COMPATIBILTY OF THE BIOCONTROL AGENT *Penicillium frequentans* Pf909 WITH BIOLOGICAL AND CHEMICAL PESTICIDES USED ON STONE FRUIT PRODUCTION

Short running title: Integration of biological and chemical pesticides

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Abstract

The compatibility of *Penicillium frequentans* with modern inputs in plant protection like fungicides (chemicals and biologicals) and insecticides is a pre-requisite for developing integrated disease management strategies. The combination of *P. frequentans* and *Bacillus amyloliquefaciens*, each with different mechanisms of action, competition and antibiosis, respectively, did not improve the biocontrol of brown rot when each treatment was applied individually. *P. frequent*ans and *B. amyloliquefaciens* could not be combined together in the same tank, because bacteria inhibited the germination and growth of *P. frequentans* tubes in suspension. Furthermore, *B. amyloliquefaciens* also competes with *P. frequentans* once

applied on fruit surfaces. Additionally, bacteria reduced the growth of *P. frequentans* CFUs on media, at different 25ºC and 30ºC throughout the incubation periods. However, 76% of fungicides and 90% of insecticides commonly used against stone fruit pests are compatible with *P. frequentans*. Results indicated that the most commercial chemical pesticides could be simultaneously applied with Pf909 in integrated pest control, but not *B. amyloliquefaciens*.

Keywords: Biofungicide, stone fruit, *Monilinia fructicola,* antagonistic effects, integrated control

1 INTRODUCTION

Brown rot caused by *Monilinia* spp. is an economically important fungal disease of stone fruit; it causes substantial preharvest and postharvest losses (Byrde and Willetts, 1977). Cultural practices, (e.g., eliminating overwintering inocula or rototilling the orchard bed), chemical control, and physical methods (specifically, rapid cooling after harvest) are the main strategies used against brown rot. Fungicides are commonly used for controlling brown rot in Spanish peach and nectarine orchards where they are usually applied three to five times during each growing season (Usall et al., 2010). The development of resistance in *Monilinia* spp. to certain fungicides (Egüen et al., 2016, 2015), new restrictions on the application of these fungicides, and environmental considerations have led to an increased interest in the use of biocontrol agents to control brown rot (Ooijkaas et al., 1998).

Penicillium frequentans Westling is a constituent of the resident mycobiota of peach twigs and flowers (Melgarejo et al., 1985). Spray formulations of *P. frequentans* strain 909 (Pf909) are potential biocontrol products for reducing the occurrence of brown rot or twig blight, both of which are caused by *Monilinia* spp., in commercial peach orchards (De Cal et al., 2002, 1990; Guijarro et al., 2007; Melgarejo et al., 1986). Competition is the primary mode of biocontrol activity of Pf909 against *M. fructicola* (Guijarro et al., 2017). Pf909 has

been regarded as a competitive biocontrol agent (BCA) because it colonizes healthy peach tissue, thereby reducing the likelihood of an outbreak of brown rot (Guijarro et al., 2008, 2007).

To improve efficacies achieved through BCA application, there has recently been increasing interest in studying the efficacy of BCA with other existing technologies like chemicals or different BCAs combinations. Compatible interactions between chemicals and BCAs may result in additive control of each single agent (Budge and Whipps, 2001) or incompatible interactions may result in reduced disease control by the biological control agent. Synergy may be expected when two effective but independent mechanisms are involved in pathogen interaction (Di Pietro et al., 1993).

In other hand, control efficacy achieved by a combination of BCAs exhibiting different mechanisms of biocontrol may also result in antagonistic, additive or synergistic effects (Guetsky et al., 2002, 2001). *Bacillus amyloliquefaciens* CPA-8 has been reported as an effective antagonist against preharvest (Gotor-Vila et al., 2017a), and postharvest brown rot caused by *Monilinia* spp. (Yánez-Mendizábal et al., 2012) based mainly on its capability of production of powerful antifungal metabolites such us fengycinlike lipopeptides and other mechanisms of action such as competition and production of volatiles (Yánez-Mendizábal et al., 2012) The combination of different mechanisms of action, competition by Pf909 and antibiosis or volatiles by CPA8, could improve brown rot biocontrol obtained by each one. However, the combined use of BCAs also showed antagonistic interactions among BCAs when the BCAs were applied simultaneously compared with applied separately (Xu and Jeger, 2013).

The purpose of the work described here was to evaluate the compatibility of *P. frequentans* Pf909 with specific and broad-spectrum chemicals and antagonistic micro-organisms such as *B. amyloliquefaciens* CPA-8, aimed at designing efficient biological and integrated brown rot control.

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75 ## 2 MATERIALS & METHODS

76 ### 2.1 Biological material

77 *2.1.1 Fungal strain*

78 A monosporic isolate of *Penicillium frequentans* strain (ATCC 908-81) (Pf909), which was
79 identified from the microbiota of peach twigs (Melgarejo and M.-Sagasta, 1984) for further
80 characterization as a potential commercial BCA against brown rot (Melgarejo and M.-
81 Sagasta, 1984), and by its sensitivity to benomyl (De Cal et al., 1994). Pf909 conidia were
82 produced in a solid state fermentation system (De Cal et al., 2002), dehydrated in fluid bed
83 drying system (Guijarro et al., 2006) and stored at 4ºC for later application. The viability of
84 Pf909 dry conidia was evaluated by a previously described bioassay (De Cal et al., 1988)
85 before being used and only taken for biocontrol purpose when conidia viability reach 70%.
86 Pf909 conidial suspensions were adjusted to 10^6 conidia ml^{-1} using sterilized distilled water
87 (SDW) after counting the number of conidia using a hemocytometer and a light microscope
88 (Zeiss Axioskop 2; Carl Zeiss, Inc., Oberkochem, Germany).

89 A monosporic isolate of *M. fructicola* (G Winter) Honey (Mf3C), which was isolated
90 from a peach fruit in a commercial orchard in Alfarrás, Lerida, Spain was used. The isolate
91 had been collected during 2009 growing season. Conidial suspensions of Mf3C were long-
92 term stored at the INIA collection in 20% glycerol at -80ºC and grown on potato dextrose
93 agar plates (PDA; Difco Laboratories, Detroit, MI, USA) for seven days at 22º ± 2 C in the
94 dark in order to produce mycelia and conidia. The mycelia and conidia were harvested from
95 the surface of the PDA plates using a sterilized disposable scalpel, and suspended in SDW.
96 The suspension was then filtered through glass wool in order to remove the mycelia. The
97 conidial filtrate was adjusted to 10^3 (ml^{-1}) using SDW following the Pf909 protocol.

98 *2.1.2 Bacterial strain.*

Bacillus amyloliquefaciens CPA-8 (Yánez-Mendizábal et al., 2012), was originally isolated from a nectarine surface and belongs to the Postharvest Pathology Group Collection of IRTA (Spain). Stock cultures were stored at 4ºC and subcultured on solid media growth (TSA, nutrient yeast Trypto-casein soy agar) (Biokar Diagnostics number BK047HA) at 30ºC for 24 h when required. Fresh bacteria cultured overnight at 30ºC in TSA plates were harvested from the surface of the TSA plates using a sterilized disposable scalpel, and suspended in potassium 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 deionized water v/v/v pH 6.5) were used to inoculate a liquid media growth (TEC, Tryptone 10 g l^{-1} meet extract 5 g l^{-1} and sodium chloride 5 g l^{-1}). The initial inoculum was adjusted to 10^6 colony formit units (CFU ml^{-1}) with a hemocytometer and a light microscope. Bacterium was produced by liquid agitation of 50 ml TEC in 250 ml flasks in an orbital motion (Lab-Line Instruments, Inc., model 3527, Melrose Park Illinois, USA) set to 200 rev min^{-1}, for 72 h at 30ºC to obtain high endospore concentration (Gotor-Vila et al., 2017b). Therefore, CPA-8(1) (cells and supernatant) was prepared as described Yañez-Mendizabal et al. (2012), where bacterial cells were harvested from culture medium by centrifugation at 9,820 g for 12 min at 10ºC in a centrifuge (SORVALL® RC5C Plus) and re-suspended approximately at 10^7 CFU ml^{-1} in the same CPA-8 supernatant medium to include the antifungal lipopeptides synthesized by cells during the production process. CPA-8(2) (cells without supernatant) were obtained by harvesting bacterial cells by centrifugation such as described above, double washed with SDW and suspended in SDW with no lipopeptides. CPA-8(3) (supernatant free cells) was obtained by vacuum filtration system (Büchi Vacumm Controller V-800) with sterile (0.02 µm) filters of supernatant and taking only filtered supernatant medium with no bacteria cells.

