

Plant growth stage, fertiliser management and bio-inoculation of arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria affect the rhizobacterial community structure in rain-fed wheat fields

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Abstract

The goal of this study was first to assess the dynamics of the bacterial community during a growing season in three Indian rain-fed wheat fields which differ mainly through their fertilizer management and yield and then to study the effects of PGPR/AMF bio-inoculations on the bacterial community structure and wheat growth. The bacterial community structure of the rhizosphere soil (RS) and the rhizoplane/endorhizosphere (RE) was determined by PCR-denaturing gradient gel electrophoresis. Seed treatments consisted of consortia of two PGPR strains alone or combined with AMF or AMF alone. The PGPR strains were *Pseudomonas* spp. which included some or all of the following plant growth promoting properties: phosphate solubilisation and production of indole-3-acetic acid, siderophores, 1-aminocyclopropane-1-carboxylate deaminase and diacetyl-phloroglucinol. The mycorrhizal inoculum was an indigenous AMF consortium isolated from the field with the lowest level of fertilization and yield. Variation partitioning analysis of the DGGE data indicated a predominant effect of the wheat growth stage (30.4% of the variance, $P=0.001$) over the type of field (9.0%, $P=0.027$) on the bacterial community structure in the RE. The impact of plant age in the RS was less than in the RE and the bacterial community structure of the field with the highest input of fertilization was very different from the low input fields. The bio-inoculants induced a significant modification in the bacterial community structure. In the RS, the bacterial consortia explained 28.3% ($P=0.001$) and the presence of AMF 10.6% ($P=0.02$) of the variance and the same trend was observed in the RE. Plant yield or grain quality was either increased or remained unaffected. For example, protein content was significantly higher in the treated plants' grain compared to the control plants; maximum values were obtained when the PGPR were co-inoculated with the AMF. The percentage of root colonization by AMF was significantly higher in the treatments containing a mycorrhizal inoculum than in the untreated control and remained unaffected by the PGPR treatments. In conclusion, the wheat rhizobacterial community structure is highly dynamic and influenced by different factors such as the plant's age, the fertilizer input and the type of bio-inoculant. In addition, there is a distance-related effect of the root on the bacterial community. Finally, a combined bio-inoculation of diacetyl-phloroglucinol producing PGPR strains and AMF can synergistically improve the nutritional quality of the grain without negatively affecting mycorrhizal growth.

Keywords: Wheat; Rhizosphere; Growth stage; PGPR; AMF; DGGE

1. Introduction

This study is part of a project by the Indo-Swiss collaboration in biotechnology (ISCB), whose main goal is to develop new biotechnologies, such as the use of bio-inoculants to improve plant growth and soil health in marginal rain-fed regions of India. In 1995, the areas planted with rice and wheat

in India covered 43 and 25 million ha, respectively. Nearly 25% of the rice area and 40% of the wheat area are currently cultivated in rice–wheat rotations (Abrol, 1999). In this system, rice is grown in the kharif (rainy) season, followed by wheat in the rabi (winter) season. The Indian 'Green Revolution', which took place in the 1960s, has increased crop yield dramatically, by introducing high-yielding varieties and the use of large amounts of mineral fertilizers and/or pesticides. Demand for rice and wheat will grow by 2.5% per year over the next 20 years (Hobbs and Gupta, 2001). In some regions however, food grain production has stagnated or even declined for both rice and wheat crops in recent years (Dawe and Dobermann, 1999).

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The causes of this decline may include changes in biochemical and physical composition of the soil organic matter, depletion and diminution in bio-availability of soil nutrients, scarcity of surface water and groundwater, as well as poor water quality (salinity), and pest population increase (Abrol, 1999; Ladha et al., 2000; Timsina and Connor, 2001). Consequently, these areas will require an integrated management strategy, including the use of biotechnology to improve not only the crop itself, but also the interaction of its roots with soil microbial partners, such as the bacterial community and arbuscular mycorrhizal fungi (AMF).

Microbial communities in the soil or rhizosphere contribute to plant growth by recycling nutrients and making them available (Lynch, 1990), increasing root health through competition with root pathogens (Weller et al., 2002) or enhancing nutrient uptake (Smith and Read, 1997). Wheat transfers about 30% of carbon assimilates into the soil through the process of rhizodeposition and part of this below-ground translocated C is incorporated by rhizosphere micro-organisms (Kuzakov and Domanski, 2000). Therefore, bacterial abundance and turnover increase in the rhizosphere compared to the bulk soil. Within the rhizosphere microbial populations are a group of plant-beneficial bacteria referred to as plant growth promoting rhizobacteria (PGPR). PGPR are beneficial to plants via nutrient acquisition (Rodriguez and Fraga, 1999; Dobbeleire et al., 2003; Ladha and Reddy, 2003), biocontrol (Walsh et al., 2001; Chin-A-Woeng et al., 2003), plant hormone-like production, lowering of plant ethylene level (Glick, 1995; Steenhoudt and Vanderleyden, 2000) and induction of systemic resistance (van Loon et al., 1998). Other beneficial microbial partners of the plant are the ubiquitous symbiotic arbuscular mycorrhizal fungi (AMF). Beneficial effects of AMF in agriculture include improved plant nutrition (mainly via phosphorus acquisition), water stress resistance (Smith and Read, 1997), biological control against pathogens (Azcón-Aguilar and Barea, 1996) and better soil structure (Miller and Jastrow, 1990).

The rhizosphere microbial communities can be affected by a wide range of factors including plant type (Grayston et al., 1998; Germida and Siciliano, 2001), plant age (Marschner et al., 2004), distance from the soil to the root (Marilley and Aragno, 1999), soil characteristics (Latour et al., 1996; Buyer et al., 1999), agronomic practices (Lupwayi et al., 1998; Alvey et al., 2003; Kennedy et al., 2004) and mycorrhizal infection (Marschner et al., 2001). These changes in the rhizosphere community might affect plant growth negatively (e.g. root pathogen development) or positively (increase in the proportion of PGPR populations). It is, therefore, necessary to study the microbial community dynamics in the field before modifying the agricultural practice, especially when using bio-inoculants to improve soil health or crop yield. The goal of this study was first to assess the dynamics of the bacterial community during a growing season in three Indian rain-fed wheat fields which differ mainly through their fertilizer management and yield and then to study the effects of PGPR/AMF bio-inoculations on the bacterial community structure and wheat growth. The bacterial community structure

of the rhizosphere soil (RS) and the rhizoplane/endorhizosphere (RE) was determined by PCR-denaturing gradient gel electrophoresis. This molecular fingerprinting technique allows the detection of the most abundant bacterial populations, culturable and non-culturable, in the rhizosphere. The experimental fields were located in the Budaun district of Uttar Pradesh (India), where rice and wheat rotation crop practices have been carried out for over 20 years. The area does not have modern irrigation facilities and is, therefore, considered as rain-fed.

