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Spatio-temporal dynamics of bacterial communities associated with two plant species differing in organic acid secretion: A one-year microcosm study on lupin and wheat

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ABSTRACT

Plants are generally assumed to influence the surrounding soil microflora through rhizodeposition. However, the role of rhizodeposits, and especially organic acids, in structuring the bacterial communities is still poorly understood. In this study, we asked the question whether plants differing in organic acid secretion have a different impact on the soil bacterial communities, and if this is the case, to which extent this impact is due to different organic acid concentrations in the rhizosphere. To investigate this question, we compared white lupin and wheat. The former is a high organic acid-secreting species, while the latter secretes only low amounts of carboxylates. We grew the plants in large microcosms including root-free control compartments for one year (replanted every second month) and analyzed the spatio-temporal changes in soil ATP concentrations, as well as in diversity and structure of bacterial communities (using DNA- and RNA-based DGGE) along a root-soil gradient after two, six and twelve month's cultivation. Our results showed: i) that white lupin and wheat differed in their impact on soil ATP concentrations and on the structure of root bacterial communities; ii) that cultivation time was a key factor in explaining the observed differences in all the parameters studied; and iii) that the amounts of organic acids accounted for a significant proportion (15%) of the variability within root active communities. These results indicate that plants influence their associated bacterial communities in a species-specific way and that for communities living in the direct vicinity of roots (rhizoplane-endorhizosphere), a significant part of this influence can be attributed to root-secreted organic acids.

1. Introduction

The colonisation of bare soil by plants is known to induce profound changes in the soil chemical and physical properties through shoot decomposition and incorporation of the litter on the one hand, and through root growth, secretion and decay on the other hand. Rhizodeposition involves the release of a wide variety of organic compounds, usually separated into low-molecular weight compounds (e.g. amino acids, organic acids, sugars, phenolics) and high-molecular weight compounds (e.g. mucilage and extracellular enzymes) (Marschner, 1995). While some of these compounds, e.g. phenolics, can act as repellents or attractants to specific populations of soil bacteria, others, e.g. sugars and organic acids, are easily degraded by bacteria and thus represent a common food source for most soil bacterial inhabitants (Deubel et al., 2000). For a recent review on root exudate-mediated plant-soil interactions, see Bais et al. (2006). The average percentage of photosynthesized carbon allocated to the roots has been estimated to 50%, but this value is greatly changing from one species to the other (Nguyen, 2003). Moreover, it is a well-known fact that plant species differ in their impact on the structure of rhizosphere microbial communities (Westover et al., 1997; Smalla et al., 2001; Marschner et al., 2004, 2005; Sharma et al., 2005; Costa et al., 2006; Patra et al., 2006; Phillips et al., 2006), but until now, the extent to which this effect might be linked to the differential rhizodeposition of the various plant species has remained an open question. To study the carbon sources preferentially utilized by the rhizosphere microflora,

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metabolic profiling has been performed using BIOLOG-plates with complex communities (Garland and Mills, 1991). However, these cultivation-based assays do not give an unbiased picture of the substrates actually used *in situ* by the native communities, due to the fact that: i) the results mostly reflect the metabolic activity of the fast growing community members; and ii) the test does not take into account the availability of the substrates in the soil (Konopka et al., 1998; Schutter and Dick, 2001). Despite these limitations, studies based on substrate utilization patterns have suggested that carbohydrates and organic acids might be the most discriminating compounds between communities sampled from the rhizosphere of different plants (Grayston et al., 1998) or between communities sampled at different root distances or root stages (Baudoin et al., 2001). In addition to these metabolic profiling methods, a few other studies assessed the effect of complex mixtures of root exudates (Kozdroj and van Elsas, 2000; Rangel-Castro et al., 2005) and of specific compounds, like glucose or oxalic acid (Landi et al., 2006) on the structure of bacterial communities. In this latter study, the addition of both glucose and oxalic acid to the soil caused shifts in the structure of bacterial communities, and the changes were higher in oxalic acid-treated soils than in glucose-treated soils. While these observations support the hypothesis that root-secreted compounds play a key role in structuring bacterial communities, further work is needed to be able to bring conclusive evidence of this role, e.g. by comparing plants differing greatly in their rhizodeposition, as well as by measuring the amounts of the root-secreted compounds of interest *in situ*, as the composition and quantity of exudates do not only depend on the plant species, but have also been shown to vary a lot in function of the soil (Veneklaas et al., 2003).

In this study, we raised the question of whether plants differing in rhizodeposition would have a different impact on rhizosphere bacterial communities. We focused our attention on low molecular weight organic acids, as a readily degradable carbon source for most soil bacteria. We chose two crops differing in organic acid secretion, wheat as a low organic acid-exuding and white lupin as a high organic acid-exuding plant and used them to compare their impact on bacterial communities. We established an experimental system that aimed at mimicking the ploughing layer in agro-ecosystems (see also Le Bayon et al., 2006a,b), while still allowing standardized plant growth conditions (light, temperature, humidity). In addition to providing a good compromise between hydroponic systems/small pots in the laboratory and cultivated plots in the field, our microcosm design offered the advantage of containing root-free compartments, which allowed the definition of four levels of root proximity. Soil biomass was evaluated by measuring the ATP concentrations. In both soil and root samples, the amounts of organic acids were analyzed and the changes in bacterial communities were monitored using DNA- and RNA-based DGGE (denaturating gel gradient electrophoresis) profiling.

The aims of this work were: i) to ascertain that in the soil and growth conditions chosen, white lupin would also lead to a larger accumulation of organic acids in its rhizosphere than wheat; ii) to compare the impact of the two species on the soil biomass, as well as on the diversity and structure of the bacterial communities, considering both a temporal (cultivation time) and a spatial (root distance) gradient; and iii) to investigate to what extent the impact of plant species could be linked to the differential concentrations of organic acids in their respective rhizospheres.

