The ISME Journal (2009) 3, 1204–1217
© 2009 International Society for Microbial Ecology All rights reserved 1751-7362/09 $32.00
www.nature.com/ismej

ORIGINAL ARTICLE

Specific rhizosphere bacterial and fungal groups respond differently to elevated atmospheric CO_2

Barbara Drigo[1,2], Johannes A van Veen[1,3] and George A Kowalchuk[1,4]

[1]*Department of Terrestrial Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Heteren, The Netherlands;* [2]*Centre for Plant and Food Science, University of Western Sydney, Penrith South DC, New South Wales, Australia;* [3]*Institute of Biology, Leiden University, Leiden, The Netherlands and* [4]*Institute of Ecological Science, Vrije Universiteit, Amsterdam, The Netherlands*

Soil community responses to increased atmospheric CO_2 concentrations are expected to occur mostly through interactions with changing vegetation patterns and plant physiology. To gain insight into the effects of elevated atmospheric CO_2 on the composition and functioning of microbial communities in the rhizosphere, *Carex arenaria* (a non-mycorrhizal plant species) and *Festuca rubra* (a mycorrhizal plant species) were grown under defined atmospheric conditions with either ambient (350 p.p.m.) or elevated (700 p.p.m.) CO_2 concentrations. PCR-DGGE (PCR-denaturing gradient gel electrophoresis) and quantitative-PCR were carried out to analyze, respectively, the structure and abundance of the communities of actinomycetes, *Fusarium* spp., *Trichoderma* spp., *Pseudomonas* spp., *Burkholderia* spp. and *Bacillus* spp. Responses of specific functional groups, such as phloroglucinol, phenazine and pyrrolnitrin producers, were also examined by quantitative–PCR, and HPLC (high performance liquid chromatography) was employed to assess changes in exuded sugars in the rhizosphere. Multivariate analysis of group-specific community profiles showed disparate responses to elevated CO_2 for the different bacterial and fungal groups examined, and these responses were dependent on plant type and soil nutrient availability. Within the bacterial community, the genera *Burkholderia* and *Pseudomonas*, typically known as successful rhizosphere colonizers, were significantly influenced by elevated CO_2, whereas the genus *Bacillus* and actinomycetes, typically more dominant in bulk soil, were not. Total sugar concentrations in the rhizosphere also increased in both plants in response to elevated CO_2. The abundances of phloroglucinol-, phenazine- and pyrrolnitrin-producing bacterial communities were also influenced by elevated CO_2, as was the abundance of the fungal genera *Fusarium* and *Trichoderma*.
The ISME Journal (2009) **3,** 1204–1217; doi:10.1038/ismej.2009.65; published online 18 June 2009
Subject Category: microbial ecosystem impacts
Keywords: elevated CO_2; rhizosphere; PCR-DGGE; real-time PCR; bacterial communities; fungal communities

Introduction

Since the advent of the industrial revolution, the concentration of atmospheric CO_2 in the Earth's atmosphere has increased by 31%, and it is expected to rise at an annual rate of 0. 5% (Alley *et al.*, 2007). This rapid rise in CO_2 concentration has led to considerable interest in the potential of biological systems to mitigate the effects of rising atmospheric CO_2 concentration by enhanced carbon (C) sequestration. It is generally believed that under increasing atmospheric CO_2 conditions the largest input of C to the soil from the atmosphere is through photosynthesis (Ainsworth and Long, 2005). Particularly for C_3 plants (Long *et al.*, 2004), stimulation of plant production results in enhanced fluxes of organic compounds into the soil (King *et al.*, 2004). Changes in soil-borne C pools, acting as a C sink or source of CO_2, can potentially affect the CO_2 concentration in the atmosphere and therefore influence global climate. It is estimated that, under current atmospheric CO_2, up to 40% of the C fixed by plants can be transferred through root turnover in the soil (Lynch and Whipps, 1990). Under elevated atmospheric CO_2 levels, rhizodeposition is generally expected to increase (Darrah, 1996) and its composition to be altered (Hodge and Millard, 1998) due to

Correspondence: GA Kowalchuk, Department of Terrestrial Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), PO Box 40, 6666 ZG, Heteren, The Netherlands.
E-mail: G.Kowalchuk@nioo.knaw.nl
Received 2 November 2008; revised 11 May 2009; accepted 11 May 2009; published online 18 June 2009

changes in plant carbohydrate status (Barron-Gafford *et al*., 2005).

Rhizodeposition is the key process of C input into the soil, occurring through secretions (including enzymes and mucilage), sloughing off of dead cells, root turnover and root exudation, releasing low molecular-weight, water-soluble substances, such as sugars, aminoacids and organic acids (Nguyen, 2003). In addition, C flux can be mediated through arbuscular mycorrhizal fungi (AMF). These symbiotic fungi, which infect the majority of land plants (Smith and Read, 1997), have been shown to influence C flow in response to elevated CO_2, thereby affecting soil microbial-community abundance and structure (Jones *et al*., 1998; Drigo *et al*., 2007; Phillips, 2007).

Previous studies on the effects of elevated atmospheric CO_2 concentrations have either examined total microbial-community patterns (Griffiths *et al*., 1998; Kandeler *et al*., 1998; Marilley *et al*., 1999; Montealegre *et al*., 2002; Jossi *et al*., 2006; Lipson *et al*., 2006; Drigo *et al*., 2007), metabolic patterns (Grayston *et al*., 1998; Hodge and Millard, 1998; Tarnawski *et al*., 2006) or have focused on the structure of specific microbial communities of interest, such as *Rhizobium* species (Schortemeyer *et al*., 1996; Montealegre *et al*., 2000), *Pseudomonas* species (Marilley *et al*., 1999; Tarnawski *et al*., 2006), ß/α-Proteobacteria or Acidobacteria (Lipson *et al*., 2006) and mycorrhizal fungi (Gamper *et al*., 2005). However, there is still a general lack of knowledge with respect to the relative responses of different specific microbial groups in response to elevated atmospheric CO_2 conditions.

The aim of this study was to assess the plant-driven impact of elevated atmospheric CO_2 concentration on shifts in the composition of specific bacteria and fungi inhabiting *F. rubra* and *C. arenaria* rhizosphere in dutch coastal dune ecosystems. To address this objective, we examined the effect of elevated atmospheric CO_2 on plant-root exudation (high performance liquid chromatography (HPLC)) and on the community structure and abundance of a number of specific soil-borne groups with presumabely contrasting life-history strategies. For specific groups, analyses targeted *Pseudomonas* spp. (Lugtenberg *et al*., 2001) and *Burkholderia* spp. (Berg *et al*., 2005), as bacteria well adapted to the rhizosphere environment and actinomycetes and the genus *Bacillus*, as group showing typical bulk-soil ecological strategies (Smalla *et al*., 2001). Two important fungal genera, *Trichoderma* and *Fusarium*, were similarly examined. As an example of potential functional impacts of elevated atmospheric CO_2, we also examined the densities of selected genes involved in antibiotic production (phloroglucinol, phenazine and pyrrolnitrin) related to potentially antagonistic activity of *Pseudomonas* spp. and *Burkholderia* spp. All results were subsequently analyzed by multivariate statistical methods to estimate the relative impact of elevated CO_2 treatment in comparison to plant species and soil-origin effects on the different microbial targets (Borcard *et al*., 1992; Ter Braak and Verdonschot, 1995; Filion *et al*., 2000).

Materials and methods

DNA isolation and PCR-DGGE analyses

Plant production, incubation conditions at ambient and elevated CO_2 and harvesting procedures are explained in detail in Drigo *et al*. (2007). Briefly, 4-week-old sterilized seedlings of *F. rubra* (a mycorrhizal plant) and *C. arenaria* (a non-mycorrhizal plant) were planted in a coastal-dune soil (Middelduinen), a former beach plain soil (Kwade Hoek) or a river-dune soil (Bergharen). A total of 240 pots (750 cm^3) were filled with 1 kg of soil and adjusted to 10% volumetric water content (on the basis of dry weight). Together with 72 unplanted soils, 4-week-old seedlings of *F. rubra* and *C. arenaria* were selected for uniformity and transferred to containers (three seedlings per container), which were equally distributed and incubated in four CO_2 flow cabinets, which controlled the temperature and moisture conditions. Two cabinets were maintained at atmospheric condition of 350 p.p.m. CO_2, and two cabinets were maintained at 700 p.p.m. CO_2. A detailed description of the CO_2 flow cabinets is provided in Drigo *et al*. (2007). *F. rubra* and *C. arenaria* rhizosphere and bulk soils were harvested, respectively. at 73 and 62 days after germination, when plants were dominated by shoot growth. In *C. arenaria*, the root–soil systems were first separated from rhizomes. On harvest, roots from each pot were shaken gently to remove loosely adhering soil. Approximately 80% of the initial soil remained in the pots, and this portion was considered 'bulk soil'. Pieces of roots with remaining adhering soil were cut and taken as a combined rhizosphere sample (Drigo *et al*., 2007).

