 were higher when formulations that included any kind of coating were applied, achieving the best reductions with the solid formulations. Probably, this distinction was possible due to the low incidence of BBR in the control treatment in 2016 (<20%), which allowed to obtain higher reductions than in 2015, when incidence of BBR of unsprayed treatment was 62% and the disease was more difficult to control. Though, other dried microbial BCAs, as *Trichoderma atroviride*, *Aureobasidium pullulans*, and *Bacillus subtilis*, commercialised as biofungicides also reduced BBR satisfactorily with relatively low-medium level of disease.[25]

Incidence differences between the two seasons were possibly caused by climatic conditions, mainly due to accumulated rainfall, which was really scarce in 2016 and principally occurred at the end of the season. Actually, *B. cinerea* incidence is favoured by high humidity and long wetness duration.[26] Additionally, in the present study climatic conditions could be considered more favourable for *C. sake* CPA-1 survival and efficacy than in other growing seasons (2009 and 2010) in the same experimental field when BCA efficacy was significantly higher with coating additives.[9,27] Despite of mean averages of temperature and RH were very similar, during

2009 and 2010, accumulated rainfall was 28.0 mm and 30.2 mm, respectively, whereas during 2015 and 2016, accumulated rainfall raised to 56 mm and 34 mm, respectively. Therefore, the major amount of rainfall in the present study could benefit the survival and efficacy of the BCA and for this reason, no significant differences were observed in the efficacy of different CPA-1 treatments. Notwithstanding, during the 2016 growing season, when accumulated rainfall was lower, CPA-1 treatments with biodegradable coatings achieved high reductions of BBR incidence. This suggests that biodegradable coatings may confer the BCA a competitive advantage when environmental conditions are less favourable for the BCA because coating compounds could protect the cells under water stress conditions.

In the present study, with only three applications per season at 2.5×10^7 CFU ml^{-1}, the overall BBR reductions were in the ranges of 44% to 100% on incidence, and 68% to 100% on severity, compared to the control. Previous studies tested four and five applications of *C. sake* during the season at 5×10^7 CFU ml^{-1}, achieving the best results when CPA-1 was applied together with the commercial coating Fungicover®.[8,9] In order to reduce economic costs, *C. sake* dose had been reduced to 1×10^7 CFU ml^{-1},[9] but an increase to 2.5×10^7 CFU ml^{-1} was required to enhance the efficacy of the BCA [11]. Moreover, the number of applications had been also optimised and strategically planned based on the two early season applications that resulted effective.[9,10]

Sour rot control of CPA-1 solid formulations was not as satisfactory as BBR control. However, these results can be considered very interesting regarding that, currently, fungicides are not providing reliable solutions. In fact, sour rot management possibilities are scarce due to the epidemiology and ethology of this disease remain unknown. Nonetheless, in 2015 all treatments reduced the severity of the disease and the liquid formulation of *C. sake* significantly reduced sour rot incidence and severity compared to the control. In 2016, severity was significantly

reduced for all the treatments compared to the control, and despite of all the treatments also reduced the sour rot incidence, only the reduction achieved by potato starch formulation was significant. In a previous experiment, Calvo-Garrido *et al.*[2] also reduced significantly the severity of sour rot with *C. sake* CPA-1 treatments applied in grapevines but incidence was not significantly reduced by any treatment.

Populations of *Candida sake* decreased rapidly after flowering and pre-bunch closure, probably due to the loss of the flowers and the growth of the grapes, respectively. Other *C. sake* CPA-1 population studies also reported sharply declines of counted yeast cells after early season applications.[8,9] In general, population dynamics of the four tested CPA-1 formulations were not significantly different. However, punctual differences usually appeared among treatments immediately after the applications, and in formulations that included coatings CPA-1 commonly survived better than the liquid formulation alone. These population dynamics results showing higher survivals when film-forming formulations confirmed previous tests developed under controlled conditions.[12] Furthermore, these results are consistent with other publications that suggested a protective effect for other BCA additives such as protection from solar radiation[28] or against temperature and relative humidity fluctuations.[29]

