Enhanced shelf-life of the formulated biocontrol agent *Bacillus amyloliquefaciens* CPA-8 combining diverse packaging strategies and storage conditions

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Abstract

Two effective biocontrol products (named as BA3 and BA4) based on *Bacillus amyloliquefaciens* CPA-8 have been reported as a potential alternative to chemical applications against brown rot caused by *Monilinia* spp. on stone fruit. To have practical use, this study aimed to describe the best packaging strategies (bags or flasks, atmosphere, and temperature of storage) to not only guarantee efficacy but also stability and ease of application of the products to be handled through the normal channels of distribution and storage. In terms of the viability neither the BA3 nor the BA4 product has been compromised after twelve months of storage. However, storage at 4 ºC affected the stability and visual aspect of both CPA-8 formulations, mainly associated not only to the increase of RH but also $a_{w.}$ Moreover, it should be pointed out that flasks did not conserve refrigerated BA3 samples in a suitable way, since RH and a_w increased noticeably making their visual properties unsightly after 10 months of cold storage. At that time, the BA4 products were better preserved at 4 ºC when packaged in flasks.

Finally, this study also demonstrated that the most suitable packaging conditions for long-term storability (stored at 22 ºC) did not show any negative effect in the biocontrol efficacy of CPA-8 in nectarines artificially infected with *M. fructicola* and provide suitable product delivery and field application. In conclusion, these results contribute to the final stage of development of these two CPA-8 products, practically ready for registration, thus contributing to the environmental-friendly management of postharvest diseases in stone fruit.

Keywords: postharvest; antagonist; formulation, storage, *Monilinia* spp; peach;

1. Introduction

Biological control appeared in the recent decades as an effective alternative to chemical treatments to control postharvest decay in fruit since the occurrence of population of pathogens resistant to fungicides and public concerns on human safety and environmental protection have resulted in attempts to develop more environmental-friendly strategies (Usall et al., 2016). However, while an abundance of effective beneficial microorganisms has been widely studied to control postharvest diseases, few microorganism-based products are already available in the market (Droby et al., 2016; Glare et al., 2012). To have practical use, microbial agents should be formulated in such a way as to guarantee not only efficacy but also stability and ease of application of the product (Droby et al., 2016; Rhodes, 1993; Teixidó et al., 2011). It means that the product, apart from being well dispersed in water and easily sprayed with the standard agricultural machinery, should be handled through the normal channels of distribution and storage.

The biocontrol agent (BCA) *Bacillus amyloliquefaciens* CPA-8, formerly known as *Bacillus subtilis* (Gotor-Vila et al., 2016) has been reported as an effective antagonist against brown rot caused by *Monilinia* spp. (Casals et al., 2012; Gotor-Vila et al., 2017a; Yánez-Mendizábal et al., 2011), the wound-invading fungus that causes economically important loses of stone fruit worldwide (Mari et al., 2016; Usall et al., 2015). Recently, two shelf-stable and efficacious CPA-8-formulated products have been developed in a powder state by fluid-bed spray-drying (Gotor-Vila et al., 2017d). While both products contained the same proportion of protecting agents, they differ in the polysaccharide used as carrier material during fluidification: maltodextrin (product called 'BA3') or potato starch (product called 'BA4'). These products have been successfully tested in a wide range of stone fruit under laboratory conditions (Gotor-Vila et al., 2017d) and after preharvest application in a peach orchard (Gotor-Vila et al., 2017a). Therefore and in order to have commercial value, once CPA-8 is formulated, it must be maintained in a suitable state. This may involve careful selection of the packaging conditions

(different materials, atmosphere conditions, and storage temperatures) to control gas exchange, prevent the loss or gain of moisture and avoid contamination of the product (Costa et al., 2002; Torres et al., 2014). Product shelf-life extension is a widespread goal both, for the increasing demand of readily available products and for enhancing its economic and environmental sustainability through the entire supply chain (Alamprese et al., 2017). A longer shelf-life reduces microbial losses as well as economic and environmental impacts of the distribution logistic. This objective can be achieved by acting at different levels: production, formulation, packaging, and eventually storage during distribution and sale (Nicoli, 2012).

Depending on the needs of the product to be preserved, there are several levels of barrier packaging. Usually they are classified into low and high barriers. High barriers have a low oxygen transmittal rate, low moisture vapor transmission rate, and a high tensile strength, or puncture resistance. When high barrier packaging is paired with an oxygen absorber or desiccant, products enjoy even greater shelf-life and stability. Therefore, low barrier bags seem to be the least effective method of packaging compared to high barrier bags, flasks or vials, so formulated cells could be partially rehydrated and could begin respiration and other degradation processes (Costa et al., 2002).

An essential parameter in the study of dried microorganisms is water activity (a_w) rather than moisture content (relative humidity, RH) (Fasoyiro et al., 2016; Teshler et al., 2007). Though they are much correlated, a_w indicates how much free water is available to microorganisms opposed to water that is strongly bound to formulation components. Moreover, the atmosphere conditions inside the package (different combinations of oxygen, carbon dioxide and nitrogen) as well as the temperature of storage also play an important role in the shelf-life of the product (Alamprese et al., 2017).

Continuing with the development of the BCA CPA-8, this work aimed to study the potential use of different types of packaging to prolong bacterium viability and preservation. In order to do this, we studied the following: (i) the effect of different materials (bags or flasks), atmosphere conditions, and storage temperatures on the viability and stability of fluid-bed spray-dried CPA-8 formulations, (ii) the dispersion in water of the CPA-8 packaged formulations and (iii) the efficacy of CPA-8 after packaging and long-term storage against *Monilinia fructicola* in nectarines.

