Chapter VII

Biological control of brown rot in stone fruit using
Bacillus amyloliquefaciens CPA-8 under field conditions

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ABSTRACT

Different treatments based on the biocontrol agent (BCA) *Bacillus amyloliquefaciens* CPA-8 to control brown rot under field conditions were evaluated as alternative to chemical applications. As part of a well-designed disease program that enables the integration of BCAs into cropping systems, testing of the sensitivity of *Monilinia laxa* and *Monilinia fructicola* at different doses of CPA-8 were conducted in stone fruit. CPA-8 dose of 10^7 CFU mL^{-1} reduced more than 60.0 and 75.5 % of brown rot incidence and severity, respectively. Once in the orchard, different degree of biocontrol activity was obtained depending on the inoculum pressure, which was mainly associated with meteorological conditions. Under drastic disease pressure, neither CPA-8 treatment nor the chemicals controlled the disease at harvest and only the chemical treatment reduced postharvest brown rot incidence. However, when *Monilinia* spp. incidence was close to the standard levels recorded in the area, treatments based on CPA-8 formulations proved to be efficacious. At harvest, BA3, BA4 treatments (CPA-8 optimised products) and PF+BA3 treatment (CPA-8 combined with *Penicillium frequentans*) reduced *Monilinia* spp. incidence compared to the control (54.7-64.1 %) although less than the chemicals (90.6 %). At postharvest, almost all CPA-8-based treatments (except PF+BA3) controlled the pathogen with BA4 treatment being as much effective as the chemicals (50.3 % of disease reduction). Finally, the population dynamics of CPA-8 on treated fruit surface remained after treatment application, at harvest and at postharvest shelf-life (>10^4 CFU cm^{-2}). This study highlights the potential of *B. amyloliquefaciens* CPA-8 as alternative or complementary strategies to control *Monilinia* spp.

Keywords: Postharvest, biocontrol, formulation, dose of application, timing, population dynamics.

INTRODUCTION

Brown rot caused by *Monilinia*. spp. is the most significant postharvest disease of stone fruit causing losses as high as 80 % of the production in years when the climate conditions are favourable to disease development (Usall *et al.*, 2015). The risk of infection increases considerably during harvest, transport, and storage processes and synthetic fungicides such as cyprodinil, fenhexamid, fludioxinil, boscalid, and triazole-like compounds have been traditionally used during bloom or preharvest to prevent postharvest decays. However, the proliferation of fungicide-resistant population of pathogens and public concerns about health risk and environmental contamination have promoted the search for alternative methods to chemical applications (Droby *et al.*, 2016; Usall *et al.*, 2016a). Among the control strategies, the use of antagonistic microorganisms has been the focus of considerable research over the last decades (Droby *et al.*, 2016; Usall *et al.*, 2016b) although its use is not already routinely applied in fruit industry.

Among the biological control agents (BCAs), *Bacillus amyloliquefaciens* CPA-8, has been reported as an effective antagonist against postharvest brown rot caused by *Monilinia* spp. (Casals *et al.*, 2012; Yánez-Mendizábal *et al.*, 2011) based on its capability of production of powerful antifungal metabolites such us fengycin-like lipopeptides (Yánez-Mendizábal *et al.*, 2012b). Moreover, the volatile organic compounds (VOCs) emitted by this strain have been recently described to play an important role in controlling postharvest fruit pathogens such as *Monilinia* spp. and *Botrytis cinerea* (Gotor-Vila *et al.*, 2017a). As part of the genus *Bacillus*, its spore forming ability also provides high resistance to extreme environmental conditions, making CPA-8 a good candidate for developing stable and efficient BCA products (Gotor-Vila *et al.*, 2017c ; Yánez-Mendizábal *et al.*, 2012a).

Microbial antagonists are applied either, before or after harvest. However, the control of postharvest diseases should be focused on the orchard as the application of BCAs after harvest may be too late to effectively compete with the decay pathogens already established in fruit (Moretto *et al.*, 2014). Moreover, the efficacy of the microbial antagonist(s) can be enhanced if they are used with alternative strategies such as low risk chemical compounds (i.e. inorganic salts, plants extracts or volatile and essential oils) or physical treatments like hot water dipping, radio frequency, microwave

energy or irradiation with far UV-light, at postharvest (Karabulut *et al.*, 2010; Mari *et al.*, 2016; Palou *et al.*, 2016; Sisquella *et al.*, 2014). Otherwise, mixed cultures of microbial antagonists could provide better control of postharvest diseases over individual cultures or strains (Sharma *et al.*, 2009). Other microorganisms have been reported as effective antagonists against brown rot disease. Guijarro *et al.*, (2007) demonstrated the effective control of *Penicillum frequentans* strain 909 against *Monilinia* spp. mainly based on competition for space and nutrients (Guijarro *et al.*, 2017). Thus, the efficiency of one BCA would be enhanced with the addition of other BCAs to finally obtain a combined action which could be additive or synergistic.

Economical production of large quantities of microorganism and formulation strategies that ensure reasonable shelf-life and maintain efficacy during long-term storage are fundamental factors in the process of developing and commercialisation of BCAs (Droby *et al.*, 2016; Teixidó *et al.*, 2011). Recently, CPA-8 has been successfully formulated by fluid-bed spray-drying (Gotor-Vila *et al.*, 2017b; Gotor-Vila *et al.*, 2017c) an innovative technology (commonly used in the pharmaceutical industry) which operates with a large air volume and low temperatures. Besides, two optimised formulations proved to be efficacious against *Monilinia* spp. in a wide range of stone fruit (i.e. peach, nectarine, apricot, plum, flat peach, cherry), which enables to broaden the spectrum of action of CPA-8 making the process of BCA's commercialisation more successful (Gotor-Vila *et al.*, 2017c).

Nevertheless, an antagonist applied in the field is frequently subjected to severe environmental conditions that may drastically limit BCAs establishment on a host target site (Cañamás *et al.*, 2008). Few studies have evaluated the effect of abiotic factors interfering with the survival of CPA-8 such as temperature, relative humidity (RH) and wash-off caused by simulated rainfall (Gotor-Vila et al., unpublished results). However, small-scale experiments may change under a natural environment. The step wise screening of microorganisms for commercial use in biological control needs full field testing (including disease control in crops with complete common plant protection schedules) and a well-designed disease program that enables the integration of BCAs into cropping systems (Köhl *et al.*, 2011). In order to do this, this work aimed to assess the potential of different formulations of *B. amyloliquefaciens* CPA-8 for brown rot control under commercial peach production. Specifically, we studied (i) the suitable CPA-8 dose of treatment, (ii) the CPA-8 population dynamics on fruit once applied in the

orchard, (iii) the CPA-8-based treatments efficacy in controlling *Monilinia* spp. incidence at harvest and postharvest time and (iv) the possibility to combine CPA-8 with *P. frequentans* 909.

