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Biocontrol of soil-borne fungal plant diseases by 2,4-diacetylphloroglucinol-producing fluorescent pseudomonads with different restriction profiles of amplified 16S rDNA

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Abstract

Fluorescent pseudomonads producing the antimicrobial compound 2,4-diacetylphloroglucinol (Phl) are being studied extensively for use as biocontrol agents of soil-borne fungal diseases. Some of them can produce pyoluteorin (Plt) in addition to Phl, whereas others synthesise only Phl. Here, a collection of seven Phl^+ Plt^- pseudomonads, seven Phl^+ Plt^+ pseudomonads and seven Phl^- biocontrol pseudomonads were compared for protection of plant roots against fungal pathogens. The seven Phl^+ Plt^+ pseudomonads were identical by restriction analysis of amplified spacer ribosomal DNA (spacer ARDRA), whereas the Phl^+ Plt^- pseudomonads and especially the Phl^- biocontrol pseudomonads were quite diverse by spacer ARDRA. Collectively, the Phl^+ Plt^- pseudomonads proved superior to the Phl^+ Plt^+ pseudomonads and the Phl^- biocontrol pseudomonads for protection of tomato against Fusarium crown and root rot (in rockwool microcosms) or cucumber against Pythium damping-off (in non-sterile soil microcosms). There was no correlation between protection *in vivo* and inhibition of the corresponding fungal pathogen on plates. However, there was a significant correlation between the amount of Phl produced on plates and protection of tomato against Fusarium crown and root rot, but not with protection of cucumber against Pythium damping-off. Interestingly, the minority of strains unable to produce HCN, an extracellular protease, or both, were among those unable to protect plants in both pathosystems. A seedling assay was developed to compare pseudomonads for suppression of Fusarium crown and root rot *in vitro*, and a significant correlation was found between disease severity *in vitro* and *in vivo*. Overall, results suggest that promising biocontrol pseudomonads may be identified based on the ability to produce Phl and/or specific ARDRA-based fingerprints.

Introduction

Rhizosphere bacteria belonging to the fluorescent *Pseudomonas* spp. are receiving increasing attention for the protection of plants against soil-borne fungal pathogens (Keel and Défago, 1997; Lemanceau and Alabouvette, 1991; Weller and Cook, 1983). Several mechanisms have been proposed to account for their disease-suppressive ability including competition, antagonism, promotion of plant growth and induced resistance (Fenton et al., 1992; Keel and Défago, 1997; Leeman et al., 1995; Lemanceau et al., 1992; Weller, 1988).

Many well-studied biocontrol pseudomonads produce 2,4-diacetylphloroglucinol (Phl; Fenton et al., 1992; Keel et al., 1996), a secondary metabolite toxic to bacteria (Keel et al., 1992), fungi (Fenton et al., 1992; Keel et al., 1992), nematodes (Cronin et al., 1997b), anthelminthic worms (Bowden et al., 1965) and plants (Keel et al., 1992; Maurhofer et al., 1995) under *in vitro* conditions. Phl was found in the rhizosphere of plants inoculated with the Phl^+ strain *P.*

fluorescens CHA0 (Keel et al., 1992), as well as in the rhizosphere of wheat grown in suppressive soil (Bonsall et al., 1997). The use of mutant strains specifically impaired in Phl production has demonstrated the implication of the metabolite in the protection of plants against phytopathogenic fungi (Fenton et al., 1992; Keel et al., 1992) and bacteria (Cronin et al., 1997a) by Phl-producing pseudomonads. Indeed, introduction of genes encoding Phl production into Phl^- pseudomonads conferred the ability to protect plants (Fenton et al., 1992), and construction of pseudomonads overproducing Phl enhanced plant protection (Maurhofer et al., 1995; Sarniguet et al., 1995; Schnider et al., 1995). In agreement with these results, Phl^+ pseudomonads are widespread in suppressive soils (Keel et al., 1996; Raaijmakers et al., 1997) but not in conducive soils (Raaijmakers et al., 1997). Despite these findings, the ability to synthesise Phl confers little (Cronin et al., 1997a) or no selective advantage (Carroll et al., 1995) to *P. fluorescens* for colonisation of the rhizosphere.

The Phl biosynthetic locus is well-conserved among the fluorescent pseudomonads (Keel et al., 1996). However, their ability to produce Phl varies tremendously from strain to strain under *in vitro* conditions (Keel et al., 1996), suggesting that Phl^+ pseudomonads may vary in their capacity to protect plants from fungal pathogens. Keel et al. (1996) found that the majority of Phl^+ pseudomonads fell into one of two groups defined by Amplified 16S Ribosomal DNA Restriction Analysis (ARDRA). Members of the first group (e.g. CHA0, Pf-5 and PF) can produce another antifungal polyketide (i.e. pyoluteorin; Plt) besides Phl, whereas those corresponding to the second ARDRA group (e.g. Q2-87) do not produce Plt. A third, smaller ARDRA group was proposed for two Phl^+ Plt^- strains including *P. fluorescens* F113.

In this study, a collection of seven Phl^+ Plt^- pseudomonads, seven Phl^+ Plt^+ pseudomonads and seven Phl^- biocontrol pseudomonads were used to investigate the relationship between genotypic properties of pseudomonads, their biocontrol traits *in vitro* and their ability to suppress Fusarium crown and root rot of tomato or Pythium damping-off *in vivo*. In addition, an *in vitro* seedling assay was developed and tested for the screening of pseudomonads for biocontrol of Fusarium crown and root rot of tomato.

Materials and methods

Biocontrol pseudomonads

Experiments were carried out with 21 biocontrol pseudomonads from various geographic origins and one Phl^- strain of *Pseudomonas* sp. (i.e. P3) without any biocontrol activity. All strains are listed in Table 1. Among the biocontrol pseudomonads, seven strains can produce Phl and Plt and they are indistinguishable by ARDRA (Keel et al., 1996). They are hereafter referred to as ARDRA1 strains. Seven other biocontrol pseudomonads can produce Phl but not Plt, and they belong to ARDRA group 2 as defined by Keel et al. (1996). The last seven biocontrol pseudomonads synthesise neither Phl nor Plt, regardless of growth medium used, as indicated by HPLC analyses (carried out as described by Keel et al., 1992 and 1996). Five of the seven Phl^- biocontrol pseudomonads were isolated on the *Pseudomonas*-selective media King's B agar (KBA; King et al., 1954; for strain CSL26) or Gould's S1 medium (Gould et al., 1985; for strains CA2, TM3, CP7 and KD) as part of an extensive screening programme to identify rhizobacteria with disease-suppressive ability. They scored positive on chrome azurol S medium, which is indicative of siderophore production (Schwyn and Neilands, 1987) and were fluorescent on S1 plates under UV light (366 nm), thereby confirming that they were fluorescent pseudomonads. All of them were also fluorescent on succinate minimal medium (Meyer and Abdallah, 1978) supplemented with EDDHA (100 μg ml^{-1}).

