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Elevation of atmospheric CO_2 and N-nutritional status modify nodulation, nodule-carbon supply, and root exudation of *Phaseolus vulgaris* L.

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Abstract

Increased root exudation and a related stimulation of rhizosphere-microbial growth have been hypothesised as possible explanations for a lower nitrogen- (N-) nutritional status of plants grown under elevated atmospheric CO_2 concentrations, due to enhanced plant-microbial N competition in the rhizosphere. Leguminous plants may be able to counterbalance the enhanced N requirement by increased symbiotic N_2 fixation. Only limited information is available about the factors determining the stimulation of symbiotic N_2 fixation in response to elevated CO_2.

In this study, short-term effects of elevated CO_2 on quality and quantity of root exudation, and on carbon supply to the nodules were assessed in *Phaseolus vulgaris*, grown in soil culture with limited (30 mg N kg^{-1} soil) and sufficient N supply (200 mg N kg^{-1} soil), at ambient (400 μmol mol^{-1}) and elevated (800 μmol mol^{-1}) atmospheric CO_2 concentrations.

Elevated CO_2 reduced N tissue concentrations in both N treatments, accelerated the expression of N deficiency symptoms in the N-limited variant, but did not affect plant biomass production. $^{14}CO_2$ pulse-chase labelling revealed no indication for a general increase in root exudation with subsequent stimulation of rhizosphere microbial growth, resulting in increased N-competition in the rhizosphere at elevated CO_2. However, a CO_2-induced stimulation in root exudation of sugars and malate as a chemo-attractant for rhizobia was detected in 0.5–1.5 cm apical root zones as potential infection sites. Particularly in nodules, elevated CO_2 increased the accumulation of malate as a major carbon source for the microsymbiont and of malonate with essential functions for nodule development. Nodule number, biomass and the proportion of leghaemoglobin-producing nodules were also enhanced. The release of *nod*-gene-inducing flavonoids (genistein, daidzein and coumestrol) was stimulated under elevated CO_2, independent of the N supply, and was already detectable at early stages of seedling development at 6 days after sowing.

Keywords: Elevated CO_2; Rhizosphere; *Phaseolus vulgaris* L.; Root exudation; Malate; Malonate; Rhizobia; Nitrogen fixation; Signal flavonoids

1. Introduction

Higher atmospheric CO_2 concentrations alter the functioning of soil ecosystems mainly due to changes in plant–soil interactions. Greater photosynthetic assimilation rates under elevated CO_2 can increase root and shoot biomass, and a fraction of the additional fixed carbon (C) may be released into the rhizosphere by root exudation. An increase in root exudation is a common (Cheng and Johnson, 1998; van Ginkel et al., 2000; Allard et al., 2006) but not a general response to elevated CO_2 (Hodge and Millard, 1998; Bazot et al., 2006). Enhanced overall root exudation was caused either by a stimulation of root

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development (van Ginkel et al., 2000) or by higher root exudation rates per unit root area (Cheng and Johnson, 1998). Qualitative alterations in the composition of root exudates towards higher C/N ratios have also been reported occasionally (Grayston et al., 1998; Hodge et al., 1998).

Increased root exudation under elevated CO_2 may stimulate microbial growth and activity in the rhizosphere, which in turn may increase the competition for limiting nutrients between plants and rhizosphere microorganisms. This effect has been discussed as a possible cause for a reduced N-nutritional status of plants grown under elevated atmospheric CO_2 concentrations (Diaz et al., 1993).

Since symbiotic nitrogen fixation could counterbalance the CO_2-induced limitation of N availability in the rhizosphere, leguminous plants may play a key role in the response of ecosystems to elevated CO_2 (Marilley et al., 1999). Accordingly, the relative contribution of symbiotically fixed N_2 to total plant nitrogen increases (Zanetti et al., 1996; Schortemeyer et al., 2002; Feng et al., 2004). Marilley et al. (1999) observed that *Rhizobium leguminosarum* outcompetes N-heterotrophic microorganisms in the clover rhizosphere, leading to a shift of the bacterial community structure under elevation of CO_2 (Schortemeyer et al., 1996). Increased root exudation of chemoattractants (malate, phenolic acids, flavonoids), of *nod*-gene-inducing flavonoids and of other signal compounds involved in establishing the *Rhizobium* symbiosis, but also an improved carbon supply to the nodules (Cabrerizo et al., 2001), have been discussed as possible reasons for the stimulation of symbiotic N_2 fixation under elevated CO_2 (Marilley et al., 1999).

This study was conducted to test following hypotheses: (i) elevated atmospheric CO_2 concentrations limit N availability in the rhizosphere by plant-microbial competition because microbial growth is stimulated by increased root exudation. (ii) In leguminous plants, the lower N-nutritional status promotes symbiotic N_2 fixation by increased root exudation of signal compounds and/or improved carbon supply to the microsymbiont.

Phaseolus vulgaris L. was selected as a fast-growing model plant with a well-characterized pattern of root exudates involved in establishing the *Rhizobium* symbiosis (Bolaños-Vásquez and Werner, 1997; Werner, 2000). Plants were grown in soil culture with low and high levels of N supply in minirhizotrons (rhizoboxes), equipped with removable front lids to enable access to the surface of soil-grown roots. Root exudates and rhizosphere soil solution were collected along single roots by applying sorption media (chromatography paper, nylon membranes) onto the root surface, with a spatial resolution of 5 mm (Neumann, 2007). $^{14}CO_2$ pulse-labelling of photo-assimilates with a short duration was employed to investigate the pattern of current assimilate partitioning between plant compartments and between plant roots and soil C pools (Meharg, 1994).

2. Material and methods

2.1. Plant culture

P. vulgaris L. (*var*. Hilds Maxi GS) seeds were surface-sterilized by 10 min shaking in 30% H_2O_2 and subsequently washed with deionized water, followed by 5 h imbibition in 10 mM $CaSO_4$. Seeds were germinated in rolls of moist filter paper soaked with 2.5 mM $CaSO_4$ for 4 days in darkness and subsequent incubation with a 16 h light period under ambient and elevated CO_2 conditions with daily moistening (2.5 mM $CaSO_4$) of the filters. Three filter paper rolls per CO_2 treatment were harvested with subsequent plant biomass determination 6, 8, 10 and 12 DAS (days after sowing). Filter paper rolls were stored at −20 °C until flavonoid extraction and HPLC analysis. For the soil culture experiment, the pre-germinated seedlings were transferred to rhizobox microcosms (1 plant per box) at 7 days after sowing with six replicates per treatment. Rhizobox microcosms contained 150 g of an air-dried calcareous loess sub-soil ($CaCO_3$ 21.5%; pH ($CaCl_2$) 7.6; C_{org} 0.1%; plant available P (P_{CAL}) 5 mg kg^{-1}; N_{total} 0.02%) inoculated with 10% (w/w) of a fresh agricultural soil (Ap horizon) for microbial inoculation, and sieved to 2 mm mesh size. Fertilisation was performed by soil application of 150 mg K kg^{-1} as 0.5 M K_2SO_4; 80 mg P kg^{-1} as 0.05 M $Ca(H_2PO_4)_2$; 50 mg Mg kg^{-1} as 0.5 M $MgSO_4$; 20 μmol Fe kg^{-1} as 50 mM Fe-EDTA. Two levels of N supply as 0.5 M $Ca(NO_3)_2$ were chosen: a well N-supplied treatment (+N: 100 mg N kg^{-1} and additionally 50 mg N kg^{-1} at 10 and 15 DAS) and a low N-supplied treatment (−N: 30 mg N kg^{-1}). Soil moisture was adjusted daily to 70% of its water holding capacity by gravimetric determination and addition of distilled water. The rhizoboxes were positioned at a horizontal angle of 50° to stimulate root growth along the transparent root observation windows of the boxes.

Plants were cultivated under controlled environmental conditions in growth chambers with a 16/8 h day/night cycle, a light intensity of 300 μmol m^{-2} s^{-1}, a 25/20 °C day/night temperature regime, a relative humidity of 60%, and either 400 μmol mol^{-1} (ambient) or 800 μmol mol^{-1} (elevated) atmospheric CO_2 concentrations. Elevated CO_2 concentrations were adjusted to 800 μmol mol^{-1} ± 5% by automatic injection of pure CO_2 in one growth chamber. The elevated CO_2 concentration is in the range of predictions from emission scenarios for the next 100 years (Intergovernmental Panel on Climate Change (IPCC), 2001). The ambient CO_2 concentration of approximately 400 μmol mol^{-1}, characteristic for the region of Stuttgart, was applied in the other growth chamber.

The $^{14}CO_2$ shoot pulse labelling was performed at 14 DAS, 3 h after the beginning of the light period in a closed plexiglas chamber with 4933 kBq of ^{14}C as $Na_2^{14}CO_3$ solution for 1.5 h. All plants were labelled simultaneously; all openings of the rhizoboxes were sealed before labelling (sealing material between shoot and rhizobox: Optosil® P

plus, Heraeus Kulzer, Dormagen, Germany). The labelled CO_2 was generated outside the chamber by adding 3 ml of a 3.5 N lactic acid solution to the $Na_2^{14}CO_3$. This was introduced into the chamber by moving air (membrane pump) through the solution as described by Kuzyakov et al. (2003). A fan homogenised the atmosphere within the chamber. Afterwards, the unassimilated tracer was removed from the labelling chamber by flushing with fresh air, which was passed through an alkaline trap containing 20 ml of a 0.5 N NaOH solution to collect the unassimilated $^{14}CO_2$. Potential formation of insoluble $CaCO_3$ from respired $^{14}CO_2$ is considered to be negligible due continuous removal of CO_2 from the soil by membrane pumps (Kuzyakov et al., 2006).

