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Effects of agronomical measures on the microbial diversity of soils as related to the suppression of soil-borne plant pathogens

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Abstract

The diversity of soil microbial communities can be key to the capacity of soils to suppress soil-borne plant diseases. As agricultural practice, as well as directed agronomical measures, are known to be able to affect soil microbial diversity, it is plausible that the soil microflora can be geared towards a greater suppressivity of soil-borne diseases as a result of the selection of suitable soil management regimes. In the context of a programme aimed at investigating the microbial diversity of soils under different agricultural regimes, including permanent grassland versus arable land under agricultural rotation, we assessed how soil microbial diversity is affected in relation to the suppression of the soil-borne potato pathogen *Rhizoctonia solani* AG3. The diversity in the microbial communities over about a growing season was described by using cultivation-based – plating on different media – and cultivation-independent – soil DNA-based PCR followed by denaturing gradient gel electrophoresis (DGGE) community fingerprinting – methods. The results showed great diversity in the soil microbiota at both the culturable and cultivation-independent detection levels. Using cultivation methods, various differences between treatments with respect to sizes of bacterial and fungal populations were detected, with highest population sizes generally found in rhizospheres. In addition, the evenness of eco-physiologically differing bacterial types was higher in grassland than in arable land under rotation. At the cultivation-independent level, clear differences in the diversities of several microbial groups between permanent grassland and arable land under rotation were apparent. Bio-assays that assessed the growth of *R. solani* AG3 hyphae through soil indicated a greater growth suppression in grassland than in arable land soils. Similarly, an experiment performed in the glasshouse showed clear differences in both microbial diversities and suppressiveness of *R. solani* growth in soil, depending on the presence of either maize or oats as the crop. The significance of these findings for designing soil management strategies is discussed.

Introduction

Microorganisms in agricultural soils are known to exert profound influences on the soil's fertility status, in particular with respect to the availability of plant nutrients, as well as on the suppression of soil-borne plant diseases (Kennedy & Smith 1995). These key life support functions of soil are likely to be dependent on crucial constituents of the soil microbial community. In fact, the "health" of a soil can be defined in terms of its microbiological capacity to counteract (suppress) the activity of plant-pathogenic or plant-deleterious (micro)organisms (Van Bruggen & Semenov 2000). This suppressiveness has been conceptually divided in "general" versus "specific" suppressiveness, wherein general suppressiveness is conferred by aspecific activities of a myriad of undefined organisms (e.g. resulting in competition for essential nutrients with target pathogens), and specific suppressiveness is related to a specific activity, e.g. antagonism, of defined organisms (Van Bruggen & Semenov 2000). Specific suppressiveness can perhaps be best illustrated by the causal relationship between the decline of take-all disease in wheat by wheat monocropping, and the concomitant increase of populations of fluorescent pseudomonads that produce the antifungal antibiotic 2,4-diacetyl phloroglucinol (Raaijmakers & Weller 1998).

However, in many cases the nature of disease suppression is unknown, and the putative causal agents involved have not been identified. In addition, the majority of soil microorganisms, which for bacteria can amount to 99% of the total microbiota, is known to be non-culturable (Kuske et al. 1997; Staley & Konopka 1985), which makes an assessment of the specific or even general disease-suppressive properties present in the system inherently difficult (Ward et al. 1995).

It is, therefore, primordial to obtain a thorough understanding of how, and to what extent, the microbial diversity in soils is, or can be, affected by agricultural practices (cf. Abawi & Widmer 2000; Mazzola 1999; Nüsslein and Tiedje 1999). In addition, the ecology of the key microbial interactions that take place in the soil environment and strategies to direct these, also need scrutiny (Mazzola 1999). It is often postulated that many healthy soils (should) show a certain degree of resilience, i.e. they possess the capacity to return to their original status following a disturbance or stress (Van Bruggen & Semenov 2000). In particular, disturbance of a soil microbial system may not lead to great effects on soil functioning as a result of the functional redundancy present in the system (Turco et al. 1994; Kennedy & Smith 1995). However, this postulate might change when considering soil suppressiveness to plant diseases, provided sufficient detailed knowledge, at the level of the individual players, on microbial activities in soils becomes available. The conjecture here is that each individual organismal member of the microbial community plays its specific role in its specific niche, and this role may not be easily replaceable by the other organisms present in the system. In addition, suppression of phytopathogens may be conferred by small groups of specific microorganisms, such as the 2,4-diacetyl phloroglucinol producers (Raaijmakers et al. 1997; Picard et al. 2000).

