The impact of increased atmospheric carbon dioxide on microbial community dynamics in the rhizosphere

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Chapter 4

Specific rhizosphere bacterial and fungal groups respond differently to elevated atmospheric

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Abstract

Soil community responses to increased atmospheric CO_2 concentrations are expected to occur mostly via 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 ppm) or elevated (700 ppm) CO_2 concentrations. PCR-DGGE and quantitative-PCR were performed to analyze, respectively, the structure and size of the communities of actinomycetes, *Fusarium* spp., *Tricoderma* ssp., *Pseudomonas* ssp., *Burkholderia* ssp. and *Bacillus* spp. Responses of specific functional groups such as phloroglucinol, phenazine and pyrrolnitrin producers, were also examined by quantitative–PCR. HPLC was employed to asses changes in exuded sugars in the rhizosphere of both plant species. Multivariate analysis of group-specific community profiles revealed 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. Interestingly, within the bacterial community, the genera *Burkholderia* and *Pseudomonas*, typically known as successful rhizosphere colonizers, were strongly 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 sizes of bacterial phloroglucinol, phenazine and pyrrolnitrin producing communities were also influenced by elevated CO_2, as was the density of the fungal genera *Fusarium* and *Trichoderma*.

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 via photosynthesis (Ainsworth & 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 & Whipps, 1990). Under elevated atmospheric CO_2-levels, rhizodeposition is generally expected to increase (Darrah 1996) and its composition to be altered (Hodge & Millard 1998) due to 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, which releases 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 & Read 1997) have been demonstrated to influence C flow in response

to elevated CO_2, thereby impacting soil microbial community size and structure (Phillips 2007; Drigo *et al.* 2007; Jones *et al.* 1998).
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 & 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), β/α-Proteobacteria or Acidobacteria (Lipson *et al.* 2006) and mycorrhizal fungi (Gamper *et al.* 2005). However, knowledge is still generally lacking with the respect to the responses of different specific microbial groups in response to elevated atmospheric CO_2 conditions.
In this study, we examined the effect of elevated atmospheric CO_2 on a number of specific soil-borne groups with presumabely contrasting life history strategies. We have employed PCR-denaturing gradient gel electrophoresis community fingerprinting analysis and real-time PCR methods to investigate the effects of elevated CO_2 on several microbial groups within the root-inhabiting communities of two dominant coastal sand dune plant species, *Carex arenaria* (a non-mycorrhizal plant species) and *Festuca rubra* ssp. *arenaria* (a mycorrhizal plant species). The controlled growth chamber experiments utilized three different coastal dune soils, as well as unplanted controls. As examples of common rhizosphere genera, the size and structure of *Pseudomonas* spp. (Lugtenberg *et al.* 2001) and *Burkholderia* ssp. (Berg *et al.* 2005) were examined. As groups more typical of bulk soil environments, actinomycetes and the genus *Bacillus* (Smalla *et al.* 2001) were chosen for similar analyses. In addition, the community size of two important fungal genera was examined, *Trichoderma* ssp. and *Fusarium* ssp, using newly designed real-time PCR assays. The densities of selected genes involved in antibiotic production (phloroglucinol, phenazine and pyrrolnitrin) were also quantified to gain insight into the effects on functions related to potentially antagonistic populations. To examine changes in C flow to the rhizosphere, high performance liquid chromatography (HPLC) was employed to characterize root exudates under ambient and elevated CO_2 growth conditions. 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 (Ter Braak & Verdonschot 1995; Borcard *et al.* 1992; Filion *et al*. 2000).

