Chapter VI

Environmental stress responses of the *Bacillus amyloliquefaciens* CPA-8-formulated products on nectarines and peaches

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ABSTRACT

The efficacy of the biocontrol agent *Bacillus amyloliquefaciens* CPA-8 against brown rot caused by *Monilinia* spp. has been described and suggested as an effective alternative to chemical applications. This study aimed to describe the population dynamics of CPA-8 on the surface of nectarines and peaches after being exposed to unfavourable environmental conditions. Two CPA-8-formulated products were obtained by fluid-bed spray-drying and then applied on fruit. Although both products included 20 % sucrose plus 10 % skimmed milk as protecting agents, they differ in the carrier material used during the formulation process: maltodextrin (CPA-8-formulated product called BA3) or potato starch (CPA-8-formulated product called BA4). CPA-8 has demonstrated wide tolerance to different factors such as temperature, relative humidity and simulated rainfall. The minimal antagonist population obtained after exposure was generally higher than 10^4 CFU cm^{-2} of fruit surface, which ensures high treatment efficacy. The results also indicated that peaches were, in general, more suitable for the CPA-8 survival than nectarines. Moreover, the properties of the two CPA-8-formulated products influenced the population dynamics of the bacterium, suggesting that the BA4 CPA-8-formulated product provided higher degree of ecological fitness of CPA-8 over the fruit than the BA3 CPA-8-formulated product. The data obtained in this work led us to conclude that the integration of these CPA- 8-formulated products into cropping systems is a promising strategy to achieve higher levels of brown rot control and hence contribute to a successful handling of postharvest diseases in stone fruit.

Keywords: *Bacillus*; biocontrol; population dynamics; temperature; relative humidity; rainfall.

INTRODUCTION

Controlling fruit decays with ecologically friendly techniques is becoming popular. Using microbial antagonists has been proposed in the last decades an effective alternative to reduce or replace the chemicals applied for control of pre- and postharvest diseases (Droby *et al.*, 2016). The biological control is safe for the appearance of fungicide-resistant population of pathogens and also for the possibility of involve toxicological risks for consumers' health. The efficacy of the biocontrol agent (BCA) *Bacillus amyloliquefaciens* CPA-8, formerly known as *Bacillus subtilis* (Gotor-Vila *et al.*, 2016), has been previously described against brown rot caused by *Monilinia* spp. (Casals *et al.*, 2012; Yánez-Mendizábal *et al.*, 2011), the wound-invading fungus that causes economically important loses (reaching even as high as 80 %) of stone fruit worldwide (Mari *et al.*, 2016; Usall *et al.*, 2015). The key mode of action of CPA-8 is based on fengycin-like lipopeptides production (Yánez-Mendizábal et al., 2012) and the emission of effective volatile organic compounds (Gotor-Vila et al. 2017a). Moreover, data regarding registration purposes, such as molecular marker design (Gotor-Vila et al, 2016) and safety tests (Gotor-Vila et al., unpublished results), have been recorded. 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 (Glare *et al.*, 2012).

The main goal for developing a commercial microorganism-formulated product is to obtain large quantities of the microorganism that ensures a reasonable shelf-life (preferentially stored at room temperatures for at least twelve months) and maintains efficacy compared to fresh cells on a wide range of hosts (Droby *et al.*, 2016; Teixidó *et al.*, 2011). Recently, two efficacious CPA-8-formulated products have been developed in a powder state by fluid-bed spray-drying (Gotor-Vila et al., 2017c). While both products contained 20 % sucrose plus 10 % skimmed milk as protecting agents, they mainly differ in the carrier material (maltodextrin or potato starch) used during the formulation process. However, to guaranty the biocontrol efficacy of such BCA-based products under field conditions, the technical application thresholds have to be determined.

An antagonist applied in the field presents a number of difficulties because BCAs would have to withstand exposure to variable and frequently hostile environmental conditions for long periods of time (Cañamás *et al.*, 2008; Köhl & Fokkema, 1998; Lahlali *et al.*, 2008).

Additives are often incorporated during mass production, formulation, and storage or added later to spray tank mixes in order to enhance the activity of the microorganism at the target side (Andrews, 1992; Burges, 1998; Sui *et al.*, 2015). Moreover, another way of improving survival rates for microorganisms is by understanding microbial stress response mechanisms and using this knowledge to improve resistance to unfavourable environmental conditions (Cañamás *et al.*, 2008). Teixidó *et al*. (2006) demonstrated that modifying water potential in the culture medium can result in cells with improved tolerance to desiccation.

Few studies have evaluated the effect of abiotic factors interfering with the survival of BCAs, such as temperature, relative humidity (RH) or UV radiation (Calvo-Garrido *et al.*, 2014a; Cañamás *et al.*, 2008; Lahlali *et al.*, 2011). In the orchard, microbial populations are subjected to daily fluctuations of the mentioned factors which could be controlled during postharvest storage (Calvo-Garrido *et al.*, 2014a; Magan, 2001; Teixidó *et al.*, 2010). However, the effect of other weather phenomena such as rainfall events on BCAs has been barely studied. Calvo-Garrido *et al.* (2014b) specifically evaluated the population dynamics of the yeast *Candida sake* exposed to simulated rainfall with different rain intensities, rain volumes, and time length between rain events. These factors have been described for influencing wash off of agrochemicals on different types of crop plants (Fife & Nokes, 2002; Hunsche *et al.*, 2007).

