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Abstract

The abundance and population structure of pseudomonads in soils collected from long-(1006 years) and short-(54 years) term grapevine monocultures in Switzerland were examined across five soil horizons within the 1.20e1.35 m range. Soil samples were baited with grapevine, and rhizosphere pseudomonads containing the biocontrol genes phlD (2,4-diacetylphloroglucinol synthesis) and/or hcnAB (hydrogen cyanide synthesis) were analyzed by MPN-PCR. The numbers of total, phlDþ and hcnABþ pseudomonads decreased with depth by 1.5e2 log (short-term monoculture) and 3e3.5 log (long-term monoculture). In addition, the percentages of phlDþ (except in short-term monoculture) and hcnABþ pseudomonads were also lower in deeper horizons. RFLP-profiling of phlDþ and hcnABþ pseudomonads revealed three phlD and twelve hcnAB alleles overall, but the number of alleles for both decreased in relation to depth. The only phlD allele found in deeper horizons was also found in topsoil, whereas one hcnAB allele (k) found in deeper horizons in long-term monoculture was absent in the topsoil. This suggests that certain Pseudomonas ecotypes are adapted to specific depths. Four hcnAB alleles enabled discrimination between monocultures. We conclude that soil depth is a factor selecting phlD and hcnAB genotypes, and that the allelic diversity of the two biocontrol genes decreases with depth.
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\textbf{A B S T R A C T}

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1. Introduction

Most studies on soil microbes focus on surface soil layers (usually the first 30 cm), where root colonization and overall microbial density and activity are higher (Blume et al., 2002; Fierer et al., 2003; Jossi et al., 2006). This overlooks the fact that agriculturally relevant soil profiles can be more than 1 m deep and sustain significant microbial populations in subsurface horizons (Van Gestel et al., 1992; Fritze et al., 2000; Blume et al., 2002; Agnelli et al., 2004; Steenwerth et al., 2008). Microbial biomass and diversity tend to diminish with soil depth (Fierer et al., 2003; Agnellli et al., 2004; Allison et al., 2007), and there are indications that subsurface microorganisms may play an important role in soil formation, ecosystem biochemistry, contaminant degradation, and the maintenance of groundwater quality (Konopka and Turco, 1991; Hiebert and Bennett, 1992; Richter and Markewitz, 1995; Hase et al., 2001). However, little is known about the effect of soil depth on the diversity of key microbial communities, especially those relevant for plant growth and health.

\textit{Pseudomonas} species constitute an important bioactive group in farm soil, because of efficient root colonization and production of antifungal compounds that are important in biological suppression of soil-borne plant diseases (Weller et al., 2002; Moënne-Loccoz and Défago, 2004; Compont et al., 2005; Haas and Défago, 2005). This is especially the case for pseudomonads that produce 2,4-diacetylphloroglucinol (Phl) and/or hydrogen cyanide (HCN), which are particularly effective in biocontrol of a wide range of soil-borne diseases on various crops (Haas and Keel, 2003; Garbeva et al., 2004; Rezzonico et al., 2007).
Biosynthetic genes for Phl are clustered as phlA-G (Schnider-Keel et al., 2000; Paulsen et al., 2005), and the chalcone synthetase homolog phlD has become the genetic marker of choice for studying Phl-producing strains. Considerable diversity of Phl-producing pseudomonads in the rhizosphere has been revealed by sequencing or RFLP analysis of phlD (McSpadden Gardener et al., 2001, 2005; Ramette et al., 2001, 2006; Wang et al., 2001; Landa et al., 2006; Mazzola et al., 2004; Svercel et al., 2009). Almost all Phl-producing pseudomonads also produce HCN, whereas many HCN-producing pseudomonads are Phl-negative (Rezzonico et al., 2007). The hcnABC gene cluster encodes the HCN synthetase essential for HCN production (Haas and Keel, 2003; Moënne-Loccoz and Défago, 2004). RFLP analysis of hcn genes (especially hcnAB) has proved useful to document strain diversity (Ramette et al., 2003, 2006; Svercel et al., 2007), allowing for a lower detection limit for bacteria carrying the hcnAB genes in the soil.