2. Material and methods

2.1. Experimental sites

Two experiments were carried out in a study site located in the village Bhavnipur (Badaun District, latitude 28.02°N, longitude 79.10°E, alt. 600 m) in Uttar Pradesh, India. The first experiment consisted in analysing the rhizobacterial community dynamics during wheat growth in three different fields. It was performed during the rabi season which lasted from November 2001 to March 2002. The second experiment was performed in the rabi season of the following year, December 2002 to March 2003, and aimed to analyse the effect of PGPR/AMF bio-inoculations on the rhizobacterial community and wheat growth. The study site is rain-fed, has been cultivated in rice-wheat rotation for twenty years and has a basic irrigation system, largely dependent on monsoon rains. For wheat, standard agronomic practices were followed such as regular sowing, irrigation and weeding. Three 4000 m² fields were selected at this site. They were separated by less than 2 km and differed mainly in their fertilizer input and wheat grain yields (field characteristics are presented in Table 1). They were classified as low input low yield (LL), low input moderate yield (LM) and high input high yield (HH). Their soils have a sandy loam texture and belong to the following USDA classification: Entisol (order), Psamment (Sub order), Doarse (Family), Hyperthermic (Regime). The same wheat cultivar, UP 2338 (provided by GB Pant University of Agriculture and Technology, Pantnagar, Uttaranchal State), was grown for more than 5 years in the three fields, in rotation with rice.

2.2. Bio-inoculant characterization and seed treatment

Bacteria used in PGPR consortia were *Pseudomonas* spp. strains selected as follows: approximately 3000 strains were isolated from the rhizosphere of wheat (variety UP 2338) from LL, LM and HH fields. From this pool, 20 strains with the most promising PGP properties were then selected. These properties included phosphate solubilisation and the production of indole-3-acetic acid, siderophores, 1-aminocyclopropane-1-carboxylate deaminase and diacetyl-phloroglucinol. These strains were then tested in different consortia for plant growth promoting effects in greenhouse experiments, in pots containing soil from LL, LM and HH with wheat var. UP2338 (Gaur, thesis submitted in 2003). Bacterial consortia which improved

Table 1
Fertilizer inputs and soil physico-chemical characteristics of the study fields LL, LM and HH, before sowing in 2001

Field	Fertilizer input		pH	Total P (kg ha ⁻¹)	Av. P (kg ha ⁻¹)	Total N (kg ha ⁻¹)	Av. N (kg ha ⁻¹)	Exch. K (kg ha ⁻¹)	OM (%)	Cu (mg kg ⁻¹)	Mn (mg kg ⁻¹)	Zn (mg kg ⁻¹)	Fe (mg kg ⁻¹)
	Urea (kg ha ⁻¹)	DAP (kg ha ⁻¹)											
LL	50	0	7.2	919.0	11.4	1198.2	95.8	136.1	1.06	1.65	18.54	0.62	22.1
LM	50	0	7.4	1040.7	15.4	1219.1	99.4	190.6	1.11	3.02	24.12	1.62	20.7
HH	60	20	7.2	1672.7	28.5	1581.4	100.8	170.2	1.42	1.36	19.25	0.59	38.3

DAP, diammonium phosphate; Av. P, available phosphorus; Av. N, available nitrogen; Exch. K, exchangeable potassium; OM, organic matter.

plant growth the most were selected for the field trials. The bacterial strains were maintained in Kings' B agar slants (Difco Laboratories, Sparks, USA) and stored under glycerol. The plant growth promoting properties of the PGPR strains used in this study are summarized in Table 2. The mycorrhizal inoculum was composed of an indigenous AMF consortium isolated from the LL field and subcultured by our partners at The Energy and Resources Institute (TERI) in New Delhi. Mycorrhizal subculturing was performed in trap cultures according to Oehl et al. (2003). The host plants were *Allium cepa*, *Tagetes* spp., *Daucus carotus*, *Medicago sativa* and *Trifolium alexandrianum* for the first growth cycle and *Gossypium* spp., *Vetiveria zizanioides*, *Sorghum* spp. and *Tagetes* spp. for the second and third cycles. The substrate was composed of autoclaved Terragreen (American Aluminium Oxide, Oil dry US Special, Type IIIR from Lobbe Umweltechnik, Iselohn) and Loess from a local site near Delhi in the ratio 1:1. Wheat seeds were coated according to Sharma et al. (2003). PGPR cultures grown overnight were washed and re-suspended in a 10 mM phosphate buffer saline (PBS) solution. Optical density was adjusted to 0.6 ($\sim 10^8$ cfu ml⁻¹). PGPR and AMF consortia were prepared by adding the AMF inoculum (final concentration 150 g l⁻¹) to the bacterial suspension, along with carboxymethyl cellulose (final concentration 0.1%), to allow the bacteria and fungal hyphae or spores to stick properly to the seeds. Seeds (ratio of 1 g g⁻¹) were added in the suspension, mixed for 20 min and air dried. The number of CFU per seed counted on King's B agar was approximately 10^6 – 10^7 .

Six different seed treatments were used:

- Control, Control containing neither AMF nor PGPR strain
- M, Mycorrhiza without PGPR consortia
- R62/R81, R62 and R81 PGPR consortium alone
- R62/R81/M, R62 and R81 PGPR consortium with AMF
- R103/R110, R103 and R110 PGPR consortium alone
- R103/R110/M, R103 and R110 PGPR consortium with AMF

Seeds were sown in mid-December 2002 in the LL field, which was separated into 18 4 × 4 m plots. Each treatment was applied to three random 16 m² plots.

2.3. Sampling procedure for the bacterial community analysis

In the first experiment (2001–2002 season), samples for the bacterial community analysis were removed at different wheat growth stages: 25 days (crown root initiation growth stage), 45 days (tillering), 90 days (flowering), and 120 days (maturity). In the second experiment (2002–2003 season), the samples for the bacterial community analysis were taken at 90 days growth, which corresponds to the flowering stage. Three to four wheat plants with roots and soil cores were sampled under semi-sterile condition (all the material used for sampling was sterilised) using a soil corer, to a depth of 0 to 30 cm at eight random spots in each field or plot. Coarse stones and vegetal debris were removed. The bulk soil was separated

Table 2
Characterization of the *Pseudomonas* spp. bacterial bio-inoculants used as treatments in the second experiment, based on Gaur et al. (2004)

Strain	Origin		PGP properties				
	Field	Fraction	P	IAA	ACC	Sid	DAPG
R62	LM	RE	+	+		+	+
R81	HH	RE	+	+	+	+	+
R103	LL	RS	+	+	+	+	
R110	LL	RS		+	+		

Abbreviations: RE, rhizoplane/endorrhizosphere; RS, rhizosphere soil; '+' indicates that the strains possess the following plant growth properties: P, phosphate solubilization; IAA, indole-3 acetic acid production; ACC, 1-aminocyclopropane-1-carboxylate deaminase production; Sid, siderophore production; DAPG, diacetyl-phloroglucinol production.

from the adhering soil by shaking the plants. The root systems with adhering soil from all eight spots were separated from the aerial part of the plant using sterile scissors and pooled. The resulting composite samples were stored at -80°C for subsequent molecular analysis and determination of mycorrhizal colonization.

