Soil Biology & Biochemistry 42 (2010) 649–656

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Denaturing gradient gel electrophoretic analysis of dominant 2,4-diacetylphloroglucinol biosynthetic *phlD* alleles in fluorescent *Pseudomonas* from soils suppressive or conducive to black root rot of tobacco

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ARTICLE INFO

Article history:
Received 3 December 2008
Received in revised form
7 January 2010
Accepted 8 January 2010
Available online 22 January 2010

Keywords:
Pseudomonas
Biocontrol
Suppressive soil
2,4-Diacetylphloroglucinol
DGGE
Phylogeny

ABSTRACT

In Switzerland, similar types of rhizosphere pseudomonads producing the biocontrol compound 2,4-diacetylphloroglucinol (Phl) have been found in soils suppressive to *Thielaviopsis basicola*-mediated black root rot of tobacco as well as in conducive soils. However, most findings were based on the analysis of a limited number of *Pseudomonas* isolates, obtained from a single experiment and only from *T. basicola*-inoculated plants. Here, an approach based on denaturing gradient gel electrophoresis (DGGE) of dominant *phlD* alleles from tobacco rhizosphere provided different *phlD* migration patterns. Sequencing of *phlD*-DGGE bands revealed a novel phylogenetic cluster of *phlD* sequences found in both suppressive and conducive soils in addition to previously-documented *phlD* alleles. *phlD*-DGGE bands and alleles differed little from one plant to the next but more extensively from one sampling to the next during the three-year study. Three of the 13 bands and 12 of the 31 alleles were only found in suppressive soil, whereas five bands and 13 alleles were found exclusively in conducive soil. The population structure of *phlD*$^+$ pseudomonads depended more on the individual soil considered and its suppressiveness status than on inoculation of tobacco with *T. basicola*. In conclusion, *phlD*-DGGE revealed additional *phlD* diversity compared with earlier analyses of individual *Pseudomonas* isolates, and showed differences in *phlD*$^+$ *Pseudomonas* population structure in relation to disease suppressiveness.

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1. Introduction

Biocontrol fluorescent *Pseudomonas* spp. living in the rhizosphere can protect plants from soil-borne fungal pathogens, and often this involves the production of antimicrobial compounds, such as 2,4-diacetylphloroglucinol (Phl) (Défago and Haas, 1990; Keel et al., 1990; Weller et al., 2002). Phl inhibits growth of several phytopathogenic fungi including *Thielaviopsis basicola* (the agent of tobacco black root rot), *Gaeumannomyces graminis* var. *tritici* (wheat take-all) and *Pythium ultimum* (cotton damping-off) (Howell and Stipanovic, 1980; Stutz et al., 1986; Brisbane and Rovira, 1988). In addition, molecular and statistical analyses have shown the importance of Phl production ability in plant protection against disease (Vincent et al., 1991; Fenton et al., 1992; Keel et al., 1992; Bakker et al., 2002; Rezzonico et al., 2007; Couillerot et al., 2009).

In certain soils (termed disease-suppressive soils), disease-susceptible plants may grow without significant damage by virulent soil-borne pathogens (Stutz et al., 1986; Raaijmakers et al., 1997; Alabouvette, 1999; Mazzola, 2002). In the case of *T. basicola*-mediated black root rot of tobacco, suppressive soils have been documented near Morens (Switzerland), at field sites where the sandstone material is overlaid with shallow morainic deposits (Stutz, 1985), whereas the neighboring soils developed from sandstone are conducive (i.e. allowing plant infection and spread of the disease) to the same pathogen (Stutz et al., 1986). Phl$^+$ fluorescent pseudomonads effective at protecting plant from disease (e.g. strain CHA0) can be readily isolated from these suppressive soils (Sharifi-Tehrani et al., 1998; Wang et al., 2001; Ramette et al., 2006). However, Phl$^+$ strains are also present in significant numbers in disease-conducive counterparts (Ramette et al., 2003b), and they have the ability to protect from *T. basicola* when used as tobacco inoculants in certain soils

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0038-0717/$ – see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.soilbio.2010.01.005

(Ramette et al., 2006). In contrast, results obtained for soils suppressive to wheat take-all indicate that Phl$^+$ pseudomonads colonize wheat roots in much higher numbers compared with conducive soils (Raaijmakers et al., 1997; de Souza et al., 2003).

The presence of Phl$^+$ pseudomonads in large numbers in both disease-suppressive and conducive soils from Morens prompted analyses aimed at comparing the genetic diversity of these bacteria. Characterization of Phl$^+$ fluorescent pseudomonads has been performed by restriction analysis or sequencing of biocontrol genes such as *phlD* and hydrogen cyanide synthase-encoding *hcnBC* (Keel et al., 1996; Sharifi-Tehrani et al., 1998; McSpadden Gardener et al., 2000; Picard et al., 2000; Mavrodi et al., 2001; Ramette et al., 2001, 2003a,b; Wang et al., 2001; de Souza et al., 2003; Couillerot et al., 2009). At Morens, the majority of *phlD* and *hcnBC* alleles were found both in isolates from suppressive soils and isolates from conducive soils (Ramette et al., 2003b, 2006). However, all these results were based on the isolation of Phl$^+$ pseudomonads on plates, which underestimates *phlD* diversity (Frapolli et al., 2008), and these isolates were very limited in number.

To avoid limitations associated with the analysis of *Pseudomonas* colonies, several *phlD*-based DGGE procedures have been developed to assess populations of Phl$^+$ fluorescent pseudomonads (Bergsma-Vlami et al., 2005; Frapolli et al., 2008). The protocol of Frapolli et al. (2008) is promising in that it is the only one that allows simultaneous assessment of *phlD*$^+$ pseudomonads of the '*Pseudomonas fluorescens*' complex and of divergent group ARDRA-1 (Keel et al., 1996).

