Published in Microbial Ecology 38, issue 1 : 39 - 49, 1999
which should be used for any reference to this work

Influence of an Elevated Atmospheric CO_2 Content on Soil and Rhizosphere Bacterial Communities Beneath *Lolium perenne* and *Trifolium repens* under Field Conditions

L. Marilley,[1] U.A. Hartwig,[2] M. Aragno[1]

[1] Laboratory of Microbiology, University of Neuchâtel, Rue Emile-Argand 11, CH-2007 Neuchâtel, Switzerland
[2] Institute of Plant Sciences, Swiss Federal Institute of Technology, 8092 Zürich, Switzerland

The increase in atmospheric CO_2 content alters C_3 plant photosynthetic rate, leading to changes in rhizodeposition and other root activities. This may influence the activity, the biomass, and the structure of soil and rhizosphere microbial communities and therefore the nutrient cycling rates and the plant growth. The present paper focuses on bacterial numbers and on community structure. The rhizospheres of two grassland plants, *Lolium perenne* (ryegrass) and *Trifolium repens* (white clover), were divided into three fractions: the bulk soil, the rhizospheric soil, and the rhizoplane–endorhizosphere. The elevated atmospheric CO_2 content increased the most probable numbers of heterotrophic bacteria in the rhizosphere of *L. perenne.* However, this effect lasted only at the beginning of the vegetation period for *T. repens.* Community structure was assessed after isolation of DNA, PCR amplification, and construction of cloned 16S rDNA libraries. Amplified ribosomal DNA restriction analysis (ARDRA) and colony hybridization with an oligonucleotide probe designed to detect *Pseudomonas* spp. showed under elevated atmospheric CO_2 content an increased dominance of pseudomonads in the rhizosphere of *L. perenne* and a decreased dominance in the rhizosphere of *T. repens.* This work provides evidence for a CO_2-induced alteration in the structure of the rhizosphere bacterial populations, suggesting a possible alteration of the plant-growth-promoting-rhizobacterial (PGPR) effect.

Introduction

An increase in atmospheric CO_2 content alters functioning of soil ecosystems. Nevertheless, the major influence of a doubling of CO_2 atmospheric concentration on soil microbial communities is indirect because CO_2 concentration in soil is greater than 0.1 kPa [66], whereas the current atmospheric content is 0.035 kPa. Consequently, the main influence is thought to occur by the intermediation of the plants. Indeed, because of the competitive inhibition by O_2 of the ribulose-1,5-bisphosphate carboxylase, which is responsible

Correspondence to: L. Marilley, GSF-National Research Center, Institute of Soil Ecology, Ingolstädter Landstrasse 1, D-85758 Oberschleißheim, Germany; Fax: +49 89 3187 2705; E-mail: marilley@gsf.de

for CO_2 fixation during photosynthesis [7], the C_3 plant photosynthetic rate increases under a CO_2-enriched atmosphere [28, 47, 51]. An increased root biomass under CO_2 enrichment has been shown by several authors [25, 30, 47, 52, 53].

Soil bacterial communities probably play a key role in the response to the elevated atmospheric CO_2. Under normal conditions, 12 to 54% of the carbon fixed by photosynthesis is released into the soil by the roots [26, 27, 36, 46]. Lekkerkerk et al. [32] showed that the root-derived, easily biodegradable compounds of wheat roots increased under a CO_2-enriched atmosphere. Van Veen et al. [66] suggested quantitative and qualitative changes in rhizodeposition linked to CO_2 enrichment. Since rhizodeposition consists mainly of organic matter, these changes may influence the biomass, activity, and structure of the bacterial community, leading to changes in soil processes involving plant roots, microorganisms, and soil physico-chemical properties.

Studies on bacterial numbers and biomass in the rhizosphere and under CO_2 enrichment are not well documented. Zak et al. [70] showed an increased bacterial biomass in the soil and the rhizosphere of *Populus grandidentata.* Schortemeyer et al. [57] found in samples taken in the spring a positive effect of the elevated CO_2 on the bacterial numbers in the rhizosphere of ryegrass, but a negative effect in the rhizosphere of *T. repens.* On the other hand, Runion et al. [54] detected no changes in bacterial numbers in the rhizosphere of cotton, and inconsistent results were found by Whipps [68] in *Zea mays* and by O'Neill et al. [43] in *Liriodendron tulipifera* rhizospheres. Studies are still needed to better understand the influence of the increased atmospheric CO_2 on soil bacterial processes, and therefore on plant growth. According to Diaz et al. [17], the elevated CO_2 atmospheric content leads to an increase in substrate release into the rhizosphere. This induces a mineral nutrient sequestration by an expanding microbiota and a consequent limitation of plant growth. According to Cardon [12], the influence is also linked to the nutrient status of the soil. On the other hand, Clarholm et al. [15] and Zak et al. [70] suggested an increased protozoan grazing due to an enhanced carbon availability and therefore an increased mineralization and plant nutrient availability.

Plant growth may also be influenced directly by the rhizobacterial populations through altered plant-growth promoting effects. Even though the recent emergence of molecular methods applied to soil microbial ecology allows better description of soil and rhizosphere microbial communities [4, 9, 18, 35, 37, 38, 50, 60], there are limited data dealing with the effects of elevated CO_2 on the structure of rhizosphere microbial populations. Based on phospholipid fatty acid profile analysis of total extractable bacteria, Zak et al. [71] found no CO_2 influence on the microbial community in soils planted with *Populus grandidentata;* nor did Griffiths et al. [22] find such as influence for *L. perenne* and *Triticum aestivum* by using broad-scale DNA techniques. Other works have focused on particular groups of microorganisms. In the rhizosphere of *T. repens,* the population of *Rhizobium leguminosarum* biovar *trifolii,* determined by MPN plant infection assay of *T. repens* seedlings, was increased twofold under CO_2 enrichment [57]. Zanetti et al. [74] reported an increase in symbiotic nitrogen fixation activity for *T. repens* growth under enriched CO_2 atmosphere. The elevated CO_2 had no effect on population of nitrifiers in the rhizospheres of *L. perenne* and *T. repens* [57] and *Liriodendron tulipifera* [43].

Our main objective in this study was to analyze, with a culture-independent approach and under field conditions, how the bacterial community structure was influenced by an elevated atmospheric CO_2 content in swards of *Lolium perenne* and *Trifolium repens.* To do this, we divided the soil into three fractions: the bulk soil, the soil adhering to the roots, and the washed roots (rhizoplane and endorhizosphere). The first stage of our methodology was based on amplified ribosomal DNA restriction analysis (ARDRA) and on the phylogenetic sequence analysis of cloned 16S rRNA genes retrieved from soil and rhizosphere fractions, allowing us to determine the main bacterial groups involved in the CO_2 response. Colony hybridization with a PSM_G oligonucleotide probe served in a second stage to show the influence of the CO_2 enrichment on the population of *Pseudomonas* spp. Moreover, a cultural approach was used to determine the influence of the CO_2 enrichment on the numbers of heterotrophic bacteria.

