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Research paper

Effects of rhizopheric nitric oxide (NO) on N uptake in *Fagus sylvatica* seedlings depend on soil CO_2 concentration, soil N availability and N source

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Rhizospheric nitric oxide (NO) and carbon dioxide (CO_2) are signalling compounds known to affect physiological processes in plants. Their joint influence on tree nitrogen (N) nutrition, however, is still unknown. Therefore, this study investigated, for the first time, the combined effect of rhizospheric NO and CO_2 levels on N uptake and N pools in European beech (*Fagus sylvatica* L.) seedlings depending on N availability. For this purpose, roots of seedlings were exposed to one of the nine combinations (i.e., low, ambient, high NO plus CO_2 concentration) at either low or high N availability. Our results indicate a significant effect of rhizospheric NO and/or CO_2 concentration on organic and inorganic N uptake. However, this effect depends strongly on NO and CO_2 concentration, N availability and N source. Similarly, allocation of N to different N pools in the fine roots of beech seedlings also shifted with varying rhizospheric gas concentrations and N availability.

Keywords: amino acids, ammonium, fine roots, nitrate, nitrogen pools, soluble protein.

Introduction

Nitric oxide (NO) is a highly reactive, membrane-permeable molecule that regulates various physiological processes in plants including germination, growth, development and plant defence (e.g., Wendehenne et al. 2004, Lamotte et al. 2005, Baudouin 2011, Wimalasekera et al. 2011 and references therein). In addition, NO plays a major role in plant responses to abiotic and biotic stress (Wimalasekera et al. 2011), which includes nutrient availability (Graziano et al. 2002, Zhao et al. 2007, Simon et al. 2013), drought (García-Mata and Lamattina 2001) and salinity (Zhao et al. 2007). These effects of NO might play an important role for plants and also ecosystem functioning considering future climate changes predicted for Central Europe, such as increasing periods of summer drought and enhanced temperatures (IPCC 2013). For example, exogenous application of NO resulted in an increased tolerance to drought stress in wheat (García-Mata and Lamattina 2001).

Rhizospheric NO is produced in large amounts mainly via nitrification and denitrification by soil microorganisms in the soils of forest ecosystems (Gasche and Papen 2002, Gessler et al. 2005). The emission of NO from the soil strongly depends not only on soil moisture (Davidson et al. 2000, Ludwig et al. 2001) and temperature (Schindlbacher et al. 2004, Kitzler et al. 2006*a*), but also on nitrogen (N) availability (Davidson and Kingerlee 1997, Zechmeister-Boltenstern et al. 2002, Simon et al. 2013). However, NO is emitted only partly into the atmosphere (Gasche and Papen 2002, Gessler et al. 2005) with the fate of the major part of rhizospheric NO still being unknown (Simon et al. 2013). Another rhizospheric signalling molecule is carbon dioxide (CO_2), which is produced in the soil via root and soil microbial respiration as well as litter decomposition (Pendall et al. 2004). Its production depends—similar to rhizospheric NO levels—on soil temperature, moisture and nutrient availability

(Davidson and Trumbore 1995, Hashimoto et al. 2004, Kitzler et al. 2006*b*). Elevated rhizospheric CO_2 levels can promote plant growth (e.g., Cramer and Richards 1999, Cramer 2002, Cramer et al. 2005, He et al. 2007). For example, Cramer and colleagues (Cramer and Richards 1999, Viktor and Cramer 2003) found that biomass increased in tomato seedlings with elevated rhizospheric CO_2 levels. However, to our knowledge nothing has been reported about the interactive effects of rhizospheric NO and CO_2 levels on plant processes.

Recent evidence indicates that increasing rhizospheric NO concentration influences inorganic and organic N uptake in deciduous (i.e., beech) and coniferous (i.e., Scots pine) tree seedlings (Simon et al. 2009, 2013). Simon et al. (2009) found no effects of rhizospheric NO on gene expression patterns of putative N transporters and enzymes of glutamine synthesis. Thus, the underlying regulatory mechanisms of NO interaction with N uptake still remain unclear, although it is suggested that these NO effects are mediated by posttranslational modification of proteins (Simon et al. 2009, Astier and Lindermayr 2012). Not only rhizospheric NO but also CO_2 concentration affects N uptake by plants (Cramer et al. 1996, Van der Merwe and Cramer 2000, Viktor and Cramer 2005). For example, elevated levels of rhizospheric CO_2 increased nitrate uptake in tomato roots grown in hydroponics (Van der Merwe and Cramer 2000). However, the mechanisms mediating changes in organic N uptake by rhizospheric CO_2 concentration are unknown.

Furthermore, inorganic and organic nitrogen uptake by trees is influenced by many biotic and abiotic factors, including competition with other trees species (Simon et al. 2010) or soil microorganisms (Dannenmann et al. 2009, Simon et al. 2011) and soil N availability (Stoelken et al. 2010, Simon et al. 2013). For example, the preference of beech seedlings to take up organic over inorganic N sources is altered with N availability (Stoelken et al. 2010). With rising atmospheric CO_2 concentrations, CO_2 levels in the rhizosphere will also increase which will likely also alter soil microbial community composition and activity (Lin et al. 2000)—including NO production—which, in turn, could affect the competition for N between plants and soil microorganisms.

In previous studies (Simon et al. 2009, 2013), we have shown that rhizospheric NO concentration affects N uptake and N pools in beech and Scots pine. Building on this work, we added another rhizospheric signalling substance (i.e., CO_2) to the experimental set-up to investigate the combined effects of NO and CO_2 concentration on N uptake and N pools and how they are affected by soil N availability, which has not been elucidated so far. Thus, the aim of this study was to characterize the combined effects of rhizospheric NO and CO_2 concentration on N uptake and N pools in European beech seedlings. For this purpose, we studied N uptake and N pools in the fine roots at different rhizospheric NO levels (i.e., low or high compared with ambient) and at different CO_2 concentrations (i.e., low, ambient, high). Furthermore, we investigated the significance of N availability (i.e., low or high) for the interaction between rhizospheric NO and CO_2 concentrations. In addition, we determined nitrosothiol (RSNO) contents as a possible mediator of posttranslational modification. The experiments were conducted in an aeroponics fumigation system under controlled conditions. European beech (*Fagus sylvatica* L.) was chosen for this study, because it represents the dominant tree species of the potential natural vegetation in moist to moderately dry areas of the sub-mountainous altitude range in Central Europe (Ellenberg 1996) and is favoured by forest practitioners and governments in current afforestation programmes in Central Europe (Fotelli et al. 2001, Gessler et al. 2007).

