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Evaluation of *Pseudomonas fluorescens* for the biocontrol of fusarium wilt in tomato and flax

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***Fusarium oxysporum* is an abundant plant pathogen in the soil and is found in the rhizosphere of many plant species. The fungus has numerous formae speciales (f.sp.) that infect and cause wilt diseases of a variety of host plants. *F. oxysporum* f.sp *lycopersici* (Fol) and *F. oxysporum* f.sp *lini* (Foln) are known to infect tomato and flax, respectively. Fluorescent pseudomonads can suppress various soilborne diseases, and their efficacy has been related both to their antagonistic activities and to their rhizosphere competitiveness. In this study, the biocontrol capacity of plant growth-promoting rhizobacteria (PGPR) strains of *Pseudomonas fluorescens* against fusarium wilt of tomato and flax was evaluated both *in vitro* and *in planta*. The *P. fluorescens* strains exhibited substantial antagonistic activities against the two fungal isolates on three culture media tested: King B (KB), potato dextrose agar (PDA) and a mixture of both (KB + PDA). As compared to the untreated control, the mycelial growth decreased by 8.33 to 49.33%. Conidial germination and germ tube elongation were significantly inhibited and reduced, respectively, by 6.15 to 47.33% and by 1.63 to 45.45%. In bioassay trials, *P. fluorescens* strains significantly reduced the disease incidence and severity by 4.86 to 74.49% on tomato plants and 3.93 to 79.19% on flax plants. The different efficacy of the biocontrol agents could be due to the influence of several factors, including the efficiency of the strain, the type of pathogen and the host.**

Key words: Plant growth-promoting rhizobacteria (PGPR), *Pseudomonas fluorescens*, fusarium wilt, tomato, flax, biocontrol.

INTRODUCTION

Disease suppression by biocontrol agents is the manifestation of interactions among the plant, pathogen, biocontrol agent, microbial community on and around the plant and physical environment. Among the wide range of beneficial microorganisms, plant growth-promoting rhizobacteria (PGPR) play a vital role in the management of plant diseases (Kloepper and Schroth, 1978; Weller, 1988; Keel et al., 1992; Haas and Defago, 2005; Siddiqui, 2006; Yan et al., 2009). In microbial interactions of the rhizosphere, telluric fluorescent pseudomonads are associated with various saprophytic and/or parasitic microbial populations, according to the host plant. *Fusarium oxysporum* Schlecht., a complex group that presents many formae speciales and physiological species, is an extremely common soil fungus that occurs in the rhizosphere of many plant species. The group is often dominant within the fusarium soil populations and causes vascular wilt that result in considerable economic losses (Armstrong and Armstrong, 1981).

Fusarium vascular wilt is a severe disease that can

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affect plants of different botanical families (e.g., tomato and flax), and for some of the causative agents, such as *F. oxysporum* f.sp *lycopersici* and *F oxysporum* f.sp *lini*, there are no effective control methods. *F. oxysporum* produces chlamydospores, macroconidia and micro-conidia, and all of these stages are present in infected tissues or soil (Nelson, 1981). The systemic nature and the infectious and epidemic character of the vascular fusarium wilts limit the use of possible preventative measures and conventional disease controls (Gordon and Martyn, 1997; Duijff et al., 1998; Larkin and Fravel, 1999; De Boer et al., 2003; Fravel et al., 2003). Despite the use of resistant cultivars, the occurrence and development of new pathogenic species is a continuous problem. Therefore, the application of fungicides is a normal practice; however, this may not be very effective because the disease appears late in the growth of the crop, and the persistence of fungicides throughout the crop cycle is always doubtful. The use of systemic fungicides has remained the method used most often, despite the risk of the emergence of resistant strains.

F. oxysporum species have been used extensively as models for their interactions with PGPR, and based on the antagonistic activities of the PGPR fluorescent pseudomonads, the microbiological control against *F. oxysporum* has been the object of extensive research carried out under both controlled and natural conditions (Bakker et al., 1990; Lemanceau and Alabouvette, 1993; Alabouvette et al., 2006). The fluorescent pseudomonads are considered potential biocontrol agents of plant telluric diseases, and several studies have shown their efficacy as an inoculum (Kloepper et al., 1980; Tomashow and Weller, 1995; Lugtenberg et al., 1999; Whipps, 2001; Weller et al., 2002; Achouak et al., 2004; Hariprasad; et al., 2009; Validov et al., 2009). Several PGPR formulations are currently available as commercial products for agricultural production of beneficial crops, such as BlightBan A506, Conquer and Victus formulated with *Pseudomonas fluorescens* strains, for the pratical use on crops of almond, apple, cherry, peach, pear, potato, strawberry and tomato (Chet and Chernin, 2002; Bhattacharyya and Jha, 2012).

These beneficial strains can colonize the soil, seeds, root surfaces and other underground parts of the plants to the same extent as the phytopathogens that they control (Keel et al., 1992; Whipps, 2001).