2.4. Plant data

At harvest in the 2001–2002 rabi season, the plants from three 1 m^2 subplots per field were combined, the grain was removed from the straw, then the straw and grain weights were recorded. In the 2002–2003 rabi season, plant height and grain weight were measured from the plants in 1 m^2 surfaces in each of the 16 m^2 plots. Mycorrhizal structures were stained with 0.05% w/v trypan blue, after the roots were cleared with KOH 10% according to Brundrett et al. (1994). Mycorrhizal colonization percentage was evaluated by the grid-line intersect method (Giovanetti and Mosse, 1980). For nutrient analysis, the following procedure was applied: 1 g seed was digested in an acid mixture of HNO_3 and HClO_4 (9:4). The resulting ash was analysed for nutrient content. The concentration of potassium and the micronutrients Zn and Fe were determined by flame photometer. The concentration of organic C was determined by the Walkley and Black method (Walkley and Black, 1934). Total nitrogen was determined by Kjeldhal's method (in Allen et al., 1974) and converted into protein content through multiplication by the constant 5.7. Phosphorus content was determined with the molybdenum–ascorbic acid colorimetric method (Hansen, 1950).

2.5. DNA extraction from RS and RE samples

A fraction of the root systems with adhering soil was immersed into sterile 0.1 M sodium phosphate buffer (pH = 7) and stirred to separate the root-adhering rhizosphere soil (RS) from the rhizoplane–endorrhizosphere (RE) fraction. The washed roots (RE) were removed. The remaining suspension was considered to be the RS fraction. The roots were rinsed with sterile deionised water and dried on sterile Whatman paper (Merck AG). One gram of fresh weight RE was crushed under sterile conditions in 10 ml of phosphate buffer, using a pestle and mortar. This constituted the RE fraction.

DNA was extracted from the RS and RE fractions by the bead-beater technique (FastPrep FP120, SAVANT, BIO101,

Carlsbad, USA) using a FastDNA spin kit for soil DNA extraction (BIO101), according to the manufacturer's protocol. The extracted DNA was further purified with a GENE-CLEAN[®] II kit (BIO101) and stored at -20°C in TE buffer.

2.6. DGGE analysis

For the first season (2001–2002), two DNA extractions and DGGE analyses were carried out per field and wheat growth stage. For the second season (2002–2003), one DNA extraction and DGGE analysis was carried out per plot (three plots per seed treatment). A two-step PCR was used to amplify the V3 region, a fragment of about 200 bp of the bacterial 16S rRNA gene. The final concentration of the amplification reaction mix was: 1x Thermophilic DNA polymerase Buffer (Promega, Madison, USA), 2.5 mM MgCl_2 (Promega), 0.025 mM of each dNTP (Gibco, Cheshire, UK), 0.25 μM of each primer (Microsynth, Balgach, Switzerland) and 0.05 $\text{U}\mu\text{l}^{-1}$ Taq polymerase (Promega). For a 20 μl reaction, 0.1–1 $\text{ng}\mu\text{l}^{-1}$ (final concentration) of extracted DNA was used as a template. First, bacterial primers GM3f and GM4r were used to amplify the 8-1492 region of the 16S rRNA gene (Muyzer et al., 1995). The first PCR was carried out with an initial denaturation step at 94°C for 4 min, followed by a touchdown PCR of 11 cycles consisting of denaturation at 94°C for 30 s, annealing from 56 to 51°C for 30 s, elongation at 74°C for 1 min. The touchdown was followed by 15 cycles comprising denaturation at 94°C for 30 s, annealing at 51°C for 30 s and elongation at 74°C for 1 min. The process was completed by a final elongation step at 74°C for 10 min. Amplicons were diluted 10 times in sterile deionised water. The nested amplification was then carried out with the universal primers 338f and 520r (Ovrias et al., 1997), that target the V3 region of the 16S rRNA gene. It comprised an initial denaturation step at 94°C for 5 min, followed by 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 65°C for 30 s with a touchdown rate from 65 to 55°C during 10 cycles and elongation at 74°C for 1 min. A final elongation step at 74°C for 10 min completed the PCR.

A composite mix of different bacterial 16S rRNA gene fragments was added on each side of the DGGE gel as a reference DGGE pattern: *Pseudomonas fluorescens* ATCC 27663, *Acidovorax facilis* DSM 550, *Bacillus subtilis* ATCC 14893, *Sinorhizobium meliloti* DSM 1981 and *Aquaspirillum dispar* ATCC 27650. DGGE was performed using a 8% (w/v) acryl-bisacrylamide gel (37.5:1, Qbiogene, Illkirch, France)

with 30–60% linear urea/formamide (Fluka, Buchs, Switzerland, Qbiogene) denaturing gradient (100% denaturant corresponds to 40% formamide + 7 M urea). Five hundred nanograms of the PCR product were electrophoresed in 1× TAE buffer (Qbiogene, France) at 60 °C, with a constant voltage of 150 V during 5.5 h, using the BioRad D-Code Electrophoresis System (Bio-Rad Inc. California, USA). The gels were stained in the dark for 20 min in 0.01% Sybr Green I (Molecular Probes, Leiden, The Netherlands) in 1× TAE solution. Gels were photographed with the Multi-Analyst package (Bio-Rad Inc., California, USA). The DGGE fingerprints were normalised according to the reference patterns and were compared using the GelCompar software (Applied Maths, Kortrijk, Belgium). An example of DGGE profile is shown in Fig. 1. DGGE banding patterns were then converted to a numerical matrix, which was used in the statistical analysis. For each sample, two DGGE gels were run and the mean value of these two profiles was considered as the final fingerprint value. Each band was considered to represent a single bacterial population and the band intensity as representative of the relative abundance of the population (Fromin et al., 2002). The bands whose average relative contribution was below 1% were discarded from the analysis.

2.7. Statistical analysis

The plant data were subjected to analysis of variance (ANOVA) and the means were compared with the Tukey test, using the S-Plus software V. 6.1 (Insightful Corp, USA). Ordination methods were applied on the basis of numerical data matrices converted using the program Progiciel R (Legendre and Vaudor, 1991) and calculated with the Canoco 4.0 software (Canoco 4.0, Microcomputer Power, Ithaca, USA). The DGGE profile matrix was composed of rows of objects representing the samples and columns of species representing the DGGE band position along the vertical gel

gradient. The relative abundance of a species in a sample was derived from the DGGE band's relative intensity with respect to the sum of intensities of all bands in a pattern. Canonical Correspondence Analysis (CCA) was applied to quantify and test the effects of various sets of explanatory variables on the DGGE profile variation. For the same purpose, the plant data were first standardized and then submitted to Redundancy Analysis (RDA). Variation partitioning analysis (Borcard et al., 1992) helps to display the variability of patterns, constrained by the factors of interest. The significance of the result was tested with the Monte Carlo permutation test. Variation partitioning analysis was performed with Canoco 4.0. Mantel tests were performed with Progiciel R, to determine the correlation between plant data and DGGE numerical data matrices.