Soil genomic DNA was isolated from 500 mg (wet weight) rhizosphere soil using the PowerSoil™ DNA Isolation kit according to the manufacturer's specifications (MoBio Laboratories, Solana Beach, CA, USA). DNA was eluted in 50 μl of deionized water and stored at −20 °C until use. We randomly selected 216 DNA extracts obtained from rhizosphere soil samples (54 samples per CO_2 treatment, resulting in a total of 108 samples per plant) and 36 from unplanted soil under two different CO_2 treatments for molecular analyses of microbial communities (see below). All PCRs were carried out in a PTC-200 thermal cycler (MJ-Research, Waltham, MA, USA) in 25 μl volumes containing 2.5 μl of 10 × PCR buffer, 2.5 μl of bovine serum albumin (BSA; 4 mg ml^{-1}), 0.75 μl of each primer (30 pM), 2.5 μl of dNTPs mix (8 mM) and 0.056 U of Expand High Fidelity polymerase (Roche, Mannheim, Germany). To reduce variation caused by pipetting errors, PCR mixtures were prepared using a Corbett Robotics CAS-1200 precision liquid handling sys-

Table 1 Primers, PCR, DGGE and real-time PCR conditions used in this paper

Primers	*Amplification protocol*[a]	*Types of analyses*	*Detection of*	*Reference*
Acti 243/1378 968-gc/1378	65 °C; 35 cycles Touchdown 65–55 °C; 35 cycles	DGGE gradient[b] (45–65% denaturant)	Actinomycetes[c]	Heuer *et al.* (1997)
Acti 243/Eub518	65 °C; 40 cycles	Real-time PCR		Lueders *et al.* (2004)
PsF/PsR 968-gc/PsR	64 °C; 35 cycles Touchdown 65–55 °C; 35 cycles	DGGE gradient[b] (45–65% denaturant)	*Pseudomonas* spp.[c]	Widmer *et al.* (1998) Garbeva *et al.* (2004)
PsF/PsR	64 °C; 40 cycles	Real-time PCR		This study
Burk3/1378 Burk3-GC/BurkR	Touchdown 62–58 °C; 45 cycles 64 °C; 35 cycles	DGGE gradient[b] (50–60% denaturant)	*Burkholderia* spp.[c]	Salles *et al.* (2002)
Burk3/BurkR	64 °C; 40 cycles	Real-time PCR		This study
BacF/1378 968-gc/1378	65 °C; 35 cycles Touchdown 63–55 °C; 35 cycles	DGGE gradient[b] (45–65% denaturant)	*Bacillus* spp.[c]	Garbeva *et al.* (2003)
BacF/1378	63 °C; 40 cycles	Real-time PCR		This study
Alfie1-GC/Alfie2	50 °C; 40 cycles	Real-time PCR	*Fusarium* spp.	Yergeau *et al.* (2005); This study
UtR/UtF	55.5 °C; 40 cycles	Real-time PCR	*Trichoderma* spp.	Hagn *et al.* (2007); Hoppener-Ogawa *et al.* (2009)
PRND1/ PRND2	68 °C; 40 cycles	Real-time PCR	PRN	De Souza *et al.* (2003) This study
Phl2a/Phl2b	65 °C; 40 cycles	Real-time PCR	DAPG	Raaijmakers *et al.* (1997); This study
PHZJR1/PHZJR2	68 °C; 40 cycles	Real-time PCR	PCA	Bergsma-Vlami *et al.* (2005); This study

Abbreviations: DAPG, 2,4-diacetylphloroglucinol production locus; DGGE, denaturing gradient gel electrophoresis; PCA, phenazine 2-carboxylic acid production locus; PRN, pyrrolnitrin production locus.
[a]PCR protocols are given as annealing temperature; number of cycles. The remaining of the procedure is given in the text.
[b]100% denaturant is defined as 40% (v/v) formamide and 7 M urea.
[c]The PCR-DGGE were obtained from a nested approach with the prime sets indicated in the table.

tem (Sydney, NSW, Australia). Table 1 summarizes the primers, thermocycling regimes and electrophoresis conditions used to analyze different target communities. All PCRs were carried out according to the touchdown protocols described in Table 1. In addition to the target soil DNA, a negative control sample (without DNA) was included with every PCR run. PCR products were examined by standard 1.5% (w/v) agarose 0.5 TBE (Tris/Borate/EDTA) gel electrophoresis with ethidium-bromide staining, to confirm product integrity and estimate yield. Approximately 0.5 μg of PCR product was used for DGGE analysis, using the method of Muyzer *et al.* (1993) as modified by Kowalchuk *et al.* (2002), using the linear gradients indicated in Table 2. DGGE was carried out using a D-Code Universal Mutation Detection System (Bio-Rad, Hercules, CA). All gradient gels were topped with 10 ml of acrylamide containing no denaturant, and electrophoresis was carried out at 60 °C and 200 V for 10 min followed by an additional 16 h at 70 V. Gels were stained in ethidium bromide and digital images captured using an Imago apparatus (Gentaur, Brussels, Belgium) on UV transillumination. Owing to the number of samples, multiple DGGE gels were run for the three different soil origins (Bergharen, Middelduinen and Kwade Hoek) and the different plants species (*C. arenaria* and *F. rubra*). Each sample was assessed in at least two different runs to confirm the reproducibility of the DGGE fingerprint across gels. To facilitate comparative statistical analysis, all gels of the same community were combined into a composite image using Corel PHOTO-PAINT 12 before further analysis (Corel Corporation, Fremont, CA, USA, 2003). Gel images were normalized with respect to migration pattern of the standard lanes of each gel, before pattern comparison using the Image Master 1D program (Amersham Biosciences, Roosendaal, The Netherlands) with rolling-circle ($r = 10$) background subtraction and automated band detection. Matching of bands was carried out in reference to a hypothetical composite lane containing bands at all positions found across each dataset. Owing to potential PCR bias and the non-quantitative nature of DGGE band densities, bands were scored on a presence or absences basis. DGGE fingerprints were hereby transformed into binary codes and used in statistical analysis as 'species' presence–absence matrices. The influence of plant species (*C. arenaria* versus *F. rubra*), soil origin (Bergharen, Kwade Hoek and Middelduinen), CO_2 concentration (ambient versus elevated) and the

Table 2 Bacterial isolates used for the production of standard curves in the quantification of actinomycetes, *Pseudomonas* spp., *Burkholderia* spp., *Bacillus* spp., *Fusarium* spp., *Trichoderma* and potential producers of PRN, DAPG and PCA

Species/strains Closest neighbor(accession number/% identity)	*Origin and source*	*Detection target*	*Reference*
Streptomyces coelicolor (AL645882/100%)	*Festuca rubra* rhizosphere soil Bergharen, The Netherlands	Actinomycetes	This study
Pseudomonas fluorescens (PINR2)	Tobacco, Italy (JM Raaijmakers)	*Pseudomonas* spp.	Keel *et al.* (1996)
Burkholderia cepacia (AY741358/98%)	*Festuca rubra* rhizosphere soil Bergharen, The Netherlands	*Burkholderia* spp.	This study
Bacillus subtilis (AL009126.1/100%)	*Festuca rubra* rhizosphere soil Bergharen, The Netherlands	*Bacillus* spp.	This study
Fusarium oxysporum (AF124843/100%)	*Festuca rubra* rhizosphere soil, The Netherlands	*Fusarium* spp.	This study
Trichoderma harzianum (EF672343/100%)	(Semi)-natural grassland soil Bergharen, The Netherlands (S. Hoppener-Ogawa)	*Trichoderma* spp.	Hoppener-Ogawa *et al.* (2009)
Pseudomonas fluorescens (CHA0) (AJ278806)	Tobacco Morens, Switzerland (G Defago)	PRN	Keel *et al.* (1992)
Pseudomonas fluorescens (Q2-87) (U41818)	Wheat, Washington, USA (LS Thomashow)	DAPG	Bangera *et al.* (1999)
Pseudomonas fluorescens (PHZ24)	Tomato Dijon, France (P Lemanceau)	PCA	De Souza *et al.* (2003)

Abbreviations: DAPG, 2,4-diacetylphloroglucinol production locus; PCA, phenazine 2-carboxylic acid production locus; PRN, pyrrolnitrin production locus.
Standards were made from full-length PCR-amplified 16S-, 18S-rRNA genes and antibiotic production genes from the strains indicated.

interactions between these three factors on the community structure, as examined by PCR-DGGE, was tested by distance-based redundancy analysis (Legendre and Anderson, 1999). Jaccard's coefficients of similarity were first calculated between samples and used to compute principal coordinates in the R-package (Casgrain and Legendre, 2001). When necessary, eigenvectors were corrected for negative eigenvalues using the procedure of Lingoes (1971), and all principal coordinate axes were exported to Canoco version 4.5 (Ter Braak and Šmilauer, 2002) and treated as 'species' data. To test the effects of the three groups of factors (plants, soil origin and CO_2), these were entered as dummy binary variables. One group of factors was entered as the explaining variables in the model, whereas the other two groups of factors were entered as co-variables. The significance of such models was tested with a Monte Carlo test based on 999 permutations restricted for split-plot design, with whole plots being the CO_2 flow cabinets. Further distance-based redundancy analyses were also carried out as described above on subsets of the whole dataset, by either analyzing plant species or soil origins separately.