Effective colonisation, high population and viability of BCAs on fruit surfaces have been considered important aspects in the successful control of postharvest diseases. If appropriate environmental conditions are not consistently available, BCA populations may fail to reduce disease incidence and severity, and may not recover as rapidly as pathogen populations when conducive conditions occur (Garrett *et al.*, 2006). The environmental conditions mentioned directly influence the capability for growth and establishment of BCAs on the fruit surface. Thus, it is important to identify the environmental niche in which an individual BCA can actively grow as this enables abiotic threshold criteria and hence design application programs to achieve high treatment efficacy (Teixidó *et al.*, 1998).

The aim of this work was to assess the persistence of the BCA *B. amyloliquefaciens* CPA-8 on the surface of nectarines and peaches after being treated with two CPA-8-formulated products and exposed to different environmental conditions. In order to do this, we studied the main factors which could affect the CPA-8 survival under field conditions: (i) temperature, (ii) relative humidity, and (iii) wash-off caused by simulated rainfall.

MATERIALS AND METHODS

Microorganism and culture conditions

B. amyloliquefaciens CPA-8 was isolated from a nectarine surface and belongs to the Postharvest Pathology Group Collection of IRTA (Lleida, Catalonia, Spain). Bacteria were subcultured 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) at 30 °C for 24 h when required.

Fresh bacteria cultured overnight at 30 ºC in NYDA plates 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 prepare an appropriate volume of inoculum to inoculate a 2 L (BioFlo/CelliGen 115, Eppendorf, New Brunswick, Canada) and 5 L (BIOSTAT-A modular fermenters, Braun Biotech International, Melsungen, Germany) laboratory scale bioreactors containing growth medium previously described by Yánez-Mendizábal *et al.* (2012a) and optimised by Gotor-Vila, *et al.* (2017c). The initial concentration was adjusted at 10^6 CFU mL^{-1}. CPA-8 cells were grown for 68-72 h at 30 °C to obtain high endospore concentration (Gotor-Vila *et al.* 2017c). Agitation was set to 300 rev min^{-1}, the air feeding rate was 0.33 vvm and antifoam (1 mL per litre) was added if needed (30 % Simethicone emulsion USP, Dow Corning®, USA).

CPA-8-formulated products

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 approximately at 10^{10} CFU mL^{-1} in the same CPA-8 supernatant medium to include the antifungal lipopeptides synthesised by the bacterium during the production process (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) according to the protocol developed by Gotor-Vila, *et al*. (2017b) and by Gotor-Vila, *et al*. (2017c). Briefly, CPA-8 cells were mixed with the protective substances 20 % sucrose plus 10 % skimmed milk 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 (CPA-8-formulated product called 'BA3') and potato starch (CPA-8-formulated product called 'BA4').

Persistence of CPA-8-formulated products on nectarines and peaches under different environmental conditions

Treatment of fruit with the CPA-8-formulated products

For each treatment, condition and sampling time assessed, 20 fruits were randomly selected without visible injuries and rots and as much as homogeneous in maturity and size. Each setup consisted of four replicates with five fruits each. CPA-8 suspensions in water were prepared from the CPA-8-formulated products BA3 or BA4 and adjusted at 10^7 CFU mL^{-1}. The suspensions were sprayed on nectarines and peaches until run off by using a manual backsprayer (ARPI 18 L, CA Bovi, Lleida, Catalonia, Spain) and then air-dried for 2 h at room temperature. The spray system was kept close to the fruit to reduce bacterial movement by aerosol from the application side.

Effect of temperature

The effect of two temperatures of storage (0 and 20 ºC) on CPA-8 populations was evaluated on 'Rome Star' peaches and 'Big Top' nectarines previously treated with the BA3 and BA4 CPA-8-formulated products as described above. 20 fruits (peaches or nectarines) per treatment, condition, and sampling time were placed on packing trays and stored in two climatic chambers programmed at the temperatures mentioned. The population dynamics of CPA-8 was assessed after 0, 1, 2, 5 and 7 days or after 0, 2, 7, 15, 30 and 60 days in fruit stored at 20 and 0 ºC, respectively.

Effect of RH

The effect of three different values of RH (40, 60 and 85 %) on CPA-8 populations was evaluated on 'Gladys' peaches and 'Fantasia' nectarines previously treated with the BA3 and BA4 CPA-8-formulated products as described above. 20 fruits (peaches or nectarines) per treatment, condition, and sampling time were placed on packing trays, covered with plastic chambers, sealed, and stored in climatic chambers programed at 20 ºC. The distinct RH values inside the plastic chambers were achieved by placing a dehumidifier (FDC32S, FRAL, Carmignano di Br., PD, Italy), and monitoring RH during storage with an external data logger (Testo 175H1, Testo Inc., Sparta Township, NJ, USA). The population dynamics of CPA-8 cells under the mentioned storage conditions was assessed after 0, 1, 2, 3, 6 and 9 days on peaches and after 0, 1, 2, 3, 8 and 10 days on nectarines.