2.2 Plant material

2.2.1 Skin peach extract

The skin extract of mature peaches (PSE) was used to determine the compatibility of conidial suspensions of Pf909 10^6 (ml^{-1}) and cells suspension of CPA-8 10^9 (ml^{-1}) in the *in vitro* inhibition assays. For preparing PSE, the skin of `Roig d´Albesa´ peaches was first dehydrated by lyophilization for 24 hours using a Cryodos-50 lyophilizer (Telstar, Barcelona, Spain). The lyophilized skin was then homogenized for 30 seconds using a high-speed benchtop tissue homogenizer (FastPrep®-24 Instrument, MP Biomedicals, Solon, Ohio, USA).

2.2.2 Fruit

The interaction between BCAs and inhibition effect in the control of Mf3C disease development between Pf909 and CPA-8 antagonists was determined *in vivo*, using healthy cherries (Var. Pico Colorado, 21mm diameter gauged, Béjar Industrial, Almendralejo, Badajoz, Spain) and red peaches (Var. Artemis, Orizieza Frutas S.L., Murcia, Spain) whose surfaces had been sterilized using a previously described protocol (Sauer and Burroughs, 1986).

2.3_Determination of compatibility interaction between Pf909 and CPA8 biocontrol agents

2.3.1 Compatibility between Pf909 and CPA8 in vitro.

Effect of CPA-8 on Pf909 germination and germ tubes.

The effect of CPA-8(1), CPA-8(2), and CPA-8(3) on conidial germination (conidia without previous incubation period, <0.5 h) and germ tube growth (conidia were pre-incubated for 18 h) of Pf909 in PSE solution was determined. Cell suspensions of each BCA, Pf909 ($1x10^6$ conidia mL^{-1}) and CPA-8 ($1x10^7$ cells ml^{-1}) were prepared in PSE. Aliquots of each cell suspension (25,000 Pf909 conidia in 25 µl and 250,000 bacteria cells in 25 µl) were mixed on a sterilized glass slide that was then placed in 150-mm diameter glass Petri dishes and incubated for 18 hours at 20º-25ºC in the dark. Three replicates (drops) were made. Then, 350 µl SDW was added to each drop and the solutions of cells were harvested from the glass

surface with a micropipette. The harvested solutions were then pooled in a 10-mL tube and SDW up to 4ml was added to give final concentrations of 5 x 10^3 conidia $ml^{-1}$and 5 x 10^4 bacteria cells ml^{-1}). Three 200-µl aliquots of each diluted suspension (approximately 10,000 conidia and 100,000 bacteria cells) were spread onto three separate Petri dishes of potato dextrose agar (PDA) or potato dextrose agar amended with 0.5 g l^{-1} streptomycin (PDAs). PDA was selected as the optimal media for conidial germination of Pf909, and CPA-8 was sensitive to streptomycin. After incubating the plates for five days at 25°C in the dark, the number of Pf909 CFUs was counted at 5-days of incubation. The complete assay was repeated twice.

The effect of non-pre-incubated bacterial cells (t<0.5 h) and pre-incubated (t=18 h) CPA-8(1) cells on Pf909 conidia in PSE solution at three different temperatures 25ºC, 30ºC and 33°C was also determined by counting the number of colony-forming units (CFUs) of Pf909 on PDA and PDAs such as described above. After incubating the plates for five days at three different temperatures 25, 30 and 33°C in the dark, the number of Pf909 CFUs was counted. The complete assay was repeated twice.

Germination and germ tubes inhibition of Pf909 by CPA-8(1), CPA-8(2), and CPA-8(3) were measured as the difference in the number of Pf909 CFUs counted in PDA and PDAs, using the following formula (1). Controls for Pf909 inhibition were PDA and PDAs plates with a Pf909 conidial suspension which was not exposed to any CPA-8.

(1) % Inhibition of Pf909 by CPA-8 = [1-(number of Pf909 CFUs on PDA/ number of Pf909 CFUs on PDAs)]*100.

Effect of CPA-8(1) on Pf909 colony growth in Petri dishes

Pf909 was tested against CPA-8(1) on dual cultures. A 5 µl drop Pf909 conidia suspension at a concentration of 10^6 ml^{-1} (SDW+1% agar) was placed at the center of a PDA plate (9mm).

Four 5 µl drops of CPA-8(1) cells at a concentration of 10^9 ml^{-1}, were then placed at 0.5 cm from the edge of the Petri plate, equidistant to the Pf909 drop and equidistant among the CPA-8(1) drops. In the control plates these four drops did not have CPA-8(1) cells. Ten replicates were made and incubated at three different temperatures 25ºC, 30ºC and 33ºC. The inhibition of the Pf909 growth rate (cm day^{-1}) was calculated by measuring two perpendicular colony diameters in the direction of each CPA-8 opposite colonies after 2, 3, 4, 7, and ten days of incubation. Pf909 sporulation density (conidia cm^{-1}) inhibition was evaluated at the ten-day of incubation as described in Guijarro et al. (2017). The complete assay was repeated twice.

Effect of Pf909 on CPA-8 (1) growth

The effect of non-germinated and germinated Pf909 conidia on non pre-incubated CPA-8(1) cells (t<0.5 h) and pre-incubated CPA-8(1) cells (t=18 h) solution was determined in PSE solution. Cells suspension of Pf909 ($1x10^6$ conidia ml^{-1}) and CPA-8(1) ($1x10^7$ cells ml^{-1}) in PSE were prepared as described above. Three 200-µL aliquots of each suspension (approximately 10,000 conidia and 100,000 bacteria cells) were spread onto three separate Petri dishes of TSA and TSAb (TSA amended with 5mg l^{-1} benomyl). TSA was selected the optimal media for bacterial germination. Pf909 was sensible to benomyl (De Cal et al., 1994). After incubating the plates for three days at 25°C in the dark, the number of CPA-8(1) CFUs was counted. The complete assay was repeated twice.

The effect of Pf909 on CPA-8(1) cells (no-pre-incubated and pre-incubated=18h) in PSE solution at three different temperatures 25ºC, 30ºC and 33°C was also determined by counting the number of colony-forming units (CFUs) of CPA-8(1) on TSA and TSAb as described above. Six replicate plates for each solution and each growth medium were made and the complete assays were repeated twice.

Growth CPA-8(1) inhibition by Pf909 was measured as the difference in the number of CPA-8(1) CFUs counted on the TSA and TSAb plates, using the following formula (2). Controls were TSA and TSAb plates with only CPA-8(1) cell suspensions.

(2) % Inhibition of CPA-8(1) by Pf909 = [1-(number of CPA-8(1) CFUs on TSA/ number of CPA-8(1) CFUs on TSAb)]*100.

2.3.2 Compatibility between Pf909 and CPA8 in stone fruit brown rot control in vivo

Biocontrol assays on cherries

The efficacy of different treatments (Table 1) combining Pf909 conidia and/or CPA-8(1) cells on brown rot disease was determined *in vivo* using healthy cherries. After fruit sterilization, cherry surfaces were dried in a laminar air flow cabinet for 2 h. The combination of Pf909 (10^6 conidia mL^{-1}), and/or CPA-8 (10^7 cell mL^{-1}) in SDW suspension were: (1) CPA-8 (0.5h); (2) CPA-8 (18h); (3) Pf909 (0.5h); (4) Pf909 (18h); (5) CPA-8 (0.5h) and Pf909 (0.5h); (6) CPA-8 (0.5h) and Pf909 (18h); (7) CPA-8 (18h) and Pf909 (0.5h); (8) CPA-8 (18h) and Pf909 (18h). Ten fruit per treatment were sprayed with 2 mL of each suspension to "run-off" and placed under the laminar flow until dry for 2 h. Then, 50µl of a conidial suspension of Mf3C (10^3 conidia ml^{-1}, viability >90%) were located in each fruit pedicel insertion (50 conidia per wound). Control treatments were: fruit inoculated with Mf3C and not treated with BCAs and fruit not inoculated with either Mf3C or treated with BCAs. Following inoculation and drying for 2 h, the cherries were incubated for seven days at 20º–25ºC at 99%–100% RH in the dark. To maintain high humidity, each inoculated cherry was placed on a sterilized dry dish in plastic boxes lined with moist paper. Trays were placed for 4-7 days at 22 °C under fluorescent lighting (100 µE m^{-2} s^{-1} with a 16 h photoperiod). Disease incidence, as measured by the number of diseased cherries per treatment, was determined using a previously described protocol (De Cal et al., 2002). Each treatment comprised ten cherries and the complete assay was repeated twice.

