Title: Field validation of biocontrol strategies to control brown rot on stone fruit in several European countries

Short running title: Biocontrol strategies to control brown rot on stone fruit

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Abstract

BACKGROUND: Brown rot caused by *Monilinia* spp. is the most significant disease of stone fruit. New approaches to fruit production have necessitated the development of control strategies that are more eco- and consumer-friendly. An efficient field strategy to control brown rot was previously designed based on the application of two biocontrol agents (BCAs), *Bacillus amyloliquefaciens* CPA-8 (CPA-8) or *Penicillium frequentans* 909 (Pf909), with calendar-based treatment. In the present study, the strategy was validated on different stone fruit hosts in four producing countries over two seasons.

RESULTS: The results obtained were reported according to three different scenarios: Scenario 1, in which there was no presence of disease in the field; Scenario 2, in which high disease pressure occurred in the field and treatments (biologicals or chemicals) were not effective; and Scenario 3, with low or medium to high disease presence. The results were successful since, in general, BCA strategies demonstrated to control brown rot to a similar extent as chemicals strategies. We found that most of the trials conducted in this study were classed under Scenario 3 (62.5 %), while only 12.5% and 25 % of the trials were classed under Scenarios 1 and 2, respectively.

CONCLUSION: These novel findings allowed the formulation of CPA-8 and Pf909 as valuable tools for farmers to more competitively produce stone fruits and meet consumer demand for safer and more environmentally friendly products.

Key words: Biocontrol, *Monilinia* spp., Brown rot, Field applications, Stone fruit

1. Introduction

In the European Union, stone fruit, apples and oranges are the most-produced fruits; of which, Italy, Spain, and Greece are the highest producers[1] of peaches, and stone fruit in general, are

highly susceptible to *Monilinia* spp. infections, which occur mainly between flowering and harvesting season. In smaller infections, the disease does not develop fully and can remain latent until harvest time.[2] When infection levels are high, disease symptoms on fruit can appear early and cause significant losses in yields. Moreover, for infections that occur on fruit closer to harvest time, brown rot commonly develops postharvest and, in extreme situations, results in losses up to 80 %.[3] However, the risk of *Monilinia* spp. infections during postharvest is very low, and Bernat et al.[4] indicated that *Monilinia* spp. are rarely detected in packinghouse environments, and therefore the great majority of infections occur in orchards. Consequently, control of *Monilinia* spp. is most efficiently undertaken in the field, where the infections most commonly occur. Currently, commercial field control strategies are implemented as preharvest treatments that act both preventively and curatively, depending on when infections occurs. At postharvest, the application of chemical treatments is authorised in some European countries.[5] These postharvest treatments are advised as complementary to the field strategy for only mid-late infections, or in the event of adverse meteorological conditions. Conventionally, these treatments are based on synthetic chemical products.[6]

In recent years, social pressure has increased consumer demand for environmentally friendly fruit production, that is more considerate of consumers' and growers' health. In addition, several other considerations have reduced the use of pesticides, such as stricter legislation on authorised active ingredients and their allowable presence on fruit and the risk of pesticides for developing resistant strains. These concerns have spurred to search for alternatives to the chemical products being used for pest control, such as biocontrol agents (BCAs)—a promising strategy that has been the focus of considerable research over the last few decades.[7–9] Over the last few years, biocontrol research has also evolved towards more integrated approaches in production systems, with greater awareness of industry concerns.[10] However, BCAs are not yet routinely applied under commercial conditions. Currently, only Serenade Max™, based on

Bacillus subtilis; Amylo X™, based on *Bacillus amyloliquefaciens*; and Julietta™, based on *Saccharomyces cerevisiae* are authorised in some countries for field control of brown rot.

Several works have reported that *Bacillus amyloliquefaciens* CPA-8 is an effective antagonist against postharvest brown rot on peaches and nectarines.[9,11,12] Likewise, *Penicillium frequentans* strain 909 (Pf909) has also been reported as a potential biocontrol product for reducing the occurrence of brown rot and twig blight, both of which are caused by *Monilinia* spp.[6,13] Moreover, Guijarro et al.[6,14,15] showed that for Pf909 to be effective, peach surfaces have to be extensively covered by it, as it will only be effective when its concentration is substantially greater than the *Monilinia* spp. load.[14] Natural populations of *P. frequentans* during stone fruit growing season are insufficient for controlling brown rot.[16]

Both the afore-mentioned BCAs have been extensively studied, in relation to some of the major obstacles for its success as a commercial biocontrol product. These include the production of sufficient quantities of microorganisms to ensure optimum shelf life[17], broadening the spectrum of action to a wider range of hosts[11], and determining the responses to environmental stress under field conditions.[18,19] The results indicated that both BCAs are promising biocontrol products under commercial conditions. Registration is considered another major obstacle that should be taken into account during BCA development processes, as it is a requirement for their use under commercial conditions. The registration process stipulates a large number of tests concerning human and environmental safety, basic toxicological tests, and effectiveness evaluation, including semi-commercial tests.[10] In this respect, some of these tests have already been conducted on these two BCAs.[18–20] Moreover, Gotor-Vila et al.[11] evaluated CPA-8 BCA establishment on the target host and designed an efficient strategy, based on the complete common plant protection schedule usually applied to crops.

In order to have sufficient data to implement and validate the previously studied strategies[9], the present study was designed with two different formulations of each BCA (*B. amyloliquefaciens*

CPA-8 and *P. frequentans* 909) in four European countries (Belgium, France, Italy, and Spain), in two growing seasons (2016 and 2017), and on three different crops (peaches, nectarines, and cherries).

2. Material and methods

2.1. Biocontrol agent products

B. amyloliquefaciens CPA-8 (CPA-8), formerly *Bacillus subtilis*[20], was originally isolated from nectarine fruit[9] and belongs to the Postharvest Pathology Group Collection of IRTA (Lleida, Catalonia, Spain). The BCA product formulation based on CPA-8 was optimised by including a protective combination of 20 % sucrose and 10 % skimmed milk during its formulation, and two different carrier materials: maltodextrin (BS1) and potato starch (BS2). Both BCA products were produced and formulated according to Gotor-Vila et al.[11] BS1 and BS2 were evaluated in trials conducted in 2016 and 2017, using 10^7 CFU mL^{-1} as the effective dose.

