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Annex I

*Environmental fate and behaviour of the biocontrol agent
Bacillus amyloliquefaciens CPA-8 after preharvest application
to stone fruit*

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ABSTRACT

Bacillus amyloliquefaciens strain CPA-8 has been described as an effective biocontrol agent to control brown rot in stone fruit in both, preharvest and postharvest applications. However, information about the environmental fate and behaviour of this strain under field conditions is not available. In this work, dispersion of CPA-8 application was evaluated using water-sensitive papers. While a complete area coverage was obtained in leaves from treated trees, less than 1 % coverage was observed in leaves from non-treated trees. Moreover, the persistence of CPA-8 on fruit, leaves, and weed was also evaluated by using plating or contact methods and PCR confirmation. The presence of CPA-8 on fruit from treated trees was maintained during preharvest and postharvest conditions. However, a significant decrease on leaves and weed was observed 21 days after treatment. On non-treated trees, the presence of CPA-8 on leaves was detected until 180 days after treatment and on weed, the population of CPA-8 was dependent on the distance to the treated tree. High persistence of CPA-8 was detected on inert materials such as, clothes and gloves worn by handlers and on harvesting plastic boxes. Finally, more than 99 % of the samples with CPA-8 morphology were confirmed as CPA-8 using PCR. This work demonstrated a good distribution, persistence and adaptation of the CPA-8 strain to field and postharvest conditions. Monitoring dispersion and persistence is an excellent tool to decide the calendar of application and an interesting information for registering issues.

Keywords: Peach; biological control agent; antagonist; persistence; population dynamics; PCR.

INTRODUCTION

Monilinia spp. are the causal agents of brown rot in stone fruit and are responsible for economic losses during preharvest, but especially during postharvest. During favourable climatic conditions for disease development, these losses can reach up to 80 % for more susceptible late-ripening varieties (Usall *et al.*, 2015). Currently, the primary strategy to control this disease in stone fruit is based on spray programmes in the field using chemical fungicides combined with adequate cultural practices, since fungicide applications are not allowed or rarely occur (Sisquella *et al.*, 2014). Moreover, several currently used preharvest fungicides will have limited availability in the coming decade due to health and environmental reasons, leading to serious challenges for growers to produce healthy fruit in a sustainable way in the near future. These concerns, occurring at the same time that the appearance of fungicide-resistant strains, have promoted the development of alternative methods to synthetic fungicides. Biological control using microbial antagonists is considered the most promising strategy to replace the use of chemical fungicides, and its efficiency has been demonstrated in a large number of studies over the last 20 years (Wisniewski *et al.*, 2016).

Many species of the genus *Bacillus* have been reported as biological control agents (BCAs) with an effective broad spectrum activity against different plant diseases caused by soil borne (Chowdhury *et al.*, 2013; Pane *et al.*, 2012; Pastrana *et al.*, 2016), foliar (Lee & Ryu, 2016; Romero *et al.*, 2007), and postharvest pathogens (Casals *et al.*, 2010; Gao *et al.*, 2016; Hong *et al.*, 2014; Pusey & Wilson, 1984). In particular, *Bacillus amyloliquefaciens* strain CPA-8 (formerly *B. subtilis*), which was isolated from the surface of a nectarine, was demonstrated to be effective in controlling brown rot caused by *Monilinia* spp. alone or in combination with other postharvest treatments (Casals *et al.*, 2012; Yáñez-Mendizábal *et al.*, 2011a). Moreover, recent studies revealed that applications of *B. amyloliquefaciens* during the preharvest of peaches and

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nectarines substantially reduced brown rot decay during postharvest (Gotor-Vila *et al.*, unpublished results).

One of the main difficulties in commercialising a BCA is the registration procedure established by the European Commission regulation 1107/2009 (Soto-Muñoz *et al.*, 2015). This guideline concerns the commercialisation of plant protection products. Particularly, the Annex II section establishes the procedures and criteria for the approval of active substances in which the fate, behaviour, and persistence of the product in the intended environment are considered. Compliance with this regulation requires the development of monitoring methods that allow the precise identification and quantification of the microorganisms at the strain level (Alabouvette & Cordier, 2011) since applied BCAs usually belong to the same species as members of the microbiota (Lindow & Brandl, 2003).

Traditional methods for monitoring BCAs in the environment have been primarily based on dilution plating methodologies using selective or semi-selective culture media (Narayanasamy, 2013). However, this methodology is arduous because it requires a high level of expertise to differentiate among other morphologically similar species. These limitations have been solved using more specific methodologies based on DNA amplification with strain specific primers.

Many studies have used DNA-based techniques to identify the applied BCA from the non-target microbiota (Holmberg *et al.*, 2009; Nunes *et al.*, 2008; Soto-Muñoz *et al.*, 2014; Villarino *et al.*, 2016). With regards to *B. amyloliquefaciens* CPA-8, two different molecular markers were developed for its detection and identification (Gotor-Vila *et al.*, 2016). The first is based on a SCAR marker, in which a semi-specific fragment is amplified for CPA-8 and other 12 *Bacillus* strains that could be morphologically distinguished. In the second approach, a strain-specific genomic marker for CPA-8 was obtained from the *RBAM 007760* gene, which is primarily involved in bacterial surface adhesion.

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With this in mind, we established an easy and reliable methodology to study the fate and behaviour of the CPA-8 strain after its application to flat peaches under field conditions and during postharvest storage using a combination of conventional and PCR methodologies. Specifically, the objectives of this study were (a) to evaluate the dispersion of the preharvest treatment, (b) to determine the air distribution of the CPA-8 cells, (c) to quantify the population of the CPA-8 strain on the surface of peaches, leaves and weed from the treated and non-treated neighbour trees, (d) to determine the persistence of the CPA-8 strain on the personal protective equipment and motorised sprayer, and (e) to quantify the population of the CPA-8 strain on fruit during postharvest storage. Moreover, colonies phenotypically similar to *Bacillus* spp. were identified using the PCR technique.

MATERIALS AND METHODS

Biological control agent

The *B. amyloliquefaciens* strain CPA-8 (Gotor-Vila *et al.*, 2016) was obtained from the culture collection of the IRTA Centre (Lleida, Catalonia, Spain). This strain was initially isolated from the surface of a nectarine in an experimental orchard in Lleida (Catalonia, Spain) and was selected for its efficacy in controlling brown rot disease (Casals *et al.*, 2010).

