Multitrophic interactions in soil
IOBC/wprs Bulletin Vol. 29 (2) 2006
pp. 167-172

Microbial diversity in wheat rhizosphere as affected by genetically modified *Pseudomonas putida* WCS358r

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Abstract: Introduction of genetically modified micro-organisms (GMOs) in the environment can lead to perturbations of soil ecosystems. To monitor possible disturbances by GMOs, the impact of *Pseudomonas putida* WCS358r and two genetically modified derivatives, which constitutively produce phenazine-1-carboxylic acid (PCA) or 2,4-diacetylphloroglucinol (DAPG), on the rhizosphere microflora of wheat was studied. The GMOs were introduced into soil as a wheat seed coating in 1999 till 2002. Every year the same treatment was applied in the same field plots. To compare a possible impact of the GMOs to that of common agricultural practice, a crop rotation of wheat and potato was incorporated in the field experiment and evaluated against continuous wheat cropping. The microflora was studied by extracting DNA directly from rhizosphere soil and subsequently amplifying rDNA using bacteria-specific primers. The resulting amplicons were separated using denaturing gradient gel electrophoresis (DGGE). In the first year all bacterial treatment had a transient impact on the indigenous bacterial microflora. After repeated introduction the DAPG-producing GMO had a significant effect on the bacterial microflora as from the second year, and the PCA-producing GMO after the fourth introduction. Cropping potato had a clear long-term impact that continued to exist in a subsequent wheat crop. The impact of the GMOs was never larger than that of changing crop from wheat to potato.

Rhizosphere samples of control wheat plants and of plants treated with wild-type bacteria or the GMOs were also analyzed using an Affymetrix GeneChip® containing 16S rDNA sequences of approximately 9000 bacterial operational taxonomic units. Depending on the year and the treatment differences were observed in occurrence of specific bacterial groups.

Key words: 2,4-diacetylphloroglucinol, denaturing gradient gel electrophoresis, genetically modified microorganisms, microarrays, microbial diversity, phenazine-1-carboxylic acid, 16S rDNA

Introduction

Large-scale introduction of genetically modified microorganisms (GMOs) in the environment might affect the natural soil ecosystem. In previous field experiments it was indeed demonstrated that introduction of a *Pseudomonas putida* strain that was genetically improved in its biocontrol properties through the production of the antimicrobial compound phenazine-1-carboxylic acid (PCA), resulted in a transient shift in the natural soil microflora (Glandorf et al., 2001). Since under commercial cropping conditions GMOs will be introduced not only at a single time point but probably every year in the same field, the aim of the present field study was to determine whether repeated introduction of the GMOs in the same field intensifies the earlier observed shift in the microflora. Effects observed in the earlier studies may have been specific for PCA and, therefore, in the present study a second GMO was introduced that constitutively produced the antimicrobial compound 2,4-diacetylphloro-

glucinol (DAPG) (Bakker et al., 2002; Viebahn et al., 2003, 2005). Since only a small part of the soil microflora can be cultured (Atlas and Bartha, 1998; Hugenholtz and Pace, 1996) cultivation-independent techniques were used to assess the effects of the introduced bacteria. DNA was isolated directly from rhizosphere samples, and small subunit (16S) ribosomal DNA (rDNA) was amplified with specific primers and subjected to denaturing gradient gel electrophoresis (DGGE) (Viebahn, 2005). DGGE can reveal changes in microbial communities, however, it is not suitable to identify the microorganisms that are affected by a certain perturbation. To identify bacterial species affected by the introduction of GMOs, additional molecular techniques were used. PCR amplified DNA of rhizosphere samples was hybridized to Affymetrix GeneChips® containing 16S rDNA sequences (DeSantis et al., 2003) of approximately 9000 operational taxonomic units (OTUs).

Material and methods

Bacterial strains and experimental field

For this field experiment the plant growth-promoting rhizobacterial strain *P. putida* WCS358r (Geels and Schippers, 1983) was modified with the *phz* biosynthetic gene locus of strain *P. fluorescens* 2-79 (Thomashow and Weller, 1988) that codes for the production of PCA. In addition, this strain was genetically modified with the *phl* biosynthetic gene cluster from *P. fluorescens* Q2-87 coding for the production of 2,4-diacetylphloroglucinol (DAPG) (Bangera and Thomashow, 1999; Viebahn et al, 2003). Both PCA and DAPG are anti-microbial compounds that have been implicated in suppression of plant pathogens by fluorescent pseudomonads. The genes were inserted into the chromosome of WCS358r by using a mini-transposon (Herrero et al., 1990). The constructs resulted in constitutive PCA or DAPG production by the GMOs (Glandorf et al., 2001; Viebahn et al., 2003). The GMOs were introduced into the soil as a coating on wheat seeds. To study possible synergistic effects of PCA and DAPG production, the GMOs were applied either separately or in combination. Every year the same treatments were applied in the same plots of the experimental field. Each treatment was repeated in 6 plots, and samples were collected each year at different time intervals after sowing (Viebahn et al., 2003). Effects of the GMOs on the natural microflora on plant roots were compared to effects of the parental strain. To compare effects caused by the GMOs with effects caused by common agricultural practice such as crop rotation, one treatment consisted of rotation plots, where in 1999 and 2001 wheat, and in 2000 and 2002 potatoes were grown.

