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Nonsymbiotic nitrogen fixation in 3-year-old Jeffrey pines and the role of elevated [CO_2]

P.S.J. Verburg, W. Cheng, D.W. Johnson, and D.E. Schorran

Abstract: Increased belowground labile C inputs under elevated [CO_2] could stimulate nonsymbiotic N_2 fixation, thereby enhancing growth responses of vegetation to elevated [CO_2] on nutrient-poor sites. To test this hypothesis, nonsymbiotic N_2 fixation rates in soils planted with 3-year-old Jeffrey pine (*Pinus jeffreyi* Grev. & Balf.) trees grown under 365 and 700 $\mu L \cdot L^{-1}$ atmospheric [CO_2] were measured by exposing the soil to $^{15}N_2$-enriched air for 78 d. Nitrogen fixation rates were estimated by measuring ^{15}N content of trees and soil. Compared with the ambient CO_2 treatment, the elevated CO_2 treatment did not affect biomass, N content, or $\delta^{15}N$ of individual plant parts and soils, indicating that elevated [CO_2] did not stimulate nonsymbiotic N_2 fixation. Because belowground C inputs did not increase under elevated [CO_2], the initial hypothesis could not be accepted or rejected. The results from the $^{15}N_2$ labeling study agree with other studies showing that nonsymbiotic N_2 fixation is not likely to provide a large input of N in forest ecosystems. The $^{15}N_2$ labeling technique was promising for studying N_2 fixation in plant–soil systems, but the preliminary nature of this study did not allow for firm conclusions with regard to the effects of elevated [CO_2].

Résumé : L'augmentation des apports de C labile dans le sol en présence d'une concentration élevée de CO_2 pourrait promouvoir la fixation non symbiotique de N_2 et par conséquent accroître la réponse en croissance de la végétation à une concentration élevée de CO_2 sur les sites pauvres en nutriments. Pour tester cette hypothèse, nous avons mesuré le taux de fixation non symbiotique de N_2 dans des sols où avaient été plantés des pins de Jeffrey (*Pinus jeffreyi* Grev. & Balf.) âgés de 3 ans et cultivés en présence de concentrations de CO_2 atmosphérique de 365 et 700 $\mu L \cdot L^{-1}$ en exposant le sol à de l'air enrichie avec $^{15}N_2$ pendant 78 j. Le taux de fixation de N a été estimé en mesurant le contenu en ^{15}N dans les arbres et le sol. Comparativement à la concentration ambiante de CO_2, la concentration élevée de CO_2 n'a pas affecté la biomasse, le contenu en N et la valeur de $\delta^{15}N$ ni dans le sol, ni dans les différentes parties des plants, indiquant que la concentration élevée de CO_2 n'a pas favorisé la fixation non symbiotique de N_2. L'hypothèse de départ ne peut être ni confirmée, ni infirmée étant donné que les apports de C dans le sol n'ont pas augmenté avec une concentration élevée de CO_2. Les résultats de l'expérience de marquage avec $^{15}N_2$ concordent avec ceux des autres études qui montrent qu'il est peu probable que la fixation non symbiotique de N_2 fournisse un apport significatif de N dans les écosystèmes forestiers. L'utilisation de N_2 marqué est une technique prometteuse pour étudier la fixation de N_2 dans les systèmes plantes–sol mais la nature préliminaire de cette étude ne permet pas de tirer de conclusions fermes concernant les effets d'une concentration élevée de CO_2.

[Traduit par la Rédaction]

Introduction

Over the past few decades, several studies have shown N accretion rates in forest soils that are larger than what can be expected based on atmospheric inputs (Binkley et al. 2000). Some of these anomalously high N accumulation rates have been ascribed to nonsymbiotic N_2 fixation (Vitousek et al. 1983; Bormann et al. 1993; Johnson and Todd 1998). Although there is direct evidence that nonsymbiotic N_2 fixation can be an important N input for some agricultural crops (Chalk 1991), its importance for natural forest ecosystems is still being debated (Binkley et al. 2000). The highest potential N_2 fixation rates measured were associated with decaying wood (Aho et al. 1974; Roskoski 1980; Crawford et al.