2. Materials and methods

2.1. Production and formulation of the CPA-8-based products

B. amyloliquefaciens CPA-8 was isolated from a nectarine surface and belongs to the collection of Postharvest Pathology Group of IRTA (Lleida, Catalonia, Spain). Bacteria cultured overnight at 30 ºC on nutrient yeast dextrose agar (NYDA: 8 g L^{-1} nutrient broth, 5 g L^{-1} yeast extract, 10 g L^{-1} dextrose and 20 g L^{-1} agar) and suspended in potassium phosphate buffer (PB, 70 mL KH_2PO_4 0.2 M; 30 ml K_2HPO_4 0.2 mol L^{-1} and 300 mL deionized water v/v/v pH 6.5) were used to inoculate 2 L (BioFlo/CelliGen 115, Eppendorf, New Brunswick, Canada) laboratory-scale bioreactors containing growth medium based on soy flour protein PROSTAR 510A (Brenntag Química, S.A.U., Barcelona, Catalonia, Spain) at 20 g L^{-1} according to the protocol optimised by Gotor-Vila et al. (2017d). CPA-8 cells were harvested by centrifugation at 9820 *g* for 12 min at 10 ºC in an Avanti J-20 XP centrifuge (Beckman Coulter, CA, USA) and resuspended at 10^{10} CFU mL^{-1} in the same CPA-8 supernatant medium to include the antifungal lipopeptides synthesised by the bacterium (Yánez-Mendizábal et al., 2012b).

Two CPA-8 formulated products were obtained by using a fluid-bed spray-dryer (HüttlinGmbH, Bosch Packaging Technology Company, Schopfheim, Germany) as previously described by Gotor-Vila et al. (2017b; 2017d). Briefly, CPA-8 cells were mixed with the protective substances

20 % sucrose plus 10 % skimmed milk and with 3.5 g of pregelatinised potato starch as binder and then fluid-bed spray-dried with 300 g of powdered carrier material previously loaded into the drying camera. Two different carriers were used: maltodextrin to obtain the CPA-8-formulated product called 'BA3', and potato starch for the CPA-8-formulated product called 'BA4'.

2.2. Packaging conditions

To assess the most suitable packages to maintain the viability of the CPA-8 formulated products, four different packaging materials were tested: two film-recovered plastic flasks and two different bags, one aluminium-based bag and other one transparent and impermeable. The bags were packaged at two different atmosphere condition: air (sealed by a manual sealer) and 99.9 % vacuum by using a hermetic sealer (Engarvac Basis, Vacarises, Barcelona, Catalonia, Spain). For the composition, properties, and technical specifications of each package see Table 1. Each flask contained 20 g of CPA-8 formulation. Otherwise, the bags contained 2 g each and were discarded after sampling. Products were stored at both, 4 and 22 ºC. The experiment was repeated twice.

2.3. Shelf-life: CPA-8 viability, moisture content, and water activity

Shelf-life of CPA-8 formulations subjected to different packaging conditions was determined after 0, 1, 2, 4, 6, 8, 10, and 12 months of storage at both, 4 and 22 ºC. For CPA-8 viability evaluation, three replicate samples (0.5 g) of each packaging material condition and temperature were sampled and rehydrated in 5 mL of distilled water. Samples were shaken vigorously for 1 min and then allowed to rehydrate for other 9 min in static. Ten-fold dilutions of each suspension were plated on NYDA to determine cell concentration per gram (CFU g^{-1}) (Gotor-Vila, et al. 2017b; 2017d). To calculate the moisture content of the CPA-8 dried products, samples of 0.5 g each were placed in aluminium-weighing boats and dried in a convection oven at 100 °C for 24 h.

The dry matter was calculated based on the weight loss after drying and expressed as relative humidity percentage (% RH). The water activity (a_w) of each formulation was checked with an Aqualab (Decagon Devices Inc, Pullman, WA, USA) a_w-meter to an accuracy of ± 0.003.

2.4. Dispersion of the CPA-8 formulations

The dispersion in water of the two CPA-8 products (BA3-formulated with maltodextrin and BA4-formulated with potato starch) was evaluated after storage at the best packaging conditions (Table 2) by measuring changes in transmittance with an spectrophotometer set at λ = 700 nm (Spectrophotometer 2000UV, Connecta S.A., Barcelona, Catalonia, Spain). Samples of 0.1 g for each packaging-atmosphere-temperature setup were rehydrated in 20 mL of distilled water, shacked vigorously for 1 min and then allowed to rehydrate for other 9 min in static (Gotor-Vila, et al. 2017b; 2017d). The first measurement was done after 5 s of agitation with three consecutive lectures. A second measurement was done after 1 min in static with three consecutive lectures as well. The rate of dispersion of each formulation was calculated on the basis of the following formula: [(t2-t1)/t2] x 100; where for each formulated product, (t1) is the measurement after agitation and (t2) is the measurement after leaving in static for 1 minute. The experiment was repeated.

2.5. CPA-8 biocontrol efficacy

The biocontrol activity of CPA-8 formulations after storage at the best packaging conditions (Table 2) to control brown rot caused by *M. fructicola* (CPMC3, Postharvest Pathology Group Collection of IRTA) was tested in 'Fruit future' nectarines. Treatments were prepared from non-stored formulations and from those packaged for 12 months at either, 4 and 22 ºC. Moreover, the efficacy was compared to CPA-8 72 h-old fresh cells and water as the untreated control (CK).

Fruit with no visible injuries and similar in size and maturity were selected, wounded in the equator with a sterile nail (3 mm wide and 3 mm deep) and then inoculated with 15 µL of a pathogen conidial suspension adjusted at 10^3 conidia mL^{-1} by hemocytometer. Conidia of each pathogen were transferred to 5 mL of sterile distilled water amended with Tween-80 (one drop per litre). After air-drying, 15 µL of each CPA-8 formulation suspended in distilled water (10^7 CFU mL^{-1}) were applied. Five fruits constituted a single replicate and each treatment was replicated four times. The percentage of decayed fruit (disease incidence) and the mean lesion diameter (cm) (disease severity) were determined after 5 days of storage at 20 ºC and 85 % RH.

2.6. Statistical analysis

Data on CPA-8 viability for each packaging condition was log-transformed and plotted on figures in which values are the averages of six determinations and bars indicate the standard deviation. Regarding the rate of dispersion obtained for each formulation, data were transformed to the arcsine of the square root to achieve a normal distribution. For efficacy trials, severity values were also transformed (Log x+1) to normalize the data. Differences in cell dispersion as well as the brown rot incidence and severity data were evaluated using analysis of variance (ANOVA) with the JMP®8 statistical software (SAS Institute, Cary, NC, USA). In case of no homogeneity of variances, the non-parametric Wilcoxon test was applied. Statistical significance was judged at the level $P<0.05$. When the analysis was statistically significant, the Tukey's HSD test was used for mean separation. Non-transformed data are represented.

