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RESEARCH PAPER

Elevated CO_2 concentration around alfalfa nodules increases N_2 fixation

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Abstract

Nodule CO_2 fixation via PEPC provides malate for bacteroids and oxaloacetate for N assimilation. The process is therefore of central importance for efficient nitrogen fixation. Nodule CO_2 fixation is known to depend on external CO_2 concentration. The hypothesis of the present paper was that nitrogen fixation in alfalfa plants is enhanced when the nodules are exposed to elevated CO_2 concentrations. Therefore nodulated plants of alfalfa were grown in a hydroponic system that allowed separate aeration of the root/nodule compartment that avoided any gas leakage to the shoots. The root/nodule compartments were aerated either with a 2500 $\mu l\ l^{-1}$ ($+CO_2$) or zero $\mu l\ l^{-1}$ ($-CO_2$) CO_2-containing N_2/O_2 gas flow (80/20, v/v). Nodule CO_2 fixation, nitrogen fixation, and growth were strongly increased in the $+CO_2$ treatment in a 3-week experimental period. More intensive CO_2 and nitrogen fixation coincided with higher per plant amounts of amino acids and organic acids in the nodules. Moreover, the concentration of asparagine was increased in both the nodules and the xylem sap. Plants in the $+CO_2$ treatment tended to develop nodules with higher %N concentration and individual activity. In a parallel experiment on plants with inefficient nodules (fix^-) the $+CO_2$ treatment remained without effect. Our data support the thesis that nodule CO_2 fixation is pivotal for efficient nitrogen fixation. It is concluded that strategies which enhance nodule CO_2 fixation will improve nitrogen fixation and nodule formation. Moreover, sufficient CO_2 application to roots and nodules is necessary for growth and efficient nitrogen fixation in hydroponic and aeroponic growth systems.

Key words: Alfalfa, amino acid, $^{13}CO_2$, H_2 evolution, *Medicago sativa*, N_2 fixation, nitrogen fixation, nodule CO_2 fixation, PEPC, xylem sap.

Introduction

Numerous studies have shown that legumes react to increased CO_2 concentrations around the shoots with an orchestrated increase in root and nodule growth (Phillips *et al.*, 1976; Murphy, 1986; Aranjuelo *et al.*, 2009). With relatively few contradictory reports, nodule specific activity remains unchanged (Cabrerizo *et al.*, 2001) and the higher N demand of the more intensely growing shoots at high CO_2 concentrations is met by the improved nitrogen fixation capacity of more and bigger nodules. Improved assimilate supply to nodules has no short-term effect on nodule specific activity (Vance and Heichel, 1991) and, in turn, an erratic assimilate supply to the nodules is buffered through nodule carbon pools like starch, α-polyhydroxybutyrate, and glycogen (Wang *et al.*, 2007). A decline in nodule activity at night is apparently more a reaction to lower temperatures than to a lower assimilate supply (Schweitzer and Harper, 1980). Rather than a lack of available sugar, nodule specific activity appears to be limited by the ability of the nodule to cleave sucrose through sucrose synthase (Gordon *et al.*, 1999; Baier *et al.*,

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Abbreviations: *ANA*, apparent nitrogenase activity measured as H_2 evolution in an N_2/O_2 mixture (80/20, v/v); *TNA*, total nitrogenase activity measured as H_2 evolution in an Ar/O_2 mixture (80/20, v/v); *EAC*, electron allocation coefficient *(1–ANA/TNA)*; AA, amino acids; OA, organic acids.

2007) and to form organic acids (Vance, 1998; Wang *et al.*, 2007). In particular, malate formation is important, since malate is the principal source of energy for the bacteroids (Driscoll and Finan, 1993), and at the same time functions as a carbon skeleton for N assimilation after reconversion to oxaloacetate (Rosendahl *et al.*, 1990). Moreover, malate might be involved in a putative osmoregulatory function of the nodule oxygen diffusion barrier that controls microaerobic conditions in the nodule infected zone (Minchin, 1997). The microaerobic conditions inside the nodule are part of the reason that nodule carbon metabolism is shunted towards organic acid, namely malate, formation. Phosphoenolpyruvate (PEP) rather than being decarboxylated, is transformed into oxaloacetate and malate by the combined activity of carbonic anhydrase (CA) (Atkins *et al.*, 2001), phosphoenolpyruvate carboxylase (PEPC) (Vance *et al.*, 1994), and malate dehydrogenase (MDH) (Schulze *et al.*, 2002). For PEPC and MDH, nodule-enhanced forms have been described (Suganuma *et al.*, 1997; Miller *et al.*, 1998) and CA shows nodule-specific expression in various legumes (de la Pena *et al.*, 1997; Atkins *et al.*, 2001). In fact, overexpression of nodule-enhanced MDH (neMDH) in alfalfa nodules not only increased nitrogen fixation per plant but also the specific activity of individual nodules (Denton *et al.*, 2002). The importance of the biochemical pathway towards malate in nodules is highlighted by the fact that PEPC and MDH activity occur alongside nitrogenase expression and activity in emerging nodules (Vance *et al.*, 1983; Egli *et al.*, 1989). PEPC is found to be 10–15-fold greater in nodules than in roots and can comprise up to 2% of the soluble protein fraction of nodules (Vance and Stade, 1984; Vance *et al.*, 1994). The post-translational regulation of PEPC activity occurs through reversible phosphorylation (Schuller and Werner, 1993). Studies with labelled CO_2 reveal that nodules do indeed have considerable CO_2 fixation rates (Warembourg and Roumet, 1989) and the down-regulation of PEPC activity in nodules through an antisense strategy impairs nitrogen fixation (Schulze *et al.*, 1998). Although leaf PEPC has a low K_m for CO_2 concentration, *in situ* saturation of the enzyme capacity might strongly depend on the ongoing drainage of its products (Willmer *et al.*, 1990; Kromer *et al.*, 1996). PEPC is tightly regulated in part by the nodule malate concentration (Zhang *et al.*, 1995). Christeller *et al.* (1977) have shown that nodule CO_2 fixation in lupin is a function of external CO_2 concentration. The apparent saturation is reached between 20–40 ml l^{-1} CO_2 in the air around the nodules. However, these measurements were made on excised nodules, in which the use of malate might progressively decline due to less N_2 fixation and N assimilation. The CO_2 concentration in the soil gaseous phase is high, and depends strongly on microbial activity. Concentrations of up to 5000 µl l^{-1} have been reported (Buyanovsky and Wagner, 1983). In experimental systems with sand culture but, in particular, in aeroponic and hydroponic systems, CO_2 concentrations around roots and nodules are often very low since the systems need to be intensely aerated to secure the available oxygen for the nodules and roots. This aeration is usually made with ambient air (around 360 µl l^{-1} CO_2) and, in particular, the roots of young plants do not add any significant additional CO_2 from respiration. There are some scattered reports that nodule activity is increased through long-term high CO_2 concentrations around the roots and nodules (Mulder and Van Veen, 1960; Grobbelaar *et al.*, 1971; Yamakawa *et al.*, 1997, 2004). Such experiments, however, necessitate the strict separation of the shoots and a root/nodule compartment to avoid the CO_2 feeding of leaves and thus a mixture of shoot and root effects. The hypothesis of the present paper was that long-term high CO_2 concentration around the roots and nodules (2500 µl l^{-1} versus zero µl l^{-1}) would improve the nitrogen fixation of young alfalfa plants due to increased CO_2 fixation, resulting in a better provision of organic acids for driving N_2 fixation and supporting N assimilation in nodules. Particular emphasis was put on the avoidance of any side-effect through any accidental additional CO_2 feeding of the shoots.