Effect of simulated rainfall

Two different trials were conducted in order to evaluate the population dynamics of CPA-8 on 'Summer lady' or 'Sweet Dream' peaches and 'Diamond Ray' or 'Alba Red' nectarines exposed to simulated rainfall. 20 fruits (peaches or nectarines) were used per treatment, condition, and sampling time. (i) In a first approach, the effect of three rain intensities (60, 100 and 150 mm h^{-1}) and four rain volumes (0 'non-exposed', 20, 60 and 120 mm) were analysed on fruit previously treated with the BA3 and BA4 CPA-8-formulated products as described above. (ii) In a second approach, the effect of an establishment time of the CPA-8 cells on the surface of the fruit (0, 1, 3 and 7 days of incubation at 20 ºC and 85 % RH) prior exposition to the most stressful rainfall conditions (determined in the first experiment) was evaluated.

Rainfall simulation was performed based on the protocol reported by Calvo-Garrido, *et al.* (2014b) using a rainfall simulator consisting of a metallic box 100x50x20 cm with a drop generation system at the bottom (2.5 mm diameter drops and 50 mm separation among each drop). Water fell freely 1.5 m above the metal grids in which the fruit was located. To avoid the continuous impact of rain drops on the same fruit part, a moving fan was placed in front of the rain curtain. Rain intensity was regulated by maintaining a constant water layer above the droppers and its uniformity was measured regularly before and after each rain event.

CPA-8 population dynamics

Five fruits were sampled per replicate and four replications were analysed from each treatment per condition tested. 25 peel disks were randomly removed with a cork borer (16 mm in diameter) from the surface of each fruit. Then, the 125 peel disks were placed together into sterile plastic filter bags (BagPage 400 mL, Interscience BagSystem, St Nom la Brètech, France) and mixed with 100 mL of PB. Each bag was homogenised in a stomacher blender (Masticator Basic 400 mL, IUL SA, Torrent de l'Estadella, Barcelona, Catalonia, Spain) set at 12 strokes sec^{-1} for 90 s. Serial ten-fold dilutions of the washings were made and plated on NYDA medium. Colonies were counted after incubation for 24 h at 30 ºC. Population dynamics of CPA-8 were collected as CFU mL^{-1} and finally expressed as CFU cm^{-2} of fruit surface.

Statistical analysis

Data from CPA-8 populations under each evaluated condition was log-transformed and expressed as Log_{10} (CFU cm^{-2}) to achieve a normal distribution. In case of the establishment of the CPA-8 cells before wash-off caused by simulated rainfall, CPA-8 population reduction was finally expressed as Log_{10} (N/N_0), where N = populations in the sample exposed to rainfall (CFU cm^{-2}) and N_0 = mean value of the population in the four replicates non exposed to rainfall (CFU cm^{-2}). For every trial, different conditions 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 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 separation of means.

RESULTS

Effect of temperature on the persistence of CPA-8-based products on nectarines and peaches

The population dynamics of CPA-8 on nectarines and peaches after storage at two different temperatures are shown in Fig. 1. The viabilities were practically unchanged regardless of the temperature. After 7 days of storage, fruit kept at 20 ºC did not show significant differences between neither the fruit nor the treatments (Fig. 1a). Along this time, the maximum reduction in CPA-8 viability (0.21 log units) was observed in peaches treated with the BA3 CPA-8-formulated product (from $1.16 \cdot 10^5$ to $7.15 \cdot 10^4$ CFU cm^{-2}) (Fig. 1a). After 60 days of storage at 0 ºC, no differences were observed in peaches. However, CAP-8 populations on nectarines moved from $9.4 \cdot 10^4$ to $6.22 \cdot 10^4$ (0.18 log units) and from $1.01 \cdot 10^5$ to $6.81 \cdot 10^4$ CFU cm^{-2} (0.17 log units) in the case of BA3 and BA4 products, respectively (Fig. 1b). CPA-8 populations obtained after 60 days of storage at 0 ºC were lower in nectarines than in peaches, decreasing even more in the case of nectarines treated with the BA3 CPA-8-formulated product ($6.22 \cdot 10^4$ CFU cm^{-2}) (Fig. 1b).