3. Results

3.1. Effect of the packaging conditions on the shelf-life of the CPA-8 formulated products

3.1.1. CPA-8 viability

CPA-8 viability just after formulation was $9.3x10^9$ and $1.3x10^{10}$ CFU g^{-1} (10.0-10.1 log units) for BA3 and BA4 products, respectively. After 12 months of storage, it ranged between 7.5-$9.9x10^9$ CFU g^{-1} (9.9-10.0 log units) for BA3 product and 6.6 $x10^9$-1.0 $x10^{10}$ CFU g^{-1} (9.8-10.0 log units) for BA4 product (Fig.1). Several samples were discarded due to the spoiling of the physical properties (see visual aspect in Fig. 2): all flasks stored for 10 and 12 months at 4 ºC and 12-months stored bags02-04 (for the BA3 product); and the flask01 stored for 12 months at 4 ºC and bags02-04 stored for 10 months at 4 ºC plus all 12-months stored bags at 4 ºC (for the BA4 product). However, some of these samples could be finally analysed for RH content and a_w. These findings indicate that for both, BA3 and BA4 products, the average shelf-life did not generally differ for any of the samples, indicating that there was no main effect on viability regarding the diverse packaging strategies employed: different materials, vacuum or not-vacuum conditions and the two tested temperatures.

3.1.2. Moisture content

The results for moisture content for all samples stored in different packages under different conditions are shown in Table 3. As a whole, different trends were observed as a function of the storage temperature. For BA3 products, the initial value of moisture (6.6 % RH) increased considerably during storage at 4 ºC. Although the RH level remained quite stable after the first 2-4 months of the assay (6.0-8.2 % RH), it was rapidly increased for samples packaged in flasks for 10 months (10.1-10.4 % RH). Due to caking of product (Fig.2), these samples could not be analysed after that time of storage. Otherwise, at 22 ºC, the moisture level was practically unchanged after one year of storage in which even a little decrease was observed until values <5.2 % RH.

Similar results were detected for the BA4 products. However, these products were not as much sensible as the BA3 products to the moisture content since the initial value was considerably

208 higher (10.0 % RH). For samples contained in flasks, no relevant changes were observed and
209 even a little decrease in RH (<8.2 % RH) was detected after 12 months of storage (except in the
210 refrigerated flask01, with less protecting barriers). This reduction could be also observed for
211 bags stored at 22 ºC (<7.5 % RH) while in the samples bagged and maintained at 4 ºC, the
212 moisture content increased until levels of 10.1-12.9 % RH after 10 months of storage. Two
213 months later, just the bag01 (aluminium bag subjected to vacuum conditions) could be analysed
214 (10.6 % RH).
215 According to these results, most relevant differences were observed for refrigerated flasks,
216 which could not prevent the gain of moisture content allowing the caking of the product.
217
218 *3.1.3. Water activity*
219 Similar to the moisture content, changes were observed for a_w as a function of storage
220 temperature and packaging condition. Table 4 shows the changes in a_w of samples over twelve
221 months of storage. The initial a_w values of the BA3 and BA4 products were 0.292 and 0.331,
222 respectively. For the BA3 product, the alterations were mainly detected in samples contained in
223 either flasks or bags at 4 ºC. The a_w progressively increased until 12 months of storage with
224 values between 0.500 and 0.633, except in both flasks (a_w of 0.611 and 0.631), in which no
225 more than 10 months could be analysed due to the caking (Fig.2c). Otherwise, all samples
226 stored at 22 ºC did not reveal considerable alterations in either, a_w or visible consistency. A
227 minor decrease in a_w could be detected for all these samples (a_w ranged between 0.246 and
228 0.292).
229 For the BA4 product, similar results were observed. While no changes were recorded for
230 samples kept at 22 ºC (a_w of 0.263-0.324 at the end of the assay), a considerable increase in a_w
231 was observed at 4 ºC. For samples contained in flasks, it was much detected after 10 months of
232 storage, with a_w values of 0.436 and 0.373, for the flask01 and flask02 respectively. For bags,

the rise in a_w was more noticeable. From 4 to 10 months of storage, the a_w raised values 0.437-0.586; after 12 months, the consistency of the sample was practically lost (Fig.2b) and just the bag01 (aluminium bag with vacuum atmosphere) could be analysed (a_w of 0.468).

Unlike the results obtained for moisture contents evaluation, most relevant differences were observed for bags stored at 4 ºC. In these cases, bags recovered with aluminium and sealed to get vacuum atmosphere better prevent the increase of a_w, and therefore, the caking of the product.

3.2. Dispersion in water of the packaged CPA-8 formulations after storage

A comparative study of the dispersion in water of the most suitable packaging conditions (Table 2) for the CPA-8-based products BA3 (maltodextrin as carrier) and BA4 (potato starch as carrier) was done by transmittance analysis comparing their rate of precipitation. As it was observed in the figure 3, packaged samples containing the BA3 product precipitated much less (so they were better dispersed in water) than the packages containing the BA4 product: rates of precipitation ranging between 0.9-2.0 % and 31.1-43.0 % for BA3 and BA4 products, respectively. These results showed a significant difference between both products analysed with regard to the dispersion rate of the carrier used. However, inappreciable (BA4 product) or any differences (BA3 product) were observed in terms of packing conditions. Comparing packaged samples and those recently formulated (0h), no changes were observed either.

3.3. Biocontrol efficacy of the packaged CPA-8 formulations after storage

The effect of the best packaging strategies for CPA-8 products (BA3 and BA4) after long-term storage on the control of brown rot decay in nectarines artificially inoculated with *M. fructicola* (compared to untreated fruit and fruit treated with CPA-8 fresh cells) is shown in figure 4. After five days at 20 ºC and 85 % RH, all formulated CPA-8 products maintained similar antagonistic

activity than fruit treated with fresh cells. The percentage of disease incidence in untreated fruit was 95.5%, with a mean lesion diameter of 1.5 cm. In contrast, for fruit treated with each selected packaged formulation, the disease incidence ranged between 5 and 35 %, with >63.2 % disease reduction compared to the untreated control. Regarding the disease severity, the lesion diameter in fruit treated with the CPA-8 packaged formulations was in all cases <1 cm, in which even 100 % of disease reduction (compared to the untreated fruit) was observed. No statistical differences were observed among the different packaging strategies evaluated.

