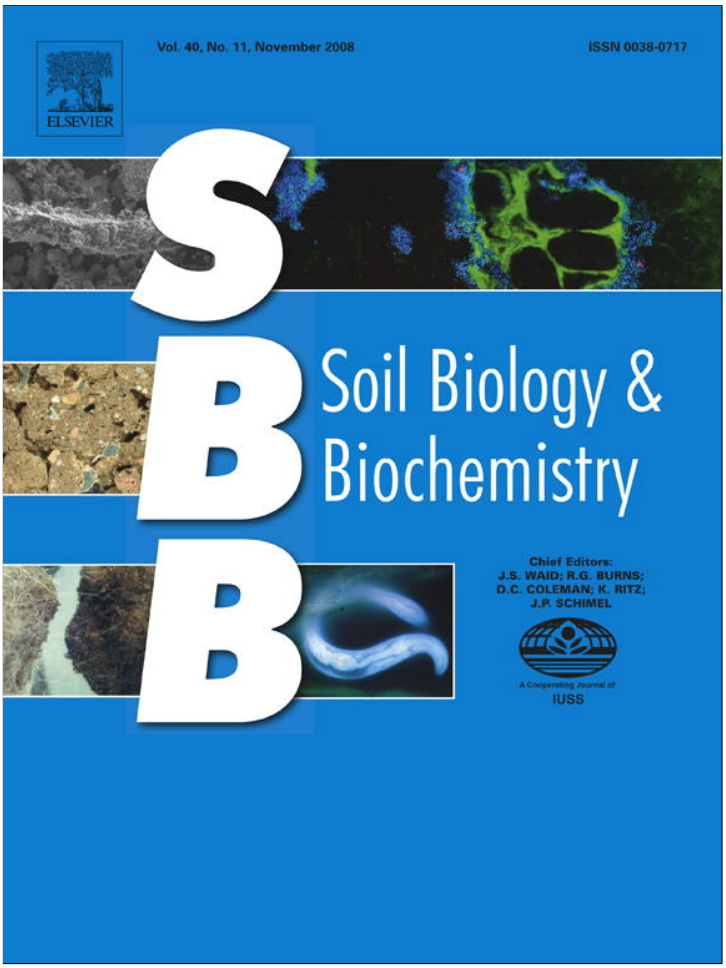

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Ammonium versus nitrate nutrition of *Zea mays* and *Lupinus albus*: Effect on root-derived CO_2 efflux

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ABSTRACT

Identification of the mechanisms contributing to nitrogen (N) fertilizer-induced changes in CO_2 efflux from soil under agricultural crops has been extremely challenging because of difficulties in separating root and microbial contribution to total CO_2 efflux. In this study we present the evidence that high costs of nitrate reduction result in a strong increase of root-derived respiration and the magnitude of an increase differs between the species with various contribution of shoots and roots to the nitrate reduction process.

Fertilization of *Lupinus albus* and *Zea mays* with nitrate or ammonium and pulse labeling of plants in $^{14}CO_2$ atmosphere allowed evaluation of the effect of N type on total and recently assimilated CO_2 efflux from soil. Addition of nitrate to planted soil increased recently assimilated CO_2 efflux by 168% in *Lupinus albus* (nitrate reduction site – in roots) and by 121% in *Zea mays* (nitrate reduction site both, in shoots and roots) in comparison with control. Ammonium-induced CO_2 increase amounted for 82% in *Lupinus albus* and for 73% in *Zea mays*. Clear diurnal changes in CO_2 efflux from planted soil at constant day/night temperature showed fast response of below-ground processes to photosynthesis. Both approaches for root-derived CO_2 assessment: ^{14}C pulse labeling and difference of CO_2 from planted and unplanted soil showed similar results: the form of N supply and the location of the nitrate reduction site have a strong significant effect on the amount of root-derived CO_2 respiration.