A suite of traditional and advanced methods is currently available for the assessment of microbial diversity in soils (Akkermans et al. 1995; Van Elsas et al. 1997, Tiedje et al. 1999). These methods range from those purely based on assessments of culturable microbial populations, often a minority in soils (Staley & Konopka 1985), to those directly accessing the total soil microflora, i.e. including the non-culturable fractions (Borneman et al. 1996; Gelsomino et al. 1999; Van Elsas et al. 1998, Ward et al. 1999). Other methods that focus on microbial functions (Alef & Nannipieri 1995) enable a view of functional diversity, without specifically assessing the underlying microorganisms. Moreover, the recent strategies to produce and apply DNA micro- arrays containing suites of probes that can report on the phylogenetic and functional status of soils, offer great potential to foster our understanding of the composition and functioning of the microbial communities (Ball & Trevors, 2002; Hurt et al. 2001).

Even with the advent of these advanced soil monitoring methods, the nature of the soil microbiota, its dynamics, activities and interactions, are still largely enigmatic to date (Tiedje et al. 1999). In order to understand the effects agronomical measures have on soil functioning and health, it is primordial that the key effects on the soil microbiota – such as interactions, shifts in community structure and activities – are elucidated. It is, therefore, important to focus on these aspects of microbial communities in the soil systems under study, and to apply a combination of powerful methods that enable the dissection of the soil microbial system into its individual components. This should be at the functional and phylogenetic level, with an additional focus on the spatial and temporal relationships between the individual organisms and functions analysed (Tiedje et al. 1999).

With respect to disease suppressiveness in soil, we hypothesize that the microbial diversity in soils (both evenness and richness) may be affected by the (long-term) cropping history of that soil. To investigate this hypothesis, an experimental site was recently selected where permanent grassland could be studied adjacent to farmland under rotation or under permanent maize cropping. The questions asked were:

1. which groups of microorganisms are dominantly present in grassland versus arable land?
2. which microorganisms are associated with the suppressiveness of potato disease caused by *Rhizoctonia solani* AG3?
3. are the levels of microbial diversity, expressed in its terms evenness and richness, and disease suppressiveness in these soils correlated?

In this paper, we discuss the strategies used to answer these questions on the significance of soil microbial diversity for disease suppressiveness, we describe the current state-of-knowledge and the results obtained to date, and we briefly discuss the future possibilities of the myriad of novel approaches that are currently available.

Experimental procedures

Fields and field treatments

The experimental work for this study was performed at the long-term ecological site at Wildekamp, Bennekom, The Netherlands. The soil at this site is a loamy sand rich in organic matter and with slightly acid to near-neutral pH (5.6–6.5). The site consists of long-term (>20 yrs) grassland fields adjacent to arable land under common agricultural rotation, which includes oats, maize and the phytopathogen-sensitive cash crop potato. In 2000, oats was grown in the arable land rotation plots, followed by maize in 2001. In addition, arable land under continuous maize cropping was also included. A number of treatments were established before the growing season of 2000, using triplicate 10 × 10 m plots per treatment in a randomized block design. In the first year of study reported here, we focussed on the treatments arable land under rotation or with continuous maize, versus permanent grassland.

Mesocosm experiment

In a mesocosm experiment performed in the glasshouse, the effects of oats versus maize on microbial diversity and suppressiveness towards *R. solani* AG3 were assessed. Soil obtained from the Wildekamp field arable land plots, including arable land under continuous maize and arable land under rotation (oats had been grown prior to collection of soil), was used in triplicate 500-g pots. The treatments consisted of planting oats or maize seedlings in each soil type (5 per pot). Plants were grown at 20 °C under a 16 h day/8 h night regime and sampled after 60 days.

Soil sampling and processing

Field soil was sampled by obtaining a large number (100) of individual surface (0–12 cm depth) samples per plot, from triplicate soil plots that had received the same treatment. The soil samples derived from each plot were composited and homogenized. One subsample (10 g) of the composite sample was then homogenized and serially diluted as described (van Elsas et al. 1986). The mesocosms were sampled by carefully removing plants with soil from their containers.

To analyze the rhizosphere, soil surrounding the plants (grass, maize, oats) was carefully removed by manual shaking. Care was taken to preserve the soil tightly adhering to the roots. The root parts were then obtained by cutting, and roots plus tightly-adhering soil were shaken as described (Van Elsas et al. 1986). The resulting rhizosphere soil suspensions were used for dilution spread-plating. Root-adhering soil was also directly separated for soil DNA extractions (see later).

Plate enumerations

Conventional dilution spread-plating was performed to assess the total and specific culturable bacterial and fungal colony-forming units (CFU). For this purpose, different media were used: potato dextrose agar (PDA; Difco, Detroit, MI, USA) was used to obtain total fungal counts. Medium R2A (Difco, Detroit, MI, USA) was used for total bacterial counts. *Pseudomonas*-specific medium S1 (Miller et al. 1990) was employed for enumerating total fluorescent *Pseudomonas* spp. Organisms of the *Burkholderia cepacia* complex were enumerated on TB-T agar (Hagedorn et al. 1987). The numbers of bacilli were determined on 1/10 strength tryptone soya agar (TSA; Oxoid), after pasteurisation of soil samples for 10 min at 80 °C. Finally, chitin-oatmeal (COA; Miller et al. 1990) medium was used for enumeration of actinomycetes. All plates were incubated at 20 °C for up to 14 days, and colonies appearing on them were regularly counted. The plates for assessment of the total bacterial numbers were counted every 2nd day. This way, the numbers of fast-growing organisms (copiotrophs, or r-strategists) and those of slow-growing ones (oligotrophs, or K-strategists) were determined.