Effect of RH on the persistence of CPA-8-formulated products on nectarines and peaches

CPA-8 populations were generally maintained after storage at three different RH values: 85, 60 and 40 % (Fig. 2). When the nectarines were treated with the BA3 CPA-8-formulated product, a reduction from $2.85 \cdot 10^4$ to $1.59 \cdot 10^4$ CFU cm^{-2} (0.25 log units) and from $2.85 \cdot 10^4$ to $1.36 \cdot 10^4$ CFU cm^{-2} (0.32 log units) was observed in fruit stored for 10 days at 85 and 60 % of RH, respectively (Fig. 2a). Similarly, when the nectarines were treated with the BA4 CPA-8-formulated product and stored for 10 days, the CPA-8 populations were reduced from $3.69 \cdot 10^4$ to $1.66 \cdot 10^4$ CFU cm^{-2} (0.35 log units) in case of fruit exposed to 85 % of RH and from $3.69 \cdot 10^4$ to $1.69 \cdot 10^4$ CFU cm^{-2} (0.34 log units) when the RH was set at 60 % (Fig. 2b). Conversely, no differences were observed on peaches at the mentioned relative RH values (Fig. 2c-d). Moreover, when either nectarine or peaches were subjected to 40 % of RH, the CPA-8 populations remained unchanged after 10 or 9 days of storage, respectively (Fig 2). On the whole, peaches provided better CPA-8 maintenance than nectarines. It is noteworthy the growth profile of CPA-8 on peaches, in which CPA-8 seems to need a preadaptation step of 3 days prior stabilisation. Moreover, peaches treated with the BA3 CPA-8-formulated product and kept for 9 days at 40 % of RH ($6.31 \cdot 10^4$ CFU mL^{-1}) showed lower CPA-8 populations than peaches exposed to either, 85 or 60 % of RH ($1.06\text{-}1.19 \cdot 10^5$ CFU mL^{-1}) (Fig. 2c).

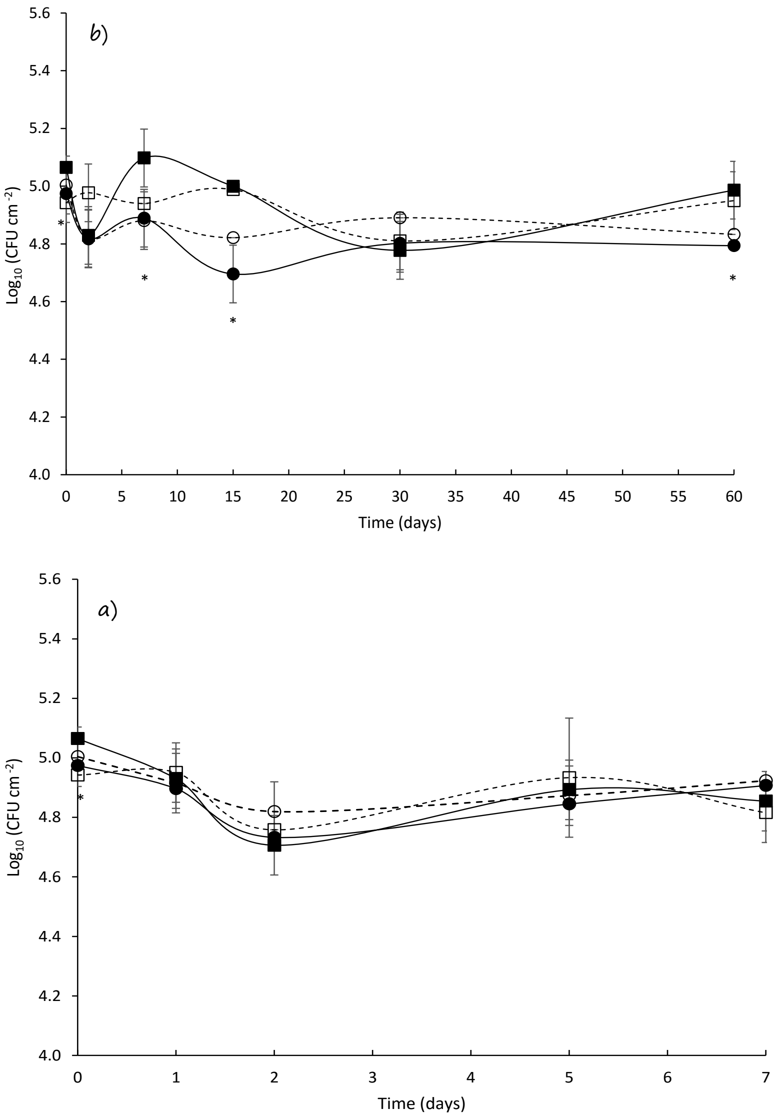

Figure 1. Effect of temperature on the population dynamics of CPA-8 at different sampling times on nectarines and peaches after storage at 20 (a) and 0 ºC (b). Nectarines treated with the BA3 CPA-8-formulated product (—●—) and with the BA4 CPA-8-formulated product (- - ○ - -) and peaches treated with the BA3 CPA-8-formulated product (—■—) and with the BA4 CPA-8-formulated product (- - □ - -). Values are the averages of four determinations and bars indicate the standard deviation. (*) means that these values are statistically significant according to Tukey's HSD test.