The percentage of variation in the dataset related to the different factors was determined by variation-partitioning analysis (Borcard *et al.*, 1992), using the same strategy as for distance-based redundancy analyses. The percentage of variation explained by a factor was the trace of the analysis constrained using this factor (redundancy analysis) divided by the trace of the unconstrained analysis (phenazine 2-carboxylic acid (PCA)). Unexplained variation was calculated by subtracting the trace of the overall amount of explained variation by the different factors in the model from the total inertia.

Total exuded-sugar analysis

The HPLC procedure outlined by Stipanovic *et al.* (1998) was used to analyze sorbitol, mannitol, trehalose, glucose, fructose, melibiose and sucrose concentrations in the rhizosphere of *C. arenaria* and *F. rubra* grown at ambient and elevated CO_2 conditions. A total of 80 pot leachate samples (40 of *F. rubra* and 40 of *C. arenaria*), each comprised of 100 mg of freeze-dried water-extracted exudates, were harvested 73 and 62 days after germination, respectively. All the samples were shaken for 30 min in a capped 125 ml Erlenmeyer flask with 15 ml of glass beads, 10 ml of 3:1 hexane:ethyl acetate and 100 µl of 10% HCl. The solution was filtered over a glass-filter funnel into a 50 ml pear-shaped flask, and the beads and residue were rinsed three times with 3 ml 3:1 hexane:ethyl acetate. The solvent was left to evaporate in a hot water bath and the residue in the flask was re-dissolved with four 150-µl washes. Each wash was transferred to a maxi-clean silica cartridge (Alltech, Breda, The Netherlands). The silica cartridge was dried using compressed air, and the soil solutions were eluted with 5 ml of isopropyl alchol:acetonitrile:water:ethyl acetate (35:21:39:5). The eluent was filtered through a 45-µm nylon filter and transferred to a crimp top vial for HLPC analysis. Twenty microliters of each sample were injected into a DIONEX HPLC system (DIONEX Corp., Sunnyvale, CA, USA), equipped with a single wavelength absorbance detector and a 250 mm $\times$ 4.6 mm i.d. ALLtima C-18 column (Alltech, Breda, The Netherlands). The column was eluted with ethanol mix at a flow rate of 1.25 ml min^{-1} and kept at 55 °C during analysis according to the protocol described by Stipanovic *et al.* (1998). Detection was carried out at 272 nm. Standards of sorbitol, mannitol, trehalose, glucose,

fructose, melibiose and sucrose were used to assess the retention times of individual sugar. As control treatments, the effect of mechanical root damage on sugar concentration in the rhizosphere and the natural abundance of the sugars in the bulk soil at ambient and elevated CO_2 were considered. To detect the effect of root damage on exudation patterns, roots were washed and separated into main and lateral roots and a metal blade was used to damage the root tissue. The material was immediately freeze-dried after injury, ground and extracted for sugar analysis.

The concentration (S) of each single sugar in the rhizosphere soil of *C. arenaria* and *F. rubra* plants grown in Bergharen, Kwade Hoek and Middelduinen at ambient and elevated CO_2 was calculated by subtracting the natural abundance of each singular sugar (S_n) as recovered in the bulk soil and the sugar concentration due to root damage (S_r). S_{tot} represents the total sugar concentration derived from HPLC analysis:

$$[S] = [S_{tot}] - ([S_n] - [S_r]) \quad (1)$$

To determine the differences in sugar concentrations between ambient and elevated CO_2 treatment in the different rhizosphere soils, the data were examined by using analysis of variance. The analyses were carried out according to a split-plot design as described by Filion *et al.* (2000). The F statistic used for testing the significance of main effects of the CO_2 treatment applied to whole plots (CO_2 flow cabinets) was obtained by dividing the treatment mean square by the mean square for CO_2 flow cabinets nested within CO_2 treatments. The error term, to test for the interactions between CO_2 and soil origin or plant species, was based on the mean square of the interaction between these treatments and cabinets nested in CO_2 (Filion *et al.*, 2000). The analyses were carried out in Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA). Normality was tested using the Shapiro–Wilks test and by inspection of residuals, and variance homogeneity by Levene's test. When data failed to satisfy one of these tests, an appropriate transformation was applied (log or square-root transformation). Tukey's honestly significant difference method and the modified version for unequal sample size (Unequal N honestly significant difference in Statistica) were used for post-hoc comparisons with a 0.05 grouping baseline. Analyses of variance were carried out in Statistica 8.0 to test for significant differences between the soil characteristics and the effect of elevated CO_2. A block structure was used for the ambient and elevated CO_2 data sets. After analysis of variance, least significant differences were calculated at a significance level of $P=0.05$.

Real-time PCR

Quantitative real-time PCR assays were carried out in 25-μl reaction volumes with the ABsolute QPCR SYBR green mix (AbGene, Epsom, UK) on a Rotor-Gene 3000 (Corbett Research) to quantify actinomycetes, *Burkholderia* spp., *Pseudomonas* spp., *Bacillus* spp., *Fusarium* spp. and *Trichoderma* spp. Small subunit (SSU) rRNA gene densities were quantified using the primers and conditions described in Table 1. Quantitative real-time PCR assays were also carried out for the detection of the pyrrolnitrin (PRN), 2, 4 diacetylphloroglucinol (DAPG), phenazine 2-carboxylic acid (PCA) production loci (Table 1). All mixes were made using a CAS-1200 pipetting robot (Corbett Research). Standards were made from full-length PCR-amplified 16S-, 18S-rRNA and antibiotic production genes from the pure isolates described in Table 2. By using 10-fold increments, the standard concentrations were adjusted from 10^9 to 10^1 gene copies per μl for each species. In all the runs, the amplification efficiency (Eff) was calculated using the software manufactured by Corbett Research by the formula:

$$\mathrm{Eff} = 10^{(-1/\mathrm{slope})} - 1$$

The efficiency of the different real-time PCR ranged from 90–100% ($-3.6 > \mathrm{slope} > -3.1$). The length of the amplicons ranged between 500 to 800 bp and secondary structures were not encountered in any of the runs. The threshold of each single run was placed above any baseline activity and within the exponential increase phase. The cycle thresholds (C_T) were determined by a mathematical analysis of the resulting curve using the software manufactured by Corbett Research. The C_T values of the non-template controls were always around 40, indicating no amplification. Dissociation curves were determined for quantitative real-time PCR products to confirm product integrity, and random samples were run on 2% agarose gels to confirm the absence of spurious PCR products.

Most of the samples, and all standards, were assessed in at least two different runs to confirm the reproducibility of the quantification. The SSU rRNA gene numbers were analyzed using analysis of variance as described previously (see sugar analysis).

Results

Effects of elevated CO_2 on Pseudomonas *spp.,* Burkholderia *spp., actinomycetes and* Bacillus *spp. community profiles*

Plant species, soil origin, CO_2 concentration and the interactions between these factors all explained a significant ($P<0.001$) part of the variation in *Pseudomonas* spp. and *Burkholderia* spp. communities (Table 3 and Figure 1; Supplementary Figure S1A, B). Variation partitioning of PCR-DGGE datasets for these communities showed that CO_2 treatment, soil origin and plant species together explained about 55% of the variation in community profiles for *Pseudomonas* spp. and 62% for

Table 3 Main PCR-DGGE distance-based redundancy analyses results for plant species (*Carex arenaria* and *Festuca rubra*), soil origin (Middelduinen, Kwade Hoek and Bergharen) and ambient and elevated CO_2 concentrations on *Pseudomonas*, *Burkholderia*, actinomycetes and *Bacillus* spp. communities based on 999 Monte Carlo permutations test and split-plot analysis of variance

Explanatory variables	*Co-variables*	*Pseudomonas spp.*		*Burkholderia spp.*		*Actinomycetes*		*Bacillus spp.*	
		P-*value*	*Trace*	P-*value*	*Trace*	P-*value*	*Trace*	P-*value*	*Trace*
CO_2	Soil origin × plants	0.001	0.300	0.001	0.354	1.000	0.001	1.000	0.001
Soil origin	Plants × CO_2	0.037	0.050	0.012	0.028	0.001	0.980	0.001	0.970
Plants	Soil origin × CO_2	0.001	0.197	0.001	0.226	0.550	0.002	0.314	0.004
Soil origin × plants × CO_2	none	0.001	0.547	0.001	0.608	0.001	0.983	0.001	0.975

Abbreviation: DGGE, denaturing gradient gel electrophoresis.