Biocontrol assays on peaches

The efficacy of Pf909 and/or CPA-8(2) and CPA-8(3) on brown rot was determined in vivo using healthy peaches (Table 2). Healthy fruit were sterilized in the same manner as described for cherries. Three 1 mm^3 artificial wounds, with 2 cm between each wound, were made on all fruit surfaces with a sterilized nail. Each fruit was sprayed with 0.5 mL of a conidial suspension of each Pf909 and/or CPA-8(2) and/or CPA-8(3) treatment combination to "run-off" (Table 4). After spraying, peach surfaces were allowed to dry for 3 h and 50µl of a conidial suspension of Mf3C (10^3 conidia mL^{-1}, viability >90%) were located in each fruit wound (50 conidia per wound). Fruit were incubated for 4-7 days as in the cherries assay. The daily lesion length (diameter in cm day^{-1}) was calculated from the individual measurements of the colony's diameter on fruit during on each day of incubation, using regression analysis (Villarino et al., 2016). The percentage of brown rot incidence and lesion length was recorded for each fruit at the end of the assay. The experiment included six fruit per treatment and each fruit had three wounds. The complete experiment was repeated twice.

2.4 Determination of compatibility between Pf909 and different commercial pesticides

The compatibility of Pf909 to commercial pesticides (fungicides and insecticides) used on pest stone fruit in Europe (Table 3) was determined by the sensitivity of Pf909 to such chemicals. The compatibility of Pf909 to commercial pesticides was assessed by an automated quantitative method (Broekaert et al., 1989) to establish the dose-response curves. In this method, Pf909 dry conidia were grown in microplate wells and their growth was monitored spectrophotometrically. The 21 commercial fungicides and 19 insecticides (Table 3) were serially diluted, ranging from 1/2 up to a 1/1000 dilution in 1% acetone from the recommended field application dose. In each well of the microtiter plates, 100 µL of each pesticide solution was mixed with 100 µL of Pf909 conidia suspension at the field dose application (2×10^6 conidia/mL) in Czapek Broth (Difco Laboratories, Detroit, USA). Absorbance was measured with a microplate reader (Multiskan Plus PV. 2.01) at 492 nm

252 wavelength. The first measurement was made just after filling the plate (A_0). The microplates
253 were then incubated under continuous agitation in the dark at 25 °C for 72 h, with one daily
254 monitoring of absorbance. The assay was repeated at least twice with six wells for each
255 antifungal compound dilution including the blanks. Growth inhibition (Broekaert et al., 1989)
256 was determined based on the equation $\left(\frac{\Delta C - \Delta T}{\Delta C}\right)x100$

257 where ΔC is the corrected absorbance of the blank standard solutions at 492 nm and ΔT is the
258 corrected absorbance of the test microculture. The corrected absorbance values equal the
259 absorbance at measured after 72 h of incubation (A_{72}) minus the absorbance measured before
260 incubation (A_0). Half maximal effective concentration (ED_{50}) value, defined as the
261 concentration of an antifungal compounds that inhibited mycelia growth by 50%, was
262 estimated by linear regression of the absorbance OD_{492} versus the antifungal concentration
263 (Mondal et al., 2005). Pf909 was considered sensitive to pesticide when its ED_{50}<commercial
264 field doses.

265 **2.5 Statistical Analysis**

266 Data were analyzed by one-way analysis of variance (ANOVA) using a computerised
267 statistical program (Statgraphics® Centurion XVI version 16.1.03). Prior to analysis,
268 sporulation density, cfu, % inhibition, and brown rot incidence data were (1+ log (1/x)), log
269 (x+1) and arcsine (x/100) transformed respectively, in order to improve homogenecity of
270 variances. When the results of the F-test were significant ($p \leq 0.05$), the means were compared
271 by Student-Newman-Keuls multiple range test (Snedecor and Cochran, 1980). Since
272 replicated experiments yielded similar results, data from each assay was pooled and analysed.

273 Regression analyses were computed using the linear model regression in Statgraphics
274 CENTURION XVI in order to obtain a standard curve with the highest correlation
275 coefficients between the dual interaction Pf909 vs CPA-8 in dual culture and the mycelia
276 growth. Model selection was performed on the basis of the significance of the estimated

277 parameters, R^2 (coefficient of determination for lineal regression analysis), the adjusted
278 coefficient of determination, the mean absolute error (average of the absolute values of the
279 residuals), and mean square error (Almeida et al., 2002).

280

281 **3 RESULTS**

282 **3.1 Determination of compatibility interaction between Pf909 and CPA8**

283 ***3.1.1 Compatibility between Pf909 and CPA8 in vitro.***

284 *Effect of CPA-8 on Pf909 germination and germ tubes*

285 All CPA-8 treatments inhibited conidial germination (no-pre-incubation<0.5h) and germ
286 tubes (pre-incubation=18h) of Pf909 at all CPA-8 incubation times in PSE (Figure 1).
287 CPA-8 treatments reduced Pf909 conidial germination (no-pre-incubation<0.5h) at the same
288 level on PDA and PDAs. The Pf909 CFUs reduction due to the effect of CPA-8(1) and CPA-
289 8(2) on Pf909 conidial germination was greater on PDA than it was on PDAs (Figure 1) when
290 both BCAs were applied at the same time in PSE. However, the highest reduction (> 90%
291 inhibition) on germ tubes (pre-incubation =18h) of Pf909 was observed with CPA-8(1) on
292 PDA at all CPA-8 incubation times (Figure 1a), followed by CPA-8(3) on both media (Figure
293 1c) after 18 h of bacterial pre-incubation. The lowest reduction (< 40% inhibition) on germ
294 tubes (pre-incubation=18h) of Pf909 was observed with CPA-8(2) on both media when both
295 BCAs were applied at the same time in PSE (Figure 1b).

296 The reduction of Pf909 CFUs due to the effect of CPA-8(1) on Pf909 conidial
297 germination was recorded at 25º and 30ºC with non-pre-incubated and pre-incubated cells of
298 CPA-8(1) (Figure 2). No Pf909 CFUs was observed at 33ºC.

299 *The effect of CPA-8 on Pf909 colony growth*

300 Reductions of Pf909 colony growth (cm) and sporulation density (conidia cm^{-1}) due to the
301 effect of CPA-8 (1) were recorded at 25º and 30ºC on PDA (Figure 3). Higher reductions of
302 Pf909 colony growth were recorded at 25ºC than at 30ºC (Figures 3a and 3b).
303 *Effect of Pf909 on CPA-8 growth*
304 Neither the no-pre-incubated nor the pre-incubated conidia of Pf909 reduced the CPA-8(1)
305 growth on TSA (Figure 4). Non-significant CPA-8 (1) reduction was observed on TSAb
306 (Figure 4). The reduction of Pf909 by CPA-8(1) was recorded at all incubation temperatures
307 (Figure 5).
308 ***3.1.2 Compatibility between Pf909 and CPA8 in stone fruit brown rot control in vivo***
309 All Pf909 and CPA-8(1) treatments reduced the incidence of brown rot on cherries, except
310 non-preincubated conidia of Pf909 (Table 1). The highest brown rot control was recorded on
311 cherries treated with non-preincubated and preincubated CPA-8(1), combined and
312 uncombined with preincubated Pf909 conidia (Table 1). Combinations of both BCAs did not
313 improve the disease control from each BCA alone. However, the disease control obtained on
314 cherries treated with both CPA-8(1) treatments combined with non-preincubated Pf909 was
315 less effective than from each CPA-8(1) alone (Table 1).
316 All Pf909 and/or CPA-8(2) treatments reduced the incidence of brown rot and daily
317 lesion length on peaches, except Pf909 treatments combined with preincubated cells of CPA-
318 8(2) (Table 2). However, only CPA-8(3) incubated for 18 hours [treatment 2] and fruit treated
319 with non-preincubated Pf909 and CPA-8(3) [treatment 5] before MF3C inoculation presented
320 a significant reduction in brown rot incidence on peaches (Table 2). Pf909 treatments
321 combined with preincubated CPA-8(3) did not reduce brown rot disease on peaches (Table 2).
322 **3.2 Determination of compatibility between Pf909 and different commercial pesticides**
323 Pf909 was only sensitive to 24% of the fungicides and 10% of the insecticides used. ED_{50} of
324 Pf909 was lower than field commercial doses of five fungicides (a.m. ciproconazole,
325 iprodione, fluidioxonil and *Bacillus subtilis*) and two insecticides (a. m. deltametrin and

clorantriamiesprol) (Table 3). Furthermore, ED50 of Pf909 was much closer to field commercial doses of three fungicides (a.m. oxicloruro Cu, sulfur, and thiram) and one insecticide (a.m. fenoxicarb) (Table 3).