Ecophysiological index

To express the distribution of the fast- versus slow-growing bacteria (r- versus K- strategists) in the soil samples in a single number, the Eco-Physiological (EP) index as proposed by De Leij et al. (1993) was calculated (using four classes, i.e. colonies grown after 2, 4, 6 and 8 days) according to the following equation:

$$H' = -\sum (p_i \times \log_{pi})$$

wherein p_i represents each of the classes defined by the rate of colony appearance on plates and is given as a proportion of the total population appearing on the plate. The more even the distribution of the classes, the higher the EP-index is. For instance, for 4 classes, $H'_{max} = 0.65$ and $H'_{min} = 0$ (De Leij et al. 1993).

Soil DNA extraction

Bacterial DNA was obtained from soils by using the procedure of Van Elsas et al. (1997), after Smalla et al. (1993). Fungal DNA was extracted using the protocol described by Van Elsas et al. (2000). DNA was routinely checked for purity and molecular size using conventional gel electrophoresis (Sambrook et al. 1989). All extracts were sufficiently pure to yield PCR products with the primer systems described below.

Molecular methods

The bacterial and fungal soil DNA extracts were used as targets in PCR amplifications of specific regions of the 16S and 18S rRNA genes, respectively, using bacterial- and fungal-specific PCR. Thus, the following primer systems were used: (1) primers for total bacteria (Heuer et al. 1997), (2) primers for total fungi (Van Elsas et al. 2000), (3) Nested, group-specific primers for the following bacterial taxa: *Pseudomonas*, *Bacillus*, *Burkholderia* and Actinomycetes. The *Pseudomonas* and *Bacillus*-specific primer systems enrich for sequences that indicate the presence of the constituents of these two groups. A full account of the development and application of these primer systems is described elsewhere (Garbeva et al. 2002a, b). The *Burkholderia*-specific amplification system has been recently described (Salles et al. 2002). The Actinomycete-specific primer system was applied in accordance with Heuer et al. (1997).

The PCR products obtained with the bacterial and fungal primer systems were sequenced using conventional automated capillary sequencing in ABI-Prism instruments. Subsequently, the sequences were analyzed using the sequence analysis programme BLAST-N accessible via the worldwide web (NCBI site). The closest hits to sequences present in Genbank are reported. This analysis furnished a baseline with respect to the dominant microbial types found in the arable land versus the grassland habitats (60 sequences per habitat analysed). Furthermore, DGGE fingerprinting of total bacterial, total fungal, *Burkholderia* and Actinomycete communities was performed essentially as described (Gelsomino et al. 1999; Heuer et al. 1997; Salles et al. 2002; Van Elsas et al. 2000). DGGE fingerprinting of *Pseudomonas* and *Bacillus* communities both used gradients of 45 to 65% of denaturants (Garbeva et al. 2002a, b).

The DGGE gel pictures were digitised and used for analysis by the Molecular Analyst software (Bio-Rad, Veenendaal, The Netherlands). The unweighted pair group method with mathematical averages (UPGMA; Dice coefficient of similarity) option of the programme was used for clustering and relatedness trees were constructed by using Treecon (Van der Peer, Ghent, Belgium). Diversity indices were determined on the basis of the molecular fingerprint using the Shannon-Wiener diversity (index SW′) option of the programme.

In vitro test for soil suppressiveness to Rhizoctonia solani

A method adapted from De Boer et al. (1998) was used to assess the suppressiveness of soil of hyphal growth of *R. solani*. Briefly, a 6-mm PDA disc containing *Rhizoctonia solani* AG3 biomass was placed on top of soil samples from the different treatments, that were either left untreated or had been microwave-treated. After one and two weeks of incubation at 20 °C, the mycelial extension through soil was measured. The results are expressed as the % of the mycelial extension in the untreated soil relative to that obtained in soil treated by microwave heating.

Size and diversity of bacterial populations in grassland versus arable land

A primary question of this study was concerned with the ability of the type of crop used to affect the belowground (microbial) diversity of soils. We, thus, contrasted permanent grassland, in which the soil had been in continuous contact with plant roots for a long period of time, with the arable land in which varying crops had been present only during the respective growing seasons. The sizes of the culturable microbial populations, and the structures of the total microbial communities were examined.