Restriction analysis of amplified spacer ribosomal DNA (spacer ARDRA)

A modified ARDRA was performed to compare the 22 pseudomonads, as described by Natsch et al. (1997). The primers used amplify the 16S-23S spacer region, as well as flanking regions in the 16S and 23S rDNA genes, and restriction of amplified DNA was carried out with *Taq*I (Natsch et al., 1997). This spacer ARDRA is more discriminative than the original ARDRA (Vaneechoutte et al., 1992). Banding patterns on agarose gels were analysed using Jaccard's pairwise coefficient of similarity (Jaccard, 1908) and clustering was performed with the unweighted pair group method with average (UPGMA), using Systat 5.05 (SPSS Inc., Evanston, IL, USA).

Table 1. Strains of fluorescent *Pseudomonas* sp. used in the study

Pseudomonads[1]	Geographic origin[2]	Isolation	Biocontrol ability[3]	Reference
Phl$^+$ Plt$^+$ strains				
CHA0	Morens (MS1), CH	Tobacco root[4]	Tobacco (Tb), wheat (Ggt), cucumber (Pu)	Stutz et al., 1986
Pf1	Morens (MS1), CH	Tobacco root	Tobacco (Tb), cucumber (Pu)	Keel et al., 1996
PINR3	Albenga, Italy	Tobacco root	Cucumber (Pu), tomato (FORL)	Keel et al., 1996
Pf-5	Texas	Cotton rhizosphere	Cotton (Pu, Rs), cucumber (Pu)	Howell and Stipanovic, 1979
PF	Oklahoma	Wheat leaf	Wheat (St)	Levy et al., 1992
PGNR2	IITA, Ghana	Tobacco root	Cucumber (Pu), tomato (FORL)	Keel et al., 1996
PGNL1	IITA, Ghana	Tobacco root	Cucumber (Pu), tomato (FORL)	Keel et al., 1996
Phl$^+$ Plt$^-$ strains				
C*1A1	Morens, CH	Cucumber root	Cucumber (Pu, Ps), cotton (Rs)	Fuchs and Défago, 1991
TM1A3	Morens, CH	Tomato root	Cucumber (Pu, Ps), cotton (Rs)	Fuchs and Défago, 1991
TM1B2	Morens, CH	Tomato root	Cucumber (Pu, Ps), cotton (Rs)	Fuchs and Défago, 1991
PITR2	Albenga, Italy	Wheat root	Cucumber (Pu), tomato (FORL)	Keel et al., 1996
PILH1	Albenga, Italy	Tomato root	Cucumber (Pu), tomato (FORL)	Keel et al., 1996
Q2-87	Quincy, Washington	Wheat rhizosphere	Wheat (Ggt)	Vincent et al., 1991
Q6-87	Quincy, Washington	Wheat root	Wheat (Ggt)	Keel et al., 1996
Phl$^-$ strains				
2-79	Lind, Washington	Wheat rhizosphere	Wheat (Ggt), Kentucky bluegrass (Mp)	Weller and Cook, 1983
Q69c-80	Quincy, Washington	Wheat rhizosphere	Wheat (Ggt)	Harrison et al., 1993
CA2	Albenga, Italy	Cucumber root	Cucumber (Pu), tomato (FORL)	This study
TM3	MS12, Morens, CH	Tomato root	Cucumber (Pu)	This study
CSL26	Coimbatore, India	Cucumber root	Tomato (FORL)	This study
CP7	Aroop, Punjab, PK	Cucumber root	Cucumber (Pu)	This study
KD	Hongzuh, China	Wheat root	Cucumber (Pu)	This study
P3	Alp Lü, CH	Barley root	No biocontrol ability documented	Voisard et al., 1989

[1] All Phl$^+$ Plt$^+$ strains belong to ARDRA group 1 and the Phl$^+$ Plt$^-$ strains to ARDRA group 2 as defined by Keel et al. (1996).
[2] Soils located near Morens (naturally suppressive to black root rot of tobacco) were described by Stutz et al. (1986). Quincy soil is suppressive to take-all of wheat, whereas the Albenga soil (Tamietti et al., 1993) and the IITA soil (Ghana) are suppressive to Fusarium wilt of tomato. CH, Switzerland; PK, Pakistan.
[3] FORL, *Fusarium oxysporum* f. sp. *radicis-lycopersici*; Ggt, *Gaeumannomyces graminis* var. *tritici*; Mp, *Magnaporthe poae*; Ps, *Phomopsis sclerotioides*; Pu, *Pythium ultimum*; Rs, *Rhizoctonia solani*; St, *Septoria tritici*; Tb, *Thielaviopsis basicola*.
[4] Root samples consisted of either root macerates or roots previously washed of soil.

Growth conditions for bacteria and fungi

Bacterial inoculants for the experiments were prepared as follows. The pseudomonads were grown in liquid Luria-Bertani (Maniatis et al., 1982) supplemented with $MgSO_4.7H_2O$ (0.25 g l^{-1}) at 27 °C with shaking (140 rpm) and the cultures were used to inoculate KBA. After a 24 h incubation at 27 °C, the cells were harvested and resuspended in sterile distilled water. The cell suspension was adjusted optically to 10^{10} colony-forming units (CFU) per ml, on the basis that a cell suspension with an OD of 0.125 (600 nm) contained approximately 10^8 CFU per ml.

The crown and root rot fungus *F. oxysporum* Schlecht. f. sp. *radicis-lycopersici* (FORL) strain Forl22 (obtained from C. Alabouvette, INRA, Dijon, France) and the damping-off fungus *P. ultimum* Trow strain 67-1 (obtained from Allelix Agriculture, Mississauga, Canada) were routinely cultivated and maintained on 1.5% malt agar (MA; Keel et al., 1989). FORL inoculant for the experiments was prepared by inoculating malt broth (2% malt) with malt plugs of the fungus and incubating the culture for 7 days at 24 °C with shaking (120 rpm). After a 15-min centrifugation at 4,000 rpm the fungi (microconidia and mycelium) were resuspended in sterile distilled water and homogenised for 15 s with a blender. Their concentration was assessed by microscopic counts of microconidia using a Thoma grid (Brand GmbH, Wertheim, Germany). In the seedling biocontrol assay performed *in vitro*, an additional step (filtration through cheese cloth) was implemented to remove the

mycelium and the spore suspension was adjusted to 10^7 microconidia ml^{-1}.

Malt plugs of *P. ultimum* were used to inoculate autoclaved millet seeds (3 plugs per 25 g of seed) previously watered with 10 ml distilled water (Maurhofer et al., 1992). The solid culture was incubated at 20 °C in the dark for seven days, ground aseptically and used directly as inoculum.

