

ORIGINAL ARTICLE

Phenazine antibiotics produced by fluorescent pseudomonads contribute to natural soil suppressiveness to *Fusarium* wilt

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Natural disease-suppressive soils provide an untapped resource for the discovery of novel beneficial microorganisms and traits. For most suppressive soils, however, the consortia of microorganisms and mechanisms involved in pathogen control are unknown. To date, soil suppressiveness to *Fusarium* wilt disease has been ascribed to carbon and iron competition between pathogenic *Fusarium oxysporum* and resident non-pathogenic *F. oxysporum* and fluorescent pseudomonads. In this study, the role of bacterial antibiosis in *Fusarium* wilt suppressiveness was assessed by comparing the densities, diversity and activity of fluorescent *Pseudomonas* species producing 2,4-diacetylphloroglucinol (DAPG) (*phlD*⁺) or phenazine (*phzC*⁺) antibiotics. The frequencies of *phlD*⁺ populations were similar in the suppressive and conducive soils but their genotypic diversity differed significantly. However, *phlD* genotypes from the two soils were equally effective in suppressing *Fusarium* wilt, either alone or in combination with non-pathogenic *F. oxysporum* strain Fo47. A mutant deficient in DAPG production provided a similar level of control as its parental strain, suggesting that this antibiotic does not play a major role. In contrast, *phzC*⁺ pseudomonads were only detected in the suppressive soil. Representative *phzC*⁺ isolates of five distinct genotypes did not suppress *Fusarium* wilt on their own, but acted synergistically in combination with strain Fo47. This increased level of disease suppression was ascribed to phenazine production as the phenazine-deficient mutant was not effective. These results suggest, for the first time, that redox-active phenazines produced by fluorescent pseudomonads contribute to the natural soil suppressiveness to *Fusarium* wilt disease and may act in synergy with carbon competition by resident non-pathogenic *F. oxysporum*.

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Introduction

The natural selection has resulted in numerous examples of genetic resistance in plants to above-ground pathogens, but curiously enough not to many below-ground pathogens. Cook *et al.* (1995) postulated that plants have developed an entirely different strategy to defend themselves against soil-borne pathogens. This strategy involves the ability of plants to selectively stimulate and support

populations of soil and rhizosphere microorganisms that are antagonistic to their pathogens. Natural disease-suppressive soils are the best examples in which the activities of specific soil and rhizosphere microorganisms keep susceptible plants almost free of infection in spite of ample exposure to virulent inoculum of soil-borne pathogens. Natural disease-suppressive soils occur worldwide and provide an enormous resource for the discovery of novel beneficial microorganisms and traits (Weller *et al.*, 2002). For most of the disease-suppressive soils, however, the consortia of microorganisms and mechanisms involved in pathogen control have not yet been identified.

The disease-suppressive soils have been classified as 'long-standing suppressive soils' and 'induced suppressive soils' according to the longevity of

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disease suppression (Hornby, 1979; Weller *et al.*, 2002). Long-standing suppressiveness is a biological condition naturally associated with the soil, its origin is not known and it appears to survive in the absence of plants. In contrast, induced suppressiveness is initiated and sustained by crop monoculture in the presence of the pathogen (Weller *et al.*, 2002). Soils suppressive to take-all disease of wheat and barley, caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici*, are referred to as take-all decline soils and are well-known examples of induced suppressiveness. The *Fusarium*-wilt suppressive soils from Châteaurenard (France) and Salinas Valley (CA, USA) are among the best examples of long-standing suppressive soils.

Fusarium wilts are caused by plant pathogenic *Fusarium oxysporum* leading to significant yield losses in many crops worldwide. *Fusarium*-wilt suppressive soils substantially limit the disease incidence or severity, but are not suppressive to diseases caused by non-vascular *Fusarium* species or other soil-borne pathogens (Alabouvette, 1986). The microbiological nature of the disease suppression in *Fusarium*-wilt suppressive soils was unequivocally shown by a range of approaches, including treatments with moist heat, methylbromide or γ -irradiation leading to loss of soil suppressiveness, and by experiments in which suppressiveness was transferred to wilt-conducive soils by mixing small amounts (0.1–10% wt/wt) of the suppressive soil into the conducive soil (Scher and Baker, 1980; Alabouvette, 1986; Weller *et al.*, 2002). *Fusarium*-wilt suppressive soils maintain their activity when brought into the greenhouse or laboratory, which greatly facilitates the identification of the microorganisms, mechanisms and genes involved in disease suppressiveness.

Among the bacterial and fungal genera proposed to contribute to *Fusarium* wilt suppressiveness are *Alcaligenes* (Yuen and Schroth, 1986), *Trichoderma* (Sivan and Chet, 1989), Actinomycetes (Amir and Amir, 1989), *Pseudomonas* (Kloepper *et al.*, 1980; Scher and Baker, 1982; Lemanceau and Alabouvette, 1993) and non-pathogenic *F. oxysporum* (Alabouvette, 1986; Larkin *et al.*, 1996). Detailed studies on *Fusarium*-wilt suppressive soils from Châteaurenard (France) and Salinas Valley showed that the suppressiveness was attributed, in particular, to the activity of non-pathogenic *F. oxysporum* and fluorescent pseudomonads (Scher and Baker, 1982; Alabouvette, 1990; Lemanceau and Alabouvette, 1991; Duijff *et al.*, 1999). For the non-pathogenic *F. oxysporum*, competition for carbon and induced systemic resistance were the main modes of action proposed so far (Steinberg *et al.*, 2007). For the fluorescent pseudomonads, siderophore-mediated competition for iron was shown to be a major mechanism in the control of *Fusarium* wilts (Scher and Baker, 1982; Lemanceau *et al.*, 1992). Particularly interesting from these studies was that the association between non-pathogenic *F. oxysporum*

and fluorescent pseudomonads provided enhanced disease suppression (Lemanceau *et al.*, 1993). Work by Duijff *et al.* (1999) supported and extended the earlier observations that disease suppression by the non-pathogenic *F. oxysporum* is related to reductions in both population density and metabolic activity of the pathogen on the root surface, and that competition for iron contributes to the suppression by *Pseudomonas* and enhances the biological activity of the non-pathogenic *F. oxysporum*.

In contrast to other disease-suppressive soils, the role of antibiosis in *Fusarium*-wilt suppressive soils has not been studied to date, in spite of the fact that several antibiotic compounds produced by antagonistic bacteria have shown growth-inhibitory activity against pathogenic *F. oxysporum*. For example, phenazine antibiotics were shown to play a key role in the biocontrol activity of several *Pseudomonas* species against *F. oxysporum* on diverse crops (Anjaiah *et al.*, 1998; Chin-A-Woeng *et al.*, 1998, 2000). Also the antibiotic 2,4-diacetylphloroglucinol (DAPG) has substantial activity against pathogenic *F. oxysporum* (Schouten *et al.*, 2004), and populations of DAPG-producing pseudomonads were highly enriched in a soil naturally suppressive to *Fusarium* wilt of peas (Landa *et al.*, 2002). Following similar molecular-based approaches used to unravel the role of antibiosis in take-all decline soils (Raaijmakers *et al.*, 1997; Raaijmakers and Weller, 1998; McSpadden-Gardener *et al.*, 2000; Weller *et al.*, 2002; Souza *et al.*, 2003a) and in soils suppressive to black root rot of tobacco (Wang *et al.*, 2001; Ramette *et al.*, 2003, 2006; Frapolli *et al.*, 2007), the objectives of this study were to determine the frequency, diversity and biocontrol activity of DAPG (*phlD*⁺)- and phenazine (*phzC*⁺)-producing pseudomonads in the *Fusarium*-wilt suppressive soil from Châteaurenard (France) in comparison with the conducive soil of Carquefou (France). The results showed that densities of *phlD*⁺ populations were not significantly different between the two soils but that their genotypic diversity differed significantly. However, the suppressive ability of isolates representative of the *phlD*⁺ diversity found in the two soils was similar. In contrast, *phzC*⁺ populations were only detected in the *Fusarium*-wilt suppressive soil and isolates representative of their diversity significantly improved the biocontrol activity achieved by non-pathogenic *F. oxysporum*. These results indicate, for the first time, that phenazine-producing pseudomonads are enriched in the *Fusarium*-wilt suppressive soil from Châteaurenard and, when reintroduced in association with non-pathogenic *F. oxysporum*, significantly enhance the soil suppressiveness to *Fusarium* wilt. Bioassays with a phenazine-deficient mutant showed that phenazine production is involved in this augmentation of disease suppression. Collectively, these results suggest a role of redox-active phenazine antibiotics in the natural soil suppressiveness to *Fusarium* wilts. A model is

presented depicting the versatility of the mechanisms and the complex interplay between consortia of antagonistic microorganisms in *Fusarium*-wilt suppressive soil.