MATERIALS AND METHODS

BCA isolation, production and formulation

B. amyloliquefaciens CPA-8, formerly *Bacillus subtilis* (Gotor-Vila *et al.*, 2016), was originally isolated from a nectarine surface and belongs to the Postharvest Pathology Group Collection of IRTA (Lleida, Catalonia, Spain). Bacteria were produced and formulated by fluid-bed spray-drying according to the works conducted by Gotor-Vila *et al.* (2017b) and Gotor-Vila *et al.* (2017c). CPA-8 was mass produced in 2 L (BioFlo/CelliGen 115, Eppendorf, New Brunswick, Canada) laboratory scale bioreactors containing optimised growth medium based on extracted defatted soy flour, DSF (100 g L^{-1}) or protein PROSTAR 510A (20 g L^{-1}). Therefore, 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 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. Then, the protecting agents were added to the cell solution, mixed with 3.5 g of pregelatinised potato starch as binder, and fluidised with 300 g of carrier material. The dried product was obtained by using a fluid-bed spray-dryer (HüttlinGmbH, Bosch Packaging Technology Company, Schopfheim, Germany) with a 0.8 mm nozzle in bottom-spray position and applying a spraying air pressure of 80 kPa. Inlet air temperature was set to 65 °C which resulted in a maximal product temperature of 42 °C depending on the spraying rate, which ranged between 4 and 4.5 g min^{-1}.

CPA-8 fresh cells (treatment called BA) and formulated CPA-8 cells (non-amended or amended with $MgSO_4 \cdot 7H_2O$ as protectant) were dried by using potato starch as carrier material and applied in the field trials conducted in 2014 (treatments BA1 and BA2, respectively). For the growing season of 2015, optimised formulations were developed based on the protective combination of 20 % sucrose 10 % skimmed milk and fluidised with either, maltodextrin (treatment BA3) or potato starch (treatment BA4), as carrier material.

CPA-8 dose of treatment optimisation

A laboratory assay to determinate the most suitable dose applicable for CPA-8 treatments was conducted on 'Big Top' nectarines (*Prunus persica* var. nectarine (Ait.) Maxim.) and 'Corona' peaches (*P. persica* (L.) Batch) against *Monilinia laxa* or *Monilinia fructicola*, respectively. The treatments were based on the optimised CPA-8 formulations (BA3 and BA4) adjusted at 10^6, $5 \cdot 10^6$ and 10^7 CFU mL^{-1}. Their efficacy was compared to untreated fruit (water as control, CK). Fruit with no visible injuries and similar in size and maturity was 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}. After air-drying, a 15 µL suspension of each treatment was applied. Five fruits constituted a single replicate and each treatment was replicated four times. The percentage of fruit infected (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.

Field trials and experimental design

Four field trials were carried out in four peach commercial orchards located in Lleida area (Catalonia, Spain): Alguaire, Alfarràs, Sudanell and Albesa over two growing seasons, 2014 and 2015 (Table 1).

Table 1. Characteristics of the orchards used in 2014 and 2015 seasons for CPA-8 field experiments.

Orchard	Location	Coordinates	Variety	Growing season
Orchard01	Alguaire	41.755150N-0.591865E	'Jesca' Peaches	2014
Orchard02	Alfarràs	41.833624N-0.531087E	'Xuclà' Peaches	2014
Orchard03	Sudanell	41.551752N-0.574049E	'Red Jim' Nectarines	2015
Orchard04	Albesa	41.779151N-0.629172E	'Roig d'Albesa' Peaches	2015

Different cultivars of peach and nectarine were used (Table 1). Plots were distributed in a completely randomised block design with four replicates per treatment. Each replicate consisted in 3-4 trees (depending on the number of fruits per tree). Barrier trees (non-treated trees) were used to separate treatments and replicates. Treatments consisted of CPA-8 formulations, alone or combined with *P. frequentans* (PF) formulations, and two control treatments: one based on chemical applications (Q) and another one based on non-

treated trees (CK). The CPA-8 concentration was adjusted according to CPA-8 dose optimisation (section above). During 2014 season, a treatment based on CPA-8 fresh cells was also applied. *P. frequentans 909*, originally obtained from peach twigs in an experimental orchard in Madrid (Spain) was identified and provided by the Department of Plant Protection of INIA (Madrid, Spain). Oil diluted PF formulations used in this study were provided by Bayer CropScience Biologics GmbH, (Malchow, Germany). The PF dose of application in the orchard was adjusted at 10^6 conidia mL^{-1}. The treatments and timing of applications are summarised in Table 2.

All treatments were applied four times following the recommended schedule for controlling brown rot in the area: 30, 14, 7 and 3 days approximately before harvest. The date of the applications was arranged according to the weather events. All treatments were applied in the morning with a backpack sprayer (operating pressure of 15 bar and hollow cone nozzle of 1.8 mm). Each tree was sprayed for 16 s (approximately 3 L). Orchards received the standard cultural and crop protection practices until 45 days before harvest. A weather station (Decagon Services Inc., Pullman, WA, USA) was placed into the field trials to hourly record weather observations of temperature (T), relative humidity (RH) and rainfall (mm).

Table 2. Treatments applied in CPA-8 field experiments.

Orchard	Treatment	Treatment description	Date of application
	CK	No treatment applied	-
	Q	Cyproconazole	04.09.14
		Iprodione	09.09.14
Orchard01		Tebuconazole	25.09.14
	BA	CPA-8 fresh cells	04.09.14/09.09.14/25.09.14
	BA1	Non-amended CPA-8 formulated cells	04.09.14/09.09.14/25.09.14
	BA2	CPA-8 formulated cells with 10 % $MgSO_4 \cdot 7H_2O$ as protectant and potato starch as carrier	04.09.14/09.09.14/25.09.14
	CK	No treatment applied	-
	Q	Cyproconazole	05.09.14
		Iprodione	19.09.14
Orchard02		Tebuconazole	26.09.14
	BA	CPA-8 fresh cells	05.09.14/19.09.14/26.09.14
	BA1	Non-amended CPA-8 formulated cells	05.09.14/19.09.14/26.09.14
	BA2	CPA-8 formulated cells with 10 % $MgSO_4 \cdot 7H_2O$ as protectant and potato starch as carrier	05.09.14/19.09.14/26.09.14

Table 2. (continued)

