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The microbial community in the rhizosphere determined by community-level physiological profiles (CLPP) and direct soil– and cfu–PLFA techniques

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Abstract

The composition of the microbial community in the rhizosphere was investigated using three different techniques: direct soil–phospholipid fatty acid (PLFA), cfu–PLFA (using culturable bacteria only) and community-level physiological profiles (CLPP) using Biolog GN microtitre plates. All three techniques showed that the rhizosphere bacterial community differed from the bulk soil community, when pea plants were grown in pots. The two PLFA techniques also indicated that the effect of roots on microbial communities was greater than differences in the PLFA pattern between different bulk soils. The difference was most prominent using the direct soil–PLFA technique. Both PLFA techniques indicated that gram-negative bacteria were relatively more abundant in the rhizosphere, while PLFAs indicative of gram-positive bacteria were relatively more common in bulk soil samples. The results obtained with the direct soil–PLFA method did not differ between rhizosphere samples taken along a pea root, while this was the case with both the cfu–PLFA technique and the CLPP method. According to both techniques, the differences in community composition were greatest near the root tip. Different plant species exhibited different microbial community composition in the rhizosphere, as judged from all three methods, when four different plant species were compared. In all cases, *Trifolium* differed most from the other species (three different grasses). Although all three methods were found to be suitable for rhizosphere studies, the CLPP method appeared less suitable than the two PLFA methods, since less of the variation in the data could usually be explained.
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Keywords: Rhizosphere; Microbial community structure; PLFA; CLPP

1. Introduction

Actively growing roots release organic compounds into the rhizosphere such as sloughed off cells, secretions, lysates and exudates (Lynch and Whipps, 1990; Bowen and Rovira, 1991). These compounds support growth of the microbial community in the rhizosphere

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(Söderberg and Bååth, 1998) and will result not only in an increased population density, but also in a community structure distinct from that in the bulk soil (Curl and Truelove, 1986; Bowen and Rovira, 1991; Kent and Triplett, 2002). Rhizosphere microbial communities are important for the functioning of the ecosystem, both in relation to direct interactions with plants (both beneficial and deleterious to growth) and with regard to nutrient and organic matter cycling.

Monitoring entire microbial communities in soil has traditionally been a very time-consuming task. The development of techniques for direct extraction of total community DNA and RNA from environmental samples and the subsequent analysis using techniques such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) and terminal-restriction fragment length polymorphism analysis (T-RFLP) has made studies of microbial communities much easier (Gelsomino et al., 1999; Øvreås, 2000). However, DNA-based techniques, although of great potential, still have problems, e.g. in the reproducibility of nucleic acid extraction and in the selectivity of the PCR step. There is, therefore, still a need for conventional methods of studying the microbial community. Several methods that do not give the species composition exactly, but instead a "fingerprint" related to the microbial community structure have been described. Two such methods are the determination of phospholipid fatty acid (PLFA) profiles (Zelles, 1999) and the determination of community-level physiological profiles (CLPP) using Biolog GN microtitre plates (Garland and Mills, 1991). The PLFA (Tunlid et al., 1989; Priha et al., 1999; Joner et al., 2001; Montealegre et al., 2002; Söderberg et al., 2002) and the CLPP methods (Garland, 1996; Smalla et al., 1998; Hodge et al., 1998; Baudoin et al., 2001, 2002) have been used in connection with rhizosphere studies.

In the present study, we used two different techniques for PLFA assessment—one based on the extraction of lipids directly from soil, indicating the total microbial community composition, and other based on the extraction of lipids from the culturable part of the bacterial community only (cfu–PLFA), together with the CLPP technique to study the microbial community of the rhizosphere. Several different aspects were studied. First, the rhizosphere effect of one plant species (pea) in different soils was studied, in order to ascertain whether the root effect was greater than effects due to soil differences. In a second study, the changes in the microbial community along a root were studied and compared with the bulk soil community. Both these studies were carried out under controlled plant growth conditions, where the bulk soil consisted of unplanted soil. As a comparison, a field study was performed to determine if differences between the rhizosphere microbial communities of different plants were more prominent than differences between rhizosphere communities and bulk soil communities.

2. Materials and methods

2.1. Comparison of the effects of the rhizosphere and different soil compositions

Soils were sampled from 11 sites in late spring. Different agricultural soils with a history of both conventional and organic management practices were used, and included clay, silty clay, sandy loam and silt loam soils. The pH (H_2O) varied between 5.6 and 8.2, while the organic matter content (measured as loss on ignition at 600 °C) varied between 2.6 and 8.1% (Table 1).