CryoSEM observations revealed that CPA-1 cells were better distributed on leaves tissues when coating formulations were used. Actually, visual appearance of treatments showed that when the liquid formulation of CPA-1 was applied without coating, the product was distributed in droplets and most of the surface was not covered. Additionally, film over grapes was visually appreciated before harvest when grapevines were treated with both novel solid formulations, both including biodegradable coating in their composition. Previously, the survival and efficacy of *C. sake* against *B. cinerea* have been improved by adding coating-forming solids to fresh cells under controlled conditions.[30]

5 CONCLUSION

In conclusion, the present study is the first to demonstrate the efficacy of solid formulations of *C. sake* CPA-1 under field conditions. The fluidised-bed spray-dried BCA formulation of CPA-1 controlled *B. cinerea* on grapes as the liquid formulation. Control was achieved without any detrimental effects on biocontrol efficacy despite of the stress applied to the cells during the dehydration process. Therefore, viable cells maintained their biocontrol activity because this drying process allows drying the BCA without high-heat damage. The addition of biodegradable coatings during the drying process favoured the distribution and enhanced the efficacy of the product on vegetable surfaces. The film-forming ability of these novel formulations was demonstrated at harvest since a visible film was formed. The findings of this work highlight the potential use of these two improved formulations of *C. sake* CPA-1 for larger scale field applications.[12]

6 ACKNOWLEDGEMENTS

The authors would like to thank Cristina Solsona, Celia Sánchez, Andrea Berge and Dani Lastrada for their technical#

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8 TABLES

Table 1 Phenological stage dates and details of population sampling for both growing seasons (2015 and 2016)

2015			2016
Phenological stage and treatment date	**Population sampling dates**	**Sample unit**	**Phenological stage and treatment date**
Flowering 08 June	08, 09, 11 June 15, 22 June 29 June	2 g of flower organs 20 ml^{-1} phosphate buffer 40 berries 50 ml^{-1} phosphate buffer 20 berries 50 ml^{-1} phosphate buffer	Flowering 20 June
Pre-bunch closure 30 June	30 June 01, 02, 03, 07 July 17 August	20 berries 50 ml^{-1} phosphate buffer	Pre-bunch closure 05 July
Additional treatment 25 August	25, 26, 27, 28 August 01, 08, 16 September	20 berries 50 ml^{-1} phosphate buffer	Additional treatment 02 August

Table 2 Means separation of significantly different *C. sake* treatments observed during the population dynamics assay. Results are expressed as Log CFU g^{-1}. Means were separated according to Tukey's test ($P < 0.05$). The treatments were: CS (liquid formulation); CS+FC (liquid formulation plus 35 g L^{-1} of Foodcoat®); MAL (maltodextrin solid formulation); and PS (potato starch solid formulation).

Date	CS	CS + FC	MAL	PS
11/06/2015	4.41 B	4.89 AB	4.79 AB	4.99 A
30/06/2015	2.92 C	4.34 AB	4.21 B	4.65 A
01/07/2015	2.99 B	4.15 A	4.02 A	3.80 A
02/07/2015	2.50 B	3.57 A	2.96 B	3.53 A
25/08/2015	5.38 A	4.92 B	5.23 AB	5.26 AB
26/08/2015	4.17 C	4.56 B	4.90 A	4.98 A
27/08/2015	3.96 C	4.25 C	5.02 A	4.60 B
28/08/2015	3.82 C	4.23 BC	4.73 A	4.29 B
01/09/2015	3.47 B	4.03 AB	4.67 A	4.02 AB
16/09/2015	3.61 A	3.92 A	3.54 A	2.41 B

