Balance between resilient fruit surface microbial community and population of *Monilinia* spp. after biopesticide field applications

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All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Belen Guijarro and Antonieta De Cal. Sample collections were performed by Carla Casals and Neus Teixidó. The first draft of the manuscript was written by Antonieta De Cal, Belen Guijarro, Inmaculada Larena y Paloma Melgarejo, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations of interest: none

Abstract

The microbial diversity on the host plant surface must be maintained because population diversity and quantity are essential to avoid disease development. It would be necessary examine the patterns and mechanisms associated with the massive and reiterative introduction of a microbial pest control agent. The effect of inundative releases of the biopesticide contained *Penicillium frequentans* for the control of *Monilinia* spp populations, and the effect on the fruit surface microbiota on 18 stone fruit orchards located in four European countries for more than two crop seasons against brown rot were studied. *P. frequentans* was monitoring after application in order to assess whether was persistent or not in the environment. Hydrolysis of fluorescein diacetate and denaturing gradient gel electrophoresis were used to studied *P. frequentans* effects on fungal and bacteria non-target on fruit surface. Effect of *P. frequentans* formulations on the populations of *Monilinia* spp. on fruit was also recovered in different orchards. *P. frequentans* population on stone fruit surfaces showed a large range between 100 CFU/cm^2 to 10,000 CFU/cm^2, where postharvest recovered populations were more than 10-100-fold than preharvest. Population of *P. frequentans* varied between orchards and years, rather than by formulations. *P. frequentans* formulation reduced *Monilinia* spp. population and brown rot and latent infections caused by this pathogen at preharvest and harvest time, while stabilizing or increasing antagonist populations and avoiding non-target impacts. However, fungicides reduced significantly the microbial activity on peach surfaces.

Keywords: non target effect, risk assessment, biocontrol, microbiota, *Penicillium frequentans*.

1. Introduction

Penicillium frequentans Westling is an indigenous specie from Mediterranean area and is also found in different types of climates worldwide (Melgarejo et al., 1985; Ramirez, 1982). *P. frequentans* has been isolated from various substrates and habitats such as water, wood pulp, food products (cereals, seeds and dried fruits, fresh fruits and fruit juices, dairy products), and soils (Domsch et al., 1983). *P. frequentans* strain 909 (Pf909) is common constituent of the resident microbiota of Spanish peaches, was isolated from of peach flowers and have previously demonstrated good efficacy to control brown rot caused by *Monilinia* spp. in stone fruit (Melgarejo et al., 1986). Species present naturally in the pathosystems should be good candidate for biocontrol agents (BCA) (Köhl et al., 2019). Pf909 can be used as an effective and safe biological control agent (BCA) against brown rot because it is able to adapt to different environmental conditions such as a broad range of pH, it was resistant to UV damage, low and high temperatures, and desiccation conditions (Guijarro et al., 2017a). Suspension concentrate of Pf909 conidia are potential biocontrol products 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 (Guijarro et al., 2017b). Natural background level of *P. frequentans* during stone fruit growth season (10–10^2 CFU per flower or fruit) are insufficient for controlling brown rot (De Cal and Melgarejo 1992; Guijarro et al., 2008). Peach surface had to be extensively covered by Pf909, where it will only be effective when its concentration is high enough and substantially greater than that of *Monilinia* (Guijarro et al., 2008).

BCAs could disturb indigenous microbial populations if they were applied to plant in sufficient numbers (Brimner et al., 2003; Winding et al., 2004), such as had been observed after chemical treatments (De Cal and Melgarejo, 1992). One of the most important concerns associated with the use of BCAs in plant protection is the possible disruption of microbial processes and the important ecological functions associated therewith. Many BCAs produce metabolites that inhibit or suppress other organisms as *Trichoderma atroviride* strain SC1 used against the fungus *Armillaria mellea* by the production of cell wall-degrading enzymes and antibiotics (Lu et al., 2004; Pertot, 2016). The host microbial communities play very important roles in the equilibrium of the ecosystem, especially in the maintained of non-disable microorganism population (Massart et al., 2015). To conserve or improve biodiversity is a general objective of sustainable cropping and enhancing abundance and efficacy of the natural enemies existing community as a priority for this production system (IFOAM Anonymous, 2014; Zehnder et al., 2007).

Non-target effects can be defined as effects of the introduced BCA on organisms other than the target organisms or on biogeochemical cycles (Winding et al., 2004). For environmental risk analysis, direct or indirect non-target effects mediated through a chain of events or interactions among organisms will cause concern. Non-target effects can be categorized in the same way as Domsch et al. (1983) categorized the side effects of agrochemicals on soil microorganisms, as negligible, tolerable, and critical, depending on the magnitude of the effect and the time needed for the system to recover. A prerequisite for introducing the BCA into the environment is generally that, in addition to effective disease suppression, the effects on non-target organisms should be at least tolerable, if not negligible (Winding et al., 2004). To this end, knowledge concerning the microbial ecology of the target habitats is necessary for reasonable risk assessment studies relating to the release of beneficial microorganisms (Anonymous, 2007, OECD).

Understanding the dynamic populations of the BCA and among the BCA and resident microbial communities in the environment is crucial not only for the efficacy of the BCA in the control of the target pathogens, but also for the environmental risk assessment.