3. Results

3.1. Wheat rhizosphere bacterial community assessment in the fields LL, LM and HH during the 2001–2002 rabi season

The straw and grain yields differed between the three fields (straw and grain yield, respectively, in t ha^{-1}): LL (1.06 and 1.55), LM (1.54 and 2.19), HH (2.01 and 2.88). The wheat rhizobacterial community was very dynamic during the growth season, as strong shifts in the DGGE patterns were observed between the different wheat growth stages. In order to test the effects of environmental factors on the structure of the bacterial community, the DGGE profiles of the RS and RE fractions were analysed by canonical correspondence analysis (CCA), as shown in Fig. 2. Symbols (DGGE profiles or objects represented as squares, triangles and circles) which lie close together are likely to have similar bacterial community profiles. Symbols close to centroids points (X in Fig. 2) represent bacterial community profiles that are likely to contain species (DGGE bands) frequently (or more abundantly) found under the conditions which correspond to the qualitative explanatory variables. In the CCA of the RE (Fig. 2(a)), the symbols are grouped by growth stage (25, 45, 90, 120 days), thus indicating a predominant effect of the wheat growth stage over the type of field on the bacterial community structure. In the RE fraction, the growth stage actually explained 30.4% ($P=0.001$) of the variance in the DGGE profiles, whereas the type of field explained only 9.0% ($P=0.027$), with a shared variance of 0.2% ($P=0.01$). In addition, the differences in bacterial community composition were less pronounced between 25 and 45 days, whereas the community at 120 days differed strongly from that of the earlier sampling dates. However, in the RS fraction, the differences between the fields were more pronounced and 15.3% ($P=0.001$) of the variance in the DGGE profiles could be explained by the type of field, against 15.6% ($P=0.01$) for the wheat growth stage (Fig. 2(b)). The percentage explanation by these factors was relatively low as 60.4 and 69.1% of the variance was not explained in the RE and RS, respectively.

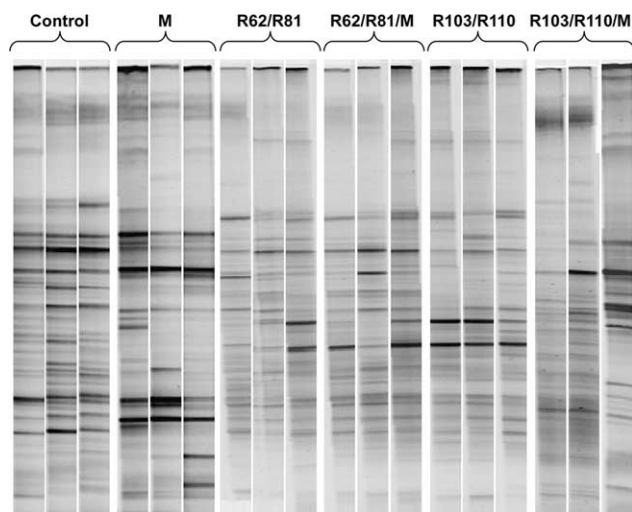


Fig. 1. Example of DGGE profiles of the rhizoplane/endorhizosphere bacterial communities for the 2002–2003 season. Each treatment is replicated three times.

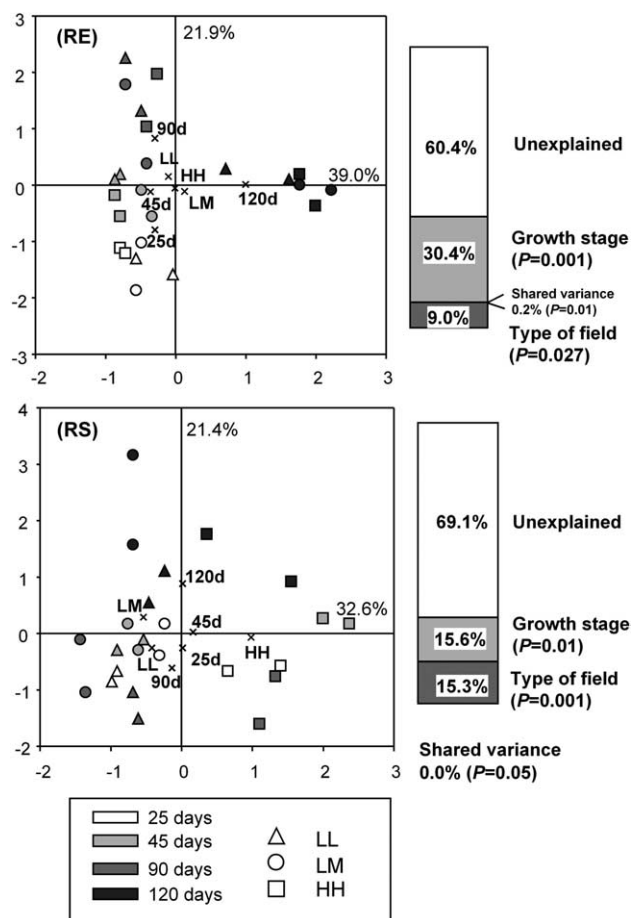


Fig. 2. Canonical correspondence analysis (CCA) of DGGE bacterial community profiles for different wheat growth stages and fields during the 2001–2002 season. Time 25, 45, 90, 120 days and fields LL, LM and HH are used as qualitative explanatory variables (centroids). The values on the axes indicate the percentage of total variation which they explain. The variance decomposition of the CCA on the bacterial community profiles is shown as a bar diagram. (a) Analysis of the community profiles of the rhizoplane/endorhizosphere: sum of all canonical eigenvalues, 1.127; total inertia, 2.846; Monte Carlo overall 999 permutation test, $P=0.001$. (b) Analysis of the community profiles of the rhizosphere soil: sum of all canonical eigenvalues, 0.881; total inertia, 2.850; Monte Carlo overall 999 permutation test, $P=0.001$.

3.2. Rhizosphere bacterial community assessment and plant data of wheat treated with PGPR and AMF in the LL field during the 2002–2003 rabi season

LL was selected because for several years this field yielded less wheat than LM and HH. In addition, the rhizobacterial community response was assessed at the flowering stage, when the total mesophilic culturable bacteria were at their highest level (Gaur, thesis submitted in 2003). Significant differences were observed between the DGGE profiles of the control and bio-inoculation treatments and there was a high homogeneity between the replicated plots (Figs. 1 and 3). In order to determine to which extent the PGPR or AMF presence affected the bacterial community structure, the DGGE profiles were analysed by variation partitioning (Fig. 3). In the RE, the PGPR consortium explained 26.1% ($P=0.001$) of the variance and

