Rhizospheric NO interacts with the acquisition of reduced N sources by the roots of European beech (*Fagus sylvatica* L.)

Judy Simon *, Gunda Stoelken, Michael Rienks, Heinz Rennenberg

Institute of Forest Botany and Tree Physiology, Chair of Tree Physiology, University of Freiburg, Georges-Koehler-Allee 53/54, 79110 Freiburg, Germany

ARTICLE INFO

ABSTRACT

The gas phase of the soil plays an important role in plant growth and development. We investigated the effect of rhizospheric NO as a signalling compound for N uptake of beech roots. Following exposure to NO, ammonium and glutamine uptake into roots were determined using ^{15}N-labelling, and gene expression of selected transporters was analysed by quantitative real-time PCR. Uptake of both N sources increased significantly with elevated NO concentration. However, with one exception, this increase was not reflected in up-regulation of expression of the respective transporters.
© 2009 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

Nitric oxide (NO) is a small, highly reactive, membrane-permeable molecule synthesised and released by soil microorganisms in the course of nitrification and denitrification [1,2] and produced by eukaryotic organisms in various metabolic processes [3,4]. NO production in soils depends on soil water content [5], temperature and N availability [2]. Forest soils with high N availability emit greater amounts of NO than N limited soils [6–8]. In plants, NO is an important biological messenger participating in numerous cellular and physiological processes [9]. It induces changes in gene expression, up-regulates jasmonic acid and ethylene synthesis as well as photosynthesis [10,11]. In the context of plant N assimilation, NO is produced during the process of nitrate reduction and stimulates nitrate reductase activity as a feedback reaction [4,12,13]. Therefore, NO production may be regulated via nitrate reductase interacting with photosynthesis and primary metabolism [13]. Although exogenously applied NO enhances drought tolerance and increases fitness and competitiveness of plants [14], it is not known whether microbe-produced NO enters plant roots and acts as a signal in the competition between soil microorganisms and plant roots [15,16]. Here, we take the first step investigating a possible role of NO as a signalling compound for N uptake in the rhizosphere. We developed a combined root spray and fumigation facility for NO and show, for the first time, that NO in the rhizosphere increases the acquisition of reduced N compounds by beech roots.

2. Materials and methods

2.1. Experimental design

Two-year old non-mycorrhizal beech seedlings (provenance Swabian Alb, Germany) from a commercial tree nursery (Schlegel & Co. Gartenprodukte GmbH, Riedlingen, Germany) were incubated in a greenhouse equipped with a combined root spray and fumigation facility (gas-phase controlled aeroponics (Supplementary Fig. S1). Average temperature ranged from 14.5 °C (night, 8 h) to 19.5 °C (day, 16 h). Artificial illumination at plant height was 200 μE m^{-2} s^{-1} as typically observed in beech understorey. Roots were exposed for 72 h to one of three NO concentrations, i.e. 54 ± 39, 170 ± 42, or 1495 ± 43 ppb NO (daily average). These NO concentrations represent the range of NO concentrations measured in the gas phase of forest soils (mean level 250 ppb NO [17]). NO was mixed with 3000 ppm CO_2, which is the average CO_2 concentration in beech forest soil [17]. During a three day pre-incubation, roots were sprayed with an artificial soil solution, mimicking the composition of soil solution in acidic beech forest with high N availability, which contained 50 μM NH$_4$Cl, 300 μM KNO$_3$, and

* Corresponding author. Fax: +49 761 2038302.
E-mail address: judy.simon@ctp.uni-freiburg.de (J. Simon).

100 μM glutamine [18]. Nutrient solution was applied at a rate of 1.2 L 10 min^{-1}. After 72 h exposure to NO, seedlings were incubated in the artificial soil solution with ^{15}N-labelled ammonium or with ^{15}N/^{13}C-labelled glutamine, or non-labelled control solution. Nitrate uptake was not analysed because under the conditions applied it is not taken up by beech roots [18]. After 2 h, roots were excised, washed (0.5 mM CaCl$_2$), weighed, frozen in liquid N$_2$, finely ground and stored at -80 °C until analysis. Previous studies showed that ^{15}N is not exported to the shoot in significant amounts under these conditions [19].

2.2. Quantification of ^{15}N in roots

^{15}N and ^{13}C levels of oven-dried (48 h, 60 °C) root material (0.5–1.0 mg) were determined using an elemental analyser coupled to an isotope ratio mass spectrometer as previously described [18]. Isotope ratios are expressed as delta notation (‰) and rates of uptake were calculated from the increase in ^{15}N and ^{13}C abundance [18].