2. Materials and methods

2.1. Experimental design, plant material and sampling

Microcosms consisted of a central PVC tube (15 cm diameter and 35 cm height) and four small perpendicular ones (“side-arms”, 4 cm diameter and 20 cm long) (for details and a scheme of the microcosms, see Le Bayon et al., 2006a,b). After collection in the field, the soil was air-dried, sieved through a 2 mm mesh, and remoistened to its water holding capacity (20% water content, w:w) prior to filling the microcosms (central tube and side-arms, reaching a total of 7 kg per microcosm) at a bulk density of around 1.3 g cm^{-3}. Root penetration from the central tube to the side-arms was prevented by a 25 μm-nylon mesh (Nitex 03-25/19, Sefar, Rüschlikon, Switzerland) between the side-arms and the central tube. This system allows the exclusion of roots from the side-arms, although root hairs can pass through the nylon mesh because of their small diameter (Dittmer, 1949). The soil used was a chromic Luvisol (5–30 cm, A horizon) containing 50% of sand, 30% of silt, 20% of clay, 0.97% of organic carbon, 0.31% of mineral carbon, with a carbon:nitrogen (C/N) ratio of 11.8, a pH_{H2O} of 7.8, a pH_{KCl} of 7.02, and 1.4 g kg^{-1} total phosphorus. Such high pH values in a Luvisol are probably due to the extensive agricultural practices previously conducted on the sampled field, especially the use of fertilizers and manure spreadings (Baize and Girard, 1998). A total of 27 microcosms was used for this study (3 replicates, 3 plant treatments and 3 cultivation times). The three plant treatments were: i) lupin (*Lupinus albus* L. cv. Amiga); ii) wheat (*Triticum aestivum* L. cv. Arina); and iii) no plant (control). Four seeds of either white lupin, or wheat were planted into the microcosm central tube, and the microcosms were then placed into a growth chamber (Normoflex, KR 11C/200S10, Schaller Uto AG, Bern, Switzerland) for cultivation. All microcosms were kept in the growth chamber under the following conditions: photoperiod 16/8 h (day/night), temperature 24/20 °C (day/night), 50% humidity, daylight intensity 8000 lux (mixed fluorescent tubes, 36W 830 and 36W 840). For watering (both in sides arms and at the top of microcosms), we used a nutrient solution without P (to stimulate cluster root formation and organic acid secretion) containing (in mg l^{-1}): $Ca(NO_3)_2.4H_2O$ (118), K_2SO_4 (32.6), $MgSO_4.7H_2O$ (40), FeNaEDTA (3.67), KCl (0.93), H_3BO_3 (0.46), $MnSO_4.H_2O$ (0.42), $CuSO_4.5H_2O$ (0.06), $ZnSO_4.7H_2O$ (0.072), $Mo_7O_{24}(NH_4)_6$ (0.93). Nitrate was added to prevent the establishment of the nitrogen-fixing symbiosis. The mycorrhizal status of wheat was not analyzed. Plants were replanted every second month (at the beginning of flowering stage) to ensure continuous growth over the one-year cultivation period. Both lupin and wheat grew well under these experimental conditions: after one year of cultivation, the cumulative production of above ground biomass per pot was 55.69 ± 1.51 g d.w. yr^{-1} and 47.58 ± 2.02 g d.w. yr^{-1} for lupin and wheat, respectively (no significant changes in productivity were observed between the different sampling dates, data not shown). Root biomass reached 0.340 ± 0.11 g d.w. yr^{-1} for wheat versus 0.277 ± 0.16 for lupin cluster roots and 0.253 ± 0.17 g d.w. yr^{-1} for lupin non-cluster roots respectively. While the shoots were cut and removed from the system at each harvest, the roots remained in the microcosms and became part of the soil organic matter. These old roots were entirely degraded in two months, so that no trace of them could be seen when harvesting the next generation. After 2, 6 and 12 months (respectively 1, 3 and 6 cultivation cycles), three microcosms per plant treatment were harvested. Roots were collected from the central tube of the microcosms (in lupin microcosms, roots were separated into cluster roots (CR) and non-cluster roots (NCR)). The rhizosphere soil (still adhering to the roots after gentle shaking) was collected by rubbing roots carefully on a 1 mm mesh sieve and collecting the sieved soil. A bulk soil sample was also collected in the microcosm main compartment, as well as in the four side-arms (10–15 cm from the main compartment). The four side-arm samples of each microcosm were pooled together. Overall, 36 samples were collected at each of the three harvest times: 6 per lupin microcosm (cluster roots and their rhizosphere soil, non-cluster roots and their rhizosphere soil, bulk soil from the main compartment and bulk soil from the side-arms), 4 per wheat microcosm (roots and their rhizosphere soil,

bulk soil from the main compartment and bulk soil from the side-arms) and 2 per control microcosm (bulk soil from the main compartment and bulk soil from the side-arms).

2.2. Soil biochemical analyses

Organic acids were extracted and quantified according to Le Bayon et al. (2006b). In brief, soil and root samples were extracted in sterile water by shaking at 1400 rpm for 30 min (Thermomixer compact, Eppendorf, Huber, Rheinach, Switzerland). After centrifugation, the supernatant was filtered at 0.2 μm and analyzed by HPLC. Organic acids were separated on a cation-exchange column (300 × 7.8 mm, 10 μm, H^+ form, Benson, Reno, Nevada, USA) using isocratic elution with 20 mM H_2SO_4. Absorbance was monitored at 210 nm. Calibration curves with standard organic acids (Fluka, Switzerland) were performed for quantification of citrate, malate and fumarate, which were by far the most abundant organic acids in root and soil fractions from lupin and wheat. ATP was measured (in soil samples only) by bioluminescence, according to Maire et al. (1999).