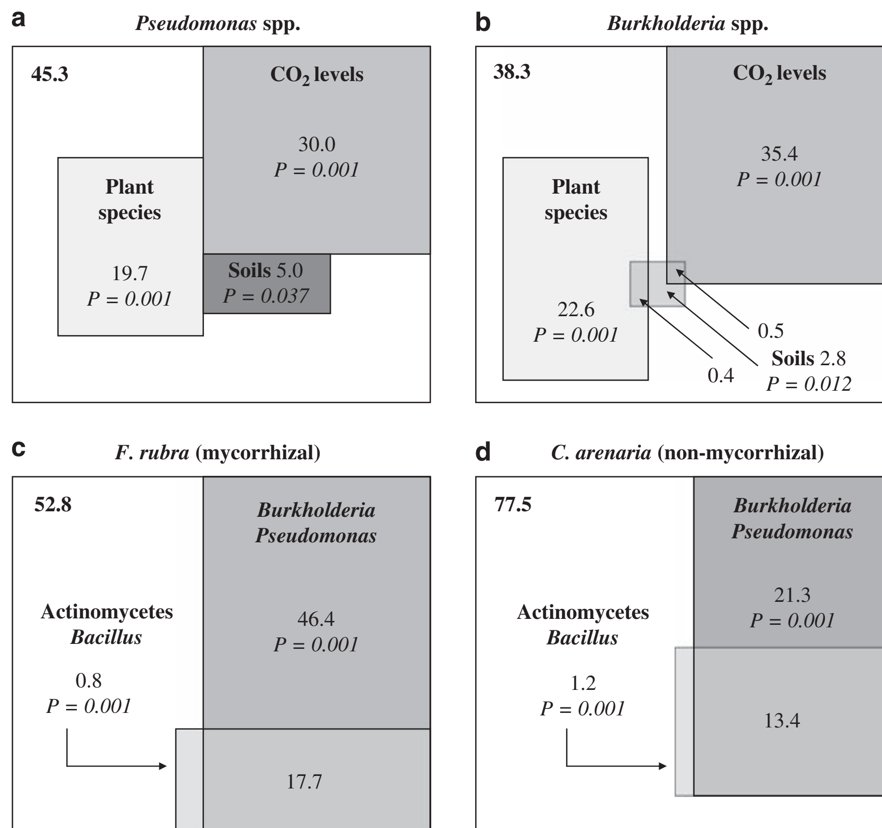

Figure 1 Variation-partitioning representation of PCR-DGGE (PCR-denaturing gradient gel electrophoresis) analyses of (**a**) *Pseudomonas* spp. and (**b**) *Burkholderia* spp. rhizosphere communities under (**c**) *Festuca rubra* (mycorrhizal) plants only and (**d**) *Carex arenaria* (non-mycorrhizal) plants only, grown in Bergharen (river dune) Kwade Hoek (former beach) and Middelduinen (coastal dune). The numbers list the percentage of variance accounted for CO_2 levels, plant species, soil origin, unexplained variance and their interactions. The significance of all of the different factors on the microbial community structure was tested by db-RDA (distance-based redundancy analysis) based on 999 Monte Carlo permutations and split-plot analysis of variance (see material and methods). The area of the white square that is not covered by any of the other rectangles is proportional to the unexplained variance.

Burkholderia spp. (Table 3 and Figures 1a, b). These variables all explained a significant part of the variation, but their relative contribution differed between these two groups of organisms. For *Pseudomonas* spp., the greatest degree of variation was explained by elevated CO_2 (30%), whereas soil origin and plant species explained about 5 and 20%, respectively, of the total variation. For *Burkholderia* spp., the greatest degree of variation was explained by elevated CO_2 (35%) and plant species (22%), whereas soil origin explained only 2.8% of the total variation. For actinomycetes and *Bacillus* spp., soil origin was the only significant explanatory factor (98 and 97%, respectively; Table 3; Supplementary Figure S1C, D). Plant species, soil origin and the interactions between these factors did not explain a significant (Table 3) part of the variation for actinomycetes and *Bacillus* spp.

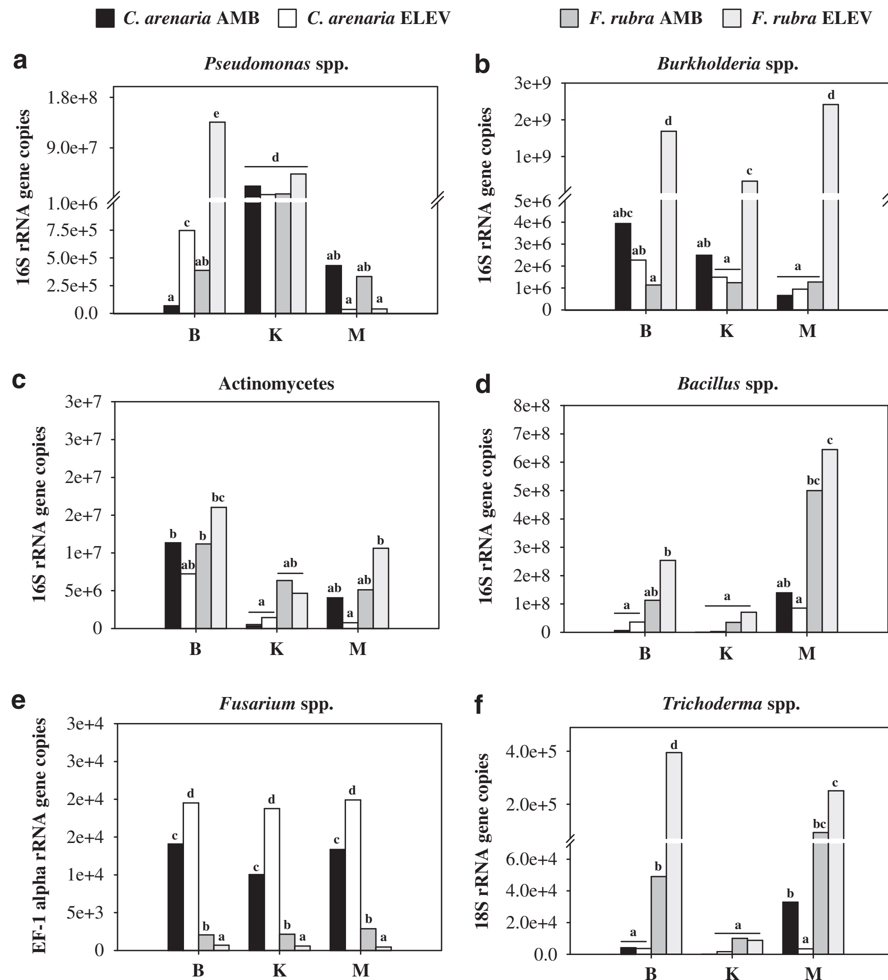

Figure 2 (**a**) *Pseudomonas* spp., (**b**) *Burkholderia* spp., (**c**) actinomycetes, (**d**) *Bacillus* spp., (**e**) *Fusarium* spp. and (**f**) *Trichoderma* spp. SSU (small subunit) ribosomal RNA genes abundance in the rhizosphere of *Carex arenaria* and *Festuca rubra* grown at elevated (ELEV) and ambient (AMB) CO_2 in Bergharen, river dune (**b**), Kwade Hoek, former beach (K) or Middelduinen, coastal dune (M) soils. Different letters within the graph refer to significantly different averages based upon a Tukey HSD (honestly significant difference) test.

Effect of elevated CO_2 on the rhizosphere community densities

The SSU rRNA gene abundances for *Pseudomonas* spp. and *Burkholderia* spp., differed with respect to plant species, soil origin and elevated CO_2 treatments (Figures 2a, b). In Kwade Hoek and Middelduinen soil, elevated CO_2 had no effect on the *Pseudomonas* spp. and *Burkholderia* spp. rhizosphere community abundances associated with *C. arenaria* ($F_{1,2} = 6.79$, $P = 0.12$), but *Burkholderia* spp. target numbers in the *F. rubra* rhizosphere were affected by elevated CO_2 ($F_{1,2} = 533.40$, $P = 0.002$). In Bergharen soil, *Pseudomonas* and *Burkholderia* communities ($F_{2,4} = 10.30$, $P = 0.026$) had a significant CO_2 and soil interaction, indicating that the effects of CO_2 elevation were soil dependent and affected the two plants rhizospheres to a similar extent.

More complex interactions between CO_2 concentration, plant species and soil origins were also observed. For *C. arenaria*, *Burkholderia* 16S-rRNA gene abundance decreased or remained stable with elevated CO_2 in all soils, whereas the opposite pattern was observed for *F. rubra*. *Pseudomonas* populations in *C. arenaria* and *F. rubra* increased or remained stable in Bergharen and Kwade Hoek soils, but decreased in Middelduinen soil.

The 16S-rRNA gene abundances for *Bacillus* spp. differed with respect to plant species ($F_1 = 20.080$; $P < 0.001$) and soil origin ($F_2 = 9.69$; $P < 0.001$), whereas actinomycetes community abundances were not significantly affected by any variable (Figures 2c, d). For both plant species, elevated CO_2 had no effect on the community abundance for actinomycetes ($F_{1,2} = 1.019$, $P = 0.42$) or *Bacillus* spp. ($F_{1,2} = 0.366$, $P = 0.61$).

SSU rRNA gene abundances for Fusarium *spp. and* Trichoderma *spp.*

The SSU rRNA gene abundances for *Trichoderma* were generally much higher for *F. rubra* than for *C. arenaria* and differed with respect to soil origin.