The soil samples were sieved (8 mm mesh size) and put into 1.5 l pots. Each pot was sown with three pea seeds (*P. sativum* L.). The seedlings were thinned to one seedling per pot after 2 weeks. Pots without plants were kept as controls for root-free bulk soil. The pots were placed outdoors at sowing from June to August and watered regularly, but no nutrients were added. The roots (four pots per soil were bulked to give one

Table 1
Soil characteristics

Soil number	pH	Organic matter (%)	Soil texture
1	5.6	5.2	Silt loam
2	5.7	5.1	Sandy loam
3	5.8	4.9	Sandy loam
4	5.9	8.1	Sandy loam
5	6.1	3.6	Silt loam
6	6.7	4.3	Sandy loam
7	7.0	5.4	Clay
8	7.7	4.1	Silty clay
9	7.7	5.7	Clay
10	7.8	4.4	Silty clay
11	8.2	2.6	Sandy loam

sample) were sampled after 9 weeks (the time of flowering). Samples were also taken from root-free bulk soil (one pot per soil).

To collect rhizosphere soil, the contents of the pots were placed on a plastic sheet and the plants were gently removed. Small root samples with adhering soil were taken from the whole root system of the plants from four pots (including root tips) and bulked into one composite sample. The soil in the rhizosphere was then removed by allowing the roots to soak in distilled water. Measurements of direct soil–PLFAs and cfu–PLFAs were then made.

2.2. Variation in the microbial community along the root

A silty loam soil (pH (H_2O): 6.3, 4.4% organic matter) was sieved (2 mm mesh size) and then mixed with pure quartz sand (Silversand 50, Ahlsell Mineral) in the proportions 3:1. Plastic pots (1.5 l) were filled with the soil mixture and pea seeds (*Pisum sativum* L.) were sown. The plants were grown in a greenhouse with a 16/8 h light/dark cycle at 22/20 °C. The water content was kept constant by watering every second day. The plants were harvested after 3 weeks. The contents of the pots were placed on a plastic sheet and the plants were gently removed. The collection of rhizosphere soil was performed in a cold room (4 °C) to prevent the rhizosphere soil from drying. Every root with rhizosphere soil that was used was divided into six equally large zones along the main axis of the root. Zone 1 was defined as the zone including the root tip (approximately 3 cm long). To obtain a rhizosphere sample from zone 1, 6–10 segments including root tips were used. From the other zones, 2–6 root segments were used to obtain enough rhizosphere soil. Soil from pots without any plants was used for bulk soil samples. The bulk soil samples were kept at 4 °C for the same time (approximately 15 min) as the rhizosphere soil processing time. Measurements of direct soil–PLFAs, cfu–PLFAs and CLPP were then made.

2.3. Comparison of the rhizosphere and bulk soil microbial communities of field-grown plants

This study took advantage of the Swedish experimental area of the BIODEPTH project (Mulder et al., 2002), where different man-made grassland communities have been established. Samples were taken from four sites containing monoculture communities (*Dactylis glomerata* L., *Phalaris arundinaceae* L., *Phleum pratense* L. and *Trifolium pratense* L.), as well as from two mixed two-species communities (mixture of *D. glomerata* and *P. arundinaceae*, and of *P. pratense* and *T. pratense*). Duplicate samples were collected. Since no differences were found for single plant species between monocultures and two-species communities, they were treated as replicates, resulting in four samples per plant species.

In monocultures as well as in mixtures, three individual plants of a given species were randomly collected and a core, 5 cm diameter and 15 cm deep, was collected underneath the above ground parts of the plant. The samples were separated into bulk and rhizosphere soil by shaking the plant vigorously on a 3 mm mesh sieve. Roots were removed from the bulk soil (soil not adhering to the roots), while roots and adhering soil were collected as rhizosphere soil. It was, however, impossible to remove all the roots from the bulk soil. Measurements of direct soil–PLFAs, cfu–PLFAs and CLPP were then made.

2.4. Measurements of direct soil–PLFAs and cfu–PLFAs

Direct soil–PLFA measurements were made on rhizosphere or bulk soil that had been freeze dried. To assess the PLFA composition of the cultivated bacteria (cfu–PLFA), plate counts were made on agar plates (0.2% tryptic soy agar) at 20 °C. Two-week-old agar plates on which the number of cfu was high (approximately 100) were flooded with citrate buffer (0.15 M, pH 4.0), and a 1.5 ml portion of this bacterial suspension was collected for lipid extraction. This is similar to the method used by Pennanen et al. (1998) and Kozdrój and van Elsas (2001).