In grasslands, pseudomonads are known to be an important constituent of both surface and subsurface communities in deep soils (LaMontagne et al., 2003), but little is known about the prevalence of important genotypes, especially for those with the potential to suppress pathogens, in deep agricultural soils. This is of particular relevance with deep-rooted perennial crops, where cumulative rhizosphere effects on soil bacteria may be expected (especially in subsurface layers, provided the crop is grown long enough. This is of particular relevance with deep-rooted perennial crops, where cumulative rhizosphere effects on soil bacteria may be expected (especially in subsurface layers, provided the crop is grown long enough.

2. Materials and methods

2.1. Soil sampling

Two neighboring vineyards at Bevaix (near Neuchâtel, Switzerland) and described by Svercel et al. (2009) were used. One was under short-term (54 years) monoculture and the other under long-term (1006 years) monoculture. The current grapevines were of the same variety (cv. Chasselas) but grafted onto different rootstocks (3309 in short-term and 5c in long-term vineyard) and of different ages (respectively 9 and 28 years old). Both vineyards had a green cover.

In July 2003, two soil profiles (i.e., SA and SB) were excavated in the short-term vineyard and two others (i.e., LA and LB) in the long-term vineyard (details of field locations are given in Fig. S1). All four profiles displayed the same five soil horizons (although not necessarily of the same thickness; Fig. 1) based on morphology analysis. Soils were classified as anthropic brunisols. grapevine roots were present in each horizon.

At each of the four profiles, about 10 kg of soil were taken from each soil horizon. Plant roots and stones were removed, soil was homogenized, air dried and stored at 4 °C until analysis. Part of the soil was used for analysis (Geology Institute of the University of Neuchâtel, Switzerland) of chemical and physical properties (Tables 1 and S1).

2.2. Plant experiment

Soil was used to fill 300 cm² plastic pots with drainage holes at the bottom. Each of the 10 treatment combinations (i.e., 5 soil horizons × 2 monoculture types) was studied using 8 pots for each of the 2 profiles. One grapevine baiting-plant (Vitis riparia Michx × Vitis rupestris Scheele 3309 accession RAC 1.1, corresponding to current grapevine rootstock in the short-term vineyard), obtained as described by Svercel et al. (2009), cultivated in semi-sterile conditions and checked for lack of Pseudomonas spp.
presence prior to the experiment, was transplanted in each pot. The pots were placed (randomized block design) for 3 wk in a growth chamber at 22 °C, 70% relative humidity, a photoperiod of 16 h light (80 mE m⁻² s⁻¹) and 8 h darkness. Distilled water was used to maintain soil water content at 20% w/w from the start of the experiment.

At 3 wk, each root system and closely-adhering soil (as described by Svercel et al., 2009) was transferred into a 15-ml tube containing 9.5 mL of 0.9% NaCl solution. Tubes were shaken for 40 min at 250 rev min⁻¹, vortexed for 10 s and then the sample suspension was used to inoculate (15 µL added per well) four wells of a 96-well microtitre plate containing 135 µL per well of King's B⁺ medium (Raaijmakers et al., 1997) for fluorescent Pseudomonas selection. Serial 10-fold dilutions for each inoculated well were made in the microtitre plate, which were incubated for 3 d at 27 °C with continuous shaking (150 rev min⁻¹). Pseudomonas growth was estimated visually based on turbidity and Pseudomonas number by the most probable number (MPN) technique (Garthright and Blodgett, 2003).

Sterile glycerol was then added to all wells (to achieve 40% concentration), microtitre plates were sealed (Greiner Biotech, Germany) and stored at ~80 °C. This procedure was appropriate, as fluorescent Pseudomonas species are readily culturable, even though particular strains may lose cell culturability under adverse conditions (Mascher et al., 2003; Möenne-Loccoz and Défago, 2004).

### 2.3. MPN-PCR

The proportion of rhizosphere pseudomonads carrying phd and hcnAB genes was determined using an MPN-PCR approach (Svercel et al., 2009). Briefly, 20 µL of bacterial suspension from each Pseudomonas-positive well was transferred into 150 µL of lysis solution (KCl 50 mM; Tween 20 0.1%; Tris—HCl 10 mM, pH 8.3) (Keel et al., 1996) in a PCR microtitre plate (Simport Plastics, Belloe, Canada). The suspension was centrifuged at 4025 g for 2 min and incubated for 10 min at 99 °C. The heat-lysed suspension was frozen at ~20 °C for 30 min. After thawing, 4-µL aliquots of supernatant were taken for PCR.