Material 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. In brief 4-weeks-old sterilized seedlings of *F. rubra* and *C. arenaria* were planted in a coastal dune soil (Middelduinen) , a former beach plain soil (Kwade Hoek) and a river dune soil (Bergharen). 240 pots (750 cm^3) were filled with 1kg of soil and wetted to 10% volumetric water content (based on dry weight). Uniform 4-week-old seedlings of *F. rubra* and *C. arenaria* were selected and transferred to containers (three seedlings per container) and were equally distributed and incubated in the CO_2 flow cabinets together with 72 unplanted soils (Drigo *et al.* 2007). *F. rubra* and *C. arenaria* rhizosphere and bulk soils were harvested respectively at 73 and 62 days after germination as described in 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 and 36 from unplanted soil under the two different CO_2 treatments and analysed those for the composition of the actinomycetes, *Pseudomonas* spp., *Burkholderia* spp. and *Bacillus* spp. communities. All PCRs were performed in a PTC-200 thermal cycler (MJ-Research, Waltham, MA) in 25 µL volumes containing 2.5 µL of 10 × PCR buffer, 2.5 µL of bovine serum albumine (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 system (Sydney, Australia). Table 1 summarizes the primers, thermocycling regimes and electrophoresis conditions used to analyze the 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 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 performed 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 200V for 10 min followed by an additional 16h at 70V. Gels were stained in ethidium bromide and digital images captured using an Imago apparatus (Gentaur, Brussels, Belgium) upon UV transillumination. Due 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 prior to further analysis (Corel Corporation, 2003). Gel images were normalized with respect to migration pattern of the standard lanes of each gel, prior to 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 performed in reference to a hypothetical composite lane containing bands at all positions found across each dataset. The DGGE fingerprints obtained were binary coded 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 interactions between these three factors on the community structure, as examined by PCR-DGGE, was tested by distance-based redundancy analysis (db-RDA, Legendre & Anderson 1999). Jaccard‘s coefficients of similarity were first calculated between samples and used to compute principal coordinates

Table 1: Bacterial isolates used as standard curves for the detection of actinomycetes, *Pseudomonas* spp., *Burkholderia* spp., *Bacillus* spp., *Fusarium* spp., *Trichoderma* and the PRN, DAPG, PCA functional genes. Standards were made from full-length PCR-amplified 16S-, 18S-genes and antibiotic production genes from the pure isolates and strains

Primers	Amplification protocol[1]	Types of analyses	Detection of	Reference
Acti 243/1378 968-gc/1378 Acti 243/Eub518	65°C; 35 cycles Touchdown 65°C to 55°C; 35 cycles 65°C; 40 cycles	DGGE gradient[2] (45-65% denaturant) real-time PCR	Actinomycetes[3]	Heuer *et al.* (1997) Luedres et al. (2004)
PsF/PsR 968-gc/PsR PsF/PsR	64°C; 35 cycles Touchdown 65°C to 55°C; 35 cycles 64°C; 40 cycles	DGGE gradient[2] (45-65% denaturant) real-time PCR	*Pseudomonas* spp.[3]	Widmer *et al* (1998) Garbeva *et al.* (2004) This study
Burk3/1378 Burk3-GC/BurkR Burk3/BurkR	Touchdown 62°C to 58°C; 45 cycles 64°C; 35 cycles 64°C; 40 cycles	DGGE gradient[2] (50-60% denaturant) real-time PCR	*Burkholderia* spp.[3]	Salles *et al.* (2002) This study
BacF/1378 968-gc/1378 BacF/1378	65°C; 35 cycles Touchdown 63°C to 55°C; 35 cycles 63°C; 40 cycles	DGGE gradient[2] (45-65% denaturant) real-time PCR	*Bacillus* spp.[3]	Garbeva *et al*. (2003) This study
Alfie1-GC/Alfie2	50.0°C; 40 cycles	real-time PCR	*Fusarium* spp.	Yeargeau *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. in preparation
PRND1/ PRND2	68°C; 40 cycles	real-time PCR	Pyrrolnitrin production locus (PRN)	De Souza *et al.* (2003) This study
Phl2a/Phl2b	65°C; 40 cycles	real-time PCR	2,4 Diacetylphloroglucinol production locus (DAPG)	Raaijmakers *et al*. (1997) This study
PHZJR1/PHZJR2	68°C; 40 cycles	real-time PCR	Phenazin 2-carboxylic acid production locus (PCA)	Bersma-Vlami *et al* (2005) This study

[1] PCR protocols are given as: annealing temperature; number of cycles. The remaining of the procedure is given in the text.
[2] 100% denaturant is defined as 40% (v/v) formamide and 7 M urea.[3] The PCR-DGGE were obtained from a nested approach with the primes sets indicated in the table.

