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Prevalence of fluorescent pseudomonads producing antifungal phloroglucinols and/or hydrogen cyanide in soils naturally suppressive or conducive to tobacco black root rot

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Abstract

Certain soils from Morens, Switzerland, are naturally suppressive to *Thielaviopsis basicola*-mediated black root rot of tobacco, and fluorescent pseudomonads are involved in this suppressiveness. Here, we compared two conducive, one moderately suppressive and one suppressive soil from Morens. Disease levels on tobacco after heavy *T. basicola* inoculation varied from 29% to 85% for the two conducive soils, 10% to 78% for the moderately suppressive soil and 11% to 42% for the suppressive soil, depending on time of the year. In the absence of *T. basicola* inoculation, disease levels were between 0% and 40% and varied also in time. Fluorescent pseudomonads were isolated from the rhizosphere and roots of tobacco subjected to *T. basicola* inoculation and characterized for production of the biocontrol metabolites 2,4-diacetylphloroglucinol (Phl) and HCN. No difference in population size was found between the suppressive and the conducive soils for total, Phl^+ and HCN^+ fluorescent pseudomonads colonizing the rhizosphere or roots of tobacco. Yet, the percentage of Phl^+ isolates was significantly higher (30–32% vs. 6–11%) in the rhizosphere and roots for plants grown in the suppressive soil compared with the moderately suppressive and conducive soils. Different restriction profiles for *phlD*, one of the Phl biosynthetic genes, were often found when analyzing Phl^+ isolates colonizing the same plant. Most *phlD* alleles were recovered from both suppressive and conducive soils, except one allele found only in root isolates from the suppressive soil.

Keywords: 2,4-Diacetylphloroglucinol; Rhizosphere; Biocontrol; *Pseudomonas*; *Thielaviopsis basicola*

1. Introduction

Soil-borne plant pathogens generally encounter a natural, yet limited, antagonism through the activity of certain indigenous saprophytic microorganisms [1,2]. In addition to this widespread phenomenon, a pathogen-specific disease suppression can occur in the so-called suppressive soils, in which 'the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil' [3]. Cook and Baker [2] further distinguished between induced and long-standing disease suppressiveness. The first category encompasses soils where disease suppressiveness is induced and sustained by a specific crop monoculture. The best known instance of induced suppressiveness is the decline of take-all in wheat fields where the incidence and severity of the disease mediated by *Gaeumannomyces graminis* var. *tritici* decreases after a few years of wheat monoculture [4]. In Washington State, this biological control was associated with the presence of indigenous populations of root-colonizing fluorescent pseudomonads producing the antimicrobial metabolite 2,4-diacetylphloroglucinol (Phl; [5,6]). The genetic polymorphism of the polyketide synthase gene *phlD*, implicated in Phl biosynthesis and present in all Phl^+ fluorescent pseudomonads [7], was shown to be a useful marker of genetic and phenotypic diversity of such bacteria [8–10]. Mavrodi et al. [8] reported that up to four different *phlD* alleles could be found in a single wheat monoculture field.

For long-standing (syn. natural) disease-suppressive

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soils, specific soil characteristics sustain reduced damage on susceptible plants over the years, even under different cropping histories [2]. Besides the fact that such suppressiveness could be attributed to abiotic factors [11], the implication of indigenous microbial antagonists has generated much interest due to potential biocontrol applications [12,13]. Such a case of long-standing or natural suppressiveness was identified in Morens, Switzerland, where pathogenic *Thielaviopsis basicola* (Berk. and Broome) Ferraris (synanamorph *Chalara elegans* Nag Raj and Kendrick) strains (the causal agents of black root rot) were present in soil, but caused no or little damage to susceptible tobacco host plants [14]. Although soil suppressiveness may involve many different groups of plant-beneficial microorganisms [15], in the case of Morens it was attributed mainly to the presence and activity of fluorescent pseudomonads [14] since (i) such bacteria were massively isolated from Morens suppressive soils, (ii) they inhibited strongly the pathogenic fungus in vitro, (iii) conferred a suppressive ability to a disease conducive soil when inoculated to the latter, and (iv) moist heat treatment of a suppressive soil led to a loss of disease suppressiveness [14,16]. The antagonistic activity of strain CHA0, such a fluorescent pseudomonad isolated from a Morens suppressive soil [14], was studied in detail and shown to rely on the production of secondary metabolites with antimicrobial activity against *T. basicola*. Among the numerous metabolites the strain produces, Phl [17] and HCN [18] have been shown to play a key role in the protection of tobacco against *T. basicola* in a gnotobiotic system mimicking Morens soils and where iron sufficiency was provided by the presence of vermiculitic clay minerals [19,20]. Although those key metabolites were shown to contribute substantially to disease suppression in controlled conditions, little is known about the occurrence and diversity of the natural population of Phl^+ and/or HCN^+ fluorescent pseudomonads in Morens soils suppressive or conducive to black root rot mediated by *T. basicola*.

