BIOCONTROL OF ROOT ROT OF STRAWBERRY CAUSED BY *PHYTOPHTHORA CACTORUM* WITH A COMBINATION OF TWO *PSEUDOMONAS FLUORESCENS* STRAINS

L. Agustí, A. Bonaterra, C. Moragrega, J. Camps and E. Montesinos

Institute of Food and Agricultural Technology-CIDSAV- XaRTA, University of Girona, Campus Montilivi 17071 Girona, Spain

SUMMARY

Pseudomonas fluorescens strains EPS817 and EPS894 protecting strawberry plants against *Phytophthora cactorum* root rot and producing different bioactive metabolites were used in combination to enhance the level of biocontrol achieved by each strain used alone. Moreover, it was confirmed by chemical analysis that the strains have clear physiological differences in terms of biocontrol potential. EPS817 produced 2,4-diacetylphloroglucinol and HCN, and EPS894 produced phenazine-1-carboxylic acid and pyoluteorin. Interaction studies between bacterial strains and *P. cactorum* revealed a significant inhibition of cyst germination *in vitro*. Scanning electron microscopy confirmed that each strain inhibited *P. cactorum* cyst germination on the strawberry root surface. Bacterial strains were able to colonize and survive on strawberry roots individually and together in a substrate potting mixture, thus indicating compatibility between strains. Biocontrol experiments performed in strawberry potted plants under greenhouse conditions revealed that the mixture of both strains not only reduced the disease severity of *Phytophthora* root rot but also the variability within experiments compared to single strain application.

Key words: Phytophthora cactorum, *Fragaria* x *ananassa*, fungal germination inhibition, biocontrol, BCA.

INTRODUCTION

Phytophthora cactorum causes crown, collar and root rot and leather rot of fruit of strawberry (*Fragaria* x *ananassa* Duchesne) (Erwin and Ribeiro, 1996). Although diseases caused by *P. cactorum* usually occur sporadically, they can potentially cause considerable damage and crop losses up to 50% can be reached (Ellis *et al.*, 1998).

Corresponding author: E. Montesinos
Fax: +34.972418399
E-mail: emonte@intea.udg.edu

Traditionally, the control of *P. cactorum* consists of preventive applications of chemical fungicides. However, biological control is an environmentally friendly strategy that offers an alternative or a complement to the use of chemical control. The use of biopesticides is considered one of the most promising methods for rational and safe crop-management practices that have been developed at the commercial level for several plant diseases (Handelsman and Stabb, 1996; Whipps, 2001; Montesinos and Bonaterra, 2009). Effective strategies to control *Phytophthora* spp. in strawberry using *Serratia plymuthica* (Kurze *et al.*, 2001), *Gliocladium* and *Trichoderma* (Vestberg *et al.*, 2004) or *Trichoderma* spp. (Porras *et al.*, 2007a, 2007b) have been reported.

Microbial biopesticides may fail to survive in the field under adverse conditions, or the variation of their activity due to biotic (host species, nutritional status, pathogen) and abiotic (temperature, relative humidity) factors gives inconsistency to disease control efficacy. So, a challenge in biocontrol development is to overcome this inconsistent performance improving its effectiveness under a wide range of conditions. This may be accomplished by the discovery of new antagonists, or combining antagonists with other control methods or combining antagonists with different mechanisms of biocontrol (de Meyer *et al.*, 1998; Steddom *et al.*, 2002; Spadaro and Gullino, 2005).

Combinations of strains permit a more extensive colonization of the rhizosphere and increase the expression of important biocontrol traits affecting the pathogen under a broader range of environmental conditions than strains applied individually (Pierson and Weller, 1994; Guetsky *et al.*, 2001; Meyer and Roberts, 2002; Roberts *et al.*, 2005). Previous studies of pathosystems different from *P. cactorum*-strawberry suggest that the use of more than one biocontrol agent (BCA) may be a way to reduce variability in their effect (Dunne *et al.*, 1998; Raupach and Kloepper, 1998; Whipps, 2001; Guetsky *et al.*, 2001, 2002; Jetiyanon and Kloepper, 2002). However, an important requisite for designing effective strain mixtures is the use of candidates that are compatible and that complement rather than interfere with the antagonistic activity of each other (Dunne *et al.*, 1998; Lutz *et al.*, 2004). An interesting choice could be *Pseudomonas fluo-*

rescens strains that have been reported to be suppressive to soil-borne pathogens. Several strains have attributes that make them suitable as BCAs of *P. cactorum*, e.g. good rhizosphere colonization, production of a wide spectrum of bioactive metabolites, and an aggressive competence with other microorganisms (O'Sullivan and O'Gara, 1992; Weller, 2007). Moreover, some mixtures of fluorescent pseudomonads resulted in enhanced protection of take-all of wheat (Weller and Thomashow, 1994; Pierson and Weller, 1994) and of *Fusarium* wilt of radish (Raaijmakers *et al*., 1995). However, the use of mixtures of fluorescent pseudomonads with different mechanisms of action in the biocontrol of *P. cactorum* in strawberry has not yet been reported.

P. fluorescens EPS817 and EPS894 were selected from a strain collection in our laboratory for their high efficacy in controlling infections by *P. cactorum* on different plant materials (strawberry leaves and potted plants) (Agustí, 2007). These strains have different origin since EPS817 was isolated from cherry roots and EPS894 from apple leaves. Genes related to the biosynthesis of 2,4-diacetylphloroglucinol (Phl) were detected in EPS817, and for the synthesis of pyoluteorin (Plt) and phenazine-1-carboxylic acid (PCA) in EPS894 (Badosa, 2001). Accordingly, it was hypothesized that a mixture of these strains would broaden the range of conditions under which biocontrol of *P. cactorum* would be feasible.

The aim of the present work was to evaluate the efficacy of a mixture of strains EPS817 and EPS894 for the biocontrol of *Phytophthora* root rot in strawberry plants. The activity on *P. cactorum* cyst germination *in vitro* and *in planta*, as well as the compatibility between strains in strawberry roots was studied. Additionally, the secondary metabolite production of these strains was determined.

MATERIALS AND METHODS

Pathogen and bacterial strains. *P. cactorum* strain 489 isolated from strawberry lesions, supplied by the Plant Production and Health Laboratory of Huelva (Andalucia, Spain) was used in these experiments. Sporangia and zoospores were obtained from 18-day-old cultures grown on tomato agar (12.5 g tomato concentrate, 2.85 g $CaCO_3$ and 16 g agar per liter) [modified from Dhingra and Sinclair (1987)] at 22 ± 1ºC under a 16-h light photoperiod. Sporangia of *P. cactorum* were collected by adding sterile distilled water to culture plates and scraping the culture surface. The suspension was cooled at 5ºC for at least 3 h to induce sporangia maturation, followed by 1 h at room temperature to let sporangia release the zoospores. The desired concentration of zoospores (10^4-10^5 zoospores per ml) was adjusted and was determined with a hemocytometer (Thoma, Germany).

