Monitoring the fate and ecosystem effects of genetically modified
Pseudomonas putida producing phloroglucinol and phenazine in wheat
rhizosphere.

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Introduction
There is increasing interest in applying microorganisms to control soil-borne plant pathogens. Performance and activity of such microorganisms was frequently inadequate. Combining several modes of action against plant pathogens in one single organism by genetic modification might improve their efficacy. Despite long-term experience with introducing non-modified microorganisms, concern about the ecological impact of large-scale release of genetically modified microorganisms (GMMs) has been raised. The impact of these genetically improved biocontrol strains may affect the soil microbial ecosystem. To date, field studies with genetically modified bacteria have focused mainly on microorganisms with markers, which are not expected to affect the indigenous soil microflora. Effects of GMMs have been studied mainly in microcosm experiments, however, these microcosms lack the full biotic and abiotic components of a field environment.

Pseudomonas putida WCS358r was modified to produce the antifungal compound phenazine-1-carboxylic acid (PCA) (Thomashow et al. 1990), or the antifungal and antibacterial compound 2,4-diacetylphloroglucinol (DAPG) (Bangera and Thomashow, 1999). These strains were applied as seed coating on wheat seeds. Field experiments were performed in the years 1997, 1998, 1999 and 2000. Our objective was to determine activity, survival and ecosystem effects of genetically improved biocontrol bacteria on the fungal and bacterial rhizosphere microflora, using cultivation-independent 18S and 16S rDNA analysis

Material and Methods

Strains
Pseudomonas putida WCS358r is a plant growth-promoting rhizobacterial strain with disease-suppressive properties, based on the production of its fluorescent siderophore (Bakker et al. 1986). A rifampin resistant derivative of WCS358, WCS358r, was used as the parental strain. The phzABCDEFG genes from P. fluorescens 2-79 (Mavrodi et al. 1998), under control of the P_tac promoter were introduced on a mini-Tn5 LacZ1 transposon into WCS358r, resulting in two derivatives, GMM 2 and GMM 8, with different levels of PCA production (Glandorf et al. 2001). Likewise, WCS358r was modified to produce DAPG by inserting, on a kanamycin-resistant mini-Tn5 lacZ1 transposon, the phlABCDEFG genes (Bangera and Thomashow 1999). The gene cluster contained its own promoter, however, the phlF gene, encoding a repressor of DAPG synthesis, was disrupted to promote constitutive production of DAPG. The DAPG producing derivative of WCS358r was labeled as GMM P. Bacteria were grown on Kings medium B (KB) containing the appropriate antibiotics.
Field experiment

In 1997 and 1998, the experiments were conducted on a site located at De Uithof, Utrecht, The Netherlands. A randomized block design was used with four treatments, with six replicates each, resulting in a total of 24 plots, each of 1 m². The four treatments were seeds treated with WCS358r, GMM 2, GMM 8, and non-bacterized seeds (control). In 1999 repeated introductions of GMMs were started. Again a randomized block design was used, with six replicates for each treatment. The treatments were seeds treated with WCS358r, GMM 8, GMM P, a mixture of GMM 8 and GMM P, and non-treated seeds. Each year following 1999 the same treatments were applied to the same plots. Per plot about 1750 wheat seeds were sown manually in 11 rows of 1 m length at a depth of 2-3 cm. Plots were separated from each other by 60 cm bare strips. The experimental field was fenced to block rabbits from entering the site and bird entry was prevented using nets.

Ecosystem effects

Total DNA was extracted from soil samples. Ribosomal DNA was amplified using primers specific for bacterial 16S rDNA or fungal 18S rDNA. Fungal PCR products were digested with TaqI and bacterial PCR products with HinfI. The fragments were separated on polyacrylamide gels. Dendrograms representing percentage similarity of banding patterns were constructed by UPGMA cluster analysis using the algorithm of Nei and Li or Dice.

The 1997 samples were further studied by specifically zooming in on the Fusarium population by sequencing Fusarium-like clones selected by ARDRA from a clone-library (Leeflang et al., 2002).

Results

Survival

In all years populations of WCS358r and the GMMs decreased from about $10^7$ CFU per gram of rhizosphere sample to $10^2$-$10^4$ CFU per gram at harvest, and to near the detection limit ($10^2$-$10^3$ CFU/g rhizosphere sample) one month after harvesting (131 or 139 days after sowing). In general no indications were found that the fitness of the GMMs was affected by the genetic modification, as numbers of CFUs of the parental strain and the GMMs were comparable. Also no differences were observed between numbers of the GMMs on rifampicin-containing KB with or without kanamycin, suggesting that the phz and phl genes were stable in the bacterial chromosome throughout the growing season.