In this study, we assessed the disease-suppressive status of four Morens soils previously identified as suppressive or conducive to black root rot of tobacco [16,21], isolated and characterized fluorescent pseudomonads from the rhizosphere and roots of tobacco, and assessed the relationship between disease suppressiveness and population levels of Phl^+ and/or HCN^+ fluorescent pseudomonads. This was completed by a comparison of *phlD* alleles recovered from both types of soils.

2. Materials and methods

2.1. Plant, fungus and culture conditions

Tobacco seeds are small and not suitable for direct planting into soil under farming conditions. Here, tobacco plants (*Nicotiana glutinosa* L.) were grown at 22°C for 4 weeks in sand in a growth chamber (70% RH) with a photoperiod consisting of 16 h of light (80 mE m^{-2} s^{-1}) and 8 h of darkness (18°C) before transplanting into soil, as described [14]. Seedlings were watered routinely with Knop's nutrient solution [22]. *T. basicola* (Berk. and Br.) Ferraris strain ETH D127 [14] was grown on malt agar (15 g malt extract l^{-1}, 17 g agar l^{-1}; Difco Laboratories, Detroit, MI, USA) at 24°C for 3 weeks. Endoconidia were suspended in sterile distilled water and separated from chlamydospores and mycelium by filtration through sterile glass wool. Endoconidial suspensions were used to inoculate sand or soil at transplanting [14].

2.2. Analysis of disease suppressiveness of Morens soils

Four soils (described in Table 1) were studied in this work, i.e. soils MS8 (disease-suppressive), MS7 (moderately suppressive, i.e. whose level of suppressiveness can vary depending on disease pressure), MC6 and MC10 (disease conducive). Soil samples were taken from 10–30 cm depth using sterilized shovels. Each soil was kept in large plastic bags at 15°C before use (within the following 10 days). Root residues and stones were removed manually using sterile gloves. The disease suppressiveness of a soil can be related to its resistance to disease development following an artificial infestation with *T. basicola* [16]. The method is the same as the one previously described [16,21]. Briefly, 4-week-old tobacco plants were transplanted into soil in 150-cm^3 plastic pots with drainage holes at the bottom. The soil was subsequently inoculated with 5 ml of *T. basicola* suspension to reach either 10^3 or 10^4 endoconidia cm^{-3} soil. Water was used as non-inoculated control. Soil water content was adjusted to 25% w/w (retention capacity for these soils) by watering pots every 2 days with distilled water. Disease severity was recorded 3 weeks after inoculation as the percentage (x) of root surface covered by *T. basicola* chlamydospores. Root disease level was rated visually using a height-class disease scale [14] based on midpoints of disease level intervals, as follows: 0% (no disease), 5% ($0 < x \leq 10\%$), 17.5% ($10 < x \leq 25\%$), 37.5% ($25 < x \leq 50\%$), 62.5% ($50 < x \leq 75\%$), 82.5% ($75 < x \leq 90\%$), 95% ($90 < x < 100\%$), and 100% (dead plant). Six to 10 pots (depending on the experiment), each containing one plant, were used per treatment.

2.3. Isolation of fluorescent pseudomonads

Since the focus was on fluorescent pseudomonads potentially implicated in disease suppressiveness, bacteria were isolated in experiments started with diseased plants. Indeed, preferential *Pseudomonas* proliferation on roots in conducive soils can take place as a consequence of more extensive root lesions and may generate a bias with respect to population size and diversity, as discussed below. Five-week-old tobacco plants were transferred each to a 700-

Table 1
Characteristics[a] of Morens soils

	MC6	MC10	MS7	MS8
Clay[b] (%)	15.7	15.5	16.8	12.3
Silt (%)	37.0	30.7	31.5	25.4
Sand (%)	47.3	53.8	51.7	62.3
pH [water]	6.8	7.4	6.1	7.6
Organic matter (%)	2.1	2.2	1.6	1.9
N total [Kjeldahl] (%)	0.16	0.18	0.14	0.15
C/N ratio	13	12	11	13
P [EDTA$(NH_4)_2CO_3$] (ppm)	222	193	115	229
CEC[c] [$BaCl_2$ triethanolamine] (cmol kg^{-1})	9.8	10.9	9.8	8.9
Saturation CEC (%)	72	88	50	93
Ca [$BaCl_2$ triethanolamine] (cmol kg^{-1})	2.84	3.91	1.89	3.58
Mg [$BaCl_2$ triethanolamine] (cmol kg^{-1})	0.29	0.50	0.25	0.22
K [$BaCl_2$ triethanolamine] (cmol kg^{-1})	0.59	0.65	0.49	0.54
Na [$BaCl_2$ triethanolamine] (cmol kg^{-1})	0.14	0.08	0.11	0.07
B [hot water] (ppm)	1.1	1.3	0.5	1.3
Cu [EDTA$(NH_4)_2CO_3$] (ppm)	6.4	6.4	2.8	4.0
Zn [EDTA$(NH_4)_2CO_3$] (ppm)	40.5	7.6	2.4	5.5
Mn [EDTA$(NH_4)_2CO_3$] (ppm)	235	504	211	362
Fe [EDTA$(NH_4)_2CO_3$] (ppm)	498	505	476	550