Materials and methods

Plant material and growth conditions

One-year-old mycorrhizal European beech (*Fagus sylvatica* L.) seedlings (provenance Swabian Alb, Germany) were purchased from a commercial tree nursery (Schlegel and Co. Gartenprodukte GmbH, Riedlingen, Germany) and grown in 15 × 15 × 20 cm pots containing commercial potting soil (Floradur, Floragard Vertriebs GmbH für Gartenbau, Oldenburg, Germany) for 2–4 months prior to the experiments. Artificial illumination (mercury vapour lamps, 1000 W) at seedling height (~50 cm) was 110 ± 22 μmol m^{-2} s^{-1} as frequently found in the understory of beech forests. Air temperature and relative humidity were, on average (16/8 h, day/night), 23.3 ± 1.9 °C/21.4 ± 2.3 °C and 45.1 ± 8.3%/50.9 ± 9.2%. Seedlings were watered three times a week to ensure sufficient water supply.

Experimental design

Prior to the experiments, seedling roots were cleaned of adherent soil particles after removal from the pots. Seedlings were transferred to an aeroponic fumigation system (a combined roots spray and fumigation facility) in the greenhouse (see Simon et al. 2009 for details). Average temperature and humidity ranged from 19.2 ± 1.4 °C (night, 8 h) to 24.1 ± 1.1 °C (day, 16 h) and from 64.3 ± 15.2 to 85.9 ± 2.3% (day/night, 16/8 h), respectively. At seedling height, ambient illumination in the greenhouse was supplemented with 106 ± 56 μE m^{-2} s^{-1} to maintain a 16/8 h (day/night) light regime. Beech roots were exposed for 72 h to one of the three NO concentrations (i.e., low, ambient or high), in combination with one of the three CO_2 concentrations (i.e., low, ambient or high) (see Table 1 for mean concentrations). Nitric oxide and CO_2 gas was purchased from Air Liquide Deutschland GmbH (Kornwestheim, Germany). Ambient rhizospheric NO and CO_2 concentrations were close to the mean average of 250 ppb NO and 3000 ppm CO_2 observed in a forest stand under natural conditions (Rudolph and Conrad 1996). During exposure to one of the nine possible combinations of NO and CO_2 levels each, beech roots were sprayed with an artificial nutrient solution with either low (containing 1 μM NH_4Cl, 100 μM KNO_3, 25 μM glutamine and 25 μM arginine) or high N availability (containing 50 μM

Table 1. Mean rhizospheric NO (ppb) and CO_2 concentrations (ppm) supplied to the fine roots of beech seedlings with low or high N solution (mean ± SD). Asterisks indicate significant differences ($P < 0.050$) between low and high N availability.

	NO (ppb)	CO_2 (ppm)
Low N availability		
Low	12 ± 6*	700 ± 31*
Ambient	150 ± 88	1390 ± 201*
High	991 ± 40	3854 ± 442*
High N availability		
Low	32 ± 13*	998 ± 143*
Ambient	229 ± 16	2937 ± 162*
High	1727 ± 450	8567 ± 361*

NH_4Cl, 300 µM KNO_3, 100 µM glutamine and 100 µM arginine). These artificial nutrient solutions mimicked soil solutions at original field sites in typical forests with low (Dannenmann et al. 2009) or high soil N availability (Stoelken et al. 2010) and contained the following further nutrients: for the low N solution, 10 µM $AlCl_3$, 90 µM $CaCl_2 \cdot 2H_2O$, 7 µM $FeSO_4 \cdot 7H_2O$, 6 µM K_2HPO_4, 50 µM KCl, 24 µM $MnCl_2 \cdot 4H_2O$, 20 µM NaCl, 70 µM $MgCl_2 \cdot 6H_2O$; and the high N solution, 20 µM $Al_2(SO_4)_3$, 75 µM $CaCl_2 \cdot 2H_2O$, 4 µM $FeCl_3 \cdot 6H_2O$, 14 µM KCl, 10 µM $MnCl_2 \cdot 4H_2O$, 40 µM $MgCl_2 \cdot 6H_2O$, 4.5 µM Na_2HPO_4, 20 µM NaCl. The concentrations of amino acids used in this study are within the range of estimates reported by Inselsbacher et al. (2011). Nutrient solutions were applied at rates of 0.3 l per plant per hour continuously over the entire duration of the experiment (i.e., 72 h). During the experiment, nitrite synthesis in the nutrient solutions was negligible (Simon et al. 2013).

^{15}N uptake experiments and harvest

After exposure to NO and CO_2, seedlings were taken out of the fumigation system and placed into brown bottles containing 250 ml artificial nutrient solution (the whole root of the plant was submerged in the solution) with either low or high N availability (see above for details). Solutions contained all four N sources (i.e., ammonium, nitrate, glutamine or arginine) but only one was ^{15}N labelled (amino acids also ^{13}C-labelled; all ≥98%); non-labelled controls were used to account for the natural abundance of ^{15}N in the roots ($n = 4$ replicates for each N compound or control, combination of NO and CO_2 concentrations and low or high N solution). Glutamine and arginine were chosen because they represent the most abundant amino acids in the fine roots of beech seedlings (Dannenmann et al. 2009). After 2 h incubation, the submerged parts of the fine roots were excised, washed twice in 0.5 mM $CaCl_2$, dried with cellulose paper and the fresh weight was determined. Further fine roots samples were taken randomly from seven seedlings for each treatment (NO/CO_2 combination and low or high N availability), shock-frozen in liquid N_2 and stored at −80 °C until analyses of N pools. Fine roots were defined as <2 mm diameter.