2.3. Molecular fingerprinting of bacterial communities

After the recovery of rhizosphere soil from roots (see above, Section 2.1), 0.5 g of soil or root samples were placed in FastRNA™ matrix tubes D for RNA and matrix tubes E for DNA (Bio101, QBiogene) and frozen in liquid nitrogen. They were kept at −80 °C until analysis. DNA and RNA extractions (bead-beating technique using a FP120 FastPrep™ cell disruptor, Savant Instruments), PCR, as well as DGGE (Denaturating Gel Gradient Electrophoresis) were performed in all samples as described in Weisskopf et al. (2005). Briefly, after extraction of DNA and reverse-transcription of extracted RNA, a PCR amplification of the V3 region of the 16S rDNA was performed, using the forward universal primer 338f (5′-ACTCCTACGGGAGGCAGCAG-3′) and the reverse universal primer 518r (5′-ATTACCGCGGCTGCTGG-3′) (Ovreas et al., 1997). DGGE was performed with a 8% (w:v) acryl-bisacrylamide (37.5:1, Qbiogene) gel with 30–60% linear urea/formamide (Fluka/Qbiogene) denaturing gradient (100% denaturant corresponds to 40% formamide +7 M urea). PCR products (600–800 ng) were concentrated in a vacuum concentrator centrifuge, loaded on the DGGE gel and submitted to electrophoresis at 60 °C with a constant voltage of 150 V during five hours. The gels were stained in the dark for 20 min in 0.01% Sybr Green I (Molecular Probes, Leiden) in 1× TAE solution and photographed with the Multi-Analyst package from Bio-Rad (Bio-Rad). Images were normalized according to the reference patterns and the profiles were compared using the GelCompar software (Applied Maths). DGGE profiles were then converted into a numerical matrix for further statistical analyses (see below).

2.4. Statistical analyses

ANOVA's were carried out on organic acids (only for root samples, because of the high number of zeros in the soil samples), as well as on ATP values and bacterial community diversity (Shannon–Wiener index). For organic acids, the model included three factors: i) plant species (lupin, wheat and control); ii) cultivation time (2, 6 and 12 months); and iii) root type (cluster roots and non-cluster roots). For the ATP concentrations and the diversity of bacterial communities, the model also included three variables, but this time, since samples from both root and soil origin were analyzed, "root proximity" (bulk soil from the side-arms, bulk soil from the main compartment, rhizosphere soil and root) replaced "root type", in addition to the factors "species" and "cultivation time". To analyse the DGGE profiles, principal component analyses (PCA) and redundancy analyses (RDA) were performed. In all cases, matrices were first arcsinus transformed (percentage). Furthermore, since PCA and RDA are not appropriate for the analysis of matrices with a high number of zeros (Legendre and Legendre, 1998), matrices were Hellinger transformed (Legendre and Gallagher, 2001). To obtain a general view on DGGE profiles, a global PCA on all DNA- and RNA-based profiles was carried out. Then, to measure the effects of plant species, cultivation time, root type, as well as organic acids on the bacterial communities, partial RDAs were calculated on six subsets of samples: i) DNA-based root profiles; ii) RNA-based root profiles; iii) DNA-based rhizosphere soil profiles; iv) RNA-based rhizosphere soil profiles; v) DNA-based bulk soil profiles (from the main compartment and from the side-arms); and vi) RNA-based bulk soil profiles. These partial RDAs allowed the extraction of the variation in the DGGE profiles explained by sets of explanatory variables and shared by these data sets (see Borcard et al., 1992; Okland and Eilertsen, 1994 for details; Jossi et al., 2006 for an example with DGGE data). For analyses on root profiles, we used four sets of explanatory variables: i) plant species; ii) root type (cluster *vs.* non-cluster); iii) organic acids; and iv) cultivation time. The same sets of variables were used for the analysis on rhizosphere soil samples. For bulk soil samples; i) plant species; ii) position (main compartment *vs*. side-arms); iii) organic acids; and iv) cultivation time were used as explanatory variables. All analyses were performed using the statistical software R 2.4.1 (R Development Core Team, 2007).

3. Results

3.1. Organic acid secretion differed greatly between lupin and wheat

The HPLC analysis revealed that citrate, malate and fumarate were by far the most abundant organic acids retrieved from root and soil samples. Other carboxylates (e.g. *trans*-aconitate) were also detected, but only in traces (not shown) and this is why we focused on citrate, malate and fumarate. The analysis of variance on citrate, malate and fumarate retrieved from root samples is shown in Table 1. This analysis could not be performed on bulk soil samples (see below); for rhizosphere soil samples, see Fig. 1. In root samples, the species had a highly significant influence on the amounts of citrate, malate and fumarate. Cultivation time also had a significant effect on all three organic acids (there was a general increase of organic acid contents with cultivation time, data not shown), while the effect of root type (cluster *vs.* non-cluster) was surprisingly only significant for fumarate and malate. This is probably due to the fact that the citrate concentrations varied greatly between the sampling dates. After twelve months (see below, Fig. 1), root type had a highly significant effect on citrate, but no effect anymore on malate and fumarate. Fig. 1 shows the amounts of citrate, malate and fumarate after twelve month cultivation. Only results for the root samples (Fig. 1A) and for the rhizosphere soil samples (Fig. 1B) are presented, because organic acids were scarcely detectable in the bulk soil samples (citrate was not detected, malate was recovered in 4

Table 1
Significant effects of treatments on the amounts of organic acids retrieved from root samples

	df	Citrate		Malate		Fumarate	
		F	*P*	*F*	*P*	*F*	*P*
S	1	128.22	***	83.82	***	73.06	***
RT	1	0.78		7.10	*	16.69	***
T	2	9.34	**	4.01	*	5.64	*
Residuals (MS)	22	0.57		0.95		0.23	

Data were log-transformed. The analysis performed was a three-way ANOVA with plant species (S), root type (RT) and sampling time (T) as explanatory variables. df: degrees of freedom; MS: mean square; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