Fusarium densities showed an opposite trend with respect to plant species (Figures 2e, f). For *Trichoderma* ($F_{1,2}=215.06$, $P<0.01$) and *Fusarium* ($F_{1,2}=1011.34$, $P<0.001$), there was a significant CO_2–plant species interaction, indicating that CO_2 elevation had different effects on the two plants rhizospheres. More complex interactions between CO_2 concentration, plant species and soil origins were also observed. For *C. arenaria*, *Fusarium* 18S-rRNA gene abundance increased with elevated CO_2 across all three soil origins, but for *F. rubra*, these patterns were exactly the opposite.

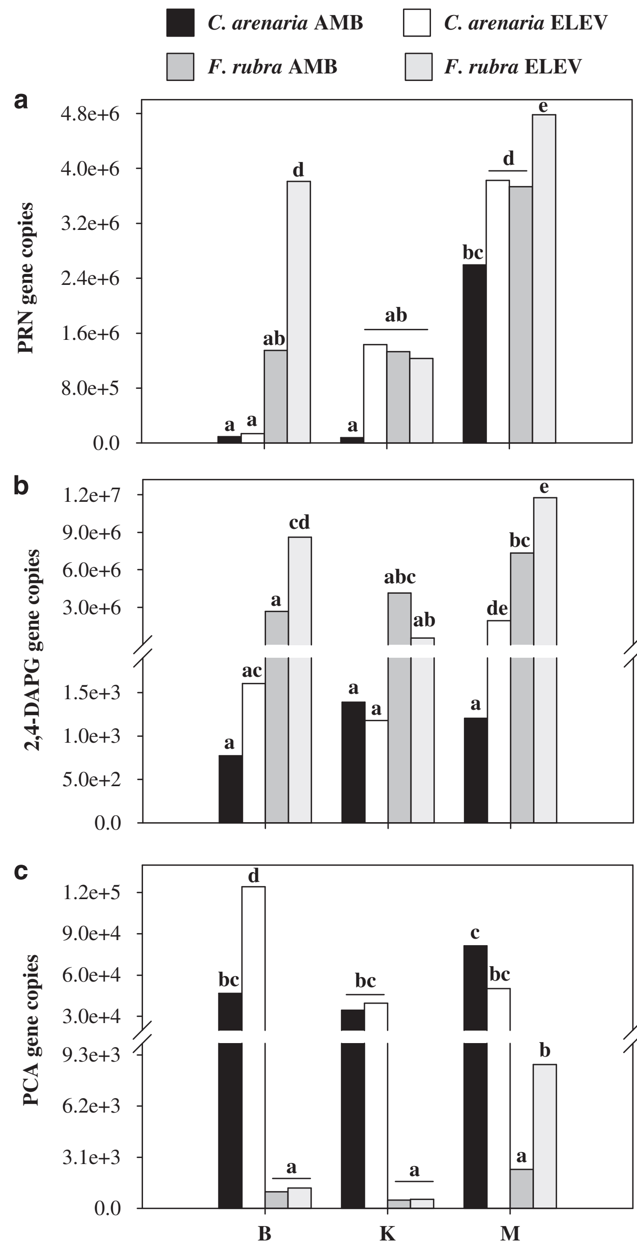

Figure 3 (**a**) Pyrrolnitrin production locus (PRN), (**b**) 2,4 diacetylphloroglucinol production locus (2,4-DAPG) and (**c**) phenazine 2-carboxylic acid production locus (PCA) *Pseudomonas* and *Burkholderia* spp. antibiotics functional genes in the rhizosphere of *Carex arenaria* and *Festuca rubra* grown at elevated (ELEV) and ambient (AMB) CO_2 in Bergharen, river dune (**b**), Kwade Hoek, former beach (K) or Middelduinen, coastal dune (M) soils. Different letters within the graph refer to significantly different averages based on a Tukey HSD (honestly significant difference) test.

Effect of elevated CO_2 on gene densities associated with PRN, DAPG and PCA production

The density of genes associated with DAPG and PRN production was generally much higher in *F. rubra* than in *C. arenaria* and differed with respect to soil origin (Figures 3a, b). Genes responsible for the production of PCA were present at low densities in the rhizospheres of *F. rubra* plants grown in Bergharen and Kwade Hoek soil, but relatively high in all *C. arenaria* rhizosphere samples (Figure 3c). For the DAPG-, PRN- and PCA-associated genes, there was a significant CO_2 elevation effect ($F_{1,2}=15.766$, $P<0.028$; $F_{1,2}=11.35$, $P<0.03$ and $F_{1,2}=11.659$, $P<0.028$, respectively), with the effects of CO_2 being dependent on the plant species and soil origin. Elevated CO_2 apparently increased the abundance of DAPG- and PRN-producing communities in both plant rhizospheres grown in Middelduinen, but only in *F. rubra* for Bergharen soil.

Effect of elevated CO_2 on total soluble exuded sugars

On being averaged across all treatments and community types, the mean of total soluble sugars increased significantly under elevated CO_2 in the rhizosphere of both plant species (plant–CO_2: $F_{1,2}=86.40$; $P<0.001$) (Figure 4). Interestingly, although both plant species increased their total exudation of sugars (glucose, fructose, sucrose, mannitol, melibiose and sorbitol) at elevated CO_2, this effect was generally greater in the rhizosphere of *F. rubra*. For *F. rubra*, the exudation of trehalose, a major product of AMF, was also increased by four-fold at elevated CO_2, compared with ambient CO_2 conditions (Figure 4). As expected, no trehalose exudation was detected for the non-mycorrhizal plant, *C. arenaria*, at either level of atmospheric CO_2.

Discussion

Response of specific soil-borne microbial groups to plant growth at elevated concentrations of atmospheric CO_2

Specific bacterial community responses. Distance-based redundancy analysis of community profiles showed that increased atmospheric CO_2 exerted differential influences on the specific bacterial groups in the rhizosphere samples associated with *C. arenaria* and *F. rubra*. Large effects of elevated CO_2 were observed within the *Pseudomonas* and *Burkholderia* communities, but no significant effects were apparent for actinomycetes and *Bacillus* spp.

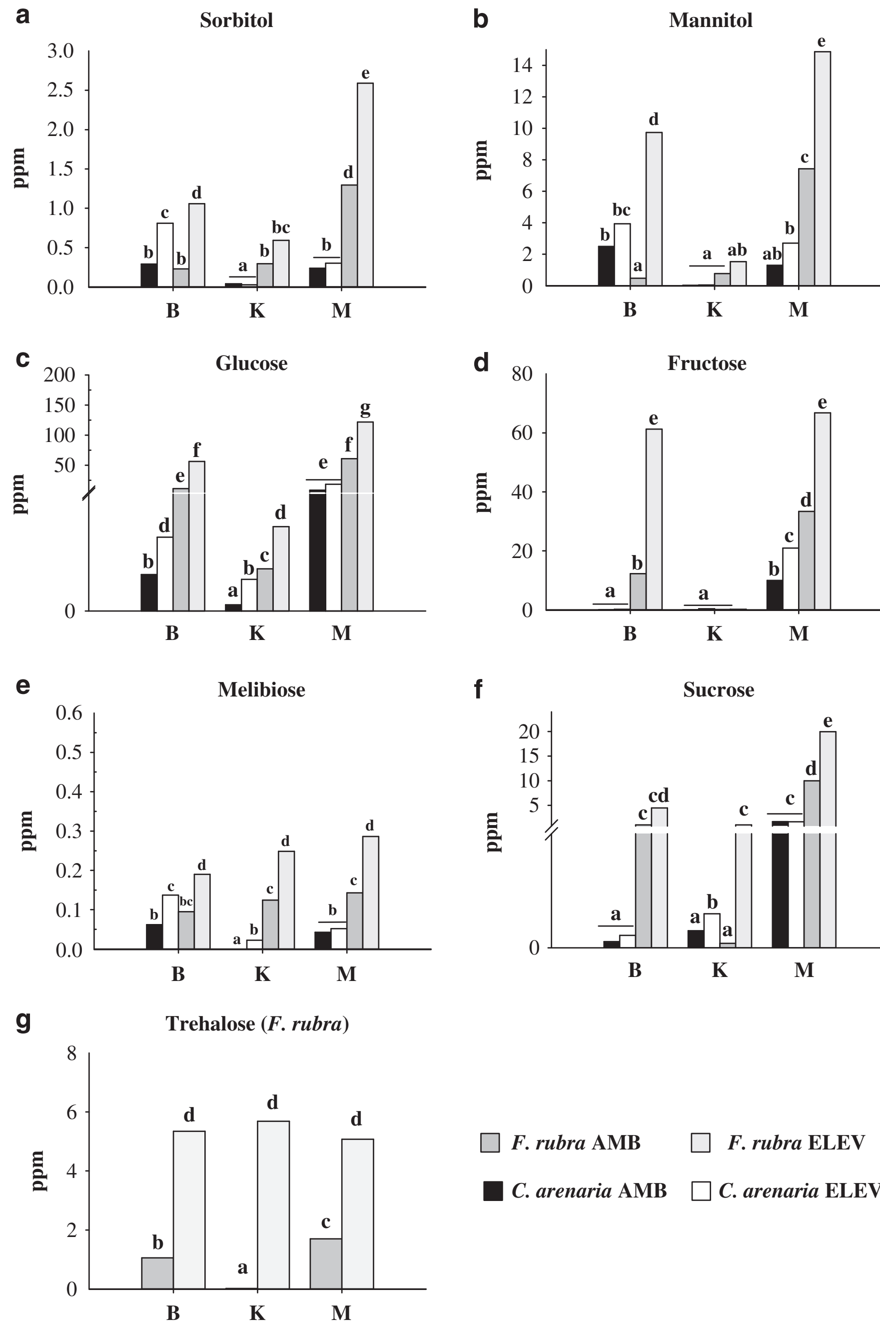

Figure 4 Means of (**a**) sorbitol, (**b**) mannitol, (**c**) glucose, (**d**) fructose, (**e**) melibiose and (**f**) sucrose at elevated CO_2, in *Festuca rubra* and *Carex arenaria* rhizosphere as determined by HPLC (high performance liquid chromatography) analysis. (**g**) Means of trehalose at ambient and elevated CO_2 in *F. rubra* rhizosphere, in *Carex arenaria* trehalose was hardly detected. Different letters within the graph refer to significantly different averages based on a Tukey HSD (honestly significant difference) test.