Materials and Methods

Study Site and CO_2 Fumigation

The study site was located at 550 m above sea level in Eschikon, canton Zürich (Switzerland). It consisted of three control (C1, C2, C3: ambient pCO_2) and three fumigated (F1, F2, F3: 60 Pa CO_2) open-air rings, situated at least 100 m apart and 18 m in diameter. Free-air-CO_2-enrichment (FACE) technology [33] was used to release CO_2 into the open field rings during the photoperiod. The experiment [25, 74] started at the end of May 1993 and lasted during the entire growing seasons. Monocultures of *Lolium perenne* cv Bastion and *Trifolium repens* cv Milkanova were cultivated in

plots measuring 2.8 × 1.9 m and fertilized with P_2O_5 (12 g m^{-2} yr^{-1}), K_2O (29 g m^{-2} yr^{-1}), Mg (1.6 g m^{-2} yr^{-1}), and N (14 g m^{-2} yr^{-1}). To counterbalance differences in nutrient availability, the amount of P–K–Mg fertilizers was increased by 35% in rings C3 and F3. The swards were harvested four times a year in 1995 and five times in 1996 and 1997. The soil was a fertile, eutric cambisol with pH (H_2O) values between 6.5 and 7.6 and consisted of 28% clay, 33% silt, 36% sand, and between 2.9% and 5.1% organic matter [25].

Soil Sampling and Preparation of Rhizosphere Fractions

Soil sampling and partitioning were performed as described by Marilley et al. [38]. Soil cores were collected from *L. perenne* and *T. repens* monocultures and transported cooled to the laboratory. Within the same day, soil cores were separated into three fractions: the bulk soil (BS), the rhizospheric soil (RS), and the rhizoplane–endorhizosphere (RE) [38]. The fractions were then placed at 4°C for the cultural methods and at −20°C for the DNA extractions.

Enumeration of Heterotrophic Bacteria

Samples for the determination of most probable numbers (MPNs) of cultivable heterotrophic bacteria were collected in 1995 and 1996. Inoculations were performed the day after the soil sampling and partitioning. 10 g of BS fraction and 5 ml of homogenized suspensions of RS and RE fractions were added to 90 ml and 5 ml, respectively, of a sterile 0.9% (w/v) NaCl solution. The dry weight of BS fraction was measured after an overnight incubation in a vacuum oven at 70°C. The values were the mean of two 10 g fresh weight subsamples. The dry weight of fraction RS was determined by drying 10 ml of the rhizospheric soil suspension at 70°C during 2 days. The MPN of heterotrophs from fraction RE was displayed as function of the fresh weight of roots measured before the pounding step [38].

The suspensions were shaken at 150 rpm for 30 min at room temperature. The samples were then tenfold serially diluted in a medium containing ionic concentrations of nutrients similar to those found in the soil solution [2]. This culture medium was modified and the following composition was used: 0.2 g L^{-1} NH_4NO_3, 0.69 g L^{-1} $CaSO_4$, 0.406 g L^{-1} $MgCl_2$ and 2 g L^{-1} yeast extract. To this solution, 1 ml of trace element solution [3], 110 µl of Fe/EDTA solution (1.4 g L^{-1} Na_2EDTA, 5 g L^{-1} $FeSO_4 \cdot 7H_2O$, and 0.05% (v/v) concentrated H_2SO_4), 680 µl of KH_2PO_4 0.1% (w/v), 0.5 ml of 1 M KOH, and 40 ml of 0.5 M Tris-HCl (pH 7.0) were added to a final volume of 1 L. After autoclaving, the medium was supplemented with a filter-sterilized solution of glucose to obtain a final concentration of 1 g L^{-1}. MPN of heterotrophic bacteria obtained on this medium was increased more than fivefold in comparison with the MPN obtained on 4 g L^{-1} nutrient broth. Eight parallel 200 µl microplate wells were filled with each dilution. No growth occurred in the absence of yeast extract and glucose. Wells containing noninoculated medium served as a control of cross-contamination. Microtiter plates were incubated at 15°C for 5 days and wells were scored positive when a bacterial pellet was visible. MPN values were calculated using the program of Schneider [56].

Construction of 16S rDNA Libraries

Soil and rhizosphere DNA was extracted and purified following the method described by Ogram [45]. Microorganisms were lysed by a direct procedure based on a lysozyme digestion, SDS treatment, and heat shocks. DNA was then purified by CTAB treatment, extraction with chloroform, centrifugation in polyethylene glycol, and ammonium acetate precipitation. Since *Taq* DNA polymerase may be inhibited by humic compounds [61, 62], the CTAB, $CHCl_3$, and ammonium acetate steps were repeated for extracts that still showed a dark brown color and for those that were difficult to amplify by PCR.

The amplification by PCR of 16S rDNA and the construction of cloned 16S rRNA gene libraries have been already described by Marilley et al. [38]. The amplicons were purified from low-melting agarose gels and cloned in pGEM-T linear vector (Promega Corp., Madison, WI) and *Escherichia coli* XL1 competent cells.

Amplified Ribosomal DNA Restriction Analysis

After construction of 16S rDNA libraries, plasmids were extracted from overnight cultures of transformed *E. coli* by using the alkaline lysis method followed by chloroform extraction [55]. Twenty-nine cloned 16S rRNA genes per fraction were then amplified by PCR and digested as described by Marilley et al. [38] using 2 U of the restriction enzymes *Hae*III and *Rsa*I, and 6 U of *Taq*I. The clones with identical restriction profiles with the three endonucleases were clustered in the same operational taxonomic unit (OTU).

Colony Hybridization

The construction of the clone libraries was performed as described above, except for slight modifications: four to eight 20 µl PCR products were pooled, precipitated with ethanol following standard protocols [55], and resuspended in 20 µl of autoclaved TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). PCR products were then purified using a QiaexII Kit (Qiagen GmBH, Hilden, Germany) according to the manufacturer's instructions. The purified amplicons were resuspended in 20 µl of sterile distilled water before being ligated in the pGEM-T vector (Promega).

About 90 transformed *E. coli* XL1 colonies were randomly picked and inoculated with sterile toothpicks on LB agar [55] supplemented with 150 µg ml^{-1} ampicillin. To control the size of the ligated inserts, 24 plasmid preparations per sample were performed and separated by electrophoresis on a 0.8% (w/v) agarose gel. Transfer of bacterial colonies to Hybond-N^+ nylon membranes (Amersham, Buckinghamshire, UK) was performed by standard methods [55]. Filters were then air-dried and the DNA fixed by an UV exposure of 70 J cm^{-2} at 254 nm. To remove bacterial cell debris from colony blots, membranes were washed for 1 h at 42°C in a solution containing 2× SSPE (0.3 M NaCl, 20 mM NaH_2PO_4, and 2.5 mM EDTA [pH 7.4]), 0.1% (w/v) SDS, and 100 µg ml^{-1} pronase, followed by an incubation for 30 min at 65°C in 2× SSPE and 0.1% (w/v) SDS.