Culturable bacteria and fungi

The results of the enumerations obtained on plates over one growing season are presented, as average values, in Table 1. The numbers of total bacteria and total fungi remained fairly stable in all samples, and only small differences were detected between samples taken from the same treatments in February, May, September or November 2000 (data not shown). Moreover, the average total bacterial as well as total fungal counts from the grassland were slightly but not significantly higher in the rhizosphere than in the corresponding bulk soil; both counts were higher than

Table 1. Culturable bacterial and fungal populations in agricultural fields in one growing season (sampled in Feb, May, Sep and Nov 2000)*

Type	Arable land		Grassland		Remarks
	Bulk	Rhizosphere**	Bulk	Rhizosphere	
Total fungi	5.43	5.70	5.68	6.05 b	–
Total bacteria	7.35	7.80 a	7.72	7.78	–
Pseudomonads	5.98	6.60 a	5.55	5.75	
Bacilli	5.60	5.80	5.80	5.80	May sample arable land outlier
Actinomycetes	5.65	6.20	6.05	6.22	–
Burkholderia cepacia complex	5.92	6.80 a	5.67	6.11 b	–

*Average values over the season are reported. No significant declining or increasing trends were noted ($P < 0.05$). a and b indicate significant rhizosphere effects.
**Measured only in Nov 2000 ($P < 0.05$).

those obtained in the arable land bulk soil. In the November sampling of the arable land, the counts obtained from the rhizospheres were also higher than the corresponding bulk soil counts.

The bacterial group-specific counts showed varying trends. On average, in the grassland the bacterial counts in rhizosphere soil were equal to (bacilli), or higher than, the ones in the corresponding bulk soil. Per habitat, and with the exception of one sample, the numbers of pseudomonads or bacilli in bulk soil were not different over the season (data not shown; Table 1 shows average counts over the growing season). For pseudomonads, an expected effect of the rhizosphere was visible. Similarly, the population densities of members of the *Burkholderia cepacia* complex did not shift drastically over the season, with average values revolving around log 6 cfu per g soil (Table 1), and consistent rhizosphere effects were also noted. A very similar picture was obtained for the actinomycetes, which also did not show a significant trend over the growing season and were stimulated, albeit not to a significant extent, by the rhizosphere.

Fast versus slow-growing bacteria (r versus K strategists) and ecophysiological indices

Per treatment, the percentages of fast- and slow growing bacteria in the arable land and grassland were very similar in the samples of February, May and September 2000 (Table 2A; averages shown). In all samples, low percentages of fast-growing and higher percentages of slow-growing bacteria were found. Fast-growing bacteria were consistently more numerous in the grassland rhizosphere and bulk soil than in the arable land (bulk) soil. However, this difference was not significant for grassland bulk soil. The results obtained in November 2000 were quite different from those obtained in the earlier samples. The percentages of fast-growing bacteria were higher than those of the slow-growing bacteria throughout, and results were remarkably similar between samples from arable land and grassland (Table 2A).

As expected from the above, the EP indices calculated for the grassland (bulk) soil were consistently higher than the ones obtained for the arable land bulk soil (Table 2B). Surprisingly, this was also true for the November sampling. EP indices obtained for the grassland rhizospheres were similar to or, on two occasions, even higher than those from the grassland bulk soil. Thus, a higher evenness between the different eco-physiological bacterial types in both the grassland rhizosphere and bulk soil versus the arable land bulk soil was indicated.

PCR-DGGE assessments of microbial diversity; total bacteria and fungi

The phylogenetic diversity of the bacterial and fungal microflora in the arable land and the grassland were determined using direct PCR followed by DGGE fingerprinting. First, the differences between the three replicate plots of each treatment were consistently much smaller than the differences between treatments,

Table 2. Fast- and slow-growing bacteria in arable land versus grassland over a growing season

A. Percentages of fast versus slow growing bacteria*

Sample date	Fast/Slow	Arable land	Grassland	
			Bulk	Rhizosphere
Feb/May/Sep	Fast	10.4 a	16.9 ab	23.5 b
	Slow	33.3	33.0	33.9
Nov	Fast	30.6	31.2	32.0
	Slow	10.3	11.5	17.3

% of total colonies. Fast: colonies appearing after 2 days; Slow: colonies appearing after 8 days. Remaining colonies appeared after 4 and 6 days respectively.
a, b indicate statistically different classes ($P < 0.05$).

B. Eco-physiological (EP) indices H′ of culturable bacteria*

Sample date (yr 2000)	Arable land	Grassland	
		Bulk	Rhizosphere
Feb	0.56 s	0.58 ss	0.62 sss
May	0.49 s	0.56 ss	0.55
Sep	0.47 s	0.57 ss	0.55
Nov	0.39 s	0.45 ss	0.49 sss

*Calculated on the basis of four classes, i.e. colonies appearing after 2, 4, 6 and 8 days.
s, ss, sss: significantly different values ($P < 0.05$), with s < ss < sss.

resulting in a clear clustering along treatment. Thus, the total bacterial community structures were different between the bulk soils of, on the one hand, the arable land under rotation and that under continuous maize, and, on the other hand, the grassland (Figure 1). In addition, there were clear and consistent differences between the arable land under rotation and that under permanent maize. The mean numbers of bands in the total bacterial PCR-DGGE analyses were higher in the grassland (37 bands) than in both types of arable land (rotation – 28 bands; permanent maize – 29 bands). There were also differences, along the same lines, between the fungal community fingerprints (data not shown). The Shannon-Wiener indices calculated on the basis of the results obtained for total bacteria, indeed, indicated a significantly higher diversity in the grassland plots than in the arable land plots (Table 3), with a smaller difference between arable land under rotation and arable land under permanent maize.