Tomato microcosm experiment

The ability of the pseudomonads to protect tomato against Fusarium crown and root rot was studied in rockwool microcosms. Seeds of tomato (*Lycopersicon esculentum* Mill.) cv. Bonny Best (Peptoseed Co., Saticoy, CA, USA) were surface-disinfected in 1% sodium hypochlorite for 30 min, rinsed several times in sterile distilled water (Fuchs et al., 1997) and germinated on 0.85% water agar at 24 °C for three days. The seedlings were transplanted to rockwool cubes (type AO 36/40-10/10, Grodan, Hedehusene, Denmark) placed in individual trays soaking in sterile OTCMG nutrient solution (A. Wigger, OTCMG, Geneva, Switzerland) throughout the experiment. The nutrient solution was prepared by mixing four solutions: solution I [per litre: $Ca(NO_3)_2 \cdot 4\ H_2O$, 147 g], solution II [per litre: K_2SO_4, 29 g; $MgSO_4 \cdot 7H_2O$, 60 g; KH_2PO_4, 13 g; KNO_3, 75 g; $NH_4H_2PO_4$, 11 g], solution III [per litre: $MnSO_4 \cdot H_2O$, 0.31 g; $Na_2B_4O_7 \cdot 10H_2O$, 0.32 g; $CuSO_4 \cdot 5H_2O$, 60 mg; $ZnSO_4 \cdot 7H_2O$, 0.13 g; $Na_2MoO_4 \cdot 2H_2O$, 12 mg; 65% nitric acid, 0.1 ml], and solution IV [per litre: EDDHA (Sigma), 0.4 g (dissolved in 8 ml 1N NaOH); Fe-EDDHA (Sequestrene 138 Fe, Novartis), 2.5 g]. During the first five days after transplanting, the nutrient solution (OTCMG1) was made using 32.5 ml of solution I, 30 ml of solution II, 50 ml of solution III, 50 ml of solution IV, and 4.84 litres of sterile distilled water. From day 6 on, the nutrient solution (OTCMG2) was made using 36 ml of solution I, 37.5 ml of solution II, 50 ml of solution III, 50 ml of solution IV and 4.83 litres of sterile distilled water.

Inoculation of the nutrient solution was performed immediately before soaking the rockwool cubes. Pseudomonads and FORL were added at the rate of approximately 4×10^7 CFU and 10^6 microconidia per ml of nutrient solution, respectively. Each treatment was studied in triplicate, and each replicate consisted of one rockwool cube with 12 plants. The experiment followed a randomised block design and was repeated once. The trays were placed in a growth chamber set at 70% air humidity with 16 h of day (22 °C) and 8 h of dark (18 °C). Disease severity was assessed 21 days after transplanting, using the following scale: 0, healthy seedlings; 1: one or two brown marks on the roots or the crown; 2, several small brown marks or a few large lesions; 3: extensive root necrosis but plant still alive; 4, plant dead. Fresh weight and length of shoots were recorded.

Cucumber microcosm experiment

Protection of cucumber against Pythium damping-off was assessed in non-sterile soil microcosms prepared using a clay loam soil from St Aubin (County Fribourg, Switzerland). Seeds of cucumber (*Cucumis sativus* L.) cv. Chinesische Schlange (R. Geissler AG, Zürich, Switzerland) were surface-disinfected in 1% sodium hypochlorite for 30 min and rinsed several times in sterile distilled water. They were germinated on water agar at 24 °C for two days (Keel et al., 1989). Inoculation of soil was performed with 2.5 mg of fungal inoculum and about 4×10^7 CFU of pseudomonads per g of soil. The soil was kept two days at 18 °C before filling the pots (200 g of soil per 190 cm^3 pot) and transplanting the seedlings. Each treatment was studied with six replicates, and each replicate consisted of one pot with four plants per pot. The experiment was repeated once. The pots were placed in a growth chamber set at 70% air humidity with 16 h of day (22 °C) and 8 h of dark (18 °C). Plant fresh weight was recorded 12 days after transplanting.

Phenotypic characterisation of pseudomonads in vitro

Production of hydrogen cyanide on KBA was studied qualitatively, using the HCN-indicator paper of Castric and Castric (1983). Production of fluorescent siderophore on S1 plates was scored under UV light (366 nm) after 24 h of growth at 27 °C. Extracellular protease is responsible for biocontrol properties of certain bacteria (Dunne et al., 1996) and was assessed using skim milk agar (Gerhardt et al., 1981). Halo formation around colonies was rated after a three-day incubation at 24 °C. Production of fluorescent siderophore and extracellular protease were scored as follows: 0, none; 1, little; 2, strong; 3, very strong (Keel et al., 1996). Manganese can be beneficial to the health of plants challenged with *Fusarium*, and consequently manganese reduction has been investigated as a possible biocontrol trait in rhizobacteria (Elmer,

1995). In the current study, the ability of pseudomonads to reduce manganese was assessed on manganese dioxide medium (Elmer, 1995) at eight weeks after inoculation, using the following scale: 0, none; 1, distance between colony and edge of halo < 3 mm; 2, distance between colony and edge of halo ≥ 3 mm. All determinations were done at least twice.

Fungal inhibition assays in vitro

Inhibition of FORL and *P. ultimum* was studied on KBA, potato-dextrose agar (PDA; Difco) and MA, as described by Schmidli-Sacherer et al. (1997). Several media were chosen, because the ability of biocontrol pseudomonads to inhibit fungal growth depends on characteristics of the growth medium (Maurhofer et al., 1995). Malt plugs of the fungi were added to the centre of the plates immediately (KBA) or 24 h (other media) after inoculation of the pseudomonads (5-μl spots). The plates were incubated for two (*P. ultimum*) or eight to nine days (FORL) at 24 °C. The distance between the edge of bacterial colonies and the fungus was measured. Experiments were performed in triplicate and each replicate consisted of one plate per treatment for each medium.

Seedling biocontrol assay in vitro

A seedling biocontrol assay was developed to investigate the ability of pseudomonads to prevent infection of tomato seedlings by FORL under *in vitro* conditions. The assay was derived from that used by Sanchez et al. (1975) to differentiate between formae speciales of *F. oxysporum* pathogenic to tomato and is analogous to the radicle assay proposed by Kloepper (1991) to screen rhizobacteria for suppression of damping-off of cotton caused by *Rhizoctonia solani*. Seeds of tomato were surface-sterilised (as described above) and inoculated by soaking them for 15–20 min in a bacterial cell suspension containing approximately 10^{10} CFU ml^{-1} (30 seeds in 2 ml). The seeds were then placed in Petri dishes containing 1% water agar previously spread with 200 μl of a spore suspension of FORL (10^7 microconidia ml^{-1}). The plates were incubated eight days in a growth chamber (70% air humidity; 16 h of day at 22 °C and 8 h of dark at 18 °C) before scoring the incidence of crown and root rot using a 3-class scale (0, healthy seedlings; 1, one or two brown marks on the roots or the crown; 2, extensive root necrosis or seedling dead). Each treatment was studied in triplicate and each replicate consisted of one plate with 10 seeds per plate. The experiment was done three times.