Amplification of phd was performed using forward primer B2BF (25-mer 5'-ACCCACCCGGAGATCGGTATCG-3') and reverse primer BPR4 (26-mer 5'-CGCGGAGATCGATAGAAAAAGT-3') (McSpadden Gardner et al., 2000), and that of hcnAB with forward primer PM2 (31-mer 5'-TGCGCCGATCGGCTGTTGCGGCACG-3') and reverse primer PM7-26R (26-mer 5'-CCGTGCTCTGTGCTGACGG-3') (Svercel et al., 2007). Primers were synthesised by MWG Biotech (Basel, Switzerland). Amplifications were carried out in 12-µL reaction mixtures containing 4 µL of lysed bacterial suspension, 1× PCR buffer (Amersham Pharmacia, Uppsala, Sweden), bovine serum albumin (0.5 g L⁻¹; Fluka, Buchs, SG, Switzerland), 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 PCR procedure was an initial denaturation (2 min at 94 °C) followed by 35 cycles of 94 °C for 30 s, 60 °C (phd) or 67 °C (hcnAB) for 30 s, and 72 °C for 60 s and final extension at 72 °C for 10 min.

Amplifications were performed with a PTC-100TM cycler (MJ Research Inc., Watertown, MA), and the resulting amplicons were separated in 1.5% agarose gels in 0.5× Tris-borate-EDTA (TBE) buffer at 160 V for 1 h. Population sizes of phd and hcn pseudomonads were estimated based on wells yielding the corresponding PCR products in gel electrophoresis, using the MPN calculations of Garthright and Blodgett (2003).

### 2.4. Statistics

Each of the 10 treatment combinations (i.e., 5 soil horizons × 2 monoculture types) was studied using 2 replicates (i.e., 2 soil
profiles). Pseudomonas numbers (expressed per g fresh root) were log_{10}-transformed and percentages were arcsine-transformed prior to statistical analyses. Analysis of variance was followed when appropriate with Tukey’s tests. Statistics were done at P < 0.05, using SYSTAT (version 9, SPSS Inc., Chicago, IL). Correlation analysis was performed using Pearson coefficient and Bonferroni probability.

### 2.5. PCR/RFLP analysis of dominant phlD and hcnAB alleles

To identify dominant phlD^{+} or hcnAB^{+} alleles in Pseudomonas populations, the last phlD-positive and/or hcnAB-positive MPN wells (often from the same dilution for different samples) were used. A total of 160 plants were tested for each locus. Restriction analysis was done with 5 μL of amplified product and 1.5 U of HaelliI, TaqI or MspI (Boehringer, Manheim, Germany). Reactions were incubated 3 h at either 37 °C (HaelliI and MspI) or 60 °C (TaqI) and then stored at −20 °C. Restriction fragments were separated by electrophoresis in ethidium bromide-stained 2.5% agarose gels. A 100-bp ladder (GIBCO-BRL Life Technologies Inc., Gaithersburg, MD) was used as a size marker. For each sample (i.e., each plant), two wells were tested per locus and they always gave the same result. The digested products were compared with RFLP profiles of reference strains for phlD (McSpadden Gardener et al., 2001, 2005) and hcnAB (Svercel et al., 2007, 2009).

### 3. Results

#### 3.1. Comparison of soil horizons

In each vineyard, soil chemistry was very similar when comparing both soil profiles, as indicated by principal component analysis (data not shown). The exception was horizon H4 under short-term monoculture, where Sr, Ca and CaCO3 contents varied extensively between the two soil profiles (Table 1).

Copper sulfate applications over the years resulted in accumulation of Cu in the topsoil layers of both vineyards (Table 1). Other differences related to soil depth included noticeably the contents in N and organic C. As many as 14 of the 16 quantitative variables studied (as well as texture) could discriminate between soils of the 1006-year-old versus 54-year-old monocultures in at least one soil horizon (Tables 1 and S1). When the five horizons were taken individually, the number of discriminant variables ranged from 4 (horizon H2) to 7 (H4).