Materials and methods

Microorganisms and soils used

Characteristics of the *Pseudomonas* reference strains are given in Table 1. All *Pseudomonas* strains were cultivated on KMB agar (King *et al.*, 1954) at 25 °C. The flax pathogen *Fusarium oxysporum* f. sp. *lini* Fohn3 and the non-pathogenic strain *F. oxysporum* Fo47 were obtained from the MIAE Culture Collection (INRA, Dijon, France), and grown on Difco potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) at 24 °C. Soil naturally suppressive to *Fusarium* wilt was obtained from Châteaurenard, and the conducive soil from Carquefou. The main characteristics of these soils have been described earlier (Lemanceau *et al.*, 1988). Briefly, the soils from Châteaurenard and Carquefou are calcic silt-clay and sandy-loam soils, respectively. The calcium carbonate content and pH of the Châteaurenard soil (37.4% CaCO₃, pH 7.9) are much higher than those of the Carquefou soil (8.4% CaCO₃, pH 5.3). These two characteristics account for the very low concentration of extractable iron of the Châteaurenard soil (18.8 p.p.m.), 15 times lower than that of the Carquefou soil (279.9 p.p.m.), even though both soils share a comparable concentration of total iron, that is, 11% and 9% for the Châteaurenard and Carquefou soils, respectively. Biocontrol assays were performed in a soil collected from a fallow field in Dijon (France) for which the proper-

ties were described in Latour *et al.* (1996). The phenazine-producing strain *P. fluorescens* 2-79 and its phenazine-deficient mutant 2-79Z were kindly provided by D Mavrodi and L Thomashow (Washington state University, Pullman, WA, USA) (Khan *et al.*, 2005). DAPG-deficient mutant 17-8D was obtained by plasposon mutagenesis of strain Phl60rif, and its inability to produce DAPG was confirmed by RP-HPLC analysis (data not shown). The deficiency in phenazine production by mutant 2-79Z was previously demonstrated by Khan *et al.* (2005).

*Frequency of indigenous *phlD*⁺ and *phzC*⁺ *Pseudomonas* in soil and rhizosphere*

Fluorescent *Pseudomonas* harboring the *phlD* (*phlD*⁺) or *phzC* (*phzC*⁺) genes were isolated and enumerated in bulk soil and in the rhizosphere of flax and tomato according to the methods described by Raaijmakers *et al.* (1997) and Souza *et al.* (2003a). Briefly, surface-sterilized seeds of flax (*Linum usitatissimum* L. cv. Opaline) and tomato (*Lycopersicon esculentum* Mill. cv. H63-5) were sown in the wilt suppressive and conducive soils, and the plants were grown for 4 weeks under controlled conditions (12:12 h light/dark photoperiod under photosynthetic active radiation of 215 μE m⁻² s⁻¹, 25:22 °C light/dark thermoperiod). For each of the two soils, five pots (3 l) were used for flax and five for tomato; each pot contained 2 l of soil and five seeds. One series of five pots was kept uncultivated (bulk soil). Rhizosphere and bulk soil samples were obtained as described earlier (Raaijmakers *et al.*, 1997) and dilution plated onto KMB agar supplemented

Table 1 Reference strains of 2,4-diacetylphloroglucinol (DAPG) and phenazine-producing *Pseudomonas* spp. used in this study

Strain	Origin	Species	Source or reference
<i>DAPG producer</i>			
PWB233	Wheat, The Netherlands	<i>Pseudomonas</i> sp.	Bergsma-Vlami <i>et al.</i> (2005)
PWB532	Wheat, The Netherlands	<i>Pseudomonas</i> sp.	Bergsma-Vlami <i>et al.</i> (2005)
PPB2310	Potato, The Netherlands	<i>Pseudomonas</i> sp.	Bergsma-Vlami <i>et al.</i> (2005)
PPB3512	Potato, The Netherlands	<i>Pseudomonas</i> sp.	Bergsma-Vlami <i>et al.</i> (2005)
PSB211	Sugar beet, The Netherlands	<i>Pseudomonas</i> sp.	Bergsma-Vlami <i>et al.</i> (2005)
PSC2218	Sugar beet, The Netherlands	<i>Pseudomonas</i> sp.	Bergsma-Vlami <i>et al.</i> (2005)
PSC415	Sugar beet, The Netherlands	<i>Pseudomonas</i> sp.	Bergsma-Vlami <i>et al.</i> (2005)
F113	Sugar beet, Ireland	<i>P. fluorescens</i>	Shanahan <i>et al.</i> (1992)
Q2-87	Take-all suppressive soil, wheat, USA	<i>P. fluorescens</i>	Vincent <i>et al.</i> (1991)
Q8r1-96	Take-all suppressive soil, wheat, USA	<i>P. fluorescens</i>	Raaijmakers and Weller (1998)
CHAO	Black root rot suppressive soil, tobacco, Switzerland	<i>P. fluorescens</i>	Stutz <i>et al.</i> (1986)
Pf-5	Cotton, USA	<i>P. fluorescens</i>	Howell and Stipanovic (1979)
Phl60rif	Take-all suppressive soil, wheat, The Netherlands	<i>Pseudomonas</i> sp.	Souza <i>et al.</i> (2003b)
17-8D	DAPG-deficient mutant of Phl60rif	<i>Pseudomonas</i> sp.	Unpublished data
<i>Phenazine producer</i>			
2-79	Take-all suppressive soil, wheat, USA	<i>P. fluorescens</i>	Weller and Cook (1983)
2-79Z	Phenazine-deficient mutant of 2-79; <i>phzD::lacZ</i>	<i>P. fluorescens</i>	Khan <i>et al.</i> (2005)
PGS12	Corn, Belgium	<i>P. aureofaciens</i>	Georgakopoulos <i>et al.</i> (1994)
PCL1391	Tomato, Spain	<i>P. chlororaphis</i>	Chin-A-Woeng <i>et al.</i> (1998)
30-84	Take all suppressive soil, wheat, USA	<i>P. aureofaciens</i>	Pierson and Thomashow (1992)
PAO1	Burned patient, Australia	<i>P. aeruginosa</i>	Holloway (1955)

with cycloheximide (100 mg l^{-1}), chloroamphenicol (13 mg l^{-1}) and ampicillin (40 mg l^{-1}) (Simon and Ridge, 1974). After 48 h of incubation at 25°C , colonies were counted and fluorescent pseudomonads were differentiated from non-fluorescent colonies under UV light (wavelength, 365 nm). The numbers of fluorescent pseudomonads that harbored the *phlD* gene or the *phzC* gene were determined by colony hybridization followed by PCR analysis as described earlier (Raaijmakers *et al.*, 1997; Souza *et al.*, 2003a). The *phlD* and *phzC* probes were obtained by labeling of a 629-bp *phlD* fragment obtained from the reference strain *P. fluorescens* Phl60rif, and of a 522-bp *phzC* fragment from the reference strain *P. fluorescens* 2-79. Digoxigenin labeling of the DNA probes was carried out by random priming using the DIG DNA labeling kit (Roche, Meylan, France). Primers B2BF and BPR4 (McSpadden-Gardener *et al.*, 2001) were used to amplify the 629-bp *phlD* fragment and primers PHZJR1 5'-CAGGGCCG(G/C)(A/G)(C/T)ATTTCTCG GTTCT-3' and PHZJR2 5'-GCGCGGGTCGCACAGG CTTTTGTA-3' for the 522-bp *phzC* fragment. The hybridized probes were immunodetected with anti-digoxigenin-alkaline phosphatase-Fab fragments and visualized with the colorimetric substrates nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolylphosphate, as described by the supplier (Roche). The colonies that were positive in hybridization were purified and subjected to PCR analysis with primers specific for *phlD* or *phzC*. For statistical analysis, population densities were \log_{10} transformed and significant differences were assessed by analysis of variance followed by Student–Newman–Keuls tests (SAS Institute Inc., Cary, NC, USA).