Orchard	Treatment	Treatment description	Date of application
	CK	No treatment applied	-
	Q	Cyproconazole	15.07.15
		Tebuconazole	29.07.15
		Tebuconazole	05.08.15
		Fembuconazole	12.08.15
	BA3	CPA-8 formulated cells with 20 % sucrose plus 10 % skimmed milk as protectants and maltodextrin as carrier	15.07.15/29.07.15/05.08.15/ 12.08.15
Orchard03	BA4	CPA-8 formulated cells with 20 % sucrose plus 10 % skimmed milk as protectants and potato starch as carrier	15.07.15/29.07.15/05.08.15/ 12.08.15
	PF	Oil diluted formulation of *P. frequentans*	15.07.15/29.07.15/05.08.15/ 12.08.15
	BA3+PF	BA3 was applied in the 1st and 2nd applications; PF was applied in the 3rd and 4th applications	15.07.15/29.07.15/05.08.15/ 12.08.15
	PF+BA3	PF was applied in the 1st and 2nd applications; BA3 was applied in the 3rd and 4th applications	15.07.15/29.07.15/05.08.15/ 12.08.15
	CK	No treatment applied	-
	Q	Cyproconazole	19.08.15
		Tebuconazole	02.09.15
		Tebuconazole	07.09.15
		Fembuconazole	10.09.15
	BA3	CPA-8 formulated cells with 20 % sucrose plus 10 % skimmed milk as protectants and maltodextrin as carrier	19.08.15/02.09.15/07.09.15/ 10.09.15
Orchard04	BA4	CPA-8 formulated cells with 20 % sucrose plus 10 % skimmed milk as protectants and potato starch as carrier	19.08.15/02.09.15/07.09.15/ 10.09.15
	PF	Oil diluted formulation of *P. frequentans*	19.08.15/02.09.15/07.09.15/ 10.09.15
	BA3+PF	BA3 was applied in the 1st and 2nd applications; PF was applied in the 3rd and 4th applications	19.08.15/02.09.15/07.09.15/ 10.09.15
	PF+BA3	PF was applied in the 1st and 2nd applications; BA3 was applied in the 3rd and 4th applications	19.08.15/02.09.15/07.09.15/ 10.09.15

CPA-8 population dynamics

To compare CPA-8 populations on fruit among the different treatments applied in the field during 2015 season, 20 fruits (five fruits from each replicate) were sampled according to Table 3. Samples were taken periodically in the orchard, at harvest, and at postharvest (harvested fruit stored for 4-6 days of shelf-life at 20 °C and 85 % RH). 25 peel disks were randomly removed with a cork borer (16 mm in diameter) from the surface of every fruit. Then, the 125 peel disks of each replicate were placed together into sterile plastic filter bags (BagPage 400 mL, Interscience BagSystem, St Nom la Brètech, France) and mixed with 100 mL of phosphate buffer (PB, 70 mL KH_2PO_4 0.2 mol L^{-1}; 30 mL K_2HPO_4 0.2 mol L^{-1} and 300 mL deionised water v/v/v pH 6.5). 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 nutrient yeast dextrose agar medium (NYDA: 8 g L^{-1} nutrient broth, 5 g L^{-1} yeast extract, 10 g L^{-1} dextrose and 20 g L^{-1} agar). Colonies were counted after incubation for 24 h at 30 °C. Population dynamics of CPA-8 were collected as CFU mL^{-1} and finally expressed as CFU cm^{-2} of fruit surface.

Table 3. CPA-8 Population dynamics schedule.

Orchard	Date of sampling	Harvest	Shelf-life
Orchard03	15.7.15/17.07.15/22.07.15/28.07.15/29.07.15/31.07.15/ 04.08.15/05.08.15/07.08.15/12.08.15/12.08.15/14.08.15	14.08.15	20.08.15
Orchard04	19.08.15/21.08.15/26.08.15/01.09.15/03.09.15/04.09.15/ 07.09.15/07.09.15/09.09.15	14.09.15	18.09.15

Efficacy trials

Disease incidence in the field

At the commercial harvest time, all fruits from one full tree (250 fruits on average) for each treatment and replicate were evaluated taken into account the total number of fruits (healthy and affected by *Monilinia* spp.) in the tree and also in the ground. The evaluation was carried out in the tree in the middle in case of three trees per replicate or when there were four trees per replicate, one tree of the middle ones was selected.

Disease incidence after harvest

At harvest time, 100 healthy fruits per replicate were randomly collected from each treatment and placed in packing trays (20 fruits each) to avoid contact between them and consequent contaminations. The number of fruits affected by *Monilinia* spp. were then recorded after 5-7 days of shelf-life storage at 20 °C and 85 %, conditions that favour rot development.

Statistical analysis

Data from *Monilinia* spp. incidence (and *Monilinia* spp. severity in the case of CPA-8 dose trials) were submitted to an 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 t-student LSD test was used for separation of means (though the LSD test control the comparison–wise type I error rate rather than experiment-wise type I error rate). To achieve a normal distribution, the arcsine square root transformation of the data from efficacy trials was performed, if needed, prior to analysis. Non-transformed means are presented. Finally, data from CPA-8 populations was log-transformed and expressed as Log_{10} ($CFU\ cm^{-2}$) and plotted in figures where the error was represented by the mean standard deviation (±SD) of four replications of each sampling date.