2.2. Collection of root exudates in apical root zones, plant harvest and soil sampling

Collection of rhizodeposition of total ^{14}C, carboxylates, sugars and amino acids from apical root zones was performed by application of filter papers as sorption media onto the root surface (Neumann, 2007). Filter discs (5 mm diameter, water uptake capacity 63 µl cm^{-2}), moistened with distilled water, were placed onto the surface of subapical root zones (at 0.5–1.5 cm distance from the root tip) of 6 lateral roots appearing at the root observation windows of the rhizobox microcosms, for trapping root exudates released from the roots 12, 15, 18 and 21 DAS. The rhizodeposition was collected 3 h after the beginning of the light period. After 30 min the first set of filter discs was discarded and replaced by new ones in order to minimise the collection of previously accumulated rhizosphere products. During incubation, the filters were occasionally re-moistened with 10–20 µl of distilled water. After 2 h the filter discs were transferred from the root surface into 1.5 ml reaction vials and stored at −20 °C until further analysis. The 2 h-short-term collection of root secretes was performed to reduce microbial degradation of root exudates and to recover a high proportion of root exudates (Neumann and Römheld, 2000; Neumann, 2007). At the end of the culture period, the 6 apical root segments used for exudate collection were harvested separately. At each sampling date, plants were harvested and separated into shoots and roots for fresh weight determination. The total root adhering soil was sampled as rhizosphere soil. The nodule tissue was then separated from the roots. The weight and the number of red-coloured nodules were determined. All tissue samples were frozen immediately in liquid N_2 and stored at −20 °C for subsequent analysis.

2.3. HPLC analysis of carboxylates in root exudates, root tissue and nodule tissue

Extraction of root exudates from the combined collection filters was performed by addition of 50 µl of an 18 mM KH_2PO_4 solution (pH 2.31; used as HPLC eluent) per filter disc according to Neumann (2007). The extract was used for high performance liquid chromatography (HPLC) analysis. Carboxylates were extracted from lateral root tissue and nodule tissue according to Neumann et al. (1999). The apical root segments and the nodule tissue were ground in 5% (v/v) H_3PO_4 (50 mg root tissue FW ml^{-1}). After centrifugating the homogenate, the supernatant was diluted 10-fold with the HPLC eluent and subjected to HPLC analysis.

HPLC analysis of carboxylates from filter papers and root tissue was performed isocratically on a reversed-phase C-18 column (GROM-SIL 120 ODS-5 ST, particle size 5 µm; length 250 mm, ID 4.6 mm) with a Hypersil ODS guard column (20 mm, ID 4.6 mm; GROM, Herrenberg, Germany) in the ion-suppression mode (Neumann, 2007). The carboxylates were identified and quantified by comparing the retention times and peak areas with those of known standards and by recording absorption characteristics.

2.4. Analysis of total amino acid and sugar concentrations in root exudates

Amino acid and sugar concentrations were determined in filter paper extracts from root exudates of the collection dates at 12 and 18 DAS, the same extracts that were prepared for HPLC analysis of carboxylates. The total amino acid concentration was analysed fluorometrically after Jones et al. (2002), using a HITACHI Fluorometer F-2000 (Colora Messtechnik, Lorch, Germany) with glycine as a standard. Since the *o*-phthaldialdehyde-*β*-mercaptoethanol procedure also detects NH_4^+ in the samples, NH_4^+ contamination was determined via continuous-flow fluorometry (Husted et al., 2000) and NH_4^+ was subtracted from the total amino acid concentration according to Jones et al. (2002).

Total sugar concentrations were determined according to a modified method of Blakeney and Mutton (1980). Glucose was used as standard. To determine reducing sugars and sucrose, 62.5 µl of the filter paper extracts, adjusted to pH 4.8 with 0.1 M NaOH, was mixed with 50 µl 0.2 M sodium acetate buffer (pH 4.8) and 12.5 µl invertase solution (10 mg Yeast Invertase Grade VII + 50 ml H_2O + 50 ml 0.2 M sodium acetate buffer). The mixture was incubated for 2 h at 30 °C to hydrolyse sucrose to glucose and fructose. Six hundred and twenty five microlitres of colour reagent (0.03 M hydroxybenzoic acid hydrazide, 0.05 M trisodium citrate, 0.01 M calcium dichloride and 0.5 M sodium hydroxide) was added to the sample solution and boiled for 4 min. The cooled coloured solution was centrifuged at 2500 rev min^{-1} and the supernatant was measured spectrophotometrically at 415 nm.

2.5. Plant N analysis, microbial biomass and ^{14}C analysis

Aliquots of plant material (oven-dried at 60 °C) and soil (oven-dried at 105 °C) were ground (ball mill) prior to analysing radioactivity and total plant N (C-N Analyser,

Carlo Erba, Mönchengladbach, Germany). Samples of ground shoot and root samples (0.1 g) and soil material (rhizosphere and bulk soil: 0.5–1 g) were subjected to combustion analysis (Biological Oxidizer OV 500; Zinsser Instruments, Frankfurt/M., Germany); the scintillation cocktail Carbomax® was used to determine radioactivity by liquid scintillation counting (Tri Carb 2000 CA, Canberra Packard Co. Ltd, Frankfurt/M., Germany). ^{14}C in root exudates was determined in the extracts from the combined filter discs applied onto 6 apical root zones with the scintillation cocktail (Rothizint® 22 ×; Roth, Karlsruhe, Germany), with a mixture of 400 µl extract solution and 4 ml scintillation cocktail.

Microbial biomass C (C_{mic}) and N (N_{mic}) was determined by the chloroform-fumigation-extraction method (Vance et al., 1987). Microbial biomass was analysed in duplicate. Fumigated and non-fumigated rhizosphere soil samples (4 g) were extracted with 16 ml of 0.5 M K_2SO_4 on a horizontal shaker for 30 min at 250 rev min^{-1}. After shaking and centrifugation (30 min at 4560 rev min^{-1}), the concentrations of extractable C (E_C) and N (E_N) in aliquots of the supernatants were measured on a Dimatoc-100 TOC/TN analyser (Dimatec GmbH, Essen, Germany). The ^{14}C activity of another aliquot was determined by scintillation counting (scintillation cocktail Rothizint® eco plus, Roth, Karlsruhe, Germany), with a 1 ml aliquot of the K_2SO_4 extraction solution added to 10 ml of the scintillation cocktail. The additional extractable C and N, and ^{14}C counts obtained from the fumigated soils, were taken to represent the microbial C and N biomass. The conversion factors $k_{E_C} = 0.45$ (Joergensen, 1996) and $k_{E_N} = 0.54$ (Joergensen and Müller, 1996) were applied. The conversion factor 0.45 was also used to calculate ^{14}C in microbial biomass. All ^{14}C measurements were conducted with two replicates. The ^{14}C partitioning in shoot, root, rhizosphere soil, bulk soil, as well as in root exudates and in the microbial biomass in rhizosphere soil was calculated as a percentage of the total recovered ^{14}C in each plant soil system.

2.6. Extraction and analysis of phenolic compounds (flavonoids)

The phenolic compounds in germination filters were extracted by continuously shaking the filter paper rolls in 250 ml HPLC-grade methanol/ethyl acetate (1:1, v/v) for 15 h at 4 °C. After vacuum evaporation of the solvents, the extracts were resuspended in 1 ml 100% methanol.

The total phenolic content in extracts of the filter paper rolls was determined spectrophotometrically at 725 nm according to a modified method after Swain and Hillis (1959). To 350 µl sample (sample in methanol diluted 1:4 with distilled water), 25 µl of Folin and Ciocalteau's reagent was mixed. After 3 min, 50 µl of a saturated sodium carbonate solution (Na_2CO_3) and 75 µl distilled water were added, with subsequent mixing and an additional 15 min waiting period until a blue colour developed. The samples were centrifuged for 2 min at 12000 rev min^{-1} to separate precipitates from the solution. Absorbance at 725 nm was recorded immediately. Calibration standards were prepared with coumestrol. The average phenolic content of 4 filter paper rolls without plants (blank) was subtracted from the results.

For flavonoid analysis a 20 µl portion of the solution (diluted 1:2 with methanol) was injected into an HPLC system equipped with an Eurospher −100 °C18 column (250 × 3 mm, 5 µm, Knauer, Berlin, Germany) and separated at a flow rate of 500 µl min^{-1}. Gradient elution was performed by varying the proportion of solvent A [100% methanol] and solvent B [5% v/v acetic acid]. The sequence was 30–40% of solvent A during 10 min, 40–60% A during 10–55 min and 55–80% A during 55–60 min. The detection wavelength was set to 270 nm for the first 36 min and 360 nm for the remaining time. The flavonoids in root exudates were identified and quantified by comparing the retention times and peak areas with those of known standards identified as effective *nod*-gene inducers (Bolaños-Vásquez and Werner, 1997) in root exudates of *P. vulgaris*. For identification, wavelength scans and LC/MS analysis were also performed on a Hewlett Packard HPLC Model 1100, using a diode array detector (240–600 nm) and a solvent gradient consisting of a 0.01 M ammonium formate buffer (pH 4.0) [A] and 100% methanol [B]. A Phenomex Luna C18:1 (250 × 3 mm, 5 µm, Phenomenex Inc., Aschaffenburg, Germany) column was used for separation at 500 µl min^{-1} with gradient elution (min/% A): 0/70; 30/40; 35/30; 40/30. MS analysis of separated compounds was performed with a VG Platform II Quadropole electrospray mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray interface (ESI). Measurements were carried out in the ESI-mode (ESI-source temperature 120 °C). The capillary voltage was set to 3.5 kV, cone voltage to 40 V and HV lense voltage to 0.5 kV.