Table 3. Shannon-Wiener indices (SW′) based on DGGE analyses of total bacteria in arable land versus grassland*

Sample	SW′
Arable land under rotation	4.63 a
Arable land, continuous maize	4.93 a
Grassland	5.86 b

*SW′ indices determined using the Molecular Analyst software. A higher SW′ value indicates a significantly higher diversity (a < b; $P < 0.05$).

Group-specific bacterial patterns

Group-specific PCR-DGGE (*Pseudomonas*, *Bacillus*, *Burkholderia* and Actinomycetes) was applied using the soil DNA extracts from all treatments and sampling points. With all PCR systems, clear differences were found between the patterns of the grassland soil and those of the arable land soil under rotation. In addition, one grassland plot was clearly different from the other two when considering the group-specific DGGE profiles.

Overall, in all samples a higher apparent diversity of *Pseudomonas* spp., measured by numbers of bands, was detected in the bulk and rhizosphere soil of the grassland than in the arable land. In the grassland, 6 to 10 dominant bands were consistently detected, and these were identified as affiliated, at 98 to 100% similarity, with sequences of *P. fluorescens*, *P. putida*, *P. syringae*, *P. migulae*, *P. veronii*, *P. rhodesiae* and *Pseudomonas* sp. In the arable land samples taken in February and May, up to three dominant bands were detected, of which one consistently appearing band was affiliated with *P. rhodesiae*, while in September and November up to six dominant bands were observed. These data are fully described elsewhere (Garbeva et al. 2002b).

The diversity of organisms detected with the *Bacillus* enrichment system could be described along the same lines. A consistently higher diversity of bacilli was found in the grassland (average number of bands: 19) than in the arable land (average: 11). A relative stability of the patterns was found in the grassland, whereas an increase of diversity was noted in the arable land, from low diversity noted in the February sample (6 bands) to considerably higher diversity in the samples taken later during the growing season (10–15 bands).

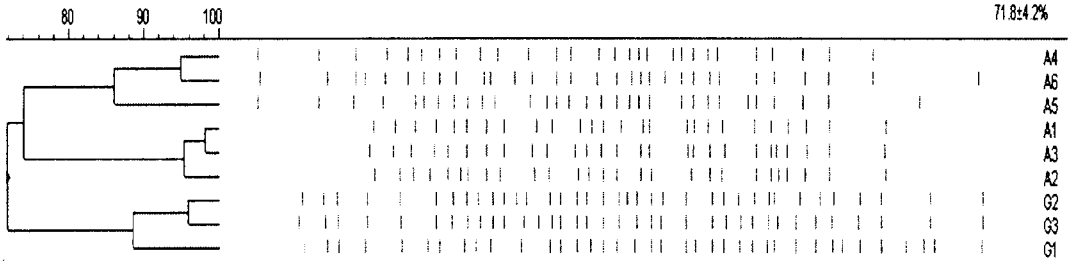

Figure 1. Dendrogram made with UPGMA representing the similarity of bacterial PCR-DGGE profiles generated with DNA from bulk soil – arable land under rotation (A1, A2, A3), arable land under maize monocropping (A4, A5, A6) and permanent grassland (G1, G2, G3). The scale indicates the % of similarity.

Differences between grassland (average band number: 11) and arable land (band number: 9) were also noted for the community structures of *Burkholderia* species in the different samples, with a consistently (although not significantly) higher diversity found in the grassland. Sequences related to a range of *Burkholderia* species, including *B. cepacia*, *B. glathei*, *B. phenazinium* and *B. andropogonis*, were identified in the grassland (described in Salles et al. 2002).

The Actinomycete group also showed higher band numbers in the grassland (average 24) than in the arable land (20). However, the differences between grassland and arable land were not large. Also, these parameters were rather stable over the season in both habitats.

Analyses of directly obtained 16S and 18S rDNA libraries

The analysis of the bacterial-specific sequences obtained from the arable and grassland DNA samples showed that between 31% (grassland bulk soil) and 38% (grassland rhizosphere soil) of the sequences generated were related (levels of similarity >89%), to sequences from unidentified clones or from uncultured organisms in the database. However, phylogenetic analyses of all of these sequences allowed their clustering into clear phylogenetic groups. Thus, all 16S rDNA sequences were related to the α-, β-, and γ-subgroups of the Proteobacteria, the Actinomycetes, the *Acidobacterium* phylum and *Bacillus*. On the other hand, only between 16 (grassland bulk soil) and 25% (arable land bulk soil) of all sequences clustered at a similarity level > 97% to any of the sequences from the database. This suggested considerable novelty among the sequences found. A full account of these data, including data from second samplings, is in preparation. Below, salient features are discussed.