1. Introduction

The nitrogen (N) requirements of plants can be met by both nitrate (NO_3^-) and ammonium (NH_4^+) ion assimilation (Lasa et al., 2002). Utilization of nitrogen in either form may affect the carbohydrate metabolism and energy economy of the plant (Blacquiere, 1987). NO_3^- ions can be accumulated in vacuoles, and so most plant species can transport nitrates to leaves for reduction and assimilation and are able to tolerate high nitrate concentrations without any sign of toxicity. However, NH_4^+ salts absorbed by the plant must be rapidly metabolized into organic nitrogen compounds as many plants tolerate few or no excess ammonium ions (Barker et al., 1996; Chaillou et al., 1994). So almost all NH_4^+ ions are assimilated in roots. This difference in the site for N assimilation leads to a difference in the demand of carbon (C) skeletons, which are provided in part by the phosphorylating cytochrome (TCA) cycle, and hence to a difference in the respiration rate (Lasa et al., 2002).

However, there are still active debates on the effect of the N source on root respiration, as attempts to explain it experimentally have led to arguable results supporting different hypotheses. Some authors suggest that, when compared to NO_3^- nutrition, NH_4^+ nutrition stimulates the rate of root respiration, attributing this increase to the stimulation of alternative pathway activity (Barneix et al., 1984; Blacquiere, 1987; Lasa et al., 2002). There are two pathways involved in respiration: the phosphorylating cytochrome and the non-phosphorylating alternative pathway. The physiological role of the latter is not clear but several authors suggest that this alternative pathway could avoid the overreduction of the electron transport chain and the subsequent production of reactive oxygen species (Purvis and Shewfelt, 1993). Thus, this pathway could allow oxidation of TCA cycle reductant, maintaining TCA cycle carbon flow for provision of biosynthetic intermediates for NH_4^+ ion assimilation.

On the other hand, NO_3^- coming to the plant before assimilation have to be firstly reduced to NH_4^+, and this process, together with assimilation, is among the most energy-intensive processes in plants, in some cases followed by an additional CO_2 evolution (Atkins et al., 1979; Aslam and Huffacker, 1982; Ninomiya and Sato, 1984; Warner and Kleinhofs, 1992; Blacquiere, 1987; Tischner, 2000). The process proceeds in two steppes: conversion of NO_3^- to NO_2^- and the following conversion of NO_2^- to NH_4^+. In illuminated

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leaves, these processes are coupled to photosynthetic electron transport. However, in roots and during darkness, reducing equivalents are generated by oxidation of carbohydrates with subsequent evolution of CO_2 (Aslam and Huffacker, 1982; Ninomiya and Sato, 1984).

Depending on the species, the site of NO_3^- reduction could be located in shoots or roots (Andrews, 1986; Oaks and Hirel, 1985; Pate and Layzell, 1990; Schilling et al., 2006; Silveira et al., 2001; Vuylsteker et al., 1997). By this property, plants are divided into three groups: species reducing NO_3^- predominantly in roots, species reducing NO_3^- predominantly in shoots, and those that do both. The C costs for reduction of NO_3^- to NH_4^+ depend on the site of nitrate reduction in plants.

In this study we use the term "root-derived CO_2" for the sum of actual root respiration and CO_2 derived from microbial activity in the immediate vicinity of the root (rhizomicrobial respiration) and "SOM-derived respiration" for CO_2 evolved after microbial decomposition of soil organic matter in root free soil. We selected maize and lupine since the two species have different sites of nitrate reduction: *Zea mays* reduces half of the NO_3^- in shoots and half in roots and *Lupinus albus* reduces the major part of the NO_3^- in roots (Pate, 1973). The objective of the present work was to confirm or refute that feeding lupine and maize with NH_4^+ reduces root-derived efflux from soil compared to feeding with NO_3^-. Three nitrogen treatments were applied to each species: nitrate fertilizer, ammonium fertilizer, and a control treatment without any N fertilizer. A nitrification inhibitor was used to prevent microbial conversion of NH_4^+ to NO_3^- in soil. Pulse labeling of plants in a $^{14}CO_2$ atmosphere was applied to quantify the effect of both fertilizers on recently (^{14}C) and total assimilated C. The difference between total CO_2 efflux from the plant-soil system and microbial respiration from bare soil incubated at the same conditions was compared with the results of the principal method of labeling for root-derived CO_2 quantification.