Quantification of ^{15}N and ^{13}C abundance and total nitrogen levels in fine roots

For quantification of ^{15}N and ^{13}C abundance and total nitrogen levels in fine roots, ~1.0–2.0 mg of oven-dried (48 h, 60 °C), finely ground material were weighed into tin capsules (IVA Analysentechnik, Meerbusch, Germany) and analysed using an elemental analyser (NA 2500, CE Instruments, Milan, Italy) coupled via a Conflo II interface to an isotope ratio mass spectrometer (Delta Plus, Thermo Finnigan MAT GmbH, Bremen, Germany). The δ values were corrected using acetanilide as a laboratory standard that was used in different weights to determine isotope linearity of the system. The laboratory standard was calibrated against different international standards: USGS-40 (glutamic acid, $\delta^{13}C_{PDB} = -26.39‰$) and USGS-41 (glutamic acid, $\delta^{13}C_{PDB} = 37.63‰$) for $\delta^{13}C$ and USGS-41 (glutamic acid, $\delta^{15}N_{air} = 47.600‰$) for $\delta^{15}N$. Net N uptake (nmol N g^{-1} fw h^{-1}) was calculated based on the incorporation of ^{15}N into the fine roots. ^{13}C-based uptake of the amino acids was generally lower than those based on ^{15}N indicating (i) the degradation of amino acids during the incubation time (2 h) in the artificial solution and/or on the root surface and/or (ii) the respiration of amino acid-derived carbon inside the roots.

Quantification of N pools in fine roots

Fine root samples were ground to a homogenous powder in liquid nitrogen. For the analysis of each N pool, ~50 mg of ground root material was used ($n = 7$ for each NO/CO_2 concentration and N availability). Nitrate was quantified according to Dannenmann et al. (2009) by mixing fine root materials extracted in 1 ml distilled water with 0.1 g washed polyvinylpyrrolidone (PVP, Sigma-Aldrich Inc., Steinheim, Germany) by shaking for 1 h at 4 °C in order to bind the phenolic constituents of the extract. Afterwards samples were boiled at 95 °C and centrifuged at 14,000*g* for 10 min. Determination of nitrate concentration was performed using an ion chromatograph (DX 120, Dionex, Idstein, Germany) coupled to an autosampler (AS 3500, Thermo Separation Products, Piscataway, NJ, USA) and equipped with the PeakNet software package (version 4.3, Dionex). An anion mixture of Cl^-, NO_3^-, PO_4^{3-} and SO_4^{2-} in distilled water was used as a standard. Amino acids and ammonium were extracted from fine roots according to Winter et al. (1992) in 200 µl HEPES buffer (pH 7.0) containing 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethansulfonic acid), 5 mM ethylene glycol tetraacetic acid (EGTA), 10 mM NaF and 1 ml methanol/chloroform (3.5 : 1.5 v/v). For quantification of total amino acids concentration, aliquots of 50 µl of the extracts were added to 50 µl aliquot ninhydrin reagent (1 : 1 mixture of solution A containing 3.84 g citric acid and 0.134 g $SnCl_2$ in 40 ml 1 M NaOH, then filled up to 100 ml with distilled water, pH 5.0 and solution B containing 4 g ninhydrin in 100 ml ethylene glycol monomethyl ether). One millilitre of 50% isopropanol was added. After incubation for 15 min at room temperature, optical density of the samples was measured at 570 nm using a UV-DU650 spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA). Glutamine was used as a standard. For the quantification of ammonium, extracts (see above) were shock-frozen in liquid N_2 and freeze-dried for 4 days. Ammonium concentration was measured according to Dannenmann et al. (2009) in 50 ml aliquots using a Waters Acquity UPLC-System (Water Corp., Milford, MA, USA). Ammonium sulfate was used as a standard. Concentration of total soluble proteins was quantified according to Dannenmann et al. (2009). For this purpose, root material was extracted in 1 ml extraction buffer containing 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 15% v/v glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT) and 0.1% Triton X-100. After incubation for 30 min at 4 °C followed by centrifugation (14,000*g*), 500 µl aliquots of the supernatant were transferred to new tubes. After adding 500 µl of trichloroacetic acid (10%), samples were incubated for 10 min at room temperature and then centrifuged (14,000*g* at 4 °C for 10 min). The pellets were dissolved in 1 ml of 1 M KOH. To quantify total soluble protein concentrations in the fine roots, 1 ml of Bradford reagent (Amresco Inc., Solon, OH, USA) was added to 50 µl aliquots of the extracts. After incubation for 10 min in the dark, the optical density was measured with a UV-DU650 spectrophotometer (Beckman Coulter Inc.) at 595 nm. Bovine serum albumin (BSA, Sigma A-6918) was used as a standard. Structural N was calculated by subtracting soluble protein-N, total amino acid-N, ammonium-N and nitrate-N from total N.

Determination of RSNO content by chemiluminescence method

The total RSNO content was determined with KI/I_2 as a reducing reagent using a Sievers 280i nitric oxide analyser (NOA, Analytix, Boldon, UK). Samples were pre-treated with acidified 20 mM sulfanilamide (in 1 M HCl) in the ratio 9 : 1 to remove nitrite interference and allow the specific measurement of RSNO. Proteins were extracted using phosphate buffer (137 mM NaCl, 0.027 mM KCl, 0.081 mM $Na_2HPO_4 \cdot 2H_2O$, 0.018 mM NaH_2PO_4) and were injected into the purging vessel of the NOA containing 3.5 ml of acidified KI/I_3 solution at 30 °C. Recorded mV signals were plotted against a calibration curve produced using solutions with known concentrations of *S*-nitrosoglutathione (GSNO) (Enzo Life Sciences GmbH, Lörrach, Germany).

Statistical analysis

Data were tested for normality (Kolmogorov–Smirnov test) and variance. Where necessary, data were transformed to satisfy the assumptions of normality and variance. The combined effects of NO × CO_2 levels on N uptake and N pools were tested in plants supplied with either low or high N availability, separately, using two-way analyses of variance (ANOVAs) followed by Tukey post hoc tests. Differences were considered significant at $P \leq 0.050$. SigmaPlot 11.0 (Systat Software GmbH, Erkrath, Germany) was used for all statistical analyses.