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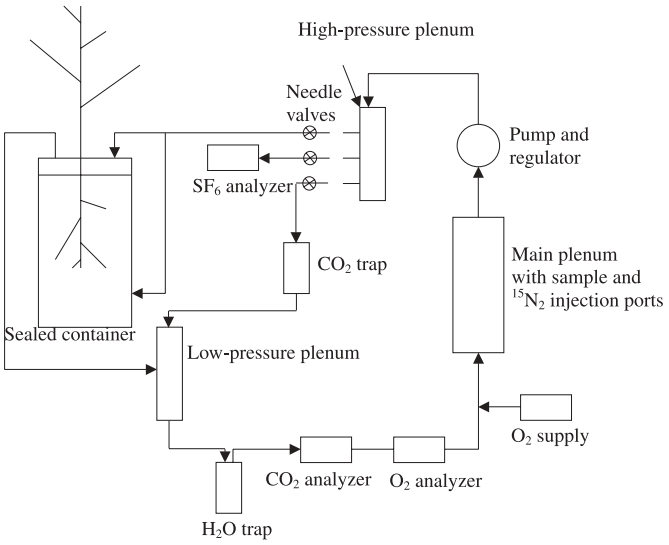
Fig. 1. Configuration of the ^{15}N labeling system. Although only one pot is shown, all eight labeled pots were connected to the same main and secondary plenums. The $^{15}N_2$ gas was fed through the top and bottom of each soil container to ensure that $^{15}N_2$ was distributed throughout the soil. Prior to injecting, the $^{15}N_2$ gas was fed through a liquid argon trap to condense N_2O, NH_3, and NO, but not $^{15}N_2$. SF_6 samples taken from different parts of the system indicated that complete air mixing throughout the system took less than 3 h.



1997). Nonsymbiotic N_2 fixation in forest ecosystems could potentially be important in regulating growth responses of vegetation to elevated [CO_2]. Several studies of the effects of elevated [CO_2] have documented an apparent increase in N uptake (Johnson et al. 1995, 1997; Zak et al. 2000). This raises interesting questions regarding the source of N for trees under elevated [CO_2]: was the additional N under elevated [CO_2] derived from: (1) increased mineralization, (2) increased soil exploration, or (3) nonsymbiotic N_2 fixation (Bormann et al. 1993)? Hypotheses 1 and 2 have often been used to explain the additional N (Johnson et al. 1997; Zak et al. 2000). Hypothesis 3 is intriguing, as it would be a potential source of N that would not require extra root exploration, which may be difficult to accomplish in a mature, closed-canopy forest ecosystem, where the soil has been explored by tree roots for many decades. Nitrogen fixation requires a supply of labile C (e.g., Arnone and Gordon 1990; Mulder 1975). Several studies have shown that symbiotic N_2 fixation increases under elevated [CO_2] as a result of increased C availability (e.g., Norby 1987; Arnone and Gordon 1990), but this effect has not been demonstrated for nonsymbiotic N_2 fixation. Elevated [CO_2] can result in increased inputs of labile C into the soil by increasing root exudation and root litter production (Cheng and Johnson 1998; Van Ginkel and Gorissen 1998; Verburg et al. 1998). This increase in labile C might stimulate nonsymbiotic N_2 fixation and thus N availability. Since growth responses to elevated [CO_2] are often limited by low nutrient availability (e.g., Johnson et al. 1995; Oren et al. 2001), nonsymbiotic N_2 fixation could enhance biomass production under elevated [CO_2] by helping to alleviate N limitation on nutrient-poor sites.

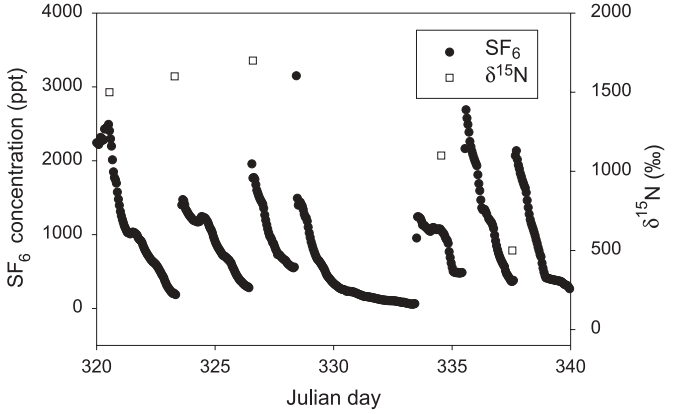
Currently, very few studies exist that have measured nonsymbiotic N_2 fixation directly. Potential N_2 fixation rates measured using the acetylene-reduction method show much lower N_2 fixation rates than estimates based on field studies (Tjepkema 1978; Grant and Binkley 1987; Hendrickson 1990; Barkmann and Schwintzer 1998). In this paper, we investigate the effects of elevated [CO_2] on nonsymbiotic N_2 fixation by exposing the soil of Jeffrey pine (*Pinus jeffreyi* Grev. & Balf.) to $^{15}N_2$-enriched air (Sims et al. 1986; Warembourg 1993; Bremer et al. 1995). We hypothesized that nonsymbiotic N_2 fixation is stimulated by elevated [CO_2], because of enhanced belowground labile C inputs from root exudation and root litter production.