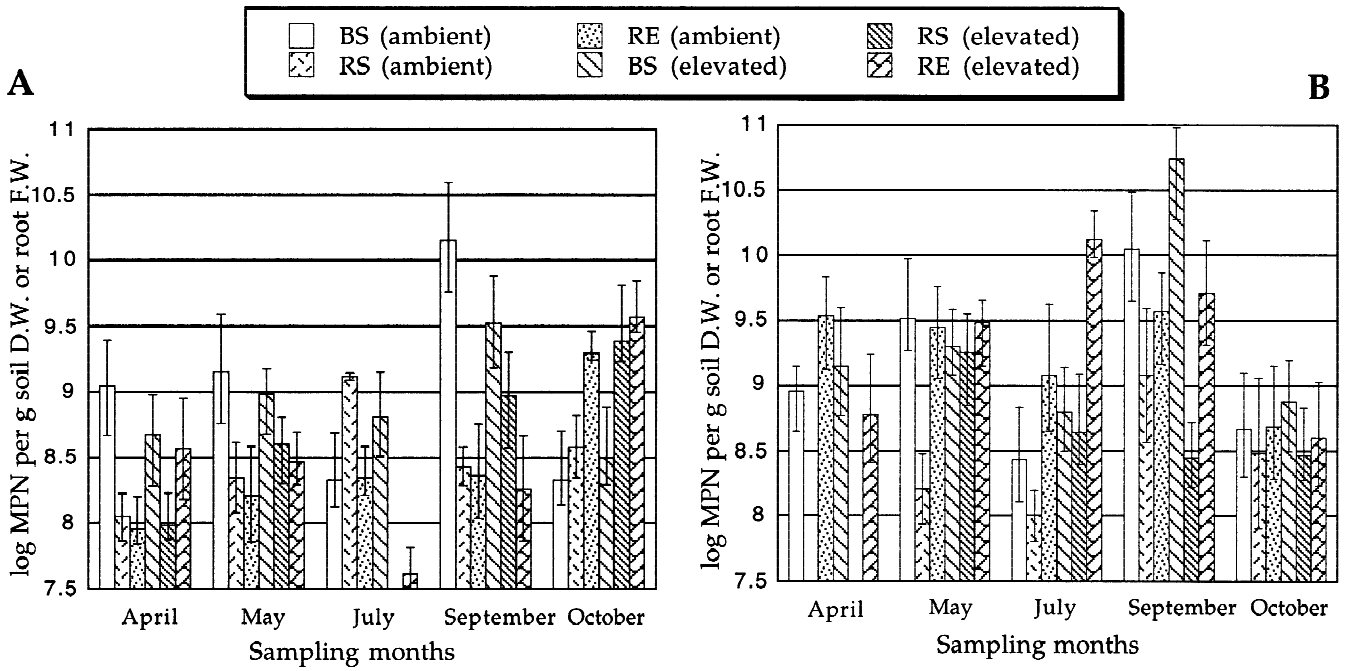

Fig. 1. Most probable numbers (MPNs) of heterotrophic bacteria in BS, RS, and RE fractions of (A) *Lolium perenne* and (B) *Trifolium repens* monocultures under ambient and elevated atmospheric CO_2. MPNs in CO_2-enriched RS fraction of *L. perenne* (July) and in fractions RS of *T. repens* (April) were not determined. BS, bulk soil; RS, rhizospheric soil; RE, rhizoplane and endorhizosphere. Error bars are confidence intervals (0.05) with 10,000 bootstraps.

The oligonucleotide probe used was PSM_G (5′-CCTTCCTCC-CAACTT-3′; *E. coli* positions 440 to 454), which is complementary to a region of the 16S rDNA conserved in the genus *Pseudomonas* and a few other bacteria [1, 10]. It was synthesized and HPLC purified by Microsynth (Balgach, Switzerland) following standard protocols. The oligonucleotide was labeled using the DIG Oligonucleotide 3′-End Labelling Kit (Boehringer Mannheim, Penzberg, Germany), according to the manufacturer's instructions.

Membranes were prehybridized for 1 h at the hybridization temperature (36°C) in 5× SSC (750 mM NaCl and 75 mM sodium citrate [pH 7.0]), 0.02% (w/v) SDS, 0.1% (w/v) *N*-lauroylsarcosin, and 1% (w/v) blocking reagent (Boehringer). Hybridization was performed overnight in the solution described above supplemented with 1 pmol ml^{-1} of the oligonucleotide probe. After two low-stringency washes for 5 min at room temperature in 2× SSC and 0.1% (w/v) SDS, membranes were washed twice for 15 min at the hybridization temperature in 5× SSC and 0.1% (w/v) SDS. The hybridized probes were immunodetected with anti-digoxigenin conjugated to alkaline phosphatase (Fab fragments, Boehringer) and visualized with the colorimetric substrates NBT and BCIP, as described in the manufacturer's protocol (Boehringer).

Results

Enumeration of Heterotrophic Bacteria

Elevated atmospheric CO_2 did not influence bacterial numbers in the BS fractions of *L. perenne* and *T. repens* (Fig. 1). Numbers of heterotrophs in the RS fraction of *L. perenne* were increased under CO_2 fumigation, reaching a sixfold increase in October. In the RS fraction of *T. repens*, the results showed an increase in samples retrieved in May and July, but not at the end of the growing season, in September and October. Elevated CO_2 had no influence on heterotrophic bacteria in the RE fraction of *L. perenne*, in which differences between control and enriched samples were inconsistent. Heterotrophic bacteria were fivefold less numerous under enrichment in RE fraction of *T. repens* (April) but were tenfold increased in July; MPNs performed in September and October did not show any CO_2 influence.