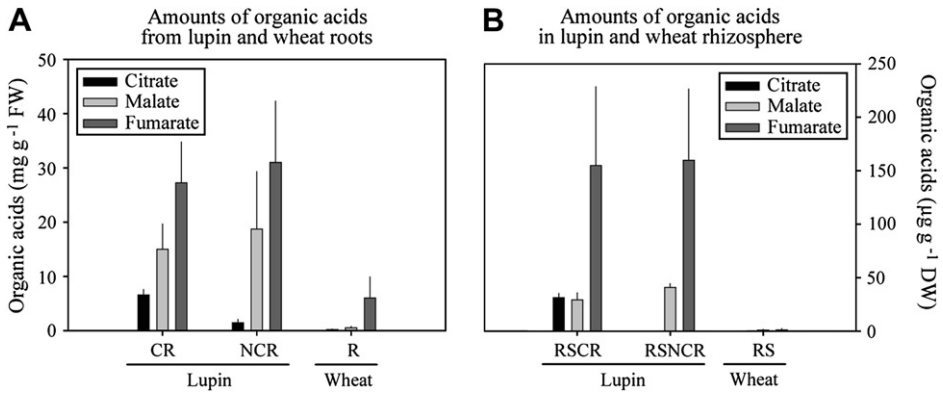

Fig. 1. Citrate, malate and fumarate contents in root (mg g^{-1} FW) and rhizosphere soil (µg g^{-1} DW) samples after twelve months. CR: cluster roots of lupin; NCR: non-cluster roots of lupin; R: roots of wheat; RSCR: rhizosphere soil of lupin cluster roots; RSNCR: rhizosphere soil of lupin non-cluster roots; RS: rhizosphere soil of wheat roots. A two-way ANOVA was performed on root and rhizosphere soil samples (except for citrate in soil samples because of the high number of 0), with species and root type (cluster *vs.* non-cluster) as explanatory variables. Species, but not root type, had a significant effect on malate ($P < 0.001$ for both root and rhizosphere samples), and fumarate ($P < 0.01$ for root and $P < 0.001$ for rhizosphere samples). In root samples, species and root type had a significant effect on citrate ($P < 0.001$ for both).

samples (out of 54) and fumarate was present at a very low concentration (average of 2.3 µg g^{-1} (SE = 0.6))). There was no species effect on fumarate concentrations. Far greater amounts of organic acids were retrieved from lupin samples than from wheat samples. While fumarate and malate were ubiquitously present in root and soil samples, citrate was much more abundant in cluster roots (CR) than in non-cluster roots (NCR) or wheat roots (R). In the rhizosphere soil samples (Fig. 1B), organic acids were present in very low amounts, compared to the root samples (values in mg g^{-1} for roots and in µg g^{-1} for rhizosphere soil samples). Citrate was only retrieved from cluster roots (RSCR), while malate was present in both cluster (RSCR) and non-cluster (RSNCR) roots of lupin, but barely detectable in wheat (RS).

3.2. Plant species, root proximity and cultivation time influenced the soil ATP concentrations

In order to see how the soil biomass would be affected by the continuous cropping of lupin and wheat, we measured the ATP contents of soil samples collected at different root proximity levels and at three different cultivation times. Our results (Fig. 2A) indicate that plant species, root proximity and cultivation time significantly influenced the soil biomass (Table 2). In the rhizosphere soil samples (grey bars), higher ATP concentrations were measured in lupin microcosms as compared to wheat microcosms during the whole year of cultivation, while no such tendency could be observed for bulk soil samples (black bars). Moreover, in lupin microcosms, there was a clear decrease in ATP levels with root distance, from the rhizosphere soil up to the bulk soil collected in the main compartment of the microcosms (BS). This was not observed in wheat microcosms. Surprisingly, the bulk soil collected from the microcosm side-arms displayed a relatively high ATP concentration in all the microcosms. The fact that this was also observed in the control microcosms suggests that this effect was not due to the plant but to other parameters.

3.3. Root proximity and cultivation time influenced the diversity of present (DNA-based profiles) and active (RNA-based profiles) communities

As a further descriptor of bacterial communities, diversity was measured by calculating the Shannon–Wiener index based on the number and the intensity of the bands in DNA- and RNA-based profiles. For DNA- and for RNA-based community profiles (Fig. 2B,C), both cultivation time and root proximity had significant effects on the diversity of bacterial communities, while diversity was not significantly affected by plant species (Table 2). In DNA-based community profiles (Fig. 2B), the detectable diversity increased steadily over the year of cultivation in all the samples, while this was not observed in RNA-based community profiles (Fig. 2C). In lupin cluster roots, the diversity of RNA-based community profiles (Fig. 2C) followed a temporal pattern: at the beginning of the experiment, a higher diversity was observed in the rhizosphere soil of cluster roots (RSCR) as compared to the root fraction (CR), but this tendency gradually changed to reach the opposite situation after twelve months, where a higher diversity was observed in the root fraction (CR) than in the rhizosphere soil fraction (RSCR). This root proximity effect did not extend to the rhizosphere soil samples, whose community diversity was generally (all sampling times and plant species considered) not higher than that of the bulk soil samples. In order to see if the diversity of bacterial communities could be linked to the root and soil contents of organic acids, we submitted the data to a Pearson's correlation analysis. While no significant correlation was observed for RNA-based community profiles, the diversity of DNA-based community profiles (root and soil samples taken together) was negatively correlated with citrate and fumarate (correlation coefficients: citrate: −0.20, $P < 0.05$; fumarate: −0.24, $P < 0.05$).

3.4. Plant species, root type, and rhizosphere organic acid concentrations influenced the structure of root active bacterial communities

In order to analyse the variability between bacterial communities sampled at different time points from lupin, wheat and control microcosms, we performed principal component analysis (PCA) on all the DNA- and RNA-based DGGE profiles. This analysis showed a clear difference between DNA- (triangles) and RNA-based (circles) profiles on the first axis (Fig. 3). This separation between "present" and "active" communities was much reduced in the root samples (white symbols) compared to the bulk soil samples (black symbols) or even to rhizosphere soil samples (grey samples). DNA-based community profiles were more similar to each other than RNA-based community profiles (triangles were grouped more closely than circles). In RNA-based community profiles (circles), root samples were separated from soil samples along the second axis, while this separation was less pronounced for DNA-based community profiles (triangles).