[a]Soils were analyzed by the Swiss soil testing service, Nyon (Switzerland). From 1992 to 1999, the cropping history for MC6 was wheat, barley, maize, wheat, barley, beet, wheat, maize; for MC10, beet, wheat, barley, tobacco, beet, wheat, tobacco (2 years consecutively); and for MS8, pasture, wheat, maize, wheat, pasture (3 years consecutively), beet. For MS7, cropping history (only available from 1997 on) was wheat (2 years consecutively), and maize.
[b]All were sandy loams.
[c]CEC, cation exchange capacity.

cm^3 pot containing sterile sand and the sand was inoculated with *T. basicola* ETH D127 to reach a final concentration of 10^4 endoconidia cm^{-3} sand. Pots were placed in plates filled with 1 cm of Knop solution. At 3 weeks, disease level of infected plants reached 50–60% of blackened roots. Diseased plants were transplanted into 700-cm^3 pots containing soil collected in April 1999 (one plant per pot and four pots for each of the four soils). All pots were arranged in a randomized complete block design. Three weeks later, fluorescent pseudomonads were isolated from the rhizosphere (i.e. the soil adhering closely to the root surface) up to 2 mm from the root surface and from the root itself (i.e. the root surface and internal root tissues), as follows. Plants were shaken to remove loosely adhering soil, and root systems (with closely adhering soil) were transferred individually into a sterile tube containing 25 ml of 0.9% NaCl solution. Tubes were shaken for 30 min at 350 rpm, and vigorously vortexed 10 s, before making decimal dilutions in 0.9% NaCl solution subsequently used to isolate rhizosphere pseudomonads. The roots were recovered from the tubes, blotted dry briefly and macerated in 25 ml 0.9% NaCl solution using a sterile mortal and pestle. The root suspension was serially diluted and used to isolate root pseudomonads. All samples were plated in duplicate onto King's medium B (KB; [23]) agar amended with cycloheximide (100 μg ml^{-1}; Fluka, Buchs, Switzerland), chloramphenicol (13 μg ml^{-1}; Fluka) and ampicillin (40 μg ml^{-1}; Sigma Chemicals, St. Louis, MO, USA) (i.e. medium KB^+; [24]). Colonies were counted after 2 days incubation at 27°C. Fluo-

Table 2
Disease level for tobacco grown in four Morens soils[a] inoculated with 0 (control), 10^3 or 10^4 endoconidia cm^{-3} of the pathogen *T. basicola* in June 1998, August 1998 or April 1999

Soil	June 1998		August 1998						April 1999					
	10^4 cm^{-3}		0		10^3 cm^{-3}		10^4 cm^{-3}		0		10^3 cm^{-3}		10^4 cm^{-3}	
MC6	49 ± 14[b]	ab[c]	6 ± 2	bc	15 ± 2	b	29 ± 4	a	8 ± 2	f	53 ± 5	bc	85 ± 3	a
MC10	60 ± 3	a	0	c	14 ± 2	b	39 ± 8	a	33 ± 6	de	61 ± 3	b	79 ± 4	a
MS7	10 ± 3	b	0	c	8 ± 2	b	10 ± 4	b	38 ± 3	cd	54 ± 4	bc	78 ± 4	a
MS8	11 ± 11	b	0	c	10 ± 2	b	12 ± 2	b	0	f	36 ± 3	cd	42 ± 5	cd

[a]Soils were sampled at three different times and planted with 4-week-old tobacco plants. Microcosms were subsequently inoculated with 10^3 or 10^4 endoconidia of *T. basicola* cm^{-3} soil or were not inoculated. The number of plants used per treatment was six (June 1998) or 10 (August 1998 and April 1999).
[b]Overall percentage of diseased tobacco roots (mean ± S.E.M.), using the eight-class scale of Stutz et al. [14].
[c]Within each of the three experiments, different letters indicate statistical differences at $P < 0.05$ (Tukey's HSD tests) between soils and levels of pathogen inoculation. Each experiment was analyzed separately.

rescence on KB was checked under UV light (366 nm). Bacteria were stored at −80°C in 40% glycerol.