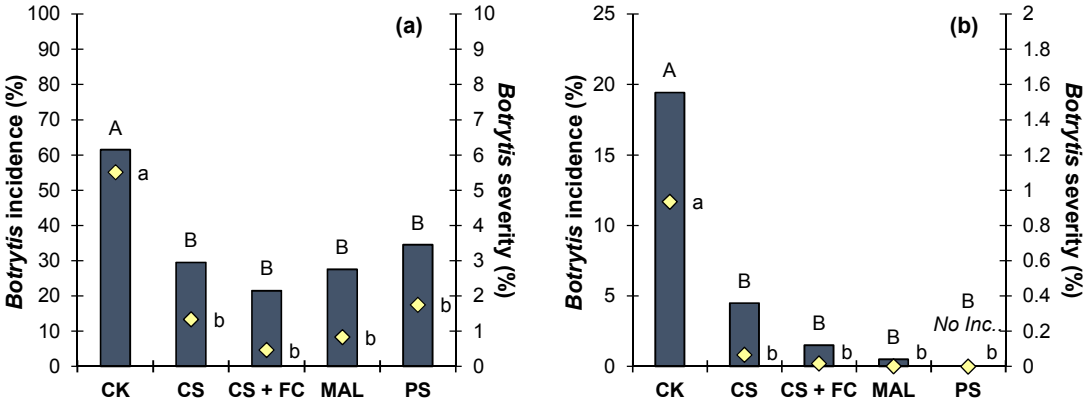

Fig. 1 Efficacy of *C. sake* CPA-1 treatments against *Botrytis* bunch rot at harvest during the 2015 (a) and the 2016 (b) growing seasons. Incidence (■) and severity (◇) represented in bars and diamonds, respectively, were evaluated on 50 bunches per replicate and four replicates per treatment. The treatments were: CS (liquid formulation); CS+FC (liquid formulation plus 35 g L^{-1} of Foodcoat®); MAL (maltodextrin solid formulation); and PS (potato starch solid formulation). Untreated vineyards were used as control treatment (CK). Two *C. sake* applications were conducted to Macabeu vines at early-season key phenological stages and an additionally application was conducted at late-season. *No inc.* indicates that no incidence was observed in that treatment. Mean values of incidence or severity linked by the same letter (upper or lower case, respectively) are not significantly different ($P < 0.05$) according to Tukey's test.

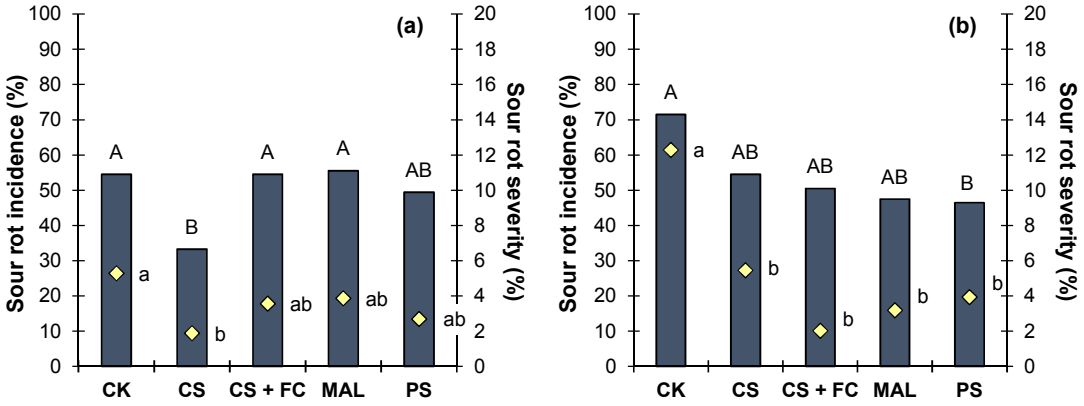

Fig. 2 Efficacy of *C. sake* CPA-1 treatments against sour rot at harvest during the 2015 (a) and the 2016 (b) growing seasons. Incidence (■) and severity (◇) represented in bars and diamonds, respectively, were evaluated on 50 bunches per replicate and four replicates per treatment. The treatments were: CS (liquid formulation); CS+FC (liquid formulation plus 35 g L^{-1} of Foodcoat®); MAL (maltodextrin solid formulation); and PS (potato starch solid formulation). Untreated vineyards were used as control treatment (CK). Two *C. sake* applications were conducted to Macabeu vines at early-season key phenological stages and an additionally application was conducted at late-season. Mean values of incidence or severity linked by the same letter (upper or lower case, respectively) are not significantly different ($P < 0.05$) according to Tukey's test.