Genetic diversity of phlD⁺ and phzC⁺ pseudomonads
Restriction-fragment length polymorphism (RFLP) analysis of the 629-bp *phlD* PCR product amplified from the indigenous isolates and reference strains was performed using the restriction enzymes *Hae*III, *Msp*I and *Taq*I (McSpadden-Gardener *et al.*, 2001). For RFLP analysis of the 522-bp *phzC* fragments, the restriction enzymes *Fnu*4HI, *Nci*I, *Nla*IV, *Nde*II, *Dde*I and *Cfr*13I were used. Restriction patterns were determined by electrophoresis at 80 V in TAE buffer with 3.5% (w/v) Metaphor agarose (Tebu, Le Perray-en-Yvelines, France). A similarity matrix was calculated using the pair wise Jaccard similarity coefficient (Jaccard, 1908). Cluster analysis was performed by the unweighted pair group method using arithmetic averages allowing the delineation of *phlD* and *phzC* genotypes. As *phlD* genotypes were earlier shown to correlate almost perfectly with those defined by BOX-PCR (Mavrodi *et al.*, 2001; McSpadden-Gardener *et al.*, 2001; Landa *et al.*, 2002, 2006, Weller *et al.*, 2002; De La Fuente *et al.*, 2006), BOX-PCR was only performed for *phzC⁺* isolates. The protocol used was described by

Rademaker *et al.* (1997), except for the *Taq* Polymerase. Four units of *Taq* DNA polymerase (Q-BIOgene, Illkirsch, France) were used in each 25 μl BOX-PCR. BOX-PCR fragments were separated overnight at 40 V in a 1.5% (w/v) Seakem LE agarose gel (Tebu) in TAE buffer. For each BOX-PCR fingerprint, a binary data matrix with 79 bands being either present (1) or absent (0) was established. A similarity matrix was calculated using the pair wise Pearson product-moment correlation coefficient. Cluster analysis was performed by unweighted pair group method using arithmetic averages. Isolates presenting Pearson product-moment correlation coefficients ≥ 0.75 were assigned to a BOX-PCR genotype named with a same letter (A–J) and, within these genotypes, those having a correlation coefficient equal to 1 were assigned a BOX-PCR genotype named with the same number (that is, A1). Next to RFLP and BOX-PCR analyses, *phlD* and *phzC* PCR fragments were cloned (pGEMT Easy Vector System II, Promega, Charbonnières, France) and sequenced (Genome Express, Meylan, France). BLAST analyses and CLUSTAL W (Thompson *et al.*, 1994) were applied for multiple sequence alignments. Phylogenies were determined by neighbor-joining method (Saitou and Nei, 1987) using the Kimura '2-parameters' correction (Kimura, 1980) with the pair wise gap removal option. To estimate tree node validity, results of 1000 bootstrapped data sets were determined.

Biocontrol efficacy of phlD⁺ and phzC⁺ pseudomonads

The *phlD⁺* and *phzC⁺* *Pseudomonas* isolates were grown on KMB agar plates for 48 h at 25°C and cells were harvested and washed once in sterile distilled water. The cell density was determined spectrophotometrically at 600 nm and adjusted to 10^7 CFU g^{-1} of fresh soil. Inoculums of the pathogenic *F. oxysporum* f. sp. *lini* Foln3 and non-pathogenic *F. oxysporum* Fo47 were prepared as described earlier (Lemanceau and Alabouvette, 1991). The fungal suspensions were adjusted to 10^5 conidia g^{-1} of soil fresh weight for *F. oxysporum* Fo47, and 10^3 conidia g^{-1} of soil fresh weight for *F. oxysporum* f. sp. *lini* Foln3. Flax plants were grown in environment-controlled growth chamber (15:9 h light/dark photoperiod under photosynthetic active radiation of $215 \mu\text{E m}^{-2} \text{ s}^{-1}$, $26:20^\circ\text{C}$ light/dark thermoperiod). The relative humidity in the growth chamber was maintained at 60% during the night and 80% during the day. Each flax plant was grown individually in a plastic tray cell filled with 50 ml of autoclaved (three successive treatments at 100°C for 1 h, each one separated by 24 h) Dijon soil placed on top of a draining layer of extruded clay beads. Surface sterilized flax seeds were sown in the filled plastic tray cells and inoculated with conidial and bacterial suspensions. Each treatment consisted of three replicate trays of 16 plants arranged in a randomized complete block experimental design.

Plants showing the typical symptoms of *Fusarium* wilt were recorded twice a week for a period of 6 weeks, and cumulated numbers of diseased plants were used to determine the area under the disease progress curve (AUDPC) according to the methods described by Steinberg *et al.* (2004). Differences in disease severity between treatments were determined by analysis of variance followed by Fisher's PLSD-test (SAS Institute Inc.).

Results

Frequency of antibiotic-producing pseudomonads in disease suppressive and conducive soils

Population densities of fluorescent pseudomonads harboring the biosynthesis gene *phlD* for DAPG or *phzC* for phenazine production were assessed in bulk soils and in the rhizosphere of tomato and flax seedlings grown in the *Fusarium* wilt suppressive (Châteaurenard) and conducive (Carquefou) soils. *phlD*⁺ pseudomonads were not detected in the two bulk soils (detection limit was 3.3×10^1 CFU g⁻¹ soil fresh weight). In contrast, *phzC*⁺ pseudomonads were detected in the wilt-suppressive bulk soil at an average density of 3.9×10^3 CFU g⁻¹ soil fresh weight, representing 1.3% of the total population of fluorescent pseudomonads. *phzC*⁺ pseudomonads were not detectable in the conducive soil (data not shown). When flax or tomato seedlings were cultivated in these soils, *phlD*⁺ pseudomonads were detected in the rhizosphere of both crops at densities ranging from 4.1×10^3 to 4.7×10^5 CFU g⁻¹ root fresh weight (Table 2), representing on an average 3.7–26.1% of the total population of culturable fluorescent pseudomonads. Owing to variations among replicates, no significant differences in the population densities of *phlD*⁺ pseudomonads were found between the wilt suppressive and conducive soils, either in flax or tomato rhizospheres. *phzC*⁺ pseudomonads were detected in the rhizosphere of flax and tomato grown in the wilt-suppressive

soil at average densities of 1.1×10^3 CFU g⁻¹ and 1.3×10^4 CFU g⁻¹ root, respectively, representing on an average 0.6–13.9% of the total rhizosphere population of culturable pseudomonads. In the rhizosphere of flax and tomato plants grown in the wilt-conductive soil, *phzC*⁺ pseudomonads were not detected (Table 2).