Materials and methods

We filled 16 acrylic containers (49 cm in length, 28 cm in width; square in cross section) with approximately 35 kg of soil and planted 3-year-old Jeffrey pine trees. The soil was a mixture of two parts (by vol.) local sandy topsoil (0–15 cm; frigid Typic Xeropsamment, derived from granitic parent material) and one part organic surface horizon material collected from a local Jeffrey pine stand. Volumetric soil moisture was monitored three times per week using Time Domain Reflectrometry probes installed 5 cm above the bottom of the containers. If needed, water was added to obtain a soil moisture content of 15%. Moisture content in any of the pots was never below 11%. Prior to the CO_2 treatment, the trees were kept at ambient [CO_2] inside a naturally lit greenhouse at the Desert Research Institute in Reno, Nevada, from May until the end of September. Day : night temperatures inside the greenhouse were 28 °C : 23 °C; maximum photon flux density was 1866 μmol photons·m^{-2}·s^{-1}.

Prior to the CO_2 treatment, 8 out of 16 containers were covered with acrylic sheets, and openings around the stems were sealed using paraffin wax to separate the shoot from the root compartment. Plumbers' putty was put around the wax to ensure an airtight seal. Three holes were drilled in the top; one for watering, one inlet, and one outlet for $^{15}N_2$-enriched air. All holes contained a threaded brass fitting sealed with Teflon tape to prevent air leaks. The watering hole was closed by an airtight cap between water additions. An extra air inlet was placed 2 cm above the bottom of the container to facilitate distribution of the ^{15}N label to the soil. The top outlet was connected to a pump continuously pulling air through the soil (Fig. 1). Airflow through the bottom inlet was checked regularly to ensure that the airways were not obstructed. The inlets of all pots were connected to a 10-L main plenum, where the $^{15}N_2$ was added. Two other plenums served as buffers to compensate for pressure changes as a result of the gas additions. Air coming from the low-pressure plenum was sampled hourly for [O_2] and [CO_2]. Excess CO_2 was removed using a soda lime trap. We injected known amounts 99% $^{15}N_2$ (Cambridge Isotope Labs, Andover, Massachusetts) at least twice each week during the experiment. The $^{15}N_2$ gas was delivered at a flow rate of 100 mL·min^{-1} to the main 10-L plenum using a mass flow controller, and the amounts added were based on timed injections. The $^{15}N_2$ was fed through a liquid argon trap prior to entering the main plenum to condense N_2O, NH_3, and

Fig. 2. The changes in SF_6 concentration and $\delta^{15}N$ signal during 20 d of the 78-d ^{15}N labeling experiment. Samples for $^{15}N_2$ were taken manually from the main plenum. Air for SF_6 concentrations was sampled automatically from the high-pressure plenum directly connected to an SF_6 analyzer (see Fig. 1).