RESULTS

Optimisation of the CPA-8 dose of treatment

CPA-8 dose affected brown rot caused by *M. laxa* and *M. fructicola* on artificially infected nectarine and peach fruit, respectively. Results showed that in general, higher CPA-8 doses ($5\cdot10^6$ and 10^7 CFU mL^{-1}) better controlled the pathogen (Fig.1). When CPA-8 was applied at 10^7 CFU mL^{-1}, the percentage of disease reduction compared to the control ranged, in peaches and nectarines, from 60.0 to 77.8 % and from 75.5 to 80.0 % according to the two disease parameters studied, disease incidence and disease severity, respectively. Subsequently, CPA-8 treatments were prepared at 10^7 CFU mL^{-1} for further applications under field conditions.
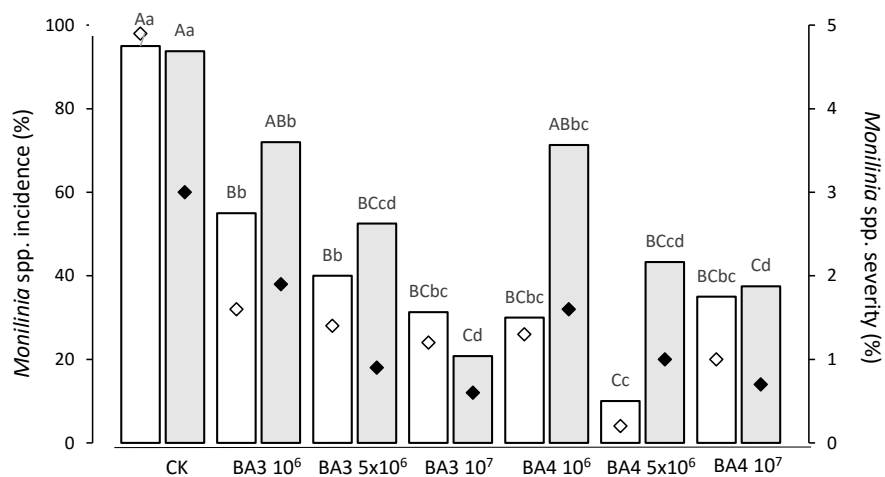
Figure 1. Antagonistic activity of CPA-8 treatments at different doses (CFU mL^{-1}) on nectarines and peaches artificially infected with *Monilinia* spp. and stored at 20 ºC and 85 % RH for 5 days. *M. laxa* in nectarines is represented by (☐) and (◇) and *M. fructicola* in peaches by (☐) and (◆). Uppercases and bars refer to disease incidence (%) and lowercases and diamonds refer to disease severity (cm). Within the same pattern, different letters indicate significant differences (*P*<0.05) according to the LSD test. The treatments were: CK (control, without CPA-8); BA3 (fluid-bed spray-dried CPA-8 cells formulated with sucrose and skimmed milk as protectants and maltodextrin as carrier); BA4 (fluid-bed spray-dried CPA-8 cells formulated with sucrose and skimmed milk as protectants and potato starch as carrier).

Meteorological data

Meteorological conditions occurred during the field experiments were collected and showed in Fig 2. The average temperature in the Orchard01 and Orchard02 were 20.2 and 19.9 ºC, respectively (2014 season late ripening varieties), lower than those obtained in the Orchard03 and Orchard04 (25.9 and 21.1 ºC) in 2015 (middle and late ripening varieties, respectively). The maximum temperature was recorded in the Orchard04, reaching 40 ºC. Regarding the RH recorded, both seasons showed similar values in average (68.8-83.0 %), although higher percentages were registered in 2014. Among the four orchards studied, the Orchard03 recorded the lowest % RH on average (68.8 %). Moreover, the 2014 season, was consistently wetter and colder than 2015 season, with more than ten times rain volume registered (96 and 64 mm of rain in the Orchard01 and Orchard02 opposed to less than 9 mm in the Orchard03 and Orchard04). It is worth mentioning the hail and heavy localised rain happened in the Orchard01 (05.09.2014) and Orchard03 (31.07.2015), damaging considerably the fruit.
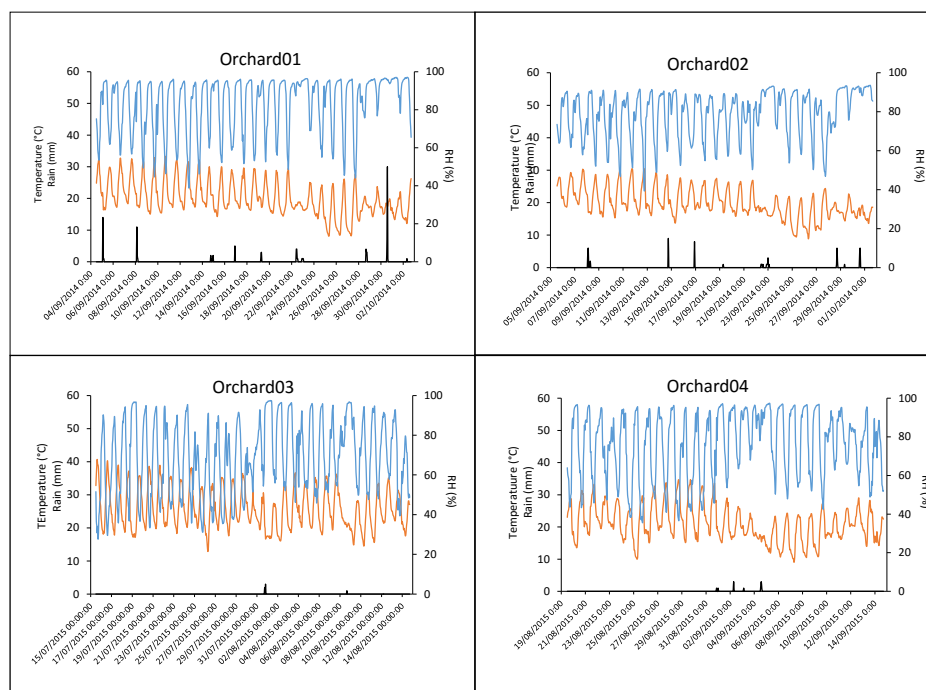
Figure 2. Meteorological data in the orchard during the treatments' application. The figure represents the temperature (°C) in red, the RH (%) in blue and the rain volume (mm) in black.

CPA-8 population dynamics

The population dynamics of CPA-8 on the fruit surface was estimated periodically after each treatment application in the orchard, at harvest, and after postharvest shelf-life (2015 season) (Fig. 3). In this season, CPA-8 treatments were applied on middle-season nectarines (Orchard03) and late-season peaches (Orchard04). In general, CPA-8 was largely maintained on fruit. CPA-8 CFU cm^{-2} were high immediately after application and then slightly declined over time until next application.

Populations of CPA-8 in the Orchard03 (Fig. 3a) were similar when comparing the different CPA-8 treatments and ranged from 2.77-3.43 ·10^4 CFU cm^{-2} (after the first application) to 4.10-4.95 ·10^4 CFU cm^{-2} (at harvest), except in the combined treatment BA3+PF, in which CPA-8 population decreased 1.18 log units during that time (probably due to both, physical removal caused by the heavy rain recorded in July