Community Structure

To construct the 16S rDNA libraries, 29 clones for ARDRA (sampling in 1995) and approximately 90 clones for colony hybridization (sampling in 1997) were arbitrarily selected for each fraction. Clones retrieved from ring C1 (ambient pCO_2) in 1995 were partially sequenced and phylogenetically assigned to the closest 16S rDNA sequence in the EMBL, allowing calculation of a frequency of chimera formation of 4.5% [37], which is in the same order of magnitude as those given in other works [9, 18, 41, 49]. The ligation and trans-

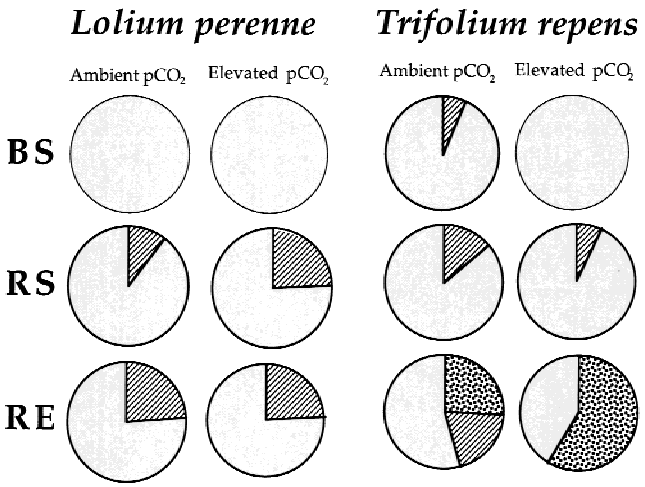

Fig. 2. Distribution of 16S rDNA operational taxonomic units (OTUs) retrieved from *Lolium perenne* and *Trifolium repens* monocultures based on restriction profile analysis with *Hae*III, *Rsa*I and *Taq*I. (▨), clones phylogenetically related to *Pseudomonas* spp. (▩), clones phylogenetically related to *Rhizobium leguminosarum.* BS, bulk soil; RS, rhizospheric soil; RE, rhizoplane and endorhizosphere.

formation steps performed for the colony hybridization were controlled by agarose gel electrophoresis of 24 plasmids per fraction. Of the analyzed clones, 6.3% were shown to have an unexpected size.

Soil samples for ARDRA were collected in October 1995 from rings C1 and F1. Clones showing identical restriction profiles with the three restriction enzymes used served to define OTUs. The frequency of clones phylogenetically related to *Pseudomonas* spp. increased as the proximity to the roots of *L. perenne* and of *T. repens* (ambient pCO_2) increased (Fig. 2). The RE fractions of *T. repens* were dominated by clones related to *Rhizobium leguminosarum.* The elevated atmospheric CO_2 content led to a greater dominance of *Pseudomonas* spp. in the RS and RE fractions of *L. perenne,* while their dominance decreased under CO_2 enrichment in the RS and RE fractions of *T. repens.* The presence of clones related to *R. leguminosarum* increased under CO_2 enrichment in the RS and in the RE fractions of *T. repens.*

Samples for detection of *Pseudomonas* spp. were collected in October 1997 from rings C1, C2, C3, F1, F2, and F3. Their frequency was assessed by colony hybridization of the 16S rDNA clone libraries with the oligonucleotide probe PSM_G (Fig. 3). The specificity of the hybridization was controlled on the 174 16S rDNA clones retrieved in 1995 from ring C1. The phylogenetic position of the above-mentioned OTUs was determined after partial sequencing [37]. Among these 174 clones, 34 had restriction profiles matching those of clones showing more than 92% of homology with fluorescent pseudomonad 16S rDNA sequences. The hybridization signals were positive with these 34 clones, and negative with all the others. Moreover, *Pseudomonas synxantha* (LMG 2190), *Pseudomonas synringae* (LMG 5066), *Pseudomonas putida* (LMG 2257), and *Pseudomonas fluorescens* (DSM 2005) positively hybridized, contrarily to *Escherichia coli* XL1.

The results of the detection of *Pseudomonas* spp. by colony hybridization showed a selective rhizosphere effect toward pseudomonads in both plants, except for the RE fraction of *T. repens,* where their frequency decreased. The CO_2 enrichment led to an increased presence of *Pseudomonas* spp. in *L. perenne* fractions. In fractions of *T. repens,* their frequency decreased as the CO_2 fumigation was applied.

Discussion

In this study, we focused on *L. perenne* and *T. repens* mainly to compare our data with other studies performed on the FACE field experiment in Eschikon (Switzerland) [23, 25, 29, 30, 42, 57, 72, 74]. Moreover, the plants have different rhizosphere structures. *L. perenne,* a grass, lacks symbiotic nitrogen fixation but establishes a rhizospheric sheath surrounding its roots, whereas rhizospheric soil of *T. repens* consists of discrete soil particles adhering to the roots.

Impact of Elevated CO_2 on Soil Heterotrophic Bacteria

Our data did not show any CO_2 influences on the most probable numbers of heterotrophic bacteria in the BS fractions (Fig. 1). In a prairie ecosystem such as the FACE facility in Eschikon, soil carbon input is mainly the result of dead plants and rhizodeposition. Jongen et al. [30] measured in the FACE study site of Eschikon an increased C:N ratio of *L. perenne* roots grown in monoculture at elevated pCO_2, while they detected no changes in the C:N ratio of clover roots. From the perspective of microbial numbers, Zak et al. [70] suggested that an increased carbon input into the soil could be counterbalanced by an increased C:N ratio, leading to unchanged microbial numbers. It is likely that in a grass monoculture under elevated CO_2 the availability of soil mineral nitrogen decreases because of the increased C:N ratio of plant-derived organic matter introduced into the soil [24].