P. frequentans Westling (Pf909) *(ATCC 908-81)* is a constituent of the resident mycobiota of peach twigs and flowers, originally from a commercial orchard in Zaragoza (Spain)[21], and was provided by the Department of Plant Protection of INIA (Madrid, Spain). Pf909 was previously proposed as a potential commercial BCA against brown rot.[6,14] Formulations of Pf909 conidia are potential biocontrol products, used to reduce the occurrence of peach and nectarine brown rot caused by *Monilinia* spp. at pre- and postharvest by competitively excluding the pathogen from twigs and the fruit surface.[15] The production of pure dried conidia powder followed the description of Guijarro et al.[13] and was made under pilot plant process conditions by Bayer CropScience Biologics GmbH, (Malchow, Germany). Pf909 is a dried conidia powder with a concentration of $1.8x10^{11}$ conidia g^{-1} and a viability range of 94– 98 %. The dry conidia powder was suspended into two different liquid oil formulations containing carriers and adjuvants to

improve dispersion and persistence of the conidia on fruit surfaces (confidential data owned by Bayer Crop Science).

In 2016, two different formulations of products based on Pf909 were evaluated: PF1 and PF2, both applied in orchards using an effective dose of 10^6 conidia mL^{-1}. In 2017, only PF2 formulated product was tested at 10^6 conidia mL^{-1} and 10^7 conidia mL^{-1} (PF2×10).

2.2. Efficacy trials

2.2.1. Field trials and experimental design

A total of 16 field trials were carried out by three officially recognised organisations (Opennatur S. L. and IRTA in France and Spain, BC (Europe) S.r. – BIOGARD in Italy, and PCfruit in Belgium) in accordance with the principles of Good Experimental Practises (GEP). Trials were designed, run, and assessed following the relevant EPPO guidelines[22–24] in different orchards located in Belgium, France, Italy, and Spain in 2016 and 2017 (Table 1). In each trial, the strategies evaluated in 2016 consisted of 1) CPA-8 formulation 1 (BS1); 2) CPA-8 formulation 2 (BS2); 3) Pf909 formulation 1 (PF1); Pf909 formulation 2 (PF2); 5) Non-treated trees (Untreated); and 6) the chemical fungicide strategy usually applied in each trial area (Table 2). In the 2017 growing season, the treatments applied were the same as in 2016, except for Pf909 where PF1 was not applied and the treatments evaluated were PF2 and PF2 formulations, but 10 times more concentrated (PF2×10). All treatments were conducted using the same strategy based on four applications in the field: 30, 14, 7, and 3 days before harvest, according to the commercial schedule and to the results obtained by Gotor-Vila et al.[11] Application dates were arranged depending on climatology events, and all treatments were applied in the morning using spraying equipment. Each tree was sprayed to run off. Plots were distributed in a completely randomised block design with four replicates per treatment. Each replicate consisted of 3-4 trees (depending on the number of fruits per tree). Buffer trees (non-treated trees) were used to separate treatments and replicates.

Orchards received treatments standard to cultural and crop protection practises until 45 days before harvest, then only insecticide treatments until harvest. A weather station was placed in or near the field trials to record hourly weather observations of temperature (T), relative humidity (RH), and rainfall (mm).

2.2.2. Disease incidence in the field

Evaluation was performed according to the EPPO 1/38[2][24] for *Monilinia laxa* and PP 1/222[1].[23] At commercial harvest time, disease incidence on nectarines, peaches, and cherries was recorded in the field. For peaches and nectarines, one full tree per treatment was evaluated and the total number of fruits, healthy, and affected by *Monilinia* spp. were recorded. A similar procedure was conducted for fruits located on the ground. According to the EPPO 1/222[1][23] for cherries, 400 fruits per replicate were evaluated. In all cases with three trees per replicate, the evaluation was carried out on the central tree. When there were four trees per replicate, one of the two trees located in the middle was selected. Finally, the number of fruits infected by *Monilinia* spp. was expressed as the incidence of infected fruit.

2.2.3. Disease incidence in postharvest period

Evaluation was carried out according to the EPPO PP 1/222[1] describing storage diseases of stone fruit.[25] One hundred healthy peach or nectarine fruits per replicate were randomly collected from each treatment at harvest time and placed in packing trays (20 fruits each) to avoid contact among them and consequent cross-contaminations. Fruits were stored at 0 °C and 90 % RH for 7 days, plus 7 days of shelf-life at 20 °C and 85 % RH. The number of fruits affected by *Monilinia* spp. was recorded after 5 and 7 days of fruit incubation at 20 °C. For cherries, the methodology was the same, except for the number of fruits evaluated per replicate, where 400 fruits, in bunches, for each replicate were evaluated.

2.3. CPA-8 and Pf909 populations on the fruit surface

To ensure that CPA-8 and Pf909 biocontrol agent applications on peaches and nectarines were well-conducted and their presence and viability were maintained until harvest; fruit from each trial was sampled after the first treatment application (AFTA) once treatment was dried out, and at harvest time (HT), three days after last application. At both samplings, 20 fruits from each treatment (five fruits per replicate and treatment) were picked for determination of BCA populations.

The CPA-8 population (BS1 and BS2) determination on peaches and nectarines was based on the random removal of 25 peel disks from each fruit using a cork borer (16 mm in diameter) and 5 fruit per replicate. 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 using a Stomacher blender (Masticator Basic 400 mL, IUL SA, Torrent de l'Estadella, Barcelona, Catalonia, Spain) set at 12 strokes s^{-1} for 90 s. Serial ten-fold dilutions of the washings were prepared and plated onto 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). Two replicate Petri dishes were used for each replicate and dilution. Colonies were counted after 24 h of incubation at 30 °C. Populations of CPA-8 were recorded as CFU mL^{-1}, and finally expressed as CFU cm^{-2} of fruit surface. For cherries, the methodology was slightly different. Ten fruits per replicate were weighed and immersed in 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), contained in 500 mL flasks. Erlenmeyer flasks containing the samples were shaken on a rotary shaker at 150 rpm for 20 min and then sonicated for 10 min in an ultrasound bath (Selecta; Abrera, Barcelona, Spain), to increase detachment of microorganisms from the berry surface. Finally, the colony determination was conducted as

previously explained for peaches and nectarines. Then, the population of CPA-8 was expressed as CFU g^{-1} of fruit.