P. fluorescens strains EPS817 and EPS894 characteristics are listed in Table 1. Bacteria were stored at -80ºC in 20% glycerol Luria-Bertani broth (LB) (Maniatis *et al.*, 1982). Bacterial suspensions were prepared from 24-h LB agar cultures grown at 23ºC inoculated into 10 ml of LB broth in culture tubes, and incubated on a rotatory shaker at 150 rpm at 23ºC. Cells of each *P. fluorescens* strain were then pelleted by centrifugation at 8,000 *g* for 15 min, resuspended in sterile distilled water. The final concentration was adjusted to 10^8 or 10^9 CFU ml^{-1} depending on the experiment, using a calibration curve between viable cell concentration and optical density at 600 nm.

Plant material and maintenance. Strawberry plants of cv. Diamante maintained at approximately 2ºC until their utilization, were planted in 500 ml pots in non-sterile peat (Potgrond P, Germany):perlite (Europerl, Spain) (3:1) mixture and used for disease suppression assays and for obtaining collar root extract and roots. The experiments were conducted under greenhouse conditions at 25 ± 4ºC with a 16-h photoperiod for two months.

Bacterial strain characterization. Bacterial strains EPS817 and EPS894 were characterized for the production of secondary metabolites, cell wall-degrading enzymes and inorganic phosphate solubilization ability.

Table 1. Relevant characteristics of the two strains of *Pseudomonas fluorescens* used in the present work.

Strain	Origin	Presence of biosynthetic genes[x]			Antagonistic activity against *P. cactorum*		
		Phl	PCA	Plt	*In vitro*[y]	*Ex vivo*[z]	*In planta*[w]
EPS817	Cherry root	+	-	-	+	+	d
EPS894	Apple leaf	-	+	+	++	+	+

[x]The presence of genes related to biosynthesis of Phl, 2,4-diacetylphloroglucinol; PCA, phenazine-1-carboxylic acid; and Plt, pyoluteorin was determined by PCR using the primers and conditions previously described by Raaijmakers *et al.* (1997); Mavrodi *et al.* (2001); Badosa (2001); Rodríguez-Romero *et al.* (2008).
[y]*In vitro* antagonistic activity against *P. cactorum* infection on potato dextrose media and Mueller-Hinton media. +, indicates an inhibition zone higher than 5 mm, and ++, indicates an inhibition zone higher than 10 mm (Agustí, 2007).
[z]Efficacy against *P. cactorum* infection on strawberry detached leaves higher than 60% (Agustí, 2007).
[w] Efficacy against *P. cactorum* infection on strawberry plants. +, indicates an efficacy higher than 70 % in different assays, and d, indicates a variable efficacy in the different assays (Agustí, 2007).

Table 2. Germination (%) of encysted zoospores of *Phytophthora cactorum* in strawberry collar root extract on PTFE membrane cylinders upon interaction with *P. fluorescens* EPS817, EPS894 and their mixture.

Treatment†	EPS817				EPS894				Mixture			
	Exp.1*		Exp.2		Exp.1		Exp.2		Exp.1		Exp.2	
Non-treated	51.0	a	59.2	a	51.0	a	59.2	a	51.0	a	59.2	a
Direct interaction	19.5	c	21.3	c	22.0	b	18.9	b	21.7	b	19.2	c
No interaction	20.4	c	19.9	c	29.1	b	25.3	b	29.3	b	22.5	c
Cell-free medium	20.2	c	22.1	c	13.0	b	18.4	b	25.1	b	16.2	c
Cell-free medium + nutrients	27.2	b	33.6	b	20.9	b	27.3	b	31.5	b	37.9	b

† The experiment was performed with strawberry collar root extract. Inserts with a 0.45 μm pore size membrane filter were used to separate bacteria or cell-free bacteria culture supernatants and encysted zoospores. Encysted zoospores at 10^4 zoospores ml^{-1} were filled in the inserts. Bacteria at 1×10^8 CFU ml^{-1} were filled either inside or outside the inserts. Cell-free culture supernatants from single bacteria cultures with or without nutrients were filled outside the inserts.

* Two experiments were done composed of 3 repetitions per treatment. Means within the same column followed by different letters are significantly different ($P \leq 0.05$) according to the Duncan test. Cysts germination was determined after 24 h of incubation at 25 ºC.

1-indole-3-acetic acid (IAA) and related compounds were identified as described by Brito Alvarez *et al.* (1995). Siderophore production was determined using chrome azurol S (CAS) agar (Schwyn and Neilands, 1987). Hydrogen cyanide (HCN) production was assessed by the picrate method (Sneath, 1966). Production of salicylic acid (SA) was identified in stationary-phase cultures (48 h, 28ºC) in SSM liquid medium (Leeman *et al.*, 1996). Cellulase, mannanase, xylanase and β-1,3-D-glucanase production was tested on tryptone-soy agar (TSA) (Oxoid, UK) amended with 1 mg ml^{-1} of specific chromogenic (azurine-dyed, cross-linked; AZ-CL) substrate (Megazyme, Australia) for each enzyme test (Nielsen *et al.*, 1998). Chitinolytic activity of bacterial strains was assessed using a chitin medium as described by Frändberg and Schnürer (1997). Inorganic phosphate solubilization activity was assessed using three different media contained $Ca_3(PO_4)_2$ described by Nautiyal (1999).

Production of secondary metabolites. Production of PCA, Phl and Plt in strawberry collar root extract (SCRE) and potato dextrose broth (PDB) (Oxoid, UK) was determined with high performance liquid chromatography (HPLC) and nuclear magnetic resonance (^{1}H-NMR). To prepare the SCRE, a collar from a healthy young strawberry plant was triturated in a mortar and suspended in 50 ml of distilled water. After discarding plant fragments, the suspension was sterilized by filtration with a 0.22 μm pore size membrane filter and kept at 4ºC in darkness. Liquid cultures of the bacterial strains were incubated 48 h on SCRE or PDB at 25ºC and centrifuged 15 min at 8,000 *g*. To extract metabolites for HPLC analysis, a 10 ml aliquot of each supernatant was acidified to pH 3.0 with 10% trichloroacetic acid and extracted with a solid-phase hydrophobic adsorbent column (RESPREP SPE Cartridges: Bonded reversed phase, C_{18}, 3 ml syringe volume). The column was washed with 0.5 ml H_2O milliQ and metabolites were disadsorbed with a 1 ml solution of acetonitrile and 15% H_2O milliQ. Samples of 20 μl were injected in a Waters HPLC model 610 (USA) equipped with an analytical column (Resteck, Pinnacle II C_{18}, 5 μm, 150x4.6 mm) packed with Tracer Hypersil ODS attached to a precolumn (10x0.4 mm). The column was eluted with a solvent system of acetonitrile:water acidified at pH 2.5 with trichloroacetic acid (40:60, v/v), at a flow rate of 1 ml min^{-1}. Measurements were done with a detector (model 484, Waters, USA) at λ=270 nm. Under these conditions, PCA, Phl, and Plt were detected respectively at 8.85, 9.73 and 5.98 min retention time. Metabolite extraction for NMR analysis was done as follows. A 30 ml aliquot of each supernatant was acidified to pH 3.0 with 10% trichloroacetic acid and extracted with a solid-phase hydrophobic adsorbent column (RESPREP SPE Cartridges: Bonded reversed phase, C_{18}, 3 ml syringe volume). The column was washed with 1.5 ml acetonitrile to disadsorb metabolites, the solution was reduced to dryness under vacuum and the dry residue dissolved in D_6-acetone and analyzed by nuclear magnetic resonance (^{1}H-NMR) at 200 MHz.