4 DISCUSSION

A series of chemicals and a biocontrol agent used against stone fruit brown rot was tested for compatibility with the biocontrol fungus *P. frequentans* Pf909 through *in vitro* and *in vivo* assays. One of the ways to overcome the limitations of most of the biocontrol agents under diverse environmental conditions is to apply them in mixture, alternately with chemical fungicides (Whipps, 2001) or to apply more than one biocontrol agent at a given time (Elad et al., 1994a). Biocontrol agents do not always meet commercial standards for disease management. Integrated stone fruit management (IPM) is an approach that attempts to make complementary use of cultural, chemical, biological and also other methods of disease management to achieve the best possible results. Combined application of biocontrol agents with other agrochemicals may result either in synergism or antagonism between them. The compatibility of Pf909 with modern inputs in plant protection like fungicides (chemicals and biologicals) and insecticides is a pre-requisite for developing integrated disease management strategies (Guetsky et al., 2001).

The combination of Pf909 and CPA-8, each with different mechanisms of action, competition and antibiosis, respectively, did not improve the biocontrol of brown rot when each treatment was applied individually. Control efficacy achieved by a combination of BCAs exhibiting different mechanisms of biocontrol may result in antagonistic, additive or synergistic effects. The synergistic effects of using several BCAs simultaneously, or sequentially, may be greatest if these BCAs are chosen not only for their efficacy against pathogens when applied alone, but also for complementary biocontrol mechanisms among the

351 BCAs (Abo-Elyousr et al., 2009; Guetsky et al., 2002). Many authors have suggested that
352 application of more than one antagonist, provided the antagonists have different ecological
353 requirements, will increase the reliability and decrease the variability of biological control
354 (Elad et al., 1994b, 1994a; Monier and Lindow, 2005). However, previous reports have also
355 suggested that there were antagonistic rather than synergistic interactions between the BCAs,
356 and this led to reduced disease control when the BCAs were applied simultaneously compared
357 with applied separately (Xu et al., 2011).

358 Pf909 and CPA-8 could not be combined together in the same tank, because CPA-8
359 inhibited the germination and growth of Pf909 tubes in suspension. Furthermore, CPA-8 also
360 competes with Pf909 once applied on fruit surfaces. Additionally, CPA-8 reduced the growth
361 of Pf909 CFUs on media, at different 25ºC and 30ºC throughout the incubation periods. The
362 observed antagonism or interference between Pf909 and CPA-8 when applied together may
363 be explained by direct and indirect interactions between both biological agents. The main
364 mode of action of CPA-8 is the production of antifungal substances, and specifically
365 fengycine-like lipopeptides (Yánez-Mendizábal et al., 2012), which can have a direct effect
366 on other fungi such as Pf909. The lack of disease suppression by the combined use of two
367 BCAs, compared with the use of a single BCA, with the same two mechanisms, can be
368 explained by the fact that, unlike a single BCA with both mechanisms, the combined use of
369 two BCAs failed to exploit the advantage of higher rates of colonizing healthy tissue in one
370 BCA and diseased tissue in the other. Thus, combining two mechanisms in a single BCA may
371 be more effective in reducing the size of pathogen refuge (Johnson, 2010) than with the
372 combined use of two BCAs, each with a single mechanism (Xu et al., 2011).

373 Seventy-six percent of fungicides and 90% of insecticides commonly used against
374 stone fruit pests are compatible and could be simultaneously applied with Pf909 in integrated
375 pest control. Pf909 was only sensitive to 24% fungicides and 10% insecticides used, whereas
376 ED_{50} of Pf909 was lower than field commercial doses of five fungicides (a.m. ciproconazole,

iprodione, fluidioxonil and *Bacillus subtilis*) and two insecticides (a. m. deltametrin and clorantriamiesprol) (Table 3). Furthermore ED_{50} of Pf909 was much closer to field commercial doses of three multi-site action fungicides (a.m. Cu oxychloride, sulfur, and thiram) and one insecticide (a.m. fenoxicarb) (Table 3). The results clearly could indicate that the synergistic effect of the most commercial pesticides and Pf909 would be more pronounced at lower concentrations compared to the higher concentrations of the fungicide. Some authors have also reported that the integration of lower doses of fungicides such as PCNB, Prothiocarb with *Trichoderma* spp. improved the disease control (Chet and Henis, 1985). Fungicides might have weakened the pathogen and made it vulnerable, thus allowing *G. virens* to become more virulent on a weak pathogen (Upadhyay and Mukhopadhyay, 1986). Pf909 could provide an opportunity not only to reduce chemical use but also to cope more effectively with the development of pathogen populations resistant to common fungicides (Dennis and Davis, 1979). *P. frequentans* Pf909 was less sensitive to captan, vinclozolin, iprodione, thiophanate-methyl, and thiran than was *Monilinia laxa* (De Cal et al., 1994). However, classification of product compatibility does not necessarily imply a recommendation to mix products together in tank. Culture- and country-specific regulations always have to be considered.

The integration of biological and chemical controls, such that the application of Pf909 would be applied when it was most likely to be effective, would enable a further reduction in the number of chemical sprays while achieving the same level of disease control. Also results on deleterious effects of pesticides to Pf909 indicate the need for the selection of the right fungicide without affecting native beneficial microflora. Thus, it is essential to conduct investigations before biological products are applied together as tank mixes in commercial agriculture, taking into account biocontrol mechanisms of each BCA and their individual requirements for survival.

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REFERENCES

Abo-Elyousr, K.A.M., Hashem, M., Ali, E.H., 2009. Integrated control of cotton root rot disease by mixing fungal biocontrol agents and resistance inducers. Crop Prot. 28, 295–301.

Almeida, A.M., Castel-Branco, M.M., Falcão, A.C., 2002. Linear regression for calibration lines revisited: Weighting schemes for bioanalytical methods. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 774, 215–222. https://doi.org/10.1016/S1570-0232(02)00244-1

Broekaert, W.F., Van Parijs, J., Leyns, F., JOOS, H., PEUMANS, W.J., 1989. A Chitin-Binding Lectin from Stinging Nettle Rhizomes with Antifungal Properties. Science 245, 1100–1102. https://doi.org/10.1126/science.245.4922.1100

Budge, S.P., Whipps, J.M., 2001. Potential for Integrated Control of *Sclerotinia sclerotiorum* in Glasshouse Lettuce Using *Coniothyrium minitans* and Reduced Fungicide Application. Phytopathology 91, 221–227.

Byrde, R.J., Willetts, H.J., 1977. The Brown Rot Fungi of Fruit: Their Biology and Control. Pergamon Press, Oxford, UK.

Chet, and Henis, Y., 1985. Trichoderma as a biocontrol agent against soilborne root pathogen,

425 in: Society, A.P. (Ed.), Ecology and Management of Soilborne Plant Pathogens. St-Paul,
426 USA, pp. 110–112.

427 De Cal, A., Larena, I., Guijarro, B., Melgarejo, P., 2002. Biocontrol Science and Technology
428 Mass Production of Conidia of *Penicillium frequentans*, a Biocontrol Agent Against
429 Brown Rot of Stone Fruits. Biocontrol Sci. Technol. 12, 715–725.
430 https://doi.org/10.1080/0958315021000039897

431 De Cal, A., M.-Sagasta, E., Melgarejo, P., 1990. Biological control of peach twig blight
432 (*Monilinia laxa*) with *Penicillium frequentans*. Plant Pathol. 39, 612–618.

433 De Cal, A., M.-Sagasta, E., Melgarejo, P., 1988. Antifungal Substances Produced by
434 *Penicillium frequentans* and Their Relationship to the Biocontrol of *Monilinia laxa*.
435 Phytopathology 78, 888–893.

436 De Cal, A., Pascual, S., Melgarejo, P., 1994. In vitro studies on the effects of fungicides on
437 beneficial fungi of peach twig mycoflora. Mycopathologia 126, 15–20.
438 https://doi.org/10.1007/BF01371168

439 Dennis, C., Davis, R.P., 1979. Tolerance of Botrytis cinerea to iprodione and vindozolin.
440 Plant Pathol. 28, 131–133.

441 Di Pietro, A., Lorito, M., Hayes, C., Broadway, R., Harman, G., 1993. Endochitinase from
442 *Gliocladium virens*: isolation, characterization, and synergistic antifungal activity in
443 combination with gliotoxin. Phytopathology 83, 308–313.

444 Egüen, B., Melgarejo, P., De Cal, A., 2016. The effect of fungicide resistance on the structure
445 of *Monilinia laxa* populations in Spanish peach and nectarine orchards. Eur. J. Plant
446 Pathol. 145, 815–827. https://doi.org/10.1007/s10658-016-0871-4

447 Egüen, B., Melgarejo, P., De Cal, A., 2015. Sensitivity of *Monilinia fructicola* from Spanish
448 peach orchards to thiophanate-methyl, iprodione, and cyproconazole: fitness analysis and
449 competitiveness. Eur. J. Plant Pathol. 141, 789–801. https://doi.org/10.1007/s10658-014-
450 0579-2

451 Elad, Y., Köhl, J., Fokkema, N., 1994a. Control of infection and sporulation of *Botrytis*
452 *cinerea* on bean and tomato by saprophytic yeasts. Phytopathology 84, 1193–1200.