2.4. Identification of Phl- and HCN-producing fluorescent pseudomonads

For each of the four plants used for each soil, 94 isolates (47 from the rhizosphere and 47 from the root) were selected randomly and further characterized. Strain CHA0 was included in each batch of 94 isolates as a positive control for the different phenotypic and genotypic tests. A total of 1504 fluorescent pseudomonads were screened for the presence of *phlD* by PCR (confirmed by high-performance liquid chromatography (HPLC) analysis of Phl production in vitro) and the ability to produce HCN.

phlD was PCR-amplified using 20-mer primers Phl2a (5′-GAGGACGTCGAAGACCACCA-3′) and Phl2b (5′-ACCGCAGCATCGTGTATGAG-3′) [24], as described [10]. Briefly, colonies were transferred into 100 μl of lysis solution (50 mM KCl, 0.1% Tween 20, 10 mM Tris–HCl (pH 8.3); [7]). The suspension was centrifuged at 5000 rpm for 1 min and incubated for 10 min at 99°C. The heat-lysed bacterial suspension was frozen (−20°C) for 30 min before addition of PCR mixture. The latter consisted of 1×PCR buffer (Amersham Pharmacia, Uppsala, Sweden), bovine serum albumin (0.5 g l^{-1}; Fluka), 5% dimethyl sulfoxide (Fluka), 100 μM each of dATP, dCTP, dGTP and dTTP (Amersham Pharmacia), 0.40 μM of each primer and 1.4 U of *Taq* DNA polymerase (Amersham Pharmacia). The initial denaturation (2 min at 94°C) was followed by 30 cycles (94°C for 30 s, 60°C for 30 s and 72°C for 60 s) and a final extension at 72°C for 10 min.

Production of Phl was checked (once) in $phlD^+$ isolates using reverse-phase HPLC, as described [25,26]. Ten-ml aliquots of bacterial cultures grown for 2 days in Keel's glycerol–casamino acid medium [27] were acidified (pH 3.0), mixed with 10 ml of ethyl acetate and agitated vigorously. The organic phase was separated from the aqueous phase by filtering through silicon-coated filter paper (Macherey-Nagel, Düren, Germany) and evaporated to dryness (HETOVAC, Heto Lab Equipment, Allerød, Denmark). The residues were dissolved in methanol and analyzed by HPLC using a diode-array detector (Hewlett Packard 1090, Hewlett Packard Co., Palo Alto, CA, USA) and a column (100×4 mm) packed with Nucleosil 120-5-C18 (Macherey-Nagel). All $phlD^+$ strains produced Phl.

Qualitative HCN production was determined colorimetrically using the method of Castric and Castric [28] as modified by Sarniguet et al. [29]. A fresh colony was transferred into 100 μl liquid KB amended with iron (32 μM $FeCl_3$) and the HCN precursor glycine (4.4 g l^{-1}). HCN production was checked after overnight incubation at 27°C (170 rpm). The test was done twice for each isolate.

2.5. PCR-restriction fragment length polymorphism (RFLP) analysis of phlD

Following PCR amplification of *phlD*, 5 μl of amplified product was used for restriction analysis with 1.5 U of either *Hae*III, *Cfo*I or *Msp*I (Boehringer, Mannheim, Germany). After a 2-h digestion at 37°C, restriction fragments were separated by electrophoresis in ethidium bromide-stained 2.5% agarose gels, as described [30]. A 100-bp ladder (Gibco-BRL Life Technologies Inc., Gaithersburg, MD, USA) was used as molecular size marker. A total of 52 of 205 $phlD^+$ pseudomonads were studied, and restriction analysis was done at least twice with each enzyme.

2.6. Statistical analysis of the data

Disease levels [20] and percentages of Phl- or HCN-positive isolates were arcsine-transformed, and colony forming units (CFU; expressed per gram of fresh tobacco roots) were $\log_{10}$-transformed [31] prior to statistical analyses. Analysis of variance was performed at the significance level of $P<0.05$, after having verified that data

Table 3
Rhizosphere and root colonization by fluorescent *Pseudomonas* in Morens soils

Compartment and soil	Total fluorescent pseudomonads		HCN^+ fluorescent pseudomonads				Phl^+ fluorescent pseudomonads			
	$\log_{10}$(CFU g^{-1} root)		$\log_{10}$(CFU g^{-1} root)		%[c]		$\log_{10}$(CFU g^{-1} root)		%	
Rhizosphere										
MC6	7.99 ± 0.13[a]	a[b]	6.71 ± 0.20	ab	7.5 ± 3.9	b	6.86 ± 0.15	a	7.4 ± 0.6	b
MC10	7.74 ± 0.24	a	7.49 ± 0.33	a	64.4 ± 15.3	a	6.53 ± 0.33	a	8.0 ± 3.2	b
MS7	7.45 ± 0.15	a	6.42 ± 0.18	b	10.1 ± 2.4	b	6.47 ± 0.22	a	11.2 ± 2.2	b
MS8	7.60 ± 0.21	a	7.18 ± 0.27	ab	39.4 ± 7.0	ab	7.04 ± 0.28	a	29.8 ± 7.3	a
Root										
MC6	8.65 ± 0.17	a	7.53 ± 0.34	a	11.2 ± 5.6	b	7.44 ± 0.23	a	6.4 ± 0.9	b
MC10	8.29 ± 0.16	a	7.96 ± 0.13	a	53.7 ± 12.5	a	7.01 ± 0.14	a	5.9 ± 1.3	b
MS7	8.11 ± 0.16	a	7.41 ± 0.24	a	21.8 ± 4.4	b	7.00 ± 0.17	a	8.0 ± 1.0	b
MS8	8.21 ± 0.19	a	7.51 ± 0.32	a	22.9 ± 6.5	ab	7.72 ± 0.22	a	32.4 ± 2.5	a