4. Discussion

After formulation and for practical use, biocontrol products must be packaged in such a way as to maintain the products in a suitable shelf-stable state for storage, distribution, and application in the agricultural market. The present work provides decisive information regarding the influence of different packaging materials, atmosphere conditions, and temperature of storage on both, the viability and preservability of the two optimised CPA-8-based products, BA3 and BA4.

In this study, we stored formulated samples in two type of polyethylene flasks which provide excellent barrier properties to gases, water vapour and aromas (Lee et al., 2008). Moreover, they also have high resistance to fats, oils and chemicals with excellent impact resistance and good sealability (manufacturers' recommendations, Table 1). Some of the samples were also recovered with EVOH (copolymer of polyvinyl alcohol) that gives the complex magnificent properties barrier to gases. As part of the different packaging evaluated, two kind of bags were also evaluated with different barrier materials: an aluminium bag including overlapped layers composed by polyester plus polyethylene and a polyethylene coextruded film (0.76 cm^3 m^{-2} day^{-1} oxygen permeability), and an impermeable transparent bag composed by polyamide and polyethylene (8.0 cm^3 m^{-2} day^{-1} oxygen permeability).

283 Other viable solutions to extend the shelf-life of the products are related to the use of modified
284 atmospheres that can exert a barrier effect against light, moisture and oxygen (Alamprese et al.,
285 2017; Gallagher et al., 2003). Vacuum packaging could be an attractive option for maintaining
286 adequate shelf-life of bioproducts. It is a preservation technique which lowers the oxygen in the
287 package atmosphere to levels in which metabolic processes are minimized and the rates of
288 chemical and biochemical reactions reduced (Elzein et al., 2009). Costa et al. (2002) enhanced the
289 survival of the freeze-dried *Pantoea agglomerans* cells (BCA against postharvest diseases in
290 pome and citrus fruit) stored under vacuum conditions. This is in agreement with the results
291 obtained in this study for CPA-8, in which a vacuum atmosphere seem to better prevent the gain
292 of moisture and a_w thus maintaining the physical homogeneity and consistency of the product
293 (although it did not result in a crucial factor, compared to the temperature of storage). It is also
294 worth mentioning that some vacuum packaged samples were discarded because they did not resist
295 long-term storage (probably due to either, the material of the bag, the sealing condition or even
296 the sealing system applied), which suppose a drawback in a large-scale package system.
297 Many authors have described the existence of correlation between viability and moisture content
298 and therefore, water availability. Sabuquillo et al. (2010) demonstrated that only *Penicillium*
299 *oxalicum* conidial formulations whose initial moisture contents ranged between 5 and 14 %
300 retained their viability after one-year storage at room temperatures. These values were similar to
301 those obtained for conidial formulations of *Penicillium frequentans* (Guijarro et al., 2006) and
302 *Epicoccum nigrum* (Larena et al., 2007), both of which are recognized BCAs against peach brown
303 rot. However, could such values kept stable along the time? Regarding to that, we found that
304 temperature was the most important factor that affects the shelf-life of the CPA-8 formulations
305 here studied. While initial moisture contents were lower than 10 % with a_w values of 0.292-0.331,
306 such values were considerably affected by the storage temperature, since they substantially
307 changed during the time course of the assay. Storing biological products at low temperatures has

been historically recommended to maintain the microorganism in a state of low metabolic activity. However, in this study we found that storage at 4 ºC compromise the stability and visual aspect of both CPA-8 formulations, mainly associated not only to the increase of RH but also $a_{w.}$ Moreover, it should be pointed out that flasks did not conserve refrigerated BA3 samples in a suitable way, since RH and a_w increased noticeably making their visual properties unsightly after 10 months of cold storage. At that time, the BA4 products were better preserved at 4 ºC when packaged in flasks.

In terms of the viability neither the BA3 nor the BA4 product has been compromised, probably due to the physiology of the microorganism. To successfully formulate CPA-8, the endospore form was used (Gotor-Vila et al., 2017d). The inherent stability of these structures enabled them to remain quiescent, and to withstand extreme environmental conditions such as temperature fluctuations and humidity stress (Gotor-Vila et al., 2017c). However, the physical features of the product were drastically altered, losing homogeneity that would become in an even bigger handicap if the product does not have to be expended in a single dose. Shelf-life is a period of time that corresponds, in appropriate storage conditions, to a tolerable decrease in the quality of a packaged product (Alamprese et al., 2017). This general definition emphasized the commercial significance of the terms and does not necessarily relate to its real life (understood as viability). The end of its marketability is also considered in terms of an unacceptable worsening of its peculiar physical and sensory features (Pergiovanni and Limbo, 2010) and the loss of these characteristics is sufficient to cause the product rejection by consumers.

This work revealed that samples avoid caking when stored at room rather than at cold conditions, against the well documented principle that high storage temperatures often affect negatively the survival of different BCAs and biopesticidal products (Melin et al., 2011). It is a very remarkable finding since costs of storage and transportation are significantly reduced due to the needless of refrigeration. These results are in agreement with previous reports on CPA-8, in which spray-

dried (Yánez-Mendizábal et al., 2012a), freeze-dried and fluid-bed spray-dried cells proved to be shelf-stable after storage at 22 ºC using standard laboratory plastic flasks (Gotor-Vila et al., 2017b; Gotor-Vila et al., 2017d). Taken together all data recorded in this work, higher a_w in the samples correlated with poor keeping qualities that mainly appeared in the packaging materials with less protecting barriers. Therefore, PE EVOH plastic flasks (flask02) as well as aluminium bags (less oxygen permeability) with vacuum conditions (bag01) should be considered for further trials for CPA-8 commercialization.

In previous works (Gotor-Vila, et al. 2017c), differences in solubility between the two carriers used during formulation were evidenced. Summarizing, maltodextrin (BA3 product) resulted in a much more soluble product since this polysaccharide is typically composed of an amount of reducing sugars between 3-20 % (whereas starch is close to zero) (Shamekh et al., 2002). In this study, it was also observed that BA3 samples stored at 4 ºC (in either flasks or bags) the moisture content, and therefore a_w, increased much more compared to the BA4 product stored at 4 ºC. Therefore, we also aimed to prove the degree of dispersion in water of different packaged formulations including both carriers. As it was observed, products including potato starch (BA4) were not so easy to dissolve showing a retention time prior precipitation considerably shorter compared to the BA3 product (rate of precipitation >31.1 and >2.0 %, respectively). It was measured in terms of turbidity, a qualitative characteristic which is impacted by solids obstructing the transmittance of light through a water sample. As it was demonstrated, products including maltodextrin would have a long-rate spray; however, potato starch enables a better deposition and maintenance of the product in the surface of fruit (Gotor-Vila et al., 2017c).