Statistical design and analyses

For the *in vivo* microcosm experiments and the seedling assay *in vitro*, data from repeated experiments were pooled before analysis based on homogeneity of variances (Mandeel and Baker, 1991). Disease index for Fusarium crown and root rot was transformed to arc sines. All analyses were done at P=0.05 level, using the software Systat 5.05 (SPSS Inc., Evanston, IL, USA). First, analyses were performed with data obtained in each replication for each strain and the controls. Analyses of variance were performed followed, when appropriate, by Tukey's test to compare treatments (*a posteriori* statistics). In addition, *a priori* statistics were done using pairwise Fisher's LSD tests (Fry, 1993). Correlation analyses were performed using Pearson's correlation coefficient and Bonferroni significance test to compare plant parameters with each others. A second series of analyses were carried out using the means for each strain. Phl$^+$ Plt$^+$strains (ARDRA1), Phl$^+$ Plt$^-$strains (ARDRA2) and the Phl$^-$ biocontrol pseudomonads were compared collectively by analysis of variance, followed by Fisher's LSD test when appropriate. Correlation analyses were carried out using Pearson's correlation coefficient and Bonferroni significance test.

Results

Comparison of the pseudomonads by spacer ARDRA

Spacer ARDRA confirmed the results of Keel et al. (1996) regarding the genotypic similarity of pseudomonads with the ability to produce both Phl and Plt (ARDRA1 group) or only Phl (ARDRA2 group) (Figure 1). The strains in this latter group appeared more diverse than the seven Phl$^+$ Plt$^+$ strains, which were indistinguishable from one another by spacer ARDRA. The Phl$^-$ biocontrol pseudomonads displayed a much wider range of genotypic backgrounds. Interestingly, the Phl$^+$ strains from both ARDRA groups were closer to certain Phl$^-$ pseudomonads than to strains belonging to the other Phl$^+$ ARDRA group.

Protection against Fusarium crown and root rot of tomato and Pythium damping-off of cucumber in vivo

The disease index (0–4 scale) used to rate severity of Fusarium crown and root rot correlated well with fresh

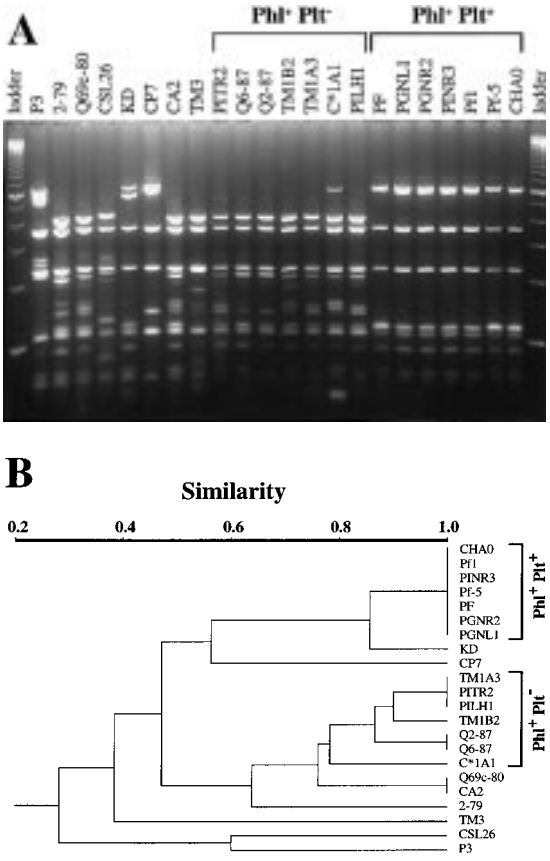

Figure 1. Banding patterns produced by the 22 fluorescent pseudomonads used in the study when studied by spacer Amplified 16S Ribosomal DNA Restriction Analysis (spacer ARDRA) (A). A 100-bp ladder (i.e. from the bottom of the gel 100 bp, 200 bp, etc.; GIBCO-BRL) is also included. For some strains, the sum of the restriction fragments exceeded the length of the amplified region (i.e. 1500 bp). Since there was apparently only one PCR product, this suggests that certain strains had several alleles of the spacer rDNA region. The results of cluster analysis of the fluorescent pseudomonads based on the results of spacer ARDRA are given in (B).

weight (r = −0.89; $P < 0.01$; n = 144) and length (r = −0.91; $P < 0.01$; n = 144) of tomato shoots. When all treatments were analysed together (*a posteriori* statistics), a total of 16 of 22 pseudomonads significantly reduced the extent of Fusarium crown and root rot of tomato in rockwool microcosms (Tukey's test; Figure 2A). As expected, strain P3 afforded no protection. This was also the case with four of the seven Phl$^-$ biocontrol pseudomonads and one of the Phl$^+$ Plt$^-$ strains (i.e. PF). However, *a priori* statistics performed using pairwise Fisher's LSD tests indicated that inoculation with strain PF did reduce the extent of Fusarium crown and root rot when compared with the treatment in which only FORL was present, although this positive effect was not significant when the Tukey test was used in *a posteriori* statistics. Collectively, Phl$^+$ strains belonging to group ARDRA2 proved statistically superior to those from group ARDRA1 or the Phl$^-$ biocontrol pseudomonads for protection of tomato against Fusarium crown and root rot (Table 2).

A total of 12 of 22 pseudomonads were effective against *Pythium*-mediated damping-off of cucumber in soil microcosms (Tukey test; Figure 2B). Strains unable to protect cucumber included six of seven Phl$^-$ biocontrol pseudomonads, three Phl$^+$ Plt$^+$ biocontrol pseudomonads (ARDRA1 group) and the negative control P3. However, all of them but P3 and the Phl$^-$ biocontrol pseudomonad TM3 actually provided some protection, as indicated by the results of *a priori* statistics. As in the tomato-FORL system, Phl$^+$ strains from group ARDRA2 reduced damping-off of cucumber more effectively than those from group ARDRA1 and the Phl$^-$ biocontrol pseudomonads (Table 2). The comparison of disease index for Fusarium crown and root rot of tomato and cucumber fresh weight indicated that there was a significant correlation between the disease-suppressive abilities of the pseudomonads in the two pathosystems (r = -0.77; $P < 0.01$; n = 22 strains).

Inhibition of FORL and P. ultimum *by the pseudomonads on plates*

All Phl$^+$ ARDRA1 strains but one (i.e. Pf1) inhibited growth of FORL on KBA, whereas none of the 15 other pseudomonads had any effect on growth of the fungus (Figure 3A). On MA however, all Phl$^+$ strains (as well as three Phl$^-$ biocontrol pseudomonads) influenced growth of FORL (Figure 3B). On PDA, only four Phl$^+$ Plt$^+$strains inhibited growth of the fungus (Figure 3C). Growth of *P. ultimum* was inhibited by all Phl$^+$ strains, regardless of whether KBA, MA or PDA was used, except that ARDRA1 strain PINR3 had no effect on the fungus on PDA (Figure 3D-F). In the case of Phl$^-$ biocontrol pseudomonads, fungal growth was affected by two strains on KBA, four on MA and five on PDA. There was no correlation between the extent of fungal growth inhibition and suppression of Fusarium crown and root rot of tomato or Pythium damping-off of cucumber, regardless of the medium used in the growth inhibition assay.