In both the permanent grassland and the arable land under rotation, sequences loosely affiliated with the Actinomycetes and Proteobacteria were most abundant. In the grassland bulk soil, 44% of the analysed sequences belonged to the Actinomycetes and 56% to the α, β and γ subgroups of the Proteobacteria. On the other hand, in the grassland rhizosphere soil, about 27% of the clones affiliated with the Actinomycetes, 70% with the α, β and γ subgroups of the Proteobacteria and 3% with the low-G + C Gram-positives. No evidence for the presence of *Bradyrhizobium* types was found. In the bulk soil of the arable land, about 53% of all sequences was affiliated with the Actinomycetes, 39% with the α- or β-subgroups of the Proteobacteria and 8% with the *Acidobacterium* group. Within the α-subgroup Proteobacteria, sequences closely related to that of the nitrogen-fixing *Bradyrizobium* sp. were found. Thus, overall, the percentage of the total sequences belonging to the Proteobacteria was higher in the grassland than in the arable land. Specifically, several representatives of the α, β and γ subgroup Proteobacteria found in the grassland (*Burkholderia cepacia*, *Ralstonia eutropha*, *Agrobacterium tumefaciens*) were not detected in the arable land.

Fungal 18S rDNA sequences were only analysed for bulk soil samples from the grassland and the arable land. Overall, the sequences were all affiliated with those of members of the Ascomycetes, the Zygomycetes and the Basidiomycetes. The sequences obtained from grassland soil were divided in 13 groups and those from arable land soil in 8 groups (not shown). The largest group in both grassland (80% of the total) and arable land (40%) contained sequences belonging to the genus *Glomus* (arbuscular mycorrhizal fungi). This group, together with one other group containing sequences related to *Mortierella* spp., were the only

Table 4. Suppressiveness of grassland versus arable land soils towards *Rhizoctonia solani* AG3 over a growing season*

Sampling date	Arable land		Grassland
	under rotation	under maize monoculture	
Sep 2000	24.6 a		21.0 b
Nov 2000	28.0 a		16.8 b
Jul 2001	31.0 a	28 a	18.0 b

*Measured as % of hyphal extension of *R. solani* AG3 in soil over that of soil following a microwave oven treatment. Per sampling: a > b ($P < 0.05$).

groups that were shared between the grassland and the arable land.

Soil suppressiveness towards Rhizoctonia solani

The extension of mycelium of *Rhizoctonia solani* AG3 was strongly suppressed by untreated soil of both the arable land and the grassland, as compared to the growth in the respective corresponding soils following microwave oven treatment. Since microwave treatment of soils will result in a strong decrease of bacterial and fungal cell numbers, this indicates a likely (micro)biological origin of the suppressive effect. Table 4 shows the data obtained for the soils under different agricultural regimes. At all sampling times, a significantly stronger growth-reductive effect was found with soil from the grassland than with that from the arable land under rotation, or from the arable land under continuous maize. Hence, the presence of grass in field soil may stimulate, or otherwise affect, the soil microbiota, in such a way that growth of the potato pathogen *R. solani* AG3 is more efficiently antagonised than in soil without grass.

Mesocosm experiment; effects of maize versus oats

A mesocosm experiment was performed in which two different crops, oats versus maize, were studied for their effects on soil microbial community structure (total bacteria, *Pseudomonas* and *Bacillus* assessed) and suppressiveness towards *R. solani* AG3. Maize and oats were, thus, both grown in soils in which the previous crop had been maize or oats.

The direct molecular assessments of microbial diversity based on PCR-DGGE showed a clear effect of the current crop in the DGGE profiles for the total bacterial communities as well as for *Bacillus* spp. In the PCR-DGGE profiles specific for *Pseudomonas*

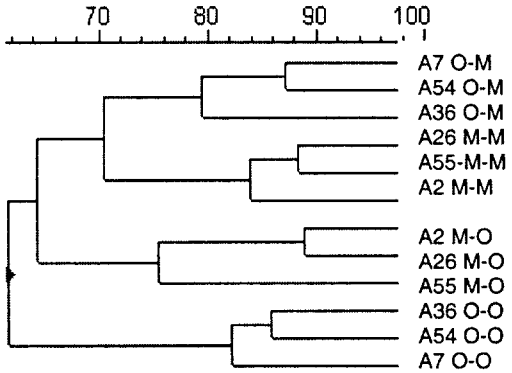

Figure 2. Dendrogram made with UPGMA representing the similarity of bacterial PCR-DGGE profiles generated with DNA from maize versus oats rhizosphere soil from the mesocosm experiment (day 60). A7, A54 and A36 represent soil samples from triplicate field plots in which oats had grown, A26, A55 and A2 represent soil from plots in which maize had grown. O-M: maize grown in glasshouse, previous crop oats, M-M: maize grown, previous crop maize, M-O: oats grown, previous crop maize, O-O: oats grown, previous crop oats. Two main cluster groups were formed with similarity of 64%; one group includes all maize-planted samples, the other one all oats-planted ones. Within these two main clusters, two smaller groups are formed; these subgroups are divided on the basis of the previous crop.