Community-level physiological profiles of soil bacteria were evaluated using the Biolog® method, which tests the ability of a microbial community to utilize different C substrates contained in a microplate (Lupwayi et al., 2009). In the last decade, several techniques based on culture-independent molecular methods have also been developed for the study of microbial environmental communities (Massart et al., 2015). For the rapid estimation of the population composition with a highly reproducible profile in a large number of samples, fingerprinting techniques such as terminal restriction fragment length polymorphism (T-RFLP) or the denaturing gradient gel electrophoresis (DGGE) were probably one of the most suitable approaches (Hunter et al., 2006). Fingerprinting techniques provided information on the diversity and dynamics of, e.g., ribotypes in an environmental sample in response to environmental triggers (Dunbar et al., 2001, 2000; Gans et al., 2005). Fluorescein diacetate (FDA) has also been used since early 90s as a measure of microbial activity, and it is generally applied to estimate total microbial activity and has been proposed to be used as a biochemical/biological indicator of microbial activity in different ecological niches (Vivian et al., 2013). The current approach tools are based on next generation technologies (NGS) are revolutionizing research on environmental microbiology and in the future will shed light into relevant further questions in BCA environmental behavior.

Biocontrol research on Pf909 has long been focused on the study of strain biology and on its interaction with pathogens (Guijarro et al., 2017a; 2017b) and host plants (Guijarro et al., 2008). Further focus on plant-associated microbial communities is necessary for better understanding the integrate role of Pf909 as a BCA with the natural microbiota on brown rot control. Therefore, unwanted, unspecific actions of the introduced Pf909 beneficial microorganism against non-target organisms have to be assessed. The objectives of these studies were to determine the potential effect of inundative releases of Pf909 for non-target species and to investigate whether their suitability differs from those of the target *Monilinia* spp. under different real field conditions, and their relationship to the success of the Pf909 in brown rot management.

2. Material and methods

2.1 Field experimental design and treatments

Eighteen field experiments were carried out along different European climatic zones in stone fruit commercial orchards located in Spain (Lleida) over three growing seasons from 2015 to 2017, and in France (Roquecourbe, Aude), Belgium (Velm and Metsteren) and Italy (Bagnacavallo) over two growing seasons from 2016 to 2017 (Table 1). Different cultivars of cherries (*Prunus avium* L.), peaches (*P. persica* (L.) Batch) and nectarines (*P. persica var. nucipersica* (L. ex Borkh.) C.K.Schneid.) were used (Table 1). Plots were distributed in a completely randomized block design with four replicates per treatment. Each replicate consisted in 3 trees. Buffer trees (non-treated trees) were used to separate treatments and replicates. Biological treatments consisted of Pf909 formulates (adjusted at 10^6 conidia/mL in 2015, 2016 and 2017 or 10^7 conidia/mL in 2017) Chemical positive control were applied based on fungicide treatments and doses (CH) according to good agriculture practice for each countries: DMI (demethylation inhibitors), SDHI (succinate dehydrogenase inhibitors), and AP (aniline-pyrimidine)-fungicides were applied every year in all orchards, except in Italy where DMIs were not applied, and in France where only DMIs were applied. QoI (quinone outside inhibitors), and PP (phenylpyrroles)-fungicides were also applied in Italy and Belgium in 2016 and 2017. One ketoreductase inhibitors (fenhexamid) and captan were also applied in Belgium orchards in 2016 and 2017. All treatments were preharvest applied four times following application schedule for controlling brown rot: 30, 14, 7 and 3 days approximately before harvest. Negative control treatment based on non-treated trees (NT) was also included.

The area of the orchards where the trials were conducted received the standard cultural and plant protection practices for the crop and agronomical conditions until 45 days before harvest. The compatibility of Pf909 with all pesticides used was previously evaluated (Guijarro et al., 2019a).

2.2 Pf909 isolation, production and formulation

2.2.1 Culture

A monosporic isolate of *P. frequentans* strain (Pf909) (ATCC 908-81), which was obtained from peach twig surfaces (Melgarejo and M-Sagasta, 1984), was used for all studies as a microbial pest control agent (MPCA). The isolate was stored at −80 °C in 20% glycerol (long-term storage) or at 4 °C on potato dextrose agar (PDA; Difco, Detroit, MI, USA) slants in the dark (short-term storage).

2.2.2 Formulation preparation

For trials using formulates of Pf909, the microbial plant active substance (MPCA) is a dried conidia powder of Pf909 with a concentration of $1.8x10^{11}$ conidia/g and a viability range of 94-98%. This MPCA was used with different liquid carriers to obtain different formulation products. The production process of pure dried conidia powder followed the description of Guijarro et al., (2006) and was made under pilot plant process conditions. A MPCA of 9.16 g was suspended in 90.84 g of carrier or formulation mixture to have a final concentration of MPCA of $1x10^{10}$ conidia/g for all formulated products. The mixture was homogenized by using an Ultra-Turrax at 3000 rpm for two min and the conidia concentration was determined by microscopic counting using a haemocytometer.

Three oil-based formulations were applied: Pf1= PSPF214-OD-A, based on plant oil;

and Pf2=PSPF214-OD-B and Pf3=PSPF214-OD-2 based on technical oil. Pf1 was applied in 2015, 2016 and 2017, while Pf2 and Pf3 were only applied in 2015 or 2016, respectively. Furthermore, Pf1 was also tested at two different application rates 10^6 and conidia/mL in 2015 to 2017 and 10^7 conidia/mL in 2017. All Pf909 formulates were developed and produced by Bayer CropScience Biologics GmbH.