The populations of Pf909 formulations were evaluated from 20 fruits per treatment (5 per replicate and treatment) suspended in sterile distilled water (SDW), shaken for 30 min at 150 rpm, concentrated by centrifugation for 10 min at 14.040 g, and resuspended in 5 mL SDW. The CFUs of Pf909 per cm^2 of fruit surface were estimated on Petri dishes containing potato dextrose agar (PDA) amended with 0.5 g L^{-1} of streptomycin to avoid bacterial growth (PDAs). One-hundred aliquots from undiluted and diluted concentrate were spread onto PDA. Five replicate dishes were used for each replicate and dilution. Three replicate Petri dishes were used for each field and were maintained in the dark at 20–25 °C for 5–7 days. The colonies were then counted and expressed as CFU cm^{-2} of fruit surface. For cherries, ten fruits from each replicate were weighed and used following the methodology described above. Finally, the population of Pf909 was expressed as CFU per g of cherry fruit.

2.4. Statistical analysis

Data on disease incidence recorded at preharvest and postharvest evaluations were analysed using ANOVA. Normality data distribution (Saphiro test) and homogeneity of variances (Levene and Barlett tests) were checked, and data were transformed (arcsin trasnfomation). In all cases, JMP®8 statistical software (SAS Institute, Cary, NC, USA) was used. Data related to CPA-8 and Pf909 populations were log-transformed and expressed as $\log_{10}$ (CFU cm^{-2}) for peaches and nectarines, and $\log_{10}$ (CFU g^{-1}) for cherries. Statistical significance was determined at $P<0.05$. When the analysis was significant, the Student's LSD test was used for separation of means (though the LSD test controls the comparison–wise type I error rate rather than experiment-wise type I error rate).

3. Results

3.1. Efficacy trials

The efficacy of CPA-8 and Pf909 preharvest treatments to control brown rot in 16 orchards from four different European countries (Belgium, France, Italy, and Spain), three crops (peaches, nectarines, and cherries), and two growing seasons (2016 and 2017), clearly depended on the level of disease pressure experienced in the untreated strategy (Figures 1-3). Hence, results have been classified in three different scenarios corresponding to different levels of disease pressure: 1) no inoculum; 2) uncontrollable disease; and 3) controllable disease.

3.1.1. Scenario 1: Low disease levels

Scenario 1 only occurred in two trials orchard 03 and orchard 06, corresponding to 'Red Jim' nectarine and 'Tardibelle' peaches; both evaluated in Spain during 2016 (Table 1). The incidence of disease recorded at HT in both trials was practically zero for all evaluated treatments (Figure 1). At postharvest, after storing fruit for 7 days at 0 °C, plus 5 days of shelf life, brown rot was again generally extremely low, less than 3 % incidence in all treatments (Figure 1), and no statistically significant differences were found among treatments including the untreated fruit treatment.

3.1.2. Scenario 2: uncontrollable disease

Scenario 2 occurred in 4 out of 16 trials. Two of them were 'Lapins' and 'Sweetheart' cherries corresponding to orchard 08 and orchard 09, respectively, in Belgium, during 2016 (Table 1); both fields were affected by hailstorms. For the 'Lapins' variety (orchard 08), disease incidence at harvest was extremely high and there was insufficient healthy fruit for postharvest evaluation (data not shown). For the 'Sweetheart' variety (orchard 09), disease incidence at the time of harvest evaluation ranged from 28 to 48 %, with no statistically significant differences among

treatments (Figure 2). At postharvest, only chemical and PF1 treatments presented statistically lower disease incidence (21% and 58 %, respectively), compared to the control (91 % of brown rot).

Trials carried out on 'Tourmaline' nectarines and 'Fidelia' peaches were also classed under Scenario 2, corresponding to orchard 01 and orchard 04, respectively (Table 1), conducted in France in 2017. In this case, both fields were affected by periods of rain near harvest. The disease incidence recorded at HT for tourmaline nectarines was close to 100 % in all treatments, and there was insufficient healthy fruit for postharvest evaluation. Results for 'Fidelia' peaches were similar, and any treatment reduced the incidence of disease, compared to the control (data not shown).

3.1.3. Scenario 3: Controlled disease

Ten out of 16 trials (62.5 %) carried out in 2016 and 2017 were included in this scenario. In general, both BCAs (CPA-8 and Pf909) successfully reduced the incidence of brown rot. Data for postharvest evaluation was globally analysed. In general, results indicated that the efficacy of BCAs was lower in comparison the chemical (data not shown). However, when data was analysed according to the scenario, no efficacy differences were found between treatments for orchards with low-medium incidence of disease, including chemical treatment. For medium-high disease incidences, chemical strategy showed higher statistical efficacy in comparison with BCAs.

In 2016, trials included in Scenario 3 conditions were conducted on 'Tourmaline' and 'Morsiani' nectarines, and 'Fidelia' and 'Corindom' peaches, corresponding to orchard 01, orchard 02, orchard 04 and orchard 05, respectively (Table 1). Results from these four orchards at HT showed that brown rot incidence in the non-treatment strategy ranged between 4 and 11 % (Figure 3). The results for orchard 01 and orchard 02 at preharvest showed that the incidence of brown rot in the non-treated control was lower than 7 %, and no significant differences were

found among all treatments evaluated (including control and chemical). In contrast, a significant brown rot reduction was observed in orchard 04 and orchard 05, with more than 50 % reductions, compared to the control, in all treatments evaluated (BS1, BS2, PF1, PF2, and chemical), and there were no differences between the treatments.

At postharvest evaluation, brown rot incidence was much higher in all four orchards, ranging between 17 and 53 % in the non-treated control. Brown rot incidence in untreated controls was lower than 35 % in orchard 01 (‘Tourmaline’ nectarine) and orchard 04 (‘Fidelia’ peaches). In orchard 04, the efficacy of most of tested BCA treatments was comparable to the chemical one. The chemical treatment statistically reduced the incidence of disease compared to the control in both orchards, (94 % and 75 % reduction in orchard 01 and 04, respectively). For orchard 04 (‘Fidelia’ peaches), BS2, PF2 and chemical strategies significantly reduced the incidence of disease, with reductions greater than 50 %, compared to the untreated control, and there were no significant differences among them.