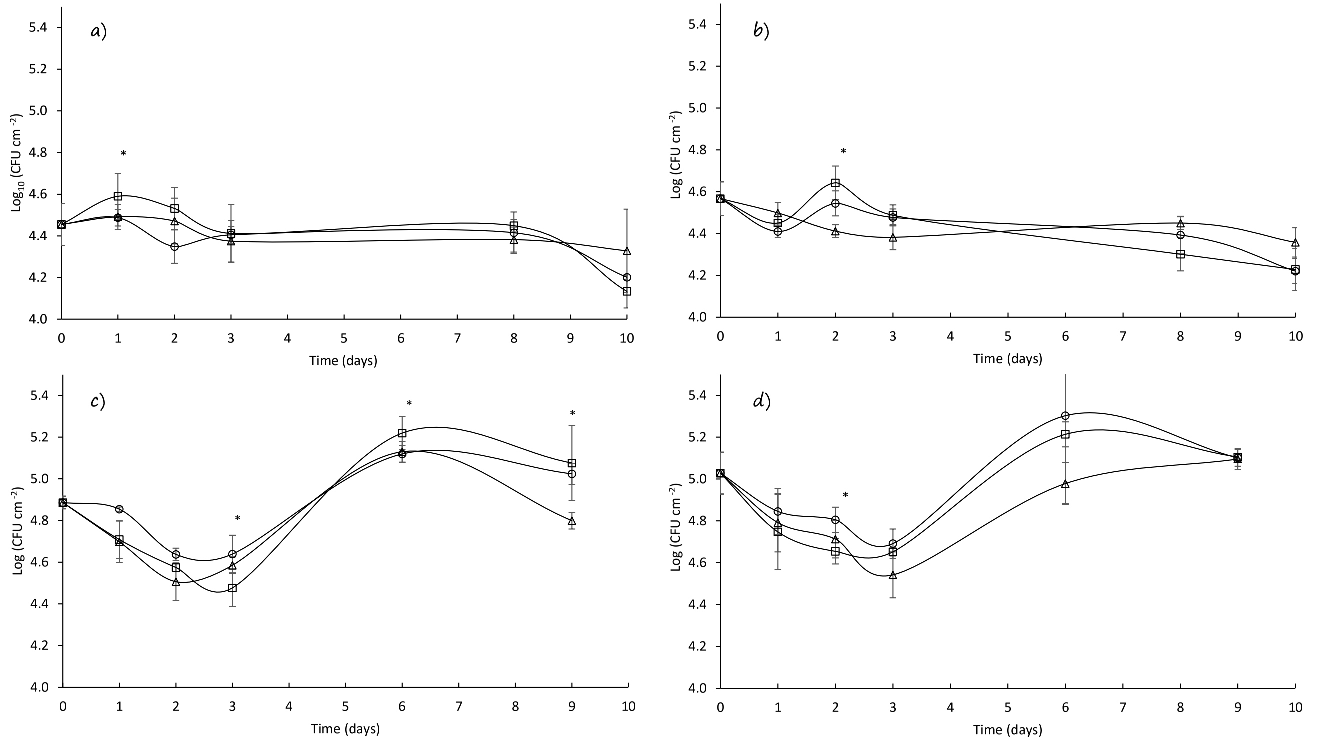

Figure 2. Effect of relative humidity (RH) on the population dynamics of CPA-8 at different sampling times on nectarines and peaches after storage at 20 ºC and 85 (—○—), 60 (—□—) and 40 % (—△—) RH. Nectarines treated with the BA3 CPA-8-formulated product (a) and with the BA4 CPA-8-formulated product (b) and peaches treated with the BA3 CPA-8-formulated product (c) and with the BA4 CPA-8-formulated product (d). Values are the averages of four determinations and bars indicate the standard deviation. (*) means that these values are statistically significant according to Tukey's HSD test.

Effect of simulated rainfall on the persistence of CPA-8-formulated products on nectarines and peaches

Effect of intensity and rain volume

CPA-8 populations on peaches and nectarines were evaluated after exposing the treated fruit to different quantities of simulated rain (rain volume) and rain intensities. In general, good CPA-8 populations were obtained after wash-off caused by simulated rainfall ($6.01 \cdot 10^{3}$-$2.87 \cdot 10^{4}$ and $1.53 \cdot 10^{4}$-$3.71 \cdot 10^{4}$ CFU cm^{-2} on nectarines and peaches, respectively). The effect of the rain volume was not different depending on each intensity. The most substantial losses were always detected after 20 mm of rain exposure whereas higher volumes caused little additional CPA-8 removal. Moreover, it is worth mentioning the dynamic populations of CPA-8 on peaches, which reached levels much higher than those obtained on nectarines.

In detail, we could observe that for nectarines, the effect of 60 mm of rain volume was also significant when an intensity of 60 or 150 mm h^{-1} was applied to fruit treated with the BA3 or BA4 CPA- 8-formulated products, respectively (Fig 3a). When the two products were compared at each rain volume, the results proved that higher CPA-8 populations were obtained in nectarines treated with the BA4 CPA-8-formulated product regardless of the rain volume applied (20, 60 or 120 mm). The results showed losses in CPA-8 populations between 0.83-1.01 and 0.32-0.53 log units for nectarines treated with the BA3 and BA4 CPA-8-formulated products, respectively, maintaining approximately 0.5 log units difference among products (Fig. 3a). Similar results were obtained for peaches, indicating that fruit treated with the BA4 CPA-8-formulated product achieved higher CPA-8 populations (losses of 0.21-0.45 log units) than that treated with the BA3 CPA-8-formulated product (losses of 0.38-.046 log units) (Fig 3b). Moreover, the effect of 60 mm of rain volume resulted again significant when either CPA-8 formulated products, BA3 or BA4, were applied on peaches and subjected to 60 or 150 mm h^{-1} (Fig. 3b). When the rain intensity was set at 100 mm h^{-1}, higher rain volumes (120 mm) also produced a significant effect (Fig. 3b).