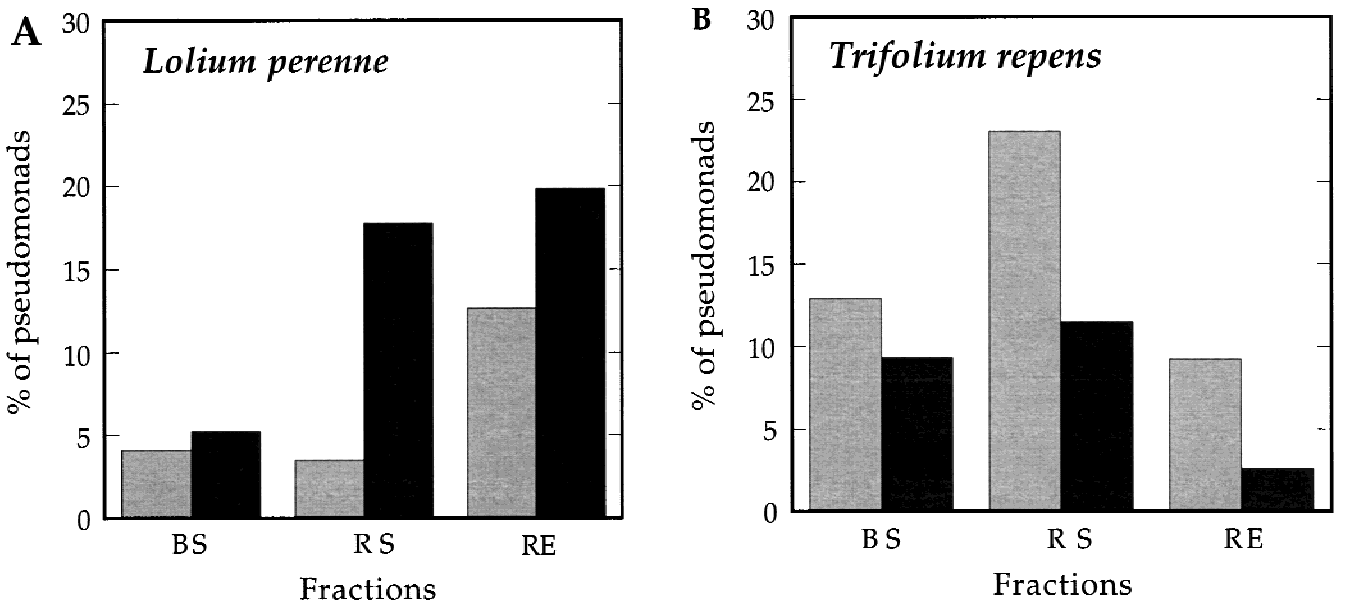

Fig. 3. Frequency of *Pseudomonas* spp., as determined by colony hybridization with the PSM_G oligonucleotide probe of 16S rDNA clones retrieved from *L. perenne* and *T. repens* monocultures. Data presented are the mean of three replica fractions. (▒), ambient pCO_2; (■), elevated; BS, bulk soil; RS, rhizospheric soil; RE, rhizoplane and endorhizosphere.

Diaz et al. [17] showed a nutritional limitation of plant growth due to CO_2-induced mineral nutrient sequestration by expanding microbiota. However, the symbiotic nitrogen fixation occurring in clover roots could counterbalance the mineral nutrient limitation. Indeed, the relative contribution of symbiotically fixed N to the total plant nitrogen increased with increasing pCO_2 [59, 72, 74].

One may also expect an increased root production under elevated pCO_2 [23], but CO_2-induced changes in the quality of the root material and an increased C sequestration into soil organic matter, as reported for the experiment in Eschikon [29, 42], may lead to unaltered microbial numbers in BS fractions. These CO_2-unaltered MPN values may also suggest that the size of the microbial population is regulated through predation by soil protozoa [70].

Impact of Elevated CO_2 on Heterotrophic Bacteria in the Rhizosphere

In the rhizosphere, bacterial populations are under the direct influence of rhizodeposition, which consists mainly of soluble secretions, cell lysates, polysaccharides, and sloughed-off cells [68]. Rhizosphere bacterial populations are supplied in this particular soil habitat with easily degradable organic compounds. Our data (Fig. 1) showed under CO_2 enrichment a consistent increase in the most probable numbers of heterotrophs inhabiting rhizospheric soil surrounding *L. perenne* roots. This result is explained by an increased amount of CO_2-induced rhizodeposition [47, 52, 66]. Qualitative changes in the composition of rhizodeposition may also be an explanation, but this feature is not well documented. Lekkerkerk et al. [32] showed under CO_2 enrichment an increase in easily digestible organic compounds released from wheat roots. Although results on microbial numbers are scarce and inconsistent [43, 67], Schortemeyer et al. [57] reported in the FACE fields in Eschikon a positive CO_2 effect in the rhizosphere of *L. perenne* sampled in May, but not in November. Our data provide evidence for a CO_2-induced increase in bacterial numbers in the rhizospheric soil, but not in the rhizoplane–endorhizosphere.

MPN values of heterotrophs in the RS fraction of *T. repens* were increased under CO_2 enrichment in samples retrieved in May and July, but not in September and October (Fig. 1). This result is not in agreement with the trends obtained in the Eschikon FACE field experiment by Schortemeyer et al. [57], who found a weakly significant decrease in heterotrophic microbial numbers. However, these authors retrieved rhizospheric soil in 1994. We suggest that *T. repens* root exudation enhanced microbial chemotaxis and growth at the beginning of the plant growing season. Gaworzewska

and Carlile [20] showed a positive chemotaxis of bacteria toward root exudates. We are aware that additional data are needed to confirm this hypothesis.

Since it is likely that the plant requirement for symbiotic nitrogen fixation increases under CO_2 enrichment, the exudation of root-mediated molecular signals, such as flavonoids and isoflavonoids, should also be enhanced under elevated pCO_2 at the beginning of the growing season. These signals act as chemoattractants at the first stage of the establishment of the *Rhizobium*–clover symbiosis [14, 65], explaining the positive CO_2 effect on bacteria numbers in the RS fraction and the tenfold increase in the RE fraction retrieved in July. In this latter fraction, bacterial counts are mainly assigned to *Rhizobium leguminosarum* counts because the *Trifolium–Rhizobium* symbiosis is highly specific [14, 65] and because the bacteroids are present in high numbers in nodules. These results are in agreement with those obtained by Schortemeyer et al. [57]. These authors showed in the rhizosphere of *T. repens* a twofold increase in *R. leguminosarum* counts in samples retrieved in May and in November. Moreover, in the same FACE study site, Zanetti et al. [74] showed that total N yield increased significantly in *T. repens* under elevated CO_2 and was assigned to an increased formation of nodules [73].

Because of the incubation conditions (5°C for 5 days), our results are representative of the soil bacterial fraction that is composed of r-strategists. Their increased presence in the RS fraction of *L. perenne* does not suggest an increase in the numbers of the whole bacterial community, even though the ratio of cultivable bacteria is enhanced in the rhizosphere of plants [5, 58].

Impact of Elevated CO_2 on the Microbial Community Structure

Molecular methods based on 16S rDNA analysis have successfully been used to describe bacterial community structure either after ARDRA of clones retrieved from an active hydrothermal vent system [41], from hypersaline environments [39], and from thermophilic compost piles [8], or after partial sequencing and phylogenetic assignments [9, 18, 60]. This allowed, for example, demonstration of the ubiquity of as-yet only molecularly detected soil bacteria, with DNA sequences related to the *Holophaga/Acidobacterium* phylum [35], or detection of new actinomycete lines of descent [50].

Even though molecular methods have the powerful advantage of bypassing the limitations linked to the low ratio of cultivable bacteria in soil and rhizosphere [11, 63], they are biased by several factors due, for example, to differences in the lysis efficiency between soil microorganisms, to the formation of chimeric DNA during PCR amplification, to the effect of genome size and *rrn* gene copy number, and to differences in PCR amplification between bacteria (for a review, see [69]). From the perspective of this work, molecular biases are discussed in previous papers [37, 38]. Moreover, we deliberately chose not to control all the clones obtained during the construction of the 16S rDNA clone library performed in 1997 because we assume that it is preferable to analyze large clone libraries with a small percentage of misligated clones than a reduced number of clones, all containing the expected insert.