#### 3.2. Effect of soil depth on abundance of phlD^{+} and hcnAB^{+} pseudomonads in short- and long-term grapevine monoculture

The abundance of total culturable fluorescent pseudomonads decreased with depth by 3 log_{10} CFU g^{-1} root under long-term monoculture (Fig. 2). There was a similar trend, which was not statistically significant, for a 1.5 log_{10} CFU g^{-1} root decrease under short-term monoculture. In both vineyards, the numbers of phlD^{+} and hcnAB^{+} pseudomonads decreased with depth by 3−3.5 log_{10} CFU g^{-1} root (long-term monoculture) and 1.5−2 log_{10} CFU g^{-1} root (short-term monoculture). The decrease in the abundance of total, phlD^{+} and hcnAB^{+} pseudomonads was also indicated by correlation analysis (Table 2). In addition, the percentages of phlD^{+} (except in short-term monoculture) and hcnAB^{+} pseudomonads were also lower in deeper soil horizons (Fig. 2), the results for the latter being confirmed by correlation analysis (Table 2). In summary, soil depth had a negative effect on the numbers of total, phlD^{+} and/or hcnAB^{+} pseudomonads, as well as the percentages of phlD^{+} (except in short-time monoculture) and/or hcnAB^{+} pseudomonads, but results were also influenced by the duration of vineyard monoculture.

#### 3.3. Dominant phlD^{+} alleles according to soil depth

When phlD amplicons from the last positive phlD MPN dilution were analyzed by RFLP and compared with previously published profiles, it appeared that three distinct phlD alleles (i.e., alleles A, D, K found in previous investigations) were detected. The number of

### Table 2

Pearson correlation coefficient between soil horizon rank (from 1 for H1 to 5 for H5) and Pseudomonas data.

<table>
<thead>
<tr>
<th></th>
<th>Long-term</th>
<th>Short-term</th>
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</thead>
<tbody>
<tr>
<td>Log-number:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Pseudomonas</td>
<td>−0.92*</td>
<td>−0.72*</td>
</tr>
<tr>
<td>hcnAB^{+} Pseudomonas</td>
<td>−0.93*</td>
<td>−0.87*</td>
</tr>
<tr>
<td>phlD^{+} Pseudomonas</td>
<td>−0.93*</td>
<td>−0.81*</td>
</tr>
<tr>
<td><strong>Frequencies:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hcnAB^{+} Pseudomonas</td>
<td>−0.93*</td>
<td>−0.88*</td>
</tr>
<tr>
<td>phlD^{+} Pseudomonas</td>
<td>−0.60</td>
<td>−0.26</td>
</tr>
</tbody>
</table>

*Statistically significant (Bonferroni probability < 0.05).
phlD alleles decreased with soil depth (Fig. 3), as follows: (i) two or three alleles were found in the top layer (H1) of both vineyards (Fig. 1); (ii) two alleles were present in middle layers H2 and H3, i.e., alleles D and K in the long-term vineyard versus alleles A and K (H2) or A and D (H3) in the short-term vineyard; (iii) only one allele (allele D) was detected in the deeper layers H4 and H5. Therefore, it appears that monoculture length had a much smaller impact than soil depth on allelic composition.

3.4. Dominant hcnAB⁺ alleles according to soil depth

Since hcnAB⁺ pseudomonads were often more abundant than phlD⁺ pseudomonads (Fig. 2), the RFLP assays for hcnAB were performed separately for the last dilution tested positive for phlD and the last one tested positive for hcnAB, so as to examine the hypothesis that many of the hcnAB restriction patterns could derive from hcnAB⁺ phlD⁻ pseudomonads. A total of 12 alleles were found, including two new hcnAB alleles (j and k) not reported before (Fig. 4). Approximately half the hcnAB alleles originated from phlD⁻ pseudomonads.

As with phlD, the number of hcnAB alleles (from phlD⁺ and/or phlD⁻ pseudomonads) decreased with soil depth (Fig. 3). Four hcnAB alleles (alleles T, V, N and b) were present only in the top layer H1 (Fig. 1), whereas allele k was found solely in the three deeper soil layers. In contrast, allele j was detected in all five soil horizons in short-term monoculture.

Unlike with phlD, monoculture length had a major impact on hcnAB diversity results. Firstly, the short-term vineyard displayed a higher number of hcnAB alleles in phlD⁺ pseudomonads in layers H4 and H5, and especially in phlD⁻ pseudomonads in layers H1 to H3 (Fig. 3). Secondly, alleles E (layers H1 to H3) and k (layers H3 to H5) were present only in the long-term vineyard and alleles X (layers H1 to H3) and j (all soil layers) only in the short-term one. Therefore, the hcnAB alleles E (from phlD⁺ pseudomonads), k and X (from phlD⁻ pseudomonads), and j (from any pseudomonad) allowed to distinguish between long- and short-term vineyards.