DNA extraction and PCR

Three to five g of roots with adhering soil were sampled from each plot and mixed with 10 ml sodium phosphate buffer (120 mM, pH 8). One g of gravel was added and samples were vortexed for 30 s. The supernatant was decanted into a new tube. One ml of the supernatant was used to extract total DNA with the FastDNA®SPIN Kit for Soil (Bio 101, Biogene, Vista, Calif.) in combination with a Ribolyser (Hybaid, Ashford, UK), as previously described (Smit et al., 2003). The extracts were suspended 1:100 in 100 µl Millipore-filtered distilled water before purification with the Wizzard® DNA Clean-up System (Promega, Madison, WI) according to the manufacturer's protocol.

Amplification of the rDNA for the DGGE was done with the primer pair F-968-GC and 1491R (Nübel et al., 1996). F-968-GC contained a 40 bp GC-clamp to stabilize the melting behavior of the DNA fragments for DGGE analysis (Sheffield et al., 1989). The PCR was performed in 10x PCR buffer 2, pH 9.2 containing 2.25 mM $MgCl_2$ (Roche Diagnostics, Mannheim, Germany), 250 µM of each of the four deoxynucleoside triphosphates, 250 nM of each primer, 2.5 U Expand Long template enzyme (Roche, Diagnostics, Mannheim, Germany) and 1 µl of appropriately diluted template DNA. The PCR conditions used in the

thermocycler (Hybaid, Ashford, UK) were: 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, and 3 min at 72°C, and finally 10 min at 72°C.

Amplification of rDNA for the microarray analysis were carried out using the primer pair 27F and 1492R (Lane, 1991), which amplify the 16S rRNA genes of a wide range of members of the domain Bacteria. The PCR was performed as described above, except that the concentration of each of the primers was 1 μM. The PCR conditions were: 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 53°C, and 1 min at 72°C, and finally 7 min at 72°C.

DGGE

PCR fragments were separated on a denaturing gradient polyacrylamide gel consisting with a urea formamide denaturant gradient of 30–60 %. A 100 % denaturing acrylamide gel contained 7 M urea and 40 % formamide. PCR products were loaded and gels were run for 17 h at 80 V at a constant temperature of 60°C in a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Veenendaal, The Netherlands). After electrophoresis gels were stained with SybrGold (Molecular Probes, Leiden, The Netherlands) and viewed under a blue light transilluminator (Clare Chemical Research, Dolores, Colorado). Images were digitalized using the GeneGenius Bio Imaging System (Syngene, Cambridge, UK). The bacterial community fingerprints of the DGGE gels were analyzed with the BioNumerics program vers. 3.5 (Applied Maths, Sint-Martens-Latem, Belgium).

Microarray analysis

Two μg DNA amplicons were prepared containing One-Phor-All Buffer Plus and 0.2 U DNAse I per μg DNA (Invitrogen, Carlsbad, CA) in a 50 μl solution, and fragmentation was performed for 10 min at 25°C, followed by 10 min at 98°C. The fragmented products were biotin-labeled for 1 h at 37°C using the Enzo®BioArray Terminal Labeling Kit (Affymetrix, P/N 900181) according to the manufacturer's protocol.

Hybridization was done according to the GeneChip® expression analysis technical manual as previously described (Masuda and Church, 2002). The arrays were scanned at 570 nm with a resolution of 3 μm with a GeneArray scanner (Affymetrix, Inc.). Data analysis was performed by using custom software, which scaled each array. Cluster analysis was performed with GeneMaths vers. 2.1 (Applied Maths, Sint-Martens-Latem, Belgium).

Results and discussion

DGGE analysis

For each year composite dendrograms representing three to four sampling dates were constructed. In 1999 all bacterial treatments clustered separately from the non-treated control plots (figure 1A, please note that the rotation plot (rp) was cropped to wheat in 1999). In the second year three clusters were apparent, one containing the samples from the plots cropped to potato, one with those treated with the DAPG-producing GMO, and one cluster containing the control treatment and samples treated with the wild type or the PCA producer (figure 1B). In 2001 again the crop rotation plots were different from the continuous wheat plots, whereas it was now cropped to wheat. All bacterial treatments affected the community composition; however, the DAPG producer had an effect differential from the wild type and the PCA-producing GMO (figure 1C). In 2002 the GMOs affected the bacterial community composition, their effects never exceeded those of changing crop from wheat to potato (figure 1D).