Lipid extraction, fractionation of lipids to collect the phospholipid fraction, and the subsequent alkaline methanolysis to transform phospholipid fatty acids into free fatty acid methyl esters followed by analysis on a gas chromatograph, were performed as described by Frostegård et al. (1993). The fatty acid nomenclature used is as follows; total number of carbon atoms:number of double bonds, followed by the position (ω) of the double bond from the methyl

end of the molecule. *Cis* and *trans* configurations are indicated by c and t, respectively. Anteiso- and iso-branching are designated by the prefix a or i. 10Me is a methyl group on the 10th carbon atom from the carboxyl end of the molecule. Cy indicates cyclopropane fatty acids. Br indicates an unknown branching position.

2.5. Measurements of CLPP using Biolog GN microtitre plates

Community-level physiological profiles were assessed using the method originally described by Garland and Mills (1991). In the experiments using pea plants, each well in the Biolog GN plates was inoculated with 150 μl of a bacterial suspension (approximately 10^5 cells ml^{-1}). The bacterial suspension was prepared by homogenizing soil with water in a knife blender, followed by low-speed centrifugation ($1000 \times g$) for 10 min. The supernatant was appropriately diluted and used as inoculum.

In the field experiment, bulk (20 g fresh weight) and rhizosphere soil (4.5 g soil and root) was mixed with 150 ml autoclaved 0.1% sodium pyrophosphate (pH 7.0) and glass beads (2 mm in diameter). This suspension was agitated for 2 h and allowed to settle for 15 min. After further dilution in 0.85% NaCl, a 1:1000 dilution (bulk soil samples) and a 1:10,000 dilution (rhizosphere samples) were used to inoculate the Biolog GN plates (150 μl per well).

The plates were incubated for up to 5 days at 20 °C and the color development was measured every 24 h using a microtitre plate reader at 590 nm. Plates with an average well color development (AWCD) of around 0.75–1 were used in the statistical calculations.

2.6. Statistical analyses

The PLFA pattern and the substrate utilization pattern were analyzed using principal component analysis (PCA). For the PLFA data, concentrations expressed as mol% of the total amount of PLFAs were used. For the CLPP data, the optical density of each well was standardized by dividing by the AWCD. Both PLFA and CLPP data were then normalized to unit variance before PCA analysis.

3. Results

3.1. Comparison of the effects of the rhizosphere and different soil types

The two different PLFA analyses used in this study showed the same differences between bulk soil and rhizosphere soil, when the data were analyzed using PCA (Fig. 1A and B). Samples taken from bulk soil in the 11 different soils had more negative score values for the first principal component than the samples taken from the rhizosphere. Less of the variation was explained by the first principal components differentiating between origin of soil (bulk and rhizosphere soil) for cfu–PLFA than direct soil–PLFA (41.7 and 25.6% for direct soil–PLFA and cfu–PLFA, respectively).

Differences due to soil type were mainly found in the second principal component of the direct soil–PLFA data (accounting for only 13.7% of the variation). This difference was consistent irrespective of whether the sample was taken from bulk or rhizosphere soil (with the exception of one soil) (Fig. 1A). This was not the case for the cfu–PLFA method, where the second component did not order the 11 different soils in a consistent way (Fig. 1B). There was a significant correlation between soil pH and the scores of the bulk soils for PC2 of the direct soil–PLFA data ($P < 0.05$). PLFAs relatively more common at high soil pH were several monounsaturated ones, like 16:1ω5 and 18:1ω7, while several branched ones, like i15:0 and i16:0, and cy17:0 and cy19:0 were relatively more common in soil with low pH (data not shown). There were no significant correlations between PC2 of the cfu–PLFA data and any of the soil characteristics.

Loadings for the individual PLFAs along PC1 showed a significant correlation ($P < 0.01$) between the direct soil–PLFA and cfu–PLFA methods, indicating that, overall, the similar results for both techniques were due to similar changes in individual PLFAs (Fig. 1C). Some PLFAs, e.g. 16:1ω5, 16:1ω7, 18:1ω7, cy17:0 and cy19:0, are considered to be typical for gram-negative bacteria (Wilkinson, 1988). These are to be found in the top right of the plot, indicating that these PLFAs were relatively more common in the rhizosphere samples than in samples from the bulk soil (Fig. 1C).