For orchard 02 and orchard 05, brown rot incidence in the control was higher than 40 % and, BS2 and PF2 treatments were effective in controlling brown rot with reductions greater than 50 %, compared to the control. Moreover, in orchard 02, PF1 also showed similar level of efficacy. However, in these orchards, the efficacy of BCAs was, in all cases, lower than that of the chemical treatments (85 % and 93 % reductions for orchard 02 and orchard 05, respectively).

In 2017, six trials conducted on nectarine, peach, and cherry were classed under Scenario 3. For nectarines and peaches from orchard 02 (‘Morsiani’ nectarine), orchard 03 (‘Red Jim’ nectarine), orchard 05 (‘Corindom’ peach) and orchard 07 (‘Groc d’Ivars’ peach) results are shown in Figure 4. At HT, brown rot incidence recorded in the untreated control was low, and ranged between 0 and 3 %, with significant differences among the treatments tested, including control and chemical treatments. In the postharvest evaluations, disease incidence was also

lower in 2017 than in 2016, and brown rot incidence in the control ranged between 10 % and 29 %, depending on the orchard. In orchards 02, 05 and 03 results showed disease incidences for BCAs statistically similar to the chemical treatments. Decay was significantly reduced to similar levels in all BCAs treatments evaluated (BS1, BS2, PF1, PF2×10) in orchards 02, 03, 05, and 07, with an average reduction of 60 %, 68 %, 32 % and 41 %, respectively, compared to the controls.

Cherry trials conducted in 2017 (orchard 08 and orchard 09 for 'Lapins' and 'Sweetheart' varieties, respectively) were included under the Scenario 3 (data not shown). The disease incidence at harvest evaluation was almost 0 %. In postharvest evaluations of orchard 08, brown rot ranged between 2 and 8 %, and no differences were detected among all treatments tested, including untreated controls and chemical treatments. The results obtained for orchard 09 indicated that the incidence of brown rot ranged between 1 and 8 %. All BCA treatments reduced the disease to similar levels (58 % reduction compared to control). However, the efficacy of chemical treatments was statistically higher (92 % reduction) than that of the PF1 treatment.

3.2. CPA-8 and Pf909 on the fruit surface

Population viability of CPA-8 and Pf909 cells were determined in trials conducted on different crops (peaches, nectarines, and cherries) in growing seasons in 2016 and 2017, at two different time points, after AFTA and at HT.

CPA-8 and Pf909 populations were determined to be similar across both seasons, in all the orchards evaluated. In general, the level of viable cells AFTA was maintained and even increased until HT (Tables 3 and 4).

CPA-8 cell populations were stable enough to compare both formulations (BS1 and BS2) in all trials and sampling times. For peaches and nectarines, populations remained between 3.84-5.15 and 3.98-5.33 log CFU cm^{-2}, for values recorded AFTA and HT, respectively. For cherries, populations remained between 3.86-5.49 and 3.87-4.83 log g^{-1} for values recorded AFTA and at HT, respectively (Tables 3 and 4).

PF1 and PF2 formulations also maintained an adequate concentration of CFU cm^{-2} on the fruit surface until harvest. On peaches and nectarines, populations were maintained and even increased until HT, and ranged between 1.61-4.28 and 2.31-4.39 log g^{-1}, AFTA and HT, respectively. For cherries, populations remained between 2.71-4.39 and 3.22-4.47 log g^{-1}, AFTA and HT, respectively (Tables 3 and 4). Although PF1 and PF2 concentrations were 10 times lower than for PF2×10, and the populations for PF1 and PF2 were, in general, lower at all evaluations conducted, the results profile was similar to that described above.

4. Discussion

The present study represents a wide validation full-field application program based on BCAs strategies to control *Monilinia* spp. under commercial conditions in different stone fruit crops (nectarines, peaches, and cherries), during two consecutive years (2016 and 2017), and in several European countries (Belgium, France, Italy, and Spain). Three accredited GEP companies have implemented field application programs (Opennatur S. L in France and Spain, BC (Europe) S.r. – BIOGARD in Italy and PCfruit in Belgium orchards). Hence, the information provided comes from different geographical locations and illustrates a wide range of conditions for evaluating the efficacy of BCAs. The GEP trials detailed in this paper provide representative data, and consequently their conclusions are absolutely robust.

The potential of *B. amyloliquefaciens* CPA-8 or *P. frequentans* (Pf909) as alternative strategies to chemical fungicides, has been related to disease incidence, and correlated more with climatological data[11] than crops, countries, and seasons. The efficacy data summarised in this biological assessment paper for CPA-8 and PF909 is in line with these patterns. In addition, OECD guidance for microbial plant protection products indicates that the levels of efficacy for microbials may be lower than that for a chemical-based pesticide, and such differences should be reflected in the label. However, it is necessary to specify that the level of efficacy depended on the disease pressure in each orchard. It is well known that brown rot incidence is mainly correlated to the climatic conditions over the season. A wide range of factors may affect the performance of microbial plant protection products. Factors such as temperature, humidity, rain, pathogen density, initial pathogen inoculum level, etc., may affect the behaviour of microorganisms in a range of different ways, according to the EPPO PP 1/276 [1].[22] Gell et al.[2] demonstrated that temperature and wetness duration were the most important factors contributing to the incidence of latent infections caused by *M. laxa* and *M. fructicola* in Spanish peach orchards, which could catalysing more than 90 % of brown rot in the post-arvest period. In addition to the weather conditions, other factors must be considered as the presence of latent infections at the pit hardening stage, which was also correlated with the incidence of fruit rot at harvest.[26]

With respect to weather effects, extremely dry seasons would suppress disease, and in contrast, long wetness periods, heavy or often rains, or even hailstorms would contribute to high levels of disease. This fact clearly affected the trials reported in this study, where the level of biocontrol efficacy depended on the disease pressure observed in each field. For this reason, the trials conducted were classified under different scenarios according to the level of disease pressure. The Scenario 1 class (low level of disease incidence) included 2 out of 16 trials, and the Scenario 2 class (high level of disease incidence), 4 out of 16 trials. In the latter scenario,