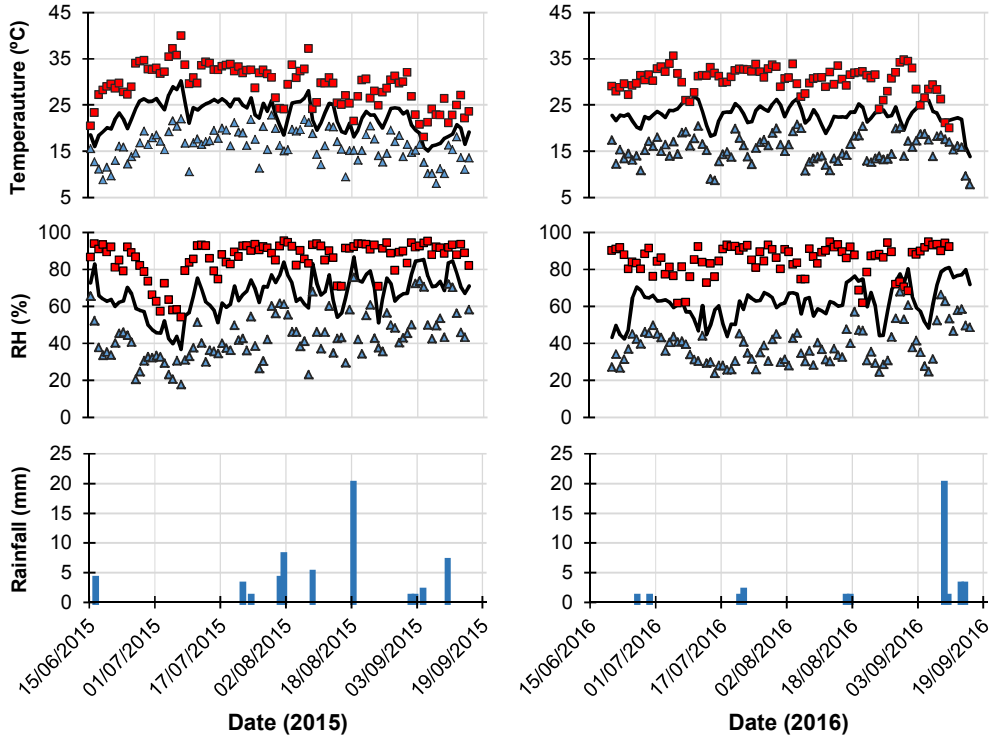

Fig. 3 Meteorological data obtained during the growing seasons in 2015 and 2016 at the experimental vineyard. Values of daily maximum (■) and minimum (▲) temperatures or relative humidity and rainfall volumes (bars) are represented. Lines (—) show the daily average temperatures and relative humidity.