2.3 Pf909 population dynamic evaluation

Fruit from the different treatments, either biological (Pf1, Pf2 and Pf3), or chemicals (CH), and non-treated (NT) were sampled for Pf909 population dynamic evaluation at 30 days before harvest (after first treatment application) and at harvest, in the eighteen commercial orchards of stone fruit in Europe over the three growing seasons (2015 to 2017). Four replicate with five (nectarines or peaches) or twenty (cherries) fruit per replicate were evaluated for each treatment. Fruit were immersed in 250 mL of sterile distilled water (SDW) in a plastic container, and shacked for 60 min at 90 rpm at room temperature (RT). Then, the liquid was treated according to Guijarro et al. (2008).

P. frequentans population was estimated as the number of colony forming units (CFU) of *P. frequentans* per cm^2 of fruit surface for peaches and nectarines and CFU per fruit in cherries. CFU of *P. frequentans* per cm^2 were estimated by calculating each fruit surface from the measurement of two of its diameters following the formula:

$$CFU/cm^2 = [CFU\ per\ fruit\ /\ 4\ \Pi\ (fruit\ mean\ diameter/2)^2]$$

Colonies were phenotypically evaluated as *P. frequentans*, and 10% of these colonies of each treatment were confirmed as Pf909 by specific strain molecular characterization (Guijarro et al., 2019b).

2.4 Pf909 effect on target microorganism (Monilinia spp.) and brown rot disease

2.4.1 *Monilinia* spp. population dynamic

Fruit were sampled and treated as described above in section 2.3., but, in this case, for assessing the *Monilinia* spp. populations. *Monilinia* spp. population was estimated as the number of colony forming units (CFU) of Monilinia per cm^2 of fruit surface for peaches and nectarines and CFU per fruit in cherries.

2.4.2 Latent infections and brown rot incidence assessment

Latent infection incidence was estimated at 30 days before harvest fruit (on immature fruit before first treatment application) and brown rot incidence on asymptomatic fruit was estimated at harvest in the eighteen commercial orchards described above. Asymptomatic inmature fruit were treated as described by Gell et al. (2008). After freezing, four replicates per treatment with five (nectarines or peaches) or twenty (cherries) fruit per replicate were placed in sealed packing trays at 22ºC, 100% RH, each containing 20 nectarines or peaches or 40 cherries for 7 days. Brown rot incidence was estimated at harvest on asymptomatic mature fruit in sealed packing trays such as described above.

Latent infection and brown rot incidence were determined by the percentage of decayed and healthy fruit by visualization the presence of *Monilinia* conidia sporulating on fruit lesions after 5-7 days of incubation. At this point, cross-infections did not occur, because it takes seven days for conidia of *Monilinia* spp. to germinate, produce mycelia, and sporulate (Byrde and Willetts 1977).

2.5 Pf909 effects on non-target microbiota

Fruit were sampled from trees treated with the Pf909 formulates (Pf1, Pf2 and Pf3), CH, and untreated (NT) at 7, 15, 30 days before harvest and at harvest in two commercial orchards of nectarines in Spain during 2015 and 2016 growing seasons (ES15.2 and ES16.2). Four replicates, with five nectarines per replicate were evaluated for each treatment. Five nectarines were immersed in 250 mL of potassium phosphate buffer SPMS (pH 7.6; 60 mM sodium phosphate monobasic salt) (Sigma Chemical Co. St Louis, MO) in a plastic container, and shook for 60 min at 110 rpm at RT. The liquid was centrifuged for 10 min at 4 ºC and 10,000 rpm and the pellet suspended in 20 mL buffer (SPMS). The pellets were lyophilized for 24 hours using a Cryodos-50 lyophilizer (Telstar, Barcelona, Spain). The fruit lyophilized pellets (FLP) were then made into a powder using a high-speed benchtop tissue homogenizer (FastPrep -24Instrument, MP Biomedicals, Solon, OH, USA) for 30 seconds for microbial density and microbial diversity analysis.

2.5.1 Fruit microbiota density analysis by FDA.

Quantification of epiphytes microbial density on nectarine surface over the crop seasons was assessed by measuring FDA hydrolysis through adaptation of Adam and Duncan procedure (2001). Briefly, 1g of FLP sample was incubated in 50 mL conical flasks containing 10 mL of 60 mM sodium phosphate buffer (pH 7.6) and 2000 ng/µl FDA (Sigma Chemical Co. St. Louise) for 60 min at 30 ºC and 90 rpm on an orbital shaker. Samples without FDA were used as controls. After incubation, reaction was quickly stopped by adding 10 mL of acetone to each flask and shacked briefly by hand. Samples were filtrated through No 1 Whatman filter paper and the filtrates measured at 492 nm in a spectrophotometer (S-20 Spectrophotometer, BOECO, Germany).

2.5.2 Fruit microbiota diversity analysis by PCR-DGGE.

Quality analysis effect of each treatment (Pf1, Pf2, Pf3, CH) was related with the structure of the fruit microbial community on each sample compared to NT. The structure of the fruit microbial community was assessed using PCR-DGGE.