Interaction studies between bacterial antagonists and *P. cactorum*. The effects of *P. fluorescens* EPS817 and EPS894 and their mixture in cyst germination was determined on strawberry collar root extract (SCRE) prepared as described above. The effects of the crude culture supernatant, direct cell interaction or nutrient depletion by the antagonists on cyst germination was determined as described by Janisiewicz *et al.* (2000). 24-well tissue culture plates with cylinder inserts provided

with a filter membrane of 0.45 μm pore size (Millicell-CM, Millipore, USA) inside each well were used. In each well and outside the insert 0.5 ml of SCRE and 0.1 ml of sterile distilled water or bacterial suspension were added alone or in mixture, depending on the treatment. Inside the insert 0.3 ml of 10^4 zoospores per ml of *P. cactorum* encysted zoospore suspension and 0.1 ml of sterile distilled water or bacterial suspension were added, depending on the treatment. Zoospores were induced to encyst by agitating the suspension in a vortex shaker for 40 sec. Bacterial suspensions were prepared using 48 h bacterial cultures on SCRE, were centrifuged for 20 min at 4,000 *g*, and the pellets were resuspended in sterile distilled water and adjusted to 10^8 CFU ml^{-1}. To prepare cell-free bacterial culture supernatants, 48 h bacterial cultures on SCRE were centrifuged for 20 min at 4,000 *g*, and supernatants were filtered (0.22-μm pore size). When single strains were tested, the cell-free culture supernatant corresponded to a single bacterial strain culture, whereas when combinations of strains were tested, a mixture of two cell-free culture supernatants from single bacterial strain cultures was added.

Five different treatments were tested consisting of: (i) co-incubation of bacteria and *P. cactorum* cyst suspension inside the insert (direct interaction), (ii) bacterial strains suspension outside the insert and *P. cactorum* suspension inside the insert (no interaction), (iii) cell-free bacterial culture supernatant outside the insert and a *P. cactorum* cyst suspension inside the insert (cell-free medium), (iv) cell-free bacterial culture supernatant amended with nutrients as glucose minimal medium (GMM) (Agustí, 2007) outside the insert and *P. cactorum* cyst suspension inside the insert, and (v) a non-treated control with *P. cactorum* suspension with SCRE inside the insert. Culture plates with the cylinder inserts placed in each well were sealed with parafilm and incubated at 20°C with a 16 h photoperiod. Observations of the insert membranes were made 24 h after the addition of *P. cactorum*. Germination was stopped by adding a 20 μl drop of lactophenol blue in each cylinder insert. Then, the cylinder inserts were removed from wells and membranes were cut with a sharp scalpel, transferred onto a glass slide and observed under a light microscope at 200X to determine cyst germination. Cysts were retained as germinated when germ tubes were longer than the cyst diameter. The mean percentage of germinated cysts at the beginning of the experiment was subtracted from the total number of germinated cysts observed at the end of the experiment. Each treatment was replicated three times and the experiment was repeated twice.

Scanning electron microscopy. Two co-inoculation methods were performed for observing the interaction between *P. fluorescens* strains EPS817 or EPS894 and *P. cactorum* on strawberry roots *in vitro* and *in planta*.

For the *in vitro* interaction study, bacterial cells of each strain and *P. cactorum* propagules were co-cultured in SCRE. Tubes containing 1.8 ml of SCRE, 0.3 ml of a bacterial strain suspension (10^8 CFU ml^{-1}) and 0.9 ml of a *P. cactorum* suspension (10^5 zoospores per ml) were incubated 24 h at 20ºC with a 16 h photoperiod, then suspensions were centrifuged at 8,000 *g* for 10 min and pellets processed as described below.

For *in planta* interaction studies, healthy strawberry plants (cv. Diamante) were removed from their pots, roots were washed with tap water and surface-disinfested by immersion in a diluted solution of sodium hypochlorite (5% active chlorine) for 1 min, rinsed three times with sterile distilled water, and placed on sterile filter paper under an air stream to remove excess water. Roots were split and immersed in tubes containing a 10^8 CFU ml^{-1} suspension of each bacterial strain for 1 h. The non-treated control consisted of roots immersed in sterile distilled water. Then, roots were immersed in a *P. cactorum* suspension adjusted at 10^5 zoospores per ml and incubated 24 h at 20ºC with a 16 h photoperiod in a controlled environment chamber. Roots were then gently removed from tubes and cut 1 cm above the tip.

Pellets from the *in vitro* interaction study and root sections were fixed in glutaraldehyde (2.5 %, v/v, in 0.1 M cacodylate buffer, pH 7.2) and dehydrated by a series of ethanol rinses (50 to 100%), dried in a critical-point drier, gold sputter-coated (Emitech K550 Sputter Coater, Quorum Technologies, UK) and observed under a scanning electron microscope (Zeiss DSM960A, Germany).

Population levels of inoculated bacterial strains on strawberry roots. For the assessment of population levels of the bacterial strains on the roots of cv. Diamante plants spontaneous mutants resistant to 100 mg ml^{-1} of nalidixic acid (strain EPS894) or rifampicin (strain EPS817) were obtained. The resistant strains were similar to the respective parental strains in colony morphology and growth rate characteristics (not shown). Strawberry plants were irrigated 7 days after planting with the corresponding strain suspension (10^8 CFU ml^{-1}), and the treatments consisted of strains alone (100 ml) or mixed (50 ml of each strain). Three replicates of five plants per treatment were arranged in a randomized experimental design, and the experiment was repeated twice. Assessment of the bacterial population level was made 21 days after the treatment. Samples of plant roots were placed in sterile plastic bags with buffered peptone water (0.1% Bacto peptone in 30 mM potassium phosphate buffer, pH 7.0), and homogenized in a stomacher for 1 min. Extracts were serially diluted in sterile distilled water, appropriate dilutions were plated onto LB agar plates amended with 100 mg ml^{-1} of rifampicin (for EPS817) or nalidixic acid (for EPS894). Colonies were counted after incubation at 23ºC for 48 h and data were transformed to $\log_{10}$ CFU g^{-1} of fresh root weight.