spp., no clear differences were detected between the samples obtained from the maize and the oats mesocosms. As expected, the cluster analysis of the total bacterial DGGE gels by UPGMA strongly revealed the effect of the crop type on community structure (Figure 2). Systems in which maize had been cropped following maize clustered together, while the systems with oats following oats formed another clustering group (Figure 2). Systems in which maize followed oats clustered, albeit clearly as a separate group, together with the systems in which maize followed maize, whereas those in which oats followed maize, clustered, again as a separate group, together with the oats-planted soils that followed oats. We, thus, concluded that the current crop strongly, and overridingly, determined the clustering of these overall bacterial community profiles, suggesting a strong direct effect of the crop type. In addition, the effect of the previous crop was still detectable, albeit at a secondary level.

An analysis of the suppressiveness of the maize- versus oats-treated soils showed that the rhizospheres of both crops enhanced the suppressiveness towards *R. solani* AG3 as compared to uncropped soil. Maize showed a significantly stronger effect than oats, and there was no significant effect of the previous crop (Table 5). Thus, much like what had been observed with grassland, cropping of soil can enhance the ant-

Table 5. Suppressiveness of arable land soil planted to maize versus that planted to oats towards *Rhizoctonia solani* AG3*

Soil sample	Type/previous crop	Current crop		
		Maize	Oats	None
Arable land	Rotation/oats	9.7 a	14.4 b	24.6 c
Arable land	Continuous maize/maize	10.5 a	19.0 b	28.0 c

*Measured as % of hyphal extension of *R. solani* AG3 in microwave-treated soil.
a < b < c ($P < 0.05$).

agonism of soil against *R. solani* AG3, and plant type will determine the extent of this effect.

Significance of findings as related to agricultural practice

The data presented in this study showed that the extent and degree of microbial diversity in soils is clearly affected by agronomical regimes such as the ones studied herein (grassland versus arable land, and planting of different crops). That cover crops (aboveground diversity) can and will affect soil microbial communities and thereby potentially affect suppressiveness to plant diseases has been known for some time (e.g. Mazzola 1999; Raaijmakers & Weller 1998; Smalla et al. 2001). A major driving force in these effects is obviously the influx of sources of carbon and energy into the grossly oligotrophic soil system (Yang & Crowley 2000). Differences in these inputs by different crop plants are likely to induce different microbial community structures (Smalla et al. 2001). Nusslein and Tiedje (1999) elegantly showed that a change in soil use from forest to pasture caused a shift in the G + C content profile of soil DNA, resulting in an estimated 49% change of the microbial community. Direct (vegetation-related) as well as indirect (soil abiotics like pH, organic matter content) effects were invoked to explain the changes in the microbiota. A clear example of the cover crop directly affecting the below-ground microbiota to the benefit of pathogen suppression was provided by the long-standing work from the laboratory of Weller and Thomashow, who showed a link between the suppression of the take-all pathogen *Geaumannomyces graminis var tritici* and the prevalence of 2,4-diacetylphloroglucinol-producing pseudomonads (Raaijmakers & Weller 1998). Similarly, Mazzola (1999) found that apple orchards established in wheat soils suppressed *Rhizoctonia solani* AG5 to a greater extent than those planted in non-wheat areas. An association of this effect with the presence of *Burkholderia cepacia* and *Pseudomonas putida* was postulated, as a reduction in disease suppression was found to coincide with a decrease of these specific populations. Wheat cropping prior to orchard establishment may, thus, have contributed to a higher incidence of these target disease suppressors, resulting in a lowered disease incidence.

On the other hand, and in spite of these promising examples, the significance, magnitude and longevity of the effects measured in our study remains enigmatic and should be the object of further research. For instance, at the level of total bacterial and fungal counts, there were relatively small differences between the grassland and the arable land. This indicates that the biological carrying capacities of the two habitats were in the same order of magnitude. Nevertheless, we cannot exclude that the small differences seen are meaningful for the suppression of plant disease, as the higher populations might affect a larger area of the soil, might be more active, or both. Also, in both plots the percentage of slow-growing bacteria was higher than that of the fast-growing ones during February, May and September. On the other hand, the EP indices in the grassland were higher than the one measured in the arable land, showing that the distribution of different eco-physiological types of culturable bacteria in the grassland was more even than that in the arable land. With respect to the significance of this finding, we can only speculate. Possibly, a more even distribution of different ecological types with pathogen-suppressive activity relates to a greater stability in terms of the capacity of soil to respond to pathogens or other stresses that play a role under different nutritional conditions, resulting in a greater stability of the suppressive effect.