2. Materials and methods

2.1. Soil

The soil, a loamy Haplic Luvisol, was taken from the top 10 cm (Ap horizon) of the Karlshof long-term field experimental station of the University of Hohenheim. Soil samples were air dried, mixed and passed through 5 mm sieve. The soil contained 1.5% C_{tot} and 0.14% N_{tot}, with 2.9% sand, 74.5% silt and 22.6% clay; its pH was 6.5.

2.2. Plants and growth conditions

Centrifuge tubes of 50 ml were filled with 50 g of soil each and were used for growing the plants. Twenty four pots remained unplanted to measure microbial respiration from bare soil.

Seeds of maize (*Zea mays* L.) and lupine (*Lupinus albus* L.) were germinated on moist filter paper in Petri dishes for 2 days. Germinated seedlings were transplanted to the PVC pots, with one seedling per pot, and were grown under controlled laboratory conditions with a 12 h/12 h day/night period at a constant day and night temperature of $25 \pm 0.5\,^{\circ}C$, and with a photosynthetically active radiation (PAR) intensity of approximately 800 $\mu mol\ m^{-1}\ s^{-1}$ at the top of the plant canopy. A constant day/night temperature was chosen to avoid the effects of changing temperature on CO_2 fluxes. During the experiment, soil water content in each pot was maintained gravimetrically at about 60% of the available field capacity by checking its weight daily. Before the labeling, the weakest plants were eliminated and only twenty-four plants similar in development and height were chosen for the following treatments. Pots with bare soil were exposed to the same incubating conditions.

2.3. ^{14}C labeling and N application

Two species were labeled with ^{14}C: 12 plants of maize were chosen and labeled in the morning on the 20th day after germination; 12 plants of lupine were labeled on the 36th day after germination.

One day before the labeling, the top of each pot was sealed with a silicone paste (NG 3170 from Thauer and Co., Dresden, Germany). The seal was tested for air leaks. Pumping the air through the soil column flushed out the CO_2 accumulated in the soil during the plant's growth.

Three nitrogen treatments were applied 4 h before ^{14}C labeling: (a) a nitrate treatment, with ^{15}N as $K^{15}NO_3$; (b) an ammonium treatment, with ^{15}N as $(^{15}NH_4)_2SO_4$; and (c) a control variant without any added nitrogen. Four plants of each species were exposed to each N treatment (^{15}N enrichment 50 atom %). Dicyandiamide (DCD) at 20 mg kg^{-1} soil was applied in solution with ^{15}N fertilizer to all the treatments in order to achieve an effective nitrification inhibition throughout the soil column (in the ammonium treatment) and to balance the side effects of the inhibitor (in the nitrate and control treatments). The amount of ^{15}N applied to a pot was calculated to produce an average concentration of 60 mg of N kg^{-1} for each N species added. Four unplanted pots were fertilized with half amount of nitrate or ammonium to estimate the effect of N fertilization on respiration of soil microorganisms.

The ^{14}C labeling process has been described in detail by Kuzyakov et al. (1999) and Kuzyakov and Cheng (2001) and Domanski et al. (2001). Briefly, sealed pots with plants were put in a plexiglas chamber, $^{14}CO_2$ was introduced to the chamber by adding 1 mL of 5 M H_2SO_4 to a $Na_2^{14}CO_3$ (1.5 MBq) solution. This allowed complete evolution of $^{14}CO_2$ into the chamber atmosphere. After a 2 h-labeling period, trapping of CO_2 from the chamber through 10 mL of 1 M NaOH solution was started to remove the remaining unassimilated $^{14}CO_2$. Then the chamber was opened. Pots with the plants were connected to an output of membrane pumps by tubes: air was pumped through every single pot from bottom to top. Another tube was connecting each pot to a CO_2 trapping tube, filled with 3 mL of 1 M sodium hydroxide (NaOH) solution. The output of the trapping tube was connected to the input of the membrane pump. Therefore, the air containing CO_2 evolved from the soil respiration was circulating in a closed system: from the plant-soil system to the trapping solution to the membrane pump and back to the plant-soil system.

2.4. Sampling and analyses

NaOH in the trapping tubes was changed for the first time 6 h after the labeling and then twice a day, in the morning and in the evening, for 6 days after the labeling, with the aim of collecting CO_2 evolved in the rhizosphere during day- and night-periods. NaOH traps were analyzed for total carbonate content and for ^{14}C activity.