Results

Influence of soil NO on N uptake depends on rhizospheric CO_2 concentration and N availability

With low N availability, the effects of NO concentration shifted depending on CO_2 concentration and N source (Figures 1 and 2). Low compared with ambient rhizospheric NO concentration resulted in higher arginine-N uptake with ambient CO_2 concentration ($P < 0.001$) but lower ammonium uptake with high CO_2 concentration ($P = 0.006$), whereas the uptake of other N sources did not change. With high to ambient NO concentration, N uptake shifted depending on the N source and CO_2 concentration: higher ammonium uptake at ambient CO_2 concentration

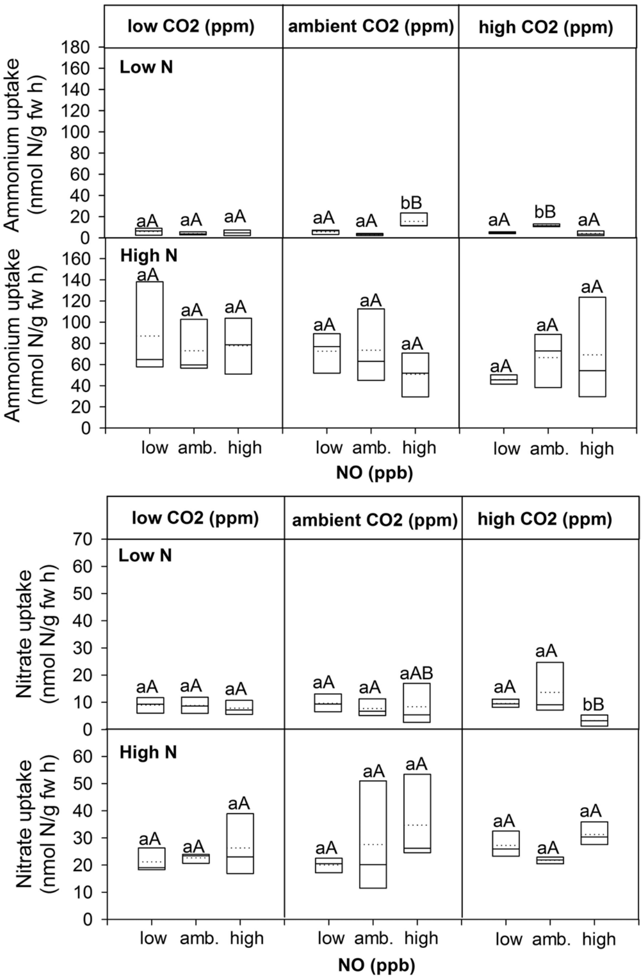

Figure 1. Effects of NO (ppb) and CO_2 (ppm) concentrations (i.e., low, ambient or high) on inorganic N uptake (i.e., ammonium, nitrate) (nmol N g^{-1} fw h^{-1}) by beech roots with varying soil N availability: low, top row; high, bottom row. Box plots show means (dotted line) and median (straight line) ($n = 4$). Different small letters indicate significant differences in N uptake between NO concentrations at a specific CO_2 concentration and soil N availability ($P \leq 0.050$). Different capital letters indicate significant differences in N uptake between different CO_2 concentrations at a specific NO concentration and soil N availability ($P \leq 0.050$).

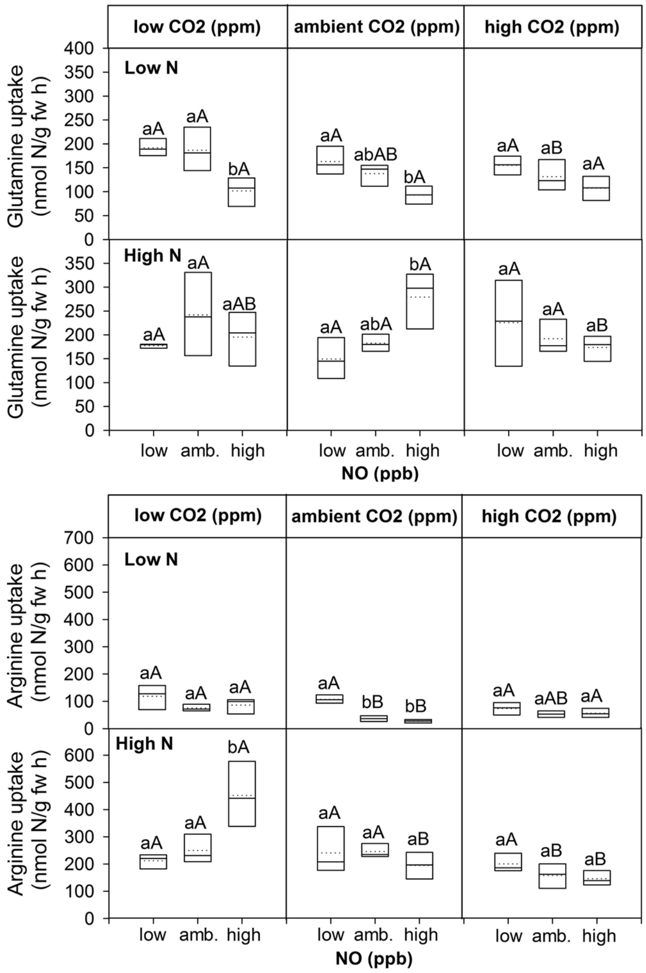

Figure 2. Effects of NO (ppb) and CO_2 (ppm) concentrations (i.e., low, ambient or high) on organic N uptake (i.e., glutamine, arginine) uptake (nmol N g^{-1} fw h^{-1}) by beech roots with varying soil N availability: low, top row; high, bottom row. Box plots show means (dotted line) and median (straight line) ($n = 4$). Different small letters indicate significant differences in N uptake between NO concentrations at a specific CO_2 concentration and soil N availability ($P \leq 0.050$). Different capital letters indicate significant differences in N uptake between different CO_2 concentrations at a specific NO concentration and soil N availability ($P \leq 0.050$).