After this descriptive analysis, we asked the question of the putative factors that might account for the variability within each of

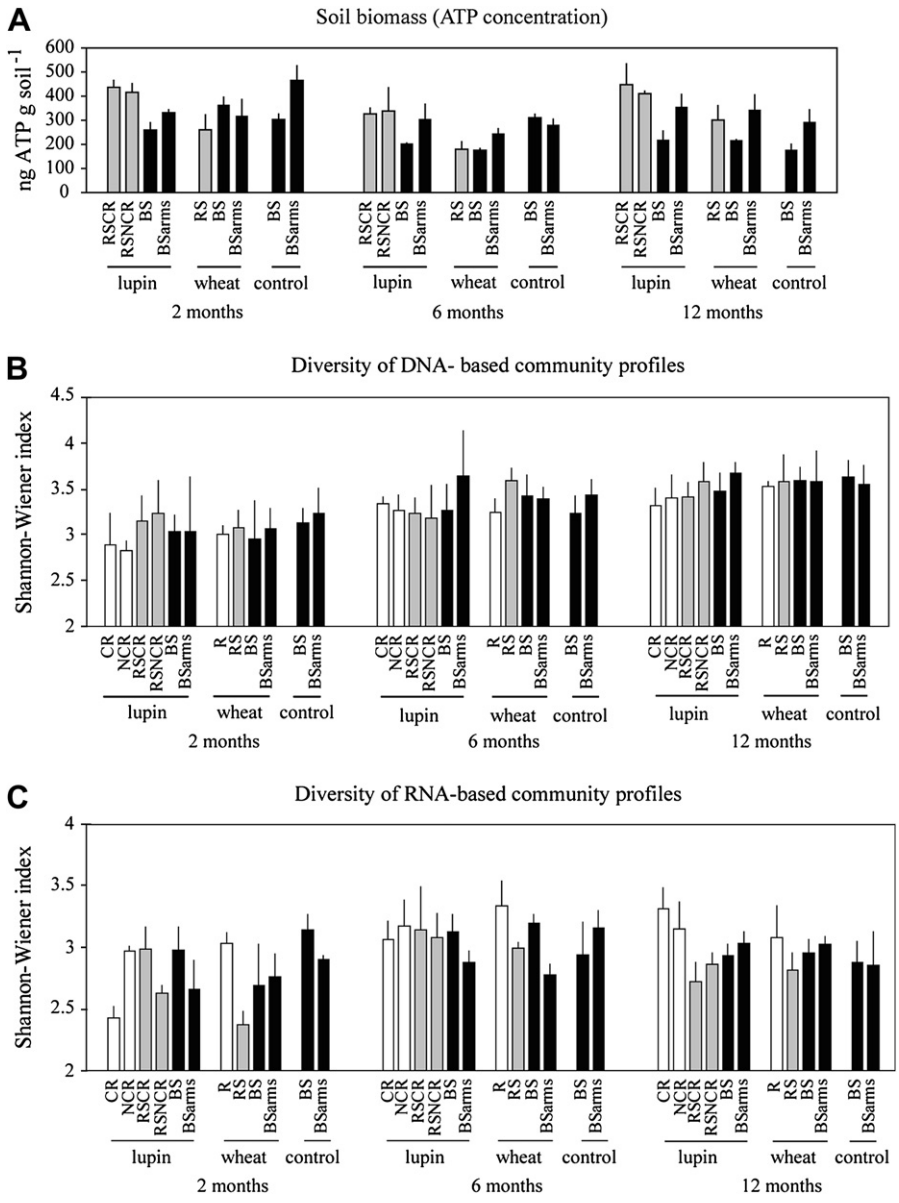

Fig. 2. Effect of time, plant species and root proximity on the soil biomass (A) and on the diversity (B, C) of bacterial communities. White: root samples; grey: rhizosphere samples; black: bulk soil samples. CR: lupin cluster roots, NCR: lupin non-cluster roots; R: wheat roots; RSCR: rhizosphere soil of cluster roots; RSNCR: rhizosphere soil of non-cluster roots; RS: rhizosphere soil of wheat roots; BS: bulk soil from the main part of the microcosm; BS_{arms}: bulk soil from the side-arms of microcosms. Control: unplanted microcosms. Error bars indicate standard deviation. See Table 2 for statistical significance of the treatments.

the different groups of communities displayed in the scatter diagram of the global PCA (Fig. 3). We tested the effect of various sets of explanatory variables including cultivation time, plant species, root type, and organic acid contents on each subset of communities. The resulting variation partitioning is shown in Fig. 4. Cultivation time was the only factor that accounted for a significant part of the variability in all subsets of samples. Its impact (in terms of percentage of variability explanation) diminished with increasing root distance in both DNA- and RNA-based community profiles. No significant effect of other explanatory variables was observed for soil samples. In contrast, in root samples, species also significantly accounted for part of the variability in DNA-based community profiles, and root type (cluster *vs.* non-cluster) as well as organic acids explained a significant portion of variability in RNA-based community profiles. Organic acids even accounted for 15% of the total variability (rank of explained variance: Malate (6.6%, $P = 0.003$) > Fumarate (6.5%, $P = 0.003$) > Citrate (5.6%, $P = 0.03$)), rising above the otherwise dominant effect of cultivation time.