2.3. Preparation of RNA and cDNA

Total RNA was extracted from fine root tips according to Kiefer et al. [20]. Amount and purity of the RNA were determined (NanoDrop® ND-1000, Peqlab Biotechnology GmbH, Erlangen, Germany). cDNA was synthesised using *SuperScriptII* Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) according to manufacturer's instructions at a final concentration of 200 nM poly-T-primer. Control reactions without the enzyme were run in parallel.

2.4. Cloning of putative nitrogen transporters and selected enzymes of the N metabolism

Sequences of orthologous genes coding for N transporters and enzymes of N metabolism were identified in silico in the *Arabidopsis thaliana* and *Populus trichocarpa* genome databases (http://www.arabidopsis.org and http://genome.jgi-psf.org/Poptr1/Poptr1.home.html). Candidate sequences from *A. thaliana* and *P. trichocarpa* were aligned using ClustalW [21], conserved regions identified, and priming sites selected. Primer pairs were tested for each target gene. PCR conditions involved denaturation for 3 min 30 s at 94 °C, primer hybridisation at 55–60 °C (32–40 cycles), polymerase reaction 30–120 s at 72 °C and endpolymerisation (5 min at 72 °C). Elongation time was kept for 1–2 min. Separation of amplified DNA segments was performed using 1–1.5% agarose gel, extracted with Quiaex II Agarose Gel Extraction Kit (Qiagen, Hilden, Germany), cloned into *pCR2.1* vector (Invitrogen, Karlsruhe, Germany), and transformed into *Escherichia coli* (Infα- or Top10, Invitrogen). Beech, poplar and *Arabidopsis* sequences were aligned using Clustal W [21] and sequenced (MWG Biotech AG, Ebersberg, Germany). Sequence identity was verified using BiologyWorkbench (http://www.workbench.sdsc.edu/) for comparison with corresponding genes in *Arabidopsis* and *P. trichocarpa* databases.

2.5. Quantitative real-time PCR

Quantification of transcript abundance for selected N transporters and enzymes were performed using gene specific primers (Supplementary Table S1). Quantification of transcript abundance for selected N transporters and enzymes of N metabolism were performed using gene specific primers (Supplementary Table S1). Specificity of the primer pairs was tested by separate sequencing (MWG Biotech AG). mRNA transcript amounts of the genes were quantified by real-time PCR using the LightCycler 480 *SYBR®* Green Master Mix (Roche, Mannheim, Germany). Ten microlitres reaction volume contained 5 μL *SYBR®* Green I Master Mix (Roche), 2.5 μL

cDNA, and 2.5 μL specific primer pair mix (0.8 μM final concentration) (Roche). Samples were centrifuged at 3000×g for 3 min before starting real-time PCR. After 5 min at 95 °C, 45 PCR cycles were conducted with a 10 s melting step at 95 °C, 15 s annealing time at 55 °C or 60 °C, and 15 s extension time at 72 °C. Using the respective purified cDNA clone in the background of the *pCR2.1* vector (Invitrogen) as a standard, efficiency of the reaction and amplified transcript amounts were calculated. Transcript levels were related to the quantity of total RNA used for reversed transcription. α-Tubulin and 18srRNA were used as house-keeping genes.

2.6. Accession numbers

Sequences can be found in the NCBI database (http://www.ncbi.nlm.nih.gov) under the following accession numbers for *Fagus sylvatica*: DQ487770 (*FsAMT1.2*), DQ487771 (*FsCAT3*), DQ487772 (*FsCAT5.1*), DQ487773 (*FsCAT5.2*), DQ487774 (*FsCAT5.3*), DQ487776 (*FsAAT*), DQ487780 (*Fsα-TUB.1*), DQ487781 (*Fsα-TUB.2*), DQ487782 (*FsGSI*), DQ487783 (*FsAAP*), DQ487784 (*FsNADH-GOGAT.1*), and DQ487785 (*FsNADH-GOGAT.2*).