[a] Mean ± S.E.M. ($n=4$ plants).
[b] Statistical differences between soils within each compartment are indicated with letters (a, b) according to Tukey's HSD tests ($P<0.05$).
[c] Percentage of positive colonies from the total pseudomonads.

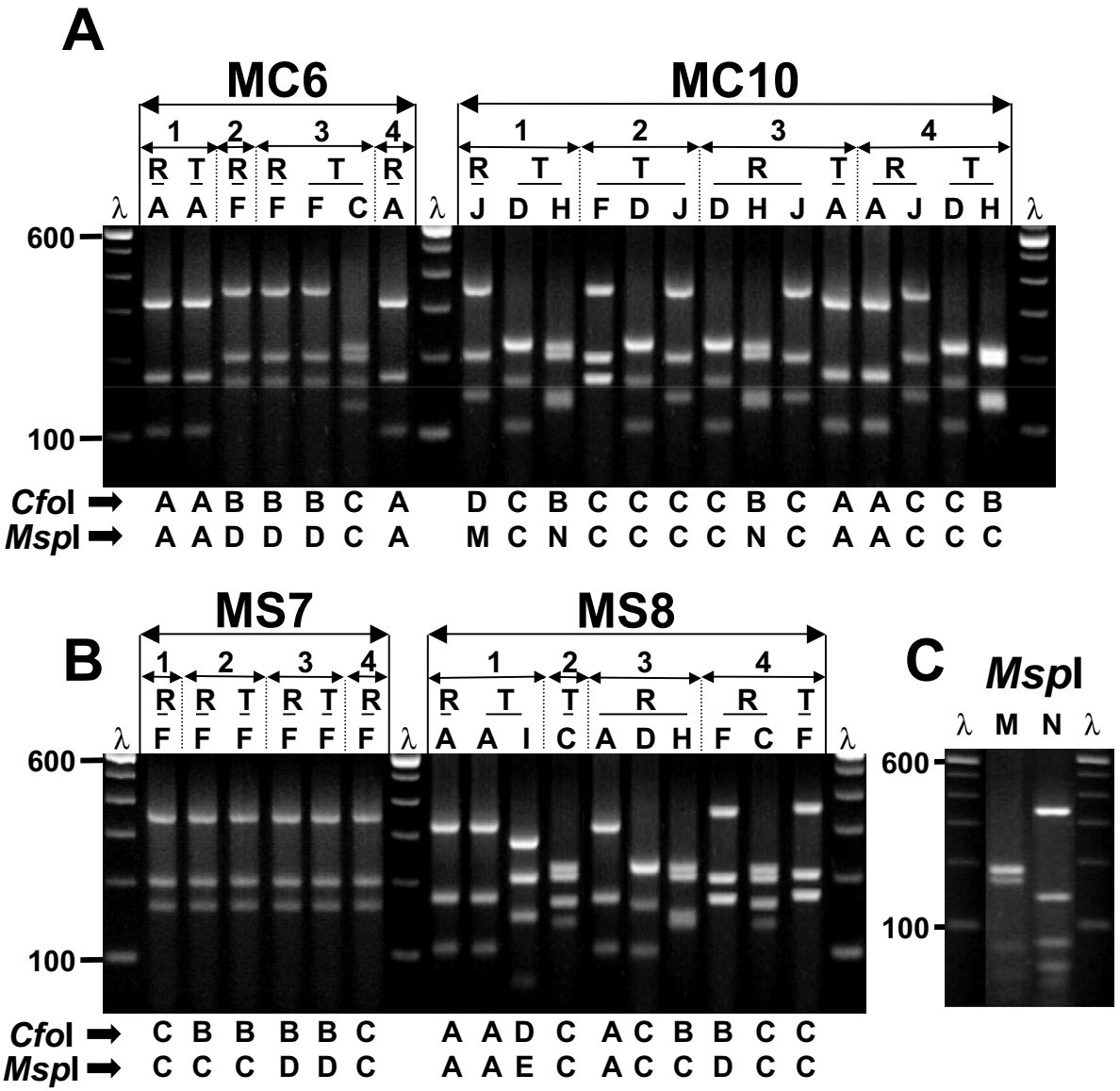

Fig. 1. *phlD* alleles found by restriction analysis with *Hae*III (A, B) for fluorescent pseudomonads from suppressive (MS8), moderately suppressive (MS7) and conducive soils (MC6 and MC10). A total of 52 Phl^+ isolates were analyzed (26 rhizosphere and 26 root isolates). For each lane, information is given above the agarose gel on (from top to bottom) the soil, the individual tobacco plant (numbers 1–4) and the compartment (R for rhizosphere and T for roots) from which the isolate originated, as well as the corresponding *Hae*III restriction pattern (designations are the same as in Wang et al. [10]). The letters directly below the agarose gel indicate the *Cfo*I and *Msp*I restriction patterns obtained with that isolate. *phlD* alleles identical to the one of the model strain CHA0 (patterns A defined for each of *Hae*III, *Cfo*I and *Msp*I) were present in all soils except the moderately suppressive soil MS7. The two new restriction patterns (M and N) found with *Msp*I in this study are shown (C). A 100-bp ladder (i.e. from the bottom of the gel 100 bp, 200 bp, etc.; Gibco-BRL) is also included (λ).

were normally distributed and variances homogeneous, using SYSTAT (version 9, SPSS Inc., Chicago, IL, USA). When appropriate, means were separated using Tukey's 'Honestly Significantly Different' tests ($P < 0.05$; SYSTAT).

3. Results

3.1. Assessment of disease suppressiveness of Morens soils

Assessment of disease levels in the absence of pathogen inoculation, in August 1998 and April 1999, indicated that disease pressure varied in time for certain soils but not others (Table 2). Analysis of black lesions on tobacco roots revealed the presence of *T. basicola* chlamydospores. For each soil, disease levels obtained at 3 weeks with a high *T. basicola* inoculum (10^4 endoconidia cm^{-3}) indicated large differences over the 1-year period studied (Table 2). Disease levels varied from 29% to 85% for the two conducive soils, 10–78% for the moderately suppressive soil MS7, and 11–42% for the suppressive soil MS8. The latter two soils displayed disease levels of 10–12% at two of the three dates, but in April 1999 only the suppressive

soil gave a disease level (42%) statistically lower than those of the conducive soils (79–85%). Disease suppressiveness was not observed at the lower *T. basicola* inoculum (10^3 endoconidia cm^{-3}), as disease levels did not differ significantly between soils.

In a distinct experiment, tobacco was infected with *T. basicola* (10^4 endoconidia cm^{-3}) while growing in sand. When transplanted into the four soils, tobacco plants displayed 50–60% blackened roots. Three weeks later, the percentage of blackened roots was higher by 15 and 28% units for the conducive soils MC6 and MC10, respectively, but lower by 10 and 12% units for the moderately suppressive soil MS7 and the suppressive soil MS8, respectively. Since root lesions have a major impact on levels of root colonization by fluorescent pseudomonads ([32,33]; our unpublished results), tobacco plants used for bacterial isolation were taken in this experiment, so that all plants had a same disease level prior to transplanting into soil.