Bacteria were cultured and formulated by fluid-bed spray-drying according to the work reported by Gotor-Vila *et al.*, (2017). CPA-8 was cultured in 2-L laboratory scale bioreactors (BioFlo/CelliGen 115, Eppendorf, New Brunswick, Canada) containing optimised growth medium containing 20 g L⁻¹ of the protein PROSTAR 510A (Brenntag Química, S.A.U., Barcelona, Catalonia, Spain). Then, CPA-8 cells were harvested by centrifugation at 9820 g for 12 min at 10 °C in an Avanti J-20 XP centrifuge (Beckman Coulter, CA, USA) and resuspended approximately at 10¹⁰ CFU mL⁻¹ in the same CPA-8 culture supernatant to include the antifungal lipopeptides synthesised by the bacteria during growth. Then, protecting agents (20 % sucrose and 10 % skim milk) were added to the cell solution, mixed with 3.5 g of pregelatinised potato starch as binder and fluidised with 300 g of maltodextrin as carrier material. A dried product was obtained by

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using a fluid-bed spray-dryer (Hüttlin GmbH, Bosch Packaging Technology Company, Schopfheim, Germany).

Preharvest treatments in the field

Field experiments were carried out in a flat peaches cv. '796' commercial orchard located in Malpartit (Alpicat, Catalonia, Spain). The BCA CPA-8 was sprayed onto flat peaches 3 days before harvest, on the 7th of July, 2016. A portable weather station was located inside the orchard for measuring temperature, relative humidity, wetness, and rate of precipitation, and was connected to a data logger for data registration.

To prepare an adequate CPA-8 concentration for the treatment, 40 g of dried cells ($9 \cdot 10^9$ CFU g⁻¹) were dissolved into 30 L of water to obtain a final adjusted concentration of 10^7 CFU mL⁻¹. Each tree was sprayed for 20 s (approximately 3 L) using a motorised sprayer (Benza P30P 2R, Ordes, Spain) at 15 bars of pressure.

The experimental design for each replicate consisted of 1 treated (T) and 3 non-treated trees (A, same row neighbour; B, same column in the next row neighbour; C, diagonal in the next row neighbour) and 3 replicates were evaluated (Fig. 1A). Each replicate was separated by 3 non-treated trees. Both treated and non-treated trees were used to monitor the dispersion, environmental Distribution, and the persistence of the CPA-8 preharvest treatment under field conditions. The sampling times were at 0 h (after letting the treatment dry) and at 1, 3 (fruit harvest for postharvest storage), 7, 14, 21, 28, 45, 60, 90, 120, 150 and 180 days after treatment. The last sampling time was in December when the leaves had already fallen.

Monitoring the dispersion and the aerial distribution of the CPA-8 preharvest treatment in the field

The dispersion of the CPA-8 treatment was monitored on the leaves and on the orchard floor using water-sensitive papers (WSPs, Singenta, Madrid, Spain) (Fig. 1B). The WSPs were positioned on the leaves of the outside and inside canopies of the treated and non-treated trees using a doubled-sided adhesive tape. The WSPs on the ground were attached to a petri plate using doubled-sided adhesive tape and were

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placed around the treated (1 WSP in each vertex of the square of the treated tree, outside of the canopy) and non-treated trees (1 WSP in line with the treated tree, outside of the canopy, with another located at medium the distance between the canopy of the treated and non-treated trees). Once the WSPs were completely dried, they were stored in a plastic bag until further analysis. WSPs were scanned with a 600 dpi imaging resolution, and the percentage of the covered surface was estimated using the ImageJ Software (ImageJ, US National Institutes of Health, <https://imagej.nih.gov/ij/>) in combination with the macro developed by Gil *et al.* (2016).

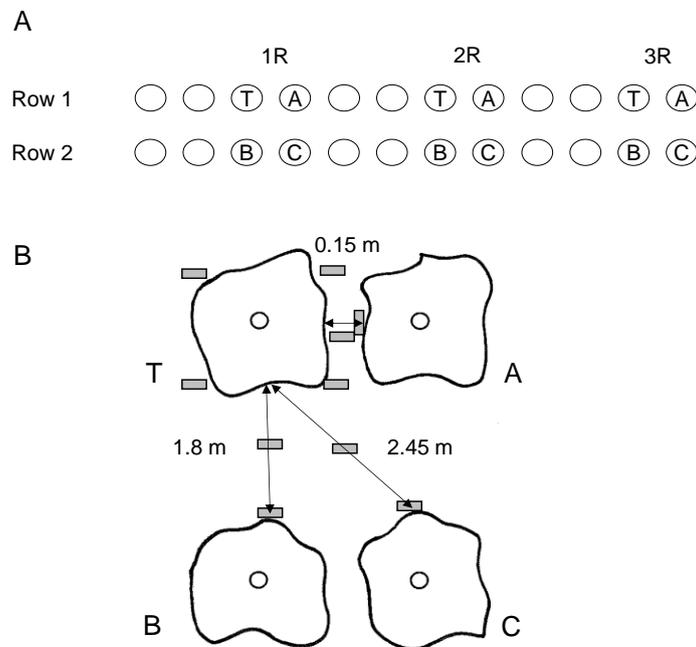


Figure 1. Experimental design of the CPA-8 treatment in the field. (A) The experimental design for each replicate consisted of 1 treated (T) and 3 non-treated trees (A, same row neighbour; B, same column in the next row neighbour; C, diagonal in the next row neighbour). A total of 3 replicates were evaluated, each one separated by 3 non-treated trees. (B) The dispersion of the CPA-8 treatment was monitored on the orchard floor using water-sensitive papers (WSPs) distributed around the canopy of the treated tree (4 WSPs), outside of the canopy of the non-treated trees (0.15, 1.8 and 2.45 m distance to non-treated trees A, B and C, respectively) and at medium distance between the canopy of the treated and non-treated trees. The same distribution was used to monitor the aerial conidia distribution.

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The aerial distribution and persistence were evaluated by gravimetry in the same points where the floor WSPs were located. Petri plates containing NYDA medium were left opened for 2 min. Plates were incubated at 30 °C for 24 h, and 10 % of the total colonies from each plate that were phenotypically similar to *Bacillus* spp. were selected for PCR identification.

Population dynamics of the CPA-8 preharvest treatment in treated trees

The population dynamics of the CPA-8 treatment was quantified in different plant tissues of the treated trees, including the fruit, leaves and weed. For the fruit, 5 fruits per replicate were harvested, and 8 pieces of peach peel (16 mm diameter) were removed with a cork borer from each fruit. These 40 pieces were dipped into 20 mL of phosphate buffer solution (0.05 mol L⁻¹ potassium phosphate, pH 6.5) in a sterile filter bag and were homogenised in a Stomacher (Seward, London, UK) set at 12 strokes s⁻¹ for 90 s. For the leaves, 10 leaves were harvested, and two pieces from each (19.6 mm diameter) were removed with a cork borer. These 20 pieces were dipped into 10 mL of phosphate buffer solution and homogenised in a Stomacher. For the weed, 1 gram of weed was collected from two different sample points, dipped into 10 mL of phosphate buffer solution and homogenised in a Stomacher. For all plant tissues, serial ten-fold dilutions of suspension samples were made thrice by dilution plating on NYDA media, which were incubated as described above. The results were expressed as CFU cm⁻² or CFU g⁻¹ depending on the sample material. Two colonies were selected from each dilution plate for PCR confirmation.