Materials and methods

Experimental design

The experimental approach was to study the effect of different CO_2 concentrations around alfalfa (*Medicago sativa* L.) roots and nodules on growth and nitrogen fixation over a longer period of time (3 weeks) avoiding side-effects through accidental additional CO_2 feeding of the shoots. This was achieved by establishing a hydroponic cultivation system which allows the CO_2 concentration in the root/nodule compartments to be continuously controlled while keeping the ambient CO_2 concentration around shoots. In the $-CO_2$ treatment, the root/nodule compartment was aerated with a CO_2-free N_2/O_2 (80/20; v/v) gas flow while in the $+CO_2$ treatment, the gas flow was enriched with 2500 µl l^{-1} CO_2. These treatments were kept for a 3-week experimental period. The effect of the treatments were studied on 'Saranac' (normal N_2-fixing plants) and 'Insaranac' (fix^- plants, fed with mineral N) to compare the effects of the CO_2 concentration around the roots and nodules. The H_2 evolution of 'Saranac' plants was measured as a parameter of nodule N_2 fixation activity during the experimental period. In addition, plant development in terms of the number of leaves and branches was recorded. At harvest, the plants were separated into shoot, root, and nodules for the determination of dry matter and N%. Amino acid (AA) export out of the nodules into the shoot was determined by analysing the xylem sap at harvest time.

In a second experiment, 'Saranac' plants were grown exactly as in the first experiment in a $-CO_2$ and a $+CO_2$ treatment to determine the CO_2 fixation and N_2 fixation capacity of the nodules at the end of the 3-week experimental period by measuring short-term nodule $^{13}CO_2$ in relation to N_2 fixation measured as H_2 evolution. In addition, the nodules were analysed for AA and organic acids (OA) concentration.

Plant growth

Cuttings of alfalfa plants cvs 'Saranac' and 'Insaranac' were made from approximately 4-week-old plants grown in nutrient solution. The cultivation of plants from cuttings enabled the selection of plants with very homogeneous development for further growth in nutrient solution culture. 'Insaranac' forms inactive nodules with regard to N_2 fixation (fix^-) (Viands *et al.*, 1979; Barnes *et al.*, 1990) whereas 'Saranac' plants have active nodules. Cuttings were

treated with hormone rooting mix, planted in containers with fine quartz sand, and maintained in a controlled environment chamber with a 16/8 h day/night cycle at temperatures of approximately 25/18 °C and a relative humidity of about 70%. The light intensity was 360 µmol m^{-2} s^{-1}. The quartz sand was kept at about 70% of its maximum water-holding capacity (21% of its dry weight) by the addition of an N-free nutrient solution of the following composition: macronutrients (mM): K_2SO_4 0.7, $MgSO_4$ 0.5, $CaCl_2$ 0.8, KH_2PO_4 0.015; and micronutrients (µM): H_3BO_3 4.0, Na_2MoO_4 0.1, $ZnSO_4$, 1.0, $MnCl_2$ 2.0, $Co(NO_3)_2$ 0.2, $CuCl_2$ 1.0, and FeNaEDTA 10. The pH was buffered with 0.25 mM MES and adjusted to 6.5 by applying KOH. In addition, at 7 d and 14 d after planting, each tray, containing approximately 50 cuttings, received 5 µmol P as KH_2PO_4 and 1 mmol N as urea. After rooting, cuttings were inoculated with *Sinorhizobium meliloti* strain 102F51. Nodules appeared 6–7 d after inoculation. Three weeks after inoculation 12 cuttings of even size were selected and carefully transferred to glass tubes (h, 600 mm; inner diameter, 20 mm) with nutrient solution. The tubes were closed with a rubber stopper at the lower side. Plants were inserted through a hole in the rubber stopper on the upper side of the tube and held in place with sponge. The hydroponic cultivation of alfalfa plants in the glass tubes is described in Schulze and Drevon (2005). The glass tubes were filled with the nutrient solution described above except for phosphorus and nitrogen. Legumes show susceptibility to P-toxicity in hydroponic culture when supplied with excessive amounts of P (Bell *et al.*, 1990; Tang *et al.*, 2001) and this agrees with our own observations on *Medicago truncatula* (Gaertn.) and *Medicago sativa* (L.). Based on pre-experiments the P-supply in our system was optimized in order to achieve intensive but not P-limited growth. Each plant received 3 µmol or 7.5 µmol P as KH_2PO_4 d^{-1} during the first or second week after transplanting, respectively. Subsequently, the P application was increased to 15 µmol P $plant^{-1}$ d^{-1}. This P supply resulted in a P concentration in the nutrient solution of 12, 30, or 60 µM, respectively. Since 'Insaranac' forms ineffective (fix^-) nodules, the plants received 2.5 mg N d^{-1} as KNO_3, while the 'Saranac' plants did not receive any mineral nitrogen. The solution was changed daily and aerated with ambient air at a flow rate of about 1.2 vols min^{-1} until the introduction of treatments.