4. Discussion

Several studies have been published investigating the impact of plant species on bacterial communities (Westover et al., 1997; Kennedy et al., 2005; Marschner et al., 2004, 2005; Phillips et al., 2006). However, most of them dealt with relatively small pots and short cultivation time. We were interested in finding a system which came closer to the field situation. Therefore, we used large microcosms allowing for the separation of four levels of root proximity and monitored the effect of two different plant species (white lupin and wheat) on the soil bacterial communities over a one-year cultivation period. Out of the large panel of root-secreted compounds, we focused our attention on organic acids, as readily available carbon source for most heterotrophic soil bacteria. Organic acid secretion has been extensively studied in white lupin for its role in cluster root metabolism and P solubilisation. When coping with phosphorus starvation, white lupin develops special root structures called cluster roots (Purnell, 1960; Lamont, 2003),

Table 2
Significant effects of treatments on the biomass and the diversity of bacterial communities

	df	Soil biomass (ATP concentration)		df	Diversity of DNA-based community profiles		Diversity of RNA-based community profiles	
		F	*P*		*F*	*P*	*F*	*P*
S	2	6.90	**	2	2.23		0.76	
RP	2	11.70	***	3	7.61	***	3.95	*
T	2	9.44	***	2	71.57	***	12.55	***
S × RP	3	5.05	**	4	1.00		0.98	
S × T	4	3.11	*	4	0.64		1.11	
RP × T	4	1.31		6	0.69		1.44	
S × RP × T	6	1.34		8	0.81		1.75	
Residuals (MS)	57	4.55		78	0.04		0.06	

ATP data were square root-transformed prior to analysis. The analysis performed was a three-way ANOVA with plant species (S), root proximity (RP) and sampling time (T) as explanatory variables. df: degrees of freedom (for ATP, only soil samples were measured, hence the lower degrees of freedom); MS: mean square; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

which secrete high amounts of organic acids, especially citrate and malate (Neumann et al., 1999; Ryan et al., 2001; Shu et al., 2005). In contrast, wheat is generally recognized as a species with a low carboxylate release (Nuruzzaman et al., 2006; Pearse et al., 2006a,b). According to the above-mentioned reports, the major organic acids retrieved from the rhizosphere of wheat were malate and citrate, with some other carboxylates present in trace amounts, such as lactate, maleate, *cis*-aconitate, fumarate, acetate, succinate and *trans*-aconitate. Since Veneklaas et al. (2003) showed that organic acid release can be greatly influenced by the soil properties, we measured the amounts of organic acids in the root and soil samples of lupin and wheat grown in our soil conditions. We observed that in our experimental conditions as well, white lupin and wheat differed greatly in this respect, with an 80-fold difference in amounts of organic acids in the rhizosphere of the two species. This confirmed the suitability of our model for studying the organic acid-mediated impact of plants on rhizosphere bacterial communities.

To monitor changes in the spatio-temporal dynamics of bacterial communities associated with the two plant species, we performed DGGE profiling on fragments of the 16S rRNA gene amplified from extracted DNA and reverse-transcribed RNA. As most microbes are in an inactive state in soils (Hu et al., 1999), whole community parameters (i.e. DNA- and fatty acid-based analyses) are probably less sensitive than those measuring some component of the active microfora. Recently developed molecular approaches such as stable isotope probing (Radajewski et al., 2000) or RNA-based analysis (Felske and Akkermans, 1998; Koizumi et al., 2003), as applied in the present work, may be more appropriate to obtain a relevant picture of metabolically active communities. As the ribosome content of cells depends on their level of activity, profiles obtained after reverse transcriptase (RT)-PCR of environmental 16SrRNA are weighted according to the actual activity of the related populations (Wagner, 1994). Thus, DNA-based profiles can be interpreted as a snapshot of the most abundant populations present at the moment of sampling, while RNA-based profiles can be viewed as a snapshot of the most abundant active populations at the moment of sampling.

4.1. The impact of plant species and root proximity on soil biomass, on bacterial diversity and on bacterial community structure

4.1.1. Impact of plant species on soil biomass

White lupin and wheat differed in their impact on soil biomass, as indicated by the ATP values measured (Table 2): in the rhizosphere soil samples, biomass was generally higher in lupin microcosms than in wheat microcosms, while this difference was not observed for bulk soil samples. Such a difference between rhizosphere soil samples of lupin and wheat was also observed in levels of organic acids (Fig. 1B), while no difference could be observed between bulk soil samples from lupin and wheat. Similar ATP contents were measured in the rhizosphere soil of cluster roots and of non-cluster roots, which is not surprising when considering that in our soil conditions, similar amounts of organic acids (with the exception of citrate, which was only detected in the rhizosphere soil of cluster roots) were found in both kinds of rhizosphere samples. This contrasts with previous literature reports, which showed higher secretion rates for cluster roots than for non-cluster roots (Neumann and Römheld, 1999; Massonneau et al., 2001). However, in most studies comparing cluster and non-cluster roots (Neumann et al., 2000; Massonneau et al., 2001), cluster roots were separated into different developmental stages, whereas such a separation was technically not possible in our soil-grown plants. Thus, mature cluster roots, which secrete high amounts of organic acids, especially citrate, are mixed with juvenile and senescent cluster roots, whose organic acid secretion levels are much lower.