(PCoA) in the R-package (Casgrain & Legendre 2001). When necessary, eigenvectors were corrected for negative eigenvalues using the procedure of Lingoes (1971), and all the PCoA 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, while the other two groups of factors were entered as covariables. 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 db-RDA analyses were also at carried as described above but on subsets of the whole dataset, by either analyzing plants species or soil origins separately. The

percentage of variation in the dataset that was related to the different factors was determined by variation partitioning analysis (Borcard *et al.* 1992), using the same strategy as for db-RDA analyses. The percentage of variation explained by a factor was the trace of the analysis constrained using this factor (RDA) divided by the trace of the unconstrained analysis (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.

Table 2: Bacterial isolates used as standard curves for the detection of actinomycetes, *Pseudomonas* spp., *Burkholderia* spp., *Bacillus* spp., *Fusarium* spp., *Trichoderma* and the PRN, DAPG, PCA functional genes. Standards were made from full-length PCR-amplified 16S-, 18S-genes and antibiotic production genes from the pure isolates and strains indicated.

Species/strains Closest neighbour(accession no./ % identity)	Origin and source	Detection target	Reference
Streptomycetes coelicolor (AL645882/100%)	F. rubra rhizosphere soil The Netherlands	Actinomycetes	This study
P. fluoresces (PINR2)	Soil The Netherlands (J.M.Raaijmaakers)	*Pseudomonas* spp.	de Souza *et al*. (2003)
Burkholderia cepacia (AY741358/98%)	F. rubra rhizosphere soil The Netherlands	*Burkholderia* spp.	This study
Bacillus subtilis (AL009126.1/100%)	F. rubra rhizosphere soil The Netherlands	*Bacillus* spp.	This study
Fusarium oxysporum (AF124843/100%)	F. rubra rhizosphere soil The Netherlands	*Fusarium* spp.	This study
Trichoderma harzianum (EF672343/100%)	Soil The Netherlands (S. Hoppener-Ogawa)	*Trichoderma* spp.	Hoppener-Ogawa et al. (in preparation)
P. fluoresces (CHA0) (AJ278806)	Tobacco Switzerland (G. Defago)	Pyrrolnitrin production locus (PRN)	Stutz *et al.* (1986)
P. fluoresces (Q2-87) (U41818)	Wheat WA, US A (L.S.Thomashow)	2,4 Diacetylphloroglucinol production locus (DAPG)	Bangera *et al*. (1999)
P. fluoresces (PHZ24)	Soil France (P. Lemanceau)	Phenazin 2-carboxylic acid production locus (PCA)	De Souza *et al*. (2003)

Total exuded sugars analysis

The high performance liquid chromatography (HPLC) procedure outlined by Stipanovic *et al.* (1998) was used to analyze sorbitol, manitol, trehalose, glucose, fructose, melibiose and sucrose concentrations in the rhizosphere of *C. arenaria* and *F. rubra* grown at ambient and elevated CO_2 conditions. 80 samples (40 of *F. rubra* and *C. arenaria*) each comprised of 100 mg of freeze-dried water-extracted exudates were harvested respectively at 73 and 62 days after germination. 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 (HEA) and 100 µl of 10% HCl. The solution was filtered over a glass-fritted filter funnel into a 50-ml

pear-shaped flask, and the beads and residue were rinsed three times with 3-ml HEA. The solvent was left to evaporate in a hot water bath, and the residue in the flask was redissolved with four 150-µl washes. Each wash was transferred to a Maxi-clean silica catridge (Alltech, Breda, and The Netherlands). The silica cartridge was dried with compressed air, and the soil solutions were eluted with 5-ml isopropyl alchol, acetonitrile, water, and 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 onto a DIONEX HPLC-system (DIONEX Corp., Sunnyvale, CA, USA), equipped with a single wavelength absorbance detector and a 250 mm × 4.6 mm i.d. ALLtima C-18 column (Alltech, Breda, The Netherlands). The column was eluted with EtOH mix at 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 at 272 nm. Standards of sorbitol, manitol, trehalose, glucose, fructose, melibiose and sucrose were used to assess the retention times of individual sugars. 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 singular 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 (Sn) as recovered in the bulk soil and the sugars concentration due to root damage (Sr). Stot represents the total sugar concentration derived from HPLC analysis:

$$(1)\quad [S] = [Stot] - ([Sn] - [Sr])$$

To determine 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 (ANOVA). 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 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). Analyses were carried in Statistica 7.0 (StatSoft Inc., Tulsa, OK). Normality was tested with a 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 (HSD) method and the modified version for unequal sample size (Unequal N HSD in Statistica) were used for post-hoc comparisons with a 0.05 grouping baseline. Analyses of variance were carried in Statistica 8.0 (StatSoft Inc., Tulsa, OK) to test 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 set. After ANOVA, 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, Sydney, Australia) to quantify actinomycete, *Burkholderia* spp., *Pseudomonas* spp., *Bacillus* spp., *Fusarium* spp. and *Trichoderma* spp. SSU rRNA gene densities, using the primers and conditions described in Table 1. Q-PCR assays were performed also for the detection of the pyrrolnitrin (PRN), 2,4 diacetylphloroglucinol (DAPG), phenazin 2-carboxylic acid (PCA) production loci (Table 1). All mixes were made using a CAS-1200 pipetting robot (Corbett Research, Sydney, Australia). Standards were made from full-length PCR-amplified 16S-, 18S- rRNA and antibiotic production genes from the pure isolates described in Table 2. Using 10-fold increments, the standard concentrations were adjusted from 10^9 to 10^1 SSU gene copies μl^{-1} for each species. Most of the samples, and all standards, were assessed in at least two different runs to confirm the reproducibility of the quantification. SSU rRNA gene numbers were analyzed using analysis of variance (ANOVA) 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; Fig. 1). Variation partitioning of PCR-DGGE datasets for these communities revealed that CO_2 treatment, soil origin, and plant species together explained about 55% of the variation in community profiles for *Pseudomonas* spp. and 60% for *Burkholderia* spp. (Table 3; Fig. 1A, B).
These variables all explained a significant part of the variation, but their relative contribution differed between groups of organisms. For *Pseudomonas* spp., the greatest degree of variation was explained by elevated CO_2 (30%), while soil origin and plant species both explained about 5-20% of the total variation. For *Burkholderia* spp., the greatest degree of variation was explained by elevated CO_2 (35%) and plant species (22%), while 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). Plant species, soil origin and the interactions between these factors did not explain a significant (Table 3) part of the variation for actinomycets and *Bacillus* spp..

Effect of elevated CO_2 on the rhizosphere community densities

SSU rRNA gene abundances for *Pseudomonas* spp. and *Burkholderia* spp., differed with respect to plant species, soil origin and elevated CO_2 treatments (Figure 2 a, b). In Kwade Hoek and Middelduinen soil, elevated CO_2 had no effect on the *Pseudomonas* spp. and *Burkholderia* spp. rhizosphere community sizes associate 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 $CO_2 \times$ soil interaction, indicating

that the effects of CO_2 elevation were soil dependent and affected the two plants rhizospheres to a similar extent.

Table 3: Main PCR-DGGE distance-based redundancy analyses results for plant species (*C. arenaria* and *F. 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	Trace	P	Trace	P	Trace	P	Trace
Plants	Soil origin × CO_2	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
CO_2	Soil origin × Plants	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	0.617	0.001	0.983	0.001	0.975

More complex interactions between CO_2 concentration, plant species and soil origins were also observed. For *C. arenaria*, *Burkholderia* rRNA gene abundance decreased or remained stable with elevated CO_2 in all soils, while the opposite pattern for *F. rubra* was observed. *Pseudomonas* communities in *C. arenaria* and *F. rubra* increased or remained stable in Bergharen and Kwade Hoek soils, yet decreased in Middelduinen soil.
SSU 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 actinomycete community sizes were not significantly affected by any variable (Figure 2 c, d). For both plant species, elevated CO_2 had no effect on community size for actinomycetes ($F_{1,2}$ = 1.019, P = 0.42) and *Bacillus* spp. ($F_{1,2}$ = 0.366, P = 0.61).