Application of different CO_2 concentrations to the root/nodule compartment

Plants were kept in the glass tubes for 4 d and aerated with ambient air to allow them to adapt. At day 5 the root/nodule compartment was sealed for the measurement of H_2 and CO_2 evolution and for the long-term application of air with different CO_2 concentrations. For that purpose the hole in the upper rubber stopper was sealed with plasticine material with a high beeswax content. The beeswax gave the material a soft and pliable consistency that ensured a tight fit to the rubber stopper and the plant stem. The material is non plant-toxic. Before sealing, stiff inflow and outflow tubing for the sealed root/nodule compartment was inserted through the hole in the upper rubber stopper and also sealed with the same material. The inflow tubing reached to the lower end of the glass cylinder while the outflow was put above any nodules on the lower side of the upper rubber stopper. The inflow and outflow of the glass tubes were subsequently connected to a gas flow (N_2/O_2, 80/20, v/v) with either zero ($-CO_2$) or 2500 µl l^{-1} ($+CO_2$) CO_2 concentration (Fig. 1). The respective gas flows were sucked through the sealed root nodule compartment at a flow rate of 200 ml min^{-1} and then directed outside the growth chamber. Root/nodule respiration resulted in a concentration of approximately 30–100 µl l^{-1} CO_2 in the outflowing gas stream in the $-CO_2$ treatment. Any possible leakage in the system would result in a dilution of the CO_2 concentration of the applied air in the $+CO_2$ treatment, while a leakage in the $-CO_2$ treatment would result in a CO_2 concentration beyond that caused by root/nodule respiration. The CO_2 content of the outflowing gas streams was under constant surveillance. No CO_2-enriched gas was able to reach the shoots. Repeated measurements of the CO_2 concentration around the shoots showed ambient CO_2 concentrations. The two CO_2 levels in the root/nodule compartment were maintained for 3 weeks.

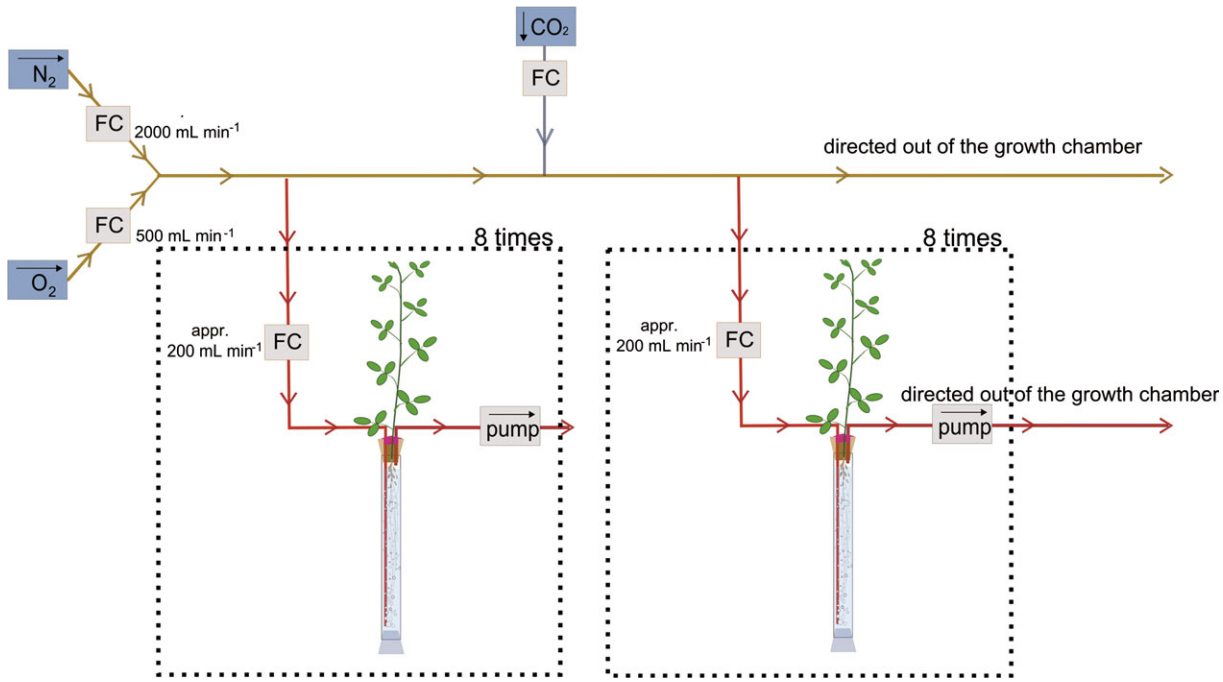

Fig. 1. Experimental set-up for the long-term application of an N_2/O_2 mixture (80/20; v/v) with different CO_2 concentrations to the root/nodule compartments. The parts enclosed in dotted lines are repeated eight times each. The gas input comes from pressurized gas bottles. The N_2 and O_2 gas was free of any CO_2 or H_2 contamination. FC, flow controller.