Consequently, restrictive conditions of simulated rainfall (intensity of 150 mm h^{-1} and rain volume of 60 mm) were chosen for subsequent assays.

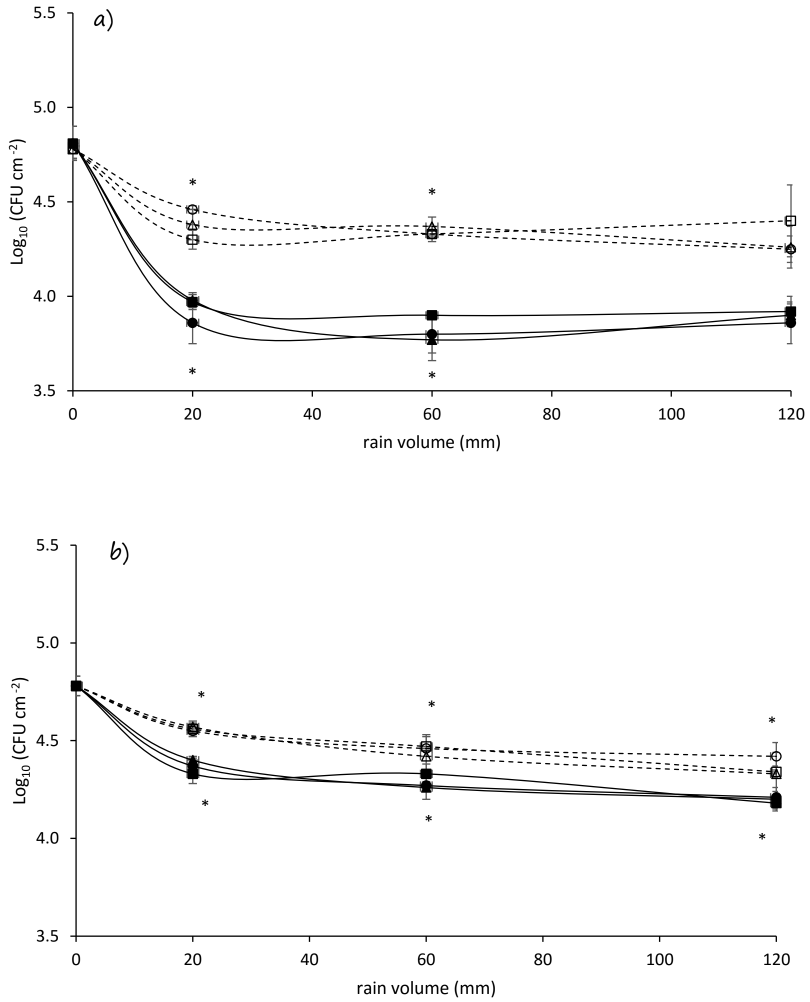

Figure 3. Population dynamics of CPA-8 on nectarines and peaches after exposure to simulated rainfall at different rain volumes (mm) and intensities (mm h -1). (a) Nectarines treated with the BA3 CPA-8-formulated product and exposed to 150 (—●—), 100 (—■—) or 60 mm h -1 (—▲—) and nectarines treated with the BA4 CPA-8-formulated product and exposed to 150 (- -○- -), 100 (- -□- -) or 60 mm h -1 (- -△- -). (b) Peaches treated with the BA3 CPA-8-formulated product and exposed to 150 (—●—), 100 (—■—) or 60 mm h -1 (—▲—) and peaches treated with the BA4 CPA-8-formulated product and exposed to 150 (- -○- -), 100 (- -□- -) or 60 mm h -1 (- -△- -). Values are the averages of four determinations and bars indicate the standard deviation. (*) means that these values are statistically significant according to Tukey's HSD test.

Effect of the establishment of CPA-8 cells prior exposure to rainfall

The occurrence of the establishment period of CPA-8 on nectarines and peaches prior exposure to simulated rainfall (intensity of 150 mm h^{-1} and rain volume of 60 mm) significantly affected the BCA survival (Fig. 4). In order to minimise the losses of CPA- 8 on treated fruit, an establishment time of 3 days was needed when the BA3 CPA-8-formulated-product was applied on nectarines, reaching a population reduction of -0.26 log units (Fig. 4a). However, in case of nectarines treated with the BA4 CPA-8-formulated product, the establishment time had to be extended up to 7 days to achieve a population reduction of -0.22 log units (Fig. 4b). When the BA3 CPA-8-formulated-product was applied on peaches, an establishment time of 7 days was also needed to minimise the CPA-8 population reduction until -0.33 log units (Fig. 4c). However, no effect was observed on peaches treated with the BA4 CPA-8-formulated-product, obtaining population reductions between -0.26 and -0.30 log units after 0, 1, 3 and 7 days of establishment (Fig. 4d).