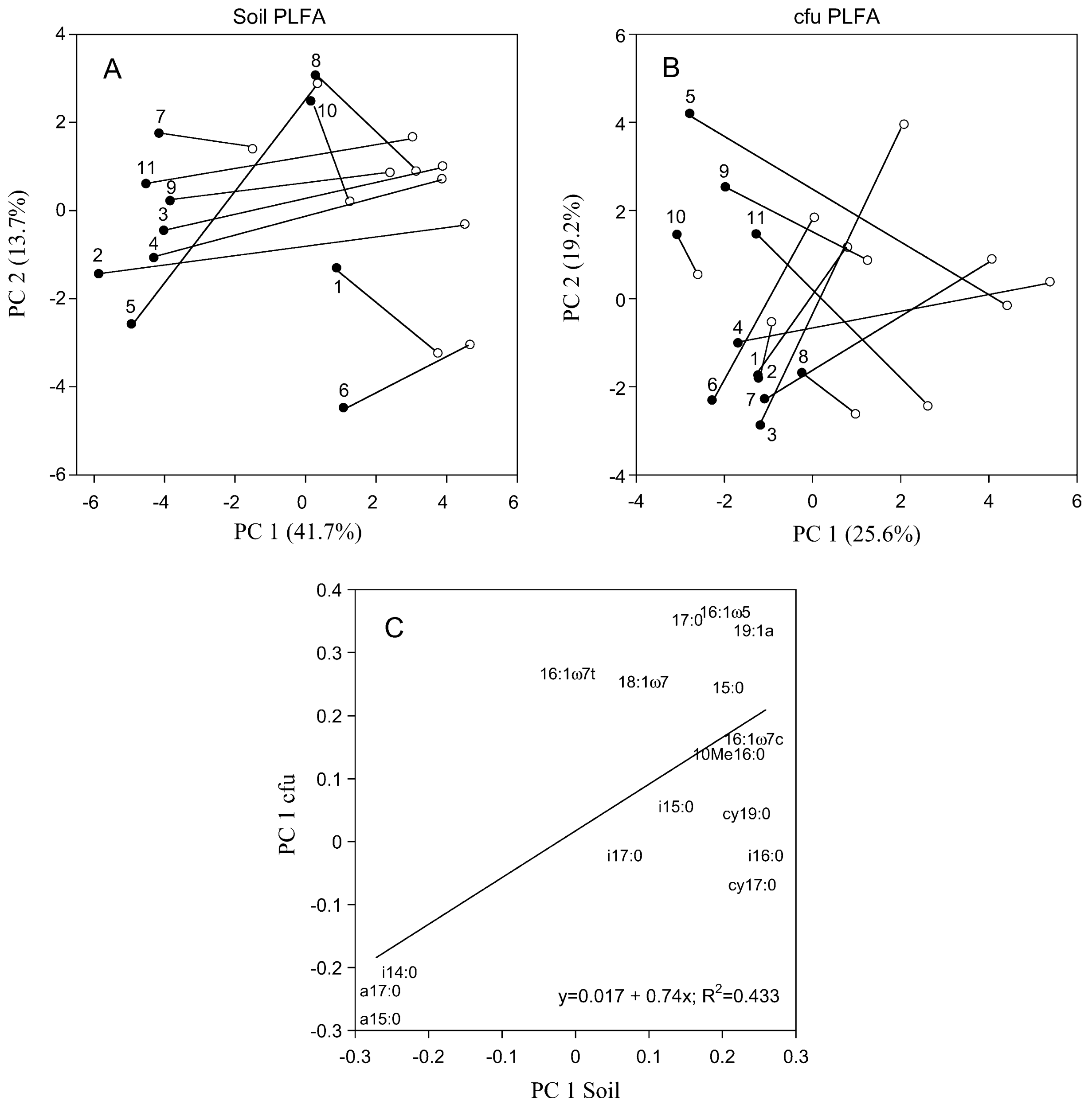

Fig. 1. The effect of different soils on the microbial community of pea rhizosphere (open symbols) and bulk soil (filled symbols). Lines indicate rhizosphere and bulk soil samples from the same soil. Numbers refer to soils in Table 1. (A) Scores for principal component analysis of direct soil–PLFAs. (B) Scores for principal component analysis of cfu–PLFAs. (C) Comparison of loadings of the first principal component of direct soil and cfu–PLFAs. Values within parentheses in (A) and (B) are the variation explained by the principal component.