Significant results concerning the biocontrol of many plant diseases have been obtained using tobacco (Maurhofer et al., 1995), cereals (Duffy et al., 1997), and horticultural plants (Kloepper et al., 1992; Duijff et al., 1998; Fravel et al., 2003) involving numerous fusarium diseases (Keel et al., 1992; Raaijmakers et al., 1995; Latour et al., 1996; Leeman et al., 1996; Weller et al., 2002). Fluorescent pseudomonad bacteria have been shown to act against pathogenic agents by synthesizing antibiotic compounds (e.g., phenazins, pyrrolnitrine and 2,4- diacetylphloroglucinol) (Keel et al., 1992), hydrogen cyanide (Maurhofer et al., 1995), lytic enzymes capable of altering the fungal cell wall (chitinase and glucanase) and other secondary metabolites (O'Sullivan and O'Gara, 1992). In addition to the antibiotic properties and the trophic competition recognized in these rhizobacteria, there is evidence that fluorescent pseudomonad strains can trigger induced systemic resistance (ISR) in plants, thus assuring a protection against a broad spectrum of phytopathogen agents (Van Loon et al., 1998).

The potential for the biological control of fusarium wilt has attracted much attention throughout the world. At present, the idea of controlling soil-borne plant pathogens, including Fusaria, with chemical pesticides or fungicides is being challenged by the approach that biological control can have an important role in sustainable agriculture (Pandey et al., 2010).

The purpose of this study was to evaluate the antifungal activity, *in vitro* and *in planta,* of *P. fluorescens* strains against *F. oxysporum* f.sp *lycopersici* and f.sp *lini*, the causative agents of fusarium wilt of tomato and flax, respectively.

MATERIALS AND METHODS

Antagonistic bacteria

On the basis of their metabolic characteristics and their biocontrol potential, the PGPR strains used in this work were selected according to their origin and their ability to produce (or not) secondary metabolites *in vitro* that are implicit in biocontrol, namely, pyoverdines (pvd), hydrocyanic acid (HCN), indole acetic acid (IAA), phosphate-solubilizing compound (phos) and phenazins (phz) (Table 1).

Five *P. fluorescens* strains, originally isolated from the rhizosphere of different plants, were selected from several PGPR strains based on their efficiency as biocontrol agents, as demonstrated by significant reductions in root and foliar diseases following their application as a seed or soil treatment in tomato, wheat, flax and palm date (Benchabane, 2005) (Table 1). The *P. fluorescens* strains CHA0 and CHA400 were kindly supplied by K. Keel (Laboratoire de Biologie Microbienne, Université de Lausanne, Switzerland). CHA0 produces a variety of secondary metabolites, promotes the growth of various crop plants and protects plants against root diseases caused by soilborne pathogenic fungi (Keel et al., 1992; De Werra et al., 2009). The genetic derivative, CHA400, differs from the wild-type strain, CHA0, by the absence of pyoverdine synthesis (Muller, 2009). Bacteria were scraped from the surface of a 24-h old King's B (KB) agar plate (King et al., 1954) and resuspended in 50 ml sdH_2O (sterilized distilled water); the density was then adjusted to 10^6 cfu/ml using a spectrophotometer (Shimadzu-m240, λ= 620 nm). Each bacterial strain was stored at -80°C in Eppendorf tubes (200 µl of sterile glycerol + 800 µl of bacterial suspension in KB liquid medium).

Plant pathogens

The *F. oxysporum* f.sp *lycopersici* isolate (Fol) (Collection of Phytopathology Laboratory, Agroveterinary Sciences Faculty, Blida University, Algeria) had originally been isolated from the roots of wilted tomato plants (Benchabane, 2005). *F. oxysporum* f.sp. *lini* (Foln) was kindly provided by P. Lemanceau (UMR Microbiologie

Table 1. Origin of the *P. fluorescens* strains.

Strain	Origin of isolation	Observation
P6	Potato rhizosphere (Algeria)	pvd +, HCN+, IAA +, phos +, phz +
B3	Wheat rhizosphere (Algeria)	pvd +, HCN+, IAA -, phos +, phz +
T9	Tomato rhizosphere (Algeria)	pvd +, HCN-, IAA +, phos -, phz +
SN1	Uncultivated soil (Algeria)	pvd +, HCN-, IAA -, phos -, phz -
D2	Date palm rhizosphere (Algeria)	pvd +, HCN+, IAA -, phos +, phz +
CHA0	Tobacco rhizosphere (Switzerland)	Reference strain, used in biocontrol and synthesize various secondary metabolites (Keel et al., 1991)
CHA400	Mutant of CHA0	pvd -

+: Positive; -: negative; pvd: pyoverdines; HCN: hydrocyanic acid; IAA: indole acetic acid; phos: Phosphatase, phz: phenazins.

du Sol et de l'Environnement, INRA- Dijon - France). Initially, the fungal pathogens were stored as a microconidial suspension in 30 % glycerol at -80°C; for all of the experiments, the fungi were maintained on potato dextrose agar (PDA, Sigma) slants and stored at 4°C. The purity of the fungal isolates was verified by monosporal cultures on PDA plates, using microscopic observations of the microconidia and macroconidia in addition to the stability of the cultural characteristics (colony shape, coloration and morphometric dimensions). The conidial suspensions were prepared as described by Steinkellner et al. (2008) with some modifications: the PDA fungal culture plates (eight days old, incubated at 28°C) were flooded with 2 ml sdH_2O and scraped into 48 ml sdH_2O. The microconidia suspensions obtained were filtered through six layers of sterile cheesecloth to separate the mycelium from the conidia and adjusted by a hemocytometer to 10^6 microconidia/ml.