2015, and the fact that non-additional CPA-8 treatment was applied after the second application). In the Orchard04 (Fig. 3b), fruit was not exposed to heavy rain so CPA- 8 cells better remained on the surface of treated fruit after the second application. Once the treatments were applied, CPA-8 populations between 5.46 and 6.62 $\cdot 10^4$ CFU cm $^{-2}$ were obtained and maintained until harvest without exceptions (8.26-9.51 $\cdot 10^4$ CFU cm^{-2}). After harvest, (shelf-life of 4-6 days at 20 °C and 85 % RH) the CPA-8 cells remained in all treatments (Orchard03 and Orchard04), reaching values generally higher than 4 $\cdot 10^4$ CFU cm^{-2} (except treatment BA3+PF in the Orchard03).
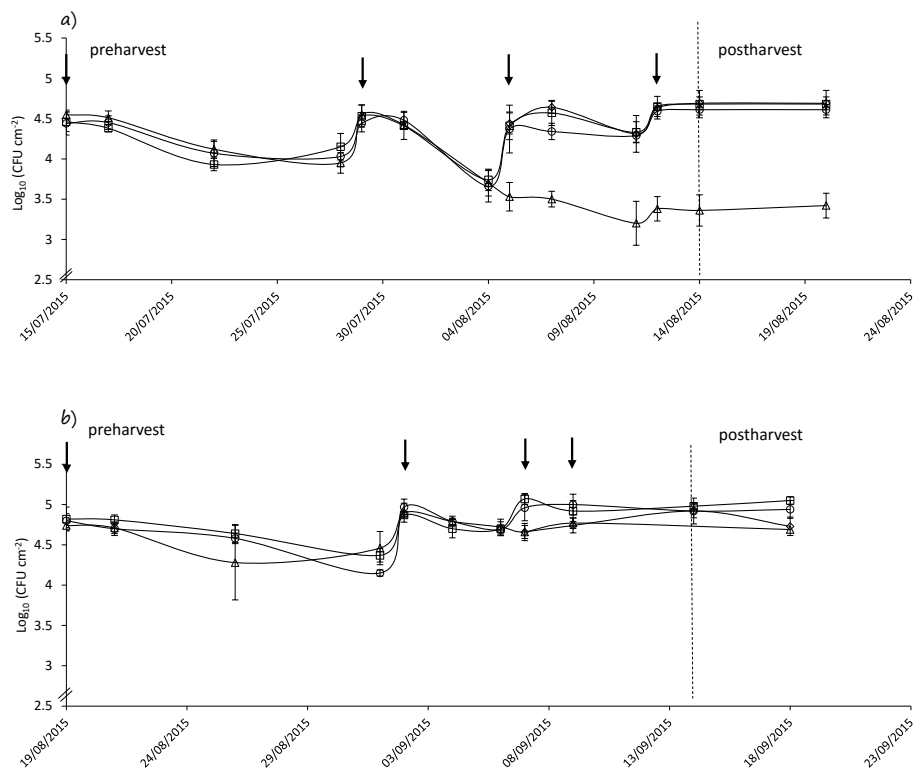

Figure 3. Population dynamics of CPA-8 cells (Log$_{10}$ CFU cm^{-2}) on fruit surface after treatment application in the orchard, at harvest, and after shelf-life (2015 season). a) Orchard03 and b) Orchard04. Arrows indicate the moment of each treatment's application and treatments were BA3 "fluid-bed spray-dried CPA- 8 cells formulated with sucrose and skimmed milk as protectants and maltodextrin as carrier" (⊖), BA4 "fluid-bed spray-dried CPA-8 cells formulated with sucrose and skimmed milk as protectants and potato starch as carrier (⊟), BA3+PF "the first two applications with BA3 and the last two with the BCA *P. frequentans*" (△) and PF+BA3 "the first two applications with the BCA *P. frequentans* and the last two with BA3 (◇). Values are the averages of four determinations and bars indicate the standard deviation.

Efficacy trials

The efficacy of CPA-8 preharvest treatments applied in four orchards in Lleida area during 2014 and 2015 seasons is described in Fig. 4. Different degree of biocontrol activity was obtained mainly due to the inoculum pressure, which is greatly depending on the meteorological conditions. When the presence of *Monilinia* spp. in the field was higher than 50 % (2014 season and the late ripening variety of 2015 season), brown rot disease could not be controlled at harvest time, not even when fruit was treated with chemical applications. Although 56.3 % disease reduction could be observed in the chemical treatment in the Orchard01 compared to the control (untreated trees), it was not statistically significant (*P*=0.09). It was not the case detected at postharvest (5-7 days of shelf-life storage at 20 ºC and 85 % RH), in which despite the high presence of inoculum, the chemicals applied successfully decreased brown rot decay, exhibiting 31.5-62.7 % of disease reduction compared to the untreated control.

Otherwise, when disease pressure was in the range of the standard levels recorded in the area (< 10 % and < 30 % at harvest and postharvest, respectively), treatments based on CPA-8 proved to be efficacious (Orchard03, middle season variety in 2015). At harvest (5.3 % disease pressure), BA3 and BA4 treatments and the PF+BA3 combined treatment significantly reduced disease incidence compared to the untreated control (54.7-64.1 % disease reduction). However, their efficacy was inferior compared to the one obtained by the synthetic fungicides applied (90.6 % disease reduction). Otherwise, at postharvest (disease pressure of 17.3 %) while all treatments controlled the pathogen (except the PF+BA3 combination), the BA4 treatment significantly controlled *Monilinia* spp. even statistically similar to chemical applications. In this case, 50.3 % of disease reduction was obtained, compared to the untreated control. It is also worth mentioning that the efficacy obtained when combining both BCAs (CPA-8 and *P. frequentans)* did not improve the efficacy of the bacterium when used alone.
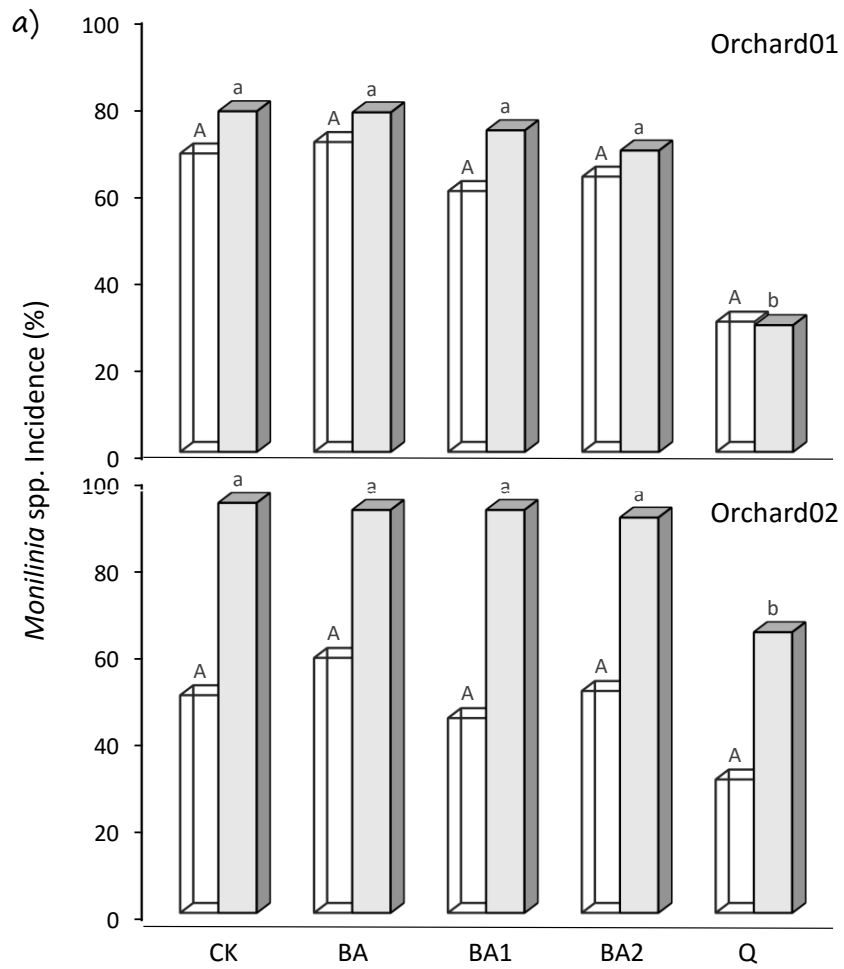
a)