3.2. Rhizosphere populations of fluorescent pseudomonads from suppressive and conducive soils

In the rhizosphere, the total number of fluorescent pseudomonads amounted to about 7.7 log CFU g^{-1} root, regardless of whether suppressive or conducive soils were concerned (Table 3). In contrast, the number of HCN^+ fluorescent pseudomonads could vary from one soil to the next, as it was lower by one log unit for MS7 (6.4 log CFU g^{-1} root) compared with MC10 (7.5 log CFU g^{-1} root). However, no relationship was found between the number of HCN^+ fluorescent pseudomonads and the disease-suppressive status of the soils. Similar results were obtained when considering the percentage of HCN^+ strains among all fluorescent pseudomonads rather than their population size. The number of Phl^+ fluorescent pseudomonads reached approximately 6.7 log CFU g^{-1} root, regardless of whether suppressive or conducive soils were concerned. Interestingly, the percentage of Phl^+ isolates was higher for the suppressive soil MS8 compared with the moderately suppressive soil MS7 and especially the two conducive soils. Standard errors indicate that inter-plant variability of this percentage was low, except for the suppressive soil MS8 (7.3%).

3.3. Root populations of fluorescent pseudomonads from suppressive and conducive soils

Fluorescent pseudomonads were also studied at the level of the root itself. The comparison between rhizosphere and root tissues indicated that generally roots were harboring three- to five-fold more total and Phl^+ fluorescent pseudomonads and two- to 10-fold more HCN^+ fluorescent pseudomonads compared with the corresponding rhizosphere (Table 3). Yet, most similarities and differences observed between suppressive and conducive soils in the rhizosphere were also found in the root compartment. No difference in bacterial abundance between suppressive and conducive soils was found for the total (about 8.3 log CFU g^{-1} root), HCN^+ (about 7.6 log CFU g^{-1} root) and Phl^+ (about 7.3 log CFU g^{-1} root) fluorescent pseudomonads in the root tissues. When expressed as a percentage of the total fluorescent pseudomonads, it appeared that the prevalence of HCN^+ fluorescent pseudomonads was lower in the conducive soil MC6 and the moderately suppressive soil MS7 than in the conducive soil MC10, whereas the suppressive soil MS8 was in an intermediate position and could not be distinguished from the others. The percentage of Phl^+ isolates was four to five times higher for the suppressive soil MS8 compared with both the moderately suppressive soil MS7 and the two conducive soils. Standard errors show that inter-plant variability for the percentage of Phl^+ pseudomonads was comparable in the rhizosphere and in roots for soil MC6, whereas for the other soils it was two to three times lower in roots compared with the rhizosphere.