In vitro antagonism

Antagonistic activities between individual *P. fluorescens* strains and *F. oxysporum* isolates (Fol and Foln) were tested in three culture media, Potato Dextrose Agar (PDA), KB (King et al., 1954) and a KB + PDA mixture (1:1 v/v), by measuring the inhibition of the mycelial growth, conidial germination and germ tube elongation. Before use, the two fungus isolates were grown separately on PDA plates for 8 days at 28°C, and the bacteria isolates were grown separately in liquid KB medium for 24 h at 26°C.

Mycelial growth

Tests for antibiosis were conducted on solid culture media plates using the dual culture technique adapted as described by Keel et al. (1996). Each bacteria strain was spotted with a loopful culture (24 h old) on four corners of a Petri plate and incubated at 26°C. Twenty-four hours later, 5-mm diameter PDA plugs containing mycelia of Fol or Foln (5 days old) were placed in the center of the plates and incubated at the same temperature. Plates inoculated with only fungal agar plugs served as a control and were observed daily until their maximal growth (8 at 10 days). The fungal growth was measured in the dual culture plates, where the colony diameter was measured in three directions on each plate, and the percent inhibition was calculated in three independent replicates as follows:

([radial growth in control - radial growth in dual culture] / radial growth in control) x 100

Conidial germination and the elongation of the germ tube

Dual inoculations of suspensions of conidia and bacteria were prepared by adding 1 ml of bacterial suspension into test tubes containing 8 ml liquid medium (PD, KB or PD + KB). After 6 h of pre-incubation at 2 °C, 1 ml of conidial suspension was added and incubated for 18 h at the same temperature in the dark while being shaken at 100 rpm. Control treatments were prepared by substitution of the bacterial suspension by 1 ml sdH_2O. The experiments were performed five times. As described by Wu et al. (2009), six (40 µl) drops of the suspension for each replicate was placed on a glass slide and mixed with a drop of acid fuchsin in lactophenol to kill and stain the conidia. The mixture was observed with a light microscope (400x magnification) calibrated to determine conidial germination and the length of the germ tubes (µm) in six randomly chosen microscopic fields per glass (200 - 300 conidia x 6). A microconidium was considered germinated if the germ tube length was at least as long as the spore. For each field, the total number of conidia was counted, and the percentage of the inhibition of conidial germination and germ tube elongation was calculated as follows:

ICG = ([GC - GD] / GC) X 100,

where ICG is the inhibition of conidial germination (%), GC is the percentage of germination in the control and GD is the percentage of germination in the dual inoculation.

IGTE = ([LC - LD] / LC) X 100

Where, IGTE is the inhibition of germ tube elongation (%), LC is the length of the germ tubes in the control treatment and LD is the length of the germ tubes in the dual treatment.

In planta bioassay

The experiments were performed independently for testing the antagonistic activities *in planta (*tomato and flax) of *P. fluorescens* strains against Fol and Foln, respectively, in a greenhouse under a day/night cycle of 16/8 h, 28°C/18°C ± 2°C and 60 - 65% relative humidity.

Soil and culture substrate

The soil, loamy sand, pH 7.6, organic matter 2% (originally from the Experimental Station of the Department of Agronomy, Blida

Table 2. Disease scale of tomato and flax wilts.

Tomato wilt	Flax wilt
0 : Plant healthy	0 = Plant healthy
1 : Plant with foliar basal wilt	1 = Partial yellowing
2: Plant with unilateral wilt	2 = Apical wilting «Shephard's Crook»
3 : Plant completely wilted	3 = Partial wilting
4: Plant completely desiccated and dead	4 = Plant completely wilted
	5 = Plant completely desiccated and dead

University), had been cultivated regularly over the last five years with tomatoes. The soil was passed through a 3 mm sieve, air-dried and added to sterilized peat (2:1). The resulting substrate was autoclaved (120°C) for 1 h on two consecutive days and used to fill plastic pots (4.5 kg substrate/pot).

Pathogen inoculation and seed bacterization

The application of pathogens and antagonists was carried out using an adaptation of the procedures described by Validov et al. (2009). The soil in the pots was inoculated with the fungal pathogens (Fol or Foln, 10^6 conidia/g of soil) using the conidial suspension prepared above 48 h prior to the bacterization. For the untreated (control) soil, the same volume of conidial suspension was replaced by sdH_2O. Certified seeds of tomato (*Solanum lycopersicum* cv. riogrande) and flax (*Linum usitatissimum* cv. opaline) were surface sterilized (3% NaOCl) for 5 min, rinsed three times in sdH_2O and dried on absorbent paper. The bacterial suspension was prepared with methylcellulose (1 %) as an emulsifier and agitated for 2 h (100 rpm) prior to use.