3. Results and discussion

3.1. Effects of NO fumigation on ammonium and glutamine uptake

With a gas phase controlled aeroponics system (see Supplementary Fig. S1) we tested whether uptake of ammonium or glutamine, at concentrations present in forest soil solution at high N availability, was affected by NO. Glutamine and ammonium uptake increased significantly at the highest NO concentration applied (1495 ppb, Fig. 1) and organic N uptake was preferred over inorganic N uptake at 1495 and 170, but not at 54 ppb NO ($P < 0.001$). These effects of NO cannot be attributed to an increase in nitrate produced inside the roots from NO influx, since root nitrate levels did not rise in response to NO fumigation (data not shown). A preference for organic N was also found for roots of adult beech trees growing in the field on alkaline substrate with low N availability [22] suggesting that this preference is a general pattern of beech roots regardless of soil N availability. Comparing absolute N uptake, our study shows higher uptake rates of all N sources compared with adult beech trees growing on a low N soil substrate [22], but similar uptake rates compared to those growing on acidic soil with high N availability [18].
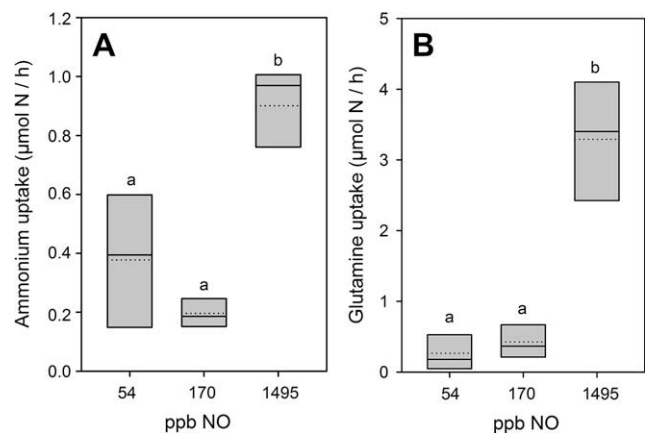

Fig. 1. Effect of NO on ammonium (A) and glutamine (B) N uptake of beech roots (μmol ^{15}N per h per g root dw). Box plots show mean ± S.D. (n = 5). Different letters for each N source indicate significant differences in N uptake at different NO concentrations ($P \leqslant 0.05$). Glutamine uptake was significantly ($P < 0.001$) higher than ammonium uptake at 170 and 1495 ppb NO.

Uptake of ammonium and glutamine was strongly affected by exposition to different NO concentrations (Fig. 1). At the highest NO level used in this study (1495 ppb), a significant increase in ammonium ($P \leqslant 0.003$) and glutamine uptake ($P \leqslant 0.001$) was observed. However, at the rhizospheric hotspots of nitrification/denitrification in close vicinity to the root surface [23] even higher NO concentrations are to be expected. These results indicate that NO can act as a signal for the N uptake of beech roots and may regulate the capacity for the uptake of inorganic and organic N sources by beech roots depending on the NO production by soil microbial activities.

3.2. Effects of NO fumigation on gene expression of putative N transporters and enzymes of glutamine synthesis

Expression analyses of genes of putative N transporters (Fig. 2) and enzymes of glutamine synthesis (data not shown) were con-

Fig. 2. Gene expression (×1000 copies/g RNA) of N transporters in roots of beech seedlings depending on NO fumigation level. (A) Ammonium transporter FsAMT1.2, (B) amino acid transporter FsAAT, (C) cationic amino acid transporter FsCAT3, (D) cationic amino acid transporter FsCAT5, and E. amino acid permease FsAAP. α-Tubulin and 18srRNA were used as reference genes and had a similar abundance in all NO concentrations. Box plots show mean ± S.D. ($n = 5$). Different letters indicate significant differences in mRNA amounts at different NO concentrations ($P < 0.05$).

ducted under varying levels of NO exposition to beech roots. The increase in organic and inorganic N uptake at the highest NO level used in this study (1495 ppb) was not reflected by an up-regulation of the expression of putative ammonium (i.e. *FsAMT1.2*) and amino acid transporter genes (i.e. *FsCAT5, AAT, and AAP*) indicating that ammonium and glutamine uptake by NO were not regulated at the level of gene expression (Fig. 2) and, thus, may be regulated at the posttranscriptional level similar to nitrate reductase [4]. Couturier et al. [24] saw a negative correlation between amounts of transcript of *AMT1.2* and glutamine concentration in poplar, which suggests that the expression of ammonium transporters is inhibited by glutamine. Similarly, Rawat et al. [25] showed for *Arabidopsis* that ammonium uptake is strongly correlated with *AMT1.2* transcript levels with glutamine functioning as a regulator. Therefore, the presence of glutamine in the spraying solution could have counteracted a potential increase in *AMT1.2* transcripts. Significant differences in expression levels of differently NO fumigated plants were only detected in amino acid transporter *FsCAT3* ($P = 0.032$) (Fig. 2). However, *FsCAT3* seems to be more specific for arginine transport, whereas *FsCAT5* seems to transport preferentially glutamine (Stoelken, unpublished results). NO fumigation did not alter transcript levels of enzymes of glutamine synthesis, i.e. glutamine synthetase and NADH-GOGAT (data not shown).