Diversity of phlD⁺ pseudomonads

For a total of 95 *phlD*⁺ isolates from the suppressive and conducive soils, and 13 *phlD*⁺ reference strains, *phlD*-RFLP analysis was performed to assess the genotypic diversity (Figure 1). The 95 indigenous isolates comprised 24 from Châteaurenard tomato rhizosphere (designated ChPhlTR), 18 from Châteaurenard flax rhizosphere (designated ChPhlLR), 27 from Carquefou tomato rhizosphere (designated CaPhlTR) and 26 from Carquefou flax rhizosphere (designated CaPhlLR). Among the indigenous *phlD*⁺ isolates, seven distinct *phlD*-RFLP genotypes could be distinguished: (i) three *phlD*-RFLP genotypes corresponded to the known genotypes F, M and O (De La Fuente *et al.*, 2006), and (ii) four genotypes, designated α , β , γ and δ , were not described earlier (Figure 1). The 42 *phlD*⁺ isolates obtained from the rhizosphere of tomato and flax grown in the Châteaurenard wilt suppressive soil were classified as the M-genotype (34 isolates) or the F-genotype (8 isolates). Most of the 53 *phlD*⁺ isolates obtained from the rhizosphere of tomato and flax grown in the Carquefou conducive soil were different from the Châteaurenard isolates and belonged to six *phlD*-RFLP genotypes, including F (6 isolates), O (4 isolates), α (1 isolate), β (6 isolates), γ (26 isolates) and δ (10 isolates) (Figure 1). Subsequent sequencing of the 629-bp *phlD* gene fragment of representative isolates of each of the *phlD*-RFLP groups followed by phylogenetic analysis strongly supported the classification based on *phlD*-RFLP analysis and confirmed that the *phlD*⁺ isolates belonging to the new RFLP groups α , β , γ

Table 2 Densities of total, *phlD*⁺ and *phzC*⁺ populations of culturable fluorescent pseudomonads in the rhizosphere of flax and tomato plants grown in the *Fusarium*-wilt suppressive soil (Châteaurenard, France) or in the wilt-conductive soil (Carquefou, France)

	Total population CFU g ⁻¹ root	<i>phlD</i> ⁺ population CFU g ⁻¹ root	<i>phzC</i> ⁺ population CFU g ⁻¹ root
<i>Flax</i>			
Suppressive soil	9.4×10^4 b (s.d. 8.9×10^4)	4.1×10^3 x (s.d. 9.2×10^3)	1.1×10^3 z (s.d. 2.4×10^3)
Conductive soil	2.3×10^5 a (s.d. 2.6×10^5)	4.7×10^5 x (s.d. 5.4×10^5)	ND ^a
<i>Tomato</i>			
Suppressive soil	1.3×10^5 ab (s.d. 1.5×10^5)	2.1×10^4 x (s.d. 4.3×10^4)	1.3×10^4 y (s.d. 1.2×10^4)
Conductive soil	1.8×10^5 ab (s.d. 2.3×10^5)	2.1×10^4 x (s.d. 3.6×10^4)	ND ^a

^aND, below the detection limit of 3.3×10^2 CFU g⁻¹ root.

Population densities are expressed as CFU g⁻¹ root fresh weight. Mean values of five replicates are given and s.d. (in parentheses) refers to the s.d. of the mean. Within a given column, means with the same letter are not significantly different ($P \leq 0.05$) according to Student–Newman–Keuls test.

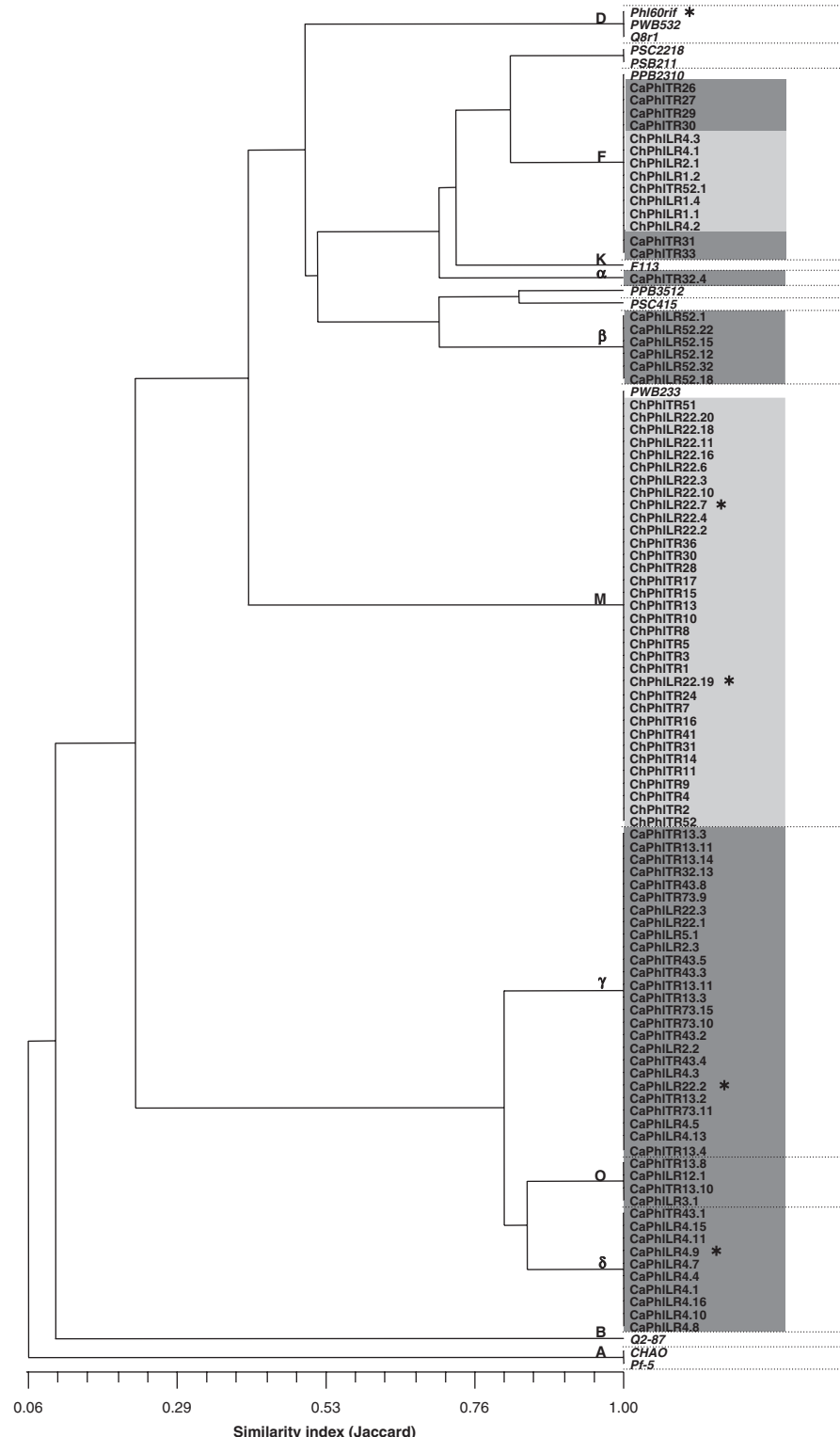


Figure 1 Diversity and phylogeny of *phlD*⁺ fluorescent *Pseudomonas* isolates obtained from the *Fusarium*-wilt suppressive soil of Châteaurenard (indicated in light gray) and the conducive soil of Carquefou (indicated in dark gray). The pair wise coefficients of similarity were clustered with the unweighted pair group method using arithmetic averages algorithm of NTSYS-pc 2.02. *PhlD*⁺ reference strains are presented in italics. The *phlD* genotype designations A, B, D, F, K, M and O are based on work of De La Fuente *et al.* (2006), whereas the novel *phlD* genotypes α, β, γ and δ were identified in this study. Isolates tested in this study for their efficacy to suppress *Fusarium* wilt of flax are indicated with an asterisk.

and δ were indeed different from the other isolates and from the *phlD*⁺ reference strains described so far (Supplementary Figure S1). RP-HPLC analysis confirmed that each of the isolates, for which the *phlD* gene was sequenced, produced DAPG *in vitro* (data not shown). These results indicate that, in spite of similar rhizosphere population densities in the suppressive and conducive soils (Table 2), most of the *phlD*⁺ isolates from the wilt-suppressive soil were genotypically different from those isolated from the conducive soil (Figure 1).