The bacteria-methylcellulose suspension was mixed with either the tomato or flax seeds and then dried overnight in a laminar air flow hood and transferred onto moist filter paper (10^7 cfu/seed). The control treatments consisted of non-bacterized dry seeds or seeds only coated with methylcellulose (1 %). Three bacterized seeds were initially sown per pot, and after emergence (12 - 16 days after plantation), only one plant was kept; the bacterial suspension (10^7 cfu/ml) was applied as a soil drench around the plant base stem. The plants were irrigated as needed with sterile water.

Assessment of disease development

The disease incidence at each observation was calculated by the number of plants that showed symptoms of wilting as a percentage of the total number of plants of each treatment per block. Subsequently, the disease ratings were plotted over time to generate the disease progress curves. Symptoms of fusarium wilt disease were assessed every 3 days using the following two scales, corresponding to each disease according to the growth characteristics of each plant: (i) tomato disease (0 - 4) and (ii) flax disease (0 - 5) (Table 2).

The plant was considered infected when a rating of 2 was recorded. The disease incidence was noted by the calculation of the infection percentage (I%) and by the assessment of the disease severity (S%) as follows:

I (%) = (number of plants infected / total number of plants observed) x 100

$$S (\%) = (\Sigma [E.a] / N.T \cdot 100)$$

Where, E is the disease scale (0 to 4) for tomato wilt and (0 to 5) for flax wilt, a is the number of plants infected at each symptomatological scale, N is the total number of plants observed and T is the maximum disease scale (4 for tomato wilt and 5 for flax wilt).

The biological control was estimated on the basis of a comparative report between the levels of the diseases amongst the positive control treatment in relation to the bacterized treatments.

Experimental design

For the experiment, each trial (with Fol or with Foln) was conducted independently in a completely randomized block design (factorial) with four replicates (four random blocks). The studies are on nine treatments per block: seven treatments corresponding to the seven bacteria strains (P6, B3, T9, SN1, D2, CHA0 and CHA400) and two controls, a positive control T(+), inoculated only with fungal conidial suspension but not bacterized, and a negative control T(-) not bacterized and not inoculated by fungi. For each trial, at least 100 tomato plants and 100 flax plants were used (25 plants per block x 4). The same design was repeated twice for each trial under the same conditions.

Statistical analysis

Statistical analyses were performed with the SPSS software (version 16.0 for windows). The data obtained were subjected to analysis of variance (ANOVA).

For the *in planta* experiments, each treatment corresponded to the means of the total trials performed. The treatment means were compared and separated using the Newman-Keuls test on the basis of the little significant amplitude (LSA) by the F values ($P \leq 0.05$). The data for the relative inhibition of mycelial growth, conidial germination and germ tube elongation were square root transformed; the infection percentage and disease severity were arcsine square root transformed before analysis.

RESULTS

In vitro antagonism activities

Inhibition of mycelial growth

The five *P. fluorescens* strains exhibited substantial antagonistic activities against the two fungal isolates on the three culture media tested, causing clear inhibition zones in the dual cultures and restricting the growth of the two formae speciales of *F. oxysporum* by 8.33 to 49.33% (T9 - Foln). As compared to the untreated

Table 3. *In vitro* inhibition of mycelial growth (%) on the three culture media tested.

Bacterial strains	PDA		KB		PDA + KB	
	(Fol)	(Foln)	(Fol)	(Foln)	(Fol)	(Foln)
B3	31.39±1.69*[a]**	28.40±1.42[b]	38.52±1.83[a]	39.78±1.99[b]	28.50±1.89[a]	22.66±1.09[b]
P6	33.72±1.74[a]	39.95±2.39[a]	26.40±1.29[b]	29.95±1.51[c]	22.30±1.23[b]	31.15±1.42[a]
T9	34.88±0.74[a]	35.33±1.67[a]	34.33±1.85[a]	49.33±2.45[a]	24.33±1.70[b]	08.33±0.45[c]
D2	14.86±1.74[b]	24.57±2.28[c]	33.40±1.76[a]	30.19±1.51[c]	25.48±1.64[a]	25.33±1.45[b]
SN1	36.75±1.14[a]	33.52±1.86[a]	36.25±1.79[a]	23.50±2.23[d]	31.18±1.78[a]	33.88±1.86[a]

Fol: *Fusarium oxysporum* f.sp. *lycopersici*; Foln: *Fusarium oxysporum* f.sp. *lini*.*: Standard deviation; **: The values followed by the same letter are not significantly different according to the Newman-Keuls test (P < 5%).

control, the mycelial growth decreased for *F. oxysporum* f.sp. *lycopersici* (Fol) by 14.86 to 36.75% on PDA, 26.40 to 38.52% on KB and 22.30 to 31.18% on KB + PD media, whereas the mycelial growth of *F. oxysporum* f.sp. *lini* (Foln) was reduced by 28.40 to 39.95% on PDA, 23.50 to 49.35% on KB and 8.33 to 33.88% on KB+PD. On the basis of the diameter of the inhibition zone, the strains were separated further into two to four groups (a, b, c or d) according to their interactions, but the level of inhibition remained statistically significant. Strain T9 exhibited more variability, with a maximal inhibition value against Foln, yet gave the minimum value against the same pathogen on KB + PDA medium (Table 3). Due to this exception, the effect of the culture media was noted but was not apparent in other interactions.