These results are in agreement with previous observations that slow-growing soil microorganisms, such as actinomycetes, were unaffected by elevated CO_2 (Zak *et al.*, 1996; Bardgett *et al.*, 1999; Jossi *et al.*, 2006). The observed impacts of elevated CO_2 were within a background of other significant sources of variation, such as effects of different plant species and soil origins, as examined by variation-partitioning analysis (Figure 1). CO_2 enrichment also influenced the abundance of the genera *Pseudomonas* and *Burkholderia*, and these effects were dependent on the soil origin and plant species (Figure 2). No such effects were observed for the abundances of the actinomycetes or the genus *Bacillus*.

Previous studies have identified *Pseudomonas* and *Burkholderia* as highly rhizo-competent genera (Vancanneyt *et al.*, 1996; Lugtenberg *et al.*,

2001; Treonis *et al.*, 2004; Berg *et al.*, 2005). In contrast, actinomycetes and *Bacillus* spp. have been implicated to be typical bulk-soil inhabitants (Smalla *et al.*, 2001), although recent studies suggest that these groups may represent only modest proportions of total soil microbial communities (Roesch *et al.*, 2007). Thus, our results provide new evidence for the previously presumed levels of interaction of these groups with plants, with rhizosphere bacteria reacting more strongly to changes in plant physiology and exudation induced by elevated atmospheric CO_2 concentrations.

Antibiotic-production genes

To gain some insight as to whether observed changes in the rhizosphere communities might have functional significance, we also targeted a number of genes known to be involved in the antibiotic production of potential plant-growth-promoting bacteria. For example, PRN is an antibiotic produced by *Pseudomonas* and *Burkholderia* species (Raaijmakers *et al.*, 2002), known to act upon plant pathogens, such *Rhizoctonia solani* and *Fusarium* spp. DAPG and PCA have also been shown to be involved in the biocontrol activity and ecological competence of several *Pseudomonas* strains. These two antibiotics are effective against the wheat root disease fungal pathogen *Gaeumannomyces graminis* var. *tritici* (take-all), as well as *Pythium ultimum* and *Fusarium oxysporum* (Raaijmakers *et al.*, 2002).

Our results showed that CO_2 enrichment influenced the densities of PRN- and DAPG-producing genes in *Pseudomonas* and *Burkholderia*, with a smaller effect on PCA-producing gene densities (Figure 3). Similar to the changes in community composition described above, the density of antibiotic producers also strongly depended on the plant species and soil origin. For instance, significant increases related to PRN, DAPG and PCA producers were observed for *C. arenaria* in Middelduinen soil. Interestingly, for *F. rubra* under elevated CO_2, large changes were observed for PRN and DAPG producers in Bergharen and \Middelduinen soils, whereas the PCA-producing *Pseudomonas* and *Burkholderia* spp. community was hardly detected, except in Middelduinen soil. Antibiotic-producing rhizosphere communities in Bergharen and Middelduinen soils showed marked CO_2 treatment responses, but no significant effects were observed in Kwade Hoek soil for either of the plants tested. These results suggest that the observed changes in bacterial communities, most notably in *Pseudomonas* and *Burkholderia*, which are known for their production of these antibiotics, may have functional consequences with respect to interactions with plant pathogens (Raaijmakers *et al.*, 2002), and that these responses are plant and soil specific. It is interesting to note that Tarnawski *et al.* (2006) observed lower frequencies of *Pseudomonas* strains capable of producing hydrogen cyanide, a potential inhibitor of root parasitic fungi, in two perennial grassland systems (*Lolium perenne* and *Medicago coerula*) under elevated (600 p.p.m. vs 360 p.p.m.) CO_2 concentrations.

Changes in plant exudation patterns in response to elevated CO_2

The elevated CO_2 conditions generally resulted in increased sugar exudation in the rhizosphere (Figure 4). The level of this increase and composition of exudates depended on plant species and soil origin. For instance, although both plants showed increased total exudation of sugars (glucose, fructose, sucrose, mannitol, melibiose and sorbitol) at elevated CO_2, this effect was on average twice as large in *F. rubra* spp. *arenaria* (caespitose) rhizospheres. In addition, we found that *F. rubra* produced four times more trehalose under elevated CO_2 conditions as compared with that at ambient CO_2 conditions. *C. arenaria* did not seem to release appreciable amounts of this compound regardless of atmospheric CO_2 level (Figure 4). Although plant litter represents the dominant pathway by which plant C is transferred to soil, living roots also contribute significantly to this process through turnover of fine roots, sloughing of living cells and exudation (Matamala *et al.*, 2003; Philips *et al.*, 2006). Furthermore, root exudation probably exerts a disproportionate impact on rhizosphere communities, as it represents the most easily accessible C available to the soil microbes (Cardon, 1996). It should be realized that analysis of root secretions in natural soils is hampered by the fact that rhizosphere microorganisms continuously consume and produce easily metabolized compounds. Thus, our exudate measurements represent more the net flux of secretion (efflux) and uptake (influx), which itself depends on the biomass, affinity and consumption rates of consumers (Tarnawski and Aragno, 2006).

Previous studies have shown that, under elevated CO_2, especially C_3 plants (as used in our study) increase their total root exudation mainly through the expansion of their root systems (Rogers *et al.*, 1994; Allard *et al.*, 2005), yielding qualitative and quantitative changes in root exudation and other forms of rhizodeposition (Paterson *et al.*, 1996; Hodge and Millard, 1998). Differences in plant exudation patterns between plants species is thought to exert differential selection in the rhizosphere, thereby shaping the size and structure of soil-borne communities (Bardgett *et al.*, 1999; Smalla *et al.*, 2001; Kowalchuk *et al.*, 2002). The AMF infections are also known to affect exudation patterns (Frey-Klett *et al.*, 2007), and the differential mycorrhizal status of the two plants in our study (*F. rubra* is mycorrhizal whereas *C. arenaria* is not; Greipsson and El-Mayas, 1999; Orlowska *et al.*,

2005) may have contributed to differences in observed exudation patterns. The results with respect to trehalose exudation are of particular relevance in this respect, as this disaccharide is an important product of AMF. Trehalose has also been implicated in the selection of potential mycorrhizal helper bacteria, including several *Pseudomonas* and *Burkholderia* species (Frey-Klett *et al.*, 2007), although it is premature to conclude that the observed changes in these genera are related to this function. The AMFs have been implicated as major conduits for C translocation from plants into the soil under different atmospheric CO_2 conditions, and our previous observation that the mycorrhizal plant (*F. rubra*) exerted greater influence on bacterial and fungal communities is consistent with this assertion (Drigo *et al.*, 2007).

It should be noted that our study only considered the sugars, and the study of other exudates, such as amino acids and organic acids, would provide a more complete picture of exudation patterns. Moreover, to fully understand the flow of C to soil, root turnover and root biomass would need to be followed over time. In our experiment, we could observe roughly an increase of root biomass of the 30% for both plants in response to the elevated CO_2 treatment.

Effects of elevated CO_2 on fungal communities

Given the important role of fungal pathogens and antagonists in the functioning of plant–soil systems (Hagn *et al.*, 2007), we also examined changes in two key fungal genera related to these activities. *Fusarium* spp. are common soil fungi, which can have important roles not only as plant pathogens, but also as saprotrophic competitors of other pathogenic fungi (Duffy *et al.*, 2004; Yergeau *et al.*, 2005). In this study, the density of *Fusarium* spp., as judged by quantitative real-time PCR targeting the 18S-rRNA gene, was significantly reduced in the rhizosphere of the mycorrhizal plant (*F. rubra*) exposed to elevated atmospheric CO_2 concentration. Interestingly, it has been suggested that AMF colonization, can reduce *Fusarium* community size (Filion *et al.*, 2000). Direct interactions, such as competition between the symbiont and the pathogen for infections sites (Muchovej *et al.*, 1991), or indirect interactions, such as alteration of root exudation and/or of the mycorhizosphere microbial community, were proposed as potential mechanisms of this observed reduction. The exact nature of these relationships requires further investigation in order to gain insight into the potential consequences of elevated CO_2 on plant disease conditions. We also observed an increase in genes associated with the production of antibiotics known to affect *Fusarium* species in response to elevated CO_2 (see above), but we are not yet able to show a causal link between this increase and the decline in *Fusarium* densities.