2.7. Statistical analysis

Plant biomass, N-nutritional status of the plants, microbial biomass, flavonoid and total phenol concentration were calculated on a dry weight basis (drying at 105 °C for soil samples and at 60 °C for plant material). Calculation of root exudation refers to the length of the apical root zones used for exudate collection, and calculation of the internal root tissue concentration of carboxylates refers to root fresh weight. The effect of CO_2, nitrogen availability and sampling time on the variables was quantified using factorial analysis of variance (ANOVA). The calculations were performed using the STATISTICA software package (Version 6.0, StatSoft®, Tulsa, USA). Discriminant function analysis was applied to assess the response of the root exudate variability to elevated CO_2, N availability and sampling time. The canonical scores for the significant roots (= axes) were correlated (Pearson correlation) with root exudate data to explain which root exudate compound

discriminates between the treatments. The Squared Mahanalobis distances were calculated to determine significant differences between group centroids. The discriminant analysis was performed using SPSS version 11 (SPSS Inc., Chicago, IL, USA). A statistical probability of $P<0.05$ was considered significant.

3. Results

3.1. Plant growth and N-nutritional status

There was no significant effect of elevated atmospheric CO_2 concentration on shoot and root biomass production of bean plants during germination in rolls of filter paper (data not shown) or in rhizobox culture, with exception of a weak increase in root biomass, mainly of N-sufficient plants at 12 days after sowing (DAS), and in shoot biomass at 21 DAS of soil-grown plants (Table 1). Nitrogen limitation led to reduced N concentrations in plant tissues of soil-grown plants, which was already detectable in early stages of plant development at 12 DAS (Table 1). Shoot nitrogen concentrations of plants with limited N supply declined below the threshold level for N deficiency of approximately $20\,mg\,g^{-1}$ shoot dry matter (Marschner, 1995) at 18 DAS, associated with limited aboveground plant biomass production. Elevated CO_2 accelerated the development of N deficiency symptoms, and N-deficient plants, grown under elevated CO_2, reached the threshold level for N deficiency already at 15 DAS (Table 1). Nitrogen concentrations in the shoot and root tissues declined under elevated CO_2, both in N-sufficient and -deficient plants (Table 1). In addition, total N content of the plants decreased in response to elevated CO_2 treatments at 15, 18 and 21 DAS (Table 1).

First nodules appeared at 18 DAS. Nodule formation was primarily confined to N-deficiency treatments, which were associated with significantly higher nodule biomass, nodule number (data not shown) and an increased fraction of leghaemoglobin-producing nodules in elevated CO_2 treatments (Figs. 2a and b).

3.2. Root exudation of sugars, carboxylates and amino acids

Sugars were the major class of low-molecular weight compounds in the root exudates released from apical root zones of *P. vulgaris*, followed by carboxylates (malate, malonate, citrate and fumarate) and total amino acids (Table 2). Three-factorial ANOVA using N, CO_2 and sampling date as factors showed that the stage of plant development as well as N supply interacted with CO_2 effects (Table 3b). At 12 DAS, a CO_2-mediated increased sugar exudation was restricted to the N-deficiency treatment (Tables 2 and 3b). This effect disappeared at later stages of plant growth. Malate exudation of N-deficient plants increased due to elevated CO_2 at 18 DAS (Table 2 and 3b). Under limited N supply, the highest carboxylate exudation was detected during early stages of the observation period (12–15 DAS). Malonate exudation of N-deficient plants increased at later stages of plant growth at 18 and 21 DAS (Table 2). In later stages of plant growth (18 DAS), an N-deficiency-induced decline of amino acid exudation was detected, but remained unaffected by elevation of CO_2. Bean plants grown under high N supply increased total sugar and carboxylate exudation in the apical root zones with increasing plant age.

Discriminant analysis (Fig. 1) including all root exudate data from the 12 and 18 DAS sampling dates yielded four axes, which added significantly to the discrimination between groups (Table 3a). The high Pearson correlation coefficients of malonate and malate revealed that these carboxylates were mainly responsible for the separation along axis 1. DF1 explained 64% of the variation within the root exudation data set, and DF2 contributed an additional 21%. Along axis 2, the elevated CO_2, low-N treatment at an early state of plant growth (12 DAS) was significantly separated from the other treatments. The separation along axis 2 was mainly caused by sugar exudation.

3.3. Belowground ^{14}C partitioning and rhizosphere-microbial biomass

One day after $^{14}CO_2$ labelling (DAL = 15 DAS), plants limited in nitrogen availability released higher amounts of assimilated ^{14}C into the apical root zone compared with plants grown under high N supply ($F_{1,20}=4.71$, $P<0.05$; Table 4). In addition, more ^{14}C was incorporated into the microbial biomass at 4 DAL (18 DAS) under N deficiency ($F_{1,20}=26.88$, $P<0.001$). Elevated CO_2 significantly increased ^{14}C exudation in the apical root zones of bean plants grown under N deficiency at 4 DAL ($N\times CO_2$: $F_{1,20}=4.36$, $P<0.05$).

In contrast, there was no effect of elevated CO_2 on ^{14}C allocation into the rhizosphere soil, on ^{14}C incorporation into the rhizosphere-microbial biomass, or on C_{mic} in the rhizosphere soil collected from the whole root system (Table 4). Elevated CO_2 temporarily increased N_{mic} in the rhizosphere soil at 15 DAS only in the N-deficiency treatments ($N\times CO_2$: $F_{1,20}=4.46$, $P<0.05$). With increasing N deficiency at later stages of plant growth (21 DAS), N_{mic} ($F_{1,20}=5.80$, $P<0.05$) and C_{mic} ($F_{1,20}=22.68$, $P<0.001$) declined in comparison to high N supply treatments.

3.4. Carboxylate concentrations in root tissue

With increasing plant age, internal carboxylate concentrations increased (Table 5). At the beginning of the observation period at 12 DAS, apical root tissue (0.5–1.5 cm) of bean plants grown under N limitation contained lower concentrations of malate ($F_{1,20}=5.51$, $P<0.05$), citrate ($F_{1,20}=5.91$, $P<0.05$) and fumarate ($F_{1,20}=11.47$, $P<0.01$) compared with plants grown under high N supply. In contrast, at later stages of N

Table 1
Shoot and root biomass (plant dry matter g) and N-nutritional status of *Phaseolus vulgaris* grown in rhizoboxes, depending on plant age (DAS = days after sowing), N supply and atmospheric CO_2 concentration

DAS	CO_2 ($\mu mol\,mol^{-1}$)	N	Shoot DW (g)		Root DW (g)		Shoot N concentration ($mg\,g^{-1}$)		Root N concentration ($mg\,g^{-1}$)		Plant N content ($mg\,plant^{-1}$)	
12	400	+	0.31 (0.013)		0.11 (0.007)		54.64 (1.08)		31.94 (0.92)		20.42 (0.99)	
	800	+	0.32 (0.018)		0.13 (0.013)		44.85 (0.80)		28.28 (0.52)		18.19 (1.14)	
	400	−	0.28 (0.008)		0.10 (0.007)		44.95 (3.61)		29.28 (0.33)		15.43 (1.13)	
	800	−	0.31 (0.006)		0.11 (0.004)		39.68 (1.10)		27.38 (0.27)		15.25 (0.55)	
		Statistics	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
		N	4.27	NS	0.57	NS	13.75	**	9.71	**	16.36	***
		CO_2	2.93	NS	4.95	*	14.12	**	23.66	***	1.51	NS
		$N \times CO_2$	0.57	NS	0.15	NS	1.27	NS	2.39	NS	1.10	NS
15	400	+	0.44 (0.027)		0.20 (0.016)		47.67 (1.74)		24.36 (0.57)		25.53 (0.86)	
	800	+	0.51 (0.031)		0.24 (0.005)		37.00 (2.03)		20.71 (0.46)		23.50 (0.37)	
	400	−	0.40 (0.020)		0.20 (0.007)		29.50 (1.59)		19.46 (0.27)		15.74 (0.87)	
	800	−	0.44 (0.044)		0.22 (0.023)		20.19 (1.02)		17.84 (0.30)		12.93 (1.34)	
		Statistics	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
		N	3.41	NS	0.50	NS	113.98	***	85.83	***	121.50	***
		CO_2	2.33	NS	4.18	NS	37.18	***	39.50	***	6.88	*
		$N \times CO_2$	0.23	NS	0.14	NS	0.17	NS	5.87	*	0.18	NS
18	400	+	0.72 (0.051)		0.27 (0.034)		37.71 (1.98)		22.80 (0.75)		32.74 (1.71)	
	800	+	0.80 (0.038)		0.35 (0.031)		28.88 (1.41)		19.60 (0.36)		29.79 (1.62)	
	400	−	0.57 (0.034)		0.26 (0.021)		16.44 (0.63)		15.77 (0.40)		13.54 (0.82)	
	800	−	0.63 (0.040)		0.28 (0.040)		10.85 (0.50)		13.55 (0.39)		10.78 (1.13)	
		Statistics	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
		N	13.87	***	1.12	NS	351.01	***	168.81	***	195.41	***
		CO_2	3.47	NS	2.40	NS	31.68	***	29.03	***	4.36	*
		$N \times CO_2$	0.04	NS	1.00	NS	1.60	NS	0.94	NS	0.01	NS
21	400	+	0.88 (0.027)		0.46 (0.018)		28.81 (1.03)		20.62 (0.30)		34.68 (0.72)	
	800	+	0.98 (0.047)		0.49 (0.028)		24.79 (0.76)		18.00 (0.53)		33.27 (1.22)	
	400	−	0.77 (0.020)		0.36 (0.015)		10.70 (0.23)		13.14 (0.33)		12.94 (0.24)	
	800	−	0.86 (0.065)		0.37 (0.024)		7.75 (0.28)		10.58 (0.30)		10.74 (0.75)	
		Statistics	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*	*F*-value	*P*
		N	6.57	**	25.20	***	698.49	***	391.89	***	743.53	***
		CO_2	5.66	*	1.42	NS	27.52	***	47.49	***	4.95	*
		$N \times CO_2$	0.01	NS	0.11	NS	0.43	NS	0.01	NS	0.24	NS