Inhibition of conidial germination and germ tube elongation

Conidial germination and germ tube elongation were significantly inhibited by the *P. fluorescens* strains. The conidial germination percentage was reduced by 9.34 to 55.95% (Fol) and by 6.15 to 29.38% (Foln). The germ tube elongation percentage was reduced by 5.42 to 45.45% (Fol) and by 1.63 to 20.89% (Foln). The antagonistic activities of the tested PGPR strains varied with respect to the target isolates of *F. oxysporum*, but in general, they were more important against Fol than Foln. The inhibition level noted with strains B3, P6 and SN1 were stronger in KB medium than in the two other media tested, whereas they were higher in PD medium for the T9 and D2 strains. For the two estimated parameters (IGC and IETG), the T9 strain resulted in the highest rates of inhibition of conidial germination and germ tube elongation against Fol (Table 4).

***In planta* bioassay**

Seven bacterial strains with notable antagonistic activities were selected for evaluation in the bioassay trials. In the pathogen control (T+) with the SN1, B3, T9 and CHA400 treatments, disease symptoms were visible from the 10th day for tomato (Figure 1) and after the 15th day for flax (Figure 2). After inoculation of the bacterial strains, the wilt disease reached considerable levels by the 17th (tomato) and 25th (flax) day, yet the plants showed a reduction in the evolution of the degree of the disease; in the control, the rate of infection rose rapidly after 17 days (> 50 %).

At the end of the two experiments, analysis of variance showed significant differences between treatments (P < 0.05), and the Newman-Keuls test differentiated five groups (tomato wilt) and six groups (flax wilt) that differed significantly from each other (Figures 1 and 2). None of the bacterial strains completely protected the plants, although all of the isolates significantly decreased the severity and incidence of the disease.

At the end of our experiments, the severity of the disease on the tomato and flax plants was reduced to different levels depending on the bacterial strain and was estimated to be 4.86 to 74.49% on the tomato plants and 3.93 to 79.19% on the flax plants (Figure 3). Similar decreases in wilt disease were observed with strains B3, D2 and CHA0 (the same Newman-Keuls group) against Fol and Foln.

The high biocontrol effects (> 60%) of the CHA400 and P6 strains were classed into two groups (Figure 2). The least amount of inhibition was noted with SN1 against the two diseases and the T9 strain against tomato fusarium wilt (Figure 3).

DISCUSSION

The use of beneficial microorganisms as biopesticides to reduce diseases on various agronomically important crops is considered one of the most promising methods in crop management practices. In the present study, we evaluated the efficacy, *in vivo* and *in planta*, of *P. fluorescens* strains as biocontrol agents against *F. oxysporum* f.sp *lycopersici* and *F. o* f.sp. *lini*.

Despite the difference in the chemical composition of the medium and its influence on the synthesis of metabolites, which can have a major function in the

Table 4. *In vitro* inhibition of conidial germination and germ tube elongation (%) on the three culture media tested.

Bacterial strain	Media	ICG (%)		IGTE (%)	
		Fol	(Foln)	Fol	Foln
	KB	22.10±3.58*c	11.33±2.08d	26.02±7.58bc	06.28±0.08e
B3	PD	18.26±1.54c	13.44±1.06d	19.36±1.28c	06.44±0.06e
	PD+KB	09.34±0.65d	16.25±1.65c	05.42±0.65e	09.43±0.65de
	KB	37.33±2.90b	29.38±1.95bc	26.62±1.82bc	20.89±1.63c
P6	PD	16.26±3.14c	12.35±2.11d	09.58±0.72de	10.58±1.18d
	PD+KB	16.58±2.78c	26.35±2.75bc	10.52±1.11d	10.36±0.31d
	KB	47.33±5.85a	15.33±1.22d	42.04±4.26a	08.38±3.82de
T9	PD	55.95±6.52a	14.69±3.52d	44.97±5.85a	07.16±1.52e
	PD+KB	17.88±2.26c	06.17±3.52e	11.12±2.45d	02.29±0.07f
	KB	23.21±3.22c	13.17±2.33e	09.12±0.82de	09.15±1.28de
D2	PD	46.65±6.18a	14.69±2.92d	45.45±1.51a	08.11±1.32de
	PD+KB	18.69±2.42c	06.15±1.52e	13.42±1.38d	01.63±0.05f
	KB	22.31±3.33c	13.15±1.56d	11.11±3.33d	07.34±1.12e
SN1	PD	16.26±1.14c	12.35±2.11d	08.25±0.74de	05.28±0.75e
	PD+KB	16.58±1.78c	26.35±2.35bc	08.38±1.08de	16.32±0.85c

ICG: Inhibition of conidial germination; IGTE: inhibition of germ tubes elongation; Fol*: Fusarium oxysporum* f.sp. *lycopersici*; Foln: *Fusarium oxysporum* f.sp. *lini.* *Standard deviation. The values followed by the same letter are not significantly different according to the Newman-Keuls test (P < 5%).