453 Elad, Y., Köhl, J., Fokkema, N.J., 1994b. Control of infection and sporulation of *Botrytis*
454 *cinerea* on bean and tomato by saprophytic bacteria and fungi. Eur. J. Plant Pathol. 100,
455 315–336.

456 Gotor-Vila, A., Teixidó, N., Casals, C., Torres, R., De Cal, A., Guijarro, B., Usall, J., Teixido,
457 N., Casals, C., Torres, R., De Cal, A., Guijarro, B., Usall, J., 2017a. Biological control of
458 brown rot in stone fruit using *Bacillus amyloliquefaciens* CPA-8 under field conditions.
459 Crop Prot. 102, 72–80. https://doi.org/10.1016/j.cropro.2017.08.010

460 Gotor-Vila, A., Usall, J., Torres, R., Abadias, M., Teixidó, N., 2017b. Formulation of the
461 biocontrol agent *Bacillus amyloliquefaciens* CPA-8 using different approaches: liquid,
462 freeze-drying and fluid-bed spray-drying. BioControl 62, 545–555.
463 https://doi.org/10.1007/s10526-017-9802-3

464 Guetsky, R., Shtienberg, D., Elad, Y., Dinoor, A., 2001. Combining Biocontrol Agents to
465 Reduce the Variability of Biological Control. Phytopathology 91, 621–627.

466 Guetsky, R., Shtienberg, D., Elad, Y., Fischer, E., Dinoor, A., 2002. Improving Biological
467 Control by Combining Biocontrol Agents Each with Several Mechanisms of Disease
468 Suppression. Phytopathology 92, 976–985.

469 Guijarro, B., Larena, I., Melgarejo, P., De Cal, A., 2006. Effect of drying on conidial viability

of *Penicillium frequentans* , a biological control agent against peach brown rot disease caused by *Monilinia* spp. Biocontrol Sci. Technol. 16, 257–269. https://doi.org/10.1080/09583150500335897

Guijarro, B., Inmaculada, L.H., Melgarejo, P., De Cal, A., 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 62, 557–566. https://doi.org/10.1007/s10526-017-9808-x

Guijarro, B., Larena, I., Melgarejo, P., De Cal, A., 2017. Adaptive conditions and safety of the application of *Penicillium frequentans* as a biocontrol agent on stone fruit. Int. J. Food Microbiol. 254. https://doi.org/10.1016/j.ijfoodmicro.2017.05.004

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. J. Appl. Microbiol. 104, 659–671. https://doi.org/10.1111/j.1365-2672.2007.03596.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. Biol. Control 42, 86–96. https://doi.org/10.1016/j.biocontrol.2007.03.014

Johnson, K.B., 2010. Pathogen Refuge : A Key to Understanding Biological Control. Annu. Rev. Phytopathol. 48, 141–160. https://doi.org/10.1146/annurev.phyto.112408.132643

Melgarejo, P., Carrillo, R., M.-Sagasta, E., 1985. Mycoflora of peach twigs and flowers and its possible significance in biological control of *Monilinia laxa*. Trans. Br. mycol. Soc. 85, 313–317.

Melgarejo, P., Carrillo, R., -Sagasta, E.M., 1986. Potential for biological control of *Monilinia*

493 *laxa* in peach twigs. Crop Prot. 5, 422–426. https://doi.org/10.1016/0261-
494 2194(86)90076-1

495 Melgarejo, P., M.-Sagasta, E., 1984. Fungal antagonism in relation to peaches, in: Woodbine.,
496 M. (Ed.), Antimicrobials and Agriculture. Butter Worths, London, pp. 122–136.

497 Mondal, S.N., Bhatia, A., Shilts, T., Timmer, L.W., 2005. Baseline Sensitivities of Fungal
498 Pathogens of Fruit and Foliage of Citrus to Azoxystrobin , Pyraclostrobin , and
499 Fenbuconazole. Plant Dis. 89, 1186–1194.

500 Monier, J., Lindow, S.E., 2005. Spatial Organization of Dual-Species Bacterial Aggregates on
501 Leaf Surfaces. Appl. Environ. Microbiol. 71, 5484–5493.
502 https://doi.org/10.1128/AEM.71.9.5484

503 Ooijkaas, L., Ifoeng, C., Tramper, J., Buitelaar, R., 1998. Spore production of *Coniothyrium*
504 *minitans* during soild-state fermentation on different nitrogen sources with glucose or
505 starch as carbon source. Biotechnol. Lett. 20, 785–788.

506 Snedecor, G.W., Cochran, W.G., 1980. Statistical Methods, 7th ed. Iowa State, USA.

507 Upadhyay, J.P., Mukhopadhyay, A.N., 1986. Biological control of *Sclerotium rolfsii* by
508 *Trichoderma harzianum* in sugarbeet. Trop. Pest Manag. 32, 215–220.
509 https://doi.org/10.1080/09670878609371066

510 Usall Rodié, J., Casals Rosell, C., Segarra Bofarull, J., Peris, M., Viñas Almenar, I., De Cal y
511 Cortina, A., 2010. Control de la podredumbre parda en melocotones y nectarinas del
512 Valle del Ebro. Vida Rural 302, 54–57.

513 Villarino, M., Melgarejo, P., De Cal, A., 2016. Growth and aggressiveness factors affecting
514 *Monilinia* spp. survival peaches. Int. J. Food Microbiol. 227, 6–12.

https://doi.org/10.1016/j.ijfoodmicro.2016.01.023

Whipps, J.M., 2001. Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. 52, 487–511. https://doi.org/10.1093/jexbot/52.suppl_1.487

Xu, X., Jeffries, P., Pautasso, M., Jeger, M.J., 2011. A Numerical Study of Combined Use of Two Biocontrol Agents with Different Biocontrol Mechanisms in Controlling Foliar Pathogens. Phytopathology 101, 1032–1044.

Xu, X., Jeger, M.J., 2013. Combined Use of Two Biocontrol Agents with Different Biocontrol Mechanisms Most Likely Results in Less Than Expected Efficacy in Controlling Foliar Pathogens Under Fluctuating Conditions : A Modeling Study. Phytopathology 103, 108–116.

Yánez-Mendizábal, V., Viñas, I., Usall, J., Torres, R., Solsona, C., Abadias, M., Teixidó, N., 2012. Formulation development of the biocontrol agent *Bacillus subtilis* strain CPA-8 by spray-drying. J. Appl. Microbiol. 112, 954–965. https://doi.org/10.1111/j.1365-2672.2012.05258.x

Yánez-Mendizábal, V., Zeriouh, H., Viñas, I., Torres, R., Usall, J., de Vicente, A., Pérez-García, A., Teixidó, N., 2012. Biological control of peach brown rot (*Monilinia* spp.) by *Bacillus subtilis* CPA-8 is based on production of fengycin-like lipopeptides. Eur. J. Plant Pathol. 132, 609–619. https://doi.org/10.1007/s10658-011-9905-0

Figure captions

Fig.1. Effect of *Bacillus amyloliquefaciens* strain CPA-8(1) cell plus supernatant suspension (A), CPA-8(2) cells (B) and CPA-8(3) supernatant (C) on colony formit unit (CFUs) of *Penicillium frequentans* strain 909 (Pf909) after no BCAs interaction (0), 0.5h interaction time (0.5), and 18h interaction time (18) after a 5-days incubation period on potato dextrose agar (PDA) (■) and PDA amended with 0.5 g L-1 streptomycin PDAs (□) at 25ºC incubation temperature. Bars followed by different letters in each figure are significantly different from each other ($p < 0.05$) according to the results of the Student-Newman-Keuls multiple range test. The values in each column charts are the average value from six replicate plates. MSE: Mean square error.

Fig.2. Effect of *Bacillus amyloliquefaciens* strain CPA-8(1) on *Penicillium frequentans* strain 909 (Pf909) in solution media at different temperatures (25, 30 and 33ºC). Data are the number of colony-forming units (CFUs) of Pf909 on potato dextrose agar amended with 0.5 g L^{-1} streptomycin (PDAs) plates after incubation with no *Bacillus amyloliquefaciens* strain CPA-8(1)(□) , CPA-8(1) suspension of 0.5h (▩), and 18h CPA-8(1) suspension (■), at three different temperatures, 25, 30 and 33ºC. Bars followed by different letters are significantly different from each other ($p < 0.05$) according to the results of the Student-Newman-Keuls multiple range test. The values in each column charts are the average value from six replicate plates. MSE: Mean square error.