Effects of N and CO_2 treatment were estimated by two-factorial ANOVA for each single harvest time.
Numbers in parenthesis represent standard error ($n = 6$). Given are the *F*-values and level of significance. NS = not significant, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Table 2
Root exudation of carboxylates (malate, malonate, citrate and fumarate), total sugars and total amino acids in 0.5–1.5 cm apical root zones (first-order lateral roots) of *Phaseolus vulgaris* grown in rhizoboxes, depending on plant age (DAS = days after sowing), N supply and atmospheric CO_2 concentration

DAS	CO_2 (μmol mol^{-1})	N	Malate[a]	Malonate[a]	Citrate[a]	Fumarate[a]	Total sugars[a]	Total amino acids[a]
12	400	+	42.7 (15.8)	0	2.2 (0.5)	3.2 (0.8)	148.3 (7.7)	53.0 (1.8)
	800	+	18.6 (7.8)	0	1.9 (0.8)	2.0 (0.4)	135.1 (10.6)	43.9 (10.2)
	400	−	83.2 (10.6)	0	6.5 (1.4)	4.9 (0.7)	134.7 (26.2)	60.3 (5.0)
	800	−	97.6 (19.4)	0	5.9 (1.1)	5.4 (1.1)	315.3 (28.5)	50.1 (7.8)
15	400	+	28.8 (12.4)	3.0 (3.1)	2.8 (0.4)	1.9 (0.6)	ND	ND
	800	+	31.1 (14.9)	0	3.3 (0.6)	2.2 (0.8)	ND	ND
	400	−	87.4 (19.7)	5.9 (5.6)	3.5 (1.2)	5.2 (1.5)	ND	ND
	800	−	105.6 (20.7)	14.7 (2.2)	6.3 (2.1)	5.0 (1.0)	ND	ND
18	400	+	58.2 (13.2)	0.5 (0.5)	9.2 (1.8)	5.3 (1.5)	256.9 (17.8)	79.8 (12.4)
	800	+	75.5 (10.5)	2.0 (2.1)	9.7 (1.9)	5.1 (0.7)	270.3 (12.3)	53.1 (8.5)
	400	−	64.4 (14.0)	31.2 (9.4)	3.7 (0.7)	4.2 (1.5)	224.5 (25.9)	34.2 (13.4)
	800	−	160.6 (39.1)	60.8 (15.5)	6.7 (1.9)	5.8 (1.7)	258.6 (34.7)	42.2 (11.5)
21	400	+	76.9 (12.0)	16.3 (3.5)	11.1 (0.7)	4.9 (0.6)	ND	ND
	800	+	91.6 (27.5)	10.2 (1.9)	9.8 (1.3)	4.8 (1.8)	ND	ND
	400	−	98.0 (22.5)	89.8 (24.3)	7.6 (2.6)	4.8 (1.1)	ND	ND
	800	−	102.6 (24.5)	84.6 (13.3)	9.0 (1.9)	3.8 (1.4)	ND	ND

Numbers in parenthesis represent standard error ($n = 6$). ND = not determined.
[a]nmol m^{-1} apical root length h^{-1}.

Table 3
Results of discriminant analysis (a) and three-factorial ANOVA (b) (factors: N, CO_2 and sampling date) including root exudation in apical root zones (carboxylates: malate, malonate, citrate, fumarate; total sugars and total amino acids) of *Phaseolus vulgaris* grown in rhizoboxes at 12 and 18 DAS (days after sowing)

(a) Discriminant function	DF1	DF2	DF3	DF4	DF5	DF6
Wilks'Lambda	0.017	0.116	0.337	0.583	0.891	0.977
Eigenvalue	5.709	1.902	0.732	0.529	0.096	0.024
Degrees of freedom	42	30	20	12	6	2
Percentage of variance	63.5	21.2	8.1	5.9	1.1	0.3
Significance of the axis	***	***	**	*	NS	NS
Canonical correlation coefficient	0.922	0.81	0.65	0.588	0.296	0.152
Pearson correlation coefficients[#]						
Malate	0.4546**	0.5229 ***	0.0881 NS	−0.7151***		
Malonate	0.7812***	0.3186 *	0.4272 **	−0.1881NS		
Citrate	−0.3130*	0.3451 *	0.5715 ***	−0.6493***		
Fumarate	−0.0188 NS	0.3579 *	0.1979 NS	−0.4400**		
Total sugars	−0.1339 NS	0.9324 ***	0.3208 *	−0.0115NS		
Total amino acids	−0.4181**	−0.0986 NS	0.1975 NS	−0.3198*		

	Malate		Malonate		Citrate		Fumarate		Total sugars		Total amino acids	
(b) Anova	*F value*	*P*	*F value*	*P*	*F value*	*P*	*F value*	*P*	*F value*	*P*	*F value*	*P*
N	15.84	***	24.06	***	0.01	NS	2.25	NS	3.74	NS	2.52	NS
CO_2	3.82	NS	2.91	NS	0.43	NS	0.05	NS	11.52	**	1.95	NS
Sampling date	4.85	*	26.83	***	11.01	**	2.29	NS	19.09	***	0.01	NS
N × CO_2	4.90	*	2.38	NS	0.33	NS	1.22	NS	11.45	**	1.55	NS
N × sampling date	0.28	NS	24.06	***	18.79	***	2.78	NS	11.07	**	6.68	*
CO_2 × sampling date	5.41	*	2.92	NS	1.25	NS	0.43	NS	3.58	NS	0.00	NS
N × CO_2 × sampling date	0.58	NS	2.38	NS	0.57	NS	0.00	NS	7.47	**	1.74	NS

(a) Discriminant function: [#]Denotes the correlation between the discriminating variables and the scores of each discriminant function.
(b) Factorial ANOVA: Given are the *F*-values and level of significance. NS = not significant, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

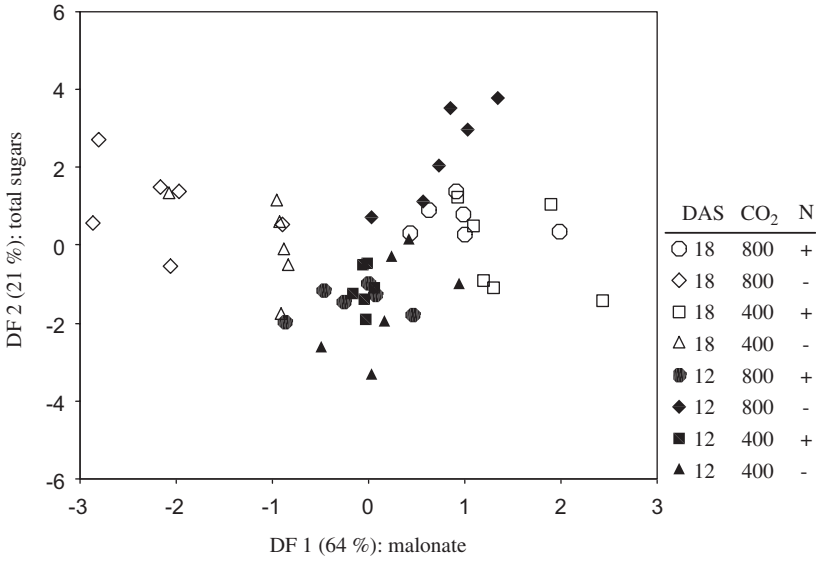

Fig. 1. Discriminant analysis including root exudation in apical root zones (carboxylates: malate, malonate, citrate, fumarate; total sugars and total amino acids) of *Phaseolus vulgaris* grown in rhizoboxes at 12 and 18 days after sowing (DAS), depending on plant age, N supply and atmospheric CO_2 concentration ($\mu mol\,mol^{-1}$). The labels of the two axes (DF1 and DF2) give the percentage of explained variance as well as the most important factor responsible for the separation of the data along the corresponding axis.

Table 4
^{14}C partitioning to root exudates of the apical root zone, to the rhizosphere soil, to rhizosphere-microbial biomass, and microbial C and N in rhizosphere soil collected from the whole root system of *Phaseolus vulgaris* grown in rhizoboxes, depending on plant age (DAS = days after sowing; DAL = days after $^{14}CO_2$ labelling), N supply and atmospheric CO_2 concentration