Diversity of *phzC*⁺ *Pseudomonas*

Twenty-nine *phzC*⁺ isolates were selected from the Châteaurenard suppressive bulk soil and from the rhizosphere of flax and tomato seedlings grown in this soil, ten from bulk soil (ChPhzS), three from flax rhizosphere (ChPhzLR) and 16 from tomato rhizosphere (ChPhzTR). Diversity was assessed by *phzC*-RFLP analysis, and by BOX-PCR and sequence analyses. Among the indigenous *phzC*⁺ isolates, five distinct *phzC*-RFLP genotypes (I, II, III, IV

and VII) could be distinguished, two of which clustered with the phenazine-producing reference strains *P. chlororaphis* PCL1391 (genotype II) and *P. fluorescens* 2-79 (genotype VII) (Figure 2). BOX-PCR analysis revealed 11 distinct genotypes, designated A1, A2, B1, B2, C1, F1–F3, F5, G1 and H1, providing an additional level of discrimination between the isolates within each *phzC*-RFLP group (Figure 2). For example, the three *phzC*⁺ isolates belonging to RFLP group VII could be discriminated in three BOX-PCR genotypes (G1, H1 and I1). Sequence and phylogenetic analyses of the *phzC* genes from 10 indigenous isolates, five reference strains, and 12 *phzC* sequences available in genomic databases showed that seven indigenous isolates clustered with the reference strains PGS12 and 30–84 (Supplementary Figure S2). Consistent with the classification based on *phzC*-RFLP and BOX-PCR analyses, isolates ChPhzTR44 and ChPhzS26 were most similar to reference strain PCL1391, and isolate ChPhzLR107 to reference strain 2-79 (Supplementary Figure S2). RP-HPLC analysis confirmed that each of the

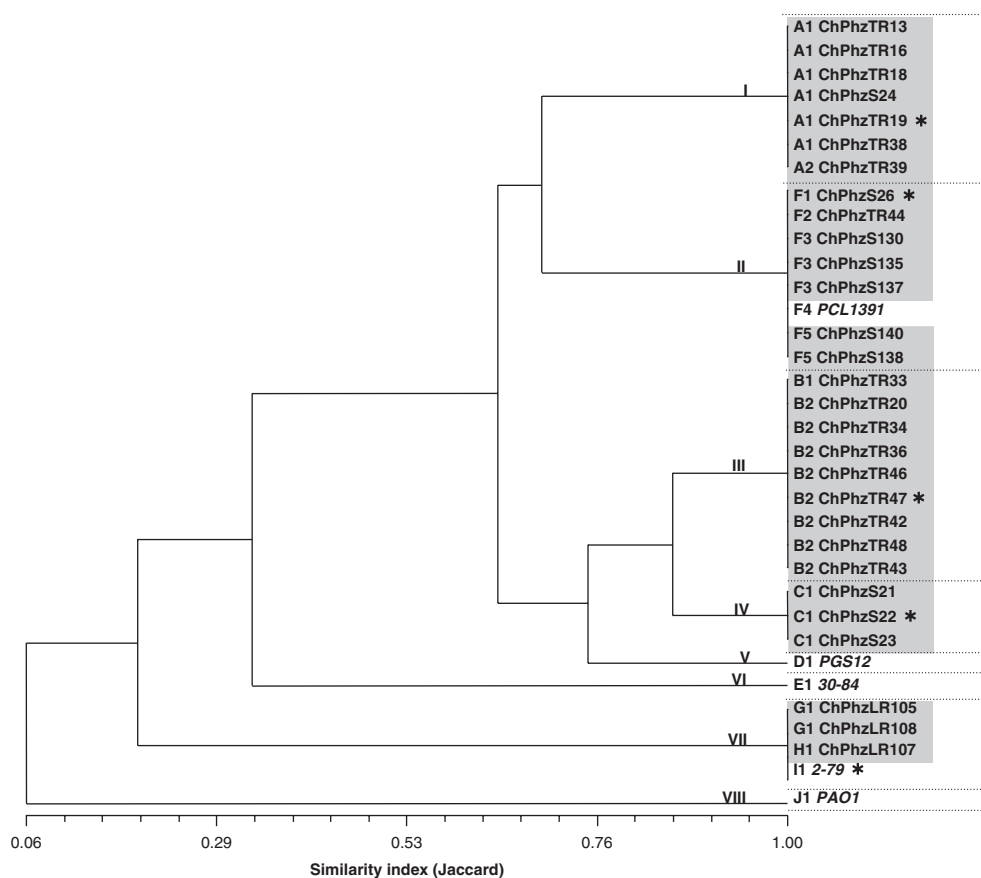


Figure 2 Diversity and phylogeny of *phzC*⁺ fluorescent *Pseudomonas* isolates obtained from the *Fusarium*-wilt suppressive soil of Châteaurenard (highlighted in light gray). The pair wise coefficients of similarity were clustered with the unweighted pair group method using arithmetic averages algorithm of NTSYS-pc 2.02. *PhzC* reference strains are indicated in italics. For the *phzC*⁺ *Pseudomonas* isolates with RFLP-genotypes I to VIII, the corresponding BOX-PCR genotypes (A1 to J1) are indicated in bold in front of the strains name. Isolates tested in this study for their efficacy to suppress *Fusarium* wilt of flax are indicated with an asterisk.

isolates for which the *phzC* gene was sequenced, produced phenazine antibiotics *in vitro* (data not shown).

Suppression of *Fusarium* wilt of flax by *phzC*⁺ and *phlD*⁺ *Pseudomonas*

Earlier studies on the Châteaurenard *Fusarium*-wilt suppressive soil showed that disease suppressiveness was ascribed to the joint activity of non-pathogenic *F. oxysporum* and fluorescent *Pseudomonas* (Lemanceau *et al.*, 2006). Therefore, the efficacy of *phzC*⁺ and *phlD*⁺ isolates to control *Fusarium* wilt of flax was tested when inoculated separately (Figures 3a and c) and in combination with the non-pathogenic *F. oxysporum* strain Fo47

(Figures 3b and d). The bacterial isolates (indicated with an asterisk in Figures 1 and 2) were chosen as representatives of the diversity of the indigenous populations: (i) an isolate of each of the five *phzC*-RFLP genotypes from the Châteaurenard suppressive soil (genotypes I–IV) plus strain 2-79 (genotype VII), and (ii) two *phlD*⁺ isolates of the *phlD*-RFLP dominant genotype M from the Châteaurenard suppressive soil, one isolate of each of the dominant *phlD*-RFLP genotypes γ and δ from the conducive soil, and reference strain Phl60rif (genotype D).

The results show that none of the five *phzC*⁺ strains was effective in disease control when applied alone (Figure 3a). However, when applied in combination with non-pathogenic *F. oxysporum* strain Fo47, they all improved the suppression