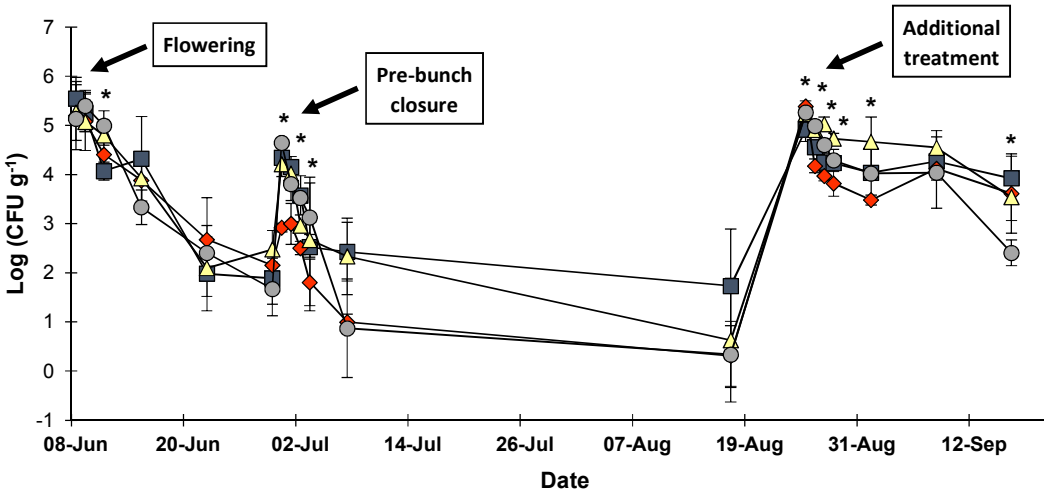

Fig. 4 Population dynamics of *C. sake* CPA-1 applied to Macabeu wine grapes in a commercial organic vineyard in 2015. Four treatments were applied at flowering, pre-bunch closure and in additional treatment after rainfall event: a liquid formulation (CS) (◆); the liquid formulation plus 35 g L^{-1} of Foodcoat® (CS+FC) (■); maltodextrin solid formulation (MAL) (△); and potato starch solid formulation (PS) (●). All BCA treatments were applied at 2.5×10^7 CFU ml^{-1}. Values represent the means of four replicates, and vertical bars represent standard errors. Treatment dates are also indicated on the top of the figure. When bars are not visible, they are smaller than symbol size. Asterisks indicate significant differences among treatments according to Tukey's test ($P < 0.05$).