DNA was extracted from each sample of FLP (0.5 g) byVWR Omega EZNA water

DNA Kit (MoBio Laboratories, Inc., USA) according to manufacturer's instructions. PCR amplification of the bacterial 16S or fungal 18S rRNA gene was performed. PCR was carried out using 25 to 50 ng of DNA template and 5 pmol of each primer in a final reaction volume of 50 µL GoTaq Green Master Mix (Promega).

Fungi DNA was amplified used a nested PCR approach that included two rounds of amplification. Primers were EF4F (TCCTCCGCTTATTGATATG) and ITS4 (GGAAGGGRTGTATTTATTAG) for the first round (Smith et al., 1999; White et al., 1990), and ITS2 (GCTGCGTTCTTCATCGATGC) and ITS1 (CTTGGTCATTTAGAGGAAGTAA) for the second (Gardes and Bruns, 1993: Smith et al., 1999). A GC-rich tail (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3´) was incorporate at the 5´end of the ITS1 primer. The PCR conditions were 94 ºC for 5 min; 35 cycles of 94 ºC for 0.5 min, 55 ºC for 0.5 min, 72 ºC for 0.5 min and a final incubation at 72 ºC for 5 min. A 999 bp DNA fragment of fungi was obtained and diluted up to 1/1000 to proceed to the nested PCR. Next PCR was performed with same PCR cycle. Final PCR product had 300 bp.

Bacteria were amplified with primer forward 341-GC (CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCTACGGGAGG CAGCAG) and reverse 907r (CCGTCAATTCCTTTGAGTTT) (Schmalenberger and Tebbe, 2003). The PCR amplification conditions were 94 ºC for 5 min; 35 cycles of 95 ºC for 1 min, 53 ºC for 1 min, 72 ºC for 2 min and a final incubation at 72 ºC for 10 min. A 550 bp DNA fragment of bacteria were amplified.

DGGE analysis was performed with a DCode DGGE system (Bio-Rad Laboratories, Hercules, CA, USA). PCR product (300 to 500 ng) was loaded into 6% (w/v) polyacrylamide gels (acrylamide/bisacrylamode 37.5/1) with denaturing gradients ranging from 20–60% for fungi and from 20–80%. DGGE was performed in 0.5 TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA [pH 8.0]) for bacteria and 1.0 for fungi at 60 ºC for 4 h at 200 V in the case of fungi and at 60 ºC, during 5 h at 150 V for bacteria. The gel was then stained with GelRed (Biotium) visualized and photographed using a gel imaging system (Bio-Rad Lab., Hercules, CA, USA).

The bands observed in the DGGE analysis were identified. For this, selected bands were excised from gels with sterile scalpel blades and DNA was purified by Wizard SV Gel and PCR Clean Up System (Promega) according with manufacture instructions. Extracted DNA (10 µL) was used as template for PCR amplification was conducted as described previously, using a non-GC-clamped version of the forward primer. The sequences recovered were aligned with bacterial and fungal gene fragments available from the National Center for Biotechnology Information (NCBI) databases.

2.6 Data analysis

All data were analysed by one-way analysis of variance (ANOVA) using a statistical program (Statgraphics-Centurion XVI version 16.1.03). Statistical significance was set at 5 %. Data of CFU per fruit cm^2 and latent infection or brown rot incidence for each treatment were log (x+1) or arcsine transformed, respectively, in order to improve homogeneity of variances before analysis. When the results of the F-test were significant ($p \leq 0.05$), means were compared using the Student–Newman–Keuls multiple range test (Snedecor and Cochran, 1980).

Using the combined data from all surveys, correlation and regression analyses were performed using Statgraphics-Centurion XVI version 16.1.03 in order to analyze the relationships between brown rot latent infection and brown rot incidence at harvest to *P. frequentans* population at preharvest and harvest, respectively.

For microbial activity evaluation, the amount of fluorescein content that was hydrolyzed by enzymatic activity in the samples was calculated by reference to a standard curve prepared from the fluorescein standards.

DGGE banding patterns were compared with Sammon's nonlinear mapping using NTSYS 5.0 and with dendrograms based on distance matrices, using the unweight-pair group method using average linkages. Unweight pair pair group method with arithmetic averages (UPGMA) cluster analysis of dice distance matrix calculated from DGGE banding patterns (based on presence/ absence).

3. Results

3.1 Pf909 population dynamic evaluation

The number of Pf909 CFU on fruit surfaces at pre and harvest were presented in Figures 1 to 4, where Pf909 formulates maintained a range of log CFU/cm^2 on fruit surface from 1.8 to 5.38.