Persistence of the CPA-8 preharvest treatment in non-treated trees

The persistence of the CPA-8 strain in non-treated trees (A, B and C) was evaluated in different plant tissues, including the fruit, leaves, and weed. The sampling method used to detect the CPA-8 strain in fruit and leaves was the contact plating method using Rodac (replicate organism direct agar contact) plates containing NYDA medium. For each sampling time and replicate, three Rodac plates were used for each tissue sampled. The colonies were incubated for enumeration and then were selected as follows: for Rodac plates, if the plate contained more than 10 colonies that were morphologically similar to *Bacillus* spp., 40 % were selected for

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PCR confirmation. Otherwise, if the plate contained less than 10 colonies, all the colonies were confirmed by PCR.

The sampling method used to detect the CPA-8 strain in weed from the non-treated trees was dilution plating, following the same protocol described previously for weed of the treated trees. The plates were incubated for colony enumeration and were selected as described above for dilution plating.

Population dynamics of the CPA-8 during postharvest storage

The population dynamics of the CPA-8 strain on the surface of flat peaches was quantified during postharvest storage (0 °C and 85 % relative humidity (RH)) at 7, 15, 21, 28 and 45 days after harvest. Moreover, the CPA-8 population was also quantified after simulating the shelf-life period during the commercialisation process (20 °C and 85 % RH for 5 days after 28 and 45 days of cold storage). The population dynamics of CPA-8 on the fruit surfaces was determined by dilution plating, which, in addition to the number of colonies selected for confirmation by PCR, was done as previously described.

Moreover, the persistence of the CPA-8 strain in the plastic boxes used to store fruit after harvest was also evaluated after 7, 15, 21, 28 and 45 days of storage at 0 °C and 85 % RH using Rodac plates as described above. At the end of the postharvest storage, the plastic boxes were cleaned by immersion for 30 s in a 5 % soap water solution and were disinfected for 1 min in a 2 % sodium hypochlorite water solution. Four days later, the same protocol was repeated but increasing the percentage of sodium hypochlorite up to 10 %. Once the plastic boxes were air dried, the persistence of the CPA-8 was evaluated using the contact plating methodology. The colonies were incubated for enumeration and selected as previously described for contact plating methodology.

Persistence of the CPA-8 preharvest treatment in the personal protective equipments (PPE) and in the motorised sprayer

The persistence and survival of the CPA-8 strain in the PPE of the treatment applicator was estimated on gloves and on the working clothes using the contact

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plating method with Rodac plates containing NYDA medium. Both right- and left-hand gloves were sampled, and the working clothes were sampled from both arms and legs, using two plates for the stomach and two plates for the back, totalling twelve Rodac plates for each sampling time. In the case of the motorised sprayer, five Rodac plates were used to detect the persistence of CPA-8 before and after treatment, and after cleaning the sprayer with water. The plates were incubated for colony enumeration as previously described for the contact planting methodology.

Colony confirmation by PCR

DNA from CPA-8 was extracted using physical methods. Colony samples (2-5 mg) were introduced into a tube and were heated in the microwave at maximum voltage for 1 min and then cooled on ice for 5 min. Samples were diluted with 20 μ L of molecular grade water (Fisher Scientific, Madrid, Spain).

To identify the CPA-8 strain, the primer pair RBAM007760F (GTACCGATTGCAACAGGTTTAGATG) and RBAM007760R (CTGTTGCCCG GTTCGTC) described by Gotor-Vila *et al.* (2016) was used to amplify a 265 bp fragment. Each reaction had a final volume of 12.5 μ L and contained 1x Kapa 2G Robust Hot Start Ready Mix (Kapa Biosystems Inc., Wilmington, MA, USA), 500 nmol L⁻¹ of each primer and 2 μ L of extracted DNA. The amplification was carried out in a GeneAmp PCR System 2700 (Applied biosystems, Madrid, Spain) thermal cycler. The cycling conditions were as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 65 °C for 45 s and 72 °C for 30 s, followed by a final extension step of 72 °C for 7 min. Two negative controls were used, one without DNA and the other with *Bacillus subtilis* (Serenade, Bayer CropScience AG, Leverkusen, Germany), and one positive control with CPA-8. The products amplified by PCR were analysed by electrophoresis on a 1.2 % agarose gel using a 100 bp DNA ladder (Nippon Genetics Europe, Dueren, Germany).

Statistical analysis

All data were analysed for significant differences using analysis of variance (ANOVA) with JMP V.8 (SAS Institute Inc.). Statistical significance was defined as

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$P < 0.05$. When the analysis was statistically significant, t-test for two sample comparison or Tukey's test for the separation of means was performed.

RESULTS

Meteorological data

Meteorological data comprising the relative humidity (RH), temperature (T), wetness (W) and rainfall (R) are shown in Figure 2 for the experimental period from 04/07/16 to 12/12/16. Specifically, July, August, and September were characterised by a very warm and dry period (T mean = 24.4 °C, T max = 39.3 °C, RH mean = 57.1 % and RH min = 22.9 %). Moreover, during these three months, it only rained four times, with a rainfall accumulation of 15 mm and a wetness accumulation of 16.6 h after raining. October was a warm month (T mean = 16.2 °C, T max = 30.6 °C, RH mean = 77.9 % and RH min = 33.7 %), and during that time it rained five times with a rainfall of 31 mm and a subsequent wetness accumulation of 79.7 hours after raining. November and December were characterised by cold temperatures and high relative humidity (T mean = 8.3 °C, T min = 0.5 °C, RH mean = 86.2 % and RH min = 48.1 %). This high RH was accompanied with a high wetness accumulation (a total of 384 h during 1.5 months) mainly due to the presence of fog rather than rain accumulation (54 mm).