SSU rRNA gene abundances of Fusarium and Trichoderma

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 (Fig 2 e, 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* rRNA gene abundance increased with elevated CO_2 across all the three soil origins, yet for *F. rubra*, these patterns were exactly the opposite.

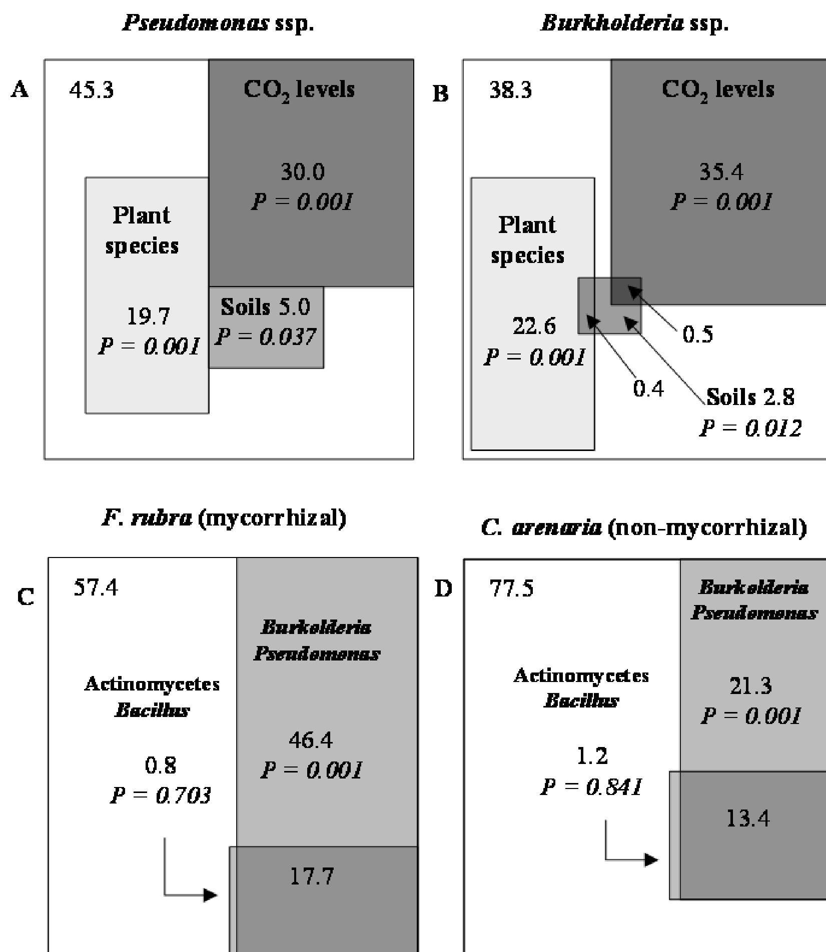

Figure 1: Variation partitioning representation of PCR-DGGE analyses with reference to (A) *Pseudomonas* spp. and (B) *Burkholderia* spp. rhizosphere communities under (C) *F. rubra* (mycorrhizal) plants only and (D) *C. arenaria* (non-mycorrhizal) plants only grown in Bergharen, (river dune), Kwade Hoek (former beach) and Middelduinen (costal dune). The numbers list the percentage variance accounted for by CO_2 levels, plant species, soil origin, unexplained variance and their intersections. The significance of all of the different factors on the microbial community structure indicated in the representation was tested by db-RDA based on 999 Monte Carlo permutations and split-plot analysis of variance. The area of each cell is proportional to the variance accounted for by the component. The area of the white square that is not covered by any of the other rectangles is proportional to the unexplained variance.

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

The density of genes associated with 2,4 diacetylphloroglucinol (DAPG) and pyrrolnitrin (PRN) production were generally much higher in *F. rubra* than in *C. arenaria* and differed with respect to soil origin. (Fig 3 a, b). Genes for the production of phenazin 2-carboxylic acid (PCA) were at low densities in the rhizospheres of *F. rubra* plants grown in Bergharen and Kwade Hoek soil, yet relatively high for all *C. arenaria* rhizosphere samples (Fig 3 c). 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 affected the size of DAPG and PRN producing communities in both plant rhizospheres grown in Middelduinen, yet only in *F. rubra* for Bergharen soil.