The objective of the current work was to compare the genetic diversity of natural populations of Phl$^+$ pseudomonads from the rhizosphere of tobacco grown in different Morens soils suppressive or conducive to *T. basicola*-mediated black root rot. Unlike in previous studies, this was done using soil samples collected in more than one year, and the effect of plant health on the diversity of Phl$^+$ pseudomonads was also considered, by performing rhizosphere experiments with tobacco seedlings transplanted into soils with or without *T. basicola* inoculation. The assessment was based on a recent *phlD*-DGGE approach involving prior enrichment of target bacteria by incubating rhizosphere extracts in semi-selective medium, which is known to recover a higher diversity of Phl$^+$ pseudomonads compared with direct DGGE analysis of rhizosphere DNA, without apparent counter-selection side-effects against particular types of Phl$^+$ pseudomonads (Frapolli et al., 2008).

2. Materials and methods

2.1. Soils and greenhouse experiments

The *T. basicola*-suppressive soils MS8 and MS16 and the conducive soils MC10 and MC112 are located within a few kilometers of each other in the Swiss region of Morens (Stutz, 1985; Stutz et al., 1986; Ramette et al., 2003b). They were collected in June 2004, November 2004 and June 2006. The soils taken in June 2006 were analyzed by the Swiss soil testing service (Nyon, Switzerland) and shown to display similar physicochemical properties (Table 1). The following conditions were used for all three experiments. Soil samples were taken from 5 to 30 cm (topsoil horizon) depth using sterilized tools and were maintained at 15 °C before use (within the subsequent 14 days). Soil was sieved (3 mm) and root residues and stones were removed. Tobacco (*Nicotiana glutinosa* L.) was grown for 4 weeks in coarse quartz sand (1.9–2.2 mm diameter) in a growth chamber (70% relative humidity) with 16 h of light (80 m Em^{-2} s^{-1}; 22 °C) and 8 h of darkness (18 °C), with weekly watering with Knop's nutrient solution (Ziegler, 1983), before transplantation into soil. Endoconidia suspension of the fungus *T. basicola* Ferraris strain ETH D127 (5 ml, to reach 10^3 of endoconidia cm^{-3} soil) was added to soil around the stems of the 4-week-old tobacco plants on the same day of transplantation in Morens soils. The same volume of distilled water was added to the non-inoculated controls. Soil water content was adjusted to 25% w/w (retention capacity for these soils) by watering pots every 1–2 days with distilled water. Sampling was done at 3 weeks.

Table 1
Characteristics of Morens soils (cambisols) used in this study.

	MS16	MS8	MC112	MC10
Clay (%)[a]	12.2	10.7	16.1	15.8
Silt (%)	28.9	29.6	29.9	30.0
Sand (%)	58.9	59.7	54.0	54.3
pH [water]	6.6	7.8	6.8	7.2
Organic matter (%)	1.6	1.3	2.2	2.1
$CaCO_3$ total (%)	0	6	0	1
Fe [$C_2H_4O_2$ 1:10] (mg kg^{-1})	293	230	251	487

[a] All were sandy loams.

The June 2004 experiment focused on *Pseudomonas* diversity only (with the four soils), whereas the November 2004 experiment dealt with plant health, *Pseudomonas* numbers and *Pseudomonas* diversity (with the four soils), and the June 2006 experiment with plant health, *Pseudomonas* numbers and *Pseudomonas* diversity (with soils MS8 and MC112). The number of pots, each containing one plant, ranged from 6 to 12 per treatment (depending on the experiment). Disease severity was recorded 3 weeks after inoculation as the percentage of root surface covered by *T. basicola* chlamydospores. The amount of root disease was rated visually using a height-class disease scale (Stutz et al., 1986) based on midpoints of disease degree intervals.

2.2. Pseudomonas enrichment and lysis

Three weeks after transplantation, tobacco plants were carefully removed from the pots. The root systems were shaken to remove loosely-adhering soil, washed with sterile distilled water and put (usually 50–100 mg roots and tightly-adhering soil) into sterile 50 ml falcon tubes filled with 10 ml of 0.9% NaCl solution. The tubes were shaken for 30 min at 350 rev min^{-1}, and they were used as rhizosphere extracts. For growth of *phlD*$^+$ *Pseudomonas* populations, rhizosphere extracts as well as serial dilutions were mixed with KB^{+++} medium (Simon and Ridge, 1974), using the following most probable number (MPN) design. A 96-well microtiter plate in which each well contained 180 µl of KB^{+++} was employed to perform decimal dilutions using 20 µl of cell extract (4 wells per dilution). The microtiter plates were covered with thermal seal film (Axygen scientific, Union City, CA) to avoid steaming and were incubated for 2 days at 27 °C under shaking at 150 rev min^{-1}. Microtiter plates were then checked for the occurrence of (i) fluorescent pseudomonads (based on fluorescence under UV light at 366 nm) and (ii) *phlD*$^+$ pseudomonads by PCR (described below). 20 µl of bacterial culture were taken from each well and replica transferred to wells containing 50 µl sterile distilled water (in 96-well PCR plates), and cells were lysed at 99 °C for 10 min in a thermal cycler. The microtiter plates were stored at −80 °C after addition of glycerol (40% final concentration).

2.3. phlD amplification from MPN wells and collection strains for DGGE analysis

PCR amplification of positive MPN wells and of KB-grown marker strains for DGGE analysis was performed as follows. The gene *phlD* was amplified using forward primer B2BF (25-mer, 5′-ACCCACCG-CAGCATCGTTTATGAGC-3′) containing a 40-bp GC clamp at the 5′ end, and reverse primer BPR4 (26-mer, 5′-CCGCCGGTATGGAA

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