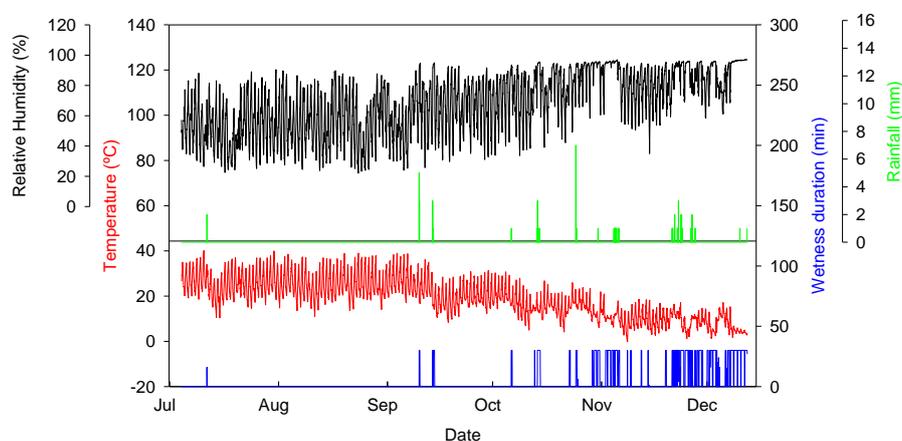


Figure 2. Meteorological data from the commercial orchard of flat peaches located at Malpartit (Catalonia, Spain). Temperature (°C, red), relative humidity (%), wetness duration (min) and rainfall (mm, green) were registered since the day of the treatment (04/07/2016) until the fall of the leaves (180 days after treatment, 12/12/2016) every 30 min.

Assessment of spray dispersion using water-sensitive papers

The spray dispersion of the CPA-8 treatment was measured using WSPs located on the leaves (Fig. 3A) and on the ground (Fig. 3B). Significant differences were observed between the area covered from the papers located on the leaves from treated trees (T) and on the leaves from the non-treated trees (A, B or C). While the application of the sprayed CPA-8 strain covered 100 % of the surface of WSPs that were inside and outside of the canopy leaves of the treated trees, less than 1 % of the area was covered in WSPs from the canopy leaves of the non-treated trees.

The dispersion of the CPA-8 treatment on the ground was represented as the relationship between the distance to the canopy of the treated and non-treated trees and the coverage area of the WSPs (Fig. 3B). It was observed that a larger area of the papers was covered for WSPs located at distances shorter than 0.5 m, corresponding to the ground WSPs around the treated trees and the non-treated tree A. The WSPs located at a medium distance between the non-treated trees B and C showed a medium area of coverage (in a range of 1 to 38 %) while the WSPs located in the most distant area (2.45 m) showed less than 5 % of area coverage.

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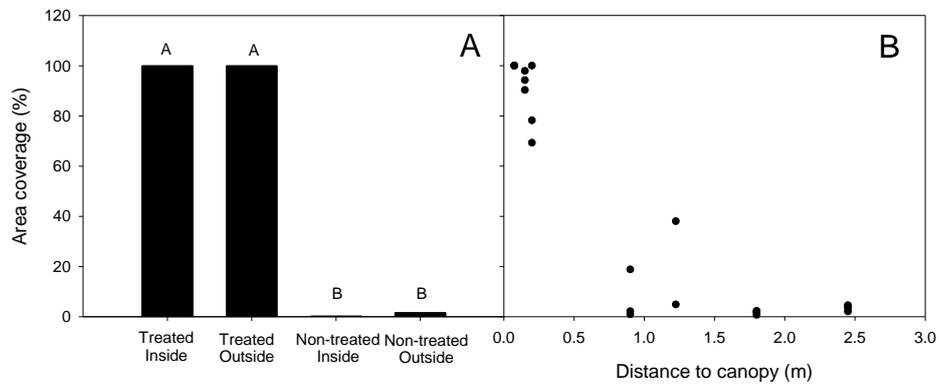


Figure 3. Percentage area coverage with CPA-8 spray treatment obtained from the analysis of water sensitive papers (WSPs) using Image J Program. The WSPs were located (A) on the leaves of the outside and inside canopy of treated and non-treated trees and (B) on the ground around the treated and non-treated trees at different distances. Different letters indicate significant differences using Tukey's test ($P < 0.05$).

Aerial distribution of the CPA-8 strain at different distances to the canopy

The aerial distribution of the CPA-8 strain was studied in relation to the application distance to the canopy and the sampling day after treatment (Fig. 4). Just after the application of the treatment (0 d), it was observed that CPA-8 was distributed randomly around both the treated and non-treated trees, even at the farthest application distance to the canopy (non-treated tree C = 2.45 m). The general pattern observed over the next sampling days was similar, with a higher colony accumulation (the sum of all colonies ranged from 77 to 160 colonies plate⁻¹) at distances shorter than 0.5 m, corresponding to the area around the treated trees and the non-treated tree A. At greater distances, CPA-8 colonies were also detected. In contrast, after 90 days of treatment it was observed a decrease in the number of aerial colonies at all checked distances. At distances shorter than 0.5 m, a decrease in the number of aerial colonies was observed, from 28 colonies plate⁻¹ to just 1 colony plate⁻¹ after 90 and 180 days after treatment, respectively. At distances greater than 0.5 m, the decrease was from 22 colonies plate⁻¹ (90 days after treatment) to 6 colonies plate⁻¹ (150 days after treatment) (data not shown), and none was observed 180 days after treatment. It was remarkable that on the last sampling day (180 days after

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treatment) only one colony of CPA-8 was detected. Figure 5A and 5B show the phenotypic morphologies of CPA-8 colonies detected from air samples of treated and non-treated trees, respectively, and Figure 5C shows the PCR confirmation of these samples.

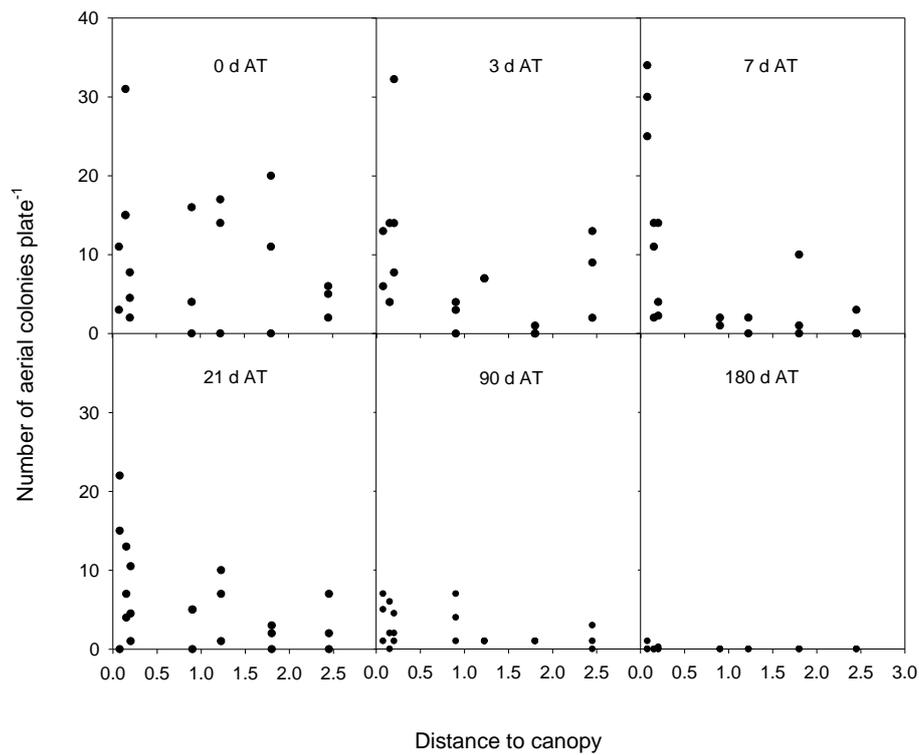


Figure 4. Quantification of aerial colonies of the CPA-8 in relation to the distance to the canopy of the treated and non-treated trees at different times after treatment (AT). Colonies were quantified using the gravimetric methodology and colonies morphologically similar to *Bacillus amyloliquefaciens* were selected for CPA-8 confirmation.