Fig. 3. Effect of *Bacillus amyloliquefaciens* strain (CPA-8(1)) on inhibition of colony diameter growth (cm) (A) and sporulation (conidia cm^{-1}) of *Penicillium frequentans* strain 909 (Pf909) (B) after a 10-days incubation period on potato dextrose agar (PDA) at 25ºC and 30ºC. The values in each point charts are the average value from ten replicate plates. R^2: coefficient of determination for lineal regression analysis on colony diameter in each treatment (A). Bars are Pf909 sporulation on colonies with (▩) or without (■) presence of CPA-8(1). Bars followed by different letters (B) are significantly different from each other ($p < 0.05$) according to the results of the Student-Newman-Keuls multiple range test. MSE: Mean square error.

Fig.4. Effect of *Penicillium frequentans* strain 909 (Pf909) on colony forming unit (CFUs) of *Bacillus amyloliquefaciens* strain (CPA-8(1)) after no BCAs interaction (0), 0.5h interaction time (0.5), and 18h interaction time (18) after a 5-days incubation period on nutrient yeast Trypto-casein soy agar (TSA) (▩) and TSAb amended with benomyl 5 mgL^{-1} (TSAb) (▨) at 33ºC. Bars followed by different letters in each figure are significantly different from each other ($p < 0.05$) according to the results of the Student-Newman-Keuls multiple range test. The values in each column charts are the average value from six replicate plates. MSE: Mean square error.

Fig.5. Effect of *Penicillium frequentans* strain 909 (Pf909) on *Bacillus amyloliquefaciens* strain CPA-8(1) in solution media at different temperatures (25, 30 and 33ºC). Data are the number of colony-forming units (CFUs) of CPA-8(1) on nutrient yeast Trypto-casein soy agar amended with 5 mg L^{-1} benomyl (TSAb) after incubation with no Pf909 (□), Pf909 conidia suspension of 0.5h (▩), and 18h Pf909 (■) conidia suspension at three different temperatures, 25, 30 and 33ºC. Bars followed by different letters are significantly different from each other ($p < 0.05$) according to the results of the Student-Newman-Keuls multiple range test. The values in each column charts are the average value from six replicate plates. MSE: Mean square error.

581 Table 1. Brown rot incidence (%) on cherries that were inoculated with *Monilinia fructicola*
582 strain 3C (Mf 3C) conidia and treated or not treated with *Penicillium frequentans* (Pf 909)
583 conidia, *Bacillus amyloliquefaciens* (CPA-8(1) cells), or a combination of both biocontrol
584 agents (BCAs). Each data is the mean of ten replicates. Data followed by different letters are
585 significantly different from each other ($p < 0.05$) according to the results of the Student-
586 Newman-Keuls multiple range test. MSE: Mean square error.

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Treatment number	**BCAs Interaction time (h)**		**Brown rot incidence %**	
	CPA-8	**Pf909**		
0	**0**	**0**	72.22 (0.41)	a
1	**0.5**	**0**	5.56 (0.03)	c
2	**18**	**0**	0.00 (0.0)	c
3	**0**	**0.5**	44.44 (0.25)	ab
4	**0**	**18**	22.22 (0.13)	b
5	**0.5**	**0.5**	33.33 (0.19)	b
6	**0.5**	**18**	0.00 (0.0)	c
7	**18**	**0.5**	22.22 (0.13)	b
8	**18**	**18**	0.00 (0.0)	c
SEM			**(0.041)**	

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Table 2. Brown rot incidence (%),daily lesion length (cm/day) on peaches that were inoculated with *Monilinia fructicola* strain 3C (Mf3C) conidia or treated and not treated with *Penicillium frequentans* strain 909 (Pf 909) conidia in combination with *Bacillus amyloliquefaciens* cells CPA-8(2) or CPA-8 supernatant CPA-8(3) after five days of incubation at 25ºC. Each data is the mean of ten replicates. Data followed by different letters are significantly different from each other ($p < 0.05$) according to the results of the Student-Newman-Keuls multiple range test. MSE: Mean square error.

Treatments		BCAs Interaction time		Brown rot incidence %	daily lesion length (cm day^{-1})
		CPA-8	Pf909		
CPA-8 (2)	**0**	**0**	**0**	100.00 (0.57) c	1.39 d
	1	**0.5**	**0**	44.44 (0.25) ab	0.33 bc
	2	**18**	**0**	35.29 (0.20) a	0.06 a
	3	**0**	**0.5**	55.55 (0.31) ab	0.30 ab
	4	**0**	**18**	44.44 (0.25) ab	0.37 bc
	5	**0.5**	**0.5**	50.00 (0.28) ab	0.28 ab
	6	**0.5**	**18**	50.00 (0.28) ab	0.29 ab
	7	**18**	**0.5**	83.33 (0.47)bc	0.47 bc
	8	**18**	**18**	83.33 (0.47)bc	0.57 c
	MSE			**(0.067)**	**0.07**
CPA-8 (3)	**0**	**0**	**0**	100.00 (0.57) b	1.39 f
	1	**0.5**	**0**	66.66 (0.38) ab	0.37 bc
	2	**18**	**0**	38.89 (0.22) a	0.11 a
	3	**0**	**0.5**	74.44 (0.41) ab	0.74 de
	4	**0**	**18**	77.77 (0.44) b	0.67 de
	5	**0.5**	**0.5**	38.89 (0.22) a	0.23 ab
	6	**0.5**	**18**	61.11 (0.35) ab	0.52 cd
	7	**18**	**0.5**	100.00 (0.57) b	0.61 de
	8	**18**	**18**	100.00 (0.57) b	0.82 e
	MSE			**(0.052)**	**0.092**

600 Table 3- Effect of commercial pesticides used in commercial stone fruit orchards for pest management on *Penicillium frequentans* (Pf909).

Application use	Commercial product	active material	Co[a] (ppm)	Regression curve	R^{2b}	ED_{50}[c] (ppm)	sensitivity
Fungicide	**ATEMI 10 WG**	ciproconazole 10%	0.20	y=-0.31x+0.22	0.77	0.06	yes
	AQUAFLO ROVRAL	iprodione 50% [sc] p/v	1.50	y=-10.85x +4.48	0.73	0.12	yes
	CAPTAM-80	n-(triclorometiltio)ciclohex-4-eno-1.2-dicarboximide	1.50	y=-1.80x +13.15	0.65	7.50	no
	CEREMONIA 25 EC	difenoconazole 25%	0.20	y=-0.45x +2.60	0.73	3.10	no
	CHORUS	ciprodinil 50%	0.50	y=-0.80x +1.15	0.65	1.25	no
	DODINA 400GR/L	guanidine dodecil acetate	1.50	y=-3.03x+1.87	0.69	3.38	no
	GEOXE	fludioxonil	0.50	y=-10.85x+5.48	0.55	0.21	yes
	IMPALA	fenbuconazole 5%	1.50	y=-3.03x+1.87	0.94	3.38	no
	LUNA EXPERIENCE	tebuconazol + fluopyram	1.50	y=-2.80x+7.20	0.75	5.80	no
	LUNA PRIVILAGE	fluopyram	0.25	y=-23.05x+12.70	0.76	1.17	no
	MICLOUTANIL	myclobutanil	0.60	y=-0.87x+4.20	0.76	3.76	no
	OXICLORURO Cu 50%	oxicloruro cu 50%	4.00	y=-0.94x+3.52	0.69	3.99	yes
	PROLECTUS	fenpirazamida	0.60	y=0.98x+2.05	0.75	2.54	no
	QUINOXIFEN 25%	5.7-dicloro-4-(p-flurofenoxi) quinolina	0.30	y=-0.98x+1.89	0.73	1.4	no
	SIGNUM	boscalida 26.7% + piraclostrobin 6.7%	0.18	y=-0.76x+1.89	0.73	1.51	no
	SOFRE 80%	sulfur 80%	0.50	y=-0.76x+3.08	0.82	2.70	no
	SWITCH	ciprodinil 37.5% + fludioxonil 25%	0.10	y=-14.95x+1.52	0.83	0.04	yes
	THIOVIT JET	sulfur 80%	5.00	y=-0.91x+5.04	0.76	5.49	no
	THIRAM 80%	disulfuro o disulfuro de tetrametil tiuram	3.00	y=-0.63x+4.09	0.78	3.77	no
	SERENADE	*Bacillus subtillis*	4.00	y=-1.09x+2.81	0.67	2.35	yes
	TRIFLOXISTROBIN (FINT)	(e.e)- metoxiimino -(2-(1-(3-trifluorometil - fenil)-etilideneaminooximetil) –fenil)	1.50	y=-045x+2.05	0.79	1.82	no
Insecticide	**APACHE**	abamectina 1.8	0.20	y=-0.96x+2.78	0.65	2.39	no
	BULLDOCK 2.5 SC	betaciflutrín 2.5%	0.70	y=0.75x +1.28	0.87	1.655	no