Population of Pf909 after first application and just before harvest during three crop seasons at Spanish orchards were shown in Fig. 1. There were significant differences between Pf909 CFU/cm^2 at the first application and harvest in 2015, 2016, and 2017 (Fig. 1), except on nectarines in 2015 in Albesa after Pf2 application (Fig. 1b). A significant 10-fold increase on Pf909 population was observed between preharvest and at harvest application (P=0.05) in each orchard (Fig. 1). Population of Pf909 was variable among years in each orchard to the same Pf1 formulate (P=0.05) (Fig. 1). The highest Pf909 population was recovered in 2015 when Pf1 was sprayed at 10^6 conidia/mL (between 3.35 log CFU/cm^2 at preharvest in ES15.2 to 4.64 log CFU/cm^2 at harvest in ES15.1) (Figs. 1a and 1b). Dynamic populations of Pf1 during 2016 (between 1.98 log CFU/cm^2 at preharvest in ES16.2 to 3.07 log CFU/cm^2 at harvest in ES16.2) (Figs. 1c and 1d) reached similar adequate levels than in 2017 when Pf1 was sprayed at 10^6 conidia/mL (between 1.80 log CFU/cm^2 at preharvest in ES17.1 to 3.55 log CFU/cm^2 at harvest in ES17.2) (Figs. 1e and 1f). However, similar Pf909 population resulted from different Pf909 formulations on fruit surface (Figs. 1a-d) when they were applied at a dose of 10^6 conidia/mL on Spanish orchards during 2015 or 2016 (P=0.05). A significant increase of Pf909 CFU/cm^2 were recorded when Pf1 was applied at 10^7 conidia/mL, specially on nectarines (between 4.23 log CFU/cm^2 at preharvest to 5.38 log CFU/cm^2 at harvest in ES17.2) (P=0.05) (Figs. 1f).

The same biological formulates (Pf1 and Pf3) applied in Spanish orchards in 2016 and 2017, were used on peach and nectarine orchards in France (Fig. 2) and Italy (Fig. 3), and on cherries in Belgium (Fig. 4) during 2016 and 2017. Population of Pf909 was variable among years in each orchard without any formulate effects. Population of Pf909 on fruit surface at harvest, after four application of each formulate along crop, was more than 10-fold higher than preharvest application at 10^6 conidia/mL (P=0.05), except in French peach orchards (Figs. 2a and 2c), Italian peach orchard 2017 (Fig. 3c), and Belgium orchards cv. Lapin in 2017 (Figs. 4c). The 10-fold higher dose of Pf1 in 2017 provided a better conidia surface cover during the application period with 2.31 to 4.81 log CFU/cm^2 after first application and 3.31 to 5.17 log CFU/cm^2 at harvest (P=0.05) (Figs. 2c-d, 3c-d, 4c-d).

3.2 Pf909 effect on the target microorganism (Monilinia spp.) and brown rot disease

3.2.1. *Monilinia* population dynamic

The effects of the three Pf909 formulates on the CFU of *Monilinia* spp. conidia on fruit surfaces were illustrated in Figs. 1 to 4, where the number of *Monilinia* spp. conidia on non-treated fruit surfaces was significantly higher at harvest than at preharvest in all orchards (P=0.05). Pf1, Pf2, Pf3, and the CH significantly reduced the pathogen population on fruit surfaces both at preharvest and harvest when it was compared with untreated fruit surfaces (P=0.05), except on Spanish nectarines in 2016 (Fig. 1d), Italian peaches (Figs. 3a and 3c), and Lapins cherries in 2016 (Fig. 4a). No significant differences were observed between pathogen population reduction by biological and chemical treatments.

3.2.2. Latent infections and disease severity assessment

Brown rot and latent infection incidence caused by *Monilinia* spp. on untreated fruit were between 2.5 to 68.75%, and 5 to 91%, respectively in Spanish orchards (Fig. 1), while between 52.63 to 66.6 % and 0 to 58.8%, respectively in French orchards (Fig. 2), between 40.0 to 55% and 0 to 36.84%, respectively in Italian orchards (Fig. 3), or between 31.5 to 55% and 0 to 45%, respectively in Belgium orchards (Fig. 4).

Pf1, Pf2, and Pf3 treatments significantly reduced latent infections and/or brown rot severity incidence on fruit at preharvest and/or harvest, respectively when they were compared with untreated fruit (P=0.05) (Figs. 1 to 4). No control was recovered on nectarines Red Jim in Spain in 2015 (Fig. 1b), where no latent infection and brown rot was observed in the untreated control, and on nectarines in Spain and France in 2017 (Figs. 1d and 2d), and on peaches Corindom in Italy (Figs. 3a and 3c). Similar disease control was recovered on biological and chemical treated fruit (P=0.05) (Figs. 1 to 4), except on nectarines in Spain, France, and Italy in 2017 (Fig. 1d, 2d, and 3d), and on peaches in Italy (Fig. 3c).

The percentage of brown rot incidence was significantly and negatively correlated with Pf909 population at harvest (r = -0.72, P = 0.00001). Furthermore, the percentage of latent infections was also significantly and negatively correlated (r = -0.54, P = 0.0001) with Pf909 population at preharvest.

In addition, the brown rot incidence was a function of the number of *P. frequentans* CFU at harvest and could be fitted by the following equation:

$$\% \text{ BR} = 52.22 - 7.36 \text{ LOG}_{10} (\text{CFU Pf909}+1) \quad (R^2 = 52.54\%)$$

3.3 Pf909 treatments on non-target microbiota

3.3.1. Fruit microbiota density analysis

Microbial activity was shown as fluorescein per gram of dry skin fruit for non-treated and treated nectarine surface samples in two orchards in Spain, ES15-2 (Fig. 5a) and ES16-2 (Fig. 5b) with Pf1, Pf2 and Pf3 biological products. The application of Pf909 at preharvest and harvest significantly ($P = 0.05$) increased microbial activity. However, chemical applications significantly ($P = 0.05$) reduced the microbial activity on peach surface compare to the untreated control in both orchards.