Fungi of the genus *Trichoderma* account for a major portion of fungal biomass in soils, and often act as important control agents for a wide range of phytopathogens (Harman and Björkmann, 1998; Hagn *et al.*, 2007). In contrast to *Fusarium* spp., *Trichoderma* density increased under elevated CO_2 in the *F. rubra* rhizosphere. This pattern mirrored that previously observed for the total fungal community size (Drigo *et al.*, 2007).

Concluding remarks

In a previous general examination of bacterial and fungal communities in the rhizosphere of plants subjected to elevated CO_2 (Drigo *et al.*, 2007), it was observed that the effects of elevated CO_2 were dependent on plant species and soil type. Here, we examined more closely the specific microbial groups affected by elevated CO_2 and showed that presumably rhizo-competent bacteria and fungi are strongly affected by increased atmospheric CO_2 in contrast to typical bulk-soil representatives. These patterns were consistent with observed changes in the density of antibiotic production genes as well as changes in exudation patterns. It should be noted, however, that our controlled experimental design did not allow for long-term adaptation to changes in atmospheric CO_2 conditions, and effects of such adaption may be significant (Klironomos *et al.*, 2005).

Although this study provides new insight into the specific plant–microbe interactions in the rhizosphere under elevated CO_2, knowledge is still rather scarce with respect to the relative flow of C to different biological groups of the plant–soil ecosystem (Olsson and Johnson, 2005; Carney *et al.*, 2007; Kreuzer-Martin, 2007). Such knowledge is critical to not only our understanding of soil food web, but also for predicting the future impacts of increasing CO_2 levels.

Acknowledgements

This study was supported by a Netherlands Research Council (NWO) grant of the Biodiversity and Global Change program (852.00.40). The authors thank Roel Wagenaar, Felix Waeckers and Wim van der Putten for expert input regarding sugar analyses, as well as critical comments toward the content of the paper. We extend our gratitude to Sachie Hoppener-Ogawa, Maria Bersma-Vlami and Jos Raaijmakers for providing strains used in this study. This is a NIOO-KNAW publication 4560.

References

Ainsworth EA, Long SP. (2005). What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. *New Phytol* **165**: 351–372.

Allard V, Newton PCD, Lieffering M, Soussana JF, Carran RA, Matthew C. (2005). Increased quantity and quality of coarse soil organic matter fraction at elevated CO_2 in a grazed grassland are a consequence of enhanced root growth rate and turnover. *Plant Soil* **276**: 49–60.

Alley R, Berntsen T, Bindoff NL, Chen Z, Chidthaisong A, Friedlingstein PB *et al.* (2007). Climate change 2007: The Physical Science Basis. Contribution of working group I to the fourth assessment report of the Intergovernmental Panel on Climate Change, Paris.

Bangera MG, Thomashow LS. (1999). Identification and characterization of a gene cluster for synthesis of the polyketide antibiotic 2, 4-diacetylphloroglucinol from *Pseudomonas fluorescens* Q2-87. *J Bacteriol* **181**: 3155–3163.

Bardgett RD, Kandeler E, Tscherko D, Hobbs PJ, Bezemer TM, Jones TH *et al.* (1999). Below-ground microbial community development in a high temperature world. *Oikos* **85**: 193–203.

Barron-Gafford G, Martens D, Grieve K, Biel K, Kudeyarov V, McLain JET *et al.* (2005). Growth of eastern cottonwoods (*Populus deltoides*) in elevated [CO_2] stimulates stand-level respiration and rhizodeposition of carbohydrates, accelerates soil nutrient depletion, yet stimulates above- and belowground biomass production. *Glob Chang Biol* **11**: 1220–1233.

Berg G, Eberl L, Hartmann A. (2005). The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. *Environ Microbiol* **7**: 1673–1685.

Bergsma-Vlami M, Prins ME, Staats M, Raaijmakers JM. (2005). Assessment of genotypic diversity of antibiotic-producing Pseudomonas species in the rhizosphere by denaturing gradient gel electrophoresis. *Appl Environ Microbiol* **71**: 993–1003.

Borcard D, Legendre P, Drapeau P. (1992). Partialling out the spatial component of ecological variation. *Ecology* **73**: 1045–1055.

Cardon ZG. (1996). Influence of rhizodeposition under elevated CO_2 on plant nutrition and soil organic matter. *Plant Soil* **187**: 277–288.

Carney KM, Hungate BA, Drake BG, Megonigal JP. (2007). Altered soil microbial community at elevated CO_2 leads to loss of soil carbon. *Proc Natl Acad Sci USA* **104**: 4990–4995.

Casgrain P, Legendre P. (2001). *The R package for multivariate and spatial analysis*. Département de sciences biologiques, Université de Montréal: Montréal, Canada.

Darrah PR. (1996). Rhizodeposition under ambient and elevated CO_2 levels. *Plant Soil* **187**: 265–275.

De Souza JT, Raaijmakers JM. (2003). Polymorphisms within the prnD and pltC genes from pyrrolnitrin and pyoluteorin-producing Pseudomonas and Burkholderia spp. *FEMS Microbiol Ecol* **43**: 21–34.

Drigo B, Kowalchuk GA, Yergeau E, Bezemer TM, Boschker HTS, Van Veen JA. (2007). Impact of elevated carbon dioxide on the rhizosphere communities of *Carex arenaria* and *Festuca rubra*. *Glob Chang Biol* **13**: 2396–2410.

Duffy B, Keel C, Defago G. (2004). Potential role of pathogen signaling in multitrophic plant-microbe interactions involved in disease protection. *Appl Environ Microbiol* **70**: 1836–1842.

Filion M, Dutilleu P, Potvin C. (2000). Optimum experimental design for Free-Air Carbon dioxide Enrichment (FACE) studies. *Glob Chang Biol* **6**: 843–854.

Frey-Klett P, Garbaye J, Tarkka M. (2007). The mycorrhiza helper bacteria revisited. *New Phytol* **176**: 22–36.

Gamper H, Hartwig UA, Leuchtmann A. (2005). Mycorrhizas improve nitrogen nutrition of *Trifolium repens* after 8 years of selection under elevated atmospheric CO_2 partial pressure. *New Phytol* **167**: 531–542.

Garbeva P, van Veen JA, van Elsas JD. (2003). Predominant *Bacillus* spp. in agricultural soil under different management regimes detected via PCR-DGGE. *Microb Ecol* **45**: 302–316.

Garbeva P, van Veen JA, van Elsas JD. (2004). Assessment of the diversity, and antagonism towards *Rhizoctonia solani* AG3, of *Pseudomonas* species in soil from different agricultural regimes. *FEMS Microb Ecol* **47**: 51–64.

Grayston SJ, Campbell CD, Lutze JL, Gifford RM. (1998). Impact of elevated CO_2 on the metabolic diversity of microbial communities in N-limited grass swards. *Plant Soil* **203**: 289–300.

Greipsson S, El-Mayas H. (1999). Large-scale reclamation of barren lands in Iceland by aerial seeding. *Land Degrad Dev* **10**: 185–193.

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.

Hagn A, Wallisch S, Radl V, Charles Munch J, Schloter M. (2007). A new cultivation independent approach to detect and monitor common Trichoderma species in soils. *J Microbiol Methods* **69**: 86–92.

Harman GE, Björkmann T. (1998). Potential and existing uses of *Trichoderma* and *Gliocladium* for plant disease control and plant growth enhancement. In: Kubicek CP, Harman GE (eds). *Trichoderma and Gliocladium, Enzymes, Biological Control and Commercial Applications*, Vol. 2. Taylor and Francis: London.

Heuer H, Krsek M, Baker P, Smalla K, Wellington E. (1997). Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Appl Environ Microbiol* **63**: 3233–3241.

Hodge A, Millard P. (1998). Effect of elevated CO_2 on carbon partitioning and exudate release from *Plantago lanceolata* seedlings. *Physiol Plant* **103**: 280–286.

Hoppener-Ogawa S, Leveau JHJ, van Veen JA, De Boer W. (2009). Mycophagous growth of Collimonas bacteria in natural soils, impact on fungal biomass turnover and interactions with mycophagous Trichoderma fungi. *ISME J* **3**: 190–198.

Jones TH, Thompson LJ, Lawton JH, Bezemer TM, Bardgett RD, Blackburn TM *et al.* (1998). Impacts of rising atmospheric carbon dioxide on model terrestrial ecosystems. *Science* **280**: 441–443.

Jossi M, Fromin N, Tarnawski S, Kohler F, Gillet F, Aragno M *et al.* (2006). How elevated pCO_2 modifies total and metabolically active bacterial communities in the rhizosphere of two perennial grasses grown under field conditions. *FEMS Microbiol Ecol* **55**: 339–350.