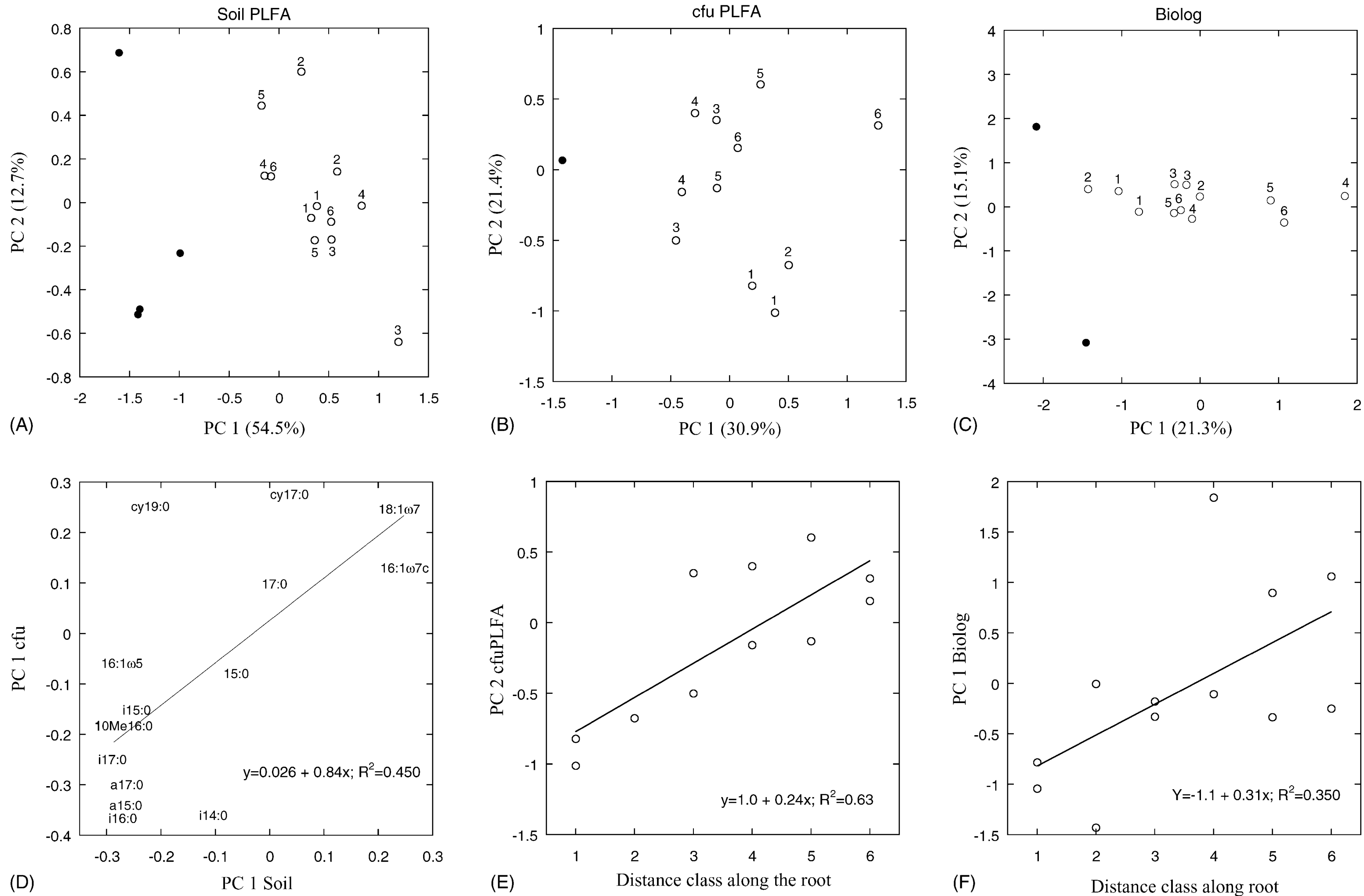

Fig. 2. Variation in the microbial community in the rhizosphere along a pea root (open symbols) and in bulk soil (filled symbols). Numbers indicate zones along the main axis of the root, where zone 1 indicates the zone including the root tip. (A) Scores for principal component analysis of direct soil–PLFAs. (B) Scores for principal component analysis of cfu–PLFAs. (C) Scores for principal component analysis of CLPP data (Biolog). (D) Comparison of loadings of the first principal component of direct soil and cfu–PLFAs. (E) Correlation between principal component 2 of cfu–PLFAs and distance along the root. (F) Correlation between principal component 1 of CLPP (Biolog) and distance along the root. Values within parentheses in (A), (B) and (C) are the variation explained by the principal component.