H_2 evolution measurements

The measurement of H_2 evolution is an indirect parameter for the determination of N_2 fixation activity of legume nodules (Hunt and Layzell, 1993). The *Sinorhizobium meliloti* strain 102F51 used here has no uptake hydrogenase (hup^-) (Blumenthal *et al.*, 1997). For the H_2 evolution measurement, the sealed root/nodule compartment was connected to an open-flow gas exchange measurement system that allowed the application of a mixture of N_2/O_2 (80/20, v/v) to the root/nodule compartment. For the measurements, the level of the nutrient solution was lowered to about 1/3 of the glass cylinder, leaving the lower virtually nodule-free part of the root system in solution. A gas flow of 200 ml min^{-1} (about 1.2 vols min^{-1}) was applied to the root compartment. A subsample (100 ml min^{-1}) of the outflowing gas was taken, dried (ice trap and $MgClO_4$) and passed through an H_2 analyser (S121 Hydrogen analyser, Qubit Systems, Canada). When a stable H_2 outflow from the root/nodule compartment was reached, this value was taken as the apparent nitrogenase activity (*ANA*). Subsequently, the composition of the air in the inflowing airstream was changed to Ar/O_2 (80/20, v/v). Argon is inert to nitrogenase and thus the whole electron flow is diverted to H^+. Consequently, H_2 evolution under argon represents the total enzyme activity (total nitrogenase activity, *TNA*). The peak value taken 3–5 min after switching to Ar/O_2 was regarded as the *TNA* value. The electron allocation coefficient (*EAC*) of nitrogenase activity was calculated as 1–(*ANA*/*TNA*). The amount of fixed nitrogen per time and per plant or unit nodule was calculated on the basis of the *ANA* and *TNA* measurements (Schulze *et al.*, 2006). *ANA*, *TNA*, and the *EAC* were measured before the introduction of the treatments, 2 d after treatment introduction, and at the end (after 3 weeks) of the experimental period.

Xylem harvest

Xylem sap was harvested and analysed for amino acid composition at the end of the experimental period. For xylem sap collection, the shoot was cut directly at the stem base. To avoid any contamination, the cut surface of the root part was rinsed for about 15 s with 1 M $CaCL_2$ solution, resulting in the closure of the phloem and the removal of the cell bleeding sap. The root was subsequently placed in a pressure chamber (Model 600 Pressure Chamber Instrument, PMS Instrument Co, Corvallis, Oregon, USA), and subjected to 300 MPa pressure. The xylem sap was collected for a period of 10 min. During the whole procedure the xylem sap was kept on ice and then immediately frozen (–20 °C).

$^{13}CO_2$ application

In a second experiment 'Saranac' plants were grown under the exact same conditions as described for the first experiment. The objective of the second experiment was to measure $^{13}CO_2$ fixation capacity of roots and nodules at the end of the experimental period. Moreover, nodule AA and OA concentration was also determined at this time. After 3 weeks of $\pm CO_2$ treatment, the nodules from both treatments were exposed to a $^{13}CO_2$ concentration of 2500 µl l^{-1} $^{13}CO_2$ (98 atom% enriched, Cambridge Isotope Laboratories, Andover MA, USA) for 15 min. Thus $^{13}CO_2$ uptake indicates the physiological capability of the nodules to fix CO_2 and the uptake is not influenced by different CO_2 concentrations around the nodules during labelling. The application was made to alternate plants from the $+CO_2$ and the $-CO_2$ treatments. The airstream was set up in the same way as to the CO_2-feeding system, i.e. the $^{13}CO_2$-enriched gas flow was sucked through the root/nodule compartment to avoid accidental feeding of the shoots. At the end of the labelling period the root/nodule compartment was thoroughly and quickly flushed with ambient air and the plants were immediately taken out of the tubes and submerged in liquid nitrogen. The plants were subsequently divided into shoots, roots, and nodules and vacuum-dried. A subsample of nodules was taken for AA and OA determination before drying. Reference plants were harvested in a growth chamber separated from the $^{13}CO_2$ application.

Nodule amino acid and organic acid concentration

Nodules were taken from intact plant roots with attached nodules and directly frozen in liquid nitrogen. The nodules collected were stored at –20 °C until analysis. For analysis of free AA and OA, nodules were homogenized with liquid N_2 using a mortar and pestle. Subsequently, 0.5 mg of the material was extracted with 3 ml of 50% ethanol (v/v) in a 40 °C water bath for 20 min. The solution was centrifuged for 30 min at 8000 *g* and 4 °C. The supernatant was immediately used for HPLC analyses after filtration (0.45 µm). AA were detected with a fluorescence detector after precolumn derivatization by orthophthaldialdehyde (Chen *et al.*, 1979). OAs were separated through HPLC and were detected by a photodiode array detector. For analytical details see Keutgen and Pawelzik (2008).

Dry matter, N, C, and ^{13}C concentration

The plants in experiments one and two were divided in shoots, roots, and nodules. The fractions were dried to a constant weight at 60 °C (experiment one) or through vacuum application (experiment two). Dried material from experiments one and two was ground to a fine powder in a pebble mill. The powdered material was subsequently analysed using a C/N analyser (NA 2500, CE-Instruments, Milano, Italy) and a mass spectrometer (Finnigan MAT, model 252, Bremen, Germany). The $^{13}CO_2$ uptake was determined by multiplying the C content of a fraction with the ^{13}C excess of this fraction over the ^{13}C% of an unlabelled reference group.

$$^{13}C_{\text{fixed}}[\text{g}] = \frac{C[\text{g}] \times (^{13}C_{\text{treatment}}\% - ^{13}C_{\text{reference}}\%)}{100}$$

Statistical analyses

Experimental data were analysed with the Sigmastat 2.03 statistical program (SPSS Inc., 1992–1997). All data sets were tested for a normal distribution. In the case of homogeneous sample variances, mean separation procedures were carried out using the *t* test.

Results

Growth, nodulation, and %N

Growth of nodulated plants with effective nodules was significantly increased in the $+CO_2$ treatment (Table 1; Fig. 2). Nodules of 'Saranac' plants were bigger and appeared pink while nodules of 'Insaranac' plants were white. Total dry matter formation in 'Saranac' was increased by 250% through the application of CO_2 to the root/nodule compartment, while this treatment was without any effect on 'Insaranac' plants, neither on total dry matter formation nor on any particular plant organ. In relative terms, the increase in shoot and roots of 'Saranac' plants was about equal but greater when compared with nodules. Plants with CO_2 application tended to form fewer and bigger nodules. There was a large variability in nodule number, nodule per plant dry matter, and individual nodule dry matter. The mean value for the nodule individual dry

Table 1. Dry matter, nodule number, and nodule individual weight of $+CO_2$ and $-CO_2$ alfalfa plants

Plants were grown for 3 weeks with different levels of CO_2 concentration in the root/nodule compartment. 'Insaranac' forms fix$^-$-nodules. Nitrogen nutrition of 'Saranac' depended on nitrogen fixation while 'Insaranac' plants received 2.5 mg N d^{-1} as KNO_3. Data are means of four replicates ±sd. An asterisk indicates a statistically significant difference from the $+CO_2$ treatment of 'Saranac' or 'Insaranac' (*t* test, $P \leq 0.05$).