Finally, and for commercial use, satisfactory control for brown rot on nectarines was obtained with all products stored under the different strategies evaluated.

In conclusion, this study revealed suitable packaging conditions for long-term storability at room temperature of the two CPA-8-based products developed (BA3 and BA4), which did not show any negative effect in the biocontrol efficacy of CPA-8 and provide suitable product delivery and field application. Thus, these products are fulfilling the last stage of their commercial development, practically ready to the registration process. The integration of BCAs into the usual cropping systems can be a promising strategy to achieve a high level of control of brown rot, thus contributing to the environmental-friendly management of postharvest diseases in stone fruit.

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Conflict of interest

No conflict of interest declared.

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Figure captions

Fig. 1. Effect of different materials, atmosphere conditions, and storage temperatures on the viability (log CFU g^{-1}) of the two formulated *B. amyloliquefaciens* CPA-8 products, BA3 (a) and BA4 (b) after twelve months of storage. The figure represents flask01 (—●—) and flask02 (—■—) stored at 4 ºC and flask01 (—○—) and flask02 (—□—) stored at 22 ºC; and bag01 (—●—), bag02 (- - -●- - -), bag03 (—■—) and bag04 (- - -■- - -) stored at 4 ºC and bag01 (—○—), bag02 (- - -○- - -), bag03 (—□—) and bag04 (- - -□- - -) stored at 22 ºC.

Fig. 2. Physical features of the products altered after storage (caking). An example of the initial aspect (a) and the loss of physical consistency in bags (b) or flasks (c) is shown.

Fig. 3. Dispersion in water of the most suitable packaging conditions for the two formulated *B. amyloliquefaciens* CPA-8 products, BA3 and BA4, after 12 months of storage. The analysis was done by transmittance analysis comparing their rate of precipitation according to the formula: [(t2-t1)/t2] x 100, where for each formulated product (t1) is the measurement after agitation and (t2) is the measurement after leaving in static for 1 minute. Non-stored formulations (0h) were also analysed. Different letters indicate significant differences (P<0.05) according to Tukey's HSD test.

Fig. 4. Antagonistic activity of the most suitable packaging conditions for the two formulated *B. amyloliquefaciens* CPA-8 products, BA3 and BA4, against artificial infection with *Monilinia fructicola* in nectarines. The percentage of fruit decayed (disease incidence) and the mean lesion diameter (cm) of brown rot (disease severity) were determined after 5 days of storage at 20 ºC and 85% RH. Uppercases and bars refers to disease incidence (%) and lowercases and diamonds refers to disease severity (cm). Within the same figure, different letters indicate significant

differences (P<0.05) according to Tukey's HSD test. All packaged formulations as well as non-stored formulations (0h) and fresh cell were compared to untreated fruit (CK).

Table 1. Properties of the packaging materials used to maintain the viability of the *B. amyloliquefaciens* CPA- 8 formulated products

CODE	Package	Atmosphere	Properties	Provider
Flask01	PE flask	Air	Polyethylene	'Alcion Plásticos' SL, Aldaia (Valencia,Spain)
Flask02	PE-EVOH flask	Air	Polyethylene plus copolymer of polyvinyl alcohol	'Alcion Plásticos' SL, Aldaia (Valencia,Spain)
Bag01	Aluminum bag 12/12/100	Vacuum	12 µm aluminum polyester plus 12 µm polyethylene plus 100 µm amino/polyethylene coextruded film	'Plastienvase' SL, Córdoba (Spain)
Bag02	Aluminum bag 12/12/100	Air	12 µm aluminum polyester plus 12 µm polyethylene plus 100 µm amino/polyethylene coextruded film	'Plastienvase' SL, Córdoba (Spain)
Bag03	Impermeable bag	Vacuum	Transparent bag 20 µm polyamide plus 50 µm polyethylene	W·.K. Thomas España SL. Rubí (Barcelona, Spain)
Bag04	Impermeable bag	Air	Transparent bag 20 µm polyamide plus 50 µm polyethylene	W·.K. Thomas España SL. Rubí (Barcelona, Spain)

Table 2. Twelve months-stored formulations used in dispersion and efficacy trials.

Formulation	CODE	Temperature (ºC)
BA3	Flask01	22
BA3	Flask02	22
BA3	Bag01	22
BA3	Bag02	22
BA3	Bag02	4
BA3	Bag03	22
BA3	Bag04	22
BA4	Flask 01	22
BA4	Flaks02	22
BA4	Flask02	4
BA4	Bag01	22
BA4	Bag02	22
BA4	Bag03	22
BA4	Bag04	22

Table 3. Effect of different materials, atmosphere conditions, and storage temperatures on the moisture content (average RH %) of the two formulated *B. amyloliquefaciens* CPA-8 products, BA3 and BA4, after twelve months of storage.

	CPA-8-based product BA3											
	Temperature of storage = 4 ºC						Temperature of storage = 22 ºC					
Time (months)	Flask 01	Flask 02	Bag 01	Bag 02	Bag 03	Bag 04	Flask 01	Flask 02	Bag 01	Bag 02	Bag 03	Bag 04
0	6.6	6.6	6.6	6.6	6.6	6.6	6.6	6.6	6.6	6.6	6.6	6.6
1	7.0	6.9	5.6	6.0	6.0	6.2	5.7	5.3	6.4	4.6	5.7	5.9
2	7.4	7.5	6.2	6.6	6.2	6.9	5.9	5.8	5.5	5.2	5.1	5.4
4	8.2	6.3	6.0	6.5	8.0	8.2	5.5	5.1	4.9	4.6	4.4	4.2
6	8.5	7.4	6.5	7.4	7.2	8.0	5.4	5.4	4.7	4.6	5.0	4.8
8	7.8	6.3	6.8	7.8	7.8	8.9	5.0	4.6	4.8	5.3	4.5	5.1
10	10.4	10.1	5.9	7.3	8.1	8.0	4.9	4.9	4.6	4.1	4.5	4.6
12	-	-	7.4	9.1	8.3	9.3	5.2	4.0	2.4	4.6	3.7	3.6
	CPA-8-based product BA4											
	Temperature of storage = 4 ºC						Temperature of storage = 22 ºC					
Time (months)	Flask 01	Flask 02	Bag 01	Bag 02	Bag 03	Bag 04	Flask 01	Flask 02	Bag 01	Bag 02	Bag 03	Bag 04
0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
1	8.9	7.3	8.0	7.0	9.1	9.9	8.3	8.6	8.7	7.4	8.3	8.2
2	7.5	7.4	7.5	8.7	9.1	9.3	7.6	6.8	7.0	6.7	8.2	7.4
4	10.2	9.3	10.3	10.2	11.1	12.6	9.4	9.0	11.8	8.6	9.1	9.1
6	9.0	8.9	10.6	11.6	11.5	12.4	8.4	8.1	9.0	8.7	8.2	8.4
8	9.9	9.1	10.9	11.8	11.5	13.8	9.5	9.3	8.4	8.3	8.2	7.9
10	9.3	9.2	10.1	11.8	11.8	12.9	7.6	8.3	7.0	7.4	7.1	5.5
12	10.0	8.2	10.6	-	-	-	7.8	7.5	7.5	7.2	6.7	6.8