The ^{14}C activity was measured in 1 mL aliquots of NaOH with 2 mL of the scintillation cocktail EcoLite$^+$ (ICN) after the decay of chemiluminescence by a liquid scintillation counter (MicroBeta, TriLux). Total assimilated $^{14}CO_2$ was determined as a difference between the $^{14}CO_2$ added to the labeling chamber and the $^{14}CO_2$ recovered from the solution with the remaining unassimilated $^{14}CO_2$.

To estimate total CO_2 efflux from the soil, CO_2 trapped in NaOH solution was precipitated with a 0.5 M barium chloride ($BaCl_2$) solution and then NaOH was titrated with 0.1 M hydrochloric acid (HCl) against phenolphthalein indicator (Zibilske, 1994).

On the 6th day after each labeling, all the plants were harvested: each shoot was cut at the base, the lid of the pot was opened, and each root-soil column was pulled out of the pot. Roots were carefully

washed with deionized water to remove soil particles. Shoots and roots were dried at 70 °C, weighed and ground with ball mill (Fa Retsch) for analysis of C_{tot}, N_{tot}, and ^{15}N content. A total of 3 g of soil were taken from each soil sample, dried at 70 °C and grounded for the same purposes. C_{tot}, N_{tot}, and the isotope ratio $^{15}N/^{14}N$ in plant and soil samples were determined using Carlo Erba NA 1500 gas chromatograph (Carlo Erba Instruments, Milano, Italy) coupled on isotope ratio mass spectrometer (Delta plus IRMS 251, Finnigan Mat, Bremen, Germany).

2.5. Statistics

The experiment was conducted with four replicates. All replicates were analyzed for ^{14}C, C- and N-contents in shoots and roots. ^{14}C data are presented as the percentage of ^{14}C assimilated during exposure of plants to the pulse labeling. All data were analyzed with SYSTAT 11.0 (SPSS Inc.). Effects of different N treatment (no N, NH_4^+-N and NO_3^--N) and sampling time (day and night) were tested using two-way analysis of variance (ANOVA). We have calculated the least significant difference (LSD 0.05) in a post hoc Newman–Keuls test to identify differing treatments.

3. Results

3.1. Dynamics of $^{14}CO_2$ efflux from a soil compartment with Lupinus albus and Zea mays

3.1.1. Lupinus albus

The maximum of isotopically enriched respiration was registered within the first 6 h after the start of the labeling (Fig. 1a). Soon after, the emission rate declined from the maximum levels of 3.4% for control, 5.8% for NH_4^+-N, and 7.3% for NO_3^--N of total assimilated $^{14}C\ d^{-1}$ to 0.9% C d^{-1} on the 3rd day.

Rhizosphere respiration of recently assimilated C (^{14}C) from the soil in all N treatments showed clear diurnal dynamics. The diurnal dynamics of recently assimilated ^{14}C in respired CO_2 were strongly pronounced for non-fertilized plants. This is especially obvious after calculation of the differences between N and control treatments. The maximum difference between control and soil with added N was found during the night periods and minimum values were found during the day (Fig. 1b).

The difference between plants fertilized by NO_3^--N and NH_4^+-N in the quantity of ^{14}C respired, was highest during the first 2 days after the labeling. After 2 days already no significant differences between N treatments were measured (Fig. 1a).

Cumulative ^{14}C respiration of roots and rhizosphere microorganisms during 6 days after the labeling reached 6.8% of assimilated ^{14}C in soil without N fertilization, 8.3% for the NH_4^+-N treatment, and 9.3% for the NO_3^--N treatment (Fig. 1c), and was significantly different ($p < 0.001$) between all N treatments.

The ^{14}C losses from the soil were recalculated per unit of root biomass (Fig. 2b) measured 6 days after the labeling. Differences between the maximum $^{14}CO_2$ emissions were chosen for the comparison between the N treatments, as it relates directly to the root-derived respiration. Although there were no significant differences in root biomass between treatments ($p = 0.1$) (Fig. 2a), strong effects of N fertilization on recently assimilated C in CO_2 were observed. Taking the control treatment without N as a 100% reference, the respiration losses of ^{14}C from the plant-soil system with lupine after 6 days amounted to 182% under NH_4^+-N and 268% under NO_3^--N ($p < 0.001$).