Parameter	Treatments			
	Saranac		Insaranac	
	$+CO_2$	$-CO_2$	$+CO_2$	$-CO_2$
Shoot dry matter (mg $plant^{-1}$)	373±160	143*±84	243±40	253±43
Root dry matter (mg $plant^{-1}$)	153±56	61*±20	109±29	114±6
Nodule dry matter (mg $plant^{-1}$)	24.3±9.8	12.1±7.9	3.7±1.7	4.6±2.3
Total dry matter (mg $plant^{-1}$)	550±220	216*±114	355 ±70	370 ±48
Nodule number	24±19	40±11	38±15	56±40
Nodule individual dry weight (mg $nodule^{-1}$)	1.60±1.06	0.31±0.22	0.11±0.07	0.12±0.12

matter was about 5-fold higher in the $+CO_2$ treatment, statistically significantly different at $P \leq 0.1$. CO_2 application had no effect on the fix$^-$-nodules of 'Insaranac' plants. 'Saranac' plants in the $+CO_2$ treatment achieved the growth advantage by progressively more leaf and branch formation during the experimental period (Figs 3, 4). Between 14–20 d after the introduction of the CO_2 treatments both parameters became significantly different. At the end of the experimental period %N concentration showed no significant difference in shoots or roots between the treatments either in 'Saranac' or 'Insaranac' plants (Table 2). However, nodules of $+CO_2$ 'Saranac' plants had about 180% N concentration when compared with nodules of the $-CO_2$ plants. Nodule %N was not affected by CO_2 application in 'Insaranac' plants.

Nitrogen fixation

N_2 fixation was determined on the basis of nodule H_2 evolution measurements which enabled the parallel monitoring of the N_2 fixation activity during the experimental period. The N_2 fixation activity did not differ between treated plants before the introduction of the different CO_2 application (Table 3). 'Insaranac' plants showed no measureable H_2 evolution during the course of the experiment. Two days after the introduction of the CO_2 treatments, a significant differentiation in N_2 fixation of the 'Saranac' plants occurred (Table 3). Nitrogen fixation in the $+CO_2$ plants was about 225% of that in the $-CO_2$ plants. The differentiation in N_2 fixation did not show significant further change from 2 d until the end of the experimental period.

Fig. 2. Nodulated alfalfa plants 'Saranac' grown for 3 weeks with either $-CO_2$ (left) or $+CO_2$ (right) application to a separated root/nodule compartment. Nodules of plants from both treatments are shown below the plants.

Amino acids in nodule and xylem sap

The higher nitrogen fixation activity of plants in the $+CO_2$ treatment is supported by a tendency towards a higher concentration of asparagine (Asn) in the nodules. Figure 5A shows the proportion of asparagine among the five most abundant amino acids measured. The amount of amino acids in nodules per plant is significantly increased in the $+CO_2$ treatment (Fig. 5B). These facts resulted in a higher total concentration of amino acids in the xylem sap (Fig. 5C). This higher total concentration was the result of particular increases in asparagine, glutamine, and aspartate.

Root/nodule CO_2 fixation

Root and nodule CO_2 fixation was determined based on $^{13}CO_2$ application to the root/nodule compartment (Fig. 1). Apparent CO_2 fixation per unit root or nodule was stronger in the $+CO_2$ treatment by approximately 3- or 4-fold, respectively (Table 4). No ^{13}C-label was detected in shoots after the 15 min labelling period.

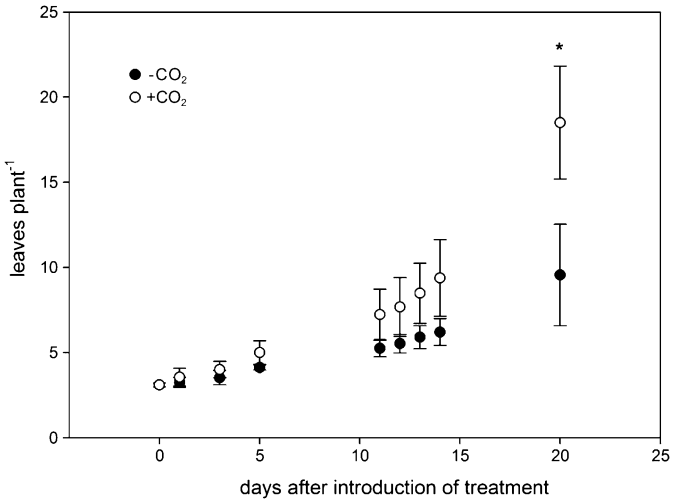

Fig. 3. Development of leaf number per plant ('Saranac') during the course of a 3-week experimental period with the application of an N_2/O_2 mixture (80/20; v/v) with either zero ($-CO_2$) or 2500 µl l^{-1} CO_2 ($+CO_2$) to the root/nodule compartment. Data are means of four replicates. Bars represent the standard deviation. An asterisk indicates a statistically significant difference from the $+CO_2$ treatment (*t* test, $P \leqslant 0.05$).