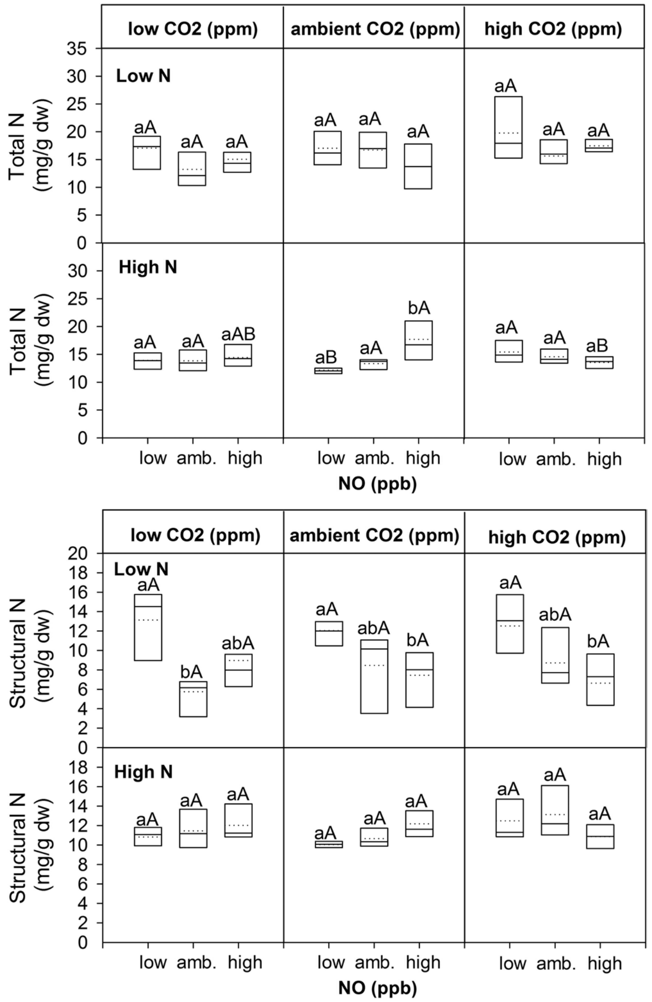

Figure 3. Effects of NO (ppb) and CO_2 (ppm) concentrations (i.e., low, ambient or high) on N metabolites (i.e., total N, structural N) (mg N g^{-1} dw) in the fine roots of beech seedlings depending on soil N availability: low, top row; high, bottom row. Box plots show mean (dotted line) and median (straight line) values ($n = 7$). Different small letters indicate significant differences between NO concentrations at a specific CO_2 concentration and soil N availability ($P \leq 0.050$). Different capital letters indicate significant differences between different CO_2 concentrations at a specific NO concentration and soil N availability ($P \leq 0.050$).

($P < 0.001$) but lower uptake of glutamine-N and inorganic N at low and high CO_2 concentration, respectively ($P \leq 0.003$).

With high N availability, inorganic and organic N uptake at low compared with ambient NO concentrations remained constant regardless of CO_2 concentration. In contrast, high compared with ambient NO concentration resulted in higher arginine-N uptake at low CO_2 concentration ($P \leq 0.007$) (Figure 2).

Influence of soil NO on N pools in fine roots depends on rhizospheric CO_2 concentration and N availability

The influence of rhizospheric NO and CO_2 concentrations on N pools was altered depending on N availability (Figures 3–5). At low N availability, low compared with ambient NO concentration led to higher amounts of structural N and ammonium-N ($P \leq 0.030$) both at low CO_2 concentration (Figures 3 and 5), but lower soluble protein-N concentration ($P = 0.016$) and amino acid-N concentration ($P = 0.036$) at low and high CO_2 concentration, respectively (Figure 4). In contrast, N pools remained constant regardless of CO_2 concentration at higher compared with ambient NO concentration (at low N availability) (Figures 3–5). At high N availability, different N pools were affected depending on CO_2 concentration in the soil compared with low soil N supply: levels of ammonium-N (at low CO_2 concentration, $P = 0.019$) and soluble protein-N (at high CO_2 concentration, $P = 0.030$) were significantly higher at low NO concentration compared with ambient NO (Figures 4 and 5). With ambient CO_2 level, soluble protein N and total N concentrations were higher