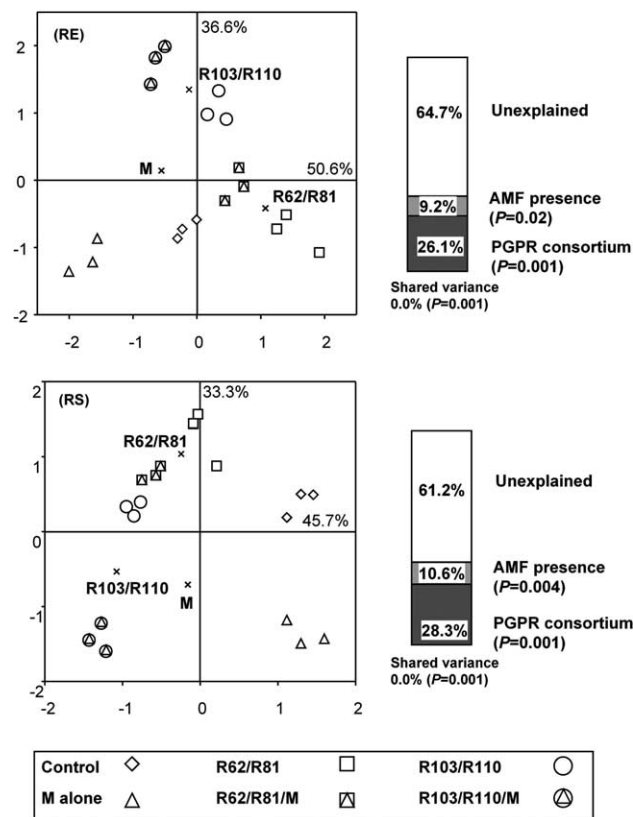


Fig. 3. Canonical correspondence analysis (CCA) of DGGE bacterial community profiles for different consortia in LL field during the 2002–2003 season. The PGPR consortium (R62/R81 or R103/R110) and the presence of arbuscular mycorrhizal fungi (M) are used as qualitative explanatory variables (centroids). The values on the axes indicate the percentage of total variation which they explain. The variance decomposition of the CCA on the bacterial community profiles is shown as a bar diagram. (a) Analysis of the community profiles of the rhizoplane/endorhizosphere: sum of all canonical eigenvalues, 0.850; total inertia, 2.408; Monte Carlo overall 999 permutation test, $P=0.001$. (b) Analysis of the community profiles of the rhizosphere soil: sum of all canonical eigenvalues, 1.001; total inertia, 2.575; Monte Carlo overall 999 permutation test, $P=0.001$.

the AMF 9.2% ($P=0.02$). The same proportion was found in the RS: the PGPR consortium explained 28.3% ($P=0.001$) of the variance in the DGGE profiles, whereas AMF explained 10.6% ($P=0.004$). As for the type of field and wheat growth stage, the percentage explanation by these factors was quite low as 61.1 and 64.7% of the variance was not explained in the RE and RS, respectively.

The percentage of root colonization by AMF was significantly higher in the treatments containing a mycorrhizal inoculum than the untreated control and was unaffected by the PGPR treatments (Table 3). In comparison with the control, several improvements were noted in the treated plants (Table 3): the plant height increased significantly ($P<0.01$) in R103/R110/M treated plants, organic carbon and protein content of the grain was significantly higher in treated plants, with maximum values obtained when the PGPR were co-inoculated with the AMF, the phosphorus concentration was doubled in the grains of plants treated with the bio-inoculants

Table 3
Wheat plant features at harvest after the 2002–2003 rabi season

Treatments	Root colonization (%)	Plant height (cm)	1000 grain weight (kg)	Grain nutrient content					
				Organic C (%)	Protein content (%)	P (mg kg ⁻¹)	K (mg kg ⁻¹)	Zn (mg kg ⁻¹)	Fe (mg kg ⁻¹)
Control	45.3a	87.7a	32.7a	23.2a	9.2a	35.4a	48.7a	0.26ab	0.48a
M alone	61.5bc	94.3a	34.7a	29.0b	11.6b	65.5bc	58.0c	0.26ab	1.35d
R62/R81	52.2ab	89.3a	38.0a	29.3bc	12.4bc	77.2d	59.5cd	0.31b	0.84b
R62/R81/M	62.6bc	94.7a	38.0a	30.8c	14.05c	68.8c	60.5d	0.28ab	1.33d
R103/R110	47.1a	90.0a	38.7a	28.4b	12.8bc	60.1b	53.8b	0.23a	0.47a
R103/R110/M	62.1c	102.3b	34.7a	33.5d	14.4c	67.2c	57.7c	0.28ab	1.17c

Values are means of 3 plot replicates. Identical letters indicate counts which are not significantly different according to the Tukey test ($P < 0.01$, $n = 3$).

and grain iron content more than doubled in treatments where AMF were applied. With respect to plant growth, the influence of PGPR and AMF was comparable, as the PGPR consortium and the AMF presence explained, respectively, 38.0% ($P = 0.001$) and 33.8% ($P = 0.001$) of the variance in the plant data (Fig. 4). The plant data consisted in plant height, grain weight and grain nutrient content, as shown in Table 3. The Mantel correlation test was carried out to determine if the DGGE profiles and the plant data were correlated. In the RS, a significant correlation (r Mantel, 0.28; $P < 0.01$) was found between the DGGE profiles and plant data. However, this was not the case in the RE ($P = 0.12$).

4. Discussion

The wheat rhizobacterial community structure in the first experiment was highly dynamic and substantially influenced by plant age, as the growth stage explained 30.4% of the variance in the DGGE profiles for the RE and 15.6% for the RS. In addition, the bacterial community structure at 120 days growth (maturity stage) differed most from the other growth

stages. These community shifts during plant growth probably resulted from a modification in the root exudation pattern, which is different at maturity (Lynch, 1990). In fact, after flowering most of the assimilated carbon is transported to the grain, the amount of rhizodeposit would therefore diminish at a later stage of wheat growth (Kuzyakov and Domanski, 2000). Plant age had a lesser impact in the RS than in the RE and explained the same proportion of variance in the DGGE profiles as the type of field. In the RS, the bacterial community structure of the HH field was very different from those of the LL and LM fields. The main difference in agricultural practice between HH and the other two fields was a higher level of fertilization. HH received 60 kg ha⁻¹ of urea and 20 kg ha⁻¹ of diammonium phosphate (DAP), compared to 50 kg ha⁻¹ of urea and no DAP in LL and LM. This higher level of fertilization is likely to have affected the rhizobacterial community, as previously reported (Liljeroth et al., 1990; Marschner et al., 2004; Sturz et al., 2004). This observation indicates that the bacterial community in the RS is likely to be more sensitive to modifications in the agricultural practices than in the RE.