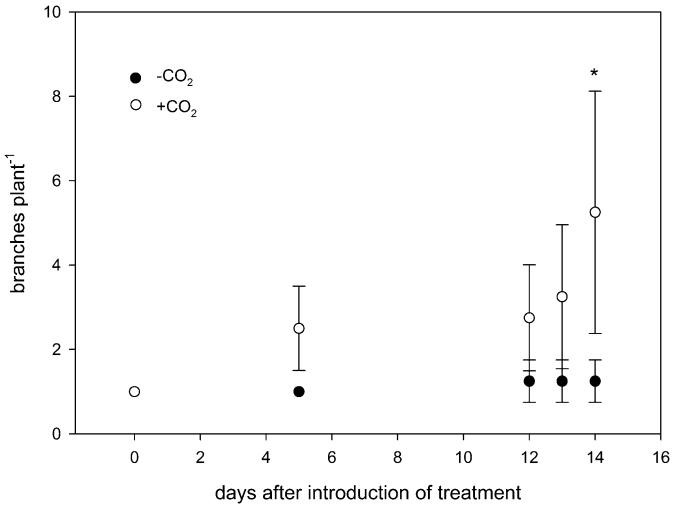

Fig. 4. Development of branch number per plant ('Saranac') during the course of a 3-week experimental period with the application of an N_2/O_2 mixture (80/20; v/v) with either zero ($-CO_2$) or 2500 µl l^{-1} CO_2 ($+CO_2$) to the root/nodule compartment. Data are means of four replicates. Bars represent standard deviation. An asterisk indicates a statistically significant difference from the $+CO_2$ treatment (*t* test, $P \leqslant 0.05$).

Nodule organic acid composition

Organic acid formation per plant was increased in the $+CO_2$ plants (Fig. 6A) by approximately 30%. This was the result of more nodule fresh weight per plant while the concentration of organic acids in nodules did not increase (Fig. 6B). Among the organic acids detected, fumarate, malate, and tartrate were the most abundant, while succinate and citrate were only found in low concentrations.

Table 2. %N concentration in shoots, roots and nodules of $+CO_2$ and $-CO_2$ alfalfa plants

Plants were grown for 3 weeks with different levels of CO_2 concentration in the root/nodule compartment. 'Insaranac' forms fix^--nodules. Nitrogen nutrition of 'Saranac' depended on nitrogen fixation while 'Insaranac' plants received 2.5 mg N d^{-1} as KNO_3. Data are means of four replicates ±SD. An asterisk indicates a statistically significant difference from the $+CO_2$ treatment of 'Saranac' or 'Insaranac' (*t* test, $P \leqslant 0.05$).

Parameter	Treatments			
	Saranac		Insaranac	
	$+CO_2$	$-CO_2$	$+CO_2$	$-CO_2$
Shoot N concentration (% N)	2.3±0.4	2.0±0.6	2.3±0.2	2.4±0.2
Root N concentration (% N)	2.8±0.2	2.7±0.3	2.8±0.2	3.1±0.2
Nodule N concentration (% N)	9.2±0.9	5.1*±0.6	4.8±0.9	5.2±1.2

Discussion

The results of our study highlight the importance of nodule CO_2 fixation for nitrogen fixation and the growth of legumes. Nodule CO_2 fixation is known to be tightly coupled to N_2 fixation, as proven, for example, by the concomitant expression of PEPC in nodules and emerging nitrogenase activity (Vance *et al.*, 1983) or through decreased N_2 fixation as a result of decreased PEPC expression in response to transformation with an antisense PEPC construct (Nomura *et al.*, 2006). The extent of nodule CO_2 fixation of lupin roots depends on the external CO_2 concentration with an apparent saturation at 20–40 ml l^{-1} CO_2 in the soil atmosphere (Christeller *et al.*, 1977). Increased nitrogen fixation and growth were found at CO_2 concentrations of about 2500 µl l^{-1} versus zero µl l^{-1} around nodules and roots in alfalfa plants. Thus, although the CO_2 concentration in the $+CO_2$ treatment is close to that found in the soil atmosphere, it might still not have been saturating for nodule CO_2 fixation. Effects of high CO_2 concentrations around nodules are reported for soybean, pea, and common bean (Mulder and Van Veen, 1960; Grobbelaar *et al.*, 1971; Yamakawa *et al.*, 2004). These reports found not only a consistent effect on N_2 fixation per plant but also on nodulation and nodule size. However, in most of these experiments a certain concomitant CO_2 feeding of shoots and thus a mix of effects on nodule and shoot CO_2 fixation cannot be completely ruled out. Our experimental set-up meant that any additional CO_2 from the root/nodule compartment reaching the shoots was avoided. Sucking the CO_2-enriched air through the root/nodule compartment rather than pressing it, would have resulted in a CO_2 dilution in the airstream in the event of any possible leakage. Repeated measurements of the CO_2 concentration in the outflowing air from the root/nodule compartments, in addition to measurements of the CO_2

Table 3. N_2 fixation of alfalfa plants ('Saranac') before and during application of different levels of CO_2 concentration to the root/nodule compartment

Plants were grown for 3 weeks with different levels of CO_2 concentration in the root/nodule compartment. N_2-fixation was calculated from nodule H_2 evolution according to Schulze *et al.* (2006). Data are means of four replicates ±sd. An asterisk indicates a statistically significant difference from the $+CO_2$ treatment (*t* test, $P \leqslant 0.05$).

Parameter	Treatments	
	$+CO_2$	**$-CO_2$**
Total N_2 fixation activity before introduction of treatments (mg N plant^{-1} d^{-1})	192±42	171±31
Total N_2 fixation activity 2 d after introduction of treatments (mg N plant^{-1} d^{-1})	283±134	126* 50
Total N_2 fixation activity 21 d after introduction of treatments (mg N plant^{-1} d^{-1})	965±279	415*±241
EAC 21 d after introduction of treatments	0.59±0.01	0.61±0.11
Specific N_2 fixation 21 d after introduction of treatments (mg N g^{-1}nodule dry matter d^{-1})	43±15	39±14
N_2 fixation activity of an individual nodule 21 d after introduction of treatments (μg N nodule^{-1} d^{-1})	61±40	11*±7