The results obtained after ARDRA and after colony hybridization with the *Pseudomonas* spp. targeted oligonucleotide probe PSM_G clearly showed a CO_2-induced increased and, respectively, decreased dominance of pseudomonads in the rhizosphere fractions of *L. perenne* and *T. repens* retrieved at an interval of two years (Figs. 2 and 3). To our knowledge, this is the first work reporting at the level of the whole bacterial community structural changes linked to an elevation of the atmospheric CO_2 content. Both the study of Zak et al. [71], based on the phospholipid fatty acid profile analysis of total extractable bacteria, and the study of Griffiths et al. [22], based on community DNA hybridization and % G+C base profiling by thermal denaturation, showed that the rhizosphere bacterial community structure of *Populus grandidentata, Lolium perenne,* and *Triticum aestivum* remained unaffected by the elevated pCO_2. These contrasting data show the importance of the molecular-based method in revealing more or less subtle structural influences, as suggested by Paterson et al. [46].

Fluorescent pseudomonads play a key role in the rhizosphere of plants. They enhance plant growth by several mechanisms, including (i) the control of phytopathogens by antibiotic production [16, 48], by competition for mineral nutrients [44], by secretion of enzymes that can lyse fungal cells [34], and by induction of plant resistance [64]; (ii) the contribution to plant mineral nutrition by secretion of plant-utilizable siderophores that bind iron [6] or by solubilizing minerals such as phosphorus [21]; (iii) nitrogen fixation [13]; and (iv) the production of phytohormones (for a review, see [21]). To influence the plant, root colonization by associative rhizobacteria is considered as a factor of primary importance [31]. Among others, bacterial chemotaxis response to root exudation is involved in this process.

Futama et al. [19] showed that soil salinity exerted an effect on root colonization by fluorescent pseudomonads through altered amino acid root exudation and therefore through altered bacterial chemotaxis. In accordance with these considerations, this work brings new insights to the plant response to elevated atmospheric CO_2 content through a possible alteration of the plant growth promoting effect by altered rhizodeposition and consequent altered composition of rhizobacterial populations.

Since the N concentration of *L. perenne* growth in monoculture decreases under elevated atmospheric CO_2 while the N concentration of *T. repens* remains unaffected and since the C:N ratio of *L. perenne* was unaltered by CO_2 fumigation when this latter plant is cultivated with *T. repens* [23], the elevated CO_2 induces an increase in the plant requirement for mineral nitrogen. This is supported by the CO_2-induced increased symbiotic nitrogen fixation [59, 72, 74] and proportion of *T. repens* in mixed cultures [23–25]. In grassland ecosystems, leguminous plants play a key role in the response to elevated CO_2. As a consequence of CO_2 enrichment, plant roots may alter the quality and the quantity of rhizodeposition, leading to a modified selective effect toward bacterial communities. This may explain the observation that *R. leguminosarum* outcompetes pseudomonads in the clover rhizosphere (Figs. 2 and 3). Montealegre et al. [40] have shown under elevated CO_2 changes in the genetic structure of the populations of *R. leguminosarum* isolated in the FACE study site of Eschikon from root nodules. These authors suggest that this CO_2 influence is linked to an alteration of the chemical signals released into the soil by clover roots. In the rhizosphere of *L. perenne,* rhizodeposition could also select populations of fluorescent pseudomonads that are more suitable for the CO_2-induced increased plant requirement for mineral nutrients.

In this work, we applied an experimental strategy targeted mainly to molecular approaches, but also to a cultural method. In soil grown with *L. perenne* and *T. repens,* the 16S rDNA-based approach allowed us to show the selective rhizospheric effect [38], to describe the main phylogenetic bacterial groups inhabiting soil and rhizosphere [37], and ultimately to provide evidence for a CO_2-induced modification of the bacterial community structure in the rhizosphere. We suggest investigation not only of the CO_2 influence on the soil bacterial processes linked to increased plant litter production, altered C:N ratios of plant residues, and rhizodeposition, such as organic matter decomposition and mineralization, but also of possible modifications of the plant-growth-promoting effects that could take place in the rhizosphere. To do this, further work dealing with genotypic and phenotypic analysis of the rhizosphere *Pseudomonas* spp. populations is urgently needed.

Acknowledgments

This work was supported by grant # 3100-040693.94/1 of the Swiss National Science Foundation. We are grateful to Andrew Ogram for communicating to us his DNA extraction method before publishing it, to the group of J. Noesberger, Institute of Plant Sciences, ETH (Switzerland), for providing access to the FACE facilities, and to Gudrun Vogt and Noémie Matile for their valuable technical assistance. Many thanks to J. Raaijmakers for having initiated us into hybridization techniques and to J. Lott-Fischer for correcting the manuscript.

References

1. Amann R, Ludwig W, Schulze R, Spring S, Moore E, Schleifer K-H (1996) rRNA-targeted oligonucleotide probes for the identification of genuine and former pseudomonads. System Appl Microbiol 19:501–509
2. Angle JS, McGrath SP, Chaney RL (1991) New culture medium containing ionic concentrations of nutrients similar to concentrations found in the soil solution. Appl Environ Microbiol 57:3674–3676
3. Aragno M, Schlegel HG (1992) The mesophilic hydrogen-oxidizing (Knallgas) bacteria. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH (eds) The Prokaryotes, A Handbook on Biology of Bacteria, Vol 1. Springer Verlag, New York, pp 344–384
4. Assmus B, Schloter M, Kirchhof G, Hutzler P, Hartmann A (1997) Improved *in situ* tracking of rhizosphere bacteria using dual staining with fluorescence-labeled antibodies and rRNA-targeted oligonucleotides. Microb Ecol 33:32–40
5. Bakken LR (1997) Culturable and nonculturable bacteria in soil. In: van Elsas JD, Trevors JT, Wellington EMH (eds) Modern Soil Microbiology. Marcel Dekker, New York, pp 47–61
6. Bar-Ness E, Chen Y, Hadar Y, Marschner H, Römheld V (1991) Siderophores of *Pseudomonas putida* as an iron source for dicot and monocot plants. In: Chen Y, Hadar Y (eds) Iron Nutrition and Interactions in Plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 271–281
7. Bazzaz FA (1990) The response of natural ecosystems to the rising global CO_2 levels. Ann Rev Ecol Syst 21:167–196
8. Blanc M, Marilley L, Beffa T, Aragno M (1999) Thermophilic bacterial communities in hot composts as revealed by cultural and molecular (16S rDNA) methods. FEMS Microbiol Ecol (28:141–149)
9. Borneman J, Skroch PW, O'Sullivan KM, Palus JA, Rumjanek

NG, Jansen JL, Nienhuis J, Triplett EW (1996) Molecular microbial diversity of an agricultural soil in Wisconsin. Appl Environ Microbiol 62:1935–1943