The bio-inoculation by PGPR or AMF, in the form of seed coating or in the soil close to the seed, may cause shifts in the community composition of either small or high magnitude (Nacamulli et al., 1997; Marschner et al., 2001; Bankhead et al., 2004; Kozdrój et al., 2004). In other cases, no significant effect has been reported (Mahaffee and Kloepper, 1997; Lottman et al., 2000; Mansfeld-Giese et al., 2002). In our study, the bio-inoculants induced a significant modification in the bacterial community structure. In addition, the type of PGPR consortium had more impact on the bacterial community structure than the presence of AMF, as roughly one third of the variance was explained by the PGPR consortium and one tenth by the AMF in both the RS and RE. The reasons for this predominant effect of the PGPR over the AMF on the bacterial community structure are unclear. It probably does not result from poor survival of the AMF inoculum, because the root colonization percentage of the AMF treated plants is significantly higher than the non-inoculated control. The high-density inoculum of the PGPR strains on the seeds may possibly have shifted the bacterial community equilibrium at early stages of plant growth. Changes in bacterial composition induced by PGPR or AMF inoculation may be undesirable if

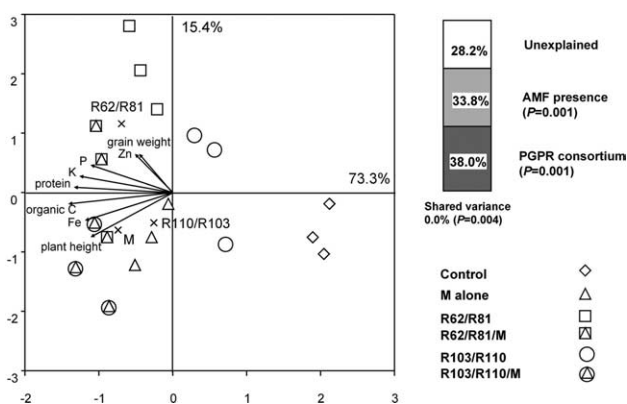


Fig. 4. Redundancy analysis (RDA) of plant data for different treatments in the LL field during the 2002–2003 season. The plant data were standardised before the analysis. Descriptors (arrows) are the plant height, grain weight, organic carbon (organic C), protein content (protein), phosphorus (P), potassium (K), iron (Fe) and zinc (Zn). The PGPR consortium (R62/R81 or R103/R110) and the presence of arbuscular mycorrhizal fungi (M) are used as qualitative explanatory variables (centroids the values on the axes indicate the percentage of total variation which they explain). The variance decomposition of the RDA of the plant data is shown as a bar diagram. Sum of all canonical eigenvalues, 0.718; total inertia, 1.000; Monte Carlo overall 999 permutation test, $P = 0.001$.

important native species are lost, thus affecting subsequent crops. However, a modification in the bacterial community structure caused by a temporary disturbance, such as a PGPR treatment, could be buffered by ecosystem resilience, which is driven by the level of diversity and interactions of the agro-ecosystem (Elliot and Lynch, 1994; Kennedy, 1999). The loss of certain bacterial species may also not change the functioning of the system, as different bacterial species can carry out the same function, a phenomenon defined as the bacterial redundancy (Kennedy, 1999; Nannipieri et al., 2003). In our experiment, even if the equilibrium of the bacterial community had been modified, the yield and grain quality remained unaffected or was increased. As suggested by Ciccillo et al. (2002), this result could mean that the negative effect caused by a modification of the bacterial community equilibrium was overcome by the beneficial effects of the bio-inoculants. Alternatively, the shift in equilibrium could have favoured the growth of beneficial populations. For example, Pandey et al. (1998) have reported that improvements in yield and plant growth resulted in part from the stimulation of N_2 -fixing bacteria in the rhizosphere of maize after the bio-inoculation by two PGPR strains. In our experiment, a positive correlation between the bacterial community profiles and the plant data could mean that an increase in plant yield after inoculation could affect the bacterial community structure, through an increased exudation rate at higher yields. Conversely, it could also mean that certain characteristics of the plant, such as grain quality, depend on the contribution of specific bacterial populations. Our bio-inoculants may therefore have modified the bacterial community equilibrium towards the selection of beneficial populations.

Even if the percentage explanation of the variance by our tested factors was statistically significant, between 60 and 69% of the variance in the bacterial community structure data remained unexplained. This means that a considerable part of the bacterial populations in the rhizosphere was affected by other factors than the growth stage, fertilizer management or the type of bio-inoculant. Our analyses were performed in field conditions meaning a greater heterogeneity between the samples even if they originated from similar fields or plots contrarily to samples that would have been analyzed from more controlled greenhouse or pot experiments. Actually, soil is a heterogeneous and discontinuous system (Nannipieri et al., 2003) and therefore other factors such as the presence/absence of specific microhabitats or root exudates within a sample might have influenced the bacterial community structure. Further research in more controlled field conditions needs to be undertaken in order to determine these other factors.

The positive effect of PGPR and AMF bio-inoculants on wheat growth, observed in previous greenhouse experiments (Gaur et al., 2004), was confirmed in the field. The bio-inoculations had a significant effect on grain quality, for instance the phosphorus content that doubled in the bio-inoculated plants. Grain iron content more than doubled in the treatments in which AMF were applied, compared to non-inoculated controls. AMF are known to improve uptake of nutrients such as phosphorus, potassium, nitrogen and

the micronutrients zinc and copper (Smith and Read, 1997) but reports on iron uptake by AMF are scarce. An increase in iron concentration in maize and sorghum mycorrhizal plants has been reported (Clark and Zeto, 1996; Caris et al., 1998). However, contrary to the observation of these authors, the soils used in this study were neither alkaline nor Fe-depleted (see Table 1). Interestingly, a synergistic effect on the level of organic carbon and protein content was observed between AMF and PGPR when they were co-inoculated. Moreover, the type of bacterial consortia and the AMF presence explained, respectively, 38.0 and 33.8% of the variance in the plant data, which means that the plant response was affected approximately equally by these two factors. Finally, even though the PGPR strains R62 and R81 produce the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG), which is known for its antifungal properties (Weller et al., 2002), the AMF growth was not affected. Indeed, the AMF root colonization percentage was similar for the control and PGPR treated plants. This confirms the findings of Barea et al. (1998); Gaur et al. (2004), who reported that 2,4-DAPG-producing rhizobacteria do not adversely affect AMF growth. It follows that, when the strains are selected appropriately, a bio-inoculant composed of AMF and PGPR may have a complementary role in promoting plant growth. This observation concurs with several earlier studies, that have reported positive interactions between AMF and a wide range of PGPR, including phosphate-dissolving bacteria (Toro et al., 1997), nodule-forming N_2 -fixing Rhizobia, free-living *Azospirillum* spp. (Barea et al., 1996; Biro et al., 2000) and *Pseudomonas* spp. (Vázquez et al., 2000), in their effect on plant growth.

While reviewing the applications of PGPR in agronomy, Lucy et al. (2004) stressed the inconsistency of results between the laboratory, greenhouse and field studies due to differences in soil type or climatic variability and the fact that plants responded better if the PGPR strains were isolated from the native rhizosphere. The positive response of wheat to PGPR and AMF inoculation, as well as the higher mycorrhizal root colonization of AMF-treated plants, might be explained by the fact that these micro-organisms were adapted to their environment in terms of soil characteristics, plant genotype and climate. Indeed, they had been selected in the wheat rhizosphere, from the same species and agricultural area. This approach might have limited the discrepancies that could have occurred between greenhouse and field trials, in the plant response to bio-inoculations.