concentration around the shoots and around the whole experimental set-up proved the validity and viability of the system with respect to specific CO_2 feeding of roots and nodules. Moreover, the observed effects were restricted to plants with efficient nodules, while plants nourished with nitrate displayed no effect on growth. In addition, a more or less equal increase in shoot and root growth through CO_2 feeding of roots and nodules was observed, while CO_2 feeding of the shoots tends preferentially to support root growth (Schulze and Merbach, 2008). A distinctly higher N_2 fixation per plant in the $+CO_2$ treatment is shown by the H_2 evolution measurements and also by the significantly higher total amino acid content in the nodules and by the higher amino acid concentrations in the xylem sap. At the end of the experimental period better nitrogen fixation per plant in the $+CO_2$ treatment was largely a result of bigger nodules with higher individual efficiency. Two days after the introduction of the treatment, nitrogen fixation per plant in the $+CO_2$ treatment was strongly increased while it was more or less constant in the $-CO_2$ treatment. This increment in H_2 evolution probably results from an increase in specific nodule activity because the emergence of active nodules takes at least 6–7 d in alfalfa; thus it is unlikely that significantly more active nodules had been formed 2 d after the introduction of the treatments. It is conceivable that CO_2 feeding accelerated the development of young nodules that were already established when the CO_2 treatment commenced. However, this is not consistent with the fact that a tendency towards lower numbers of nodules was noted in the $+CO_2$ treatment at the end of the experimental period. In our experiment, a noticeable initial increase in nodule specific activity was found, as well as the formation of increasingly larger nodules with higher per nodule

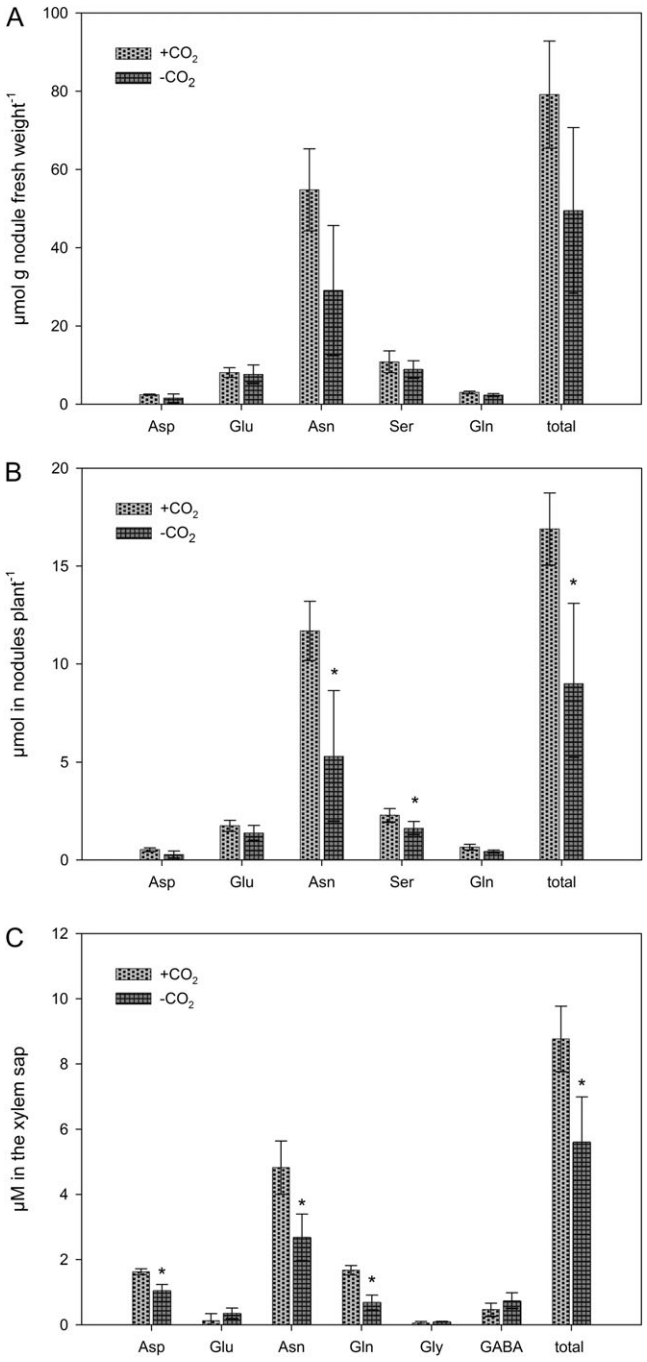

Fig. 5. Amino acids in nodules and in the xylem sap 3 weeks after beginning the application of an N_2/O_2 mixture (80/20; v/v) with either zero ($-CO_2$) or 2500 μl l^{-1} CO_2 ($+CO_2$) to the root/nodule compartment. Data are the means of four replicates. Bars represent standard deviation. An asterisk indicates a statistically significant difference from the $+CO_2$ treatment (*t* test, $P \leqslant 0.05$). (A) Concentrations of the five most abundant amino acids in the nodules. (B) Total amount in nodules per plant of the five most abundant nodule amino acids. In addition to the amino acids shown, Ala, Gaba, Tyr, Arg, Try, Lys, Val, Thr, Leu, His, Ile, Gly, and Prol. were detected in concentrations below 0.5 μmol g^{-1} nodule fresh weight. (C) Concentrations of amino acids found in the xylem sap in concentrations above 0.1 μM.

Table 4. CO_2 fixation capacity of alfalfa ('Saranac') roots and nodules after 3 weeks of growth at different CO_2 concentrations in the root/nodule compartment

CO_2 fixation was determined through a 15 min application of $^{13}CO_2$ (2500 µl l^{-1} $^{13}CO_2$) to the root/nodule compartments and subsequent measurement of ^{13}C in roots and nodules. N_2-fixation was calculated from nodule H_2 evolution according to Schulze *et al.* (2006). Data are means of three replicates ±sd. An asterisk indicates a statistically significant difference compared to the +CO_2 treatment (*t* test, $P \leq 0.05$).