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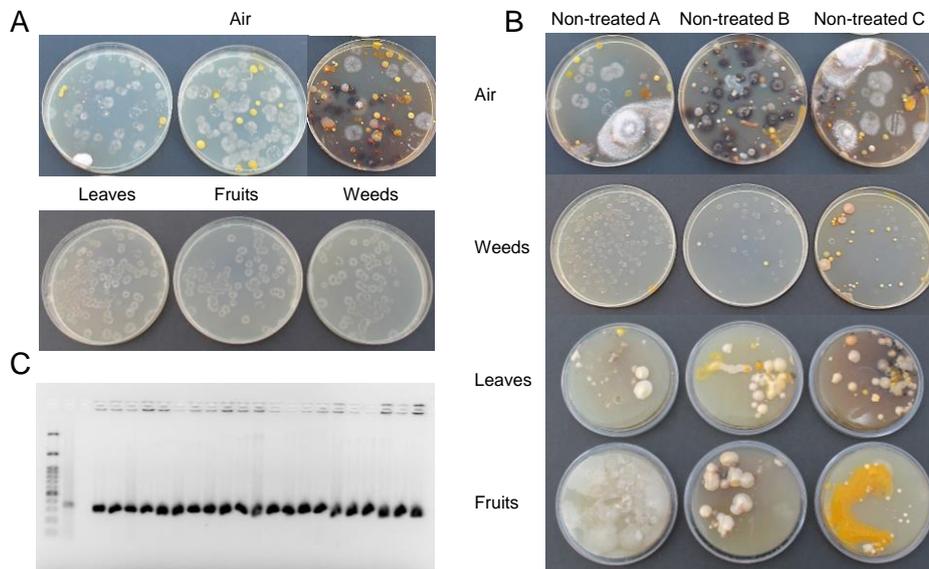


Figure 5. Plate sampling from air, leaves, fruit, and weed from (1) treated and (2) non-treated trees that showed the presence of colonies morphologically similar to *Bacillus amyloliquefaciens*. (3) Example of sample confirmation as CPA-8 by polymerase chain reaction (PCR).

Persistence of the CPA-8 strain on the treated trees

The population dynamics of the CPA-8 strain on fruit, leaves, and weed of the treated trees were measured by dilution plating and confirmed by PCR (Figs. 6 and 7). Just after the preharvest application of the CPA-8 treatment, the population level of the bacteria on fruit surfaces was $4.13 \log_{10}$ CFU cm^{-2} and remained stable for 3 and 45 days under field or postharvest conditions, respectively (Fig. 6). At postharvest, no statistical differences were observed between the population levels of CPA-8 after 28 days at cold storage and 28 days at cold storage with a shelf-life period of 5 days. However, a lower population of the CPA-8 strain was observed at 45 days at cold storage conditions ($4.00 \log_{10}$ CFU cm^{-2}) in comparison to 45 days at cold storage with a shelf-life period of 5 days ($4.24 \log_{10}$ CFU cm^{-2}).

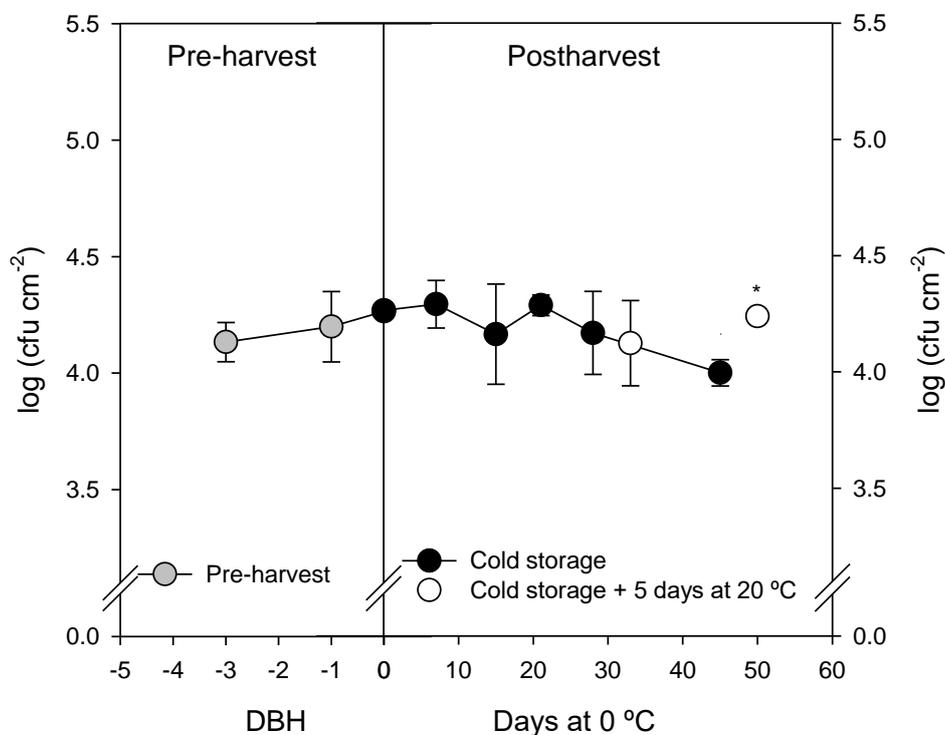


Figure 6. Population dynamics of CPA-8 on flat peaches surface quantified by dilution plating and confirmed by PCR. The CPA-8 treatment was sprayed 3 days before harvest (DBH). After harvest, fruit was stored at cold conditions (0 °C and 85 % RH) for 7, 15, 21, 28 and 45 days. After 28 and 45 days of cold storage, fruit was exposed to 20 °C and 85 RH for 5 days, simulating shelf-life conditions. Each point represents the mean of three biological replicates and error bars represent the standard deviation of three biological replicates. * indicates significant differences according to t-test ($P < 0.05$).