Kandeler E, Tscherko D, Bardgert RD, Hobbs PJ, Kampichler C, Jones TH. (1998). The response of soil micro-organisms and roots to elevated CO_2 and temperature in a terrestrial model ecosystem. *Plant Soil* **202**: 251–262.

Keel C, Schnider U, Maurhofer M, Voisard C, Laville J, Burger P *et al.* (1992). Suppression of root diseases by

Pseudomonas fluorescens CHA0: importance of the secondary metabolite 2,4-diacetylphloroglucinol. *Mol Plant Microbe Interact* **5**: 4–13.

Keel C, Weller DM, Natsch A, Defago G, Cook RJ, Thomashow LS. (1996). Conservation of the 2,4-diacetylphloroglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. *Appl Environ Microbiol* **62**: 552–563.

King JS, Hanson PJ, Bernhardt E, DeAngelis P, Norby RJ, Pregitzer KS. (2004). A multiyear synthesis of soil respiration responses to elevated atmospheric CO_2 from four forest FACE experiments. *Glob Chang Biol* **10**: 1027–1042.

Klironomos JN, Allen MF, Rillig MC, Piotrowski J, Makvandi-Nejad S, Wolfe BE *et al.* (2005). Abrupt rise in atmospheric CO_2 overestimates community response in a model plant-soil system. *Nature* **433**: 621–624.

Kowalchuk GA, Buma DS, de Boer W, Klinkhamer PGL, van Veen JA. (2002). Effects of above-ground plant species composition and diversity on the diversity of soil-borne micro-organisms. *Antonie Van Leeuwenhoek* **81**: 509–520.

Kreuzer-Martin HW. (2007). Stable isotope probing: Linking functional activity to specific members of microbial communities. *Soil Sci Soc Am J* **71**: 611–619.

Legendre P, Anderson MJ. (1999). Distance-based redundancy analysis: Testing multispecies responses in multifactorial ecological experiments. *Ecol Monogr* **69**: 1–24.

Lingoes JC. (1971). Some boundary conditions for a monotone analysis of symmetric matrices. *Psychometrika* **36**: 195–203.

Lipson DA, Blair M, Barron-Gafford G, Grieve K, Murthy R. (2006). Relationships between microbial community structure and soil processes under elevated atmospheric carbon dioxide. *Microb Ecol* **51**: 302–314.

Long SP, Ainsworth EA, Rogers A, Ort DR. (2004). Rising atmospheric carbon dioxide: plants face the future. *Annu Rev Plant Biol* **55**: 591–628.

Lueders T, Manefield M, Friedrich MW. (2004). Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ Microbiol* **6**: 73–78.

Lugtenberg BJJ, Dekkers L, Bloemberg GV. (2001). Molecular determinant of rhizosphere colonization by *Pseudomonas*. *Annu Rev Phytopathol* **39**: 461–490.

Lynch JM, Whipps JM. (1990). Substrate flow in the rhizosphere. *Plant Soil* **129**: 1.

Marilley L, Hartwig UA, Aragno M. (1999). Influence of an elevated atmospheric CO_2 content on soil and rhizosphere bacterial communities beneath *Lolium perenne* and *Trifolium repens* under field conditions. *Microb Ecol* **38**: 39–49.

Matamala R, Gonzalez-Meler MA, Jastrow JD, Norby RJ, Schlesinger WH. (2003). Impacts of fine root turnover on forest NPP and soil C sequestration potential. *Science* **302**: 1385–1387.

Montealegre CM, van Kessel C, Blumenthal JM, Hur HG, Hartwig UA, Sadowsky MJ. (2000). Elevated atmospheric CO_2 alters microbial population structure in a pasture ecosystem. *Glob Chang Biol* **6**: 475–482.

Montealegre CM, van Kessel C, Russelle MP, Sadowsky MJ. (2002). Changes in microbial activity and composition in a pasture ecosystem exposed to elevated atmospheric carbon dioxide. *Plant Soil* **243**: 197–207.

Muchovej JJ, Muchovej RMC, Gonçalves EJ. (1991). Effect of kind and method of fungicidal treatment of bean seed on infections by the VA-mycorrhizal fungus *Glomus macrocarpum* and by the pathogenic fungus *Fusarium solani*. *Plant Soil* **132**: 47–51.

Muyzer G, de Waal EC, Uitterlinden AG. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**: 695–700.

Nguyen C. (2003). Rhizodeposition of organic C by plants: mechanisms and controls. *Agronomie* **23**: 375–396.

Olsson PA, Johnson NC. (2005). Tracking carbon from the atmosphere to the rhizosphere. *Ecol Lett* **8**: 1264–1270.

Orlowska E, Ryszka P, Jurkiewicz A, Turnau K. (2005). Effectiveness of arbuscular mycorrhizal fungal (AMF) strains in colonization of plants involved in phytostabilisation of zinc wastes. *Geoderma* **129**: 92–98.

Paterson E, Rattray EA, 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.

Philips DA, Fox TC, Six J. (2006). Root exudation (net efflux of amino acids) may increase rhizodeposition under elevated CO_2. *Glob Chang Biol* **12**: 561–567.

Phillips RP. (2007). Towards a rhizo-centric view of plant-microbial feedbacks under elevated atmospheric CO_2. *New Phytol* **173**: 664–667.

Raaijmakers JM, Weller DM, Thomashow LS. (1997). Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. *Appl Environ Microbiol* **63**: 881–887.

Raaijmakers JM, Vlami M, de Souza JT. (2002). Antibiotic production by bacterial biocontrol agents. *Antoine Van Leeuwenhoek* **81**: 537–547.

Roesch LF, Fulthorpe RR, Riva A, Casella G, Hadwin AKM, Kent AD *et al.* (2007). Pyrosequencing enumerates and contrasts soil microbial diversity. *ISME J* **1**: 283–290.

Rogers HH, Runion GB, Krupa SV. (1994). Plant-Responses to atmospheric CO_2 enrichment with emphasis on roots and the rhizosphere. *Environ Pollut* **83**: 155–189.

Salles JF, de Souza FA, van Elsas JD. (2002). Molecular method to assess the diversity of Burkholderia species in environmental samples. *Appl Environ Microbiol* **68**: 1595–1603.

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.

Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S *et al.* (2001). Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: Plant-dependent enrichment and seasonal shifts revealed. *Appl Environ Microbiol* **67**: 4742–4751.

Smith SE, Read DJ. (1997). *Mycorrhizal Symbiosis*. Academic Press: San Diego.

Stipanovic RD, Williams HJ, Sattler I, Scott AI, Liu J. (1998). Preparation of two stereochemically defined isomers of deuterium labelled d-cadinene. *J Labelled Comp Radiopharm* **39**: 223–230.

Tarnawski S, Aragno M. (2006). The influence of elevated CO_2 on diversity, activity and biogeochemical function of rhizosphere and soil bacterial communities. In: Nösberger J, Long SP, Norby RJ *et al*. (eds). *Managed*

Ecosystems and CO2—Case Studies, Processes and Perspectives. Ecological Studies Serie, Vol. 187. Springer: Berlin, Heidelberg, pp 393–409.
Tarnawski S, Hamelin J, Jossi M, Aragno M, Fromin N. (2006). Phenotypic structure of Pseudomonas populations is altered under elevated pCO_2 in the rhizosphere of perennial grasses. *Soil Biol Biochem* **38**: 1193.
Ter Braak CJF, Šmilauer P. (2002). *CANOCO reference manual and CanoDraw for Windows user's guide: software for canonical community ordination (version 4.5)*. Microcomputer Power: Ithaca, NY.
Ter Braak CJF, Verdonschot P. (1995). Canonical correspondence analysis and related multivariate methods in aquatic ecology. *Aquat Sci* **57**: 255–289.
Treonis AM, Ostle NJ, Stott AW, Primrose R, Grayston SJ, Ineson P. (2004). Identification of groups of metabolically-active rhizosphere micro-organisms by stable isotope probing of PLFAs. *Soil Biol Biochem* **36**: 533–537.
Vancanneyt M, Witt S, Abraham WR, Kersters K, Fredrickson HL. (1996). Fatty acid content in whole-cell hydrolysates and phospholipid fractions of pseudomonads: A taxonomic evaluation. *Syst Appl Microbiol* **19**: 528–540.
Widmer F, Seidler RJ, Gillevet PM, Watrud LS, Di Giovanni GD. (1998). A highly selective PCR protocol for detecting 16S rRNA genes of the genus Pseudomonas (sensu stricto) in environmental samples. *Appl Environ Microbiol* **64**: 2545–2553.
Yergeau E, Filion M, Vujanovic V, St-Arnaud M. (2005). A PCR-denaturing gradient gel electrophoresis approach to assess Fusarium diversity in asparagus. *J Microbiol Methods* **60**: 143–154.
Zak DR, Ringelberg DB, Pregitzer KS, Randlett DL, White DC, Curtis PS. (1996). Soil microbial communities beneath *Populus grandidentata* crown under elevated atmospheric CO_2. *Ecol Applic* **6**: 257–262.

Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)