353 none of the evaluated treatments were effective in controlling brown rot, including the chemical
354 strategy. Orchards were deeply affected by a hailstorm causing critical wounds on fruit, in number
355 and size, which became direct routes for fungal infections, especially *Monilinia* spp. Fruit losses
356 in these fields reached 45 %, and in some cases, the entire crop was lost. As occurs under
357 commercial conditions, most of the trials classed under scenario 3 (10 out of 16 trials), comprising
358 62.5 % of the trials conducted. Under this scenario, biological or chemical products are both
359 effective for controlling brown rot. However, it is important to note that when the incidence
360 recorded in postharvest was lower than 35 %, the efficacy level of the BCA strategies was
361 comparable to that of the chemical strategy. However, when brown rot incidence recorded in
362 postharvest increased up to 35 %, all treatments were effective, with the chemical strategy
363 showing higher efficacy compared to BCA treatments. From our results, we can advise the need
364 to integrate cultural practises in the field, such as summer pruning or removing infected fruit
365 from the field. This would contribute to decreasing the level of inoculum in the field, thereby
366 improving the efficacy of the BCA strategy to similar levels as the chemical strategy. Recently,
367 another field strategy to control brown rot has been developed, based on the integration of BCAs
368 with low doses of fungicides.[27] They found that a higher level of efficacy was recorded in the
369 fungicide treatments at full dose. However, when BCAs were combined with low doses of
370 fungicides, the efficacy was not much lower. Our results were analogous to what occurred in
371 our previous study where the level of BCA efficacy was clearly dependent on the disease
372 incidence.[28] It was also concluded that when the incidence of *Monilinia* spp. was close to the
373 standard levels recorded in the area (comparable to scenario 3), treatments based on CPA-8
374 formulations proved to be efficacious.

375 Under field conditions, rapid fluctuations in water availability and temperature are
376 characteristic of farm environments and constitute the main factors limiting the development of
377 microbial populations.[29] Nevertheless, in this study, the populations of CPA-8 and Pf909

recorded on fruit surfaces did not suffer losses between the first treatment and harvest, and were sufficient to effectively control brown rot. These results corroborate the information provided by Gotor-Vila et al.[11] which highlight the ability of CPA-8 to survive largely on the fruit surface after preharvest application. Accordingly, in previous studies, the wide tolerance to temperature, relative humidity and simulated rainfall that could occur under commercial conditions in the field was also demonstrated for CPA-8 cells.[18] Cell viability under field conditions is highly relevant and should be studied for all BCAs, as their behaviour can differ according to the antagonist used. For example, in contrast to our results obtained for CPA-8, Calvo-Garrido et al.[30] found that the cell viability of *Candida sake* CPA-1 drastically declined over time after application in organic vineyards.[30] Pf909 could establish and survive actively over a broad range of climatic conditions. We have previously reported that the population of Pf909 in the peach phyllosphere grew better during late spring and summer. For applications of Pf909 on peaches[31] that are administered during preharvest periods, the environmental temperature and relative humidity are the most important factors that can affect the dynamics of Pf909 populations. Critically, the environmental temperature and relative humidity account for 87 % and 63 %, respectively, of the observed variability in the number of conidia and colony forming units (CFUs) of Pf909 on the peach surface after application. Therefore, Pf909[14] should be applied in commercial peach orchards when environmental temperatures are warm. In 2017, it was applied at concentrations ten times higher (PF2×10), but evaluation of the final population counts demonstrated that increased concentrations did not correlate with higher efficacy in controlling brown rot. Guijarro et al.[14] also proposed that in order to increase the size of the Pf909 populations on fruit, larger populations of Pf909 should be applied to the flowers during bloom (because the peach blossoms carry low indigenous epiphytic populations) and at preharvest. Applying Pf909 under these conditions encourages the growth and sporulation of the indigenous *P. frequentans* populations and increases the size of populations

on the flower and fruit surfaces. Knowledge of the conditions that influence fungal sporulation is very useful, as sporulation is the key factor in proliferation and dispersal.[32]

Brown rot control is more difficult due to legislation issues regarding registered active ingredients and limits on residues on fruit surfaces. Combined with consumer demands for more eco-friendly and health-conscious fruit production, these pressures drive the need for alternative treatments to synthetic fungicides. Although postharvest biocontrol has been extensively studied for the last 35 years, some constraints affect its commercial feasibility.[33] Little information is available regarding the integration of BCAs into conventional cropping systems. The successful results obtained in this study have taken science one step further, demonstrating that a field program strategy based on CPA-8 or Pf909 formulated products effectively control brown rot in most cases, at similar levels of efficacy to chemical strategies.

The method of integrating of BCAs into conventional crop production management could affect their viability or efficacy. Therefore, in this report, we also checked whether the standard practises commonly used in field management could interfere with the viability of BCAs. Moreover, our BCAs are compatible with the majority of chemical products that are applied to stone fruit orchards under conventional production strategies.[34] In cases where BCAs cannot match the efficacy of chemical strategies, our BCAs could be combined with the chemical products, and applied in alternating cycles. In fact, in conventional agriculture, there is an increasing tendency to compliment the use of chemical products with those based on BCAs, so an important requirement is for BCAs to be compatible with chemical products. Moreover, it has to be note that this integration will minimise the risk of fungicide resistances in comparison with the application of fungicide programmes, which, often, their design is based on products with the same mode of action, being not sustainable for controlling pathogens that have a high risk of fungicide resistance. In this context, the integrated disease management that includes the

BCAs evaluated in this paper (not commercial available) would be a long-term commercially preferable strategy to control brown rot.

Although the results obtained in this study were successful, some constraints on BCA efficacy are apparent when the incidence of disease in the field is medium to high. In these cases, BCAs were effective in reducing the incidence of brown rot, but at a lower efficacies than chemical strategies. Basic information relating to the expected disease pressure in each orchard is necessary for the successful use of BCAs in brown rot control applications. Tools such as prediction models for disease infection risks are indispensable and will be useful for this purpose. Depending on the scenario predicted, the deficiencies of control agents could be overcome by integrating their application with other cultural practices or chemical applications, where necessary, in control strategies adapted to each situation, for maximum benefits. However, until now, information provided by our prediction model indicates the infection risk by *Monilinia* spp. in the field along the growing fruit season, but not the prediction for the final level of disease at harvest. For decisions making to determine the optimal strategy to be applied in each case the technical criteria will be fundamental.