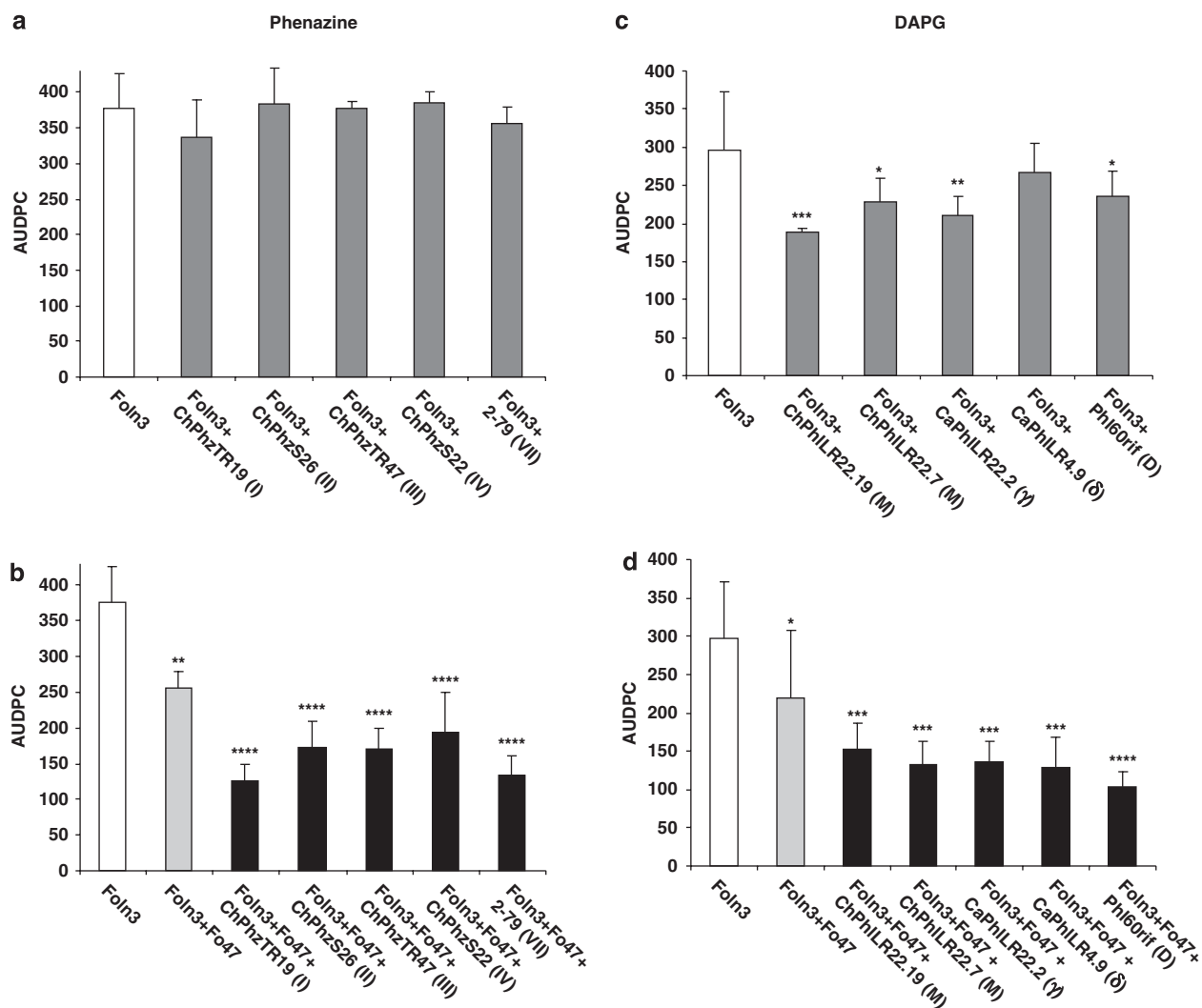


Figure 3 Protection of flax against *Fusarium* wilt by *phzC*⁺ *Pseudomonas* isolates obtained from the wilt-suppressive soil (a and b) and by *phlD*⁺ *Pseudomonas* isolates obtained from the wilt-suppressive or conducive soil (c and d). For each of the *Pseudomonas* isolates tested, the corresponding genotypes as defined in Figures 1 and 2 are indicated between brackets. Plants were grown in soil infested with pathogenic *F. oxysporum* f.sp. *lini* Foin3 (10^3 conidia g^{-1} soil fresh weight), and inoculated with cell suspensions of the *Pseudomonas* isolates (10^7 CFU g^{-1} soil fresh weight) and non-pathogenic *F. oxysporum* Fo47 (10^5 conidia g^{-1} soil fresh weight) separately or in combination. The area under disease progress curve represents the cumulative disease severity monitored over a period of 6 weeks of plant growth. Error bars represent the s.d. of the mean. Fisher's PLSD-test was used for pair wise comparison of the treatment with the infested control (Foin3): * $P \leq 0.1$; ** $P \leq 0.05$; *** $P \leq 0.01$; **** $P \leq 0.001$.

achieved by Fo47 (Figure 3b). Regardless of their origins, four of the five *phlD*⁺ strains significantly reduced *Fusarium* wilt of flax when applied alone (Figure 3c) and, when co-inoculated with Fo47, all enhanced the level of disease control (Figure 3d). To further resolve the role of DAPG and phenazine in disease control and in the synergy with non-pathogenic *F. oxysporum* strain Fo47, biosynthesis mutants deficient in DAPG (mutant 17-8D) or phenazine (mutant 2-79Z) production were tested and their biocontrol efficacy compared with their respective wild-type strains. The level of protection achieved by the two wild-type strains did not differ from that of their respective antibiotic-deficient mutants (Figures 4a and c). In the presence of Fo47, however, differential effects were observed between the DAPG and phenazine producers.

DAPG-producing strain Phl60rif and its DAPG-deficient mutant 17-8D both enhanced as efficiently the disease suppression by Fo47 (Figure 4d). In contrast, phenazine-producing strain 2-79 was more effective in combination with Fo47 than its phenazine-deficient mutant 2-79Z (Figure 4b). Collectively, these results suggest that phenazines, but not DAPG, contribute to the enhanced disease suppression achieved in combination with non-pathogenic *F. oxysporum*.

Discussion

To unravel the role of antibiosis in the natural soil suppressiveness to *Fusarium* wilt, this study used strategies that were earlier applied for take-all