3.1.2. Zea mays

For all N treatments, the maximum intensity of $^{14}CO_2$ efflux was reached between 26 and 30 h after $^{14}CO_2$ application (Fig. 3a). The emission rate declined rapidly from the maximum levels of 3.2% for NH_4^+-N and control, and 5.6% for NO_3^--N of total assimilated $^{14}C\ d^{-1}$,

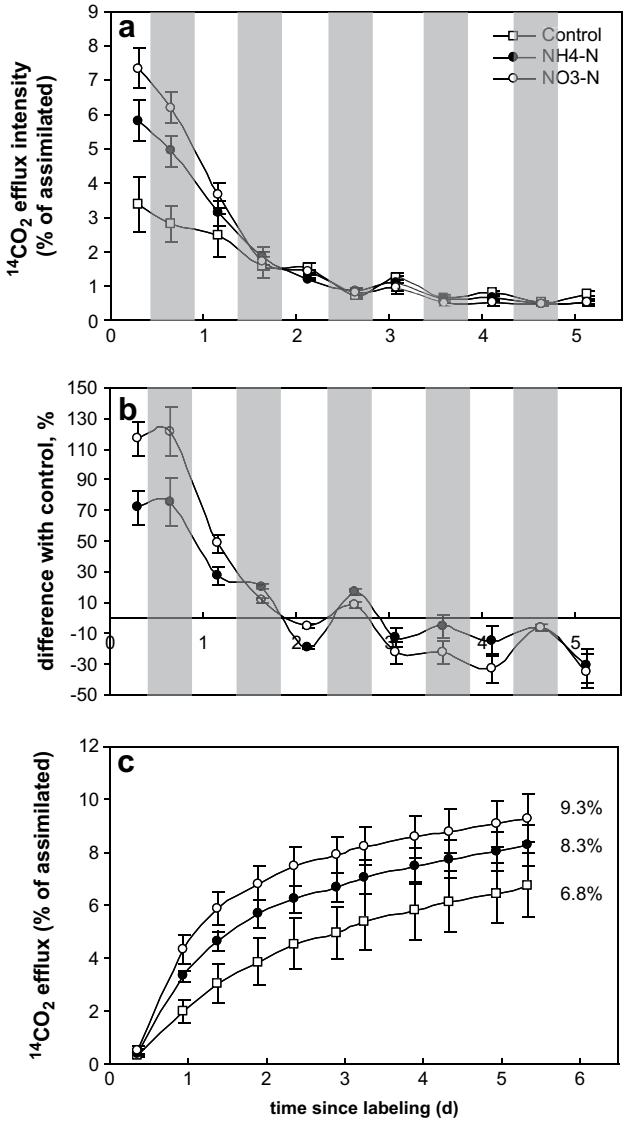

Fig. 1. (a) Dynamics of $^{14}CO_2$ from the soil (±SE) with *Lupinus albus* under three N treatments: control, NH_4^+-N, and NO_3^--N for 5.5 days after $^{14}CO_2$ pulse labeling of shoots; (b) differences between control without N and soil with NO_3^--N and NH_4^+-N applied, as % of control. Day (white) and night (gray) periods are shown; (c) cumulative $^{14}CO_2$ efflux (±SE) from the soil with *Lupinus albus* under different N treatments.

to the value of about 0.9% C d^{-1} on the 4th day. The diurnal dynamic of respired ^{14}C was more clearly observed than in the case of lupine. The differences between control and soil with applied N repeated the shape of the lupine curve (Figs. 1b and 3b), with maximum values at night and minima during the day. The absolute difference in the quantity of ^{14}C respired between plants fertilized by NO_3^--N and NH_4^+-N was again highest during the first days after the labeling (Fig. 3a).

Cumulative ^{14}C respired by roots and rhizosphere microorganisms during the 6 days of the experiment reached 6.0% for the control, and 5.6% and 7.2% for the NH_4^+-N and NO_3^--N treatments respectively (Fig. 3c). The difference in cumulative respiration between the two types of N applied was significant ($p < 0.001$), but no difference was observed between NH_4^+-N and the control ($p > 0.05$).