Orchard01

Orchard02

Monilinia spp. Incidence (%)
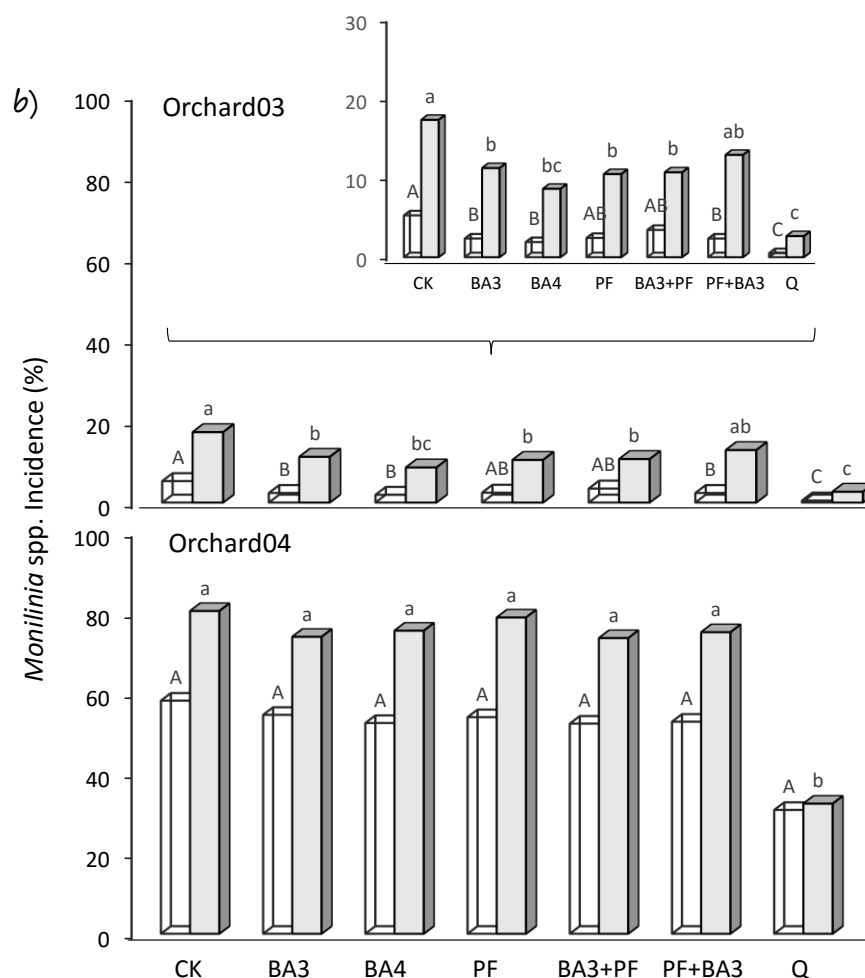
CK   BA   BA1   BA2   Q

Figure 4. Efficacy trials of CPA-8 treatments during 2014 (a) and 2015 (b) season. (▢) represents *Monilinia* spp. incidence (%) at harvest time and (▢) represents *Monilinia* spp. incidence (%) at postharvest (after 5- 7 days of storage at 20 °C and 85 % HR). The treatments tested were BA 'CPA-8 fresh cells', BA1 'non-optimised fluid-bed spray-dried CPA-8 cells formulated without protectants', BA2 'non-optimised fluid-bed spray-dried CPA-8 cells formulated with $MgSO_4$ as protectant and potato starch as carrier', BA3 'fluid-bed spray-dried CPA-8 cells formulated with sucrose and skimmed milk as protectants and maltodextrin as carrier', BA4 'fluid-bed spray-dried CPA-8 cells formulated with sucrose and skimmed milk as protectants and potato starch as carrier', PF '*P. frequentans* formulation', BA3+PF 'the first two applications with BA3 and the last two with the BCA *P. frequentans*', PF+BA3 'the first two applications with the BCA *P. frequentans* and the last two with BA3', Q 'chemical control' and CK 'no treatment applied'. Within the same pattern, different letters indicate significant differences (*P*<0.05) according to the LSD test. If needed, the arcsine square root transformation of the data was performed. Non-transformed means are presented.

Chapter VII

DISCUSSION

The present study represents the first full field testing on BCA *B. amyloliquefaciens* CPA-8 applications. Key factors for brown rot control have been identified, contributing to the development of disease management strategies in the Mediterranean area. Moreover, this work also illustrated aspects on *P. frequentans* 909 efficacy against *Monilinia* spp. complementing few studies already available (Guijarro *et al.*, 2007; Guijarro *et al.*, 2008).

As part of a well-designed disease program that enables the integration of BCAs into cropping systems, testing of the efficacy of CPA-8 against *M. laxa* and *M. fructicola* at different doses were conducted under laboratory conditions. The results showed that the highest BCA dose tested (10^7 CFU mL^{-1}) was the most effective against brown rot development, showing more than 60.0 and 77.8 % suppression of disease incidence and disease severity, respectively. Although doses of $5 \cdot 10^6$ CFU mL^{-1} also worked well, it was preferably to apply the highest one to thus ensuring biocontrol efficacy. The established CPA-8 dose of treatment (10^7 CFU mL^{-1}) was easily achievable (around ten times lower than those applied for the commercialised *Bacillus subtilis* Serenade Max, Bayer CropScience, Germany), thus faciliting the biomass production and formulation procedures.