5. Conclusions

In the current framework, the BCAs studied showed enough potential to be commercially viable, with numerous applications as a reliable tool in conventional stone fruit production, especially in organic production where chemical fungicides are not permitted. Therefore, in the near future, further experiments will focus on improving the level of BCAs efficacy by integrating BCAs and chemical products in the same brown rot control strategy laying groundwork for the complementary and simultaneous use of BCAs and chemical products, towards a more sustainable and competitive future in agriculture.

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7. Conflict of Interest Declaration

The authors declare that they have no conflict of interest.

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568 Table 1. Orchard characteristics used to validate the biocontrol strategy in different European
569 countries.

Crop	Country	Variety	Orchard	Location	Growing season	Trials conducted
Nectarines	France	'Tourmaline'	Orchard 01	43.220833N-2.651666E	2016 / 2017	2
	Italy	'Morsiani'	Orchard 02	44.427430N-11.94748E	2016 / 2017	2
	Spain	'Red Jim'	Orchard 03	41.552120N-0.573463E	2016 / 2017	2
Peaches	France	'Fidelia'	Orchard 04	43.220277N-2.653888E	2016 / 2017	2
	Italy	'Corindom'	Orchard 05	44.440169N-11.96811E	2016 / 2017	2
	Spain	'Tardibelle'	Orchard 06	41.586239N-0.740606E	2016	1
		'Groc d'Ivars'	Orchard 07	41.834309N-0.532591E	2017	1
Cherries	Belgium	'Lapins'	Orchard 08	50.779583N-5.136766O	2016 / 2017	2
		'Sweetheart'	Orchard 09	50.842866N-5.173066O	2016 / 2017	2

570

571 Table 2. Chemical treatment description applied at each orchard.

		Active ingredient		
Country	**Orchard**	**2016**	**2017**	**Application time (days before harvest)**
Belgium	Orchard 08	Captan	Captan	30
		Boscalid+pyraclostrobin	Boscalid+pyraclostrobin	14
	Orchard 09	Tebuconazole	Tebuconazole	7
		Fenhexamid	Fenhexamid	3
France	Orchard 01	Fenbuconazole	Fenbuconazole	30
		Tebuconazole	Fenbuconazole	14
	Orchard 04	Tebuconazole	Tebuconazole	7
		Fenbuconazole	Fenbuconazole	3
Italy	Orchard 02	Cyprodinil+fludioxonil	Cyprodinil+fludioxonil	30
		Boscalid+pyraclostrobin	Boscalid+pyraclostrobin	14
	Orchard 05	Boscalid+pyraclostrobin	Boscalid+pyraclostrobin	7
		Boscalid+pyraclostrobin	Boscalid+pyraclostrobin	3
Spain	Orchard 03	Cyproconazole	Cyproconazole	30
	Orchard 06	Fluopyram+tebuconazole	Fluopyram+tebuconazole	14
	Orchard 07	Fluopyram	Fluopyram	7
		Fenbuconazole	Fenbuconazole	3

572

573

574 Table 3. Population of *Bacillus amyloliquefaciens* CPA-8 and *Penicillium frequentans* Pf909 for
575 BS1-, BS2- and PF1-, PF2-, PF2×10-formulated products, respectively. Evaluations were
576 conducted after the first treatment applications in 2016 and 2017.

		BCAs population (log CFU cm^{-2}) *							
		2016				**2017**			
Crop	**Variety**	BS1	BS2	PF1	PF2	BS1	BS2	PF2	PF2×10
Peach	'Corindom'	3.85	3.84	2.48	2.55	4.74	4.77	2.08	3.98
	'Fidelia'	5.03	5.07	2.52	2.71	4.97	4.96	2.24	3.55
	'Groc d'Ivars'	-	-	-	-	4.88	4.96	2.31	4.23
	'Tardibelle'	5.15	5.03	1.86	2.15	-	-	-	-
Nectarine	'Morsiani'	3.99	4.12	2.00	2.10	4.30	4.33	1.61	2.31
	'Red Jim'	4.65	4.78	1.88	1.99	4.30	4.42	1.80	3.62
	'Tourmaline'	5.01	4.97	1.91	1.92	4.94	4.98	4.28	5.57
Cherries*	'Lapins'	4.44	4.45	2.71	3.14	4.31	3.86	2.89	4.42
	'Sweetheart'	5.49	5.43	2.73	3.07	4.22	4.29	4.39	4.20

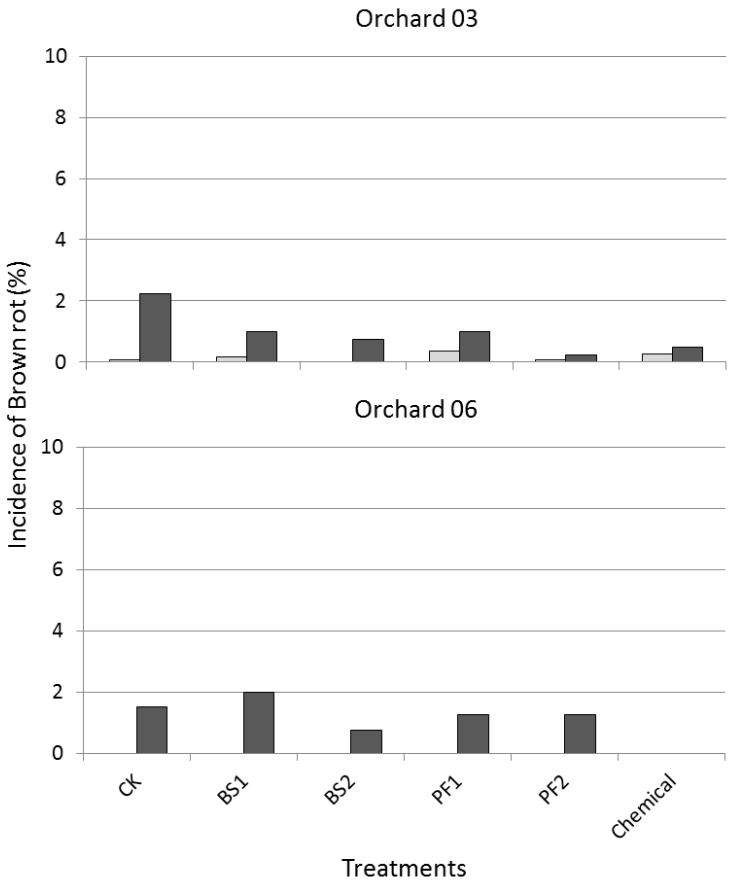
577 *On Cherries BCAs population was expressed as $\log_{10}$ CFU g^{-1}.