Biological control of *P. cactorum* on strawberry plants. The effect of single or mixed strain treatments on the biological control of *P. cactorum* was assessed on cv. Diamante plants. The first treatment was conducted at the moment of planting by dipping plant roots in the bacterial suspension (10^9 CFU ml^{-1}). Non-treated control plants were irrigated with distilled water. Treated plants were placed into 500 ml pots containing peat:perlite (3:1) mixture and maintained in a greenhouse at 25±4ºC and a 16 h photoperiod. Treatments were repeated at 15, 23, 38 and 53 days by irrigation as with the experiment for population assessment as described for *P. fluorescens* strains bacterization in rooted plants (Bonaterra *et al.*, 2003). Thirty days after the first treatment, plants were inoculated with *P. cactorum* by placing 40 ml of an infested substrate around the roots (1×10^6 propagules per plant). The *P. cactorum*-infested substrate was prepared by placing mycelium plugs of a 15-day-old *P. cactorum* culture in a sterilized 10 liter mixture of wheat bran:vermiculite (1:1) containing 600 g of soybean flour and 1700 ml of distilled water. The inoculated substrate was incubated at 22±1ºC and a 16 h light photoperiod for 28 days, as described by Kurze *et al.* (2001). Inoculated potted plants were kept in perforated transparent plastic bags for 24 h to reach high relative humidity. Plants were maintained in the greenhouse at 25±5ºC with a 16 h photoperiod. Three replicates of five plants per treatment were arranged in a randomized experimental design, and two independent experiments were conducted. Disease levels were assessed 45 days after pathogen inoculation by observing of the extent of necrosis inside the collar root and according to the following scale: 0 = no necrosis, 1 = up to 1/3 of the collar necrosed, 2 = up to 2/3 of the collar necrosed, and 3 = up to 3/3 of the collar necrosed. Disease severity was calculated using the following formula:

$$S = \frac{\sum_{i=1}^{n} I_i}{n \cdot 3} \cdot 100$$

where S is the disease severity per repetition, I_i is the necrosis index per plant, and n is the number of plants per replicate.

Data analysis. Data were submitted to analysis of variance (ANOVA) and the treatment means were separated using Duncan's multiple range test at $P \leq 0.05$. The coefficient of variation between experiments was calculated for all combined and individual treatments in the biocontrol assay. The analysis was performed with the GLM procedure of the SAS software (version 8.2, SAS Institute, USA).

RESULTS

Characterization of selected bacterial strains. Strains EPS817 and EPS894 produced siderophores but not the phytohormone IAA. Strain EPS817 produced also HCN. HPLC and NMR analysis confirmed that EPS817 produced Phl and EPS894 produced PCA and Plt in both PDB and SCRE media (Fig. 1). Neither strain was able to solubilize inorganic phosphate nor showed hydrolytic activity on the cell wall polymers tested (cellullase, mannanase, xylanase and β-1,3-D-glucanase).

Interaction studies between bacterial antagonists and *P. cactorum*. Germination of *P. cactorum* cysts was inhibited in SCRE medium when cells of EPS894, EPS817 or a mixture of both were added (Table 2). Cyst germination was 51-59% in the absence of treatment and around 20% in the presence of EPS894, EPS817 or their combination at a ratio of 10^4 CFU per cyst. Inhibition of germination was also observed with cell-free SCRE culture supernatant filtrate of bacterial strains. There were no significant differences in the reduction of

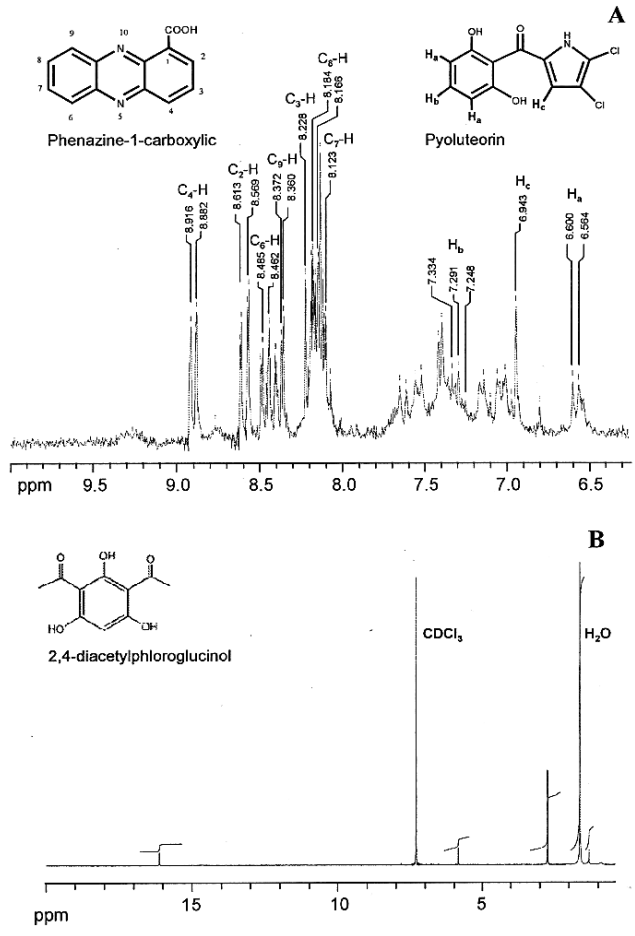

Fig. 1. Proton nuclear magnetic resonance spectrum of metabolites synthesized by *Pseudomonas fluorescens* EPS894 (A) and EPS817 (B).

cyst germination among the three treatments; bacterial cells interacting directly with cyst, separated with a filter and cell-free culture supernatants. No significant differences were observed between the effect of single strains and their combination on cyst germination. However, when cell-free culture supernatants of either strain EPS817 or EPS894 were amended with nutrients, cyst germination increased compared to non-amended cell-free culture supernatant, but it was lower than in the non-treated control. Hence, the amendment of nutrients in the cell-free culture supernatant, for both bacterial strains, only partially restored cyst germination.