BCA timing experiments conducted in four fields and over two seasons gave consistent information about the relevance of *Monilinia* spp. incidence. As it was observed, different degree of biocontrol activity was obtained depending on the inoculum pressure. Brown rot incidence was lower in the Orchard03 than in the Orchard04 and in both orchards studied in 2014 season, as it was evidenced by minor disease incidence in the untreated control. This difference may be associated with meteorological conditions. Since outbreaks of brown rot are dependent on prevailing environmental conditions, Gell *et al.* (2008) demonstrated that temperature and wetness durations were the two most important weather factors that contribute to the incidence of latent infections caused by *M. laxa* and *Monilinia fructigena* in Spanish peach orchards and that could account for more than 90 % of brown rot. They also suggested that RH is even more influential than temperature. Thus, longer fruit wetness duration most likely accounted for the significantly higher incidence of *Monilinia* spp. on fruit in 2014

season and in the Orchard04 in 2015. Under such drastic circumstances (> 50 % *Monilinia* spp. incidence), not even four chemical applications were sufficient for an effective control of brown rot at harvest time. Otherwise, when *Monilinia* spp. incidence was in the range of the standard levels recorded in the area (Orchard03), treatments based on CPA-8 formulations proved to be efficacious. Such standard levels have been considered over four growing seasons (2013-2016) including 26 field trials comprising early and late varieties. These data revealed that 90 % of the cases showed disease incidence at harvest lower than 10 %. Regarding postharvest studies (shelf-life at 20 ºC and 85 % RH), the percentage of trials which presented < 30 % of *Monilinia* spp. incidence raised the 77 % (Casals, personal communication). Therefore, results obtained from the Orchard03 could be included into these standard ranges of disease. At harvest (5.3 % *Monilinia* spp. incidence), BA3 and BA4 treatments and the PF+BA3 mixed culture significantly reduced *Monilinia* spp. brown rot compared to the untreated control (54.7-64.1 % disease reduction). In addition, almost all treatments controlled the pathogen at postharvest (except PF+BA3). At this moment, the BA4 treatment effectively reduced brown rot disease (50.3 % compared to the untreated control), even statistically similar to the chemical applications.

Moreover, the interaction between CPA-8 and PF did not improve the BCA effectiveness in controlling brown rot disease. Therefore, despite recommending a combination of strategies with multiple modes of action to ensure more consistent disease control and overcome fluctuations in external factors, a synergistic or additive effect was unlikely to occur. Combinations of BCAs would not have been necessary in this case.

The main differences observed in the efficacy tests among CPA-8 treatments are probably due to the formulation process. BA3 and BA4 treatments differ in the composition of the two polysaccharides used in fluidification: maltodextrin, with higher degree of solubility in water (Shamekh *et al.*, 2002), and potato starch. It seems that potato starch is an essential component in the formulation of CPA-8 since the BA4 treatment demonstrated higher efficacy than BA3 treatment at postharvest time. However, further studies should be conducted in order to better clarify this issue.

All CPA-8 formulations used in this work (except BA1 used in 2014 season) have been previously designed using diverse protective substances ($MgSO_4$, sucrose or

skimmed milk) and carrier materials (maltodextrin and potato starch) with the aim to improve biocontrol ability (Gotor-Vila *et al.*, 2017b; Gotor-Vila *et al.*, 2017c). These compounds were added to extend the shelf-life of the product over a period of up to 15 months and to increase the CPA-8 adherence over the fruits. The antagonists need to possess effective mechanisms to daily cope with the abiotic stresses to which they are commonly subjected in the orchard (Sui *et al.*, 2015). Gotor-Vila *et al.*, (unpublished results) described the persistence of BA3 and BA4 formulations directly exposed to abiotic factors that most affect the survival of BCAs under field conditions: temperature, RH and rainfall. Although satisfactory results were then reported, it is important to note that run off dynamics may change in the field under a natural environment. In this work, colonisation of treated fruit by CPA-8 appears to follow a general pattern in which high CPA-8 populations were obtained just after treatment application ($>10^4$ CFU cm^{-2}) and largely maintained until harvest and shelf-life evaluation at postharvest. These results led us to conclude that enough antagonist population level on fruit surface to subsequently obtain efficient control was achieved. Similar results were generally obtained in 2014 season, in which CPA-8 population dynamics (BA, BA1 and BA2 treatments) did not suffer losses between first application and harvest (data not shown). It is worth mentioning the ability of CPA-8 to largely survive on the fruit surface after preharvest application. It suggests that with less number of applications, the efficacy obtained for CPA-8 treatments would be the same. In contrast, many other BCAs such as the yeast *Candida sake* CPA-1, drastically declines over time once applied in the orchard (Calvo-Garrido *et al.*, 2013).

The approach to biocontrol research has evolved toward being more ecologically holistic and more oriented toward both production strategies and industry's concerns. Biocontrol products will be the alternative not only to demonstrate disease control but also to be an environment-friendly strategy that do not imply residues on fruit. However, more research is needed in integrating BCAs into cropping systems such as in rotating biocontrol with chemical pesticides and in considering these into forecast models to choose whether to apply a chemical pesticide or biocontrol (Fravel, 2005).

Application of the CPA-8-based products resulted in high efficacy when the pressure of the pathogen ranged between the standard values recorded in the area, indicating that this BCA is a good candidate for future larger-scale field applications

in different regions producers of stone fruit. Thus, even if the contribution of treatments to brown rot reduction at harvest is dependent on meteorological conditions, this study highlights the potential of *B. amyloliquefaciens* CPA-8 as alternative or complementary strategies to control *Monilinia* spp. Disease control could be achieved by combining BCAs with non-chemical control or prevention methods already existing or under development (Köhl *et al.*, 2011). Therefore, continued research in biocontrol is needed to contribute to the movement toward sustainable agriculture and to ensure available alternatives.

Acknowledgments

REFERENCES

Calvo-Garrido, C., Elmer, P.A.G., Viñas, I., Usall, J., Bartra, E., Teixidó, N., 2013. Biological control of botrytis bunch rot in organic wine grapes with the yeast antagonist *Candida sake* CPA-1. Plant Pathology 62, 510-519.

Cañamás, T.P., Viñas, I., Usall, J., Casals, C., Solsona, C., Teixidó, N., 2008. Control of postharvest diseases on citrus fruit by preharvest application of the biocontrol agent *Pantoea agglomerans* CPA-2 part I. Study of different formulation strategies to improve survival of cells in unfavourable environmental conditions. Postharvest Biology and Technology 49, 86-95.

Casals, C., Elmer, P.A.G., Viñas, I., Teixidó, N., Sisquella, M., Usall, J., 2012. The combination of curing with either chitosan or *Bacillus subtilis* CPA-8 to control brown rot infections caused by *Monilinia fructicola*. Postharvest Biology and Technology 64, 126-132.