Introduction of a DAPG-producing derivative of *P. putida* WCS358r affected the bacterial microflora after repeated introductions. The PCA-producing GMO affected the bacterial microflora differentially from the parental strain only after 4 consecutive introductions. These effects never exceeded those of changing crops from wheat to potato.

Cropping potato once had a long lasting effect on the microflora, as it was still apparent one year later.

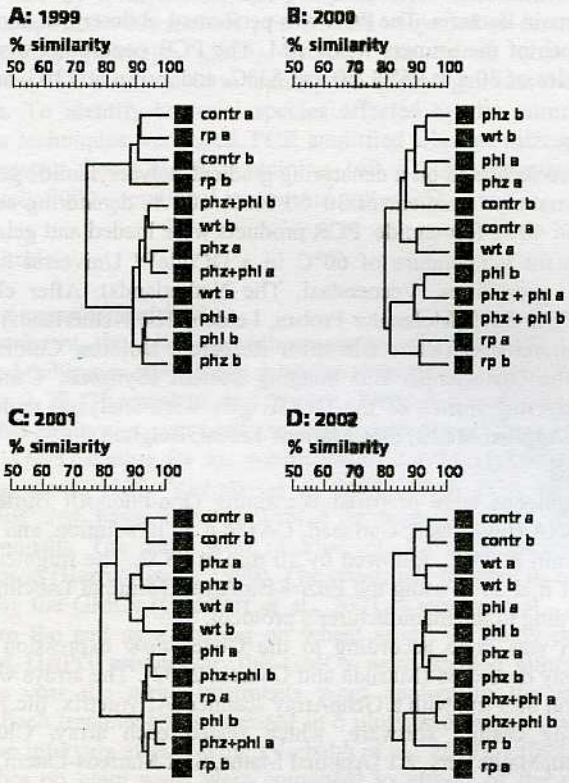

Figure 1. Composite dendrograms representing the genetic similarity of the bacterial communities of field-grown wheat plants in 1999 (A), 2000 (B), 2001 (C), and 2002 (D). Wheat seeds were treated with a control coating containing no bacteria (contr), the parental strain WCS358r (wt), the DAPG producer (phl), the PCA producer (phz) or the combination of both GMOs (phz+phl). In a rotation plot (rp) untreated wheat was sown in 1999 and 2001 and in 2000 and 2002 untreated potato was planted. The six replicates of each treatment were pooled to two replicate samples. Similarities are based on DGGE patterns generated from 16S rDNA fragments.

Affymetrix GeneChip® data

The GeneChip® data were used to construct dendrograms to identify similarity between treatments (data not shown). Two main clusters were apparent, one containing samples from the GMO-treated plots, and one from the control and wild-type treated plots. The same samples were subjected to DGGE, resulting in a similar dendrogram (figure 2). Analysis of the individual signals from the GeneChip® data revealed that bacterial diversity in the rhizosphere of control plants was lower than that in the rhizosphere of treatments with the bacteria, either wild type or GMO.

Introduction of antagonistic bacteria seemed to increase rather than decrease bacterial biodiversity in the wheat rhizosphere

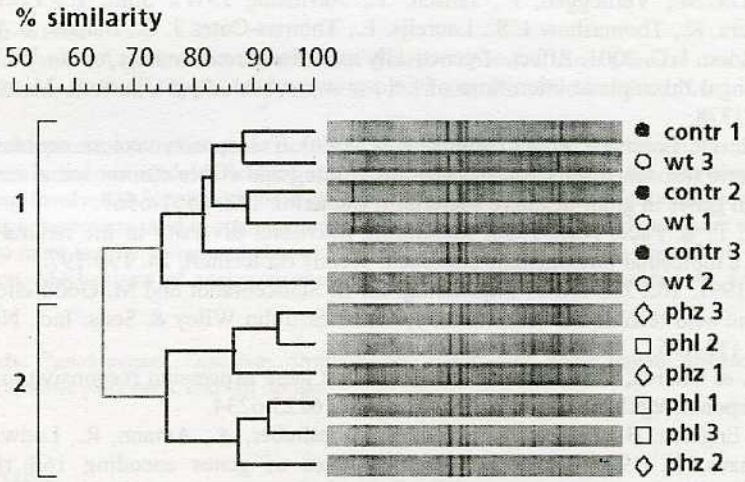

Figure 2. Dendrogram based on the genetic similarity of the bacterial communities of field-grown wheat plants in 2002 with the corresponding DGGE gel. Wheat seeds were treated with *P. putida* WCS358r (wt), the DAPG-producing derivative of WCS358 (phl), the PCA producer (phz), or a control coating containing no bacteria (contr). Significant clusters are indicated by the gray line.

Acknowledgements

Dr Linda Thomashow (USDA, ARS, Pullman, WA, USA) is gratefully acknowledged for providing the two GMO derivatives of WCS358r. We thank Bas Valstar and Fred Siesling (Botanical gardens, Utrecht University) for constructing and maintaining the experimental field site, and Christiaan Veenman and Rogier Doornbos for excellent technical assistance.

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