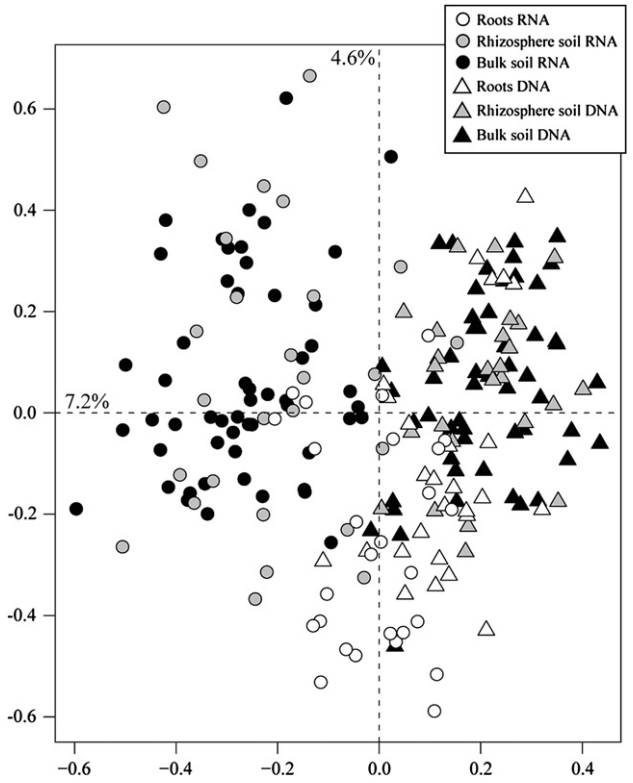

Fig. 3. Scatter diagram of the principal component analysis (PCA) of bacterial communities performed on total samples, using 16S rDNA- and 16S rRNA-based DGGE profiles. White: roots; grey: rhizosphere soil; black: bulk soil. Triangles: DNA-based profiles; circles: RNA-based profiles.

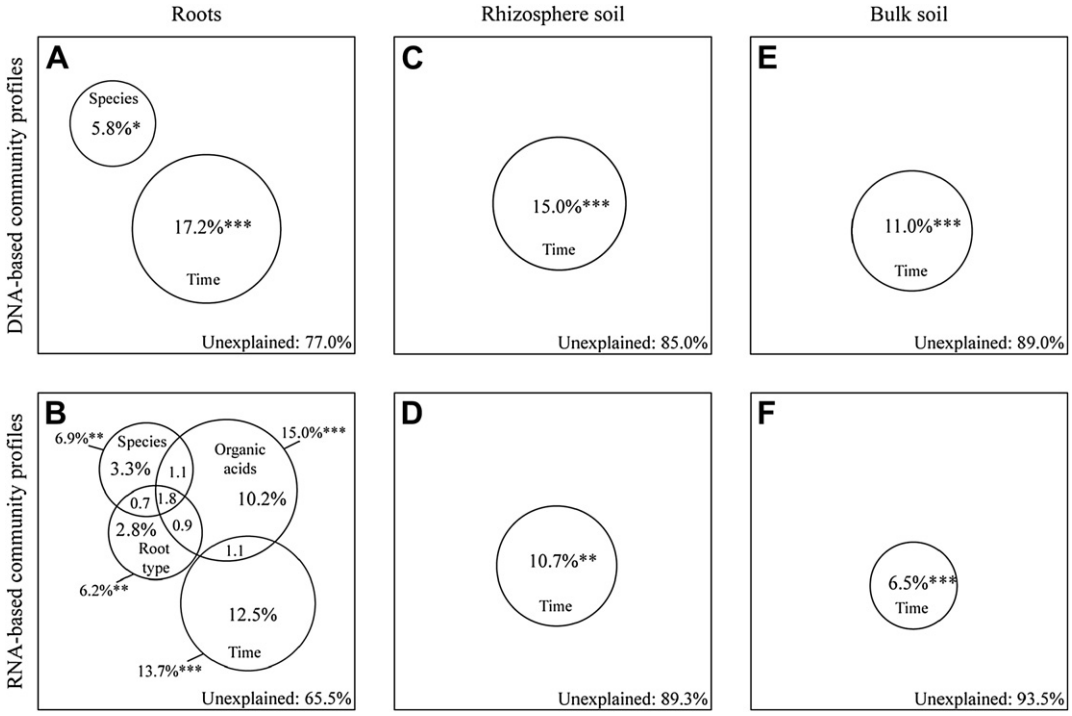

Fig. 4. Variation partitioning with partial redundancy analysis (RDA) performed on subsets of DNA- or RNA-based community profiles from root (A, B) rhizosphere soil (C, D) and bulk soil (E, F) samples. Monte Carlo permutation tests with 999 permutations were performed on each set of explanatory variables (for root and rhizosphere soil samples: i) species (wheat *vs.* lupin); ii) root type (cluster *vs.* non-cluster); iii) organic acids; and iv) cultivation time; for bulk soil samples: i) species (wheat, lupin and control); ii) position (in the microcosm *vs.* in the arms); iii) organic acids; and iv) cultivation time). Only sets of explanatory variables accounting significantly for variation are presented. Values for shared variations below 0.5% are not displayed on the figure. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

4.1.2. Impact of plant species and root proximity on bacterial diversity

In order to analyze the changes in bacterial diversity occurring along our spatio-temporal gradient, we calculated Shannon indices from the DGGE profiles of the different communities. Though initially designed for the description of the community structure of multicellular organisms (Simpson, 1949; Shannon and Weaver, 1963), diversity indices can also be used to describe microbial communities (for a review, see Hill et al., 2003). The Shannon index is the most commonly used diversity index, yet it is not free of bias when applied to DGGE data, because of the diversity and multiplicity of bacterial operons, which can give rise to an artificially high diversity index. Despite this draw-back, the Shannon index is often used to compare bacterial community profiles (Simpson et al., 1999; Ampe and Miambi, 2000; Kocherginskaya et al., 2001; Marschner et al., 2001; McCaig et al., 2001; Ogino et al., 2001). No significant effect of plant species was observed on the diversity of DNA- and RNA-based community profiles, but root proximity significantly affected both types of communities: in DNA-based community profiles, diversity increased with root distance in lupin microcosms, which is in line with previous studies that reported a decrease in diversity with increasing root proximity (Marilley and Aragno, 1999; Weisskopf et al., 2005), due to the elective and selective effect of the root. In contrast, in RNA-based community profiles, the opposite tendency was observed, especially in wheat microcosms: bacterial diversity increased with root proximity. This also confirms previous results observed in the rhizosphere of lupin (Weisskopf et al., 2005). Other studies have reported similar discrepancies between DNA- and RNA-based community profiles: while Koizumi et al. (2003) observed higher differences in the structure of RNA-based community profiles between the upper and the lower layers of a lake sediment, Mahmood and Prosser (2006) obtained quicker, finer-scaled and more reproducible shifts following treatment in ammonia-oxidizing RNA-based community profiles than in DNA-based community profiles. These reports, together with our results, highlight the necessity of including RNA-based fingerprinting techniques to the more commonly used DNA-based techniques, in order to gain a more complete understanding of the structure and function of bacterial communities.