3.2. Variation in the microbial community along the root

The three different methods describing the bacterial composition were used to investigate changes along a root. All the methods showed similar results. PCA of the data from the different methods revealed changes in the microbial community due to the soil status (bulk soil or rhizosphere soil) in PC1 (Fig. 2A–C). The explained variation of the techniques in the first

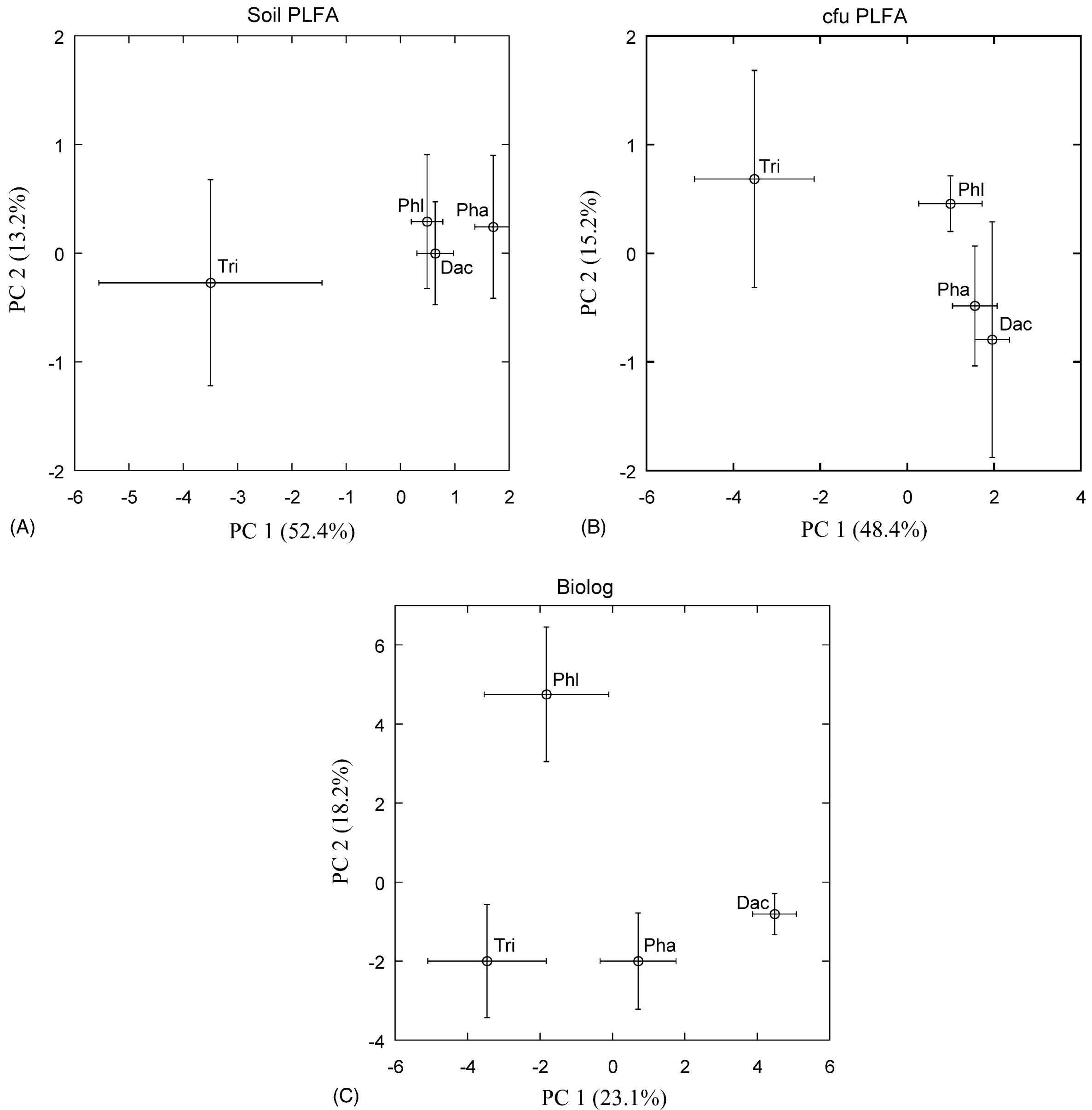

Fig. 3. Effect of different plants on the microbial community. Dac: *Dactylis glomerata*, Pha: *Phalaris arundinaceae*, Phl: *Phleum pratense* and Tri: *Trifolium pratense*. (A) Scores for principal component analysis of direct soil–PLFAs. (B) Scores for principal component analysis of cfu–PLFAs. (C) Scores for principal component analysis of CLPP (Biolog). Values within parentheses in (A), (B) and (C) are the variation explained by the principal component. Bars indicate standard errors.

axis decreased in the order: direct soil–PLFA (54.5%), cfu–PLFA (30.9%) and CLPP (21.3%). Also, a correlation was found between the loadings of the individual PLFAs from the two different PLFA methods for PC1 (the principal component indicating differences between bulk soil and rhizosphere, Fig. 2D). PLFAs indicating gram-negative bacteria, e.g. 16:1ω7c, cy17:0 and 18:1ω7, were found to be relatively more common in rhizosphere samples than in bulk soil samples with both the direct soil–PLFA and cfu–PLFA methods, while PLFAs indicating gram-positive bacteria, like several branched ones, were relatively more common in the bulk soil.