- no sample evaluated

Table 4. Effect of different materials, atmosphere conditions, and storage temperatures on the water activity (average a_w) of the two formulated *B. amyloliquefaciens* CPA-8 products, BA3 and BA4, after twelve months of storage.

	CPA-8-based product BA3											
	Temperature of storage = 4 ºC						Temperature of storage = 22 ºC					
Time (months)	Flask 01	Flask 02	Bag 01	Bag 02	Bag 03	Bag 04	Flask 01	Flask 02	Bag 01	Bag 02	Bag 03	Bag 04
0	0.292	0.292	0.292	0.292	0.292	0.292	0.292	0.292	0.292	0.292	0.292	0.292
1	0.447	0.468	0.330	0.355	0.370	0.382	0.319	0.319	0.299	0.295	0.290	0.305
2	0.440	0.459	0.352	0.400	0.409	0.433	0.313	0.315	0.290	0.294	0.284	0.290
4	0.535	0.467	0.392	0.476	0.511	0.528	0.307	0.301	0.293	0.275	0.263	0.266
6	0.538	0.481	0.425	0.493	0.509	0.534	0.286	0.282	0.250	0.242	0.237	0.243
8	0.551	0.503	0.479	0.506	0.582	0.573	0.282	0.266	0.243	0.245	0.226	0.236
10	0.631	0.611	0.460	0.534	0.536	0.574	0.257	0.251	0.248	0.237	0.239	0.248
12	-	-	0.500	0.582	0.579	0.633	0.292	0.284	0.290	0.246	0.262	0.251
	CPA-8-based product BA4											
	Temperature of storage = 4 ºC						Temperature of storage = 22 ºC					
Time (months)	Flask 01	Flask 02	Bag 01	Bag 02	Bag 03	Bag 04	Flask 01	Flask 02	Bag 01	Bag 02	Bag 03	Bag 04
0	0.331	0.331	0.331	0.331	0.331	0.331	0.331	0.331	0.331	0.331	0.331	0.331
1	0.341	0.336	0.348	0.368	0.371	0.383	0.337	0.331	0.327	0.324	0.321	0.318
2	0.348	0.343	0.370	0.397	0.395	0.424	0.336	0.340	0.325	0.321	0.300	0.301
4	0.366	0.350	0.400	0.453	0.448	0.499	0.341	0.326	0.321	0.302	0.289	0.285
6	0.356	0.340	0.403	0.492	0.465	0.525	0.320	0.310	0.303	0.284	0.278	0.272
8	0.374	0.354	0.419	0.514	0.498	0.567	0.313	0.308	0.279	0.263	0.254	0.253
10	0.436	0.373	0.437	0.530	0.531	0.586	0.292	0.324	0.278	0.267	0.268	0.261
12	0.434	0.374	0.468	-	-	-	0.292	0.324	0.275	0.266	0.269	0.263