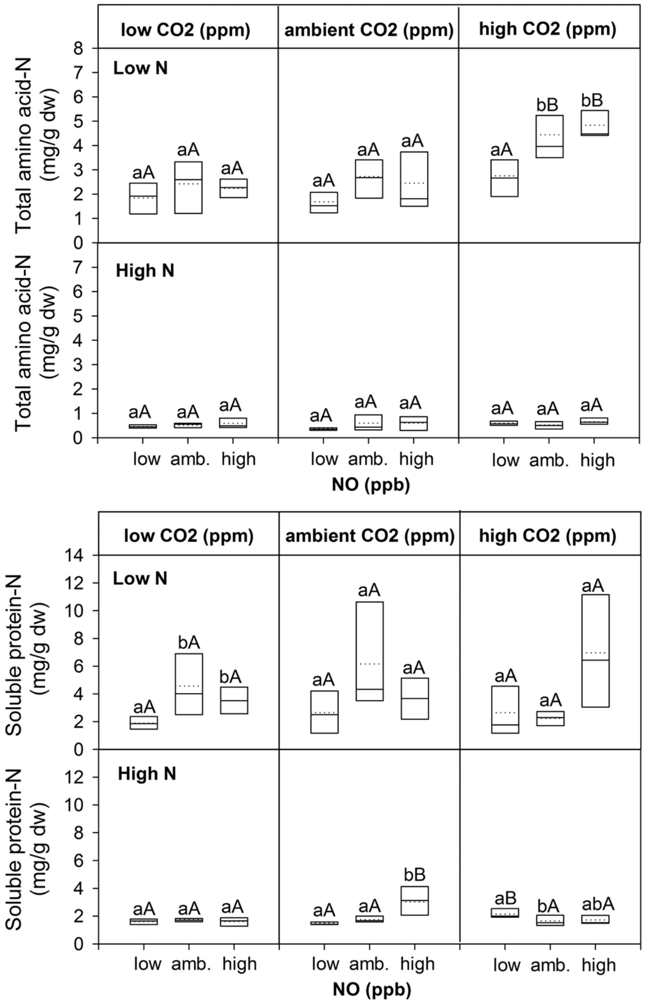

Figure 4. Effects of NO (ppb) and CO_2 (ppm) concentrations (i.e., low, ambient or high) on N metabolites (i.e., total amino acid-N, soluble protein-N) (mg N g^{-1} dw) in the fine roots of beech seedlings depending on soil N availability: low, top row; high, bottom row. Box plots show mean (dotted line) and median (straight line) values ($n = 7$). Different small letters indicate significant differences between NO concentrations at a specific CO_2 concentration and soil N availability ($P \leq 0.050$). Different capital letters indicate significant differences between different CO_2 concentrations at a specific NO concentration and soil N availability ($P \leq 0.050$).

with high compared with ambient NO concentration ($P \leq 0.023$) (Figures 3 and 4).

Interaction of rhizospheric NO and CO_2 concentration affects N uptake in fine roots

The additive effect of rhizospheric NO and CO_2 concentrations on N uptake in fine roots (Table 2) varied with N supply. With low N availability, the effect of different levels of NO on ammonium ($P < 0.001$) and arginine-N uptake ($P = 0.012$) depended on the CO_2 level, which was not found for nitrate or glutamine-N uptake. Furthermore, no significant interaction effect was found for any of the analysed N pools in the fine roots. With high N availability, the effect of NO was also influenced by CO_2 concentration with regard to organic N uptake (i.e., glutamine-N: $P = 0.025$; arginine-N: $P < 0.001$), but not inorganic N uptake. Similarly, rhizospheric CO_2 concentrations influenced the effect of NO on the levels of ammonium-N, soluble protein-N and total N ($P \leq 0.005$).

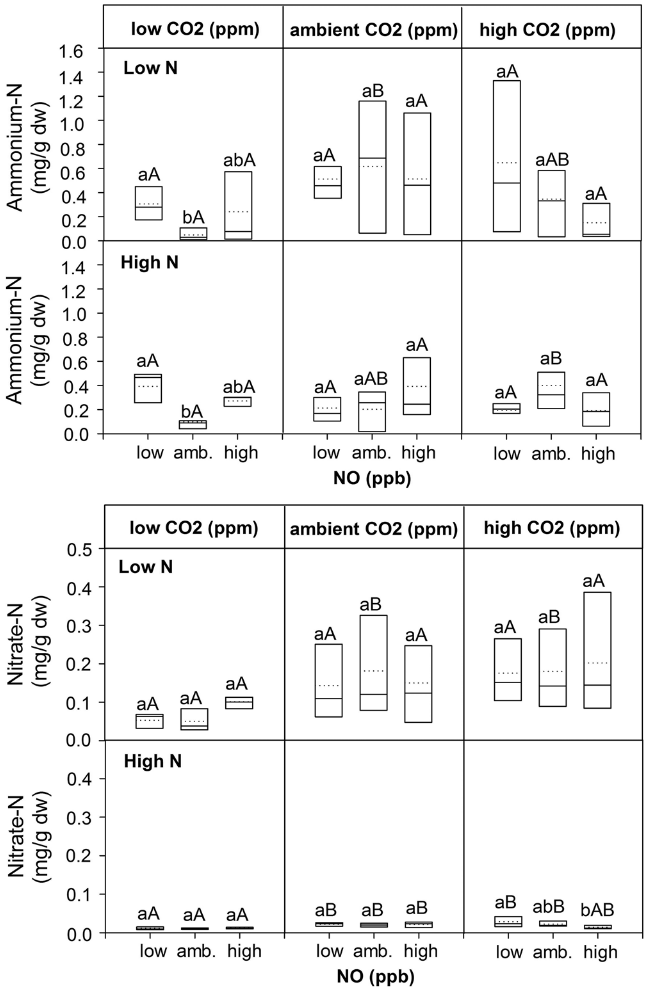

Figure 5. Effects of NO (ppb) and CO_2 (ppm) concentrations (i.e., low, ambient or high) on N metabolites (i.e., ammonium-N, nitrate-N) (mg N g^{-1} dw) in the fine roots of beech seedlings depending on soil N availability: low, top row; high, bottom row. Box plots show mean (dotted line) and median (straight line) values ($n = 7$). Different small letters indicate significant differences between NO concentrations at a specific CO_2 concentration and soil N availability ($P \leq 0.050$). Different capital letters indicate significant differences between different CO_2 concentrations at a specific NO concentration and soil N availability ($P \leq 0.050$).

In this study, the focus was on how CO_2 concentration in the soil influences the rhizospheric NO effect on plant N uptake and N pools; thus, the results of how NO influences the effect of CO_2 can be found in supplemental material (see Figures S1 and S2 available as Supplementary Data at *Tree Physiology* Online). Furthermore, we quantified the RSNO content in the fine roots as a possible posttranslational modification mechanism mediated by NO. Results showed no significant differences between different treatments with low, ambient and high NO concentration and RSNO contents often below the detection limit (see Table S1 available as Supplementary Data at *Tree Physiology* Online).

Table 2. Effects of rhizospheric NO (ppb) and/or CO_2 concentration (ppm) (two-way ANOVAs) on N uptake (nmol N g^{-1} fw h^{-1}) and N pools (mg (g dw$)^{-1}$) in the fine roots. Bold *P*-values indicate a significant effect of NO and/or CO_2 concentration on N uptake and N pools ($P < 0.050$).