The initial population of CPA-8 on leaves from the treated trees was $4.43 \log_{10}$ CFU cm^{-2} , remaining stable until 21 days after treatment (Fig. 7). At this time, the population levels of the CPA-8 strain progressively decreased until 120 days after treatment ($1.88 \log_{10}$ CFU cm^{-2}) and remained stable until the last sampling time after 180 days of the treatment ($1.68 \log_{10}$ CFU cm^{-2}).

In the case of the weed, the initial population of CPA-8 was $6.26 \log_{10}$ CFU g^{-1} fresh weight (FW) and remained stable until 21 days after treatment ($5.53 \log_{10}$ CFU g^{-1} FW) (Fig. 7). Afterwards, the CPA-8 population decreased until $3.2 \log_{10}$ CFU g^{-1} FW, detected at 90 days after treatment. It is interesting to note the differences observed in the population levels among biological replications. This could be due to

the presence of weed at the beginning of the trials and the growth of new weed over the time-course of the sampling.

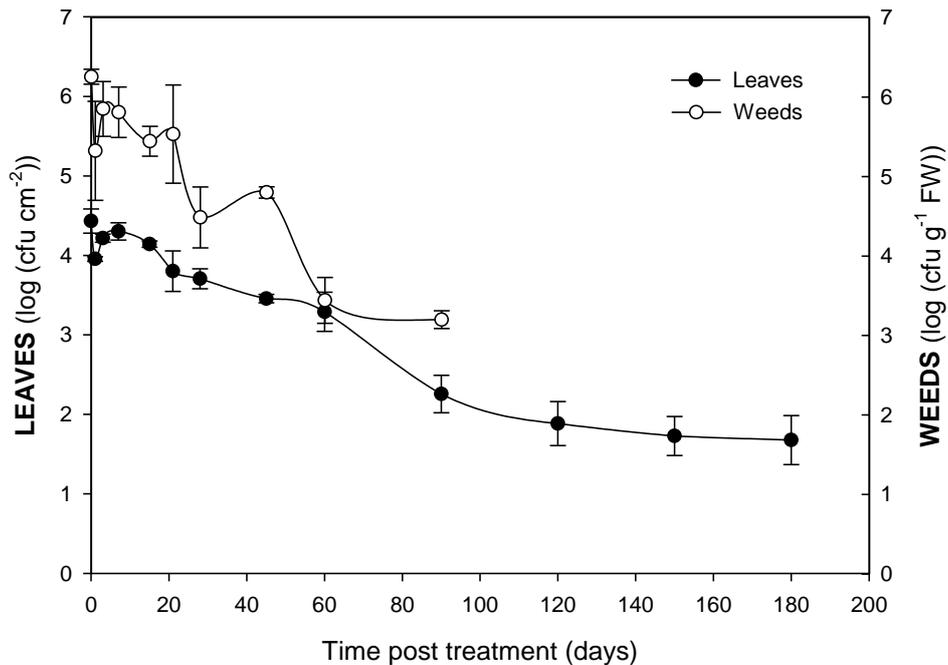


Figure 7. Population dynamics of CPA-8 on leaves (log CFU cm⁻²) and weed (log CFU g⁻¹ fresh weight) of the treated trees quantified by dilution plating and confirmed by PCR at different sampling times. Each point represents the mean of three biological replicates and error bars represent the standard deviation of three biological replicates.

Figure 5A shows the phenotypic morphologies of CPA-8 colonies obtained from the leaves, fruit and weed of treated-trees, and Figure 5C shows the PCR confirmation of these samples.

Persistence of the CPA-8 strain on non-treated trees

The persistence of the CPA-8 strain in the non-treated trees was evaluated using printings on fruit and leaves and by using dilution plating methodology for weed (Table 1 and Fig. 8). In fruit, after just 0 and 1 day of CPA-8 application, 89 % of Rodac plates showed colonies that were phenotypically similar to CPA-8, which could be mostly confirmed (100 and 87 %, respectively) (Table 1). After 3, 7 and 15 days of

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treatment, 100 % of sampled plates showed colonies that were phenotypically similar to CPA-8, and they were all confirmed as well. The persistence of CPA-8 on the surfaces of fruit under field conditions was probably longer than 15 days, however, no more sampling times were analysed due to the over-maturity of the fruit.

Table 1. Sampling plates from fruit and leaves of the non-treated area that showed similar morphology to CPA-8 and that were confirmed as CPA-8 using polymerase chain reaction (PCR).

Plant material	Days after treatment (d)	Morphologically similar to CPA-8		CPA-8 confirmed by PCR	
		Plate ratio ^a	Percentage (%)	Plate ratio ^b	Percentage (%)
Fruits (n = 9)	0	8/9	89	8/8	100
	1	8/9	89	7/8	87
	3 (harvest)	9/9	100	9/9	100
	7	9/9	100	9/9	100
	15	9/9	100	9/9	100
Leaves (n = 9)	0	9/9	100	9/9	100
	1	7/9	78	7/7	100
	3 (harvest)	9/9	100	9/9	100
	7	9/9	100	9/9	100
	15	9/9	100	8/9	89
	21	9/9	100	9/9	100
	28	9/9	100	9/9	100
	45	9/9	100	9/9	100
	60	9/9	100	9/9	100
	90	9/9	100	9/9	100
	120	6/9	67	6/6	100
150	7/9	78	7/7	100	

^a Ratio of plate number with similar morphology to CPA-8 in relation to the total number of sampled plates.

^b Ratio of plate number confirmed as CPA-8 using PCR methodology in relation to the total number of sampled plates.

In leaves sampled up until 90 days after treatment, a general pattern was observed in which most of the Rodac plates yielded colonies that were morphologically similar to CPA-8, and they were all confirmed as the CPA-8 strain (Table 1). However, after 120 days, a decrease in the number of plates containing colonies similar to CPA-8 was observed, which was even more pronounced 180 days after the CPA-8 treatment (when only one of the plates showed the presence of colonies that were morphologically similar to CPA-8). It is noteworthy that all the colonies that were morphologically similar to CPA-8 were confirmed except one, which was sampled 15 days after the CPA-8 application

Figure 8 shows the population dynamics of the CPA-8 strain on weed from non-treated trees being differentiated from the non-treated trees A, B and C. The same

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tendency in the population levels during the time course of the different non-treated trees was observed. The population levels decreased 1 day after treatment, increased between 3 and 7 days after treatment and finally start falling again until being maintained during the last sampling times (15-28 days after treatment). However, probably due to the important differences between the biological replicates, no statistical differences in the population levels during the time course were observed. The initial population levels detected in the weed of the non-treated tree A were higher (6.10 CFU g⁻¹ FW) than those detected in the non-treated trees B and C (3.96 and 3.84 CFU g⁻¹ FW, respectively). During the first sampling times (1-7 days after treatment) the population levels detected in the weed of the non-treated tree A were also higher than those detected in B and C.