DAS	DAL	CO_2 ($\mu mol\,mol^{-1}$)	N	^{14}C exudation (cm^{-1} apical root zone) (% of total recovered $^{14}CO_2$)	Rhizosphere soil				
					^{14}C (% of total recovered $^{14}CO_2$)	$^{14}C_{mic}$ (% of total recovered $^{14}CO_2$)	C_{mic} ($\mu g\,g^{-1}$ soil DW)	N_{mic} ($\mu g\,g^{-1}$ soil DW)	C_{mic} to N_{mic} ratio
15	1	400	+	3.1×10^{-4} (6.9×10^{-5})	4.4 (0.60)	0.22 (0.048)	65.1 (3.4)	20.0 (2.5)	3.6 (0.6)
		800	+	4.2×10^{-4} (8.5×10^{-5})	3.8 (0.39)	0.23 (0.055)	65.4 (4.4)	16.3 (1.6)	4.2 (0.5)
		400	–	6.9×10^{-4} (2.1×10^{-4})	4.7 (0.70)	0.30 (0.045)	62.2 (3.8)	9.9 (0.2)	6.3 (0.4)
		800	–	6.4×10^{-4} (1.4×10^{-4})	4.7 (0.72)	0.28 (0.053)	61.1 (5.8)	14.8 (2.1)	4.5 (0.6)
18	4	400	+	2.9×10^{-4} (3.4×10^{-5})	2.4 (0.50)	0.28 (0.019)	82.0 (6.3)	12.4 (0.7)	6.6 (0.4)
		800	+	2.9×10^{-4} (7.8×10^{-5})	2.3 (0.75)	0.24 (0.046)	75.3 (4.9)	12.7 (0.5)	6.0 (0.4)
		400	–	3.0×10^{-4} (2.5×10^{-5})	3.5 (0.43)	0.50 (0.051)	60.2 (7.4)	11.0 (0.7)	5.4 (0.5)
		800	–	5.5×10^{-4} (9.0×10^{-5})	2.9 (0.42)	0.42 (0.041)	64.8 (5.4)	11.7 (0.4)	5.6 (0.7)
21	7	400	+	1.8×10^{-4} (4.4×10^{-5})	2.1 (0.33)	0.23 (0.044)	99.5 (6.4)	11.8 (0.6)	8.5 (0.4)
		800	+	1.2×10^{-4} (1.8×10^{-5})	2.4 (0.30)	0.21 (0.035)	92.3 (6.4)	10.2 (0.7)	9.0 (0.4)
		400	–	1.9×10^{-4} (4.5×10^{-5})	2.1 (0.28)	0.29 (0.024)	70.0 (4.1)	9.3 (0.9)	7.8 (0.6)
		800	–	2.2×10^{-4} (3.6×10^{-5})	2.1 (0.40)	0.25 (0.030)	70.2 (4.3)	9.2 (0.8)	7.8 (0.4)

Numbers in parenthesis represent standard error ($n = 6$).

deficiency the concentrations of malate (18 DAS: $F_{1,20} = 5.49$, $P < 0.05$) and malonate (18 DAS: $F_{1,20} = 17.3$, $P < 0.001$; 21 DAS: $F_{1,20} = 6.80$, $P < 0.05$) increased in the apical root tissue.

Under N limitation, there was a trend for increased carboxylate accumulation in apical root tissue of plants grown under elevated CO_2 and malate as well as malonate accumulation in nodule tissue was significantly increased (Figs. 2c and d).

3.5. Root exudation of phenolic compounds and signal flavonoids

The effect of elevated CO_2 on release of phenolic compounds was detected in bean seedlings during germination in filter paper rolls. The concentration of total phenolics in root exudates was significantly increased up to 167% in response to elevation of atmospheric CO_2 concentration (Fig. 4). This effect already appeared at early

Table 5
Carboxylate concentration (malate, malonate, citrate, fumarate) in apical lateral root tissue of *Phaseolus vulgaris* grown in rhizoboxes, depending on plant age (DAS = days after sowing), N supply and atmospheric CO_2 concentration

DAS	CO_2 ($\mu mol\,mol^{-1}$)	N	Malate ($\mu mol\,g^{-1}$ root FW)	Malonate ($\mu mol\,g^{-1}$ root FW)	Citrate ($\mu mol\,g^{-1}$ root FW)	Fumarate ($\mu mol\,g^{-1}$ root FW)
12	400	+	1.19 (0.39)	0.05 (0.03)	0.53 (0.10)	0.007 (0.002)
	800	+	0.74 (0.18)	0.04 (0.02)	0.66 (0.23)	0.007 (0.001)
	400	−	0.45 (0.15)	0.09 (0.03)	0.33 (0.04)	0.002 (0.001)
	800	−	0.39 (0.09)	0.07 (0.02)	0.24 (0.04)	0.003 (0.001)
15	400	+	0.52 (0.08)	0.08 (0.06)	0.43 (0.08)	0.005 (0.002)
	800	+	0.79 (0.19)	0.10 (0.03)	0.53 (0.07)	0.009 (0.003)
	400	−	0.79 (0.24)	0.09 (0.04)	0.32 (0.03)	0.004 (0.002)
	800	−	1.35 (0.43)	0.28 (0.08)	0.40 (0.09)	0.007 (0.003)
18	400	+	1.99 (0.46)	0.28 (0.07)	0.66 (0.04)	0.017 (0.005)
	800	+	1.69 (0.24)	0.24 (0.02)	0.96 (0.23)	0.014 (0.003)
	400	−	2.58 (0.66)	0.73 (0.19)	0.64 (0.13)	0.015 (0.004)
	800	−	3.38 (0.48)	0.74 (0.10)	0.85 (0.22)	0.018 (0.003)
21	400	+	3.67 (1.10)	0.67 (0.15)	1.45 (0.31)	0.027 (0.008)
	800	+	3.74 (0.94)	0.73 (0.23)	2.46 (0.76)	0.040 (0.011)
	400	−	3.42 (0.60)	1.17 (0.23)	0.87 (0.24)	0.026 (0.005)
	800	−	4.13 (0.93)	1.62 (0.44)	0.99 (0.16)	0.031 (0.008)

Numbers in parenthesis represent standard error ($n = 6$).

stages of seedling development (6 DAS) during unfolding of the primary leaves and increased up to 10 DAS. In contrast, under ambient CO_2, exudation of total phenolics was not altered between 6 and 12 DAS.

Single phenolics in the root exudates were identified after HPLC separation by recording of absorption spectra in comparison with known standards and by LC–MS analysis (Figs. 3a–c). The blue-fluorescent flavonoid coumestrol was identified as the major phenolic compound in the root exudates of bean seedlings (Fig. 4). Coumestrol was released along the whole root system, and exudation was also detectable in soil-grown plants after 20 h-application of nylon membranes onto the root surface, by induction of the blue autofluorescence of coumestrol bound at the membrane surface under UV light of 360 nm (Fig. 3d). The isoflavonoids genistein, daidzein and isoliquiritigenin have been identified as important *nod*-gene inducers in the root exudates of *P. vulgaris* seedlings (Bolaños-Vásquez and Werner, 1997).

Elevated atmospheric CO_2 concentration stimulated exudation of coumestrol, genistein and daidzein already at early stages of seedling growth (6–12 DAS).

4. Discussion

4.1. Plant growth, N-nutritional status and C allocation within the plant rhizosphere soil system

The responses of shoot and root biomass to elevation of CO_2 depend not only on plant species, but also on the plant-nutritional status. In the present rhizobox experiment, *P. vulgaris* did not show significant alterations of above- and below-ground biomass production under elevation of atmospheric CO_2 (Table 1). This result confirms earlier reports on CO_2 reponses of *P. vulgaris* (Salsman et al., 1999), whereas growth of other members of the genus *Phaseolus* and of many other plant species is stimulated under elevated CO_2 (Hodge and Millard, 1998; Salsman et al., 1999). Greater starch accumulation instead of investments in plant growth, or increased carbon losses by respiration and/or root exudation, have been discussed as possible causes for the absence of growth responses to elevated CO_2 in some plant species (Drake et al., 1997; Cheng and Johnson, 1998).

Despite the absence of growth-stimulating effects, elevated CO_2 accelerated the development of N-deficiency symptoms in *Phaseolus* beans with limited N supply: growth limitation and the deficiency threshold of shoot N concentrations ($20\,mg\,N\,g^{-1}$ DM) was reached three days earlier (15 DAS) in plants grown under elevated CO_2 compared with control plants (18 DAS) (Table 1). Moreover, the total N content of plants treated with elevated CO_2 fell by approximately 20%.

$^{14}CO_2$ pulse-chase labelling, however, revealed no increase in overall root exudation of ^{14}C-labelled compounds or in ^{14}C incorporation into rhizosphere microorganisms under elevated atmospheric CO_2 concentrations (Table 4). Accordingly, no stimulation of rhizosphere-microbial biomass production was detected. These results contrast with earlier studies showing strongly enhanced root-derived C allocation towards microbial biomass (van Ginkel et al., 2000; Allard et al., 2006), which has been discussed as possible cause for N limitation of plants by increased plant-microbial N competition (Diaz et al., 1993). However, the increase we recorded in rhizosphere-microbial N (N_{mic}) due to CO_2 elevation at 15 DAS