	[NO]	[CO_2]	[NO] × [CO_2]
N uptake			
Low N availability			
Ammonium	0.112	**0.038**	**<0.001**
Nitrate	**0.016**	0.642	0.119
Glutamine-N	**<0.001**	**0.039**	0.366
Arginine-N	**<0.001**	**<0.001**	**0.012**
High N availability			
Ammonium	0.037	0.930	0.625
Nitrate	0.111	0.246	0.306
Glutamine-N	0.402	0.940	**0.025**
Arginine-N	0.146	**<0.001**	**<0.001**
N pools			
Low N availability			
Total N	**0.025**	0.068	0.325
Structural N	**<0.001**	0.884	0.102
Total amino acid-N	0.131	**<0.001**	0.080
Soluble protein-N	**0.007**	0.404	0.171
Nitrate-N	0.572	**0.006**	0.598
Ammonium-N	**0.014**	**0.018**	0.276
High N availability			
Total N	0.126	0.308	**0.001**
Structural N	0.519	0.158	0.075
Total amino acid-N	0.084	0.432	0.375
Soluble protein-N	0.374	0.175	**<0.001**
Nitrate-N	0.261	**<0.001**	0.112
Ammonium-N	0.875	0.934	**0.005**

Discussion

Effects of rhizospheric NO concentration on N uptake depends on rhizospheric CO_2 concentration and N availability

Inorganic and organic N uptake by plants is affected by rhizospheric NO concentration in deciduous and coniferous tree species (Simon et al. 2009, 2013). Furthermore, rhizospheric CO_2 can also influence N acquisition in plants as was shown by Van der Merwe and Cramer (2000) for nitrate uptake in tomato plants. In the present study, influence of rhizospheric CO_2 concentration on root N acquisition was shown, for the first time, in a woody species, not only for inorganic but also for organic N uptake (Figures 1 and 2 and Table 2). Overall, four different patterns were found depending on N supply:

1. At high N supply, N uptake increased with high compared with ambient NO concentration (i.e., arginine (at low CO_2)). In the other treatments, no effects were found for variations in NO on N uptake capacity comparing high with ambient NO concentration (Figures 1 and 2). These results are in contrast to the study by Van der Merwe and Cramer (2000) who found an increase in nitrate uptake with increasing CO_2 concentration. Rising rhizospheric dissolved inorganic carbon ($DIC = CO_2 + HCO_3^-$) can lead to an increased supply of carbon skeletons for ammonium assimilation (Cramer et al. 1996, Van der Merwe and Cramer 2000), for example by CO_2 fixation via PEP carboxylase activity in the roots as shown in *Lotus* (Fotelli et al. 2011). This would result in an increase in both ammonium and nitrate uptake which, however, was not found in our study.

At low N supply, effects of rhizospheric NO concentration on N uptake was altered depending on the specific CO_2 concentration:

2. At high CO_2 concentration, inorganic N uptake decreased at high and low compared with ambient NO levels (Figure 1). This result contrasts the findings at high N supply and suggests that soil N availability (here simulated by the artificial nutrient solution) plays a more important role in N uptake rather than CO_2 or NO concentration in the soil. This key influence of N availability was shown also in previous studies for beech and Scots pine (Stoelken et al. 2010, Simon et al. 2013).
3. At low CO_2 concentration, organic N uptake (i.e., glutamine) decreased with high compared with ambient NO concentration (Figure 2). This finding suggests that depending on CO_2 concentration, either inorganic or organic N uptake is reduced.
4. At ambient CO_2 concentration, inorganic N (i.e., ammonium) increased with higher compared with ambient NO concentration, whereas organic N uptake increased with lower compared with ambient or high NO concentration (Figures 1 and 2). Apparently, under these conditions, the influence of NO becomes more important.

Increased CO_2 levels are an indicator for enhanced microbial activity (Lin et al. 2000). High levels of rhizospheric CO_2 as a consequence of increased soil microbial biomass and activity indicate high plant–microbial competition for N under these conditions. N availability in the soil decreases due to microbial activity that may have been stimulated, for example, by enhanced labile carbon availability in the soil. Under these conditions, plant N uptake will decrease, especially at limited N availability (Dunn et al. 2006, Dannenmann et al. 2009). Lower levels of rhizospheric CO_2 indicate decreased microbial activity; thus, N turnover rates will decrease as well. Decreased activity of soil microbes also includes mycorrhiza fungi, which play a significant role in plant N acquisition (Dannenmann et al. 2009, Stoelken et al. 2010). At ambient CO_2 concentration, the increased plant N uptake, however, suggests that competition for N might be balanced under current climate conditions, but will negatively affect plants under increased CO_2 concentrations in the future. Overall, our results indicate that rhizospheric NO levels can influence organic and inorganic N uptake in beech, but shifts in rhizospheric CO_2 concentration can overrule NO effects. However, the influence of both soil gases on N uptake by beech seedlings are strongly dependent on soil N availability. The latter has also been shown for Scots pine roots at ambient rhizospheric CO_2 levels (Simon et al. 2013).

Effect of NO concentration on N pools depends on rhizospheric CO_2 concentration and N availability

Dependent on rhizospheric CO_2 concentration and N availability, total N, total amino acid-N and soluble protein-N concentrations significantly increased with increasing rhizospheric NO concentration (Figures 3 and 4). However, total N concentration did not change with increasing (Viktor and Cramer 2003) or decreasing CO_2 concentration regardless of the NO level in the rhizosphere with low N availability (Figure 3). These results indicate that the changes in N pools occurred at the expense of one another. This is unexpected and indicates a high mobility even of structural N in root cells. However, we measured only N pools in fine roots. Thus, transport of metabolites from the roots to the shoots and vice versa may also have contributed to differences in root N pools.

At high N availability and ambient CO_2 level, the increase in root total N is due to an increase in soluble protein-N levels with increasing NO from ambient to high levels (Figures 3 and 4). This finding is in contrast to the study by Simon et al. (2013) with pine seedlings in which NO-dependent changes in total N were not observed at high N availability. However, no significant differences were found at high CO_2 levels (Figure 5). This finding is in contrast to Cramer et al. (1996) who reported nitrate accumulation in barley at elevated rhizospheric CO_2 levels. Therefore, the effects of rhizospheric NO and CO_2 levels on the allocation of N to different pools in the fine roots appear to be species-specific. At high CO_2 level, the total amino acid-N concentration increased with rising NO concentration with low N availability, which could be partially due to dissolved inorganic carbon. Its enhanced influx into the roots might improve the incorporation of N into amino acids as a consequence of greater availability of anaplerotic carbon for protein synthesis (Cramer et al. 1993, Viktor and Cramer 2003, He et al. 2010). Our findings indicate that the influence of rhizosphere CO_2 concentration on N pools differs with different N sources and N availability.