No differences in bacterial composition along the root were demonstrated by the direct soil–PLFA technique (Fig. 2A). However, both the cfu–PLFAs (in PC2) and the CLPP pattern (in PC1) varied with distance along the root, since there was a significant correlation between the different distance classes along the root and the composition according to cfu–PLFA and CLPP ($P < 0.01$ and 0.05, respectively; Fig. 2E and F). The largest difference was observed between the different samples near the root tip.

3.3. Comparison of the rhizosphere and bulk soil microbial communities of field-grown plants

No difference between the composition of bulk soil bacteria and rhizosphere bacteria was seen using the direct soil–PLFA and cfu–PLFA techniques. Both rhizosphere and bulk soil samples were, therefore, used to differentiate the effects of different plants. However, when using CLPP, the bulk soil samples did not differ between plants, and thus only rhizosphere samples were used in the PCA analysis (Fig. 3C). When data were analyzed using PCA (Fig. 3), significant differences were found between the plant species with the three different methods used along PC1 ($P < 0.05$).

When using the direct soil–PLFA and cfu–PLFA techniques, soil from the *Trifolium* was significantly different from soil from the other three plant species, and when using CLPP, soil from *Trifolium* and *Phleum* were significantly different from that from *Dactylis*. The explained variation of PC1 for the three techniques decreased in the order: direct soil–PLFA (52.4%), cfu–PLFA (48.4%) and CLPP (23.1%). The order in which the plant species appeared in the first principal component of the PCA ordination was similar for direct soil–PLFA, cfu–PLFA and CLPP.

4. Discussion

All three methods indicated that the rhizosphere community differed from that in the bulk soil in the pot studies. This has of course been shown earlier (Curl and Truelove, 1986; Bowen and Rovira, 1991; Kent and Triplett, 2002). We found that the rhizosphere was more important in determining the microbial community than the soil type (Fig. 1). However, since the extent of the root effect differs between different plant species (see e.g. Fig. 3) and the effect of soil type depends on the range of soils tested, this can only be used as an indication of the importance of the rhizosphere in determining the microbial community in soil. The extent of the root effect on the composition of the microbial community has also been shown to vary depending on plant species, soil type and the way in which the rhizosphere is sampled, using both PLFA and CLPP techniques (Priha et al., 1999; Marschner et al., 2001). In some combinations of plant species and soil, the soil type was more important while in other combinations the root effect was more important.

In the present study, soil pH was the main soil factor determining differences between soils using direct soil–PLFA. The importance of pH in determining the soil PLFA pattern was recently shown by Bååth and Anderson (2003). Several of the PLFAs increasing or decreasing in concentration with soil pH in our study were also found to behave in the same way in the study by Bååth and Anderson (2003).

The rhizosphere effect was most evident in the pot experiments (Figs. 1 and 2), where the bulk soil consisted of pots without plants, while in the field study no differences between bulk and rhizosphere soil were found. Instead, for the PLFA measurements the same differences between plant species were found irrespective of whether the soil was predefined as bulk soil or rhizosphere soil. There could be several reasons for this observation. First, there are considerable difficulties in separating bulk soil from rhizosphere soil in field samples, while in laboratory studies the bulk soil samples are more easily controlled. Second, it might be that there is no “true” bulk soil in the field. Once soil has been in contact

with a root, and the community has changed accordingly, it might take a long time for it to revert to the original composition. The bulk soil in the field might, therefore, actually consist of former rhizosphere soil, having been in contact with roots at various times. Third, it is also possible that the soil mesofauna (e.g. earthworms) transport material between bulk soil and rhizosphere soil, thus making separation between the two entities difficult. Fourth, the way of sampling used for this study might have confounded the bulk and rhizosphere soil, resulting in no differences.