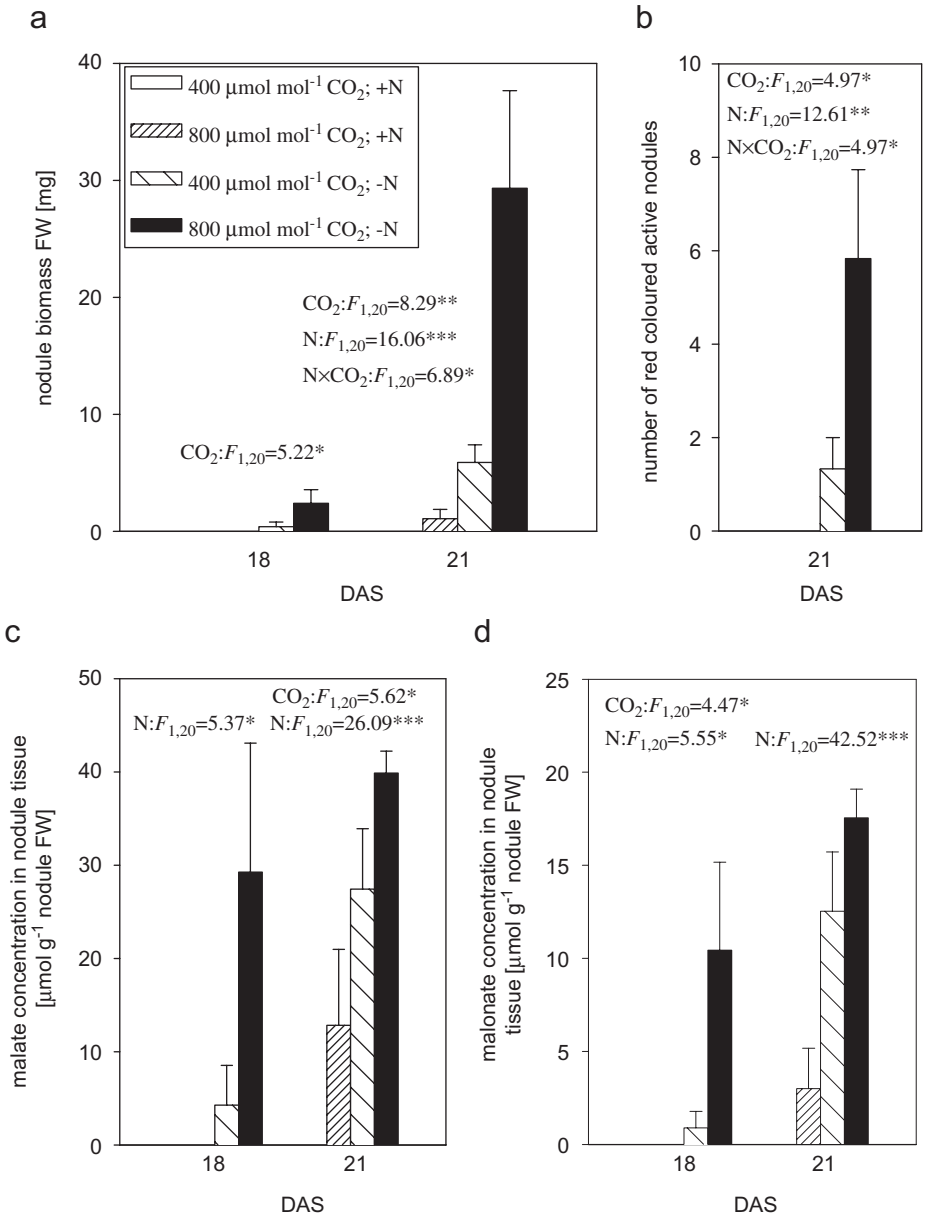

Fig. 2. Nodule biomass (a), number of red, active nodules (b) (containing leghaemoglobin), malate (c) and malonate (d) concentration in nodule tissue of *Phaseolus vulgaris* grown in rhizoboxes, depending on plant age (DAS = days after sowing), N supply and atmospheric CO_2 concentration. Presented are means and standard errors. Significant effects of the factors N and CO_2 are presented as *F*-values and level of significance ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$) estimated by a two-factorial ANOVA for each single harvest time.

indicates at least a short-term-enhanced sequestration of N by the rhizosphere-microorganisms under conditions of low N supply.

The N-nutritional status of the plants seemed to be more important for rhizosphere-microbial abundance than the CO_2 treatments. Four days after labelling (18 DAS), N deficiency increased the fraction of root-derived C allocation towards microbial biomass in the rhizosphere. The observed pattern of ^{14}C partitioning is consistent with the data reported by Bazot et al. (2006) for spring sampling of rye grass (2 DAL). This indicates that high N supply stimulated the allocation of recently assimilated C to shoots (data not shown) and reduced belowground-translocation, whereas elevated CO_2 had no effect.

4.2. Root exudation in apical root zones

Since the proportion of root-derived carbon in rhizosphere soil collected from the whole root system was unaffected by CO_2 treatments, we also investigated the localised accumulation of root exudates in 0.5–1.5 cm apical root zones of first-order lateral roots, with young root hairs as the major sites for attraction and root infection by the microsymbiont (Werner, 2000). Effects of

elevated CO_2 on root exudation were restricted to apical root zones of N-deficient bean plants (Tables 2, 3, Fig. 1). A greater proportion of assimilated ^{14}C in root exudates collected at 4 DAL = 18 DAS (Table 4), as well as the increased root exudation of total sugars (12 DAS) and of malate as the major organic acid (18 DAS) (Table 2), suggest that, under N limitation, a higher proportion of the additional photosynthates assimilated at elevated CO_2 was allocated to apical root zones. Since the exudation of total amino acids remained unchanged (Table 2), the low-molecular weight fraction of exuded compounds may exhibit a shift to higher C/N ratios under elevated CO_2 concentrations, which has been similarly reported by Grayston et al. (1998).

However, nitrogen fertilisation effects on root exudation in apical root zones were more pronounced than CO_2 effects. One day after labelling (15 DAS), the amount of assimilated ^{14}C in exudates collected from apical root zones was enhanced in the variants with limited N supply. This was also observed for carboxylate exudation during early stages of N limitation (12–18 DAS), with malate, malonate and citrate as major carboxylates. Particularly, malate is well known as a moderately potent chemo-attractant for rhizobia in the rhizosphere and it is the major carbon source for bacteriods (Yurgel and Kahn, 2004), whereas antimicrobial functions against pathogens and herbivory have been discussed for the succinate dehydrogenase inhibitor malonate (Li and Copeland, 2000). Since plants can retrieve released amino acids (Jones et al., 2004), higher re-absorption may have contributed to lower amino acid concentrations in root exudates of N-deficient plants.

With increasing plant age, high N supply enhanced the root exudation of carboxylates, total sugars and total amino acids (Table 2); it also increased the internal carboxylate concentration of the apical root tissue (Table 5). Similarly, Henry et al. (2005) reported that high N fertilisation increased C release from roots of five-weeks old *Lolium multiflorum*.

4.3. Nodule formation and carbon supply to the micro-symbiont

Elevated CO_2 induced a significantly higher number, biomass and an elevated fraction of leghaemoglobin-producing nodules. This reflects promoted *Rhizobium*–plant interactions, particularly in the N-deficient bean plants (Fig. 2). Similar results were reported by Zanetti et al. (1996), Schortemeyer et al. (2002) and Feng et al. (2004). Marilley et al. (1999) and Hartwig et al. (2000) assumed that the resulting increased symbiotic nitrogen fixation can counterbalance CO_2-induced N limitation of

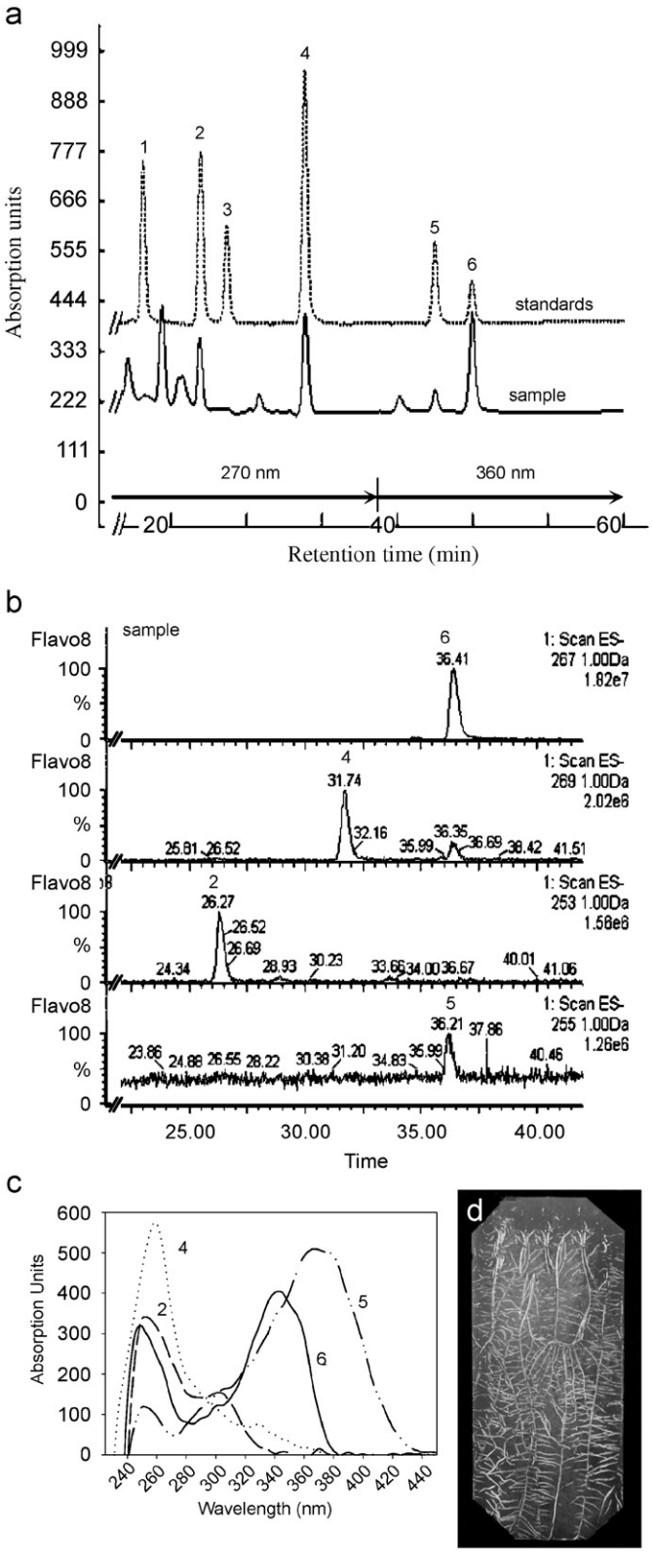

Fig. 3. High-performance liquid chromatography (HPLC) profile (a), liquid chromatography-electrospray ionization-mass spectrometry (LC–ESI–MS) $[M–H]^-$ ion (b), UV spectra (c) of major phenolic compounds (*nod*-gene-inducing flavonoids: 1 liquiritigenin, 2 daidzein, 3 naringenin, 4 genistein, 5 isoliquiritigenin, 6 coumestrol) in root exudates of *Phaseolus vulgaris* seedlings grown in filter paper 6 DAS (days after sowing). Fluorescent flavonoid root exudates collected (d) from soil-grown *Phaseolus vulgaris* in rhizoboxes by 20 h-application of nylon membranes onto the root surface. Blue auto-fluorescence of coumestrol (picture presented in grey scales, blue auto-fluorescence = white-coloured areas) induced by UV 360 nm.