3.3.2. Fruit microbiota diversity analysis

Analysis by DGGE of PCR-amplified eubacterial 16S rDNA fragments and with 18S rRNA fungi fragment along treatments on peach surface with Pf909 formulates (Pf1, Pf2 and Pf3) during two consecutives growing seasons in Spain (2015 and 2016) revealed distinctly different profiles for the different fruit surface treated with the biologicals, the chemical or untreated (data not shown). Results were confirmed by cluster analysis (Figs. 6 and 7).

Fungi and bacterial microbial epiphytic populations from 2015 and 2016 Spanish orchards evaluated were distributed into two main clusters (Figs. 6 and 7). There were no differences among Pf909 formulates in microbial population distribution. The reproducibility of the profiles from different DNA extractions belonging to the same sample was unaffected. The profiles of the fungal populations were more variable than bacterial populations and the dendrogram reflected the difficulty in grouping the application dates together (Figs. 6 and 7).

The CH (cyproconazole, tebuconazole and fenbuconazole) were grouped, and the biological applications (Pf1, Pf2 and Pf3) did not present divergence from untreated fungal samples in both years (Fig. 6a and 6b). The biological samples from fruit treated by Pf1, Pf2, and Pf3 as well as the untreated controls presented a distribution grouped by dates, except in the case of 2015 samples at seven and fifteen days before harvest, where no effect of samples date were observed (Fig. 6a).

In the case of effect of Pf909 applications on bacteria community diversity, chemical fungicides are grouping together with no effect on application dates, since biological treatments are grouping by dates (Fig. 7a and 7b).

4. Discussion

Biological brown rot control by Pf909 reduced disease incidence and *Monilinia* population growth rate, while stabilizing or increasing antagonist populations and avoiding non-target impacts. However, chemical fungicides reduced significantly the microbial activity on peach surfaces compare to the untreated control. Pesticide applications had a large effect on peach non-target epiphytic fungi, reducing populations in the field in some case by up to 50% (De Cal and Melgarejo, 1992).

To conserve or improve biodiversity is a general objective of sustainable cropping and enhancing abundance and efficacy of the natural antagonist existing community is a priority for this production system (IFOAM Anonymous, 2014). Before beginning any biocontrol program, it is also important to determine the lethal and sublethal impact of BCAs on non-target organisms (Lefebvre et al., 2011; Preetha et al., 2010), because the microbial communities play very important roles in the ecosystem. Therefore, the potential impact of any fungicide application on natural antagonist populations must be previously investigated (López et al., 2018). The influence of a single microorganism on the microbial community has already been studied for plant pathogens and/or BCAs (Kröber et al., 2014; Schreiter et al., 2014). Fungal and bacterial populations on nectarines treated with Pf1, Pf2 and Pf3 did not present divergence from both populations in untreated samples, and they showed an increasing FDA activity in the treated fruit was observed. It could suggest a general trend for disease control with increasing FDA activity in biological applications, and related to the highest levels of Pf909 populations on fruit.

FDA and DGGE helped us to demonstrate that there was no need to worry about non-target effects of Pf909 on peach epiphytic micro-organisms. The use of molecular tools enabled the presence of genes encoding for important functions to be traced, and showed that release of a relatively small quantity of a BCA did not modify the epiphytic functioning (Sessitsch et al., 2002). Another family of methods enables a global assessment of the impact of BCA introduction on the structure of the microbial communities showed that even when an impact was detected shortly after BCA introduction, the structure of the microbial communities tended to revert rapidly to their initial stage (Alabouvette et al., 2011). After a few weeks, there was no difference in the structures of the microbial communities between the infested soil and the non-infested control. Moreover, similar studies have shown that traditional agricultural practices have much more impact on microbiota than the release of a BCA. This is especially the case with chemical treatments (Vivian et al., 2013).

Pf1, Pf2, and Pf3 treatments significantly reduced pathogen population on fruit surfaces, latent infection and brown rot incidence, both at harvest and preharvest, when they were compared with untreated fruit. No significant differences were observed between brown rot control by biological and chemical treatments on cherries and up to 55% of peach and nectarine orchards. Successful brown rot biological control depends on establishing large populations of Pf909 on stone fruit surface (De Cal et al., 1990; Guijarro et al., 2018). A significant correlation was recorded between brown rot control and Pf909 population. A Pf909 population greater than 500,000 CFU/cm^2 would be necessary to reduce the brown rot incidence to below 10%. Various agro-ecological factors play an important role for the success of a BCA in the augmented site. These include fitness of the BCA (Borzoui et al., 2016), its capacity to disperse in augmented sites (Zappala et al., 2012), tolerance to abiotic factors (Hasan and Ansari 2015), availability and suitability of host (Alam Shah et al., 2016), interaction with other natural enemies (Vanaclocha et al., 2013), target specificity (Jalali et al., 2009) and tolerance to pesticides and its residual effects (Hasan and Ansari 2017; 2016; Pozzebon et al., 2010). Pf909 can be used as an effective and safe BCA because it is able to adapt to different environmental conditions (Guijarro et al., 2017a). Pf909 was also compatible with 76% of the fungicides and 90% of the insecticides commonly used against stone fruit pests (Guijarro et al., 2019a). The stability of Pf909 field populations under fungicide regimes may be also due to their highly competitive nature and their ability to exploit any ecological niche left vacant than to any tolerance to fungicides (De Cal and Melgarejo 1992).