The distribution of bacteria along roots is generally assumed to mirror the exudation pattern of the plant root (Van Vuurde and Schippers, 1980; Liljeroth et al., 1991; Maloney et al., 1997). Differences in exudation in different parts of the root may be due to both different root anatomical structure and different physiological conditions (Bowen and Rovira, 1976; Vancura, 1988; Baudoin et al., 2001). We found differences in the rhizosphere community along the root with two of the three methods used, while the direct soil–PLFA technique did not reveal any differences (Fig. 2). Conflicting results have been found earlier. Duineveld et al. (1998) found small changes in the microbial communities associated with different parts of the roots using DGGE and substrate utilization patterns. In contrast, Baudoin et al. (2001) found clear changes in microbial communities along the roots using the CLPP technique, and Marschner et al. (2001) found differences using DGGE. Yang and Crowley (2000) found many common bands in the DGGE gels from samples from different root locations, but the communities could be distinguished by differences in a few bands. It is likely that there are differences in the microbial community along the root. The conflicting results might, therefore, be partly due to the methods used. Techniques that include the whole community (like DGGE and direct soil–PLFA), might also include species that are inactive, thus decreasing the possibility of detecting differences in a minor active part. This might be the reason why differences between the rhizosphere soil and bulk soil microbial communities were only seen in weak bands from the DGGE gels, while dominant bands appeared to be common to both communities (Duineveld et al., 1998).

In the present studies, both direct soil–PLFA and cfu–PLFA measurements indicated that gram-negative bacteria were relatively more common in the rhizosphere than in the bulk soil (Figs 1C and 2D), since PLFAs indicative of this group (e.g. 16:1ω7c and 18:1ω7) were relatively more commonly found in the rhizosphere. It is well known that for instance the gram-negative *Pseudomonas* is a group of bacteria that are usually found in the rhizosphere (Curl and Truelove, 1986). Gram-negative bacteria are normally also characterized by a high reproductive rate and high activity under conditions of sufficient supply of nutrients, traits that would be an advantage in the rhizosphere. However, most of these earlier studies were performed using plate count techniques. Recent studies using whole community analysis by PCR followed by DGGE have shown conflicting results. Some studies indicate that gram-negative bacteria are relatively more common in the rhizosphere (e.g. Marilley and Aragno, 1999), while several studies indicate the contrary, namely that gram-positive bacteria are relatively more common near the root than in the bulk soil (Normander and Prosser, 2000; Smalla et al., 2001). The fact that molecular methods are still selective, especially in the DNA extraction and the PCR step, might be one explanation of these conflicting results. Our results, especially using the direct soil–PLFA technique representing the whole microbial community indicate, however, that the earlier view of a predominance of gram-negative bacteria in the rhizosphere might still be true.

Earlier comparisons of the sensitivity and reproducibility of the PLFA and CLPP techniques have usually indicated that the PLFA technique is superior in that it more easily differentiates between treatments, shows less variation between replicates, and treatments usually account for more of the variation in the data (Bååth et al., 1998; Ibekwe and Kennedy, 1998; Pennanen et al., 1998; Priha et al., 1999; Khalil et al., 2001). In the present study the CLPP- and the PLFA-based methods exhibited similar discrimination power. For example, they differentiated between the plant species in the same way (Fig. 3). However, also in our case the principal component differentiating between treatments accounted for less of the variation using CLPP than using the PLFA techniques, when discriminating between bulk and rhizosphere soil (Fig. 2) and when comparing different plant species (Fig. 3).

It is not surprising that the two techniques, cfu–PLFA and CLPP, give similar results, since

the degrading capacity of a community of bacteria (measured using CLPP) must to a certain extent reflect the species composition (measured using the cfu–PLFA technique). Both methods also probably reflect fast-growing culturable bacteria. It is less evident that a technique reflecting all cells (direct soil–PLFA) will be correlated with a technique reflecting only a minor part of the community (culturable bacteria, cfu–PLFA). However, this indicates that changes in bacterial plate counts (cfu), at least in an environment of high activity such as the rhizosphere, partly reflect changes in the total bacterial community. That this is the case is also indicated by the often found correlation between culturability and cell-specific bacterial activity (measured using thymidine and leucine incorporation rates, e.g. Bååth, 1994; Bååth and Arnebrant, 1994; Söderberg and Bååth, 1998). Furthermore, under certain conditions, cfu–PLFAs might better reflect changes in the bacterial community than direct soil–PLFAs, as in the case of differences along the root (the present study) or when the effect of the addition of plant growth promoting bacteria to the rhizosphere is studied (Probanza and Bååth, personal communication). This might also be due to the rhizosphere being a zone of high activity with rapid changes especially in the active microbial community.

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