Scanning electron microscopy analysis of the *in vitro* interaction study revealed that both strains attached to the surface of different fungal structures (Fig. 2). Bacterial cells were observed on the surface of germinated cysts, especially on the cyst and on the base of the germ tube (Fig. 2a, b). Bacteria attached to hyphae were also observed (Fig. 2a, b), but the amount of bacterial cells on the hyphal surface was lower than that observed on other fungal structures. Bacterial cells were also observed covering the surface of oogonia and sporangia

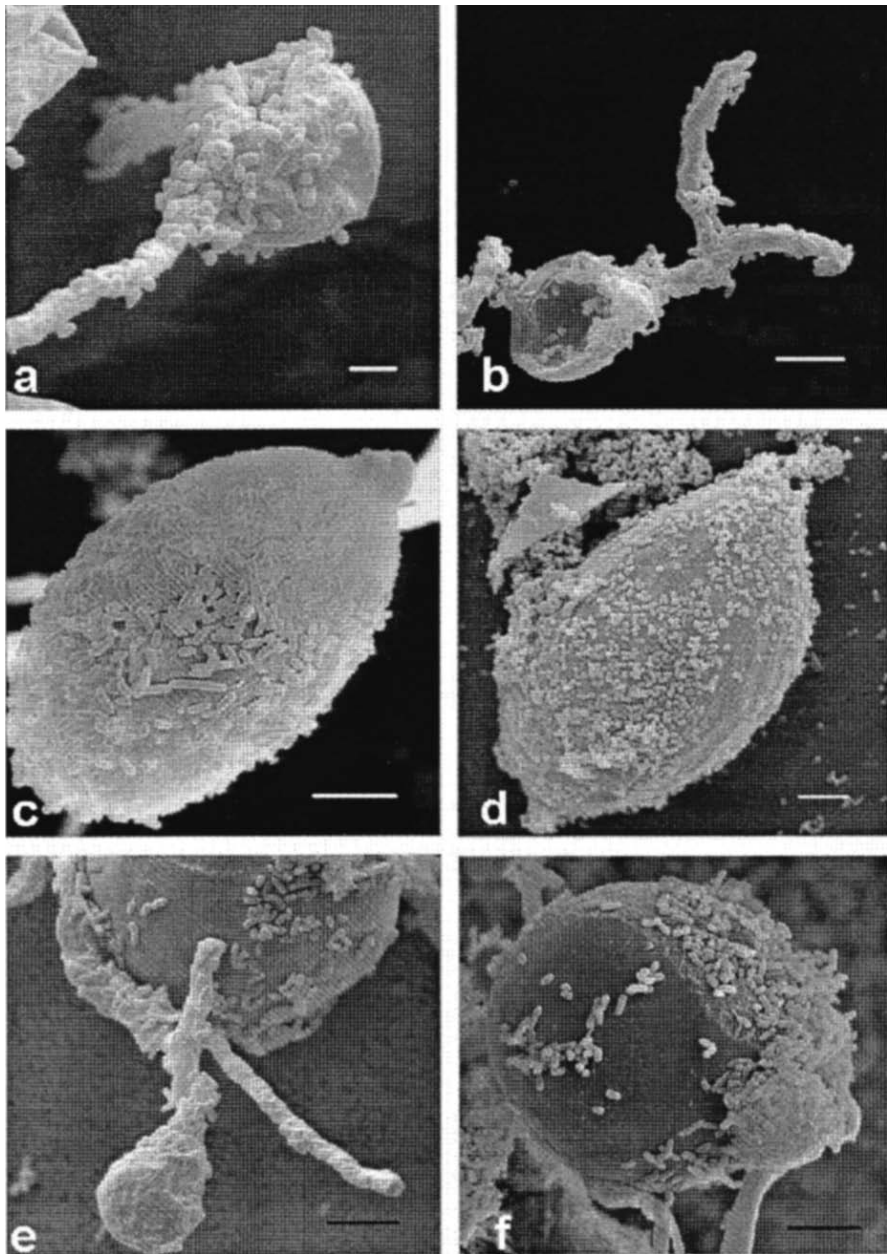

Fig. 2. Scanning electron micrographs of *P. fluorescens* strains EPS817 (left panels) and EPS894 (right panels) interacting with *P. cactorum* propagules induced to germinate *in vitro*. Micrographs were taken 24 h after inoculation. (a) Germinated cyst with attached EPS817 cells (scale bar: 2 μm). (b) Germinated cyst colonized with EPS894 cells, especially on the germinating tube (scale bar: 5 μm). (c) Sporangium with papilla completely covered with EPS817 cells (scale bar: 5 μm). (d) Sporangium covered with EPS894 cells (scale bar: 5 μm). (e) Germinated cyst and oospore with EPS817 cells (scale bar: 5 μm). (f) Oogonium and antheridium with EPS894 cells (scale bar: 5 μm).

(Fig. 2c, d, e, f). Sporangia that had not released their zoospores and were completely covered by bacterial cells were frequently observed.

Scanning electron microscopy observations were also made on strawberry roots treated with strains EPS817 and EPS894, and inoculated with *P. cactorum* (Fig. 3). Pathogen growth was observed on the surface of non-treated roots 24 h after inoculation. Encysted *P. cactorum* zoospores germinated and formed dense hyphal growth around the root. Some hyphae grew into the creases and grooves of the root epidermis, presumably to penetrate and infect the root (Fig. 3a), and hyphal tips adhered to the root surface (Fig. 3b). Poor hyphal development was observed on all bacteria-treated roots (Fig. 3c, d, e, f), showing the presence of bacterial cells close to the pathogen in epidermal creases. Bacterial cells were also observed embedded in a mucilaginous matrix on the root surface, forming microcolonies (Fig. 3c, d).

Compatibility between bacterial strains on strawberry roots. Significant differences among population levels in the two bacteria applied were observed ($P<0.0001$).