3.4. *phlD* polymorphism in Phl^+ pseudomonads

PCR-RFLP analysis of *phlD* was performed on 52 of the 205 Phl^+ isolates to gain some insights on the diversity of Phl-producing populations from suppressive and con-

Table 4
Repartition of *phlD* alleles (defined by three letters corresponding successively to their *Hae*III, *Msp*I and *Cfo*I restriction patterns) in 52 *phlD*$^+$ fluorescent pseudomonads from Morens soils

Soil	Compartment	Number of Phl^+ isolates		Number of isolates for each *phlD* allele											
		Identified	Studied	AAA[a]	CCC	DCC	FBC	FBD	FCC	HBC	HBD	HBN	IDE	JCC	JDM
MC6	Rhizosphere	14	6	3				3							
	Root	12	5	2	1			2							
MC10	Rhizosphere	15	8	1		1						3		2	1
	Root	11	10	1		3			1	1	1	2		1	
MS7	Rhizosphere	21	5				2	1	2						
	Root	15	5				2	3							
MS8	Rhizosphere	56	7	1	1	1		1		2		1			
	Root	61	6	2	2				1				1		

[a] *phlD* allele displayed by the model strain CHA0.

ducive soils. Seven different *Hae*III restriction patterns were obtained (illustrated in Fig. 1A,B). Pseudomonads displaying *Hae*III pattern F gave *Cfo*I pattern B (with *Msp*I pattern C or D) or C (with *Msp*I pattern C), those displaying *Hae*III pattern H gave *Cfo*I pattern B (with *Msp*I pattern C or N), and the isolates with *Hae*III pattern J gave *Cfo*I pattern C (with *Msp*I pattern C) or D (with *Msp*I pattern M). Thus, a total of 12 different *phlD* alleles were found.

As many as eight *phlD* alleles were present in the suppressive soil MS8, but each was also found in at least one conducive soil and sometimes also in the moderately suppressive soil MS7, except *Hae*III pattern I recovered only from root tissues in MS8 (Fig. 1B and Table 4). The number of *phlD* alleles was three for the moderately suppressive soil MS7 (including one allele, corresponding to the one displayed for plant 2 in Fig. 1B, which was never found for the three others soils) and the conducive soil MC6 (Fig. 1A), and eight for the conducive soil MC10 (Fig. 1A).

Interestingly, different *phlD* alleles could be obtained from the rhizosphere (e.g. plant 3 in soil MC10; Fig. 1A) or from the root (e.g. plant 2 in soil MC10; Fig. 1A) of a single plant. Moreover, when considering all plants of the experiment, it appears that nine of 12 *phlD* alleles were recovered from both rhizosphere and root (Table 4).

4. Discussion

Both the soils suppressive and conducive to black root rot of tobacco are known to shelter pathogenic *T. basicola* [11,14,34,35], which was confirmed here by the observation that black root rot lesions may occur on tobacco plants even in the absence of pathogen inoculation (Table 1). Levels of *T. basicola* population are not affected by the presence of non-host plants (e.g. wheat, maize) [36], in accordance with the fact that suppressiveness to *T. basicola* (unlike e.g. take-all decline) does not require a particular cropping history [16,21]. In contrast, certain environmental properties (e.g. temperature and soil humidity, [37,38]) are crucial determinants for *T. basicola* development in the field. Indeed, the ability of indigenous *T. basicola* (as well as that of introduced strain ETH D127) to cause black root rot varied when considering the August 1998 and April 1999 samplings (Table 1). Disease level in inoculated soils MS7 and MS8 was only 10–12% in June and August 1998, but rose significantly in April 1999, and at that sampling MS7 behaved similarly as the conducive soils at both inoculation levels.