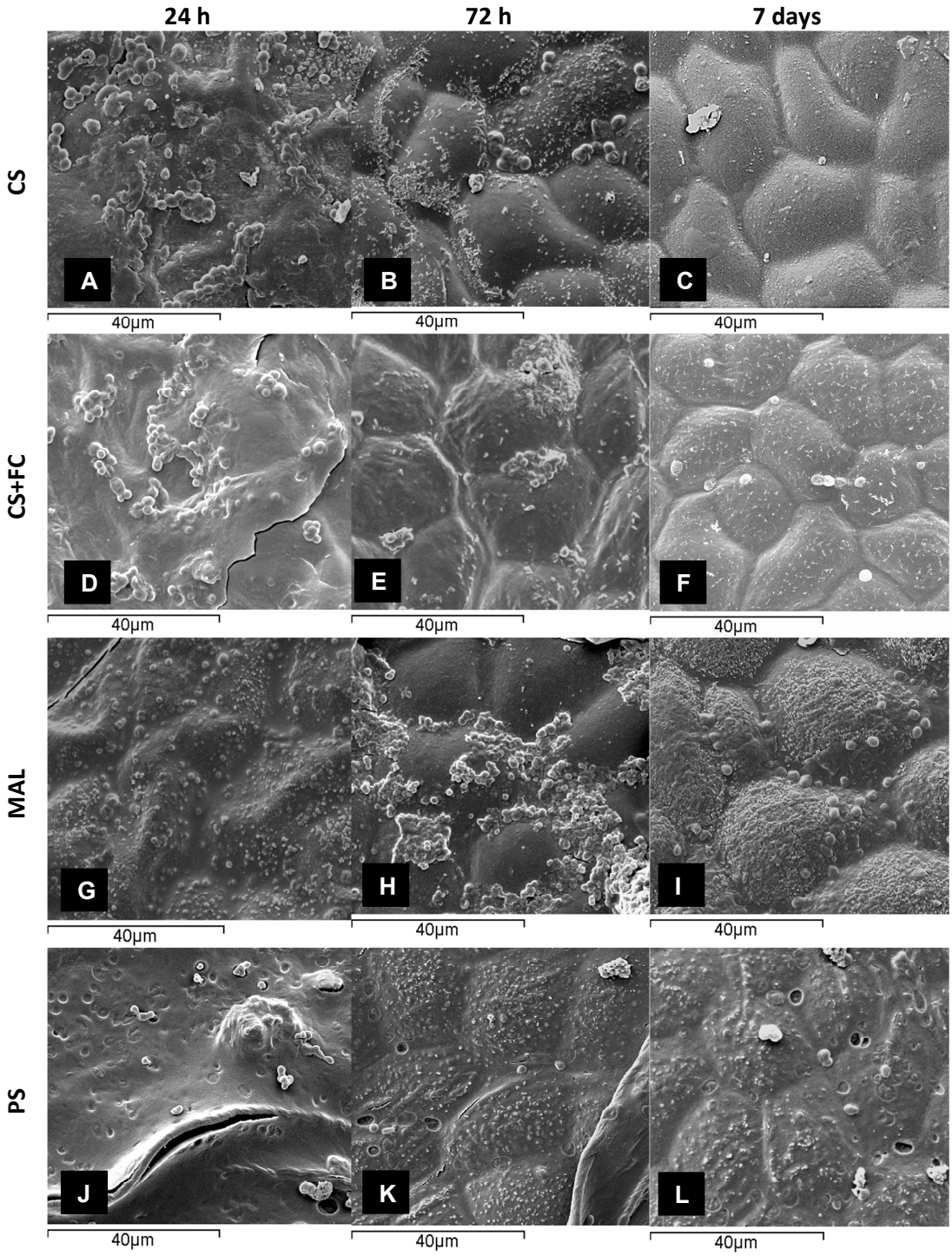

Fig 5 Representative cryoSEM images of vine leaves surface 24 h, 72 h and 7 days after the application for the different formulations: the liquid formulation (CS) (A, B and C); the liquid formulation plus 35 g L^{-1} of Foodcoat® (CS+FC) (D, E and F); maltodextrin solid formulation (MAL) (G, H and I); and potato starch solid formulation (PS) (J, K and L).