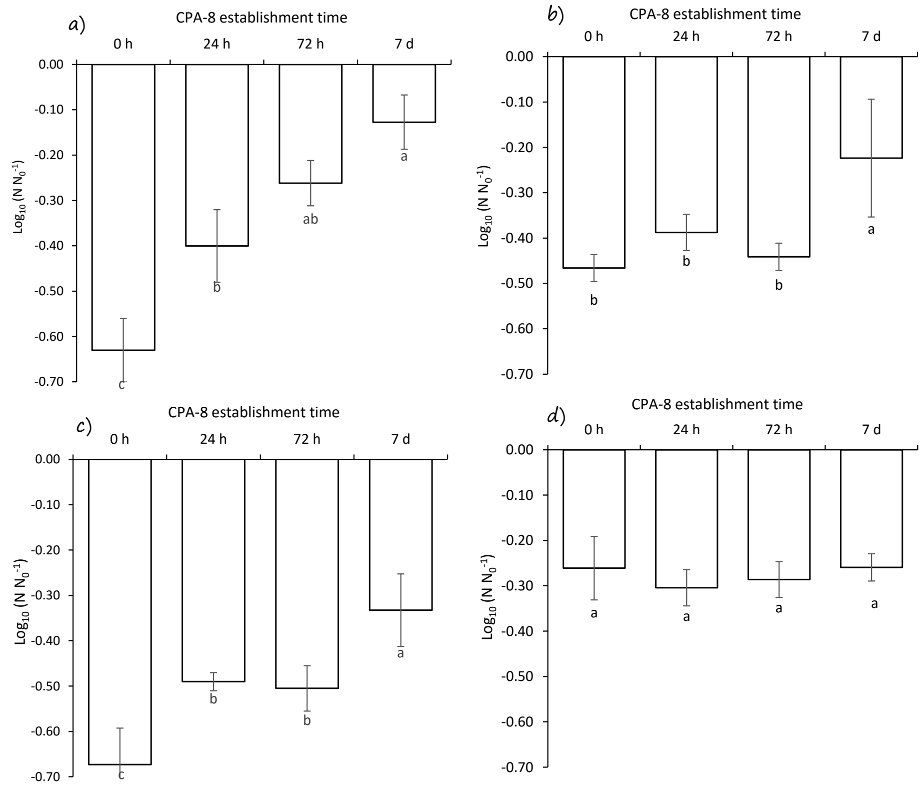

Figure 4. Population dynamics of CPA-8 on nectarines and peaches incubated for 0, 1, 3 and 7 d at 20 ºC and 85 % relative humidity (RH), prior to exposure to simulated rainfall at 150 mm h^{-1} and 60 mm of rain volume. (a) Nectarines treated with the BA3 CPA-8-formulated product; (b) nectarines treated with the BA4 CPA-8-formulated product; (c) peaches treated with the BA3 CPA-8-formulated product and (d) peaches treated with the BA4 CPA-8-formulated product. Values are the averages of four determinations and bars indicate the standard deviation. Within the same figure, different letters indicate significant differences ($P<0.05$) according to Tukey's HSD test.

DISCUSSION

The present work provides decisive information regarding the influence of the main abiotic factors on the persistence of CPA-8-formulated products in stone fruit, which may directly affect the efficacy of the treatments. The implementation of the data obtained into the management of field strategies may provide higher levels of disease control and would then contribute to a successful handling of postharvest diseases.

The BCA CPA-8 has demonstrated a wide tolerance to different environmental conditions. Temperature is one of the major environmental stresses experienced by microorganisms. Once the antagonist has been applied on a fruit surface, exposure to temperature stress can significantly reduce the survival of the BCAs, especially during preharvest applications under field conditions or even during postharvest storage, distribution, and marketing period (Sui *et al.*, 2015). While the optimum temperature of CPA-8 ranges from 37 to 30 ºC (Gotor-Vila et al., unpublished results), this study demonstrated that CPA-8 also tolerate exposure to 20 ºC and to 0 ºC (which simulates the temperature of the chambers conditioned for fruit storage), thus exhibiting a high degree of thermotolerance on nectarines and peaches. In general, the viabilities remained practically unchanged regardless of the temperature and treatment, reaching CPA-8 populations between $6.22 \cdot 10^4$ and $9.68 \cdot 10^4$ CFU cm^{-2} after 7 days or 60 d of storage, when fruit was exposed to room or cold temperatures, respectively. These results proved that the temperatures tested (0 and 20 ºC) do not restrict the storage conditions for fruit treated with the two CPA-8-formulated products assessed. These data agree with the previous work reported by Yánez-Mendizábal *et al.* (2012a) in which non-formulated CPA-8 cells also had good survival and efficacy on fruit after storage at 20 and 0 ºC.