The relationship between fluorescent pseudomonads and the development of root disease is often complex. On one hand, certain pseudomonads can effectively control the ability of fungal pathogens to induce disease [12,39,40]. On the other hand, indigenous populations of fluorescent pseudomonads are stimulated by root necrosis, presumably because release of organic compounds by the root is higher. For instance, wheat infected by the take-all fungus *G. tritici* var. *graminis* harbors five to 100 times more fluorescent pseudomonads than healthy plants [32,41], and among them *phlD*$^+$ fluorescent pseudomonads were also more abundant [33]. Similarly, fluorescent pseudomonads were 10 times more numerous on diseased roots compared with healthy roots for infected tobacco transplanted into Morens suppressive soil or for healthy tobacco transplanted into Morens conducive soil (our unpublished data). Therefore, bacterial isolation was carried out after having inoculated tobacco plants with the pathogenic fungus in an effort to limit the possibility of differential stimulation in conducive and suppressive soils. This also contributed to reduce inter-plant variability, as indigenous pathogenic populations cause variable necrosis levels from one root to the next. Indeed, the non-random distribution and temporal variation of *T. basicola* strains within a same field have been reported [42], and different degrees of root lesions may have different effects on root-associated bacterial populations. Despite this protocol, disease levels 3 weeks after transplanting were less in the suppressive soils, indicating that natural suppressiveness was effectively expressed.

No relationship was found between disease suppressiveness and the total number of fluorescent pseudomonads (Table 3). A similar situation was obtained with the number of HCN$^+$ fluorescent pseudomonads, or the percentage of HCN$^+$ isolates among fluorescent pseudomonads. Most Phl$^+$ biocontrol pseudomonads are also HCN$^+$ [10], and the usefulness of HCN production for biocontrol of black root rot has mostly been established in strain CHA0 [18], which can already protect tobacco via Phl production [17]. In addition, HCN production in certain pseudomonads can even be deleterious for plant health [43], a property perhaps linked to the amount of HCN produced on the root surface. Therefore, since HCN production may have contrasted effects on plants, it is conceivable that quantifying HCN$^+$ pseudomonads may not enable discrimination between suppressive and conducive soils.

A large number of Phl$^+$ fluorescent pseudomonads were found in disease-suppressive soils, as expected [10,14]. Surprisingly, Phl$^+$ fluorescent pseudomonads were also recovered from conducive soils, and indeed they were harbored in similar numbers in both types of soils (Table 3). In contrast, wheat plants in take-all decline soil harbored high numbers of Phl$^+$ fluorescent pseudomonads, whereas the latter were hardly found in conducive counterparts [5]. These bacteria were shown to produce Phl in the wheat rhizosphere [17,44] and to protect roots from the take-all fungus *G. graminis* var. *tritici* [5]. Here however, the percentage of Phl$^+$ isolates among fluorescent pseudomonads was significantly higher for the suppressive soil MS8 compared with the moderately suppressive soil MS7 and the two conducive soils (Table 3). Interestingly, this finding

was made for both the rhizosphere and the root compartment, in contrast with a previous study in which population dynamics of pseudomonads in the rhizosphere and at the root surface were found to differ [45]. Yet, the prevalence of the Phl$^+$ fluorescent pseudomonads was not a suitable criterion to distinguish between the moderately suppressive soil MS7 and the two conducive soils. Consequently, it may be needed to take also into account possible differences in expression levels of biocontrol genes in suppressive and conducive soils, and this issue will be addressed in future work.

Fluorescent pseudomonads correspond to a bacterial group comprised of several dozen species, if not hundreds of species [46]. When considering fluorescent pseudomonads with biocontrol activity, noticeably those producing HCN and/or Phl, a considerable level of strain diversity can be observed [7,9,10,47,48]. This includes also the ability of the strains to produce biocontrol compounds like HCN and Phl [7,49,50]. Therefore, it is not all that surprising that counts of total, HCN$^+$ and Phl$^+$ fluorescent pseudomonads were not sufficient to understand differences in disease-suppressive ability between soils. Indeed, PCR-RFLP analysis of *phlD* showed that Phl$^+$ fluorescent pseudomonads recovered from Morens were diverse (Fig. 1 and Table 4). *phlD* has been advocated as a good marker of the genetic background of Phl$^+$ fluorescent pseudomonads since it matched closely with data obtained from whole-genome fingerprinting methods, such as Random Amplification Polymorphic DNA (RAPD) or rep-PCR analysis, and key phenotypic characteristics [8–10,51]. Of the 12 *phlD* alleles found in this work, two (*Msp*I patterns M and N; Fig. 1C) were novel compared with the 16 alleles identified before [10]. In previous work done with *Pseudomonas* strains of worldwide origin, it was hypothesized that strains with different *phlD* alleles may colonize the same dicot rhizosphere at a particular geographic location [10]. The current study validated this hypothesis in the case of tobacco grown in Morens soil (Fig. 1A,B). In contrast, a small number of *phlD* alleles (usually no more than one or two per field) were found in take-all decline fields [8]. A majority of *phlD* alleles were present both in suppressive and conducive soils (Table 4). No clear relationship was found between *phlD* alleles and disease suppressiveness, except that one allele (*Hae*III pattern I) was recovered only from the disease-suppressive soil MS8 (Fig. 1B). In this context, perhaps other microbial groups (implicated or not in disease suppressiveness) could be more useful than biocontrol pseudomonads as bioindicators of the disease-suppressive status of these soils.

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