- no sample evaluated

Figure

Fig. 1a

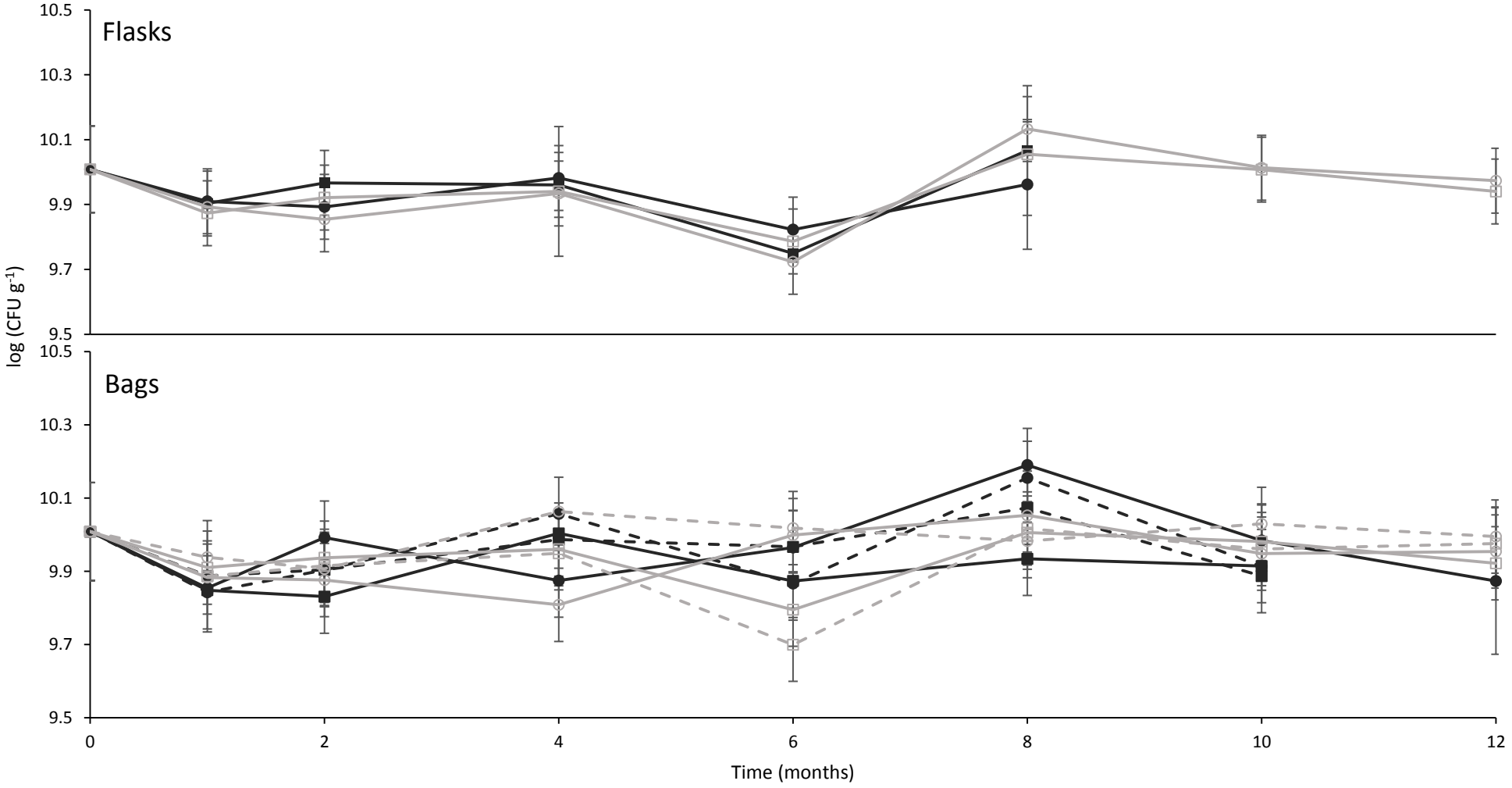

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Figure

Fig. 1b

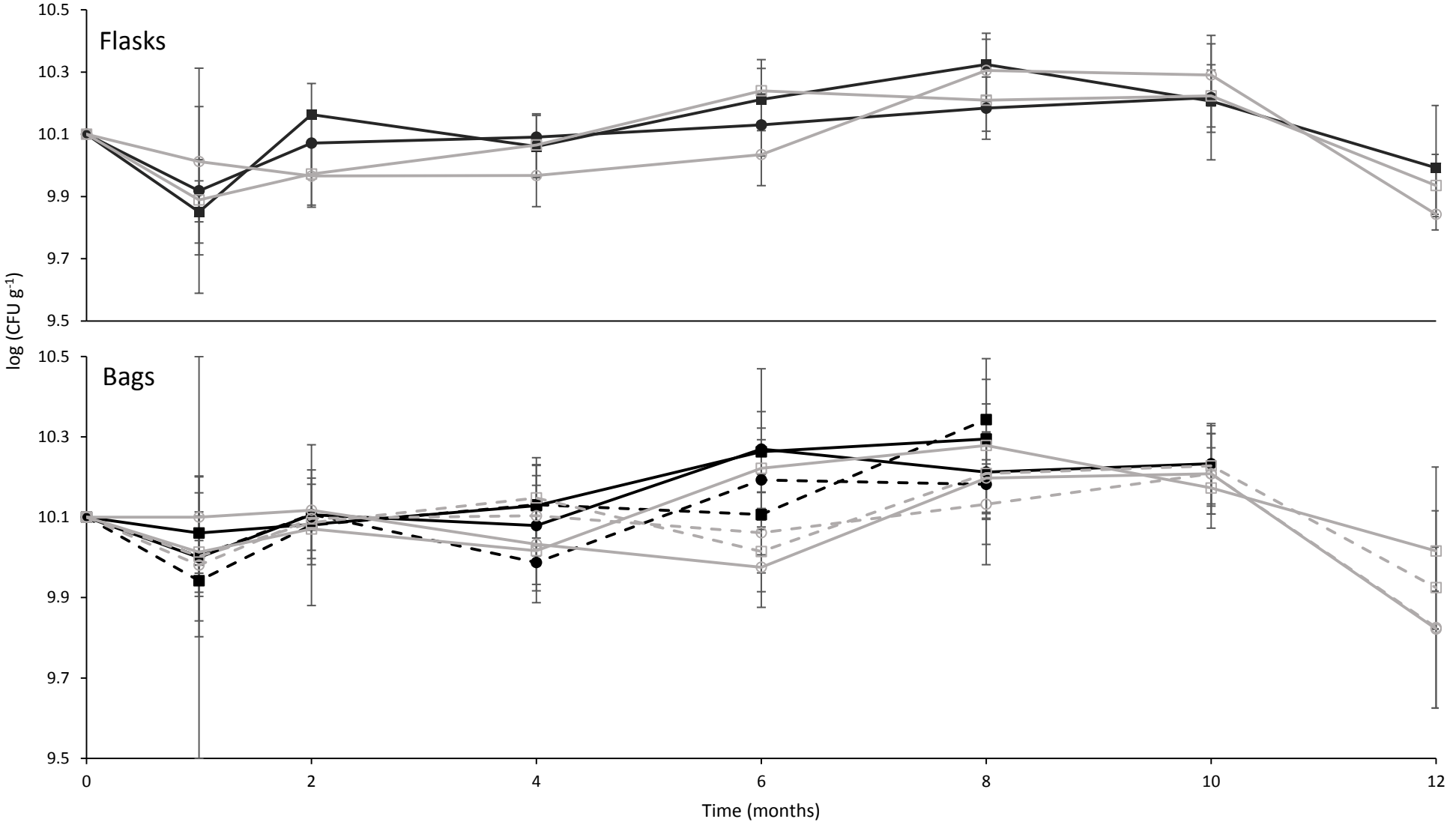

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Figure