4. Conclusion

High availability of reduced organic and inorganic N sources stimulates the rate of nitrification in the soil [26]. Under these conditions, plants such as beech which preferentially take up reduced N sources [18] need to compete for uptake with microbial activity to ensure successful N acquisition as a major growth limiting factor. In this study, we provide first evidence that NO can stimulate the uptake of reduced N sources into beech roots. Thus, competition for reduced N sources in forest soil between beech roots and microbial nitrifiers can take place by an NO mediated increase in root N uptake.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.07.052.

References

[1] Gasche, R. and Papen, H. (2002) Spatial variability of NO and NO_2 flux rates from soil of spruce and beech forest ecosystems. Plant Soil 240, 67–76.
[2] Kitzler, B., Zechmeister-Boltenstern, S., Holtermann, C., Skiba, U. and Butterbach-Bahl, K. (2005) Nitrogen oxides emission from two beech forests subjected to different nitrogen loads. Biogeosci. Discuss. 2, 1381–1422.
[3] Mayer, B. and Hemmens, B. (1997) Biosynthesis and action of nitric oxide in mammalian cells. Trends Biochem. Sci. 22, 477–481.
[4] Du, S., Zhang, Y., Lin, X., Wang, Y. and Tang, C. (2008) Regulation of nitrate reductase by nitric oxide in Chinese cabbage pakchoi (*Brassica chinesis* L.). Plant Cell Environ. 31, 195–204.
[5] Davidson, E.Y., Keller, M., Erickson, H.E., Verchot, L.V. and Veldkamp, E. (2000) Testing a conceptual model of soil emissions of nitrous and nitric oxides. BioScience 50, 667–680.
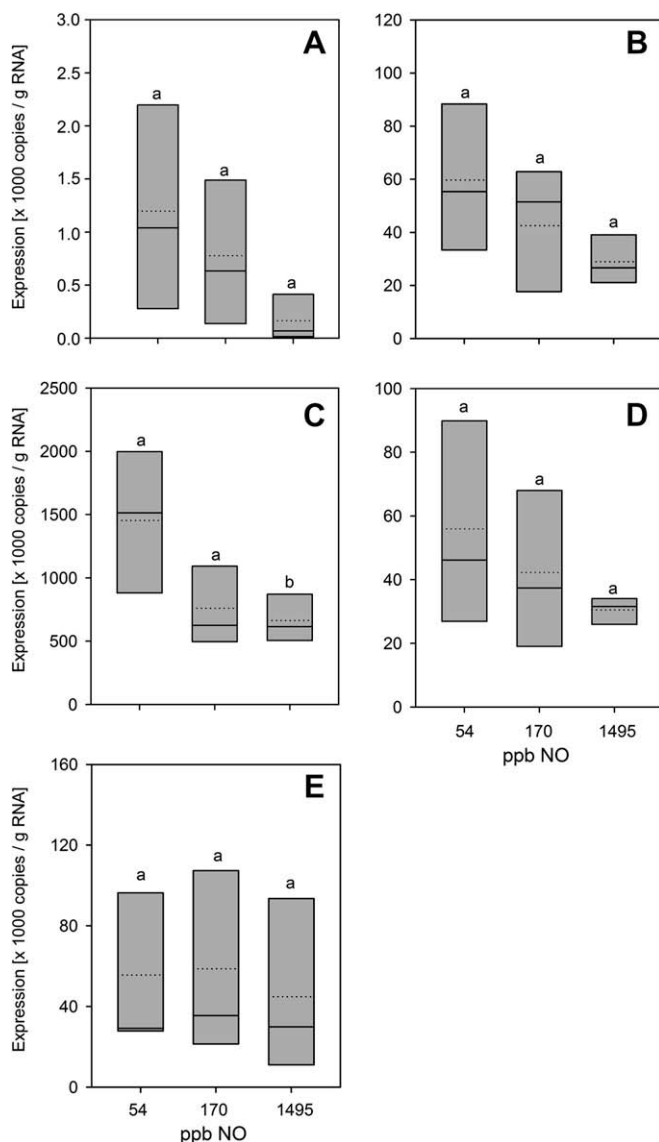
[6] Butterbach-Bahl, K., Gasche, R., Breuer, L. and Papen, H. (1997) Fluxes of NO and N$_2$O from temperate forest soils: impact of forest type, N-deposition and of liming on NO and N$_2$O emissions. Nutr. Cycl. Agroecosyst. 48, 79–90.

[7] Davidson, E.A. and Kingerlee, W. (1997) A global inventory of nitric oxide emissions from soils. Nutr. Cycl. Agroecosyst. 48, 37–50.