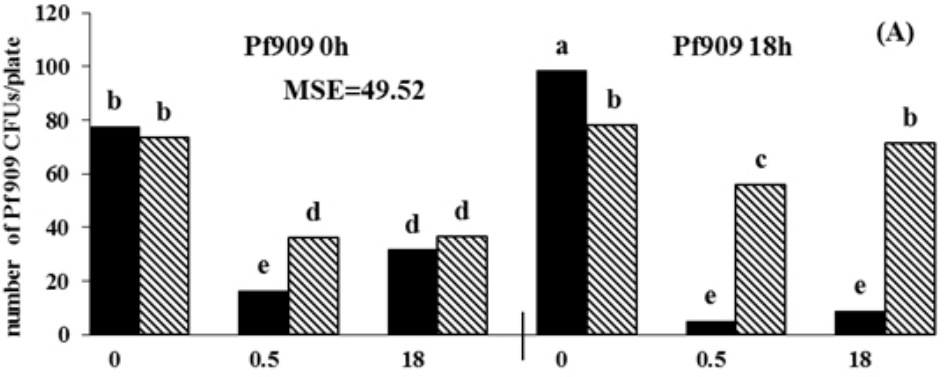
	CONFIDENTE	imidacloprid 20%	0.70	y=-0.450x +2.46	0.96	2.235	no
	NATURALIS-L	*Beauveria bassiana* 2.3% (2.3 x 10^9 conidia viable/ml)	0.02	y=-1.09x+3.29	0.65	3.835	no
	SPINTOR 480 SC	spinosad 48%	0.02	y=-0.89+3.98	0.77	3.535	no
	AUDACE	deltametrín 2.5% ((esp i)) [ec] p/v	2.00	y=-1.78x+4.95	0.69	4.06	no
	BRAVO-50	clortalonil 50% [sc] p/v	3.00	y=-1.06x+5.78	0.83	5.25	no
	DECIS PROTECH	deltametrin 1.5% [ew] p/v	8.00	y=-0.98x+6.61	0.74	6.12	yes
	FURY 10O EW	zeta-cipermetrín 10% [ew] p/v	0.20	y=0.98x+7.44	0.76	7.93	no
	KARATE ZEON	lambda cihalotrín 10% [cs] p/v	2.00	y=-1.76x+8.27	0.81	7.39	no
	PROTEUS O-TEQ	deltametrín 2% + tiacloprid 15% [od] p/v	0.60	y=-0.67x+9.10	0.88	8.76	no
	CLORPIRIFOS ETIL 75%	ethyl clorpyriphos	5.00	y=-1.01x+9.93	0.80	9.42	no
	FENOXICARB (INSEGAR)	fenoxicarb 25% p/p	4.00	y=-1.34x+4.90	0.89	4.23	no
	FOSMET 50%	ditiofosfato de o.o-dimetilo y de s-ftalimidometilo; n-(dimetoxifosfinotioil-tiometil)ftalimida.	1.50	y=-1.45x+5.89	0.84	5.16	no
	PIMOTROZINA 50%	pimotrozina 50%	5.00	y=-1.23x+7.40	0.84	6.78	no
	SPIROTETRAMAT 10%	spirotetramat 10%	0.15	y=-1.20x+3.90	0.75	3.3	no
	TIACLOPRID 480Gr/L (CALIPSO)	tiacloprid	2.00	y=-1.20x+3.90	0.83	3.3	no
	CORAGEN	clorantraniliprol 20 %	3.00	y=-1.29x+1.45	0.75	0.80	yes
	DELTAQUI	*Bacillus thuringiensis*	0.08	y=-4.34x+2.98	0.87	0.81	no

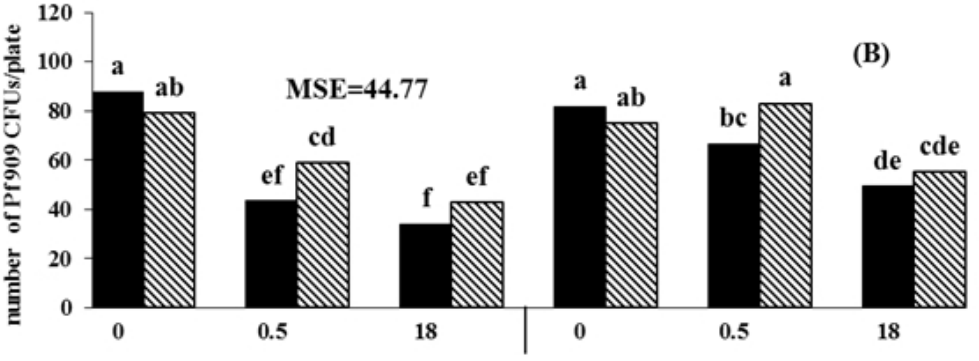
a Co, Maximum commercial pesticide doses used in stone fruit orchards. Six replicates were used by antifungal agents; y = absorbance at 492 nm; x= $\log_{10}$ antifungal concentration.

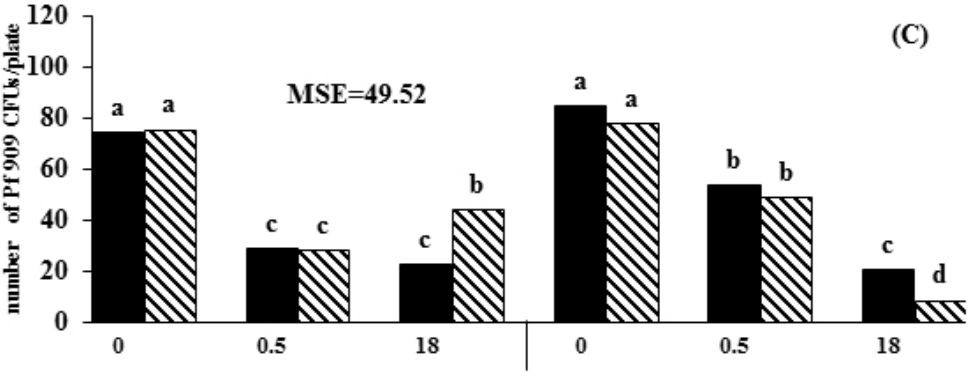
b R^2, the coefficient of determination for lineal regression analysis (Almeida et al., 2002) is the measure of success of predicting the dependent variable from the independent variables.

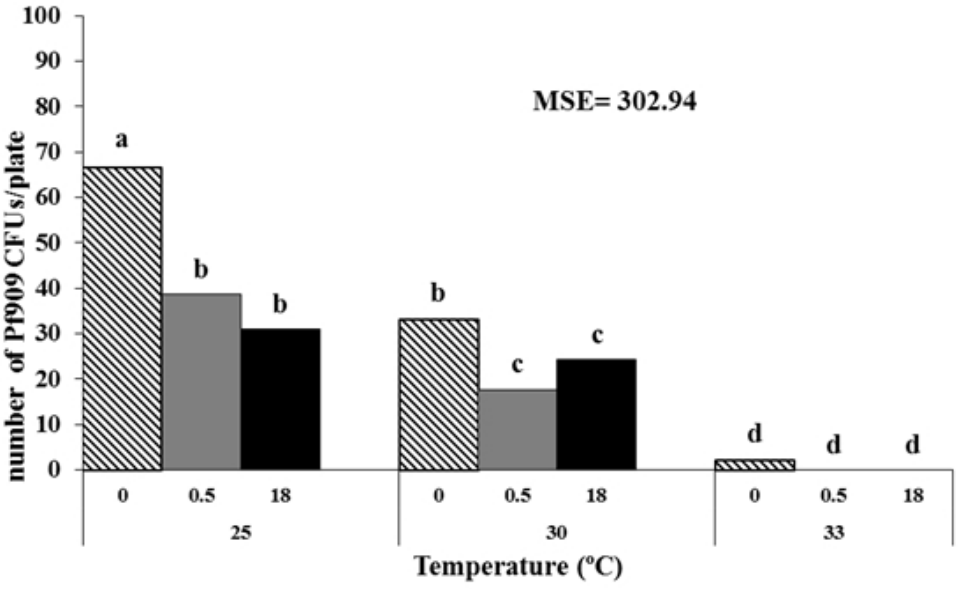
605 [c] Half maximal effective concentration (ED_{50}) value, defined as the concentration of an antifungal compounds that inhibited mycelia growth by
606 50%, was estimated by linear regression of the absorbance OD_{492} versus the antifungal concentration (Mondal et al., 2005). Pf909 was sensibility to
607 pesticide when its ED_{50}<commercial field doses.
608