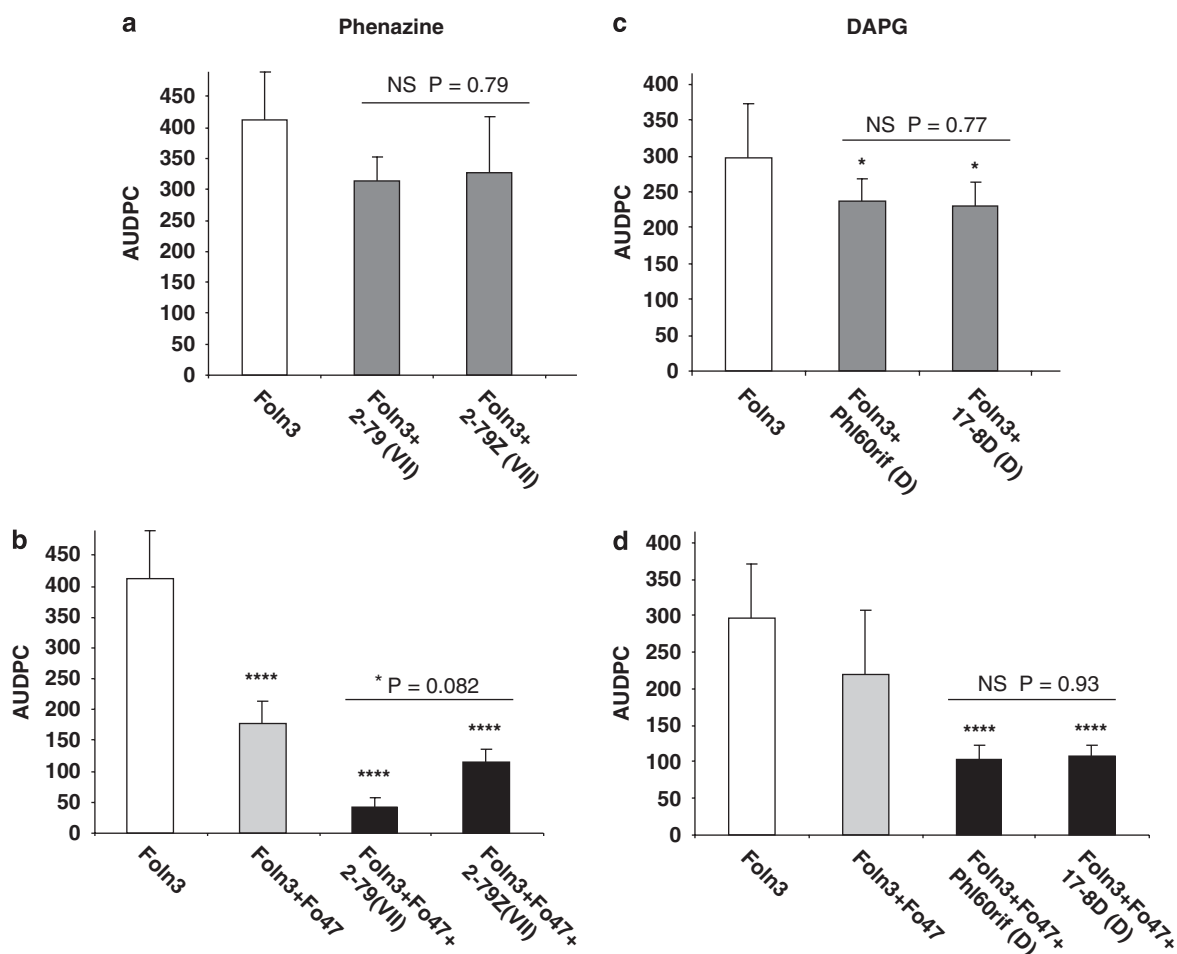


Figure 4 Protection of flax against *Fusarium* wilt by phenazine-producing strain *P. fluorescens* 2-79 and by 2,4-diacetylphloroglucinol (DAPG)-producing strain *P. fluorescens* Phl60rif. Each of the strains was tested alone or in combination with non-pathogenic *F. oxysporum* Fo47. For strain 2-79, the biocontrol efficacy was compared with that of its phenazine-deficient mutant 2-79Z (a and b); for strain Phl60rif, a comparison was made with its DAPG-deficient mutant 17-8D (c and d). Plants were grown in soil infested with pathogenic *F. oxysporum* f.sp. *lini* Folin3 (10^3 conidia g^{-1} soil fresh weight), and inoculated with cell suspensions of the *Pseudomonas* isolates (10^7 CFU g^{-1} soil fresh weight) and non-pathogenic *F. oxysporum* Fo47 (10^5 conidia g^{-1} soil fresh weight) separately or in combination. The area under disease progress curve represents the cumulative disease severity monitored over a period of 6 weeks of plant growth. Error bars represent the s.d. of the mean. Fisher's PLSD-test was used for pair wise comparison of the treatment with the infested control (Folin3): * $P < 0.1$; **** $P < 0.001$. The results of the statistical pair wise comparison between the effects of the wild-type strains and their antibiotic-deficient mutant are indicated with probability values.

decline soils (Raaijmakers *et al.*, 1997; Raaijmakers and Weller, 1998; McSpadden-Gardener *et al.*, 2000; Weller *et al.*, 2002; Souza *et al.*, 2003a) and for soils suppressive to black root rot of tobacco (Wang *et al.*, 2001; Ramette *et al.*, 2003, 2006; Frapolli *et al.*, 2007). This strategy encompasses a comparative analysis of the densities, diversity and activity of antibiotic-producing pseudomonads in two soils chosen for their opposite level of suppressiveness to *Fusarium* wilt: one being suppressive (Châteaurenard) and the other being conducive (Carquefou). These two soils were the same as those used earlier to show the role of carbon and iron competition in soil suppressiveness to *Fusarium* wilts (Lemanceau *et al.*, 1988). At this stage, the two structurally different antibiotics DAPG and phenazines were considered because of their established role in the activity of various *Pseudomonas* strains against pathogenic *F. oxysporum* (Georgakopoulos *et al.*, 1994; Anjaiah *et al.*, 1998; Chin-A-Woeng *et al.*, 1998; Schouten *et al.*, 2004) and, for DAPG-producing pseudomonads, because of their prevalence in a soil naturally suppressive to *Fusarium* wilt of peas (Landa *et al.*, 2002).

Molecular-based detection revealed that DAPG- and phenazine-producing pseudomonads were present in the rhizosphere of flax and tomato plants grown in the wilt-suppressive soil. DAPG-producing pseudomonads were found at similar densities in the conducive soil, whereas phenazine-producing pseudomonads were only detected in the suppressive soil. To our knowledge, this is the first report establishing differences in population densities of phenazine-producing pseudomonads between disease suppressive and conducive soils. Furthermore, they also show the coexistence of DAPG- and phenazine-producing *Pseudomonas* in a naturally suppressive soil. Although phenazine-producing strain *P. fluorescens* 2-79 was originally isolated from a take-all suppressive soil and was shown to efficiently suppress take-all disease through the production of phenazine-1-carboxylic acid (Thomashow and Weller, 1988), the role of phenazine-producing *Pseudomonas* populations in the natural soil suppressiveness to take-all and other soil-borne diseases could not be confirmed (Raaijmakers *et al.*, 1997; Mavrodi *et al.*, 2006). The higher density of phenazine-producing *Pseudomonas* in the suppressive soil of Châteaurenard compared with the conducive soil of Carquefou may be related to differences in the physico-chemical properties of these two soils. Indeed, owing to the high pH (7.9) and CaCO₃ content of the Châteaurenard soil, the concentration of extractable iron was shown to be 15 times lower than that in the Carquefou soil (Lemanceau *et al.*, 1988). As phenazines are redox-active antibiotics (Dietrich *et al.*, 2008) and may contribute to iron mobilization in soils (Hernandez *et al.*, 2004; Price-Whelan *et al.*, 2006; Wang and Newman, 2008), the ability of bacteria to produce these metabolites may give them

a competitive advantage under the iron-limiting conditions prevailing in the Châteaurenard suppressive soil.

At the genotypic level, the *phzC*⁺ isolates were distributed in five groups, four of them being closely related to reference strains known to produce different phenazine derivatives, including phenazine-1-carboxylic acid, 2-OH-phenazine-1-carboxylic acid and phenazine-1-carboxamide (reviewed by Mavrodi *et al.*, 2006). The *phlD* genotypes found in the two soils differed substantially, with genotype M (81% of the isolates) being dominant in the suppressive soil. Interestingly, the two genotypes M and F identified in the suppressive soil of Châteaurenard were described earlier as being dominant in European take-all decline soils (Souza *et al.*, 2003a). Phylogenetic analysis further revealed that the four *phlD* genotypes found in the conducive soil, designated α , β , γ and δ , were not described so far (De La Fuente *et al.*, 2006). Collectively, these results correspond well with the results obtained by Ramette *et al.* (2006) for the soils suppressive or conducive to black root rot of tobacco; in their work, DAPG-producing *Pseudomonas* spp. were also detected at similar densities in the conducive and suppressive soils and were genotypically diverse. However, in their study the different genotypes also differed in their biocontrol efficacy, whereas in this study the *phlD*⁺ isolates from the wilt-suppressive soil were equally effective as the isolates from the conducive soil. None of the genotypically different *phzC*⁺ isolates from the *Fusarium*-wilt suppressive soil were able to control *Fusarium* wilt of flax when inoculated separately; this includes isolate ChPhzS26 which belongs to the same *phzC*-RFLP genotype (II) as *P. chlororaphis* PCL1391, a strain that effectively suppressed crown and root rot of tomato caused by *F. oxysporum* f. sp. *radicis lycopersici* (Chin-A-Woeng *et al.*, 1998).

In contrast with suppressiveness to take-all, which has been ascribed mainly to fluorescent pseudomonads (Weller *et al.*, 2002), soil suppressiveness to *Fusarium* wilt has been related to both non-pathogenic *F. oxysporum* (Rouxel *et al.*, 1979) and fluorescent pseudomonads (Scher and Baker, 1980). Densities of indigenous populations of non-pathogenic *F. oxysporum* were shown earlier to be much higher in *Fusarium* wilt suppressive than in conducive soils (Alabouvette, 1986). Additional suppression by the combination of non-pathogenic *F. oxysporum* strain Fo47 and specific isolates of fluorescent pseudomonads was reported earlier (Lemanceau and Alabouvette, 1991; Lemanceau *et al.*, 1992, Duijff *et al.*, 1999) and proposed to account for the efficacy and stability of the natural suppressiveness (Lemanceau *et al.*, 2006). Data shown here confirm that most of the isolates tested improved the protection achieved by Fo47. On the basis of the experiments with mutants affected in antibiotic biosynthesis, DAPG bio-

synthesis does not seem to be involved in this increased protection. In contrast, this additional level of disease suppression was shown to be related to phenazine biosynthesis. Interestingly, the additional suppression provided by the bacterial and fungal combination was ascribed earlier to an interplay between carbon and iron competition achieved by these two groups of antagonistic microorganisms (Lemanceau *et al.*, 1988, 1993): pyoverdine-mediated iron competition achieved by the fluorescent pseudomonads was shown to reduce the efficacy of carbon metabolism of the pathogenic *F. oxysporum* making it more susceptible to carbon competition with non-pathogenic *F. oxysporum* (Lemanceau *et al.*, 1993). Considering that phenazines are redox-active antibiotics (Dietrich *et al.*, 2008) and may contribute to iron mobilization in soils (Hernandez *et al.*, 2004; Price-Whelan *et al.*, 2006), they also could play a role in iron competition, thereby making pathogenic *F. oxysporum* more susceptible to carbon competition with non-pathogenic *F. oxysporum*. Alternatively, phenazines also could act against pathogenic *F. oxysporum* as redox-active antibiotics leading to the accumulation of toxic oxygen radicals (Mavrodi *et al.*, 2006). Whether one or both modes of action of phenazines

operate in *Fusarium* wilt suppressiveness remains to be explored.