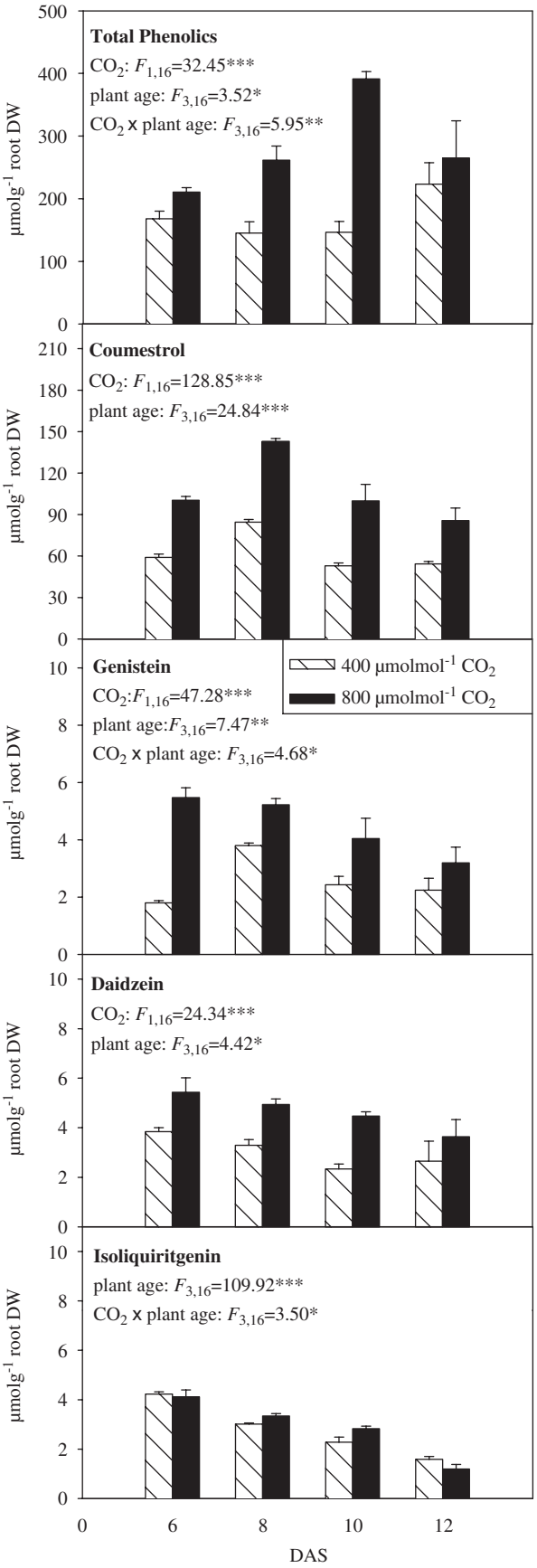

Fig. 4. Root exudation of total phenolics and *nod*-gene-inducing flavonoids (coumestrol, genistein, daidzein, isoliquiritigenin) in response to elevated CO_2 and plant age of *Phaseolus vulgaris* seedlings germinating in filter papers. Presented are means and standard errors. Significant effects of the factors CO_2 and plant age (DAS = days after sowing) are presented as *F*-values and level of significance ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$) estimated by a two-factorial ANOVA.

Trifolium repens. In the present study, however, the relative contribution of symbiotically fixed N to the total plant nitrogen under elevated CO_2 is expected to be negligible because only the early stages of nodule development were covered during the 3-week culture period. Moreover, *P. vulgaris* is considered as a legume species with poor performance of symbiotic nitrogen fixation (Piha and Munns, 1987).

Several mechanisms may explain the increased nodule formation under elevated CO_2: (1) an altered number of infection sites, (2) changes in carbohydrate supply to the micro-symbiont, and (3) increased root exudation of signal compounds.

In this study, root biomass production was not affected by elevated CO_2 (Table 1). Nonetheless, an increase of possible infection sites, such as root hairs and lateral root tips, or alterations in root hair development, cannot be excluded because root morphology was not investigated. However, enhanced malate exudation of N-deficient plants at elevated CO_2 (Table 2) and higher malate accumulation in nodules (Fig. 2c)—which also occurred to a lesser extent in the apical root tissue (Table 5)—may reflect an improved supply of nodules with carboxylates. Our findings agree with those of Cabrerizo et al. (2001), who observed higher malate, sucrose and starch concentrations in nodules. This suggests that carbon flux increases in nodules grown at enriched CO_2 concentrations. We also provide the first report on strongly increased malonate accumulation in nodules of N-limited plants exposed to elevated CO_2 (Fig. 2d). Although the exact mechanism of malonate action in nodules during symbiotic N_2 fixation remains unclear (Kim and Kang, 1994; Li and Copeland, 2000), recent findings suggest that *R. leguminosarum* bv. *Trifolii* mutants, lacking enzymes for malonate metabolization, lost the ability to establish a functional symbiosis with *Trifolium* (Kim, 2002). This points to an essential function of malonate in symbiotic N_2 fixation. The role of CO_2-induced alterations in exudation and intracellular accumulation of malonate remains to be established.

4.4. Root exudation of phenolic signal compounds for the plant–Rhizobia symbiosis

An alternative explanation for the increased nodule biomass under elevated CO_2 was hypothesised in a previous study of Marilley et al. (1999), assuming increased root exudation of phenolic signals under CO_2 enrichment, involved in the establishment of the *Rhizobium* symbiosis. Montealegre et al. (2000) also linked elevated

CO_2-mediated changes in the genetic structure of *R. leguminosarum* populations isolated from root nodules to altered chemical signals released into the soil by clover roots. Accordingly, the present study provides the first report on CO_2-stimulated root exudation of total phenolics and of *nod*-gene-inducing flavonoids (genistein, daidzein and coumestrol) in a leguminous plant (*P. vulgaris*, Figs. 3 and 4). The major compound was coumestrol, which was released over the whole root system (Fig. 3d) and may additionally function as an antimicrobial compound (Dakora and Phillips, 1996; Bolaños-Vásquez and Werner, 1997; Werner, 2000). Surprisingly, the CO_2 effect was already detectable during germination of very young seedlings (6 DAS) with emerging primary leaves, even prior to transfer into soil culture and independent of N treatments, which were applied only in soil culture (Fig. 4). Since different groups of phenolics were influenced in a similar manner, these findings suggest that the elevated phenolic exudation may not primarily be related with adaptive responses to N limitation. Excess intracellular accumulation of carbohydrates in the young seedlings without N supply, by stimulation of photosynthesis under elevated CO_2 in combination with high seed reserves, may stimulate existing biosynthetic pathways for the various phenolic compounds associated with increased root exudation. Increased intracellular accumulation of phenolics as drainage for excess carbon has been frequently reported as a response to high sugar concentrations in growth media (Yamakawa et al., 1983), limitation of N and P (Chishaki and Horiguchi, 1997; Plaxton, 1998), anthocyanin formation in autumn leaves (Ford, 1986), and also under elevated atmospheric CO_2 concentrations (Castells et al., 2002; Peltonen et al., 2005). Increased root exudation of signal flavonoids under elevated CO_2 may, therefore, be regarded as an indirect response with potential stimulatory effects on N_2 fixation. Exogenous application of signal flavonoids by seed dressing to increase symbiotic N_2 fixation is already used in commercial fertilizer preparations (Agri-Biotics Inc. Canada). Clearly, a stimulating effect of increased flavonoid exudation remains to be established in future studies.

5. Conclusions

In contrast to the initial hypothesis, assuming that elevated atmospheric CO_2 concentrations increased plant-microbial N-competition in the rhizosphere due to enhanced root exudation with a subsequent stimulated microbial growth, in the present study there was no indication for a general increase in total root exudation. Our results suggest a more selective stimulation of factors involved in establishing and maintaining the rhizobium symbiosis at elevated CO_2. This was expressed in increased exudation of signal flavonoids, elevated root exudation and increased tissue concentration of carboxylates in apical root zones and in nodules. The relative importance of these factors in stimulating symbiotic N_2 fixation, along with a potential contribution of altered root morphology, remains to be established. Further studies are necessary to investigate whether similar responses can also be expected in other leguminous plant species and under different soil conditions.

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References

Allard, V., Robin, C., Newton, P.C.D., Lieffering, M., Soussana, J.F., 2006. Short and long-term effects of elevated CO_2 on *Lolium perenne* rhizodeposition and its consequences on soil organic matter turnover and plant N yield. Soil Biology & Biochemistry 36, 1178–1187.

Bazot, S., Ulff, L., Blum, H., Nguyen, C., Robin, C., 2006. Effects of elevated CO_2 concentration on rhizodeposition from *Lolium perenne* grown on soil exposed to 9 years of CO_2 enrichment. Soil Biology & Biochemistry 38, 729–736.

Blakeney, A.B., Mutton, L.L., 1980. A simple colorimetric method for the determination of sugars in fruit and vegetables. Journal of the Science of Food and Agriculture 31, 889–897.

Bolaños-Vásquez, M.C., Werner, D., 1997. Effects of *Rhizobium tropici*, *R. etli*, and *R. leguminosarum* bv. *phaseoli* on *nod* gene-inducing flavonoids in root exudates of *Phaseolus vulgaris*. Molecular Plant-Microbe Interactions 10 (3), 339–346.