4. Discussion

The grapevine is a deep-rooted perennial plant, which provided effective circumstances to assess the effect of soil depth on root-adapted fluorescent pseudomonads. Indeed, all five horizons
studied were colonized by roots, which is typical of grapevine root development (Steenwerth et al., 2008).

When the bait plants were grown in surface horizon soil, the grapevine roots selected high numbers of cultivable *Pseudomonas* spp. from this soil layer, whereas about 1000 times fewer pseudomonads colonized roots grown in soil from the deepest horizon. Pankhurst et al. (2002) made similar findings, but soil was studied down to 80 cm only and the decline in *Pseudomonas* numbers was only 0.5 log units. The presence of pseudomonads in deeper soil layers may be assisted by their vertical transport from topsoil by water flow in soil macropores (Troxler et al., 1998; Teixier et al., 2008). For example, the *Pseudomonas* biocontrol strain CHA0, which was present at 10^8 CFU g^-1 in surface soil, was detected at 10^7–10^8 CFU g^-1 soil along macropores between 10 and 150 cm depth after heavy rainfall (Natsch et al., 1996). However, sampling of vineyard soils was done in summer 2003, an exceptionally hot and dry year in Switzerland, which suggests that pseudomonads found in deeper soil layers were indigenous and/or had been transported there much earlier. Once transported, survival of *Pseudomonas* strains may depend on effluent water properties, which can be related to soil type and time of the year (Hasse et al., 2001). Representatives of *hcnAB^+* and *phlD^+* pseudomonads were recovered at all soil depths, regardless of soil layer composition. However, the numbers of *phlD^+* and/or *hcnAB^+* pseudomonads, as well as their importance in the total *Pseudomonas* populations, decreased rather sharply with depth. The decrease in the percentage of *hcnAB^+* pseudomonads with depth (from 60% to 10%) was unexpected, as more HCN is produced under oxygen-deprived conditions (Haas and Keel, 2003; Mascher et al., 2003), but perhaps the slope situation and the presence of stones facilitated aeration of the deeper layers. Soil depth had also a negative effect on the numbers of *phlD* and *hcnAB* alleles. The disappearance of two *phlD* alleles and nine *hcnAB* alleles and the appearance of a new *hcnAB* allele with depth is in accordance with the presence of *Pseudomonas* ecotypes adapted to different soil depths and/or soil layers differing in soil chemistry.

The two vineyards studied also provided a unique opportunity to explore the significance of monoculture duration. Cumulative effects related to soil usage, cropping system and/or monoculture duration may have important long-term consequences on the ecology of root-colonizing microbes (Guemouri-Athmani et al., 2000; Diedhiou et al., 2009; Svercel et al., 2009), but this possibility remains very poorly documented as suitable experimental sites are difficult to find. Here, the main differences between the two vineyards concerned (i) the numbers of *phlD^+* pseudomonads in the first two soil horizons, in accordance with our previous results on surface layer pseudomonads in different regions of Switzerland (Svercel et al., 2009), and (ii) *hcnAB* allelic diversity. Indeed, several *hcnAB* alleles could be used to discriminate between both vineyards at various depths. Higher Ca and CaCO_3_ contents were found in the long-term vineyard than the short-term one, in accordance with Cailleau et al. (2005) who showed an accumulation of calcium and CaCO_3_ in soil profiles under 100 years old native trees. We hypothesize that differences in grapevine monoculture duration was a significant factor affecting soil chemistry, which in turn influenced the ecology of soil pseudomonads. It must also be kept in mind that the two vineyards differed in terms of grapevine age (9 years in short-term versus 28 years in long-term vineyard) and the rootstock (respectively 3309 and 5c) on which cv. Chasselas was grafted, which might also have influenced results. Morano and Kliewer (1994) showed that root growth and activity may vary according to grapevine cultivar or rootstock, and the diversity of rhizosphere bacteria can differ when comparing different plant cultivars (Okubara et al., 2004; Picard et al., 2004).

In conclusion, this study is one of the first to focus on the impact of soil depth (and monoculture duration) on rhizosphere pseudomonads harboring important biocontrol genes, and soil depth had major consequences on allelic diversity of *phlD* and *hcnAB*. From a practical perspective, our study indicates that deep soil could also serve as a reservoir of possible PGPR bacteria for grapevine monoculture.

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**Appendix. Supplementary data**


**References**