Figure 5B shows the phenotypic morphologies of the CPA-8 colonies detected from leaves, fruit and weed of treated-trees, and Figure 5C shows the PCR confirmation of these samples.

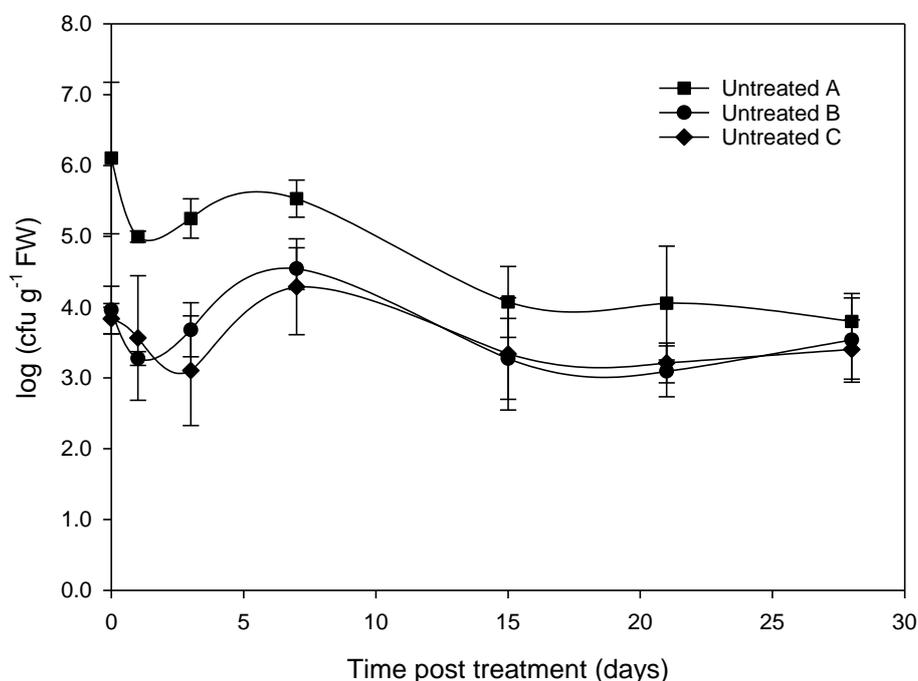


Figure 8. Population dynamics of the CPA-8 on weed of non-treated trees (A, B and C) quantified by dilution plating and confirmed by PCR at different sampling times. Each point represents the mean of three biological replicates and error bars represent the standard deviation of three biological replicates.

Persistence of the CPA-8 strain on the personal protective equipment, on the motorised sprayer and postharvest plastic boxes

On the gloves and clothes worn by handlers, the CPA-8 strain was detected and confirmed on all the plates from the day of the treatment until the last sampling time (180 days after treatment) (data not shown). In the case of the motorised sprayer, CPA-8 was not detected before the treatment (data not shown). However, once CPA-8 was applied, it was detected and confirmed for all sampled plates. Similar results were obtained after washing the motorised sprayer with water.

The CPA-8 strain was also detected and confirmed in all sampled plates from the plastic boxes that contained the fruit during postharvest storage (7, 15, 21, 28 and 45 days at 0 °C and 85 % RH after treatment) (data not shown). Furthermore, while the disinfection of the plastic boxes with 2 % sodium hypochlorite did not affect the

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presence of CPA-8, the re-disinfection with 10 % sodium hypochlorite partially eliminated the presence of CPA-8 colonies in 66.7 % of the total sampling plates. Seven days after the re-disinfection, CPA-8 was not detected in any plastic boxes (data not shown).

DISCUSSION

The efficacy of the preharvest and postharvest application of the BCA *Bacillus amyloliquefaciens* CPA-8 to control brown rot in stone fruit has already been demonstrated (Gotor-Vila *et al.*, unpublished results; Yáñez-mendizábal *et al.*, 2001). However, to assure its efficacy after field treatment, it is necessary to know the ecological fitness of this bacterium, which is determined by the physical and microbiological environment (Pujol *et al.*, 2007). Monitoring the ecological fitness is necessary for registration procedures since it is required to determinate its traceability, persistence and environmental impact (Montesinos, 2003). Different methodologies have been used to assess the ecological fitness of BCAs. Taking advantage of the PCR technique developed by Gotor-Vila *et al.* (2016) it was possible to distinguish CPA-8 from the non-target microbiota.

In this study, more than 1800 samples that were phenotypically similar to CPA-8 were analysed, and only 10 of them gave a negative result by PCR confirmation. These results demonstrated that the plate counting methodology was suitable for monitoring the CPA-8 strain primarily due to the characteristic colony morphology, thus making it difficult to confuse within the other microbiota. No colonies phenotypically similar to CPA-8 strain were detected in the orchard before CPA-8 application. Moreover, the fast growth of CPA-8 allowed incubation times of 24 h. In contrast to the results obtained in this study, in the monitoring of the BCA *Pantoea agglomerans* CPA-2 strain in a citrus fruit orchard, the use of the PCR technique was practically indispensable, since the target microorganism was phenotypically similar to other species in the field, specifically *Pantoea ananatis* (Soto-Muñoz *et al.*, 2015).

The dispersion of the treatment is one of the most important factors to take into account, since a correct spray can improve the accuracy and the efficiency of the

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treatment, thus achieving a more uniform spray deposition and avoiding loss of product and environmental contamination (Marçal & Cunha, 2008). For this reason, it is also important to monitor the spray treatment in treated and non-treated areas. In this study, the sprayed area of the WSPs was 100 % when they were located on leaves outside and inside of the canopy of treated trees, while less than 1 % of the covered area was detected on leaves of non-treated trees. These results agreed with those obtained by Soto-Muñoz et al (2015) in a sprayer application of the BCA *P. agglomerans* CPA-2 in a citrus fruit orchard, despite the different training systems. However, both results are quite different from that obtained when using a chemical fungicide to control fruit diseases caused by species such as *Monilinia* spp. Syngenta Crop Protection AG (Basel, Switzerland) recommends at least 50-70 droplets cm⁻² (Zhu et al., 2011) and that a total coverage area of approximately 15 % should be enough for satisfactory fungicide applications (Deveau, 2016). These important differences in area coverage observed between the biocontrol applications and chemical treatments could be related with the mode of action of each product. While most chemical fungicides are systemic, *B. amyloliquefaciens* CPA-8, as other BCAs, needs a high population and viability to effectively colonise the fruit surface (Gotor-Vila et al, unpublished results). In the case of the WSPs located on the floor, an inverse relationship was observed between the distance to the canopy and the area of coverage, as well as in the number of air colonies at different sampled times. Only just after the treatment was a high density of aerial colonies observed around all the treated and non-treated areas.