Cabrerizo, P.M., González, E.M., Aparicio-Tejo, P.M., Aresse-Igor, C., 2001. Continuous CO_2 enrichment leads to increased nodule biomass, carbon availability to nodules and activity of carbon-metabolising enzymes but does not enhance specific nitrogen fixation in pea. Physiologia Plantarum 113, 33–40.

Castells, E., Roumet, C., Penuelas, J., Roy, J., 2002. Intraspecific variability of phenolic concentrations and their responses to elevated CO_2 in two Mediterranean perennial grasses. Environmental and Experimental Botany 47, 205–216.

Cheng, W., Johnson, D.W., 1998. Elevated CO_2, rhizosphere processes, and soil organic matter decomposition. Plant and Soil 202, 167–174.

Chishaki, N., Horiguchi, T., 1997. Responses of secondary metabolism in plants to nutrient deficiency. Soil Science and Plant Nutrition 43, 987–991.

Dakora, F.D., Phillips, D.A., 1996. Diverse functions of isoflavonoids in legumes transcend anti-microbial definitions of phytoalexins. Physiological and Molecular Plant Pathology 49, 1–20.

Diaz, S., Grime, J.P., Harris, J., McPherson, E., 1993. Evidence of a feedback mechanism limiting plant response to elevated carbon dioxide. Nature 364, 616–617.

Drake, B.G., Gonzàlez-Meler, M.A., Long, S.P., 1997. More efficient plants: a consequence of rising atmospheric CO_2? Annual Review of Plant Physiology and Plant Molecular Biology 48, 609–639.

Feng, Z., Dyckmans, J., Flessa, H., 2004. Effects of elevated carbon dioxide concentration on growth and N_2 fixation of young *Robinia pseudoacacia*. Tree Physiology 24, 323–330.

Ford, B.J., 1986. Even plants excrete. Nature 323, 763.

Grayston, S.J., Campbell, C.D., Lutze, J.L., Gifford, R.M., 1998. Impact of elevated CO_2 on the metabolic diversity of microbial communities in N-limited grass swards. Plant and Soil 203, 289–300.

Hartwig, U.A., Lüscher, A., Daepp, M., Blum, H., Soussana, F., Nösberger, J., 2000. Due to symbiotic N_2 fixation, five years of elevated atmospheric pCO_2 had no effect on the N concentration of plant litter in fertile, mixed grassland. Plant and Soil 224, 43–50.

Henry, F., Nguyen, C., Paterson, E., Sim, A., Robin, C., 2005. How does nitrogen availability alter rhizodeposition in *Lolium multiflorum* Lam. during vegetative growth? Plant and Soil 269, 181–191.

Hodge, A., Millard, P., 1998. Effect of elevated CO_2 on carbon partitioning and exudate release from *Plantago lanceolata* seedlings. Physiologia Plantarum 103, 280–286.

Hodge, A., Paterson, E., Grayston, S.J., Campbell, C.D., Ord, B.G., Killham, K., 1998. Characterization and microbial utilisation of exudate material from the rhizosphere of *Lolium perenne* grown under CO_2 enrichment. Soil Biology & Biochemistry 30, 1033–1043.

Husted, S., Hebbern, C.A., Mattsson, M., Schjoerring, J.K., 2000. A critical experimental evaluation of methods for determination of NH_4^+ in plant tissue, xylem sap and apoplastic fluid. Physiologia Plantarum 109, 167–179.

IPCC, Intergovernmental Panel on Climate Change, (Eds.), 2001. Climate Change 200: Third Assessment Report. Cambridge University Press, Cambridge, UK.

Joergensen, R.G., Müller, T., 1996. The fumigation-extraction method to estimate soil microbial biomass: calibration of the k_{E_N} value. Soil Biology & Biochemistry 28, 33–37.

Joergensen, R.G., 1996. The fumigation-extraction method to estimate soil microbial biomass: calibration of the k_{E_C} value. Soil Biology & Biochemistry 28, 25–31.

Jones, D.L., Owen, A.G., Farrar, J.F., 2002. Simple method to enable the high resolution determination of total free amino acids in soil solution and soil extracts. Soil Biology & Biochemistry 34, 1893–1902.

Jones, D.L., Hodge, A., Kuzyakov, Y., 2004. Plant and mycorrhizal regulation of rhizodeposition. New Phytologist 163, 459–480.

Kim, Y.S., Kang, S.W., 1994. Novel malonamidases in *Bradyrhizobium japonicum*. Journal of Biological Chemistry 269, 8014–8021.

Kim, Y.S., 2002. Malonate metabolism: biochemistry, molecular biology, physiology, and industrial application. Journal of Biochemistry and Molecular Biology 35, 443–451.

Kuzyakov, Y., Raskatov, A., Kaupenjohann, M., 2003. Turnover and distribution of root exudates of *Zea mays*. Plant and Soil 254, 317–327.

Kuzyakov, Y., Shevtzova, E., Pustovoytov, K., 2006. Carbonate re-crystallization in soil revealed by ^{14}C labelling: experiment, model and significance for paleo-environmental reconstructions. Geoderma 131, 45–58.

Li, J., Copeland, L., 2000. Role of malonate in chickpeas. Phytochemistry 54, 585–589.

Marilley, L., Hartwig, U.A., 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. Microbial Ecology 38, 39–49.

Marschner, H., 1995. Mineral Nutrition of Higher Plants, second ed. Academic Press, London, UK.

Meharg, A.A., 1994. A critical review of labelling techniques used to quantify rhizosphere carbon flow. Plant and Soil 166, 55–62.

Montealegre, C.M., Van Kessel, C., Blumenthal, J.M., Hur, H.G., Hartwig, U.A., Sadowsky, M.J., 2000. Elevated atmospheric CO_2 alters microbial population structure in a pasture ecosystem. Global Change Biology 6, 475–482.

Neumann, G., Römheld, V., 2000. The release of root exudates as affected by the plant physiological status. In: Pinton, R., Varanini, Z., Nannipieri, P. (Eds.), The Rhizosphere: Biochemistry and Organic Substances at the Soil–Plant Interface. Marcel Dekker, New York, pp. 41–93.

Neumann, G., Massonneau, A., Martinoia, E., Römheld, V., 1999. Physiological adaptations to phosphorus deficiency during proteoid root development in white lupin. Planta 208, 373–382.

Neumann, G., 2007. Root exudates and organic composition of plant roots. In: Luster, J., Finlay, R., (Eds.), Handbook of Methods used in Rhizosphere Research. Swiss Federal Institute for Forest, Snow, and Landscape Research. Birmensdorf, Switzerland, published online ⟨http://www.rhizo.at⟩.

Peltonen, P.A., Vapaavuori, E., Julkunen-Tiitto, R., 2005. Accumulation of phenolic compounds in birch leaves is changed by elevated carbon dioxide and ozone. Global Change Biology 11, 1305–1324.

Piha, M.I., Munns, D.N., 1987. Nitrogen fixation potential of beans (*Phaseolus vulgaris* L.) compared with other grain legumes under controlled conditions. Plant and Soil 98, 169–182.

Plaxton, WC., 1998. Metabolic aspects of phosphate starvation in plants. In: Lynch, J.P., Deikman, J., (Eds.), Phosphorus in Plant Biology: Regulatory Roles in Molecular, Cellular, Organismic, and Ecosystem Processes. American Society of Plant Physiologists, pp. 229–241.

Salsman, K.J., Jordan, D.N., Smith, S.D., Neuman, D.S., 1999. Effect of atmospheric CO_2 enrichment on root growth and carbohydrate allocation of *Phaseolus* spp. International Journal of Plant Sciences 160 (6), 1075–1081.

Schortemeyer, M., Hartwig, U.A., Hendrey, G.R., Sadowsky, M., 1996. Microbial community changes in the rhizosphere of white clover and perennial ryegrass exposed to free-air carbon dioxide enrichment (FACE). Soil Biology & Biochemistry 28, 1717–1724.

Schortemeyer, M., Atkin, O.K., McFarlane, N., Evans, J.R., 2002. N_2 fixation by *Acacia* species increases under elevated atmospheric CO_2. Plant, Cell and Environment 25, 567–579.

Swain, T., Hillis, W.E., 1959. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. Journal of the Science of Food and Agriculture 10, 63–68.

van Ginkel, J.H., Gorissen, A., Polci, D., 2000. Elevated atmospheric carbon dioxide concentration: effects of increased carbon input in a *Lolium perenne* soil on microorganisms and decomposition. Soil Biology & Biochemistry 32, 449–456.

Vance, E.D., Brookes, P.C., Jenkinson, D.S., 1987. An extraction method for measuring soil microbial biomass C. Soil Biology & Biochemistry 19, 703–707.

Werner, D., 2000. Organic signals between plants and microorganisms. In: Pinton, R., Varanini, Z., Nannipieri, P. (Eds.), The Rhizosphere: Biochemistry and Organic Substances at the Soil–Plant Interface. Marcel Dekker, New York, pp. 197–222.

Yamakawa, T., Kato, S., Ishida, K., Kodama, T., Minoda, Y., 1983. Production of anthocyanins by *Vitis* cells in suspension culture. Agricultural and Biological Chemistry 47, 2185–2191.

Yurgel, S.N., Kahn, M.L., 2004. Dicarboxylate transport by rhizobia. FEMS Microbiological Reviews 28, 489–501.

Zanetti, S., Hartwig, U.A., Lüscher, A., Hebeisen, T., Frehner, M., Fischer, B.U., Hendrey, G.R., Blum, H., Nösberger, J., 1996. Stimulation of symbiotic N_2 fixation in *Trifolium repens* L. under elevated atmospheric pCO_2 in a grassland ecosystem. Plant Physiology 112, 575–583.