Different N fertilizers significantly affected ($p < 0.01$) root biomass of maize with a lowest values being 0.23 g for NH_4^+-N fertilized plants and the highest being 0.38 g for the control (Fig. 4b). The ratio between treatments in the quantity of respired ^{14}C, as related per unit of root biomass (Fig. 4a), was similar to that

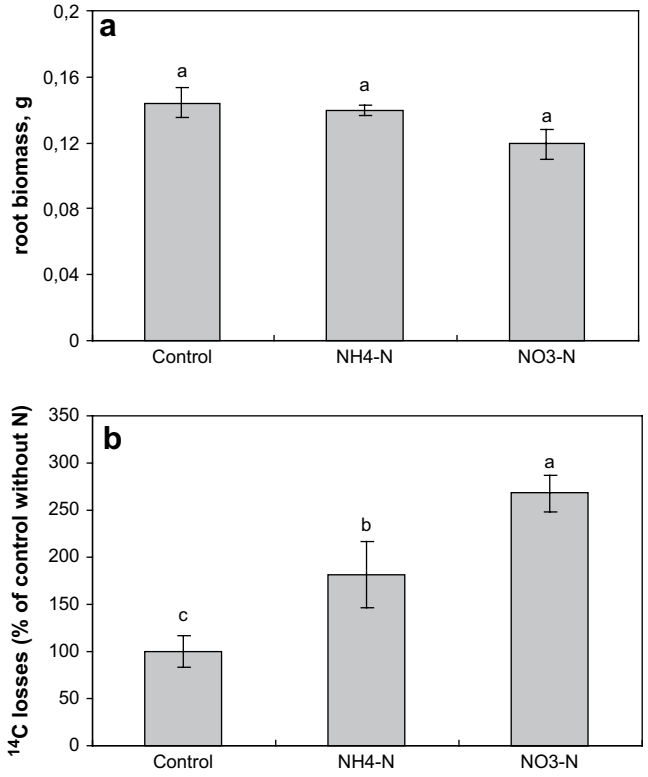

Fig. 2. (a) Belowground biomass of *Lupinus albus* (±SE) at the end of the experiment: 5.5 days after the labeling; (b) ^{14}C (peak values) respired from root-soil system with *Lupinus albus* in % of control without N, related for the unit of root biomass. Letters above indicate the significance of the differences at $p = 0.05$ between treatments.

of lupine. Losses of ^{14}C from the plant-soil system with maize reached 173% under the NH_4^+-N treatment and 221% under the NO_3^--N treatment ($p < 0.001$), again relative to the control.

3.2. Total CO_2 efflux from planted soil with Lupinus albus and Zea mays

The difference between total CO_2 efflux from the root-soil system and microbial respiration from bare soil incubated at the same conditions was used to calculate the CO_2 respired by roots and associated rhizosphere microorganisms (root-derived respiration) and to compare with the results from ^{14}C labeling for root-derived CO_2.

3.2.1. Lupinus albus

Total and root-derived CO_2 efflux from the soil in all planted treatments showed a clear diurnal dynamic (Fig. 5a,b). Average total CO_2 respired from the plant-soil system was lowest for the control (3.13 mg C d^{-1} pot^{-1}), and amounted to 4.36 mg C d^{-1} pot^{-1} for NH_4^+-N and 5.58 mg C d^{-1} pot^{-1} for NO_3^--N. The difference in total CO_2 respired from soils with different types of N applied was significant during the whole measurement period ($p < 0.05$) (Fig. 5a). On the average, the largest root-derived respiration during the day and during the night was observed under NO_3^--N even if the difference with NH_4^+-N was not significant (Fig. 5b).

CO_2 efflux from unplanted soil had no diurnal changes and the difference between N treatments was not significant ($p = 0.74$) (Fig. 5b).

The value of root-derived CO_2, calculated as a difference between total CO_2 efflux from soil with roots and microbial respiration from bare soil, was recalculated per units of root biomass and presented as a percent of the control (Fig. 5c): root-derived CO_2 from the plant-soil system with lupine was found to be 233% for the NH_4^+-N treatment and 318% for the NO_3^--N treatment ($p < 0.001$).