Effect of elevated CO_2 on total soluble exuded sugars

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; $P < 0.001$) (Fig 4). Interestingly, although both plant species increased their total exudation of sugars (glucose, fructose, sucrose, manitol, meliobiose and sorbitol) at elevated CO_2, this effect was generally greater in *F. rubra* rhizosphere. In addition, *F. rubra* exudates at elevated CO_2 contained four times the quantity of trehalose (Fig. 4) compared to ambient CO_2. As trehalose is a product of mycorrhizal fungi it points to the strong response of these symbionts to the increased atmospheric CO_2 concentration. No trehalose exudation was detected for *C. arenaria* at either atmospheric CO_2 level.

Discussion

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

Specific bacterial community responses

Distance-based redundancy analysis (db-RDA) of the community profiling patterns revealed 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. 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; and 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 sizes of the *Pseudomonas* and *Burkholderia* communities, and these effects were dependent on the soil origin and plant species (Figure 2). No such effects were observed for the sizes of the actinomycetes and *Bacillus* communities.

Previous studies have identified *Pseudomonas* and *Burkholderia* as highly rhizo-competent genera (Berg *et al.* 2005; Lugtenberg *et al.* 2001; Vancanneyt *et al.* 1996; Treonis *et al*. 2004). In contrast, actinomycetes and *Bacillus* spp. have been implicated to be common bulk soil inhabitants (Smalla *et al.* 2001). Thus, our results reflect the previously presumed levels of interaction of these groups with plants, with rhizosphere bacteria expected to react more strongly to changes in plant physiology and exudation induced by elevated atmospheric CO_2 concentrations.

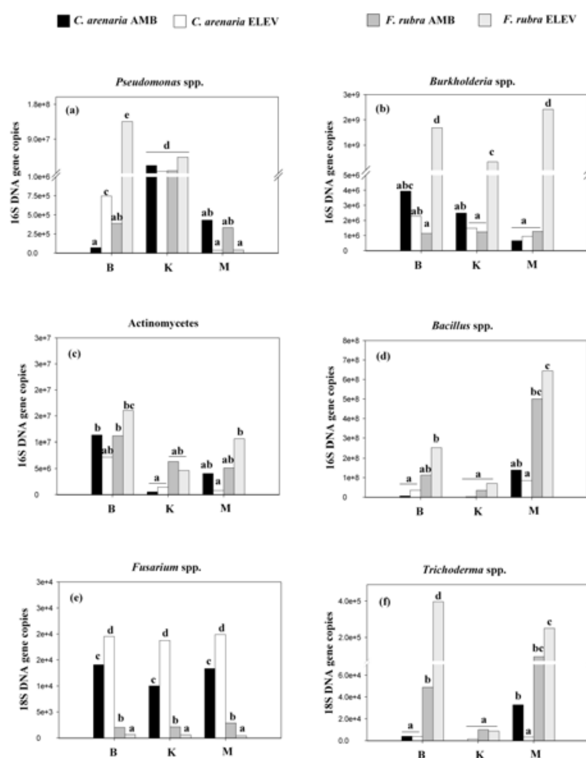

Figure 2: (a) *Pseudomonas* spp., (b) *Burkholderia* spp., (c) actinomycetes, (d) *Bacillus*, (e) *Fusarium* and (f) *Trichoderma* spp. SSU rRNA genes abundance in the rhizosphere of *C. arenaria* and *F. rubra* grown at elevated (ELEV) and ambient (AMB) CO_2 in Bergharen, river dune (B), Kwade Hoek, former beach (K) or Middelduinen, costal dune (M) soils. Different letters within the graph refer to significantly different averages based upon a Tukey-HSD test.