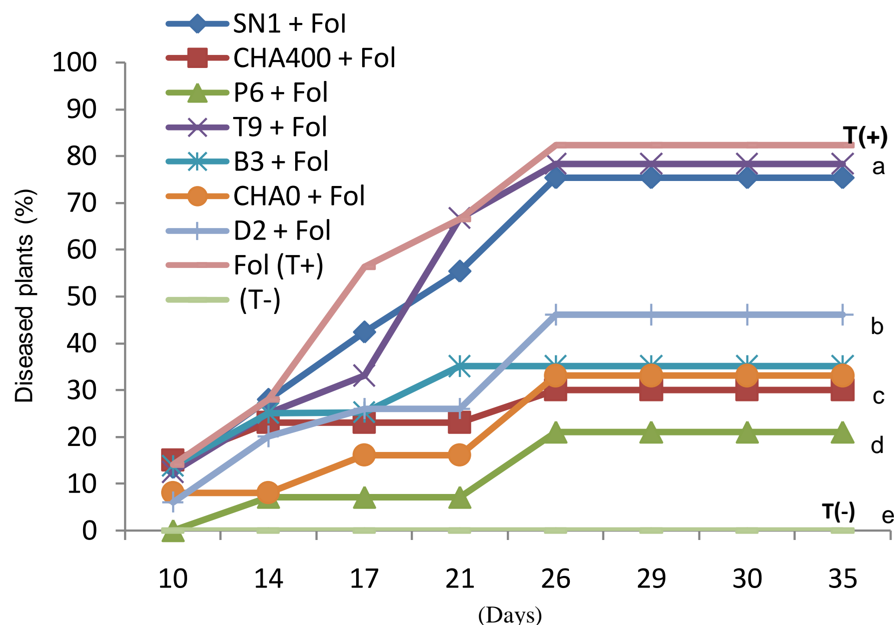

Figure 1. Disease evolution on tomato plants. The treatments followed by the same letter are not significantly different according to the Newman-Keuls test (P < 0.05). *P. fluorescens* strains: SN1, CHA400, P6, T9; B3, CHA0, D2. Fol (T+) : Positive control T(+), inoculated only with fungal conidial suspension but not bacterized. (T-): Negative control; T(-) not bacterized and not inoculated by fungi; Fol *: Fusarium oxysporum* f.sp. *lycopersici.*

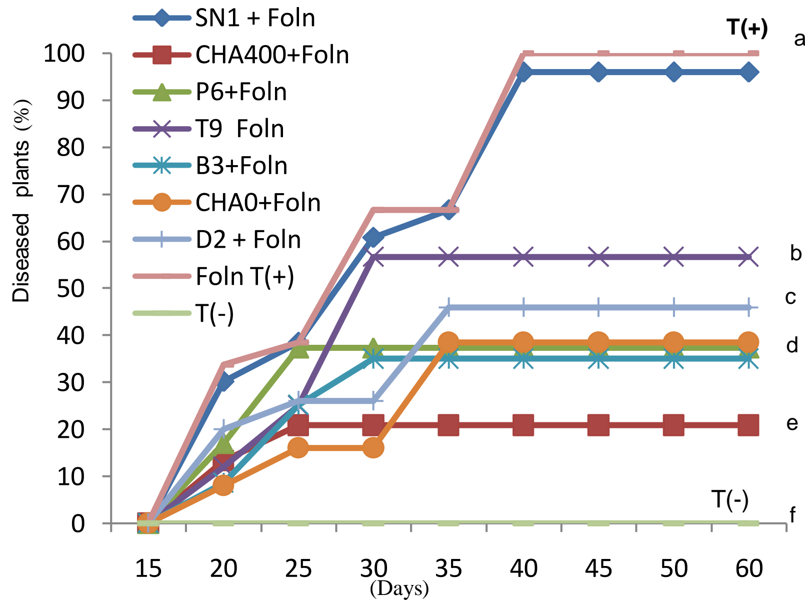

Figure 2. Disease evolution on flax plants. The treatments followed by the same letter are not significantly different according to the Newman-Keuls test ($P < 0.05$). *P. fluorescens* strains: SN1, CHA400, P6, T9, B3, CHA0, D2. Foln T(+) : Positive control, inoculated only with fungal conidial suspension but not bacterized. T(-) : Negative control, not bacterized and not inoculated by fungi. Foln : *Fusarium oxysporum* f.sp. *lini.*