Population of Pf909 on stone fruit surfaces showed a large range between 100 CFU/cm^2 to 100,000 CFU/cm^2, where all formulations at harvest were more than 10-100-fold than preharvest application in 2016 and 2017. Population of *P. frequentans* varied between orchards and years, rather than by the type of the applied formulate or fruit surface. A consistent population of *P. frequentans* Pf909, ranging from 1,000 to 10,000 CFU of *P. frequentans* per flower or fruit had already been reported by other Pf909 formulates (Guijarro et al., 2008). Colonization of peach surfaces by *P. frequentans* followed a general pattern with a higher colonization of fruit at preharvest than on the flowers at bloom (Guijarro et al., 2008). A significant increase of *P. frequentans* CFU/cm^2 were recorded when Pf1 was applied at 10^7 conidia/mL. Pf909 was dispersed well in treated trees, persisting in the ecosystem up to 2 weeks and staying genetically stable after 36 months of storage (Guijarro et al., 2019b).

Pf909 reduced brown rot and pathogen population, while avoiding non-target impacts. The formulations of Pf909 allowed to maintain a population on the fruit of the biocontrol agent above natural background, will suppose an effective tool in the sustainable control of brown rot on stone fruit.

Acknowledgements

This work was carried out with the financial support from BIOCOMES project (EU FP7 612713). The authors would like to acknowledge Raquel Castillo for her technical assistance.

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Table 1 Orchard datas used in Spain, France, Italy and Belgium between 2015 and 2017 seasons for field experiments.

Country	Growing season	Orchard	Location	coordinates	cultivar
Spain	2015	ES15.1	Albesa	41.779151N-0.629172E	Peach, var. Roig d´Albesa
		ES15.2	Sudanell	41.552120N-0.573463E	Nectarine, var. Red Jim
	2016	ES16.1	Alamús	41.615024N-0.740212E	Peach, var. Tardibelle
		ES16.2	Sudanell	41.552120N-0.573463E	Nectarine, var. Red Jim
	2017	ES17.1	Alfarras	41.8363652N-0.531158E	Peach, var. Groc d'Ivars
		ES17.2	Sudanell	41.552120N-0.573463E	Nectarine var. Red Jim
France	2016	FR16.1	Roquecourbe Minervoies	43.220277N-2.653888E	Peach, var. Fidelia
		FR16.2	Roquecourbe Minervoies	43.2208333N-2.651666666E	Nectarine, var. Tourmaline
	2017	FR17.1	Roquecourbe Minervoies	43.220277N-2.653888E	Peach, var. Fidelia
		FR17.2	Roquecourbe Minervoies	43.2208333N-2.651666666E	Nectarine, var. Tourmaline
Italy	2016	IT16.1	Bagnacavallo	44.4401694N-11.96811944E	Peach, var. Corindom
		IT16.2	Bagnacavallo	44.4274305N-11.9474888E	Nectarine, var. Morsiani 90
	2017	IT17.1	Bagnacavallo	44.4401694N-11.96811944E	Peach, var. Corindom
		IT17.2	Bagnacavallo	44.4274305N-11.9474888E	Nectarine, var. Morsiani 90
Belgium	2016	BE16.1	Velm	50.77931N-5.13162W	Cherry, var. Lapins
		BE16.2	Metsteren	50.503887N-4.469936W	Cherry, var. Sweetheart
	2017	BE17.1	Velm	50.77931N-5.13162W	Cherry, var. Lapins
		BE17.2	Metsteren	50.503887N-4.469936W	Cherry, var. Sweetheart

Fig. 1 Population of *Penicillium frequentans* Pf909 (■) and *Monilinia* spp (□) as ($\log_{10}$ CFU/cm^2) on fruit surface in three Spanish orchard during 2015 (a, b), 2006 (c, d), and 2017 (e, f): (a) ES15.1, (b) ES15.2, (c) ES16.1, (d) ES16.2, (e) ES17.1, and (f) ES17.2, after first treatment application and at harvest. Percentage of latent infection caused by *Monilinia* spp at 30 days before harvest was represented by (▲) and percentage of brown rot disease incidence at harvest by (●) on peaches (a, c, e) and on nectarines (b, d, f). The treatments were: NT (control, without treatment); CH (chemical); and Pf909 formulations: Pf1, Pf2, and Pf3 at 10^6 conidia/mL, and Pf1 at 10^7 conidia/mL in 2017 (Pf1x10). Value are the average of four determinations. Within the same pattern, different letters indicate significant differences ($P < 0.05$) according to the Student Newman Keuls test. A-D uppercases and dark grey bars refer to Pf909 population; a-d lower cases and light grey bars to *Monilinia* spp population. X-Z uppercases refer to latent infection and disease incidence (%)