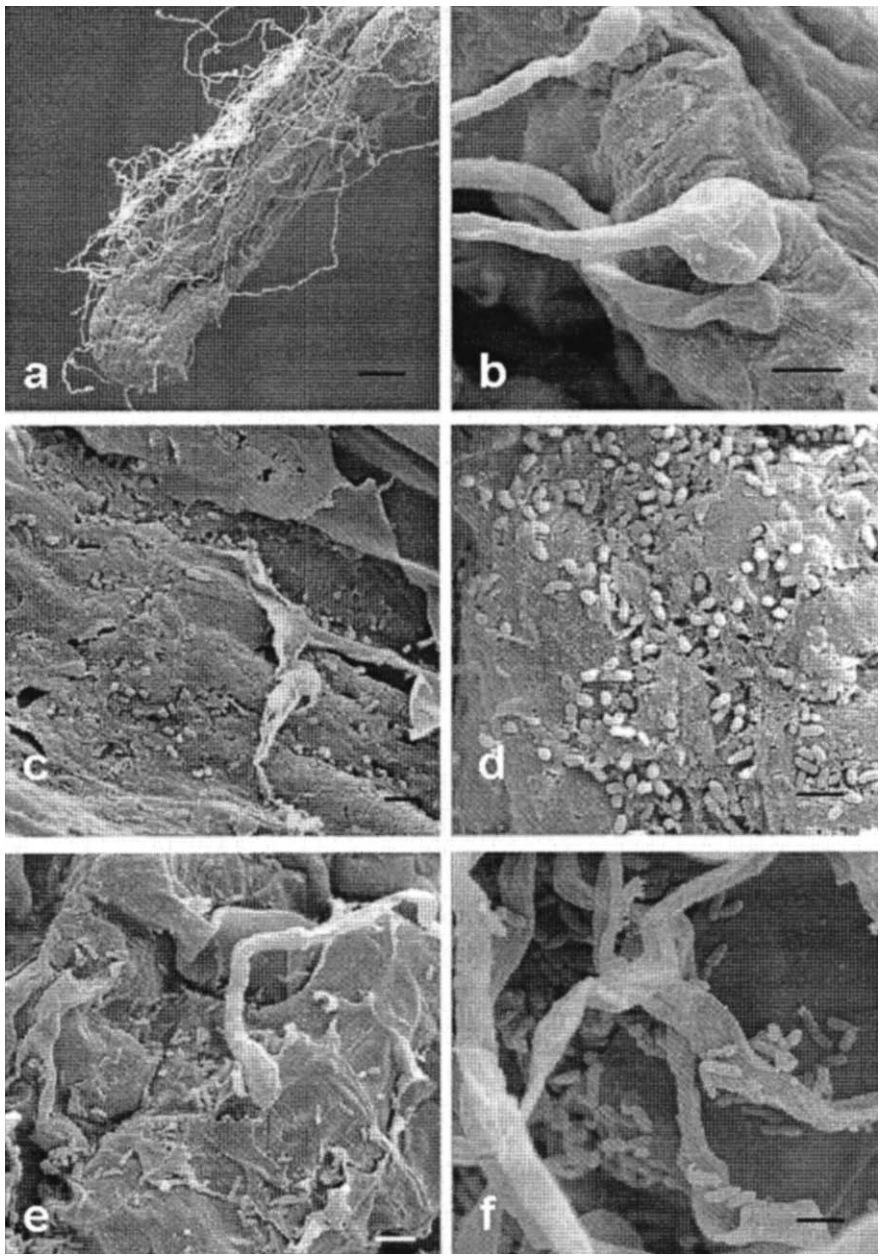

Fig. 3. Scanning electron micrographs of *P. fluorescens* strains EPS817 (left panels) and EPS894 (right panels) interacting with *P. cactorum* propagules germinating on strawberry roots (cv. Diamante). Micrographs correspond to 24 h after inoculation. (a) Encysted and germinated zoospores growing on the root tip surface without bacterial treatment (scale bar: 50 μm). (b) Non-treated root surface with *P. cactorum* propagules germinating (scale bar: 2 μm). (c) EPS817 cells on the root surface (scale bar: 2 μm). (d) EPS894 cells (scale bar: 2 μm). (e) Bacterial cells, hyphae and *P. cactorum* propagules on the surface of an EPS817-treated root (scale bar: 2 μm). (f) Hyphae and bacterial cells on the surface of an EPS894-treated root (scale bar: 2 μm).

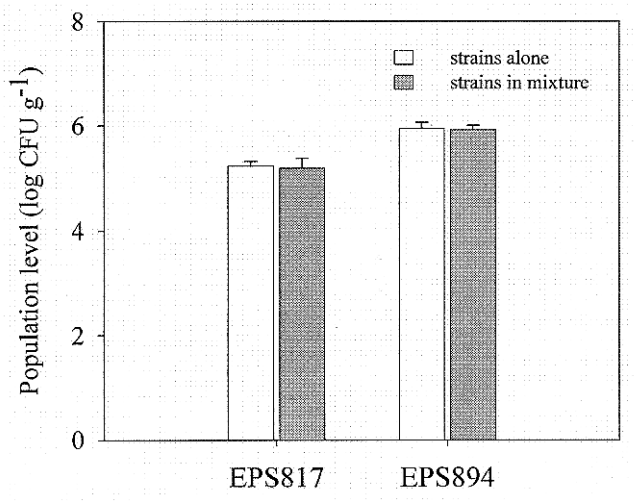

Fig. 4. Population levels of *P. fluorescens* EPS817 and EPS894 applied alone (white bars) or in mixture (grey bars) 21 days after inoculation on roots of strawberry plants. Values represent the mean of two experiments, with three replicates of five plants per treatment. Error bars indicate the confidence interval.

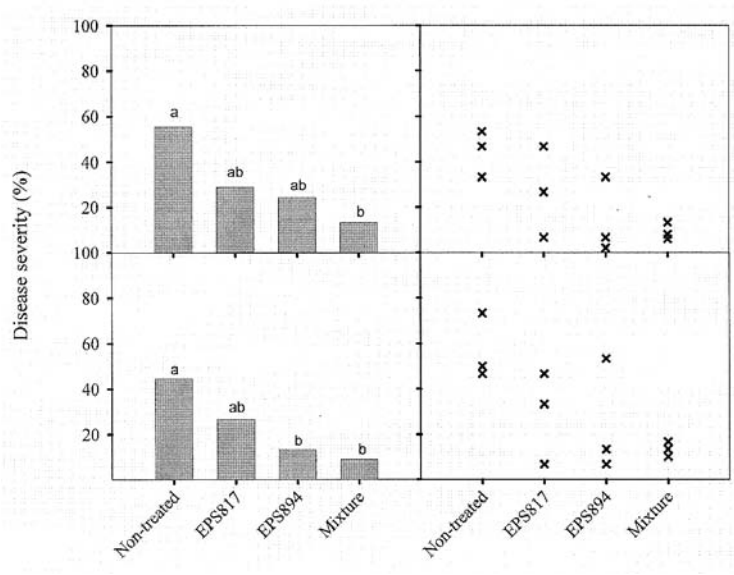

Fig. 5. Effect of *P. fluorescens* EPS817 and EPS894 applied alone or in mixture on *P. cactorum* disease severity (left panels) in potted strawberry plants of cv. Diamante. Data for each replicate and treatment within each trial are indicated to show variability (right panels). The experiment was performed twice (upper and down panels). Values are the mean of three replicates of five plants. Means headed by different letters are significantly different ($P \leq 0.05$) according to Duncan's test.

When applied alone, strain EPS894 reached a population of 8.7×10^5 CFU g^{-1} root 21 days after inoculation, whereas strain EPS817 population was 1.7×10^5 CFU g^{-1} root (Fig. 4). Population levels reached by each strain applied in mixture were not significantly different from those obtained when applied individually ($P>0.05$). In plants treated with a mixture of strains in a 1:1 ratio the population was 8.3×10^5 CFU g^{-1} root for EPS894 and 1.5×10^5 CFU g^{-1} root for EPS817. No significant differences were observed between the two experiments performed ($P=0.7485$). These results suggest that the strains are compatible.