In conclusion, the wheat rhizobacterial community structure is highly dynamic and influenced by different factors such as the plant age, the fertilizer input and the type of bio-inoculant. There is a distance-related effect of the root on the bacterial community as the bacterial community of the root-adhering rhizosphere soil was more influenced than the one of the rhizoplane/endorhizosphere by modifications in its soil environment, such as an increase in fertilizer input. In addition, the positive effect on plant growth of the bio-inoculants in this study might not only result from a direct PGP effect but also from an indirect modification of the bacterial community. This study also offers means of improving the selection of PGPR

strains for setting-up bio-inoculant formulas. For example, AMF can be combined with PGPR strains producing the antibiotic 2,4-DAPG as it was shown that these two beneficial micro-organisms do not compete with one another and can improve synergistically the nutrient quality of the grain. Moreover, it would be preferable for the PGPR or AMF strains composing the treatments to be adapted to the local management practice. Nevertheless, because the rhizobacterial community structure is highly dependent on the plant's growth stage, discrepancies in plant responses due to different field conditions could be reduced by using PGPR strains isolated from the RE fraction or having an optimal activity at different stages of wheat growth.

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References

- Abrol, I.P., 1999. Sustaining rice–wheat system productivity in the Indo-Gangetic plains: water management-related issues. *Agricultural Water Management* 40, 31–35.
- Allen, S.E., Grimshaw, H.M., Parkinson, J.A., Quarmby, C., 1974. *Chemical Analysis of Ecological Materials*. Blackwell Scientific Publication, Oxford.
- Alvey, S., Yang, C.H., Buerkert, A., Crowley, D.E., 2003. Cereal/legume rotation effects on rhizosphere bacterial community structure in west african soils. *Biology and Fertility of Soils* 37, 73–82.
- Azcón-Aguilar, C., Barea, J.M., 1996. Arbuscular mycorrhizas and biological control of soil-borne plant pathogens—an overview of the mechanisms involved. *Mycorrhiza* 6, 457–464.
- Bankhead, S.B., Landa, B.B., Lutton, E., Weller, D.M., Gardener, B.B.M., 2004. Minimal changes in rhizobacterial population structure following root colonization by wild type and transgenic biocontrol strains. *FEMS Microbiology Ecology* 49, 307–318.
- Barea, J.M., Tobar, R.M., Azcón-Aguilar, C., 1996. Effect of a genetically modified *Rhizobium meliloti* inoculant on the development of arbuscular mycorrhizas, root morphology, nutrient uptake and biomass accumulation in *Medicago sativa*. *New Phytologist* 134, 361–369.
- Barea, J.M., Andrade, G., Bianciotto, V., Dowling, D., Lohrke, S., Bonfante, P., O'Gara, F., Azcón-Aguilar, C., 1998. Impact on arbuscular mycorrhiza formation of *Pseudomonas* strains used as inoculants for biocontrol of soil-borne fungal plant pathogens. *Applied and Environmental Microbiology* 64, 2304–2307.
- Biro, B., Koves-Pechy, K., Voros, I., Takacs, T., Eggenberger, P., Strasser, R.J., 2000. Interrelations between *Azospirillum* and *Rhizobium* nitrogen-fixers and arbuscular mycorrhizal fungi in the rhizosphere of alfalfa in sterile, AMF-free or normal soil conditions. *Applied Soil Ecology* 15, 159–168.
- Borcard, D., Legendre, P., Drapeau, P., 1992. Partialling out the spatial component of ecological variation. *Ecology* 73, 1045–1055.
- Brundrett, M., Melville, L., Peterson, L., 1994. *Practical Methods in Mycorrhiza Research*. Mycologue Publications, Waterloo.
- Buyer, J.S., Roberts, D.P., Russek-Cohen, E., 1999. Microbial community structure and function in the spermosphere as affected by soil and seed type. *Canadian Journal of Microbiology* 45, 138–144.
- Caris, C., Hördt, W., Hawkins, H.J., Römhild, V., George, E., 1998. Studies of iron transport by mycorrhizal hyphae from soil to peanut and sorghum plants. *Mycorrhiza* 8, 35–39.
- Chin-A-Woeng, T.F.C., Bloemberg, G.V., Lugtenberg, B.J.J., 2003. Phenazines and their role in biocontrol by *Pseudomonas* bacteria. *New Phytologist* 157, 503–523.
- Ciccillo, F., Fiore, A., Bevivino, A., Dalmastri, C., Tabacchioni, S., Chiarini, L., 2002. Effects of two different application methods of *Burkholderia ambifaria* MCI 7 on plant growth and rhizosphere bacterial diversity. *Environmental Microbiology* 4, 238–245.
- Clark, R., Zeto, S.K., 1996. Mineral acquisition by mycorrhizal maize grown on acid and alkaline soil. *Soil Biology & Biochemistry* 28, 1495–1503.
- Dawe, D., Dobermann, A., 1999. Defining productivity and yield. *IRRI Discussion Paper Series No. 33*. IRRI Manila, Philippines, 13 pp.
- Dobbelaere, S., Vanderleyden, J., Okon, Y., 2003. Plant growth-promoting effects of diazotrophs in the rhizosphere. *Critical Reviews in Plant Sciences* 22, 107–149.
- Elliot, L.F., Lynch, J.M., 1994. Biodiversity and soil resilience. In: Greenland, D.J., Szabolcs, I. (Eds.), *Soil Resilience and Sustainable Land Use*. CAB International, Wallingford, pp. 353–364.
- Fromin, N., Hamelin, J., Tarnawski, S., Roesti, D., Jourdain-Miserez, K., Forestier, N., Teyssier-Cuvelle, S., Gillet, F., Aragno, M., Rossi, P., 2002. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. *Environmental Microbiology* 4, 634–643.
- Gaur, R., Shani, N., Kawaljeet, Johri, B.N., Rossi, P., Aragno, M., 2004. Diacetylphloroglucinol-producing pseudomonads do not influence AM fungi in wheat rhizosphere. *Current Science* 86, 453–457.
- Germida, J.J., Siciliano, S.D., 2001. Taxonomic diversity of bacteria associated with the roots of modern, recent and ancient wheat cultivars. *Biology and Fertility of Soils* 33, 410–415.
- Giovanetti, M., Mosse, B., 1980. An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infections in roots. *New Phytologist* 84, 489–500.
- Glick, B.R., 1995. The enhancement of plant-growth by free-living bacteria. *Canadian Journal of Microbiology* 41, 109–117.
- Grayston, S.J., Wang, S., Campbell, C.D., Edwards, A.C., 1998. Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biology & Biochemistry* 30, 369–378.
- Hanson, W.C., 1950. The photometric determination of phosphorus in fertilisers using the phosphovanado–molybdate complex. *Journal of Science in Food and Agriculture* 1, 172–173.
- Hobbs, P.R., Gupta, R., 2001. Resource conserving technologies for wheat in rice–wheat systems (www.cimmyt.org).
- Kennedy, A.C., 1999. Bacterial diversity in agroecosystems. *Agriculture Ecosystems & Environment* 74, 65–76.
- Kennedy, N., Brodie, E., Conolly, J., Clipson, N., 2004. Impact of lime, nitrogen and plant species on bacterial community structure in grassland microcosms. *Environmental Microbiology* 6, 1070–1080.
- Kozdrój, J., Trevors, J.T., van Elsas, J.D., 2004. Influence of introduced biocontrol agents in maize seedling growth and bacterial community structure in the rhizosphere. *Soil Biology & Biochemistry* 36, 1775–1784.
- Kuzyakov, Y., Domanski, G., 2000. Carbon input by plants into the soil. *Review. Journal of Plant Nutrition and Soil Science* 163, 421–431.
- Ladha, J.K., Reddy, P.M., 2003. Nitrogen fixation in rice systems: state of knowledge and future prospects. *Plant and Soil* 252, 151–167.
- Ladha, J.K., Fischer, K.S., Hossain, M., Hobbs, P.R., Hardy, B., 2000. Improving the productivity and sustainability of rice–wheat systems of the Indo-Gangetic plains: a synthesis of NARS-IRRI partnership research. *IRRI Discussion Paper Series No. 40*. IRRI, Los Banos, Philippines, 31 pp.
- Latour, X., Corberand, T., Laguerre, G., Allard, F., Lemancau, P., 1996. The composition of fluorescent pseudomonad populations associated with roots is influenced by plant and soil type. *Applied and Environmental Microbiology* 62, 2449–2456.
- Legendre, P., Vaudor, A., 1991. Le progiciel R. Analyse multidimensionnelle, analyse spatiale. Université de Montréal, Québec.