Fig. 2 Population of *Penicillium frequentans* Pf909 (■) and *Monilinia* spp (□) as ($\log_{10}$ CFU/cm^2) on fruit surface in two French orchard during 2016 (a, b) and 2017 (c, d): (a) FR16.1, (b) FR16.2, (c) FR17.1, and (d) FR17.2, after first treatment application and at harvest. Percentage of latent infection caused by *Monilinia* spp at 30 days before harvest was represented by (▲) and percentage of brown rot disease incidence at harvest by (●) on peaches (a, c) and on nectarines (b, d).The treatments were: NT (control, without treatment); CH (chemical); and Pf909 formulations: Pf1 and Pf3 at 10^6 conidia/mL, and Pf1 at 10^7 conidia/mL in 2017 (Pf1x10). Value are the average of four determinations. Within the same pattern, different letters indicate significant differences ($P < 0.05$) according to the Student Newman Keuls test. A-D uppercases and dark grey bars refer to Pf909 population; a-d lower cases and light grey bars to *Monilinia* spp population. x-z lower cases refer to latent infection and disease incidence (%)

Fig. 3 Population of *Penicillium frequentans* Pf909 (■) and *Monilinia* spp (□) as ($\log_{10}$ CFU/cm^2) on fruit surface in two Italian orchard during 2016 (a, b) and 2017 (c, d): (a) IT16.1, (b) IT16.2, (c) IT17.1, and (d) IT17.2, after first treatment application and at harvest. Percentage of latent infection caused by *Monilinia* spp at 30 days before harvest was represented by (▲) and percentage of brown rot disease incidence at harvest by (●) on peaches (a, c) and on nectarines (b, d). The treatments were: NT (control, without treatment); CH (chemical); and Pf909 formulations: Pf1 and Pf3 at 10^6 conidia/mL, and Pf1 at 10^7 conidia/mL in 2017 (Pf1x10). Value are the average of four determinations. Within the same pattern, different letters indicate significant differences ($P < 0.05$) according to the Student Newman Keuls test. A-D uppercases and dark grey bars refer to Pf909 population; a-d lower cases and light grey bars to *Monilinia* spp population. x-z lower cases refer to latent infection and disease incidence (%)

Fig. 4 Population of *Penicillium frequentans* Pf909 (■) and *Monilinia* spp (□) as ($\log_{10}$ CFU/cm^2) on CHERRY fruit surface in two Belgium orchard during 2016 (a, b) and 2017 (c, d): a) BE16.1, (b) BE16.2, (c) BE17.1, and (d) BE17.2, after first treatment application and at harvest. Percentage of latent infection caused by *Monilinia* spp at 30 days before harvest was represented by (▲) and percentage of brown rot disease incidence at harvest by (●) on cv. Lapin (a, c) and on cv. Sweetheart (b, d). The treatments were: NT (control, without treatment); CH (chemical); and Pf909 formulations: Pf1 and Pf3 at 10^6 conidia/mL, and Pf1 at 10^7 conidia/mL in 2017. Value are the average of four determinations. Within the same pattern, different letters indicate significant differences

($P < 0.05$) according to the Student Newman Keuls test. A-D uppercases and dark grey bars refer to Pf909 population; a-d lower cases and light grey bars to *Monilinia* spp population. x-z lower cases refer to latent infection and disease incidence (%)

Fig. 5 Effect of *Penicillium frequentans* (Pf909) applications on microbial biomass activity measure as fluorescein diacetate FDA-staining on fruit skin in two Spanish nectarine orchards: (a) ES15.2 in 2015 and (b) ES16.2 in 2016. Data are means of three replicates each of 5 fruit samples from each treatment. Different letters indicate significant differences ($P < 0.05$) according to the Student-Newman-Keuls test. The treatments were: NT (control without treatment); Pf909 formulations: Pf1, Pf2 and Pf3, at 10^6 conidia/mL and CH (chemical treatment)

Fig. 6 Cluster analysis of fungal community structures on the nectarines phyllosphere community after the introduction of *Penicillium frequentans* (Pf909) from two orchards in Spain (a) ES15.2 and (b) ES16.2, as generated from the pooled applications profiles. The UPGMA (Unweighted Pair-Group Method with Arithmetic Means) on the DNA average peak height for each base length were used to build the dendrograms of the fungal communities. Samples were obtained after application of different treatments in each orchard. The treatments were: NT (control without treatment); Pf1, Pf2 and Pf3 (Pf909 conidia formulated) and CH (chemical treatment). For every treatment sampling were made: after first application or 30 days before harvest (-30dbh); after second application or 15 day before harvest (-15dbh); after last application or seven days before harvest (-7dbh), and at harvest (-h). UPGMA cluster analysis of dice distance matrix calculated from DGGE banding patterns (based on presence/ absence)

Fig. 7 Cluster analysis of bacterial community structures on the nectarines phyllosphere community after the introduction of *Penicillium frequentans* (Pf909) from two orchards in Spain (a) ES15.2 and (b) ES16.2, as generated from the pooled applications profiles. The UPGMA (Unweighted Pair-Group Method with Arithmetic Means) on the DNA average peak height for each base length were used to build the dendrograms of bacterial communities. Samples were obtained after application of different treatments in each orchard. The treatments were: NT (control without treatment); Pf1, Pf2 and Pf3 (Pf909 conidia formulated) and CH (chemical treatment). For every treatment sampling were made: after first application or 30 days before harvest (-30dbh); after second application or 15 day before harvest (-15dbh); after last application or seven days before harvest (-7dbh), and at harvest (-h). UPGMA cluster analysis of dice distance matrix calculated from DGGE banding patterns (based on presence/ absence)