The analyses at the total microbial community level based both on the sequencing of 16S and 18S rDNA genes and on PCR-DGGE of soil DNA with the different primer systems used showed rather consist-

ent differences in the microbial communities between the arable land and the grassland plots. Although one cannot make strong extrapolations from the sequencing work due to the limited numbers of sequences analysed, the affiliation of the sequences obtained and their spread was quite different between arable land and grassland. In addition, the PCR-DGGE profiles, both the overall and the group-specific ones, showed clear differences between arable land and grassland populations. Given that the PCR-DGGE analysis is known to be geared towards the numerically dominant types within each target sequence population (Heuer et al. 1997; Gelsomino et al. 1999), it is clear that, at least at this level, differences occur as a result of the long-term agronomical management regimes used.

With respect to the suppressiveness of these soils to root diseases, we obtained direct evidence based on the reduction of hyphal extension of *R. solani* AG3, about a definite effect of agronomical regime, grassland being more suppressive to rhizoctonial growth than arable land. Similarly, the growth of maize instead of oats in the arable land soil resulted in enhanced suppressiveness, although this might relate to differences in the degree of root development, which was visibly greater for the maize than for the oats. How this relates to the putative development of microbial types that confer specific suppressiveness, is at present unclear, although we obtained evidence for a definite effect of the crops on the bacterial assemblages associated with their rhizospheres. It is known that the hyphal extension of *R. solani* AG3 through soil can be affected by the nutritional status of soil, and so this factor needs proper controls. Also, we did not (yet) find evidence for a difference in the prevalence of specific antibiotic production functions that we screened for, in the different soil treatments (data not shown). It is our ultimate aim to link the functional observations on the suppressiveness of soil systems against *R. solani* AG3, with those on the diversity of various microbial populations, attempting to single out those organisms that might be functionally involved in the suppression of fungal phytopathogens like *R. solani* AG3.

Concluding remarks – soil microbial diversity revisited

It is becoming increasingly clear that, by directly accessing the microbial diversity in agricultural soils, a handle on the soil's intrinsic potential to curb plant disease can be obtained. The data from the experiments described in the foregoing showed that in both the arable land and the grassland plots, considerable diversity can be found, which can be used as a pool of microbial functions, to be sampled for its characterization, or even its exploration and exploitation (MacNeil et al. 1999; Rondon et al. 1999). In spite of the clear-cut differences in the microbial communities in the two habitats at the phylogenetic and functional levels, it is not at all clear what these differences really indicate with respect to soil functioning in terms of the mechanisms of disease suppression in both systems. It is, therefore, necessary to get more focus on these mechanisms, for instance including in the analyses direct assessments of the antagonistic functions operational in the two habitats (Picard et al. 2000; Raaijmakers et al. 1997). This way, the suppressiveness conundrum might finally be dissected into its key components, which might be different in each particular case. Molecular analyses, in particular those in which messenger RNA is directly targeted, are the most promising for inclusion in these assessments, since they assess microbial activities, even in non-culturable cells (Hurt et al. 2001). However, a link to the culturable component of the soil microbiota, if at all possible, is recommendable.

Most importantly, the picture of microbial communities at the level of either phylogenetic or functional diversity in different soil habitats should always be placed in the context of the soil abiotics, that is, the constellation of the key abiotic factors that strongly affect microbial life in soil. It is known that key abiotic determinants, such as soil organic matter or clay content, pH, water holding capacity and temperature, can exert strong controls over microbial activities in soil (Van Veen et al. 1997). Hence, the expression of the full potential for suppressiveness of soil to phytopathogens may be largely dependent on the abiotic soil characteristics, in addition to the soil microbial community structure. Moreover, it is known that certain soil-borne pathogens, such as *R. solani*, actually can thrive in soil given appropriate conditions. Predictions with respect to the degree of suppression of soil-borne diseases on the basis of an overview of microbial communities and activities will, therefore, always have to be related to the soil abiotics. This also pertains to the longevity of the suppressive effects that can be established in soil as a result of soil treatments. In a recent study on the effect of organic matter added to soils in order to enhance the suppressiveness versus the potato pathogen *Ralstonia solanacearum*, we found that different sources of organic matter, cf piggery manure

versus compost, resulted in different longevity of the disease suppression (Schönfeld et al. 2002). We postulated that the organic matter type and distribution through soil resulted in a differential effect on the soil microbiota, which, in turn, affected *R. solanacearum* differently. It is a challenge for future work to assess the intricacies of soil management strategies with respect to the shifts these bring about in the microbial communities locally present (Mazzola 1999). The advent of soil DNA chips which can introduce additional power to these assessments (Ball and Trevors 2002; Hurt et al. 2001) will certainly turn out to represent an important step forward in this highly complex area.

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