10. Braun-Howland EB, Vescio PA, Nierzwicki-Bauer SA (1993) Use of a simplified cell blot technique and 16S rRNA-directed probes for identification of common environmental isolates. Appl Environ Microbiol 59:3219–3224
11. Campbell R, Greaves MP (1990) Anatomy and community structure of the rhizosphere. In: Lynch JM (ed) The Rhizosphere. John Wiley & Sons Ltd, Essex, pp 11–34
12. Cardon ZG (1996) Influence of rhizodeposition under elevated CO_2 on plant nutrition and soil organic matter. Plant Soil 187:277–288
13. Chan Y-K, Barraquio WL, Knowles R (1994) N_2-fixing pseudomonads and related soil bacteria. FEMS Microbiol Rev 13: 95–118
14. Chanway CP, Turkington R, Holl FB (1991) Ecological implications of specificity between plants and rhizosphere microorganisms. In: Begon M, Fitter AH, Macfayden A (eds) Advances in Ecological Research, Vol 21. Academic Press, San Diego, pp 121–169
15. Clarholm M (1985) Interactions of bacteria, protozoa and plants leading to mineralization of soil nitrogen. Soil Biol Biochem 17:181–187
16. Défago G, Keel C (1995) Pseudomonads as biocontrol agents of diseases caused by soil-borne pathogens. In: Hokkanen HMT, Lynch JM (eds) Benefits and Risks of Introducing Biocontrol Agents. Cambridge University Press, Cambridge, UK, pp 137–148
17. Diaz SA, Grime JP, Harris J, McPherson E (1993) Evidence of a feedback mechanism limiting plant response to elevated carbon dioxide. Nature 364:616–617
18. Felske A, Wolterink A, van Lis R, Akkermans DL (1998) Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils (The Netherlands). Appl Environ Microbiol 64:871–879
19. Futama H, Sakai M, Ozawa H, Urashima Y, Sueguchi T, Matsuguchi T (1998) Chemotactic response to amino acids of fluorescent pseudomonads isolated from spinach roots grown in soils with different salinity levels. Soil Science and Plant Nutrition 44:1–7
20. Gaworzewska ET, Carlile MJ (1982) Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. J Gen Microbiol 128:789–798
21. Glick BR (1995) The enhancement of plant growth by free-living bacteria. Can J Microbiol 41:109–117
22. Griffiths BS, Ritz K, Ebblewhite N, Paterson E, Killham K (1998) Ryegrass rhizosphere microbial community structure under elevated carbon dioxide concentrations with observations on wheat rhizosphere. Soil Biol Biochem 30:315–321
23. Hartwig UA, Lüscher A, Daepp M, Blum H, Soussana J-F, Nösberger J (1999) Due to symbiotic N_2 fixation, five years of elevated atmospheric pCO_2 had no effect on litter N concentration in a fertile grassland ecosystem. Plant Soil (in press)
24. Hartwig UA, Zanetti S, Hebeisen T, Lüscher A, Frehner M, Fischer B, van Kessel C, Hendrey GR, Blum H, Nösberger J (1996) Symbiotic nitrogen fixation: one key to understanding the response of temperate grassland ecosystems to elevated CO_2? In: Körner C, Bazzaz FA (eds) Community, Population, and Evolutionary Responses to Elevated CO_2 Concentration. Academic Press, New York, pp 253–264
25. Hebeisen T, Lüscher A, Zanetti S, Fischer BU, Hartwig UA, Frehner M, Hendrey GR, Blum H, Nösberger J (1997) Growth response of *Trifolium repens* and *Lolium perenne* as monocultures and bi-species mixture to free air CO_2 enrichment and management. Global Change Biology 3:149–160
26. Helal HM, Sauerbeck D (1987) Methods of sampling and investigation of rhizosphere soil. In: Methodology in Soil Research. Proc of the 20th colloqium, International Potash Institute, pp 235–246
27. Helal HM, Sauerbeck D (1989) Carbon turnover in the rhizosphere. Z Pflanzenernähr Bodenk 152:211–216
28. Idso SB, Kimball BA, Wall GW, Garcia RL, LaMorte R, Pinter PJ, Mauny JR, Hendrey GR, Lewin K, Nagy J (1994) Effects of free-air CO_2 enrichment on the light response curve of net photosynthesis in cotton leaves. Agric For Meteorol 70:183–188
29. Ineson P, Cotrufo MF, Bol H, Harkness DD, Blum H (1996) Quantification of soil carbon inputs under elevated carbon dioxide: C3 plants in C4 soil. Plant Soil 187:345–350
30. Jongen M, Jones MB, Hebeisen T, Blum H, Hendrey GR (1995) The effects of elevated CO_2 concentrations on the root growth of *Lolium perenne* and *Trifolium repens* grown in a FACE system. Global Change Biology 1:361–371
31. Kloepper JW, Beauchamp CJ (1992) A review of issues related to measuring colonization of plant roots by bacteria. Can J Microbiol 38:1219–1232
32. Lekkerkerk LJA, van de Geijn SC, van Veen JA (1990) Effects of elevated atmospheric CO_2-levels on the carbon economy of a soil planted with wheat. In: Bouwman AF (ed) Soils and the Greenhouse Effect. John Wiley & Sons, New York, pp 423–429
33. Lewin KF, Hendrey GR, Nagy J, LaMorte RL (1994) Design and application of a free-air carbon dioxide enrichment facility. Agric For Meteorol 70:15–29
34. Lim H-S, Kim Y-S, Kim S-D (1991) *Pseudomonas stutzeri* YPL-1 genetic transformation and antifungal mechanism against *Fusarium solani,* an agent of plant root rot. Appl Environ Microbiol 57:510–516
35. Ludwig W, Bauer SH, Bauer M, Held I, Kirchhof G, Schulze R, Huber I, Spring S, Hartmann A, Schleifer KH (1997) Detection and in situ identification of representatives of a widely distributed new bacterial phylum. FEMS Microbiol Lett 153: 181–190
36. Lynch JM, Whipps JM (1990) Substrate flow in the rhizosphere. Plant Soil 129:1–10
37. Marilley L, Aragno M (1999) Phylogenetic diversity of bacterial communities from different proximity to *Lolium perenne* and *Trifolium repens* roots. Appl Soil Ecol (in press)
38. Marilley L, Vogt G, Blanc MP, Aragno M (1998) Bacterial

diversity in the bulk soil and rhizosphere fractions of *Lolium perenne* and *Trifolium repens* as revealed by PCR restriction analysis. Plant Soil 198:219–224