[8] Zechmeister-Boltenstern, S., Hahn, M., Meger, S. and Jandl, R. (2002) Nitrous oxide emissions and nitrate leaching in relation to microbial biomass dynamics in a beech forest soil. Soil Biol. Biochem. 34, 823–832.

[9] Palmieri, C., Sell, S., Huang, X., Scherf, M., Werner, T., Durner, J. and Lindermayr, C. (2008) Nitric oxide-responsive genes and promoters in *Arabidopsis thaliana*: a bioinformatics approach. J. Exp. Bot. 59, 177–186.

[10] Huang, X., von Rad, U. and Durner, J. (2002) Nitric oxide induces transcriptional activation of the nitric oxide-tolerant alternative oxidase in *Arabidopsis thaliana* suspension cells. Planta 215, 914–923.

[11] Polverari, A., Molesini, B., Pezzotti, M., Buonaurio, R., Marte, M. and Delledonne, M. (2003) Nitric oxide-mediated transcriptional changes in *Arabidopsis thalina*. Mol. Plant–Microb. Interact. 16, 1094–1105.

[12] Yamasaki, H. and Sakihama, Y. (2000) Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence fort he NR-dependent formation of active nitrogen species. FEBS Lett. 468, 89–92.

[13] Rockel, P., Strube, F., Rockel, A., Wildt, J. and Kaiser, W.M. (2002) Regulation of nitric oxide (NO) production by plant nitrate reductase in vivo and in vitro. J. Exp. Bot. 53, 103–110.

[14] García Mata, C. and Lamattina, L. (2001) Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. Plant Phys. 126, 1196–1204.

[15] Jackson, L.E., Schimel, J.P. and Firestone, M.K. (1989) Short term partitioning of ammonium and nitrate between plants and microbes in an annual grassland. Soil Biol. Biochem. 21, 409–415.

[16] Wang, J.G. and Bakken, L.R. (1997) Competition for nitrogen during mineralization of plant residues in soil: microbial response to C and N availability. Soil Biol. Biochem. 29, 163–170.

[17] Rudolph, J. and Conrad, R. (1996) Flux between soil and atmosphere, vertical concentration profiles in soil, and turnover of nitric oxide: 2. Experiments with naturally layered soil cores. Atmos. Chem. 23, 275–300.

[18] Gessler, A., Schneider, S., von Sengbusch, D., Weber, P., Hanemann, U., Huber, C., Rothe, A., Kreutzer, K. and Rennenberg, H. (1998) Field and laboratory experiments on net uptake of nitrate and ammonium by the roots of spruce (*Picea abies*) and beech (*Fagus sylvatica*) trees. New Phytol. 138, 275–285.

[19] Gessler, A., Kreuzwieser, J., Dopatka, T. and Rennenberg, H. (2002) Diurnal courses of ammonium net uptake by roots of adult beech (*Fagus sylvatica*) and spruce (*Picea abies*) trees. Plant Soil 240, 23–32.

[20] Kiefer, E., Heller, W. and Ernst, D. (2000) A simple and efficient protocol for isolation of functional RNA from plant tissues rich in secondary metabolites. Plant Mol. Biol. Report. 18, 33–39.

[21] Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res.

[22] Dannenmann, M., Simon, J., Gasche, R., Holst, J., Pena, R., Naumann, P.S., Kögel-Knabner, I., Knicker, H., Mayer, H., Schloter, M., Polle, A., Rennenberg, H., and Papen, H. (2009) Tree girdling provides insight in the role of labile carbon in the competitive balance of N partitioning between soil microorganisms and adult European beech. Soil Biol Biochem. doi:10.1016/j.soilbio.2009.04.024.

[23] Schimel, J.P. and Benett, J. (2004) Nitrogen mineralization: challenges of a changing paradigm. Ecology 85, 591–602.

[24] Couturier, J., Montanini, B., Martin, F., Brun, A., Blaudez, D. and Chalot, M. (2007) The expanded family of ammonium transporters in the perennial poplar plant. New Phytol. 174, 137–150.

[25] Rawat, S.R., Silim, S.N., Kronzucker, H.J., Siddiqi, M.Y. and Glass, A.D.M. (1999) AtAMT1 gene expression and NH$_4^+$ uptake in roots of *Arabidopsis thaliana*: evidence for regulation by root glutamine levels. Plant J. 19, 143–152.

[26] Gilliam, F.S., Lyttle, N.L., Thomas, A. and Adams, M.B. (2005) Soil variability along a nitrogen mineralization and nitrification gradient in a nitrogen–saturated hardwood forest. Soil Sci. Soc. Am. J. 69, 247–256.