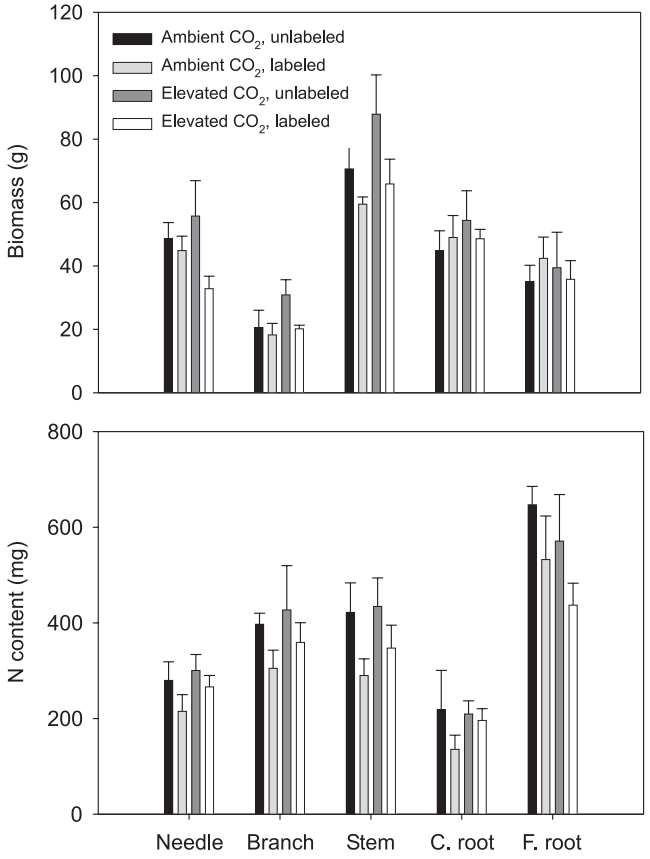


NO, but not $^{15}N_2$. With every $^{15}N_2$ injection, we also injected 50–100 cm^3 of sulfur-hexa-fluoride (SF_6) with a concentration of 2.29 ± 0.04 $\mu L \cdot L^{-1}$ as a chemically and biologically inert tracer to check for leaks in the system. The SF_6 was measured hourly throughout the duration of the experiment using a Varian 3700 gas chromatograph equipped with an electron capture detector. To confirm that all air was well mixed within the system, we measured the SF_6 content in different parts of the system including plenum, headspace of the soil containers, and tubing. Complete air mixing typically occurred within 3 h after each addition. The air was sampled regularly for $^{15}N_2$ analysis. Extra O_2 was added automatically if $[O_2]$ dropped below 15%. The soil $[O_2]$ was kept at 15% during the experiment, and soil $[CO_2]$ was kept below 3.5%. The soil $[O_2]$ was monitored using a Servomex 1440 gas analyzer, and CO_2 was monitored using a LiCOR-6262 infrared gas analyzer. For logistical reasons, we could not connect the control containers to a similar closed gas circulation system.

On 27 September, four covered and four uncovered containers were put in a naturally lit growth chamber (Taub et al. 2000) having an atmospheric $[CO_2]$ of 365 $\mu L \cdot L^{-1}$. The remaining eight containers were put in a growth chamber operating at 700 $\mu L \cdot L^{-1}$. Day:night growth temperatures were 25 °C : 15 °C. Day length was 12 h. Maximum photosynthetic photon flux density was 1100 µmol photons$\cdot m^{-2} \cdot s^{-1}$. Light, temperature, and $[CO_2]$ inside each chamber were monitored automatically and recorded every 15 min. Because of the complexity of the setup, we could not rotate chambers during the experiment to eliminate potential chamber effects. All measured environmental parameters, except for CO_2, were identical between the chambers, however, so we assumed that chamber effects were minimal.

After 78 d, the trees were harvested, and biomass was separated into live needles, branches, stems, coarse roots (>2 mm), and fine roots (≤2 mm). All biomass samples were dried at 70 °C to constant weight. The soil was separated into bulk and rhizosphere soil. The trees were carefully lifted out of the containers and, after gently shaking, any soil adhering to the intact roots was considered rhizosphere soil (Cheng and Coleman 1990). This method allowed us to collect soil that had been in immediate contact with the root system. Roots were removed from both bulk and rhizosphere soil by handpicking and rinsed to remove adhering soil particles. Soil samples were homogenized and dried at 70 °C to constant weight. The $^{15}N_2$ and total N content in air, tree, and soil samples were analyzed at the University of California, Davis, using a Europa Scientific "Integra" analyzer (Crewe, UK). The soils were analyzed for total C using a PerkinElmer 2400 CHN analyzer.

Fig. 3. Biomass and N content of needle, branch, stem, coarse roots (C. root), and fine roots (F. root) of Jeffrey pines. Eight trees were exposed to ambient $[CO_2]$ (365 $\mu L \cdot L^{-1}$) for 78 d, while the remaining eight trees were exposed to elevated $[CO_2]$ (700 $\mu L \cdot L^{-1}$). Within each CO_2 treatment, four trees were exposed to a ^{15}N-enriched soil atmosphere. The ANOVA results showed no significant main nor interactive effects of the labeling and CO_2 treatment ($P < 0.05$). Error bars represent standard errors of the mean ($n = 4$).



Effects of CO_2 on plant and soil C, N content, and $\delta^{15}N_2$ were analyzed by two-way analysis of variance (ANOVA) with "labeled vs. unlabeled" and "CO_2 treatment" as main factors using DataDesk version 6.0. Because we assumed that chamber effects were absent, we treated our experiment as a fully replicated design. Main effects were considered to be significant if $P < 0.05$.