Effect of the combination of strains on disease control. Despite the reduction in disease severity in plants treated with strains EPS817 and EPS894 applied alone, a significant effect was observed only in plants treated with EPS894 in experiment 2 (Fig. 5). The lack of statistical significance was due to the high variability observed in the individual strain treatments (coefficient of variation of 100% in EPS894, 70-75% in EPS817). However, the mixture of strains significantly decreased disease severity compared to the non-treated control with a reduction of disease severity that ranged from 76% to 80%, in both experiments. The mixture not only improved efficacy of disease suppression but also reduced variability because the coefficient of variation ranged from 35 to 43%.

DISCUSSION

In this study, two *P. fluorescens* strains protecting strawberry against *P. cactorum* and producing different bioactive metabolites were used in combination to enhance the level of biocontrol achieved by each strain used alone.

P. fluorescens strains EPS817 and EPS894 were selected for this study for their overall performance inhibiting *P. cactorum in vitro* and *ex vivo* in detached strawberry leaves, were characterized for the production of metabolites and further studied. EPS894 was effective in the biocontrol of *P. cactorum* on strawberry plants, whereas EPS817 was less efficient (Agustí, 2007). In this study we confirmed by chemical analysis that the strains have clearly physiological differences in terms of biocontrol potential. EPS817 produced siderophores, HCN and Phl in different media, whereas EPS894 produced siderophores PCA and Plt.

Interaction studies between bacterial strains and *P. cactorum* revealed a significant inhibition of *P. cactorum* cyst germination in SCRE. Since the effect of bacterial cells, either with or without direct interaction, and of cell-free culture supernatants on cyst germination was the same it was hypothesized that the inhibition would be mainly mediated by Phl and HCN in EPS817, and PCA and Plt in EPS894. However, the implication of these compounds has not been determined and will require the use of defective biocontrol mutants. Similarly, scanning electron microscopy also revealed significant inhibition of *P. cactorum* cyst germination and hyphal growth by the presence of attached bacterial cells *in vitro* and on strawberry roots.

Combination of EPS817 and EPS894 in the *in vitro* interaction study had the same effect on reduction of cyst germination than the single strains, indicating that the coinoculated strains do not interfere with each other. Accordingly, population levels of EPS817 and EPS894 on strawberry root were similar when applied alone or together, confirming the compatibility of the combination. It is hypothesized that each bacterial strain colonizes different sites of the strawberry root, thus they do not interfere with each other. This differential distribution could increase the probability of interaction between bacterial strains and *P. cactorum* at the infection site, as reported for a combination of *Trichoderma koningii* and *Pseudomonas* strains on biocontrol of take-all of wheat (Duffy *et al*., 1996).

This work has shown that EPS817 and EPS894 protected strawberry roots from *P. cactorum* rot in greenhouse experiments performed in peat:perlite mixture. Further work will assess whether this results in a soil-less system can be extended to a broader range of soil types and conditions. Also, from the practical point of view, a concentration of 2×10^7 CFU ml^{-1} of peat-perlite mixture (10^{10} CFU per strawberry plant) used for the bacterial inoculants in our treatments is within the range of doses reported generally as suitable in similar studies (Raupach and Kloepper, 1998; de Boer *et al*., 1999).

Finally, mixed application of EPS817 and EPS894 improved biocontrol of *P. cactorum* in strawberry plants compared to the individual strain treatments, and reduced the variability within the trial, thus providing more consistency in disease suppression. It is hypothesized that the different mechanisms of action between strains act complementary or synergistically. This is in agreement with other studies performed on different pathogens and plant hosts suggesting that the application of more than one antagonist with different ecological requirements and mechanisms of action would increase biocontrol reliability (Pierson and Weller, 1994; Duffy and Weller, 1995; Raupach and Kloepper, 1998; Guetsky *et al.*, 2001, 2002; Roberts *et al.*, 2005).

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REFERENCES

Agustí L., 2007. Characterization and efficacy of bacterial strains for biological control of soil-borne diseases caused by *Phytophthora cactorum* and *Meloidogyne javanica* on rosaceous plants. Ph.D. Thesis. University of Girona, Girona, Spain.

Badosa E., 2001. Anàlisi polifàsica de soques de *Pseudomonas fluorescens* potencials agents de biocontrol en malalties de fruiters. Ph.D. Thesis. University of Girona, Girona, Spain.

Bonaterra A., Ruz L., Badosa E., Pinochet J., Montesinos E., 2003. Growth promotion of *Prunus* rootstocks by root treatment with specific bacterial strains. *Plant and Soil* **255**: 555-569.

Brito Alvarez M.A., Gagné S., Antoun H., 1995. Effect of compost on rhizosphere microflora of the tomato and on the incidence on plant growth-promoting rhizobacteria. *Applied and Environmental Microbiology* **61**: 194-199.

de Boer M., van der Sluis I., van Loon L.C., Bakker P.A.H.M., 1999. Combining fluorescent *Pseudomonas* spp. strains to enhance suppression of fusarium wilt of radish. *European Journal of Plant Pathology* **105**: 201-210.

de Meyer G., Bigirimana J., Elad Y., Hyefte M., 1998. Induced systemic resistance in *Trichoderma harzianum* T39 biocontrol of *Botrytis cinerea*. *European Journal of Plant Pathology* **104**: 279-286.

Dhingra O.D., Sinclair J.B., 1987. Basic Plant Pathology Methods. CRC Press, Boca Raton, FL, USA.

Duffy B.K., Weller D.M., 1995. Use of *Gaeumannomyces graminis* var. *graminis* alone and in combination with fluorescent *Pseudomonas* spp. to suppress take-all of wheat. *Plant Disease* **79**: 907-911.

Duffy B.K., Simon A., Weller D.M., 1996. Combination of *Trichoderma koningii* with fluorescent Pseudomonads for control of take-all on wheat. *Phytopathology* **86**: 188-194.

Dunne C., Moënne-Loccoz Y., McCarthy J., Higgins P., Powell J., Dowling D.N., O'Gara F., 1998. Combining proteolytic and phloroglucinol-producing bacteria for improved biocontrol of *Pythium*-mediated damping-off of sugar beet. *Plant Pathology* **47**: 299-307.

Ellis M.A., Wilcox W.F., Madden L.V., 1998. Efficacy of metalaxyl, fosetyl-aluminum and straw mulch for control of strawberry leather rot caused by *Phytophthora cactorum*. *Plant Disease* **82**: 329-332.