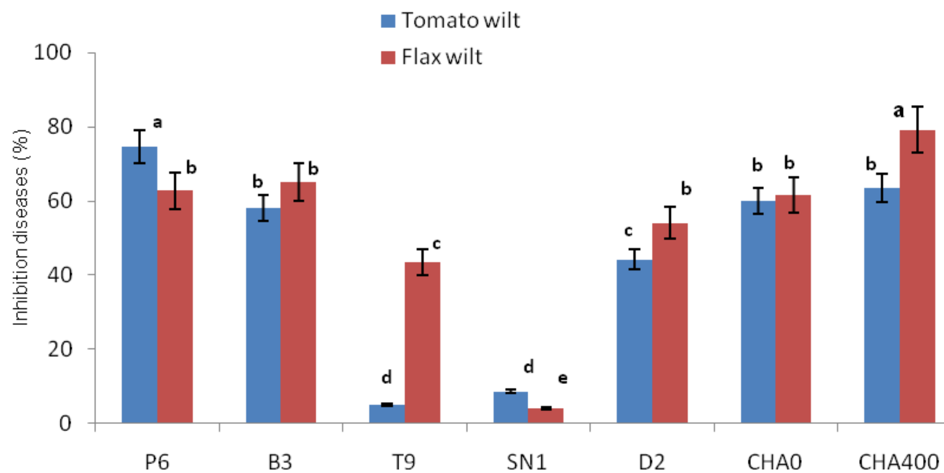

Figure 3. Biological protection rates against fusarium wilt of tomato and flax. Different uppercase letters indicated the difference in statistics according to the Newman-Keuls test ($P < 0.05$). *Pseudomonas fluorescens* strains: SN1, CHA400, P6, T9, B3, CHA0, D2.

inhibition effect; the three utilized culture media permitted the observation of antagonism for all of the five tested bacterial strains. We noted that the KB medium favored the bacterial synthesis of siderophores, whereas the PD and PD + KB media did not. The inhibition of the conidial germination and the elongation of the germ tubes observed on these two media suggested that the observed inhibition was not due to the action of the siderophores but that other mechanisms were developed by the bacteria, unlike the conidial germination inhibited on the KB media, which can be correlated with the presence of the siderophores.

It has been previously demonstrated that the antagonistic action of these same strains did not seem to be specific for the pathogenic agent but rather depended on the culture medium; in some cases, a broad-spectrum efficacy has been observed, acting at the same time on several fungal isolates of different genera (*Fusarium*,

Rhizoctonia, *Vertcillium* and *Pythium*) (Benchabane, 2005). The variability in the antagonistic activity of the *Pseudomonas* strains in the media tested suggested a diversity in the mechanisms implied in the biocontrol. In addition to the plurifactorial determinism of the antagonism, our results underline the possibility of a broad, non-specific efficacy against the two isolates of *F. oxysporum* (Fol and Foln). The difference in mycelial stimulation or inhibition in culture media results from physiological mechanisms under the culture conditions. Whereas spores are critical to the spatial dispersal of fungi, spore germination is essential to their survival and the establishment of an actively growing colony on suitable substrates. Invasive germ tube growth, such as the typical phenotype of *Fusarium* species, during surface germination is anomalous and unique to a small collection of strains or certain species (Glenn, 2006).

The signals affecting spore germination of the tomato pathogen, *F. oxysporum*, do not seem to be host-specific, as a similar pattern of microconidial germination has been found in the presence of root exudates from the host tomato plant and several non-host plants (sweet pepper, bean, barley, tobacco and cucumber). However, there are indications for a specific effect of the compounds found in tomato root exudates, and it has been reported that the germination rate of several *F. oxysporum* strains is affected differently by tomato root exudates (Steinkellner et al., 2008). No differences in the mycelial development of *F. oxysporum* have been observed in the vicinity of tomato and wheat roots, such that non-host plants can function as symptomless carriers of fusarium wilt (Steinberg et al., 1999). Perhaps the observed variation can be attributed to the biological variation of the fungal strains and/or cellular structures. However, further studies are needed to characterize the signaling compounds in plant root exudates that may be involved in the host plant interactions with F*. oxysporum*. Because spore developmental processes are critical for the reproduction and survival of fungi, spore germination for a plant pathogenic fungus may be seen as a critical developmental step in pathogenesis: germination must occur at both the correct time and location in order for infection to be successful (Seong et al., 2008).

The seven *P. fluorescens* strains used in our experiments showed varying antagonistic effects against both Fol and Foln. Theses rhizobacterial strains are characterized by different potentialities in terms of the production of metabolites implicated in biocontrol, such as the synthesis of pyoverdine (except CHA400) and the variation (+ or -) for other characteristics, especially the synthesis of phenazins and HCN production (Benchabane, 2005) (Table 1). When compared with wild type, the genetic derivative, CHA400, exhibits similar inhibition levels, although this strain lacks the ability to produce the strong siderophore pyoverdine (Keel et al., 1992), and therefore, should not take up iron. In contrast, the wild type and various mutants of the CHA0 strain complex iron by producing different siderophores, namely, pyochelin (Ankenbauer et al., 1988), salicylic acid (Meyer et al., 1992) and pyoverdine (Albrecht-Gary et al., 1994), which all form strong complexes with Fe (Müller, 2009).