One may consider that the colonization level of the indigenous *phzC*⁺ pseudomonads in the suppressive soil was lower than that of introduced phenazine-producing strains in the biocontrol experiments, and therefore may not be high enough for effective disease suppression. This complex issue highly depends on the spatial distribution of the bacteria and the level of expression of the antagonistic traits involved. Various studies performed so far show that the relationships between bacterial densities on plant roots and the level of disease control are nonlinear and have an asymptotic nature (Bull *et al.*, 1991; Johnson, 1994; Raaijmakers *et al.*, 1995; Raaijmakers and Weller, 1998). These studies further show that when indigenous or introduced *Pseudomonas* populations reach a density in the rhizosphere of 10³–10⁵ CFU g⁻¹ of root fresh weight, several fungal diseases, including *Fusarium* wilt, are controlled to some degree. At densities above 10⁵ CFU g⁻¹ of root, there is no further improvement in the level of disease control (Raaijmakers *et al.*, 1995; Raaijmakers and Weller, 1998), which is probably related to the spatial heterogeneity of the

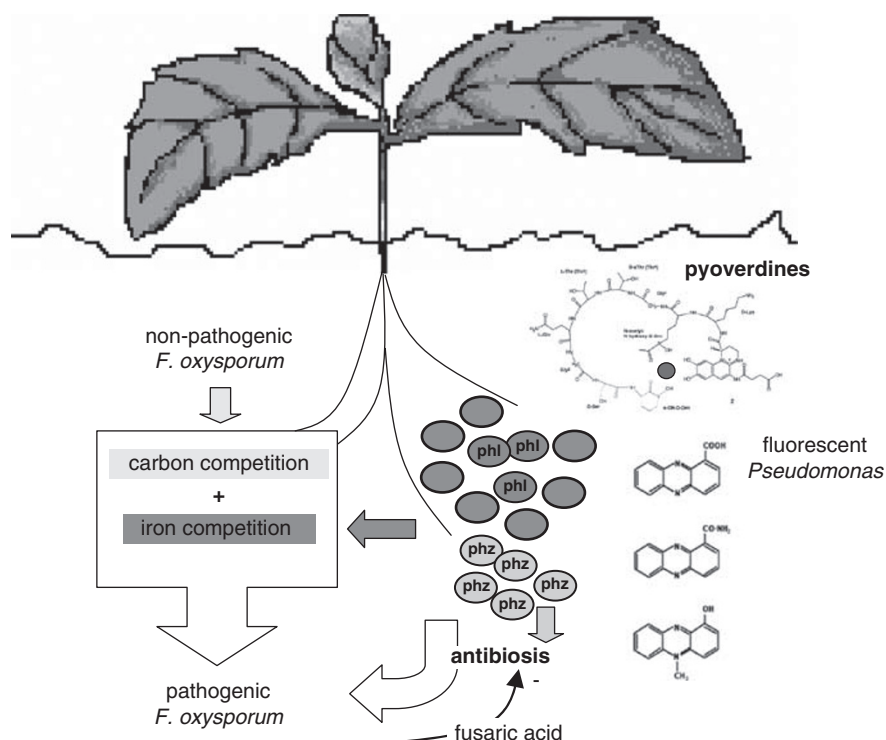


Figure 5 Schematic model presenting the proposed mechanisms that contribute to the natural soil suppressiveness to *Fusarium* wilt. The significant higher microbial biomass in the suppressive soil as compared with the conducive soil contributes to a higher level of carbon competition in the suppressive soil. On this background of general competition, the higher density of non-pathogenic *F. oxysporum* in the suppressive soil further increases the carbon competition. The suppressive soil also differs from the conducive soil by its lower concentration of extractable iron, due to its high pH and CaCO₃ content, making pyoverdine-mediated iron competition between the pathogen and the fluorescent pseudomonads stronger in the suppressive than in the conducive soil. Carbon and iron competition act in synergy to suppress the saprophytic growth of pathogenic *F. oxysporum*, leading to a reduced activity and rate of root infection. The results of this study suggest that also redox-active phenazine antibiotics may play a role in *Fusarium* wilt suppressiveness and may act in synergy with the carbon competition achieved by non-pathogenic *F. oxysporum*. In return, fusaric acid produced by *F. oxysporum* may affect the activity of the fluorescent pseudomonads by interference with the expression of antibiotic biosynthesis genes.

indigenous/introduced bacteria and the level of colocalization of the bacteria and the pathogen. These quantitative dose–response relationships were shown for several *Pseudomonas* strains and mechanisms, including phenazine-mediated antibiosis by strain 2-79 (Bull *et al.*, 1991), DAPG-mediated antibiosis (Raaijmakers and Weller, 1998), siderophore-mediated competition for iron and induced systemic resistance (Raaijmakers *et al.*, 1995). Therefore, the population densities of the indigenous *phzC*⁺ pseudomonads reported in our study do reach population densities within the range, where at least some level of disease control can be expected. This is certainly the case for *phzC*⁺ *Pseudomonas* populations in the tomato rhizosphere but to a lesser extent for flax. However, when acting in synergy with other antagonistic microorganisms and mechanisms, these relatively low population densities may contribute to disease control (Olivain *et al.*, 2004).

The observation that DAPG production did not play a significant role in the enhanced disease suppression achieved by strain Phl60rif in combination with Fo47 may have been due to various factors. One important factor in this respect is the choice of the *phlD* genotype tested, which may substantially affect the level of disease control (Raaijmakers and Weller, 2001; Ramette *et al.*, 2006). Therefore, more *phlD* genotypes and their respective DAPG-deficient mutants need to be tested to more conclusively assess the contribution of the antibiotic DAPG in the natural soil suppressiveness to *Fusarium* wilt disease. Furthermore, DAPG biosynthesis may also have been repressed by specific environmental conditions (Duffy and Défago, 1999) or by fusaric acid (Figure 5; Notz *et al.*, 2002; Duffy *et al.*, 2004), a phytotoxin produced by pathogenic *F. oxysporum* and non-pathogenic *F. oxysporum* strain Fo47 (Schouten *et al.*, 2004). However, given that also phenazine production can be repressed by fusaric acid (Van Rij *et al.*, 2004, 2005), it seems unlikely that this phytotoxin played a major role in the multitrophic interactions occurring in the biocontrol assays.

In conclusion, the data presented in this study provide, for the first time, evidence that phenazine-producing pseudomonads are enriched in the *Fusarium*-wilt suppressive soil of Châteaurenard, and when combined with non-pathogenic *F. oxysporum*, significantly enhance disease suppressiveness either directly through antibiosis and/or indirectly through iron competition (Figure 5). The suppressive soil of Châteaurenard shows a high pH and CaCO₃ content, and hosts an abundant microflora. These microbial characteristics lead to strong competition for carbon, whereas the physico-chemical characteristics of the Châteaurenard soil contribute to a strong competition for the poorly available iron. Non-pathogenic *F. oxysporum*, which are much more abundant in the suppressive

than in the conducive soil, successfully compete for carbon sources with pathogenic *F. oxysporum* with which they share similar trophic requirements. At the same time, fluorescent pseudomonads decrease the iron availability for pathogenic *F. oxysporum*, which are more susceptible to iron starvation than non-pathogenic *F. oxysporum* (Figure 5). We postulate that redox-active phenazine antibiotics play a role in the control of pathogenic *F. oxysporum* by indigenous fluorescent pseudomonads, and when acting in synergy with carbon and iron competition contribute to the longstanding nature of the soil suppressiveness to *Fusarium* wilt disease.

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