These changes in diversity in function of root proximity mainly occurred between the immediate root environment (rhizoplane-endorhizosphere) and the soil samples, while only few differences were observed between the rhizosphere and the bulk soil samples. This could be due to the fact that in our conditions, very low amounts of organic acids were measured in the rhizosphere soil samples (see Fig. 1B), which might be sufficient to generally stimulate bacterial activity and thus lead to an enhanced biomass but not sufficient to induce changes in the diversity of bacterial communities. It should be noted however, that the low amounts of organic acids retrieved in the soil samples could be the result of a very rapid turnover through bacterial breakdown, rather than reflecting low levels of secreted organic acids (Jones, 1998; Weisskopf et al., 2006a).

4.1.3. Impact of plant species on bacterial community structure

When looking at the effect of plant species on the structure of bacterial communities, we observed that it was only significant for root communities, accounting for 5.8% of the total variability between DNA-based community profiles and for 6.9% of the total variability between RNA-based community profiles. This is in line with previous reports that demonstrated the influence of plant species on global communities (Westover et al., 1997; Marschner et al., 2004, 2005; Phillips et al., 2006) as well as on specific phylogenetic or functional groups of rhizosphere bacteria (Costa et al., 2006, 2007; Patra et al., 2006). Surprisingly, there was no effect of plant species in rhizosphere soil communities, in contrast to what was observed by Jossi et al. (2006), where the effect of plant species was also clearly visible in the soil communities. Though taken as

a global factor in our statistical analysis, the fact that plant species encompasses many parameters of diverse nature should be kept in mind: apart from the rhizodeposition (organic acids, but also flavonoids, which are secreted in high amounts into the rhizosphere of white lupin (Weisskopf et al., 2006b)), the general fitness and health of the plant, its nutrient uptake and the induced changes in rhizosphere nutrient availability are factors that are all likely to have a significant impact on the rhizosphere microbial communities.

4.2. Cultivation time as a major factor influencing bacterial communities

In all parameters studied (soil global biomass, diversity and community structure of DNA- and RNA-based community profiles), the cultivation time was a significant factor accounting for the observed variability. Moreover, the percentage of variability between bacterial communities explained by the cultivation time decreased with increasing root distance (Fig. 4). This was not surprising, since each harvest time corresponded to a different plant generation, as well as to different plant individuals that are expected to induce greater changes in their direct vicinity than at a larger distance. Interestingly, cultivation time also explained a significant fraction of the variability in bulk soil communities. As this subset of data comprises not only the root-free side-arm compartments, but the bulk soil samples from the main compartment as well, this might be due to the supply of organic matter through degradation of old roots, which can be expected to influence the bacterial communities. This recurrent significance of the time factor highlights the necessity of working on a large time scale. Previous studies have been carried out in laboratory to investigate the impact of the plant species on the structure of microbial communities but in most cases, these were short- or middle-termed experiments (30 days for Yang and Crowley, 2000; 2 months for Marschner et al., 2004; 75 days for Kennedy et al., 2005; 80 days for Innes et al., 2004), with a longest cultivation time of 4.5 months in the case of Phillips et al. (2006). While these reports are very informative at their scale and may describe accurately the first steps of the establishment of rhizosphere bacterial communities associated with the arrival of a nutrient-furnishing root into the soil, in our opinion, working on a larger temporal scale is required to assess long-term changes and to come closer to the understanding of the real field situation.

4.3. Organic acids: key players in the structuring of bacterial communities?

The diversity of DNA-based community profiles was negatively correlated with the amounts of citrate and fumarate retrieved in the samples (root and soil samples taken together). This suggests that these compounds could act as elective and/or selective agents in the rhizosphere, stimulating the growth of particular bacterial populations and/or inhibiting other populations, e.g. through the acidification which is often concomitant to organic acid secretion (Jones, 1998; Weisskopf et al., 2006a), and this also indicates that they could account for the decrease in diversity with increasing root proximity. Interestingly, this correlation between organic acids and bacterial diversity of DNA-based community profiles was not corroborated by a significant effect of plant species, indicating that in our case, the organic acid concentration in the rhizosphere was a more relevant factor in explaining changes in bacterial diversity than the plant species itself. As for the community structure, organic acids explained a significant part of the variability among active root communities, accounting for 15% of total variability. This result, together with the fact that only a small part of the variation explained by organic acids overlapped with the variation explained by plant species and/or root type, confirms our hypothesis that organic acids *per se* might play an important role in the structuring of root microbial communities. Surprisingly, this effect of organic acids was not observed for DNA-based community profiles, even if their diversity was negatively correlated with the amounts of organic acids.

While the effect of plant roots on the surrounding microflora could naturally never be reduced to a single class of compounds, our results suggest nevertheless that organic acids play a significant role among root-secreted compounds in the structuring influence of plants on the rhizosphere bacterial communities, at least in the direct vicinity of roots (endorhizosphere-rhizoplane). These new findings raise further questions as to the mechanism of action of these compounds on the microbial communities, e.g. by stimulating specific populations corresponding to a given metabolic profile, by favouring r-strategists through the supply of an easily degradable carbon source and/or by inhibiting other populations through the acidification which often accompanies the secretion of organic acids (Jones, 1998; Weisskopf et al., 2006a), aspects which would require further attention in the future. Using model plants differing only in organic acid secretion (e.g. close cultivars or mutants with enhanced or blocked secretion) might prove a useful tool for a better understanding of this potential structuring role of organic acids on bacterial communities.

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