The sensitivity of the CPA-8-formulated products to dry conditions on treated nectarines and peaches was evaluated at three different RH values: 85 (suitable RH for the development of the microorganism), 60 (intermediate) and 40 % (average RH in the field in the summer time). Although bacteria are generally very sensitive to the absence of free water in the phyllosphere (Köhl & Fokkema, 1998), CPA-8 populations were largely maintained, reaching values over $1.36 \cdot 10^4$ CFU cm^{-2} in all the conditions tested. Conversely, *Pantoea agglomerans* populations rapidly declined in chambers with low RH contents (Cañamás *et al.*, 2008). Moreover, the work conducted by Calvo-Garrido *et al.* (2014a) also revealed that the population of *C. sake* on treated grape berries drastically decreased under suboptimal regimes of temperature and RH, suggesting that temperature is one of the major environmental stresses experienced by yeasts.

Regarding the mentioned factors of temperature and RH, we could observe that in general, peaches were more suitable for the CPA-8 development than nectarines. Comparing the results obtained, a slight decrease of CPA-8 populations could be observed in nectarines after 60 days of storage at 0 ºC and in nectarines exposed to the highest RH values (85 and 60 %). These results could be due to the dissimilar morphology of the peel of the fruit, which exhibits different sensibility to stress. Moreover, it was noticeable that the two CPA-8-formulated products used in this study had a considerable influence on the population dynamics of this bacterium, probably due to the composition of each carrier material used during formulation. These results indicated that the BA3 CPA-8-formulated product was more restrictive for the CPA-8 maintenance over the fruit than the BA4 CPA-8-formulated product.

The differences above observed were even more accentuated when the effect of simulated rainfall on CPA-8 populations was evaluated. The occurrence of rain had the greatest effect upon the activity of the BCAs applied (Behle *et al.*, 1997; Norris *et al.*, 2002; Pietrarelli *et al.*, 2006). Factors such as the dilution, redistribution, physical removal, and extraction from the fruit tissue should be specially contemplated (Hunsche *et al.*, 2007). In this work, wash-off of CPA-8-formulated products from the surface of nectarines and peaches was evaluated after 0 (non-exposed), 20, 60, and 120 mm of heavy rain. Results showed that CPA-8 was washed-off easily from the surface of the fruit due to the impact of the first 20 mm of rain, while higher volume of rain caused little additional removal. As it was expected, rain washed easier on the surface of nectarines than on peaches and the CPA-8 cells from the BA4 CPA-8-formulated product remained better on fruit. However, the data obtained revealed loses in CPA-8 as much as 1 log unit, reaching viabilities between $6.01 \cdot 10^{3}$ and $1.53 \cdot 10^{4}$ after rain exposure. The establishment time of CPA-8 cells on fruit between treatment and rainfall has significantly affected the wash-off of the BCA. Depending on the properties of each product applied and the type of fruit assessed, different establishment times were needed to achieve a reduction in CPA-8 cells between -0.22 and -0.33 log units, which was the minimum loss observed in all the experiments conducted. The positive effect of an establishment time prior exposure to rainfall was also observed in *C. sake* (Calvo-Garrido *et al.*, 2014b), which significantly reduced population loss and removed the effect of rain intensity. These practical observations underline an advantage of BCAs compared to chemical pesticides as treatment persistence may improve due to the colonisation ability of the microorganisms (Calvo-Garrido *et al.*, 2014b).

The two CPA-8-formulated products used in this work included the same proportion of protectants (sucrose and skimmed milk) but differed in the carrier materials employed in the formulation process. Maltodextrin and potato starch were chosen for enhancing powder recovery with no agglomerates and also for being low-cost commercial products. The composition of these two polysaccharides was different regarding the content of reducing sugars, which provided maltodextrin (Dextrose Equivalent (DE) between 3-20 % whereas starch is close to zero) with higher degree of solubility in water (Shamekh *et al.*, 2002). That property could probably determine the differences observed in the population dynamics of CPA-8.

In order to enhance biological control of postharvest diseases, the antagonist needs to possess effective mechanisms to cope with the abiotic stresses to which the microorganisms are commonly subjected (Sui *et al.*, 2015). This study provides for the first time exhaustive information to describe the persistence of two CPA-8-formulated products on nectarines and peaches directly exposed to environmental factors which most affect the survival of BCAs under field conditions: temperature, RH and rainfall. The results obtained led us to conclude that the minimal antagonist population level on fruit surface to subsequently obtain efficient control (10^4 CFU cm^{-2}) has been achieved. However, it is important to note that this study was a small-scale experiment and that run off dynamics may change on a field scale under a natural environment. Moreover, more studies should be considered to establish the impact of other stresses caused by environmental factors such as solar radiation (UV light), wind or oxidative stress probably associated with controlled-atmosphere storage.

While potato starch appears to be an essential component in the formulation of CPA- 8, further studies are now the next research step in order to better clarify this issue. These data demonstrate that the two CPA- 8-formulated products evaluated suppose a promising way to devise possible strategies to maximise the efficacy of brown rot control in stone fruit under field applications and thus widespreading the commercialisation of BCAs.

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