Parameter	Treatments	
	+CO_2	–CO_2
Root CO_2 fixation (µg C g^{-1} root dry matter h^{-1})	42±22	14±12
Nodule CO_2 fixation (µg C g^{-1} nodule dry matter h^{-1})	82±25	22*±14
Nodule CO_2 fixation per N_2 reduced (mg C g^{-1} N)	88±47	35±15

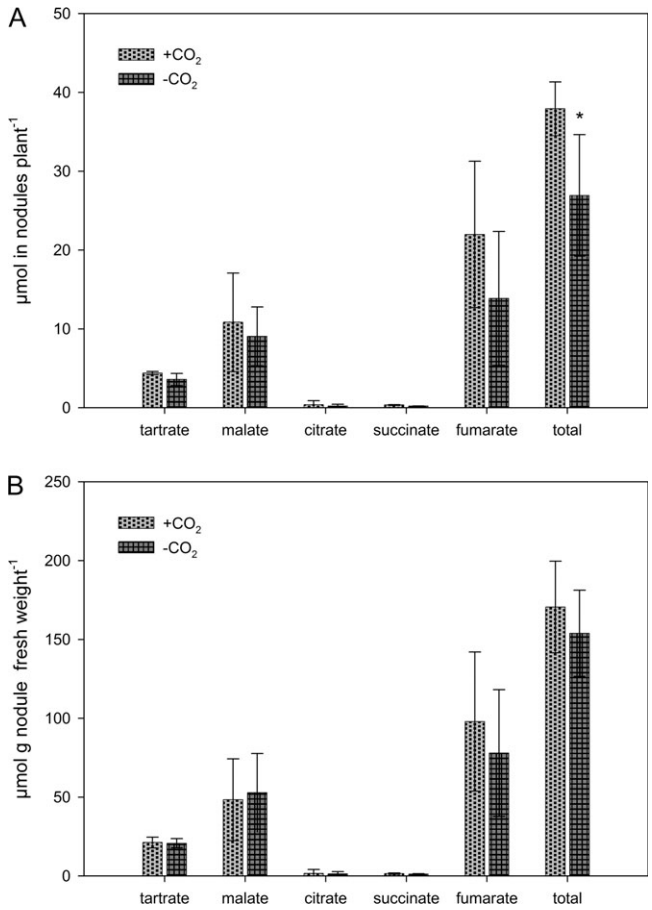

Fig. 6. Nodule organic acid compostition. Data are the means of four replicates. Bars represent standard deviation. An asterisk indicates a statistically significant difference from the +CO_2 treatment (*t* test, $P \leq 0.05$). (A) Total amount of organic acids in nodules per plant. (B) Concentration of detected organic acids in nodules.

activity. By contrast, shoot CO_2 feeding in most reported experiments shows neither a short- nor a long term-effect on nodule specific activity (Vance and Heichel, 1991; Cabrerizo *et al.*, 2001).

The observed increase in growth and nitrogen fixation was connected with the development of more new leaves and the development of additional branches. Improved C fixation may have improved C nutrition at the whole plant level, by at least partly supporting the C costs arising from nitrogen fixation and/or nodule growth. This might have resulted in the development of additional leaves and branches. Nodule growth and nitrogen fixation consumes considerable amounts of carbon (Schulze, 2004). A substantial contribution of nodule CO_2 fixation to the overall root/nodule carbon balance is indicated by several long-term experiments at the whole plant level (Warembourg and Roumet, 1989; Schulze *et al.*, 2006). Nitrogen fixation in the –CO_2 treatment could obviously not fully support the growth potential of the alfalfa plants. Better legume growth with nitrate nutrition as opposed to exclusive nitrogen fixation has been reported repeatedly (Herrmann *et al.*, 2001). Under natural soil conditions, a mixed supply of nitrogen from the nodules and soil solution is normal and apparently the optimal way to meet the plants' nitrogen requirements (Lamb *et al.*, 1995).

Increased nitrogen fixation in the +CO_2 treatment was accompanied by a higher asparagine content in nodules per plant and an increased asparagine concentration in the xylem sap. However, nodule concentration in the detected organic acids or, in particular, in malate was not improved by the +CO_2 treatment. For analysis, the nodules had been fixed in liquid nitrogen while adhering to the roots and subsequently had not been allowed to melt before extraction. Thus organic acid analysis and, in particular, that for malate, allows a one-off insight into a steady-state turnover in which the organic acids are intensely drained through uptake by the symbiosome and respiration and also through carbon skeleton provision for the increasingly available ammonium. Consequently, the equal concentrations in nodules with strongly different nitrogen fixation and therefore concurrent malate use indicate improved malate production brought about by nodule CO_2 fixation. In fact, in experiments with pea plants, it was found that organic acid concentration was higher in senescent nodules compared with active ones and also in nodules left detached yet otherwise intact for a certain period of time (Ahmed, 2007). Both observations indicate that a decrease in nodule nitrogen fixation activity is connected with organic acid accumulation, which is suggested to have a negative regulatory impact on nitrogenase activity (Roux *et al.*, 2008).

These measurements on root/nodule CO_2 fixation show that it was higher in the +CO_2 treatment, thus supporting the thesis of improved organic acid formation. In addition, the long-term +CO_2 treatment also improved the root/nodule CO_2 fixation capacity, since the measurements were made with equal $^{13}CO_2$ concentrations in both treatments (2500 µl l^{-1}). Thus sufficient CO_2 around the nodules apparently contributes to the emergence of efficient nodules in terms of CO_2 and N_2 fixation activity. At the time of the $^{13}CO_2$ fixation measurements, the shoots (and thus the shoot N demand in the +CO_2 treatment) were already considerably greater when compared with the –CO_2

treatment. This might have played a role in the measured higher CO_2 fixation indicating a possible feedback effect of shoot N demand on root/nodule CO_2 fixation.

In conclusion, our results support the thesis that short- and long-term CO_2 concentration around the nodules is of importance for nitrogen fixation activity and for the formation of efficient nodules in alfalfa. This has implications for experimental procedures measuring nodule gas exchange, in particular, in hydroponic and aeroponic systems. Measurements using pure N_2/O_2 mixtures or ambient air might underestimate nitrogen fixation. Moreover, long-term hydroponic growth with aeration of the nutrient solution with ambient air might impair the formation of optimally efficient nodules, in particular in young plants when root/nodule respiration does not sufficiently increase the nodule internal and external CO_2 concentrations. The biochemical pathway leading from nodule CO_2 fixation to malate production and use can be influenced through breeding and techniques of plant genetic transformation. Both strategies might improve nitrogen fixation activity, in particular in the early stages of growth in alfalfa plants. Moreover, agronomic measures improving soil respiration and thus CO_2 concentration in the soil atmosphere might contribute to more efficient legume growth.

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