Nitrogen availability influences the effects of rhizospheric NO and CO_2 concentration on N uptake and N pools

Nitrogen availability seems to have a greater influence on inorganic and organic N uptake compared with NO and CO_2 levels in the soil (Tables 1 and 2), although the concentrations of NO and CO_2 between low and high N availability (Table 1) differed significantly in part of the treatments (i.e., low NO and all CO_2 treatments). The key role of soil N availability on plant N uptake has already been suggested in studies by Stoelken et al. (2010) for beech and Simon et al. (2013) for pine seedlings, both of which used the same artificial nutrient solutions also used in the present study. Still, N uptake patterns differed between species. In beech seedlings, inorganic and organic N uptake appeared to be higher with high compared with low soil N availability regardless of NO or CO_2 concentration in the soil (Figures 1 and 2). These findings are consistent with the study by Simon et al. (2013) using pine seedlings with regard to inorganic and arginine-N uptake at ambient CO_2 concentration. For glutamine-N uptake, however, the opposite was observed in pine seedlings (Simon et al. 2013), indicating that beech and pine seedlings possess different strategies for N uptake under similar environmental conditions.

With regard to N pools in the fine roots, increasing N availability seems to lead to lower levels of nitrate-N, soluble protein-N and total N (Figures 3–5). These findings also indicate species-specific differences in N allocation to N pools and its dependency on soil N availability. Pine seedlings had significantly lower levels of nitrate-N with increasing N availability (Simon et al. 2013). Although nitrate-N levels in fine roots of beech seedlings were generally lower, total amino acid-N amounts were generally higher in beech compared with pine roots (Figures 4 and 5). Furthermore, increased N availability led to a decrease in soluble protein-N and total N levels in beech (Figures 3 and 4), but not in pine seedlings (Simon et al. 2013). Overall, we conclude that the effects of rhizospheric NO and CO_2 concentration on N pools in the fine roots depend on N availability and species.

Limitations of the present experiments

Mycorrhization plays a significant role for plant N acquisition (e.g., Dannenmann et al. 2009, Stoelken et al. 2010). The key influence of this symbiosis has also been reported in experiments with elevated levels of CO_2 (e.g., Constable et al. 2001, Chen et al. 2007), however, with contrasting results. Inorganic N uptake by *Plantago lanceolata* L. increased upon mycorrhization with rising CO_2 levels, but not in *Festuca arundinacea* Schreb., indicating that this positive effect of mycorrhization is species-specific (Chen et al. 2007). Since the beech seedlings used in the present study were mycorrhizal, N uptake by beech seedlings could have been promoted by mycorrhization as well. The significance of mycorrhization for the interaction of NO and CO_2 levels with N acquisition and allocation, however, remains to be elucidated.

The present experiments were conducted over 12 weeks; during this time development of beech seedlings continued. A field study on adult and young beech trees over the growing season showed that inorganic and organic N uptake varied with tree age depending on the season (Simon et al. 2011). Seedlings required more external N for new growth and leaf development due to smaller N storage pools compared with adult beech trees in spring (Simon et al. 2011). Because the present study started in July and ended in September, we cannot exclude that changes in N uptake patterns due to seasonal variation or seedling development might have influenced our results.

In the present study, we investigated the effects of rhizospheric CO_2 and NO concentration on plant N acquisition. Both molecules are known as signalling compounds in plants. However, the underlying regulatory mechanisms of rhizospheric NO on plant inorganic and organic N uptake are currently unknown. Previous studies with beech seedlings by Simon et al. (2009) showed that gene expression patterns of putative N transporters and enzymes of glutamine synthesis are not affected by rhizospheric NO levels. It has been suggested that the NO influences are regulated via posttranslational modification (Simon et al. 2009, Astier and Lindermayr 2012). In the present study, we have looked at RSNO contents as an indicator for posttranslational modification by nitrosylation (see Table S1 available as Supplementary Data at *Tree Physiology* Online). However, no modulations in total *S*-nitrosothiol content were observed from our results. Other mechanisms of posttranscriptional regulation such as phosphorylation, ubiquitination, miRNAs, etc. (Marín-González and Suárez-López 2012, Mazur and van den Burg 2012, Offringa and Huang 2013, Rojas-Triana et al. 2013) might be involved in NO mediated changes in N uptake.

Elevated atmospheric CO_2 affected plant N uptake differently depending on the studied species (Newbery et al. 1995, BassiriRad et al. 1996, BassiriRad 2000, Constable et al. 2001). In addition, it can increase or decrease nitrate reductase (NR) activity (Maeskaya et al. 1990, Hocking and Meyer 1991, Geiger et al. 1999), which is subjected to posttranscriptional regulation (Crete et al. 1997, Stitt and Krapp 1999). In the study by Constable et al. (2001), root NR activity increased in response to rising CO_2 and mycorrhizal inoculation. However, the underlying regulatory mechanisms of rhizospheric CO_2 on plant inorganic and organic N uptake still remain to be elucidated.

Conclusion

In conclusion, the effects of rhizospheric NO concentration on inorganic and organic N acquisition and on root N pools are influenced by rhizospheric CO_2 concentrations. This effect, however, was strongly regulated by N availability. Similarly, N pools shifted with NO and CO_2 concentration as well as N supply. In addition, N acquisition strategies under these influences are apparently species-specific (compare this study and Simon et al. 2013). Furthermore, the regulatory mechanisms of these influences still remain to be elucidated. Evaluating the significance of these different controls, we conclude that soil N availability has the strongest effect among the factors studied, followed by rhizospheric CO_2 and NO concentrations. The latter may be of particular significance under high competition for N between plants and microbial N transformation processes such as nitrification and denitrification. This competition might have increasing significance for the growth of plants in a future climate (Rennenberg et al. 2009).

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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