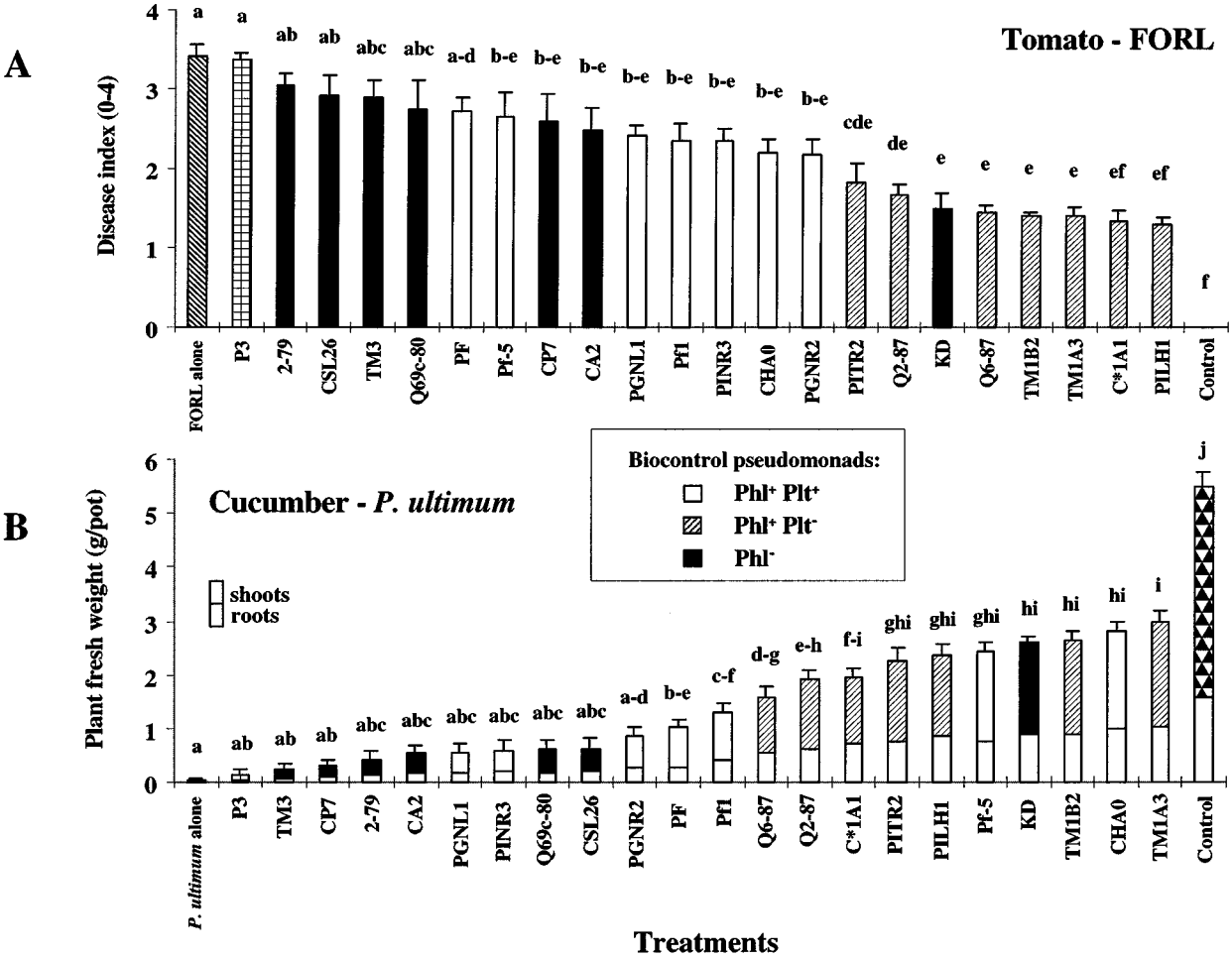

Figure 2. Effect of inoculation with 22 fluorescent pseudomonads on the severity of crown and root rot of tomato (A) and fresh weight of cucumber (B) challenged with FORL and *P. ultimum*, respectively. An uninoculated control and a treatment with only the pathogen were also studied in both systems. Error bars represent standard errors. The statistical relationship between treatments (Tukey test) is indicated with letters.

Phenotypic comparison of the pseudomonads

Correlation analysis (Figure 4) was used to identify whether there was a link statistically between the amounts of Phl or Plt produced *in vitro* and the disease-suppressive abilities of the 21 pseudomonads *in vivo* (Figure 2). The amounts of Phl and Plt recorded by Keel et al. (1996) on KBA and MA for the 14 strains producing Phl or Plt were used. In the tomato-FORL system, the only significant correlations were between disease index and Phl on KBA ($r = -0.68$; $P < 0.01$; $n = 21$) or MA ($r = -0.71$; $P < 0.01$; $n = 21$). When only the 14 Phl^+ pseudomonads were considered and when statistical analyses were carried out using log-transformed data of Phl production on MA, the coefficient of correlation reached -0.84 ($P < 0.01$; $n = 14$). In the cucumber-*P. ultimum* system however, results indicated that there was no correlation between plant fresh weight and levels of Phl or Plt produced (on KBA or MA).

Three of the seven Phl^- biocontrol pseudomonads (i.e. TM3, CSL26 and 2-79), as well as strain P3, failed to produce HCN. Each Phl^+ strain was hydrogen cyanide-positive. All pseudomonads were proteolytic, with the exception of three Phl^- biocontrol strains (i.e. TM3, CSL26 and Q69-80c). Four of the five biocontrol pseudomonads that failed to protect tomato from Fusarium crown and root rot *in vivo* (Figure 2A) were either hydrogen cyanide-negative (i.e. 2–79), lacked an extracellular proteolytic activity (i.e. Q69c-80), or both (i.e. TM3 and CSL26), and they

Table 2. Biocontrol abilities and phenotypic properties of Phl^+ Plt^+, Phl^+ Plt^- and Phl^- biocontrol pseudomonads