Erwin D.C., Ribeiro O.K., 1996. Phytophthora Diseases Worldwide. APS Press. St. Paul, MN, USA.

Frändberg E., Schnürer J., 1997. Antifungal activity of chitinolytic bacteria isolated from airtight stored cereal grain. *Canadian Journal of Microbiology* **44**: 121-127.

Guetsky R., Shtienberg D., Elad Y., Dinoor A., 2001. Combining biocontrol agents to reduce the variability of biological control. *Phytopathology* **91**: 621-627.

Guetsky R., Shtienberg D., Elad Y., Fisher E., Dinoor A., 2002. Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. *Phytopathology* **92**: 976-985.

Handelsman J., Stabb E.V., 1996. Biocontrol of soilborne plant pathogens. *Plant Cell* **8**: 1855-1869.

Janisiewicz W.J., Tworkoski T.J., Sharer C., 2000. Characterizing the mechanism of biological control of postharvest diseases on fruits with a simple method to study competition

for nutrients. *Phytopathology* **90**: 1196-1200.

Jetiyanon K., Kloepper J.W., 2002. Mixtures of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple plant diseases. *Biological Control* **24**: 285-291.

Kurze S., Bahl H., Dahl R., Berg G., 2001. Biological control of fungal strawberry diseases by *Serratia plymuthica* HRO-C48. *Plant Disease* **85**: 529-534.

Leeman M., Den Ouden F.M., Van Pelt J.A., Dirkx F.P.M., Steijl H., Bakker P.A.H.M., Schippers B., 1996. Iron availability affect induction of systemic resistance to fusarium wilt of radish by *Pseudonomas fluorescens*. *Phytopathology* **86**: 149-154.

Lutz M.P., Wenger S., Maurhofer M., Défago G., Duffy B., 2004. Signaling between bacterial and fungal biocontrol agents in a strain mixture. *FEMS Microbiology Ecology* **48**: 447-455.

Maniatis T., Fritsch E.F., Sambrook J., 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory. Cold Spring Harbor. NY, USA.

Mavrodi O.V., McSpadden Gardener B.B., Mavrodi D.V., Bonsall R.F., Weller D.M., Thomashow L.S., 2001. Genetic diversity of *phlD* from 2,4-diacetylphloroglucinol-producing fluorescent *Pseudomonas* spp. *Phytopathology* **91**: 35-43.

Meyer S.L.F., Roberts D.P., 2002. Combinations of biocontrol agents for management of plant-parasitic nematodes and soilborne plant-pathogenic fungi. *Journal of Nematology* **34**: 1-8.

Montesinos E., Bonaterra A., 2009. Pesticides, Microbial. In: Schaechter M. (ed.). Encyclopedia of Microbiology, 3rd edition pp. 110-120. Elsevier, New York, NY, USA.

Nautiyal C.S., 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiology Letters* **170**: 265-270.

Nielsen M.N., Sorensen J., Fels J., Pedersen H.C., 1998. Secondary metabolite- and endochitinase-dependent antagonism toward plant-pathogenic microfungi. *Applied and Environmental Microbiology* **64**: 3563-3569.

O'Sullivan D.J., O'Gara F., 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant-root pathogens. *Microbiological reviews* **56**: 662-676.

Pierson E.A., Weller D.M., 1994. Use of mixtures of fluorescent Pseudomonads to suppress take-all and improve the growth of wheat. *Phytopathology* **84**: 940-947.

Porras M., Barrau C., Romero F., 2007a. Effects of soil solarization and *Trichoderma* on strawberry production. *Crop Protection* **26**: 782-787.

Porras M., Barrau C., Arroyo F.T., Santos B., Blanco C., Romero F., 2007b. Reduction of *Phytophthora cactorum* in strawberry fields by *Trichoderma* spp. and soil solarization. *Plant Disease* **91**: 142-146.

Raaijmakers J.M., Leeman M., van Oorschot M.M.P., van der Sluis I., Schippers B., Bakker P.A.H.M., 1995. Dose-response relationships in biological control of Fusarium wilt of radish by *Pseudomonas* spp. *Phytopathology* **85**: 1075-1081.

Raaijmakers J.M., Weller D.M., Thomashow L.S., 1997. Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. *Applied and Environmental Microbiology* **63**: 881-887.

Raupach G.S., Kloepper J.W., 1998. Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. *Phytopathology* **88**: 1158-1164.

Roberts D.P., Lohrke S.M., Meyer S.L.F., Buyer J.S., Bowers J.H., Baker C.J., Li W., de Souza J.T., Lewis J.A., Chung S., 2005. Biocontrol agents applied individually and in combination for suppression of soilborne diseases of cucumber. *Crop Protection* **24**: 141-155.

Rodríguez-Romero A.S., Badosa E., Montesinos E., Jaizme-Vega M.C., 2008. Growth promotion and biological control of root-knot nematodes in micropropagated banana during the nursery stage by treatment with specific bacterial strains. *Annals of Applied Biology* **152**: 41-48.

Schwyn B., Neilands J.B., 1987. Universal chemical assay for the detection and the determination of siderophores. *Analytical Biochemistry* **160**: 47-56.

Sneath P.H.A., 1966. Identification methods applied to *Chromobacterium*. In: Gibb B.M., Skinner F.A. (eds). Identification Methods for Microbiologist, Part A, pp. 15-20. Academic Press, London, UK.

Spadaro D., Gullino M.L., 2005. Improving the efficacy of biocontrol agents against soilborne pathogens. *Crop Protection* **24**: 601-613.

Steddom K., Becker O., Menge J.A., 2002. Repetitive applications of the biocontrol agent *Pseudomonas putida* 06909-rif/nal and effects on populations of *Phytophthora parasitica* in citrus orchards. *Phytopathology* **92**: 850-856.

Vestberg M., Kukkonen S., Saari K., Parikka P., Huttunen J., Tainio L., Devos N., Weekers F., Kevers C., Thonart P., Lemoine M.C., Cordier C., Alabouvette C., Gianinazzi S., 2004. Microbial inoculation for improving the growth and health of micropropagated strawberry. *Applied Soil Ecology* **27**: 243-258.

Weller D., 2007. *Pseudomonas* biocontrol agents of soilborne pathogens: Looking back over 30 years. *Phytopathology* **97**: 250-256.

Weller D.M., Tomashow L.S., 1994. Current challenges in introducing beneficial microorganisms into the rhizosphere. In: O'Gara F., Dowling D.N., Boesten B. (eds). Molecular Ecology of Rhizosphere Microorganisms: Biotechnology and the Release of GMOs, pp. 1-18. VCH, Weinheim, Germany.

Whipps J.M., 2001. Microbial interacions and biocontrol in the rhizosphere. *Journal of Experimental Botany* **52**: 487-511.

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