Droby, S., Wisniewski, M., Teixidó, N., Spadaro, D., Jijakli, M.H., 2016. The science, development, and commercialization of postharvest biocontrol products. Postharvest Biology and Technology 122, 22-29.

Fravel, D.R., 2005. Commercialization and implementation of biocontrol. Annual Review of Phytopathology 43, 337-359.

Gell, I., De Cal, A., Torres, R., Usall, J., Melgarejo, P., 2008. Relationship between the incidence of latent infections caused by *Monilinia* spp. and the incidence of brown rot of peach fruit: factors affecting latent infection. European Journal of Plant Pathology 121, 487-498.

Gotor-Vila, A., Teixidó, N., Di Francesco, A., Usall, J., Ugolini, L., Torres, R., Mari, M., 2017a. Antifungal effect of volatile organic compounds produced by *Bacillus amyloliquefaciens* CPA-8 against fruit pathogen decays of cherry. Food Microbiology 64, 219-225.

Gotor-Vila, A., Teixidó, N., Usall, J., Dashevskaya, S., Torres, R., 2016. Development of a SCAR marker and a strain-specific genomic marker for the detection of the biocontrol agent strain CPA-8 *Bacillus amyloliquefaciens* (formerly *B. subtilis*). Annals of Applied Biology 169, 248-256.

Gotor-Vila, A., Usall, J., Torres, R., Abadias, M., Teixidó, N., 2017b. Formulation of the biocontrol agent *Bacillus amyloliquefaciens* CPA-8 using different approaches: liquid, freeze-drying and fluid-bed spray-drying. BioControl *in press*, doi: 10.1007/s10526-10017-19802-10523.

Gotor-Vila, A., Usall, J., Torres, R., Solsona, C., Teixidó, N. 2017c. Biocontrol products based on *Bacillus amyloliquefaciens* CPA-8 using fluid-bed spray-drying process to control postharvest brown rot in stone fruit . LWT - Food Science and Technology 82, 274-282.

Guijarro B., Hernandez-Escribano L., Larena I., Melgarejo P., De Cal A. 2017 Competition is the mechanism of biocontrol of brown rot in stone fruit by *Penicillium frequentans*. BioControl *in press*, doi: 10.1007/s10526-017-9808-x.

Guijarro, B., Melgarejo, P., Torres, R., Lamarca, N., Usall, J., De Cal, A., 2007. Effects of different biological formulations of *Penicillium frequentans* on brown rot of peaches. Biological Control 42, 86-96.

Guijarro, B., Melgarejo, P., Torres, R., Lamarca, N., Usall, J., De Cal, A., 2008. *Penicillium frequentans* population dynamics on peach fruits after its

applications against brown rot in orchards. Journal of Applied Microbiology 104**,** 659-671.

Karabulut, O.A., Smilanick, J.L., Crisosto, C.H., Palou, L., 2010. Control of brown rot of stone fruits by brief heated water immersion treatments. Crop Protection 29**,** 903-906.

Köhl, J., Postma, J., Nicot, P., Ruocco, M., Blum, B., 2011. Stepwise screening of microorganisms for commercial use in biological control of plant-pathogenic fungi and bacteria. Biological Control 57, 1-12.

Mari, M., Bautista-Baños, S., Sivakumar, D., 2016. Decay control in the postharvest system: Role of microbial and plant volatile organic compounds. Postharvest Biology and Technology 122**,** 70-81.

Moretto, C., Cervantes, A.L.L., Batista, A., Kupper, K.C., 2014. Integrated control of green mold to reduce chemical treatment in post-harvest citrus fruits. Scientia Horticulturae 165**,** 433-438.

Palou, L., Ali, A., Fallik, E., Romanazzi, G., 2016. GRAS, plant- and animal-derived compounds as alternatives to conventional fungicides for the control of postharvest diseases of fresh horticultural produce. Postharvest Biology and Technology 122**,** 41-52.

Shamekh, S., Myllärinen, P., Poutanen, K., Forssell, P., 2002. Film formation properties of potato starch hydrolysates. Starch - Stärke 54**,** 20-24.

Sharma, R.R., Singh, D., Singh, R., 2009. Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A review. Biological Control 50**,** 205-221.

Sisquella, M., Picouet, P., Vinas, I., Teixido, N., Segarra, J., Usall, J., 2014. Improvement of microwave treatment with immersion of fruit in water to control brown rot in stone fruit. Innovative Food Science and Emerging Technologies 26**,** 168-175.

Sui, Y., Wisniewski, M., Droby, S., Liu, J., 2015. Responses of yeast biocontrol agents to environmental stress. Applied and Environmental Microbiology 81**,** 2968-2975.

Teixidó, N., Torres, R., Abadias, M., Usall, J., 2011. Biological control of postharvest diseases in fruit and vegetables. In: Lacroix, C., (Ed.), Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage. Woodhead Publishing Limited, Cambridge, UK, pp. 364-402.

Usall, J., Casals, C., Sisquella, M., Palou, L., De Cal, A., 2015. Alternative technologies to control postharvest diseases of stone fruits. Stewart Postharvest Review 11 **(**4), 1-6.

Usall, J., Ippolito, A., Sisquella, M., Neri, F., 2016a. Physical treatments to control postharvest diseases of fresh fruits and vegetables. Postharvest Biology and Technology 122**,** 30-40.

Usall, J., Torres, R., Teixidó, N., 2016b. Biological control of postharvest diseases on fruit: A suitable alternative? Current Opinion in Food Science 11**,** 51-55.

Yánez-Mendizábal, V., Usall, J., Viñas, I., Casals, C., Marín, S., Solsona, C., Teixidó, N., 2011. Potential of a new strain of *Bacillus subtilis* CPA–8 to control the major postharvest diseases of fruit. Biocontrol Science and Technology 21**,** 409-426.

Yánez-Mendizábal, V., Viñas, I., Usall, J., Cañamás, T., Teixidó, N., 2012a. Endospore production allows using spray-drying as a possible formulation system of the biocontrol agent *Bacillus subtilis* CPA-8. Biotechnology Letters 34**,** 729-735.

Yánez-Mendizábal, V., Zeriouh, H., Viñas, I., Torres, R., Usall, J., De Vicente, A., Pérez-García, A., Teixidó, N., 2012b. Biological control of peach brown rot (*Monilinia* spp.) by *Bacillus subtilis* CPA-8 is based on production of fengycin-like lipopeptides. European Journal of Plant Pathology 132**,** 609-619.