	Biocontrol pseudomonads[1]		
	Phl^+ Plt^+ strains	Phl^+ Plt^- strains	Phl^- strains
Tomato-FORL system			
Disease index *in vivo* (0-4)[2]	2.40 (0.21)[3] a[4]	1.47 (0.19) b	2.58 (0.52) a
Disease index seedling assay *in vitro* (0-2)[5]	1.37 (0.21) a	1.01 (0.10) b	1.20 (0.21) ab
Inhibition FORL on KBA (mm)	1.4 (0.9) a	0 b	0 b
Inhibition FORL on MA (mm)	3.8 (4.7) a	9.2 (5.3) b	2.4 (4.2) a
Inhibition FORL on PDA (mm)	0 a	1.3 (1.8) b	0 a
Cucumber-*P. ultimum* system			
Plant fresh weight *in vivo* (g pot^{-1})[6]	1.4 (0.9) a	2.2 (0.5) b	0.9 (0.8) a
Inhibition *P. ultimum* on KBA (mm)	7.8 (1.3) a	5.6 (1.0) b	0.4 (0.7) c
Inhibition *P. ultimum* on MA (mm)	7.5 (4.3) a	10.3 (2.2) a	2.4 (3.0) b
Inhibition *P. ultimum* on PDA (mm)	3.6 (3.6) a	10.8 (2.0) b	3.3 (4.7) a
Phenotypic properties of strains[7]			
Phl on KBA (μg ml^{-1})	1.8 (4.8) a	30 (12) b	0 a
Phl on MA (μg ml^{-1})	3.3 (2.7) a	32 (16) b	0 a
Plt on KBA (μg ml^{-1})	30 (44)	0	0
Plt on MA (μg ml^{-1})	0.6 (1.6)	0	0
Extracellular protease (0-3)	2.0 (0)	1.6 (0.5)	1.1 (1.2)
Mn reduction (0-2)	0.3 (0.5)	0.7 (0.8)	0
Fluorescent siderophore on S1 (0-3)	2.9 (0.4) a	1.3 (0.5) b	2.0 (0.6) c

[1] Each group comprised seven strains.
[2] The disease index was 0 in the absence of the fungus and 3.41 when only the fungus was added.
[3] Standard deviation.
[4] The statistical relationship between treatments (LSD test) is indicated with letters a,b et c where significant differences were found.
[5] The disease index was 0 in the absence of the fungus and 1.99 when only the fungus was added.
[6] Plant fresh weight was 5.5 g pot^{-1} in the absence of the fungus and 0.04 g pot^{-1} when only the fungus was added.
[7] All 21 pseudomonads were HCN-positive, with the exception of three Phl^- strains. Data of Phl and Plt production for the 14 strains producing Phl or Plt were those obtained by Keel et al. (1996).

were among those unable to protect cucumber from Pythium damping-off (Figure 2B).

Pseudomonads differed in the extent of fluorescent siderophore produced on S1 plates, but no relationship was found with the disease-suppressive ability of the strains in the tomato-FORL or cucumber-*P. ultimum* systems. Two Phl^+ Plt^+ strains (i.e. PF and Pf-5) and four of the Phl^+ Plt^- strains (i.e. Q2-87, Q6-87, C*1A1 and PILH1) reduced manganese. No relationship was found between the ability of pseudomonads to reduce manganese *in vitro* and protection of tomato or cucumber *in vivo*.

Seedling assay for biocontrol of Fusarium crown and root rot in vitro

A total of 15 of 22 pseudomonads significantly reduced the extent of Fusarium crown and root rot of tomato in the seedling assay. As expected, strain P3 provided no protection to the seedlings. This was also the case with one Phl^- biocontrol pseudomonad (i.e. 2–79) and five of the seven Phl^+ Plt^+ strains (the two Phl^+ Plt^+ strains that protected were PF and PINR3). Collectively, Phl^+ strains belonging to group ARDRA2 proved superior to those from group ARDRA1 but not (P = 0.056) to the Phl^- biocontrol pseudomonads for protection of seedlings on the plates (Table 2). Significant correlations were observed between Fusarium crown and root rot *in vitro* and *in vivo* when considering data from all treatments studied (r = 0.81; $P < 0.01$; n = 24), the results from all 21 biocontrol pseudomonads (r = 0.55; P = 0.01; n = 21), or those obtained with Phl^+ strains only (r = 0.76; $P < 0.01$; n = 14). Indeed, the 11 pseudomonads resulting in a disease index of 1.2 or less *in vitro* comprised the 8 strains that reduced the index of Fusarium crown and root rot below 2 under *in vivo* conditions (Figure 5). However, no significant relationship was

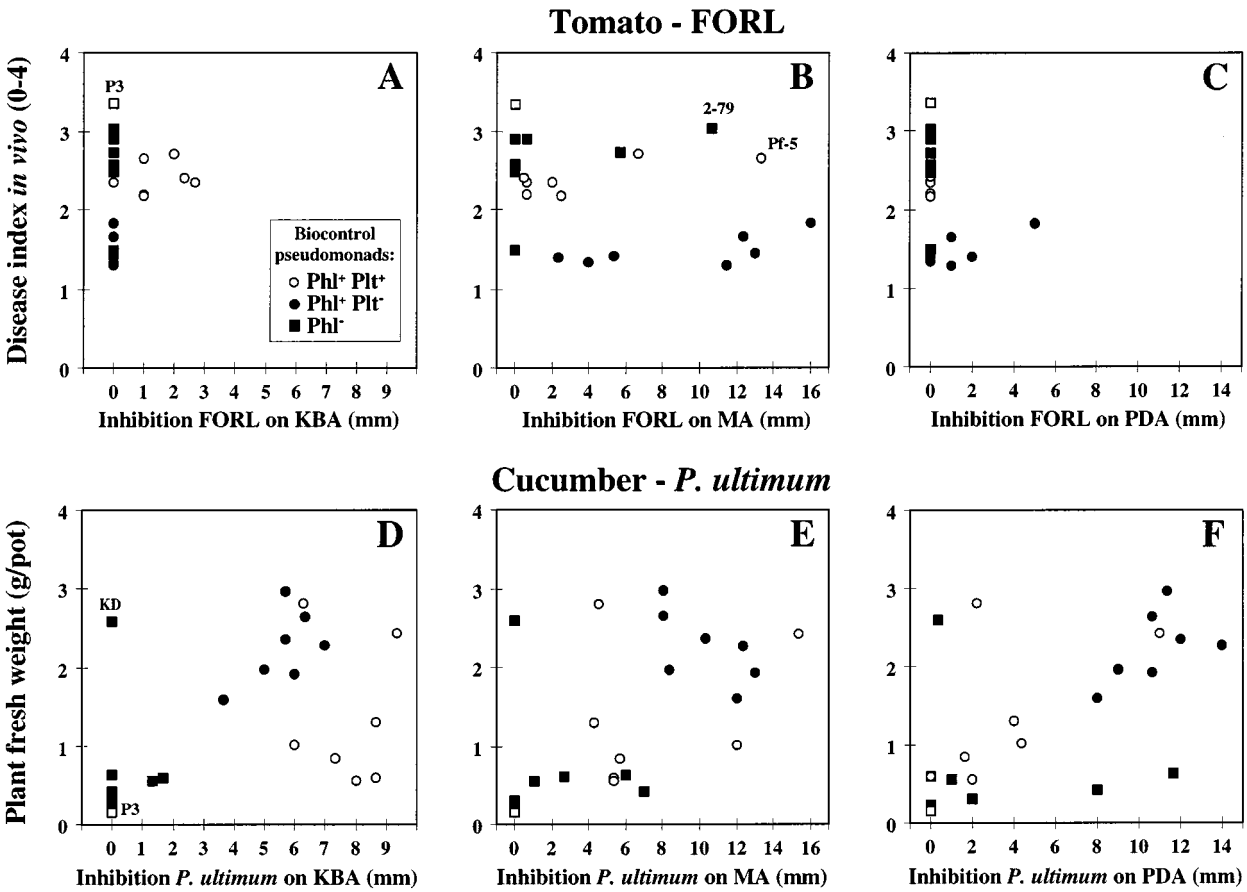

Figure 3. Relationship between inhibition of FORL (A-C) and *P. ultimum* (D-F) by the 22 pseudomonads *in vitro* and their disease-suppressive ability in the tomato-FORL and cucumber-*P. ultimum* systems, respectively. Growth inhibition was studied on KBA (A,D), MA (B,E) or PDA (C,F). There was no statistical correlation between the ability or the extent of fungal growth inhibition and Fusarium crown and root rot index of tomato or fresh weight of cucumber.

found between protection of tomato seedlings *in vitro* and inhibition of FORL or production of Phl on plates.