The infection process of *F. oxysporum* involves the following steps: germination of spores in response to root exudates, production of penetration hyphae that attach to the root surface and directly penetrate it, and invasive growth in the host plant tissue (Rodríguez-Gálvez and Mendgen, 1995). The stimulation of plant pathogens by root exudates is an integral part of the concept of inoculum potential (Lockwood, 1986), and extensive studies have been performed on the biology of *F. oxysporum* and the colonization process (Steinkellner et al., 2008). Root exudates are supposed to play a key role in determining the positive or negative outcome of an interaction in the rhizosphere by various metabolites that are known as general germination stimuli for spores (Nelson, 1991; Bais et al., 2006). At different growth stages, tomato roots exude varying amounts of sugars and organic acids, and depending on the plant age, the effect of the tomato root exudates on spore germination of the tomato pathogen *F. oxysporum* also varies (Lugtenberg et al.,1999; Steinkellner et al., 2008).

In this study, overall, the most active rhizobacteria strains were P6, B3 and T9, and their antagonistic activities can be correlated with their metabolic characters, notably their secondary metabolites (phenazins) (Benchabane, 2005). The inefficiency of the T9 and D2 strains against *F. oxysporum* f.sp *lycopersici* reinforces their absence in relation to their rhizospherical origin and their antagonist activities. However, the lack or absence of an antagonistic effect for the SN1 strain, isolated in non-rhizospherical soil, showed that, independent of the biotope around the plant, a rhizospherical origin seems to play a primary role in biocontrol and appears to have a correlation with their capacity of rhizospherical adaptation and their acquired competitiveness. However, a rhizospherical origin of the bacterial strains did not seem to play a role in their *in vitro* antagonistic activities; thus, the T9 and D2 strains isolated from tomatoes and palm date rhizospheres, respectively, did not present a specific and regular antagonism against the *F. oxysporum* isolates. Two types of disease suppression are usually distinguished: (i) specific disease suppression (Weller et al., 2002) is caused by one or a few specific (genotypes of) microorganisms, such as phloroglucinol-producing pseudomonads suppressing *Gaeumannomyces graminis* (Raaijmakers and Weller, 1998), and (ii) general disease suppression is caused by multiple microorganisms acting against multiple pathogens and is restored quickly after a major disturbance (Hoitink and Boehm, 1999). The occurrence of specifically acting antagonists can occur anywhere but seems to be most dominant in the rhizosphere soil and, thus, is not entirely congruent with pathogen suppression (Termorshuizen and Jeger, 2008).

The growth and activity of the root system induces

significant modifications in the physicochemical and biological properties of the soil surrounding the root (rhizosphere effect) due to rhizodeposition. The ability to support certain biocontrol agents varies among plant species, cultivars and genotypes (Lucy et al., 2004). The secretion of rhizodeposition is an important way for plants to respond to their environment, and root exudates mediate communication between plants and other organisms (pathogens and antagonists) and stimulate defense responses against soil-borne pathogens and/or favor the association with beneficial soil microbes. The plant species, cultivar and phenological stage also have a primary role in the modulation of the quantity and the quality of the root exudates, similar to the physiological reactions to stress factors during the development microorganisms in the rhizosphere and their adhesion on root compounds (Nelson, 1991; Lugtenberg et al., 1999).

The results from the plant (seeds) that were bacterized with *P. fluorescens* strains showed varying degrees of bioprotection that were invoked in flax and tomato, as well as other plants (Kloepper et al., 1980; Maurhofer et al., 1995; Raaijmakers and Weller, 1998). PGPR antagonize plant pathogenic fungi, mainly by the production of antimicrobial metabolites but also by the competition for iron or rhizosphere niches (Keel et al., 1992) and the stimulation of the host defenses (induced systemic resistance) (Van Loon et al., 1998). Other mechanisms are involved directly in the promotion of plant growth and modulate the biocontrol activity of the bacteria (Lemanceau and Alabouvette, 1993; De Werra et al., 2009).

In conclusion, our results show that *P. fluorescens* strains have great potential to be used as biocontrol agents for the management of the *Fusarium* species that cause fusarium wilt of tomato and flax. The *in vitro* and *in vivo* data support the hypothesis that antagonism is the main mechanism for the biological control of disease; however, the antimicrobial compounds responsible have not yet been isolated and identified.

Thus, further study is necessary for determining the mechanisms of antago-nistic action of these strains. Our results show that the *Pseudomonas* strains, selected on the basis of their recognized genetic, physiological, metabolic and ecological properties of antagonism, can constitute an efficient biological control.

The non-specific action of the bacterial strains against phytopathogenic fungi, the variability of the responses according to the environment and the diversity of the implicated mechanisms (and even redundant in some their action should be considered when selecting strains. PGPR have great potential in both phytostimulation and the biocontrol of plant pathogens, but they have not been widely applied in the field for various reasons, such as the problem of formulation for efficient application (Akhtar and Sidiqui, 2009).

Through the overlap of a number of characteristics for antagonism, the selection of candidates (bacterial strains) with different mechanisms of action should reinforce the final biocontrol effect. Furthermore, the association (co-inoculation) of different strains characterized by a variety of antagonistic features may result in a synergistic effect (or at least an additive effect) and a broader spectrum of action.

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