39. Martinez-Murcia AJ, Acinas SG, Rodriguez-Valera F (1995) Evaluation of prokaryotic diversity by restrictase digestion of 16S rDNA directly amplified from hypersaline environments. FEMS Microbiol Ecol 17:247–256
40. Montealegre CM, van Kessel C, Blumenthal JM, Hur H-G, Hartwig UA, Sadowsky MJ (1999) Elevated atmospheric carbon dioxide alters microbial population structure and competitive ability in a pasture ecosystem (submitted)
41. Moyer CL, Dobbs FC, Karl DM (1994) Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S rRNA genes from a microbiological mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. Appl Environ Microbiol 60:871–879
42. Nitschelm JJ, Lüscher A, Hartwig UA, van Kessel C (1997) Using stable isotopes to determine soil carbon input differences under ambient and elevated atmospheric CO_2 conditions. Global Change Biol 3:411–416
43. O'Neill EG, Luxmoore RJ, Norby RJ (1987) Elevated atmospheric CO_2 effects on seedling growth, nutrient uptake, and rhizosphere bacterial populations of *Liriodendron tulipifera* L. Plant Soil 104:3–11
44. O'Sullivan DJ, O'Gara F (1992) Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiol Rev 56:662–676
45. Ogram A (1998) Isolation of nucleic acids from environmental samples. In: Burlage RS, Atlas R, Stahl D, Geesey G, Sayler G (eds) Techniques in Microbial Ecology. Oxford University Press, Oxford, pp 273–288
46. Paterson E, Hall JM, Rattray EAS, Griffiths BS, Ritz K, Killham K (1997) Effect of elevated CO_2 on rhizosphere carbon flow and soil microbial processes. Global Change Biology 3:363–377
47. Paterson E, Rattray EAS, Killham K (1996) Effect of elevated atmospheric CO_2 concentration on C-partitioning and rhizosphere C-flow for three plant species. Soil Biol Biochem 28: 195–201
48. Raaijmakers JM, Weller DM (1998) Natural plant protection by 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. in take-all decline soils. Molecular Plant–Microbe Interactions 11:144–152
49. Rappé MS, Suzuki MT, Vergin KL, Giovannoni SJ (1998) Phylogenetic diversity of ultraplankton plastid small-subunit rRNA genes recovered in environmental nucleic acid samples from the Pacific and Atlantic Coasts of the United States. Appl Environ Microbiol 64:294–303
50. Rheims H, Spröer C, Rainey FA, Stackebrandt E (1996) Molecular biological evidence for the occurrence of uncultured members of the actinomycete line of descent in different environments and geographical locations. Microbiology 142: 2863–2870
51. Rogers A, Fischer BU, Bryant J, Frehner M, Blum H, Raines CA, Long SP (1998) Acclimation of photosynthesis to elevated CO_2 under low-nitrogen nutrition is affected by the capacity for assimilate utilization. Perennial ryegrass under free-air CO_2 enrichment. Plant Physiol 118:683–689
52. Rogers HH, Runion GB, Krupa SV (1994) Plant responses to atmospheric CO_2 enrichment with emphasis on roots and the rhizosphere. Env Pollution 83:155–189
53. Ross DJ, Saggar S, Tate KR, Feltham CW, Newton PCD (1996) Elevated CO_2 effects on carbon and nitrogen cycling in grass/clover turves of a Psammaquent soil. Plant Soil 182:185–198
54. Runion GB, Curl EA, Rogers HH, Backman PA, Rodriguez-Kabana R, Helms BE (1994) Effects of free-air CO_2 enrichment on microbial on microbial populations in the rhizosphere and phyllosphere of cotton. Agric For Meteorol 70: 117–130
55. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY
56. Schneider S Internet site: http://anthropologie/unige./ch/~schneider/
57. Schortemeyer M, Hartwig UA, Hendrey GR, Sadowsky MJ (1996) Microbial community changes in the rhizospheres of white clover and perennial ryegrass exposed to free air carbon dioxide enrichment (FACE). Soil Biol Biochem 28:1717–1724
58. Soerensen J (1997) The rhizosphere as a habitat for soil microorganisms. In: van Elsas JD, Trevors JT, Wellington EMH (eds) Modern Soil Microbiology. Marcel Dekker, New York, pp 21–45
59. Soussana JF, Hartwig UA (1996) The effects of elevated CO_2 on symbiotic N_2 fixation: a link between the carbon and nitrogen cycles. Plant Soil 187:321–332
60. Stackebrandt E, Liesack W, Goebel BM (1993) Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. FASEB J 7:232–236
61. Steffan RJ, Goksoyr J, Bej AK, Atlas RM (1988) Recovery of DNA from soils and sediments. Appl Environ Microbiol 54: 2908–2915
62. Tebbe CC, Vahjen W (1993) Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. Appl Environ Microbiol 59:2657–2665
63. Torsvik V, Goksoyr J, Daae FL (1990) High diversity in DNA of soil bacteria. Appl Environ Microbiol 56:782–787
64. Van Peer R, Niemann GJ, Schippers B (1991) Induced resistance and phytoalexin accumulation in biological control of *Fusarium* wilt of carnation by *Pseudomonas* sp. strain WCS417r. Phytopathology 81:728–734
65. Van Rhijn P, Vanderleyden J (1995) The *Rhizobium*–plant symbiosis. Microbiol Rev 59:124–142
66. Van Veen JA, Liljeroth E, Lekkerkerk LJA, van de Geijn SC (1991) Carbon fluxes in plant–soil systems at elevated atmospheric CO_2 levels. Ecol Appl 1:175–181

67. Whippe JM (1985) Effect of CO_2 concentration on growth, carbon distribution and loss of carbon from the roots of maize. J Exp Bot 36:644–651
68. Whipps JM (1990) Carbon economy. In: Lynch JM (eds) The Rhizosphere. John Wiley & Sons Ltd, Essex, pp 59–97
69. Wintzingerode FV, Göbel UB, Stackebrandt E (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev 21: 213–229
70. Zak DR, Pregitzer KS, Curtis PS, Teeri JA, Fogel R, Randlett DL (1993) Elevated atmospheric CO_2 and feedback between carbon and nitrogen cycles. Plant Soil 151:105–117
71. Zak DR, Ringelberg DB, Pregitzer KS, Randlett DL, White DC, Curtis PS (1996) Soil microbial communities beneath *Populus grandidentata* grown under elevated atmospheric CO_2. Ecol Appl 6:257–262
72. Zanetti S, Hartwig UA (1997) Symbiotic N_2 fixation increases under elevated atmospheric pCO_2 in the field. Acta Oecol 18:285–290
73. Zanetti S, Hartwig UA, Nösberger J (1998) Elevated atmospheric CO_2 does not affect *per se* the preference for symbiotic nitrogen as opposed to mineral nitrogen of *Trifolium repens* L. Plant, Cell Environ 21:623–630
74. Zanetti S, Hartwig UA, Lüscher A, Hebeisen T, Frehner M, Fischer BU, Hendrey GR, Blum H, Nösberger J (1996) Stimulation of symbiotic N_2 fixation in *Trifolium repens* L. under elevated atmospheric pCO_2 in a grassland ecosystem. Plant Physiol 112:575–583