Discussion

The results of the current study indicate that 10 of 14 Phl^+ pseudomonads reduced disease incidence both in the tomato-FORL and cucumber-*P. ultimum* systems (Figure 2). In contrast, only one Phl^- biocontrol pseudomonad (i.e. KD) could protect the plant in both pathosystems. Four of the seven Phl^- biocontrol pseudomonads (i.e. 2–79, Q69c-80, CSL26 and TM3) were ineffective both against FORL and *P. ultimum in vivo*. Several studies have reported that protection of wheat against take-all by strains 2–79 and Q69c-80 can be inconsistent in soil microcosms (Duffy and Weller, 1995; Pierson and Weller, 1994). The Phl^- strain CSL26 was initially selected on the basis of effective protection of tomato against crown and root rot in an experimental system similar to the one used here, but with a lower disease pressure (data not shown). Overall, these results suggest that Phl production may be a useful biocontrol trait across a range of disease pressures.

All Phl^+ Plt^+ strains (group ARDRA1) protected tomato from Fusarium crown and root rot to a similar extent (Figure 2A). In addition, the Phl^+ Plt^+ pseudomonads were indistinguishable by spacer ARDRA (Figure 1) and highly similar by Random Amplified Polymorphic DNA (RAPD) analysis (Keel et al., 1996). However, a different situation was found in the cucumber-*P. ultimum* system, where strains Pf-5 and CHA0 protected better than the other Phl^+ Plt^+ pseudomonads against damping-off (Figure 2B). In contrast, there was little (cucumber-*P. ultimum* sys-

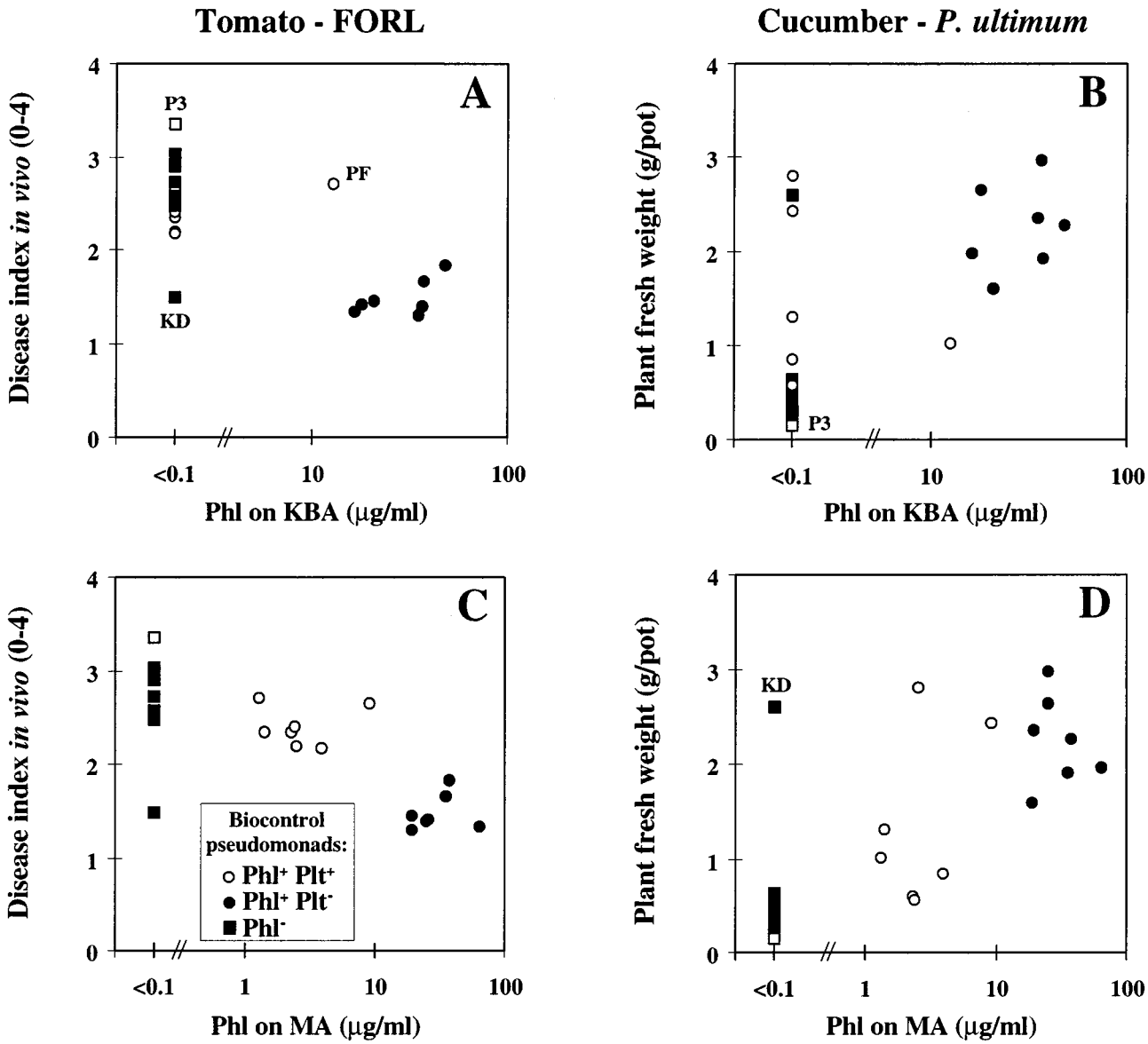

Figure 4. Relationship between production of Phl by the 22 pseudomonads *in vitro* (log scale) and disease index for Fusarium crown and root rot of tomato (A,C) or plant fresh weight (cucumber-*P. ultimum* system) (B,D). Phl production was assessed on KBA (A,B) or MA (C,D). Data of Phl and Plt production used for the 14 strains producing Phl or Plt were obtained by Keel et al. (1996). All strains that did not produce Phl are also shown for comparison, at an arbitrary value of the X axis. The correlations between disease index of Fusarium crown and root rot and Phl on KBA ($r = -0.68$; $P < 0.01$; $n = 21$) or MA ($r = -0.71$; $P < 0.01$; $n = 21$) were significant. There was no statistical relationship between Phl production *in vitro* and fresh weight of cucumber.

tem) or no difference (tomato-FORL system) between Phl^+ pseudomonads from group ARDRA2 in terms of level of disease suppression, despite the fact that these strains displayed substantial genotypic diversity as indicated by spacer ARDRA (Figure 1) and RAPD analysis (Keel et al., 1996). Collectively, Phl^+ Plt^- strains (ARDRA2) provided a greater level of protection than Phl^+ Plt^+ pseudomonads (ARDRA1) in each pathosystem. This finding will need to be confirmed with a larger collection of pseudomonads.