Activity

Detection of PCA in rhizosphere extracts was done using HPLC and mass spectrometry. Rhizosphere extracts obtained in the field trial of 1998 twelve days after sowing were fractionated using reversed phase HPLC. In extracts of control- and WCS358r-treated wheat rhizosphere no PCA was present. HPLC chromatograms of rhizosphere extracts of wheat plants treated with GMM 2 and GMM 8 had peaks with the same retention time as standard PCA and the presence of PCA was confirmed by mass spectrometric analysis of these peaks. Comparison of the heights of the PCA peaks suggests that PCA production in the rhizosphere by GMM 8 is higher than the production by GMM 2.

Ecosystem effects

Seed application of both WCS358r and the PCA-producing GMMs caused a shift in the
fungal population of wheat roots, as indicated by cluster analysis of replicate ARDRA-generated profiles of rhizosphere samples. Treatments are considered to be different, if both replicate ARDRA patterns of one treatment cluster together, apart from patterns of other treatments. In this case the replicate ARDRA patterns per treatment are more similar to each other than to other patterns. Effects on the fungal microflora as a result of bacterization with WCS358r or the GMMs seemed differential, since the ARDRA profiles from the GMM-treated samples clustered separately from the WCS358r-treated samples and from the control treatment. Effects of the GMMs could be observed up to 40 days (1997) and 89 days (1998) after sowing, whereas WCS358r-induced effects were detectable up to 12 and 40 days, respectively. In both years all treatments cluster together one month after harvest, indicating that the effects induced by the bacterial treatments were transient (Glandorf et al., 2001).

The 1997 samples were further studied by specifically zooming in on the Fusarium population using a molecular method (Leeflang et al., 2002). Seventy Fusarium-like clones, selected from a library consisting of 1000 clones, were selected and sequenced. Analysis showed that both the WCS358r and the GMM inhibited the development of Fusarium type I. This probably allowed other Fusarium types to develop and resulted in a higher diversity of different Fusarium types in the WCS358r and the GMM treatments.

In 1999 and 2000, next to effects on the fungal microflora, effects on the bacterial microflora were detected. The DAPG producing derivative of WCS358r caused a shift in the fungal microflora that lasted until the end of the growing season. For the bacterial microflora a transient shift up to 40 days was observed for the treatments with the DAPG producers. In 2000, however, no distinct clustering patterns could be observed, and similarity between replicate samples was low, suggesting that the natural heterogeneity of microbial populations exceeded possible effects of the GMMs.

Discussion

No differences in survival between the genetically modified strains and the wild type were observed, indicating that the extra metabolic load did not affect the ecological fitness of the GMMs.

In the field trials of 1997 and 1998, both introduction of the modified and wild type strains resulted in a transient effect on the composition of the rhizosphere fungal microflora, as determined by 18S rDNA analysis. This was most prominent at the beginning of these field trials, when the numbers of introduced bacteria were relatively high (Glandorf et al. 2001). The WCS358r-induced effect on the fungal microflora is probably caused by the production of pseudobactin 358, the fluorescent siderophore of WCS358 (Bakker et al. 1986). GMM-induced impact on the composition of the fungal microflora lasted longer than the WCS358r-induced impact. The GMM-induced shift in the fungal microflora was longer lasting and differed qualitatively from the shift caused by the parental strain. This indicates that the PCA produced by the GMMs also affected the composition of the fungal microflora. The detection of PCA in the rhizosphere of GMM-treated plants and not in rhizosphere samples of WCS358-treated plants and control plants supports the role of PCA in these shifts in the fungal microflora.

In 1999, introduction of the DAPG producing GMM, either as a single application or in the combination with the PCA producer, had a long lasting effect on the rhizosphere fungal microflora, as determined by 18S rDNA analysis. For the same treatments a transient effect was observed on the bacterial microflora, based on 16S rDNA analysis. It was expected that the intensity of the effects would increase with repeated introduction of the bacterial strains in the same plot. However, in 2000 no clear effects of bacterial treatments were observed on either the fungal or the bacterial microflora.

Thus in this year of the experiment effects of the introduced GMMs did not exceed those of
natural variation. In the 2000 experiment we also observed that seed treatment with bacteria resulted in increased plant growth. This plant growth promotion was independent of the ability of the bacteria to produce PCA or DAPG.

Conclusions

Our results show that introduction of PCA-producing GMMs can transiently affect the composition of the rhizosphere fungal microflora of field-grown wheat. When introduced for the first time, the DAPG-producing GMM had a longer lasting effect on both the bacterial and fungal microflora, This effect was no longer observed at the beginning of the following season, and, contrary to our expectation, no enhanced effects were observed by repeated introduction.

References