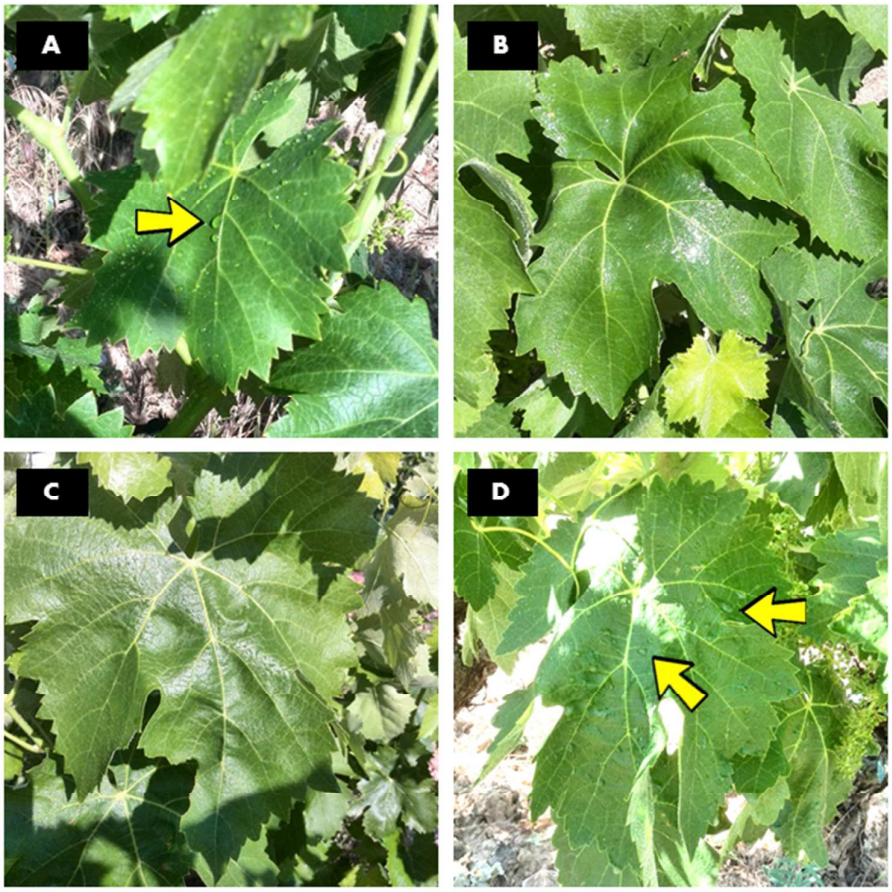

Fig. 6 Representative images of the appearance of *C. sake* CPA-1 treatments on vine leaves after their application at experimental field. Photographs of each treatment are represented: (A) the liquid formulation (CS); (B) the liquid formulation plus 35 g L^{-1} of Foodcoat® (CS+FC); (C) maltodextrin solid formulation (MAL); and (D) potato starch solid formulation (PS). Arrows indicate some of treatments drops after the application.

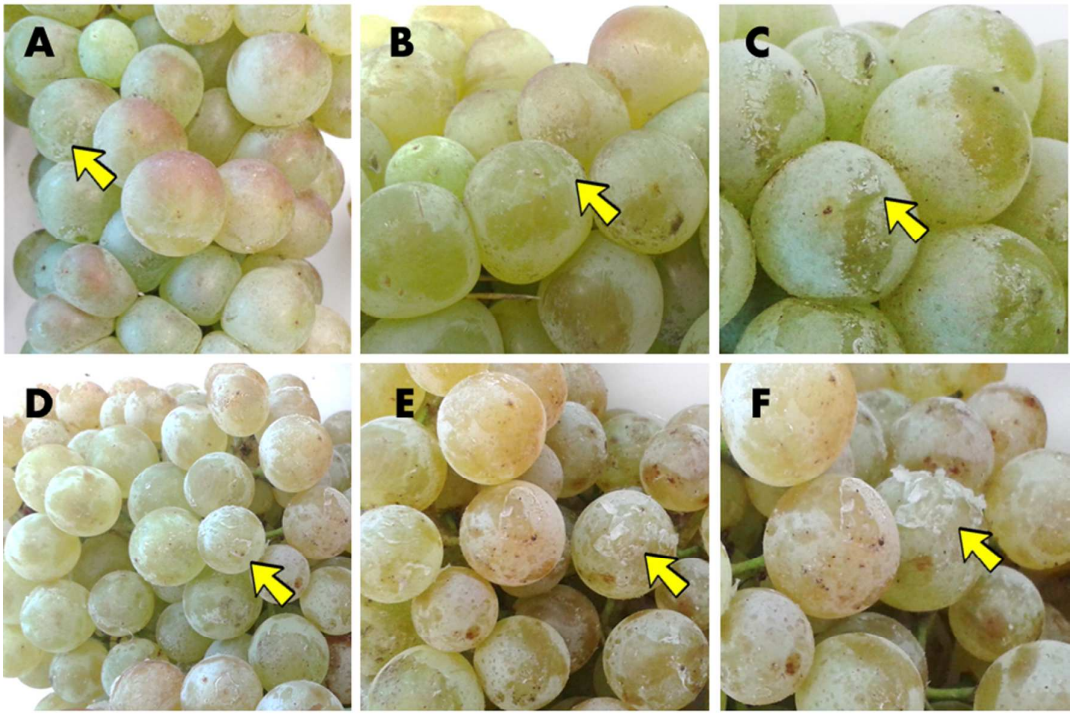

Fig. 7 Representative images of the appearance of solid film-forming formulations of *C. sake* CPA-1 on grapes at harvest in an experimental vineyard. Different perspectives of each formulation are represented: (A, B, C) maltodextrin formulation (MAL); and (D, E, F) potato starch formulation (PS). Harvest stage took place three weeks after the last applications. Arrows indicate film formed over the grapes.