A significant correlation was found in terms of plant protection in the cucumber-*P. ultimum* and tomato-FORL systems. The ability of Phl^+ biocontrol strains to protect against a range of soil-borne fun-

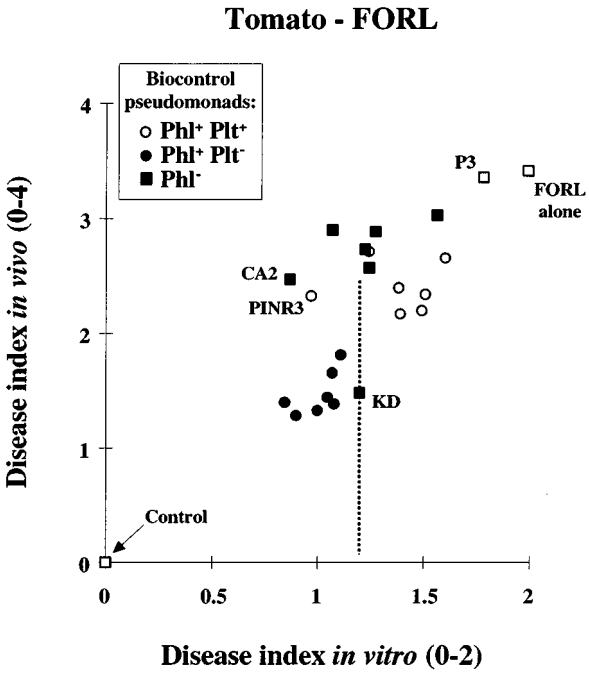

Figure 5. Relationship between disease indices for Fusarium crown and root rot of tomato in the seedling assay *in vitro* and the rockwool microcosms (*in vivo*). The dotted line corresponds to a disease index of 1.2 *in vitro*.

gal diseases is well documented (Table 1). The Phl$^+$ strain *P. fluorescens* F113 can affect phytopathogenic bacteria (Cronin et al., 1997a) and plant-parasitic nematodes (Cronin et al., 1997b) in addition to soil-borne fungi (Fenton et al., 1992). However, the ability of biocontrol strains to protect against several diseases is not restricted to Phl$^+$ pseudomonads, as for instance the Phl$^-$ strain KD proved effective in both pathosystems in the current work (Figure 2). In addition to wheat take-all, the Phl$^-$ strain 2-79 controls *Magnaporthe poae*-mediated summer patch disease of Kentucky bluegrass in greenhouse (Thompson et al., 1996).

For both fungi, correlation analyses did not reveal any significant link between inhibition of fungal growth on plates and protection of the plant. A lack of correlation between data obtained *in vitro* and *in vivo* seems to be the rule when dealing with biocontrol of soil-borne diseases (Kloepper, 1991). This conclusion was reached here even when dealing with Phl$^+$ strains from a same ARDRA group, i.e. strains that are closely related genotypically and that display similar biocontrol trait(s). Interestingly, a significant correlation was found between Phl production on plates and protection of tomato against Fusarium crown and root rot, but not with that of cucumber against Pythium damping-off. In addition, no correlation was found between production of Plt by Phl$^+$ Plt$^+$ pseudomonads (ARDRA1) and their ability to control root diseases. Plt was involved in protection of cress from Pythium damping-off by CHA0, but did not contribute to the strain's ability to suppress damping-off of cucumber (Maurhofer et al., 1994). There was no obvious link between the plant species from which pseudomonads were isolated and their ability to protect against fungal disease.

One important factor that may account for the lack of relationship between biocontrol traits *in vitro* and actual protection *in vivo* is the fact that more than one bacterial trait can contribute to biocontrol. For instance, CHA0 produces several antifungal metabolites in addition to Phl, noticeably siderophore(s), hydrogen cyanide and Plt (Keel and Défago, 1997). Hydrogen cyanide is involved in the ability of CHA0 to protect tobacco against black root rot (Voisard et al., 1989), and indeed the results of the current work indicate that the few pseudomonads that did not protect in both pathosystems comprised the few strains that were hydrogen cyanide-negative. The possibility that pseudomonads could have produced other biocontrol metabolites (reviewed by Dowling and O'Gara, 1994 and Keel and Défago, 1997) was outside the scope of the current work. For instance, Pf-5 synthesises the antifungal compound pyrrolnitrin (Sarniguet et al., 1995), and the Phl$^-$ biocontrol pseudomonad *P. fluorescens* 2–79 produces a phenazine (Thomashow and Weller, 1988). Strain KD, which was effective in both pathosystems, may produce an as yet unknown antifungal secondary metabolite or protect plants through mechanism(s) other than antibiosis.

Considering the inefficacy of fungal inhibition tests *in vitro*, novel screening strategies are needed to effectively identify strains with biocontrol potential. The results of the current work suggest that Phl production is a useful biocontrol trait and that Phl$^+$ strains from group ARDRA1 proved diverse in their ability to protect plants. The identification of new Phl$^+$ pseudomonads may be facilitated by screening rhizosphere isolates for particular ARDRA fingerprints, as this would be faster than Phl production tests. Simplified *in vitro* systems mimicking some of the interactions taking place in the rhizosphere have also been proposed e.g. inhibition of fungal pathogens on plates containing seed exudates (Stephens et al., 1993). Indeed, a significant correlation was found between inhibition of *P. ultimum in vitro* and emergence

of sugarbeet in non-sterile soil microcosms when seed exudate plates were used instead of complex laboratory media providing nutrients unrelated to those actually present in the rhizosphere (Stephens et al., 1993). This may explain why no relationship was found in the current study between inhibition of *P. ultimum* (Figure 3D-F) or Phl production (Figure 4BD) *in vitro* and cucumber weight *in vivo*. Other simplified *in vitro* systems have been designed based on protection of seedlings against pathogenic fungi (Kloepper, 1991). In the current work, a seedling bioassay was proposed with the same objective. Results indicated that the correlation between the results of the *in vitro* bioassay and biocontrol *in vivo* was significant, and the bioassay effectively identified four of the five best biocontrol strains against Fusarium crown and root rot.

In conclusion, the results of the study confirm the usefulness of Phl^+ pseudomonads for biocontrol of soil-borne diseases. Furthermore, they suggest that promising biocontrol pseudomonads may be identified by screening for Phl production ability or membership to specific ARDRA groups.

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