578

579 Table 4. Population of *Bacillus amyloliquefaciens* CPA-8 and *Penicillium frequentans* Pf909 for
580 BS1-, BS-2 and PF1-, PF2-, PF2×10- formulated products, respectively. Evaluations were
581 conducted at harvest time for trials carried out in 2016 and 2017.

		BCAs population (log CFU cm^{-2})							
		2016				**2017**			
Crop	**Variety**	BS1	BS2	PF1	PF2	BS1	BS2	PF2	PF2×10
Peach	'Corindom'	4.11	4.49	2.66	2.86	4.90	5.04	2.31	3.98
	'Fidelia'	5.23	5.24	2.47	2.49	4.83	4.77	2.55	3.75
	'Groc d'Ivars'	-	-	-	-	4.92	5.17	3.65	5.38
	'Tardibelle'	5.33	5.20	2.43	2.48	-	-	-	-
Nectarine	'Morsiani'	3.98	4.00	2.98	3.04	4.26	4.22	2.52	3.31
	'Red Jim'	4.89	4.97	2.84	3.07	5.01	5.00	2.57	3.51
	'Tourmaline'	5.02	4.93	3.21	3.43	4.87	4.87	4.39	5.84
Cherries	'Lapins'	4.07	3.87	3.22	3.47	4.83	4.59	3.47	4.42
	'Sweetheart'	4.34	4.82	3.43	3.76	4.36	4.56	4.47	4,42

582 *On Cherries BCAs population was expressed as $\log_{10}$ CFU g^{-1}.



583

584 Figure 1. Efficacy trials classed under Scenario 1 (orchards with very low disease incidence in the
585 field). Data representing the incidence of brown rot (%) recorded in orchard 03 and orchard 06
586 corresponding to 'Red Jim' nectarines and 'Tardibelle' peaches, respectively, at harvest time (□
587) or at postharvest (■), after 7 days at 0 ºC, and 90 % RH, plus 5 days at 20 ºC and 85 % RH.
588 Treatments evaluated were BS1 and BS2, using products based on *Bacillus amyloliqueaciens*
589 CPA-8; PF1 and PF2, and using products based on *Penicillium frequentans* Pf909; Chemical,
590 which used the standard chemical strategy for each country; and CK, wherein no treatment was
591 applied. No differences were found according to the LSD test ($p < 0.05$).

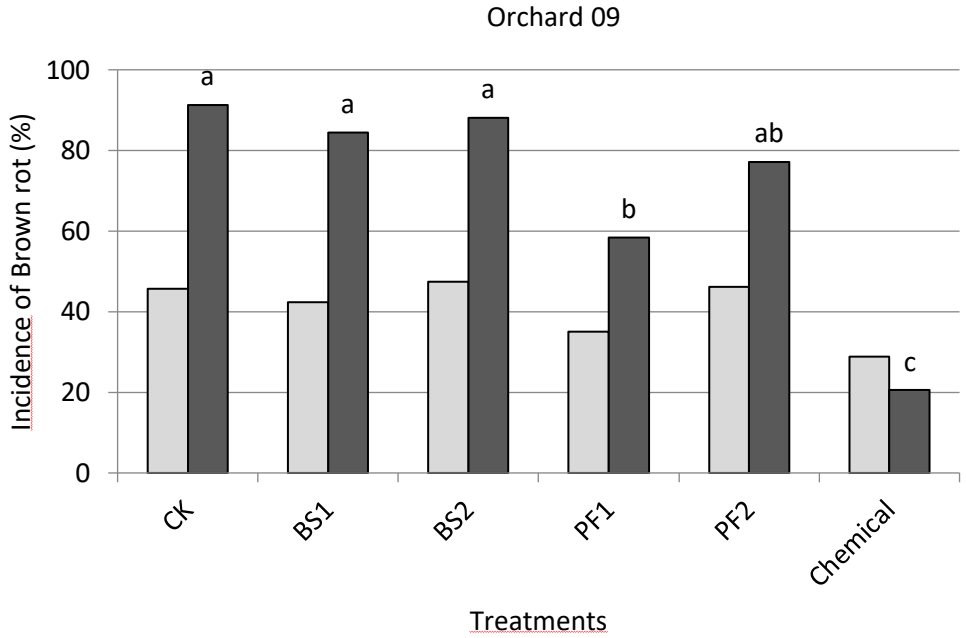


Figure 2. Efficacy trial classified under Scenario 2 (very high disease incidence). Data represent the incidence of brown rot (%) recorded in orchard 09 in 2016, corresponding to 'Sweetheart' cherries at harvest time (□), or at postharvest (■) after 7 days at 0 ºC and 90 % RH, plus 7 days at 20 ºC and 85 % RH. Treatments evaluated were BS1 and BS2, products based on *Bacillus amyloliqueaciens* CPA-8; PF1 and PF2, products based on *Penicillium frequentans* Pf909; Chemical, which used the standard chemical strategy for each country; and CK, wherein no treatment was applied. Within the same series, different letters indicate significant differences ($p < 0.05$) according to the LSD test.