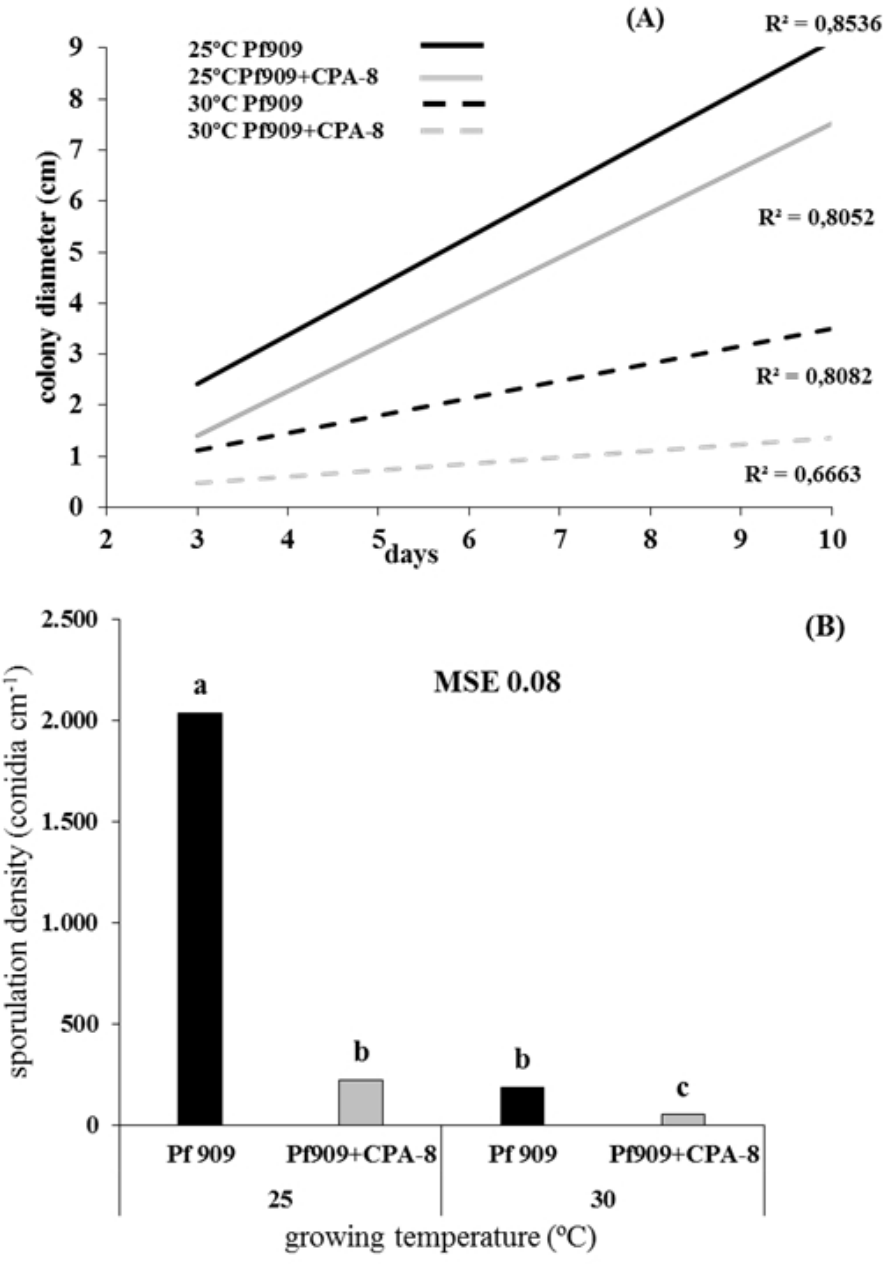
609

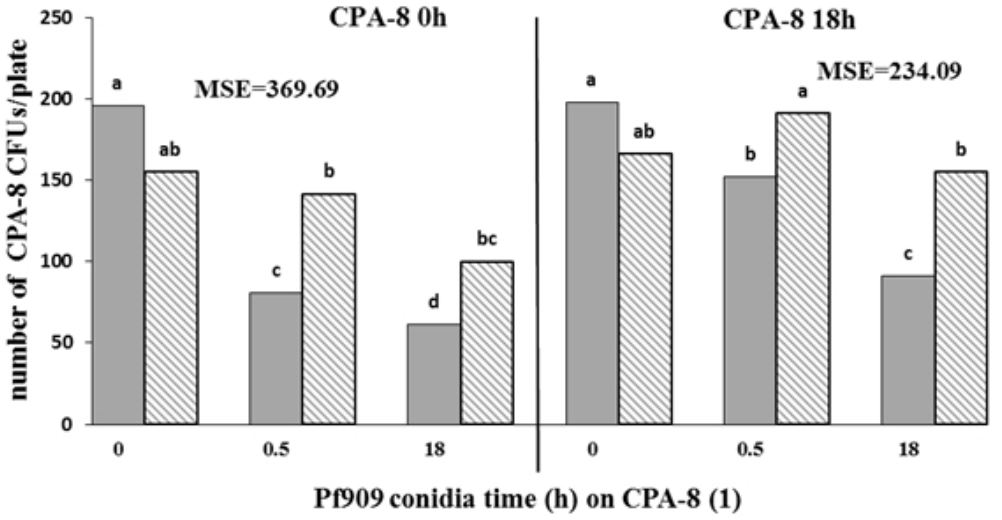

Pf909 0h
MSE=49.52
Pf909 18h
(A)
number of Pf909 CFUs/plate
120
100
80
60
40
20
0
b b
e d
d d
a b
e c
e b
0 0.5 18 0 0.5 18

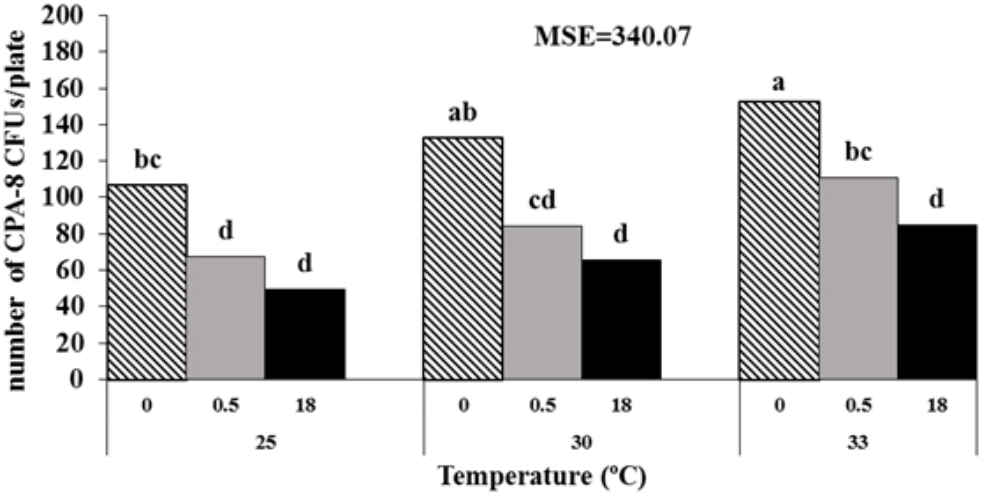

MSE=44.77
(B)
number of Pf909 CFUs/plate
120
100
80
60
40
20
0
a ab
ef cd
f ef
a ab
bc a
de cde
0 0.5 18 0 0.5 18


MSE=49.52
(C)
number of Pf909 CFUs/plate
120
100
80
60
40
20
0
a a
c c
c b
a a
b b
c d
0 0.5 18 0 0.5 18


MSE= 302.94
number of Pf909 CFUs/plate
100
90
80
70
60
50
40
30
20
10
0
a
b
b
b
c
c
d
d
d
0
0.5
18
0
0.5
18
0
0.5
18
25
30
33
Temperature (°C)


(A)
25°C Pf909
25°CPf909+CPA-8
30°C Pf909
30°C Pf909+CPA-8
R² = 0,8536
R² = 0,8052
R² = 0,8082
R² = 0,6663
colony diameter (cm)
days
(B)
MSE 0.08
sporulation density (conidia cm-1)
a
b
b
c
Pf 909
Pf909+CPA-8
Pf 909
Pf909+CPA-8
25
30
growing temperature (ºC)


CPA-8 0h
CPA-8 18h
MSE=369.69
MSE=234.09
number of CPA-8 CFUs/plate
250
200
150
100
50
0
a
ab
c
b
d
bc
a
ab
b
a
c
b
0
0.5
18
0
0.5
18
Pf909 conidia time (h) on CPA-8 (1)


MSE=340.07
number of CPA-8 CFUs/plate
200
180
160
140
120
100
80
60
40
20
0
bc
d
d
ab
cd
d
a
bc
d
0
0.5
18
0
0.5
18
0
0.5
18
25
30
33
Temperature (°C)