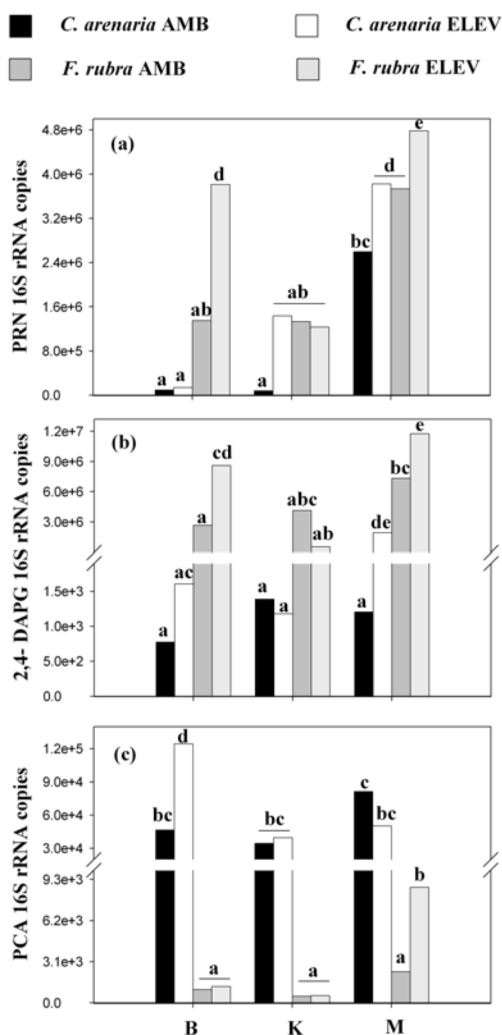

Figure 3: (a) Pyrrolnitrin production locus (PRN), (b) 2,4 diacetylphloroglucinol production locus (2,4 – DAPG), and (c) phenazin 2-carboxylic acid production locus (PCA) antibiotics functional genes in the rhizosphere of *C. arenaria* and *F. rubra* grown at elevated (ELEV) and ambient (AMB) CO_2 in Bergharen, river dune (B), Kwade Hoek, former beach (K) or Middelduinen, costal dune (M) soils. Different letters within the graph refer to significantly different averages based upon a Tukey-HSD test.