Fig. 2

Figure

Fig. 3

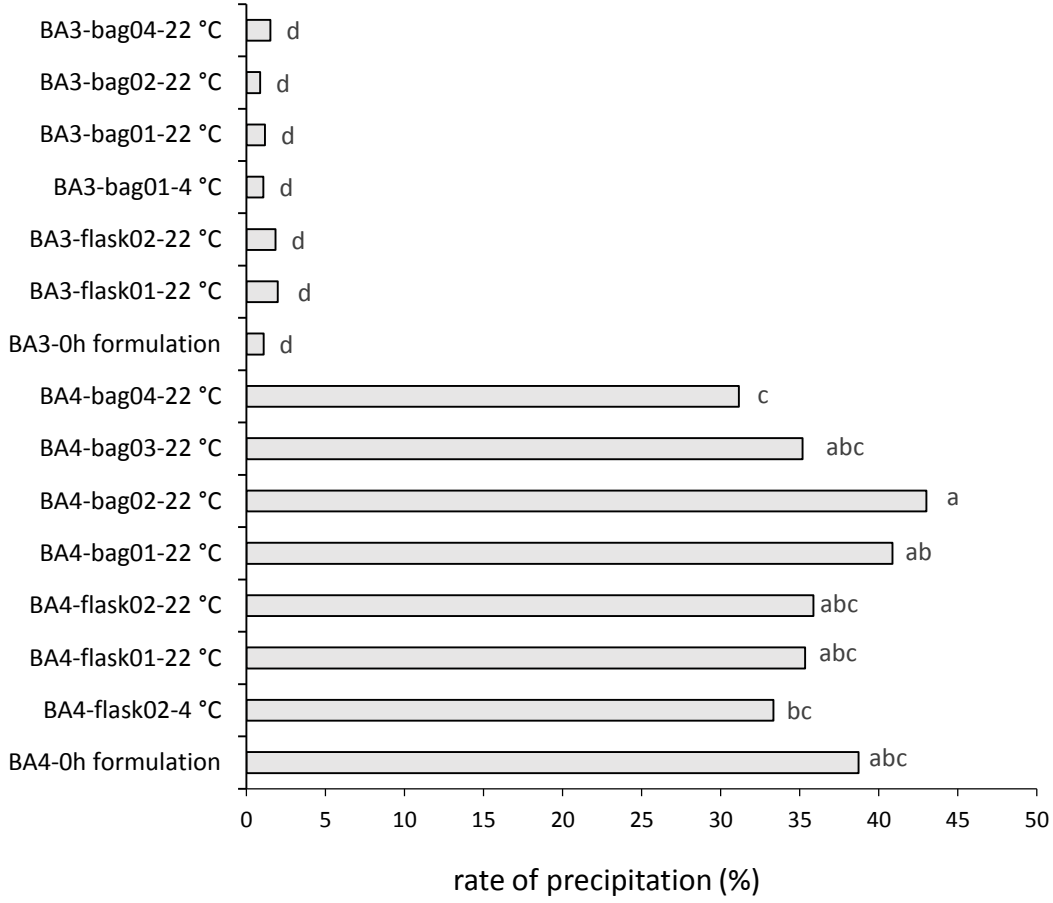

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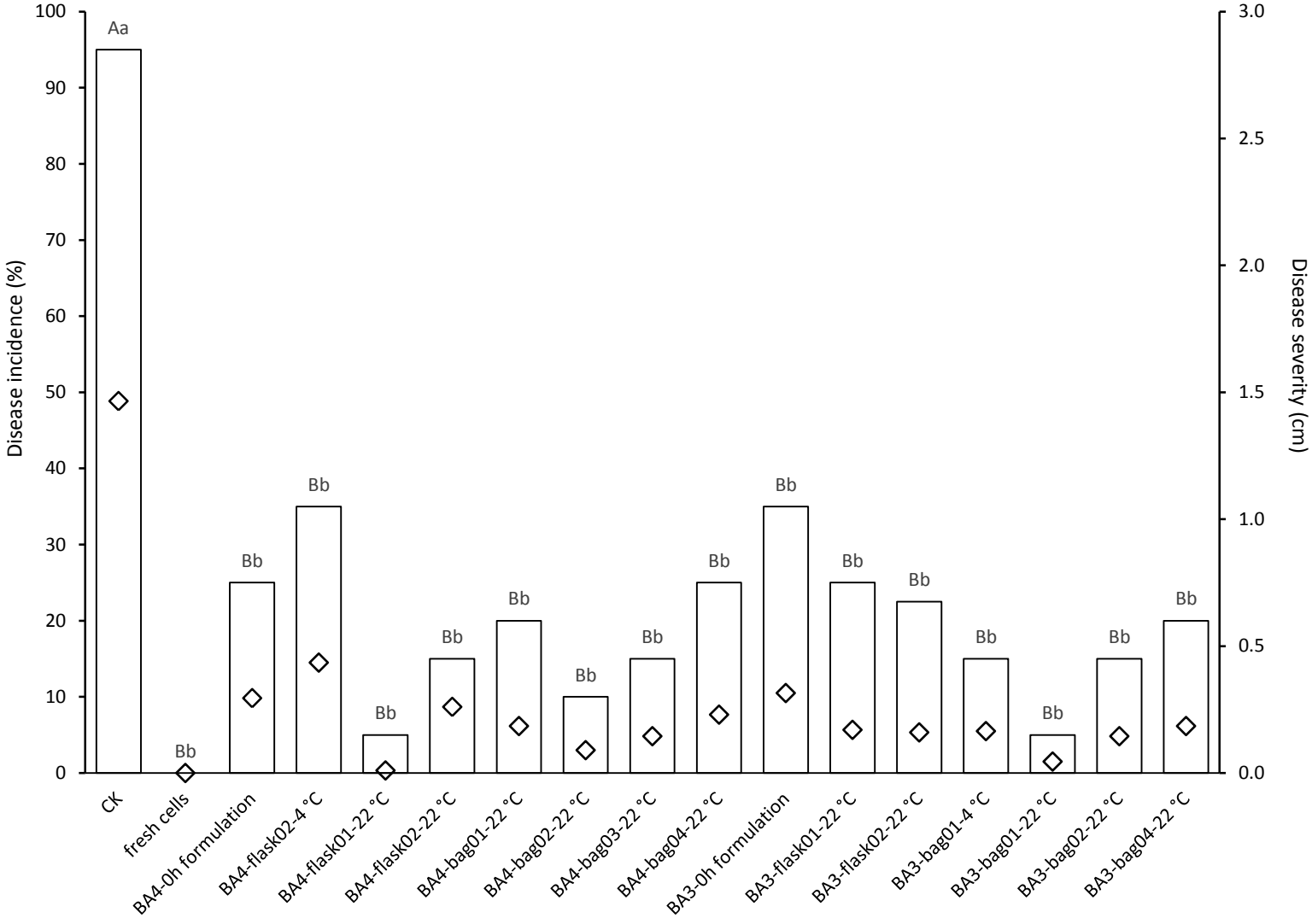
Figure

Fig. 4



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