Results and discussion

Throughout the experiment, the system maintained a $^{15}N_2$-enriched atmosphere. The SF_6 data indicated, however,

Fig. 4. The $\delta^{15}N$ values of needle, branch, stem, coarse roots (C. root), and fine roots (F. root) of Jeffrey pines, bulk soil (B. soil), and rhizosphere soil (Rh. soil). Eight trees were exposed to ambient [CO_2] (365 $\mu L \cdot L^{-1}$) for 78 d, while the remaining eight trees were exposed to elevated [CO_2] (700 $\mu L \cdot L^{-1}$). Within each CO_2 treatment, four trees were exposed to a ^{15}N-enriched soil atmosphere. The ANOVA results showed no significant main nor interactive effects of the labeling and CO_2 treatment ($P < 0.05$). Error bars represent standard errors of the mean ($n = 4$).

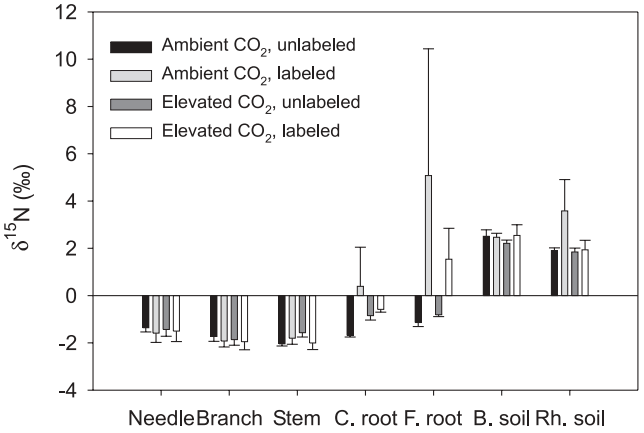
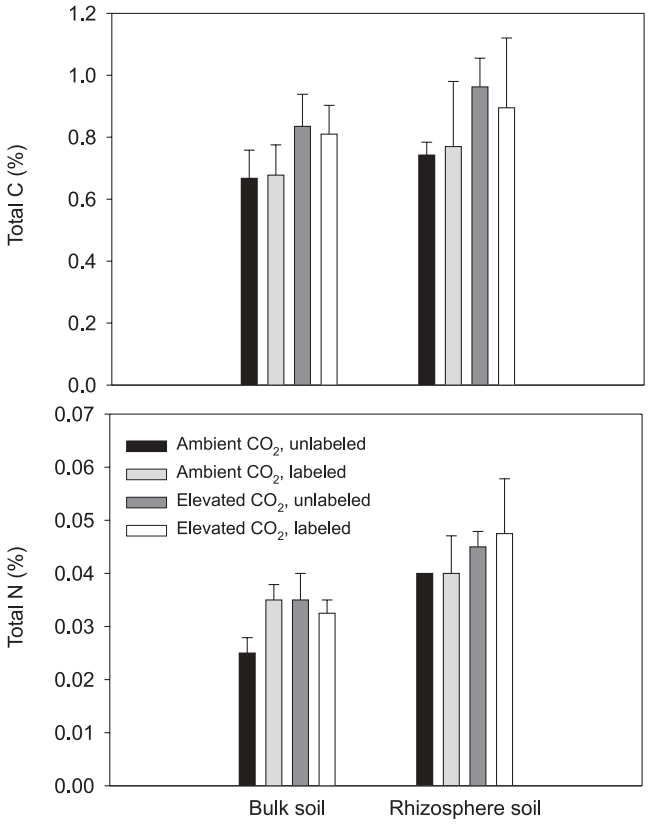


Fig. 5. Total C and N of bulk and rhizosphere soil planted with Jeffrey pines. Eight trees were exposed to ambient [CO_2] (365 $\mu L \cdot L^{-1}$) for 78 d, while the remaining eight trees were exposed to elevated [CO_2] (700 $\mu L \cdot L^{-1}$). Within each CO_2 treatment, four trees were exposed to a ^{15}N-enriched soil atmosphere. The ANOVA results showed no significant main nor interactive effects of the labeling and CO_2 treatment ($P < 0.05$). Error bars represent standard errors of the mean ($n = 4$).



that leaks were present in the system (Fig. 2). The leaks did not have any effects on measurement of relative effects of CO_2 treatments, however, because the applied label was exactly the same in each container. Using the SF_6 data, we calculated that the total air volume inside the system was 115 L. Since the natural abundance of $^{15}N_2$ equals 0.366%, the total amount of $^{15}N_2$ present was 0.42 L. Each time we injected between 0.5 and 1 L of pure ^{15}N so initially the ^{15}N% was doubled resulting in a $\delta^{15}N$ signal between 1000‰ and 2000‰ (Fig. 2). The $\delta^{15}N$ values prior to new additions were never lower than 500‰.