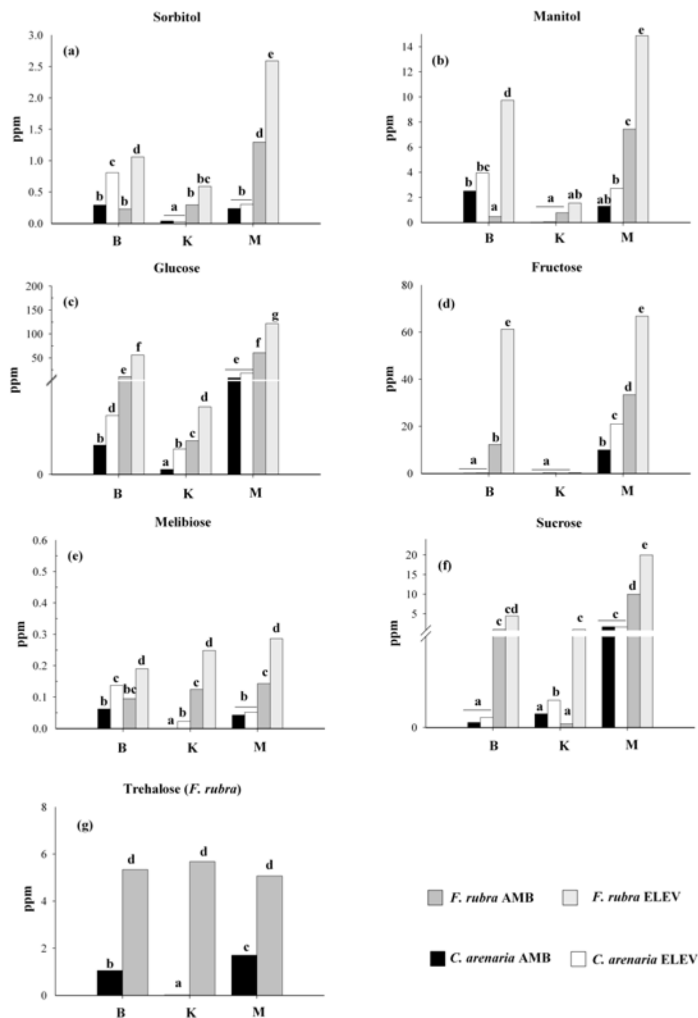

Figure 4: Mean of (a) sorbitol, (b) manitol, (c) glucose, (d) fructose, (e) meliobiose and (f) sucrose at elevated CO_2, in *F. rubra* and *C. arenaria* rhizosphere as determined by HPLC analysis. (g) Mean of trehalose at ambient and elevated CO_2, in *F. rubra* rhizosphere, in *C.arenaria* trehalose were hardly detected. Different letters within the graph refer to significantly different averages based upon a Tukey-HSD test.

Antibiotic production genes

In order 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 (PGPB). For example, pyrrolnitrin (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.. 2,4-diacteylphloroglucinol (DAPG) and phenazine-2-carboxylate (PCA) has 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 gramis* 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, with a smaller effect on PCA (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 community was hardly detected, except in Middelduinen soil. Antibiotic-producing rhizosphere communities in Bergharen and Middelduinen soils showed marked CO_2 treatment responses, yet no significant effects were observed in Kwade Hoek soil for either of the plants tested. These results suggest that the observed changes in bacterial community, most notably in genera known for the 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 (HCN), a potential inhibitor of root parasitic fungi, associated with two perennial grassland systems (*L. perenne* and *M. Coerula*) under elevated (600 ppm) versus ambient (360 ppm) CO_2 concentrations.

Changes in plant exudation patterns in response to elevated CO_2

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, manitol, meliobiose and sorbitol) at elevated CO_2, this effect was on average twice as large in *F. rubra* rhizospheres. In addition, we found that *F. rubra* produced four times more trehalose under elevated CO_2 conditions as compared to at ambient CO_2. *C. arenaria* did not appear to release appreciable amounts of this compound regardless of atmospheric CO_2 level (Fig. 5). Although plant litter represents the dominant pathway by which plant C is transferred to soil, living roots also contribute significantly to this process via turnover of fine roots, sloughing of living cells and exudation (Matamala *et al.* 2003; Phillips *et al.* 2006). It is important to note that 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 micro-

organisms 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 & 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 via 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 *et al.* 1998). Differences in plant exudation patterns between plant species is thought to exert differential selection in the rhizosphere, thereby shaping the size and structure of soil-borne communities (Smalla *et al*. 2001; Bardgett *et al.* 1999; Kowalchuk *et al.* 2002). Arbuscular mycorrhizal fungal (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 & 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 changes in these genera as described are related to this function. AMF 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).

Effects of elevated CO_2 on fungal communities

Given the important role of fungal pathogens and antagonists in the functioning of plant-soil systems (Packer & Clay 2003; 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 saprotrophic competitors of other pathogenic fungi (Duffy *et al.* 2005; Yergeau *et al.* 2005). In our study, the density of *Fusarium* spp., as judged by 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. Reduction of the *Fusarium* community size as a result of AMF colonization has been suggested by Filion *et al.* previously (2002). 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 mycorrhizosphere 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 demonstrate 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 & Björkmann1998; 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), we 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 demonstrated 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-microbial 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 & Johnson 2005; Carney *et al.* 2007; Kreuzer-Martin 2007). Such knowledge is critical to not only our understanding of soil food web, but also to predicting the future impacts of increasing CO_2 levels.

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Supplementary data

Figure S1:Representative 16S rRNA gene-based denaturing gradient gel electrophoresis profiles of (A) Burkholderia ssp., (B) Pseudomonas ssp., (C) Actinomycets and (D) Bacillus ssp. communities obtained from rhizosphere soil of (c) *C. arenaria* and (f) *F. rubra* cultivated on (Bc, Bf) Bergharen, (Kc, Kf) Kwade Hoek, (Mc, Mf) Middelduinen soils under (AMB) ambient and (ELEV) elevated CO_2 concentrations. The variation within a treatment was negligible and only a single pattern per treatment is shown for simplicity of presentation. (r) stands for bacterial reference pattern.