- Liljeroth, E., Bååth, E., Mathiasson, I., Lundborg, T., 1990. Root exudation and rhizoplane bacterial abundance of barley (*Hordeum vulgare* L.) in relation to nitrogen fertilization and root growth. *Plant and Soil* 127, 81–89.
- Lottmann, J., Heuer, H., de Vries, J., Mahn, A., Duering, K., Wackernagel, W., Smalla, K., Berg, G., 2000. Establishment of introduced antagonistic bacteria in the rhizosphere of transgenic potatoes and their effect on the bacterial community. *FEMS Microbiology Ecology* 33, 41–49.
- Lucy, M., Reed, E., Glick, B.R., 2004. Applications of free living plant growth-promoting rhizobacteria. *Antonie van Leeuwenhoek* 86, 1–25.
- Lupwayi, N.Z., Rice, W.A., Clayton, G.W., 1998. Soil microbial diversity and community structure under wheat as influenced by tillage and crop rotation. *Soil Biology & Biochemistry* 30, 1733–1741.
- Lynch, J.M., 1990. *The Rhizosphere*. Chichester, UK.
- Mahaffee, W.F., Klopper, J.W., 1997. Bacterial communities of the rhizosphere and endorhiza associated with field-grown cucumber plants inoculated with a plant growth-promoting rhizobacterium or its genetically modified derivative. *Canadian Journal of Microbiology* 43, 344–353.
- Mansfeld-Giese, K., Larsen, J., Bodker, L., 2002. Bacterial populations associated with mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices*. *FEMS Microbiology Ecology* 41, 133–140.
- Marilley, L., Aragno, M., 1999. Phylogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots. *Applied Soil Ecology* 13, 127–136.
- Marschner, P., Crowley, D.E., Lieberei, R., 2001. Arbuscular mycorrhizal infection changes the bacterial 16S rRNA gene community composition in the rhizosphere of maize. *Mycorrhiza* 11, 297–302.
- Marschner, P., Crowley, D., Yang, C.H., 2004. Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. *Plant and Soil* 261, 199–208.
- Miller, R.M., Jastrow, J.D., 1990. Hierarchy of root and mycorrhizal fungal interactions with soil aggregation. *Soil Biology & Biochemistry* 22, 579–584.
- Muyzer, G., Teske, A., Wirsén, C.O., Jannasch, H.W., 1995. Phylogenetic relationships of Thiomicrospira species and their identification in deep sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Archives of Microbiology* 164, 165–172.
- Nacamulli, C., Bevivino, A., Dalmastri, C., Tabacchioni, S., Chiarini, L., 1997. Perturbation of maize rhizosphere microflora following seed bacterization with *Burkholderia cepacia* MCI 7. *FEMS Microbiology Ecology* 23, 183–193.
- Nannipieri, P., Ascher, J., Ceccherini, M.T., Landi, L., Pietramellara, G., Renella, G., 2003. Microbial diversity and soil functions. *European Journal of Soil Science* 54, 655–670.
- Oehl, F., Sieverding, E., Ineichen, K., Mader, P., Boller, T., Wiemken, A., 2003. Impact of land use intensity on the species diversity of arbuscular mycorrhizal fungi in agroecosystems of Central Europe. *Applied and Environmental Microbiology* 69, 2816–2824.
- Ovreas, L., Forney, L., Daae, F.L., Torsvik, V., 1997. Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Applied and Environmental Microbiology* 63, 3367–3373.
- Pandey, A., Sharma, E., Palni, L.M.S., 1998. Influence of bacterial inoculation on maize in upland farming systems of the Sikkim Himalaya. *Soil Biology & Biochemistry* 30, 379–384.
- Rodriguez, H., Fraga, R., 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances* 17, 319–339.
- Sharma, A., Johri, B.N., Sharma, A.K., Glick, B.R., 2003. Plant growth-promoting bacterium *Pseudomonas* sp strain GRP(3) influences iron acquisition in mung bean (*Vigna radiata* L. Wilczek). *Soil Biology & Biochemistry* 35, 887–894.
- Smith, S.E., Read, D.J., 1997. *Mycorrhizal Symbiosis*. Academic Press, London.
- Steenhoudt, O., Vanderleyden, J., 2000. *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. *FEMS Microbiology Reviews* 24, 487–506.
- Sturz, A.V., Ryan, D.A.J., Coffin, A.D., Matheson, B.G., Arsenault, W.J., Kimpinski, J., Christie, B.R., 2004. Stimulating disease suppression in soils: sulphate fertilizers can increase biodiversity and antibiosis ability of root zone bacteria against *Streptomyces* scabies. *Soil Biology & Biochemistry* 36, 343–352.
- Timsina, J., Connor, D.J., 2001. Productivity and management of rice–wheat cropping systems: issues and challenges. *Field Crops Research* 69, 93–132.
- Toro, M., Azcón, R., Barea, J.M., 1997. Improvement of arbuscular mycorrhiza development by inoculation of soil with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability (³²P) and nutrient cycling. *Applied and Environmental Microbiology* 63, 4408–4412.
- van Loon, L.C., Bakker, P., Pieterse, C.M.J., 1998. Systemic resistance induced by rhizosphere bacteria. *Annual Review of Phytopathology* 36, 453–483.
- Vázquez, M.M., Cesar, S., Azcón, R., Barea, J.M., 2000. Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum*, *Pseudomonas*, *Trichoderma*) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants. *Applied Soil Ecology* 15, 261–272.
- Walkley, A., Black, I.A., 1934. An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. *Soil Science* 37, 29–38.
- Walsh, U.F., Morrissey, J.P., O’Gara, F., 2001. *Pseudomonas* for biocontrol of phytopathogens: from functional genomics to commercial exploitation. *Current Opinion in Biotechnology* 12, 289–295.
- Weller, D.M., Raaijmakers, J.M., Gardener, B.B.M., Thomashow, L.S., 2002. Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annual Review of Phytopathology* 40, 309–348.