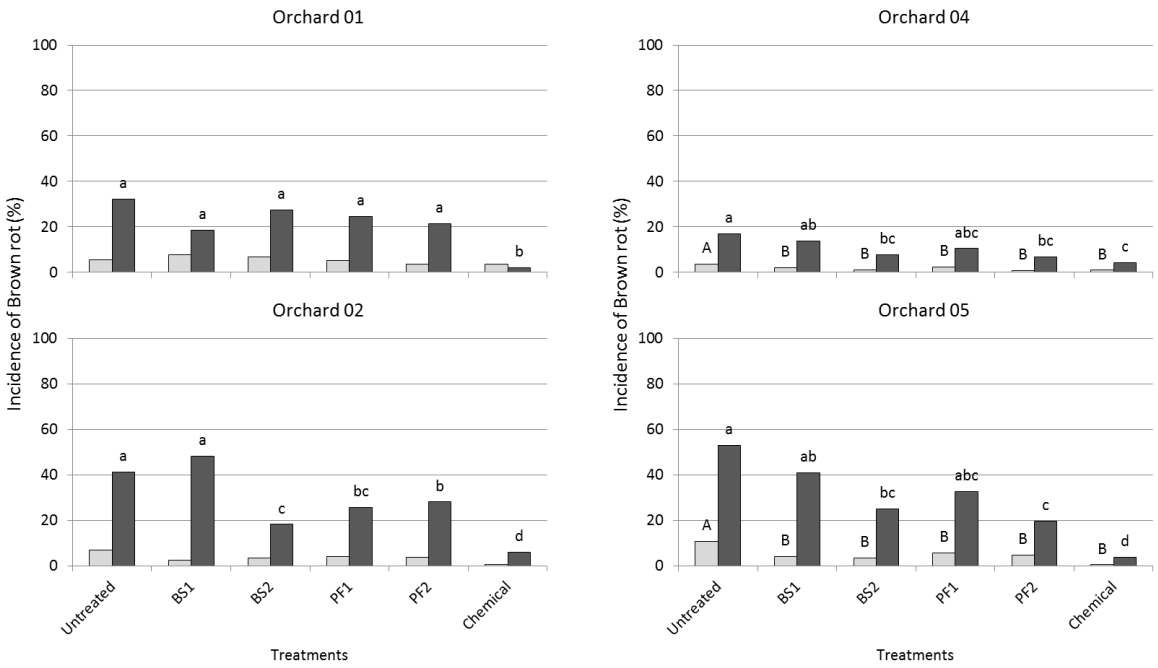
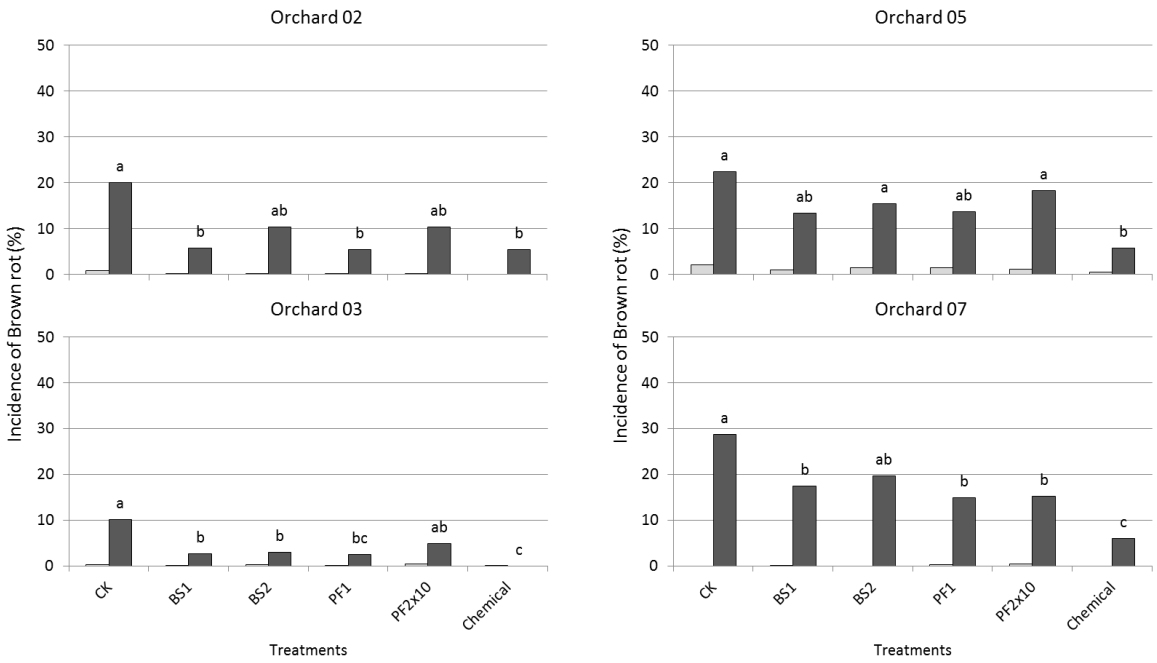


Figure 3. Efficacy trials classed under scenario 3 (controllable disease). Data represent the incidence of brown rot (%) recorded in 2016 for orchard 01 ('Tourmaline' nectarine in France), orchard 02 ('Morsiani' nectarine in Italy), orchard 04 ('Fidelia 'peach'in France), and orchard 05 ('Corindom' peach in Italy), at harvest time (□) or at postharvest (■) after 7 days at 0 ºC, and 90 % RH, plus 7 days at 20 ºC and 85 % RH. Treatments evaluated were BS1 and BS2, products based on *Bacillus amyloliqueaciens* CPA-8; PF1 and PF2 products based on *Penicillium frequentans* Pf909; Chemical, which used the standard chemical strategy for each country; and CK, wherein no treatment was applied. Within the same series, different letters indicate significant differences ($p < 0.05$) according to the LSD test.



615

616 Figure 4. Efficacy trials included in scenario 3 (controllable disease). Data represent the incidence
617 of brown rot (%) recorded in 2017 for orchard 02 ('Morsiani' nectarine in Italy), orchard 03
618 ('Red Jim' nectarine in Spain), orchard 05 ('Corimdom' peach in Italy), and orchard 07 ('Groc
619 d'Ivars' peach in Spain) at harvest time (□) or at postharvest (■) after 7 days at 0 ºC and 90 % RH
620 plus 7 days at 20 ºC and 85 % RH. Treatments evaluated were BS1 and BS2, products based on
621 *Bacillus amyloliqueaciens* CPA-8; PF1 and PF2, products based on *Penicillium frequentans*
622 Pf909; Chemical, which used the standard chemical strategy for each country; and CK, wherein
623 no treatment was applied. Within the same series, different letters indicate significant differences
624 ($p < 0.05$) according to the LSD test.

625