The population dynamics of the CPA-8 strain were evaluated at different time courses on the fruit, leaves, and weed of treated trees to observe the presence and persistence of this BCA on different plant tissues, and to evaluate its adaptation to field conditions. This study demonstrated that CPA-8 was able to maintain the same population level for 3 days under field conditions and for 45 days at postharvest storage conditions. Previous studies with the BCA *P. agglomerans* CPA-2 on citrus demonstrated that the bacterial population remained stable after preharvest applications at optimal levels for 20 days under field conditions (Cañamás et al., 2008). In addition, when fruit from inside and outside of the canopy were analysed separately, a higher bacterial population stability was observed on fruit from the

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inside part (Soto-Muñoz *et al.*, 2015). The authors attributed these results to the decreased exposure of the fruit to environmental factors, such as UV radiation, wind, humidity, and high temperature. In this study, the field conditions recorded during the trials were very warm and dry, achieving maximum temperatures of 40 °C and a minimum RH of 22 %, characteristics from a cold semi-arid climate as classified by Köppen (Peel *et al.*, 2007). Gotor-Vila *et al.*, (unpublished results) demonstrated that CPA-8 is a microorganism that is highly adapted to high temperatures under *in vitro* conditions, with stable viability on stone fruit surface exposed to 0 and 20 °C and different RH values (85, 60 and 40 %). However, the population levels of the yeast *Candida sake* on treated grapes decreased when they were exposed to high temperatures (40 and 45 °C) and 30 % RH. In the case of *Candida oleophila* strain O on the surface of apples under postharvest conditions (low temperature and ultra-low oxygen concentrations) the viability was maintained over 60 days (Massart *et al.*, 2005). The BCA *Penicillium oxalicum* isolate 121 is adapted to dry and warm conditions, having an optimal growth range between 15 and 30 °C, but it was not able to grow under 4 °C or above 35 °C (Pascual *et al.*, 1997). Overall, depending on the microorganism (bacteria, yeast or fungi) these environmental factors could affect their viability to a greater or lesser degree. This is the reason why many studies have been conducted based on the adaptation of the microorganisms to improve their efficacy (Droby *et al.*, 2016).

In the phyllosphere and in the weed, the population levels of CPA-8 showed a high dependence on environmental conditions. This study determined that population levels of CPA-8 remained stable until 21 days after treatment and then started falling. Despite the leaf surface being characterised by low nutrient availability and high exposure to environmental conditions (Lindow & Brandl, 2003), CPA-8 maintained a stable population level of 1.68 log₁₀ CFU cm⁻² until the fall of the leaves, 180 days after treatment. Some authors have correlated this decrease to the stressful environmental conditions that stimulate the entry in a viable, but unculturable bacterial state (Pujol *et al.*, 2007). In this study, the decrease in bacterial populations on leaves could be related to the rainfall events accompanied with lower temperatures. Some authors demonstrated that the first 20 mm of rain considerably

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reduced the population of the BCAs from the fruit surface (Calvo-Garrido *et al.*, 2014b; Gotor-Vila *et al.*, unpublished results). Similar tendencies were observed in the reduction of chemical treatments on grapes and vine leaves due to rainfall (Cabras *et al.*, 2001). However, in blossoms a high and stable population of the bacteria *Pseudomonas fluorescens* EPS62e was observed, and it was even able to increase in population during the first days after treatment (Pujol *et al.*, 2006; Pujol *et al.*, 2007). It is interesting to note that the weather conditions in both Mediterranean and Atlantic climates during spring were not as extreme as in the summer and can boost the colonisation of flowers to achieve an efficient control of fire blight.

Although the sprayed treatment seemed not to be dispersed on the leaves from the non-treated trees, CPA-8 was detected on both leaves and fruit over all analysed sampling times. While small drops of the treatment were not detected in WSPs at long application distances (Salyani *et al.*, 2013), CPA-8 could be most likely be detected due to the small size of the bacteria. These results indicate the high dispersion of the CPA-8 strain in the field and its efficient colonisation and adaptation on fruit and leaf surfaces. In the case of the weed, higher population levels of CPA-8 were observed in the non-treated tree A compared to B or C. Moreover, the population levels detected in the non-treated tree A were quite similar to those detected in the weed around the treated tree. These results are consistent because the non-treated tree A is closely located to the treated tree. The differences between the population levels detected in the weed of the non-treated tree A were approximately 100-fold higher than the ones detected in the non-treated trees B or C. Other authors also detected differences of approximately 10-100-fold in the BCA population levels between the treated and non-treated trees, probably because BCAs better colonised the niches compared to the indigenous microbiota (Johnson *et al.*, 2000; Lindow & Brandl, 2003; Pujol *et al.*, 2007). Pujol *et al.* (2007) even detected the BCA in non-treated flowers 15-35 m far away from the treated trees.

The persistence of the CPA-8 strain in inert materials, such as PPE (clothes and gloves) was detected until 180 days after treatment. It is necessary to remark that these PPE were conveniently stored indoors. In the case of the BCA *P. agglomerans* CPA-2, Soto-Muñoz *et al.* (2015) found that the persistence on working clothes was less than 7 days. It is known that *Bacillus* endospores are extremely persistent, being viable in soils for a long time (Nicholson *et al.*, 2000). Wood *et al.* (2015) showed that

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Bacillus subtilis was found on glass, wood, concrete, and topsoil up to 56 days after inoculation. However, the viability was reduced when these materials (except topsoil) were exposed to UV radiation. In the case of the postharvest pathogen *Monilinia fructicola*, the conidia viability on inert surfaces was higher at cold storage temperatures (0 °C) than at 20 °C (Bernat *et al.*, unpublished results). However, despite the high persistence of CPA-8, it was also found that the disinfection of postharvest plastic boxes with a 10 % of bleach solution just for one minute significantly reduced the viability of this microorganism.

In conclusion, this work revealed the good adaptation of the BCA CPA-8 to field and postharvest conditions for optimal management of *Monilinia* spp. Moreover, the ability of CPA-8 to spread from treated to non-treated trees was also demonstrated, thus allowing a better homogeneity of the treatment and colonisation of the habitat. This knowledge could be used in the near future to not only to improve the BCA-based products applied but also to design more detailed protocols for the control of disease (permitting better adjustment of the treatment doses and calendar applications). Finally, crucial information for BCA registration purposes has been obtained.

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