The labeling procedure and CO_2 treatment did not affect weight, N content, or $\delta^{15}N$ of individual plant parts and soils (Figs. 3, 4, and 5). The results from our $^{15}N_2$ labeling study support results from other studies, showing that nonsymbiotic N_2 fixation is not likely to provide a large input of N in forest ecosystems (e.g., Barkmann and Schwintzer 1998) and cannot explain large N accretion rates found in several forest ecosystems (Binkley et al. 2000). In addition, N_2 fixation did not increase under elevated [CO_2]. We initially hypothesized, however, that N_2 fixation would be stimulated by elevated [CO_2] through increased belowground labile C inputs. Since belowground C inputs did not increase under elevated [CO_2] (Fig. 5), we cannot accept or reject our initial hypothesis based on the results of this study. Several other studies have shown that elevated [CO_2] enhances rhizodeposition in a variety of natural and agricultural plant and tree species (Cheng and Johnson 1998; Van Ginkel and Gorissen 1998; Verburg et al. 1998). Microbial immobilization could have prevented uptake of the label by trees (Mulder 1975), but soils did not show any $^{15}N_2$ enrichment, indicating that no N_2 fixation had occurred. Potentially, the CO_2 treatment was too short to be able to detect significant increases in belowground C inputs in these relatively slow-growing trees.

Since this study represented a first attempt using the $^{15}N_2$ labeling technique, the experiment was compromised by pseudo-replication, a small sample size, and a relatively short duration. Also we did not cover the unlabeled containers, which may have resulted in differences in soil [O_2], an important parameter affecting nonsymbiotic N_2 fixation (Mulder 1975). Still, soils were oxic in both unlabeled and labeled containers, and the ^{15}N label should have been strong enough to detect fixation of $^{15}N_2$. The experimental limitations in combination with the high variability observed within each treatment make it difficult to draw firm conclusions from this study. Variability was especially large for the $\delta^{15}N$ values of soil and roots of the labeled, ambient CO_2 treatment. In this treatment, one tree showed $\delta^{15}N$ values of 21.1‰, 5.2‰, and 7.5‰ for the fine roots, coarse roots, and rhizosphere soil, respectively. The fraction of ^{15}N in this plant originating from N_2 fixation X was approximated by the method of Balesdent et al. (1987):

$$X = \frac{\delta^{15}N_{\text{labeled plant}} - \delta^{15}N_{\text{unlabeled plant}}}{\delta^{15}N_{\text{air}} - \delta^{15}N_{\text{unlabeled plant}}}$$

If we assume that the average $\delta^{15}N$ of the labeled air was 500‰, and that there is no isotope fractionation during N_2 fixation (Nadelhoffer and Fry 1994), the fraction of ^{15}N

originated from N_2 fixation for fine roots at ambient [CO_2] was 0.0445 or 4.45% of total ^{15}N. Multiplying this percentage with the N content of the fine and coarse roots in this tree resulted in a total amount of N_2 fixation of 12.4 mg during the 78 d. This estimate represents an upper limit, since the label was higher than 500‰ for most of the time. This overestimation may be partly offset, however, by uptake in favor of the lighter ^{14}N isotope. The only apparent difference between this tree and all other trees was that the roots showed a high infection with ectomycorrhizal fungi despite the trees not having been inoculated. Although it was just one tree, our results agree with other studies that have found various species of N_2-fixing microbes associated with ecto- and endo-mycorrhizae (Amaranthus et al. 1990; Li et al. 1992). Further study is needed to determine if the high uptake found in this tree was caused by presence of ectomycorrhizal fungi, or if other factors caused this anomalous result.

Our $^{15}N_2$ labeling technique appeared promising for measuring N_2 fixation rates. At this stage, the experimental limitations and short duration of the study prevented us from drawing firm conclusions with regard to the effects of elevated [CO_2] on nonsymbiotic N_2 fixation, and our initial hypothesis could not be accepted nor rejected. Variability in the ambient [CO_2] labeled treatment was especially large because of one tree having an abundance of ectomycorrhizal fungi. The described technique promises to be a useful tool to further investigate mechanisms of atmospheric N_2 fixation under highly controlled conditions.

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