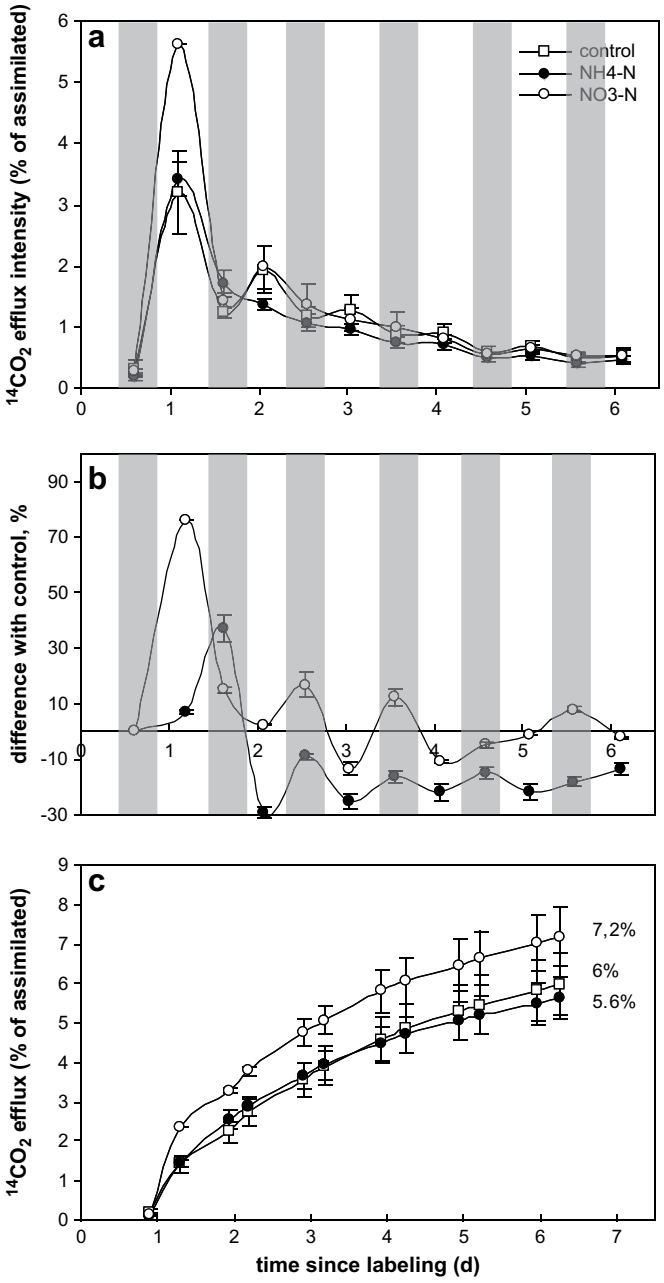

Fig. 3. (a) Dynamics of $^{14}CO_2$ from the soil (±SE) with *Zea mays* under three N treatments: control, NH_4^+-N and NO_3^--N for 6 days after $^{14}CO_2$ pulse labeling of shoots; (b) differences between control without N and soil with NO_3^--N and NH_4^+-N applied, as % of control. Day (white) and night (gray) periods are shown; (c) cumulative $^{14}CO_2$ efflux (±SE) from the soil with *Zea mays* under different N treatments.

3.2.2. Zea mays

A clear diurnal dynamic of total and root-derived CO_2 from the soil for all N treatments was observed also for the plant-soil system with maize (Fig. 6a,b). Average total CO_2 respired from the plant-soil system was lowest for the control (3.94 mg C d^{-1} pot^{-1}), while under NH_4^+-N, the efflux rate was 4.79 mg C d^{-1} pot^{-1} and under NO_3^--N, it was 5.31 mg C d^{-1} pot^{-1}. However, the difference between the two N treatments was not significant ($p > 0.05$) (Fig. 6a). The largest average root-derived respiration in the day and in the night was observed under nitrate N (Fig. 6b).

Relative to the control, the losses of CO_2 from the plant-soil system were 122% under the NH_4^+-N treatment and 164% under the NO_3^--N treatment (Fig. 4c). However the difference between the two N treatments was not found to be significant.

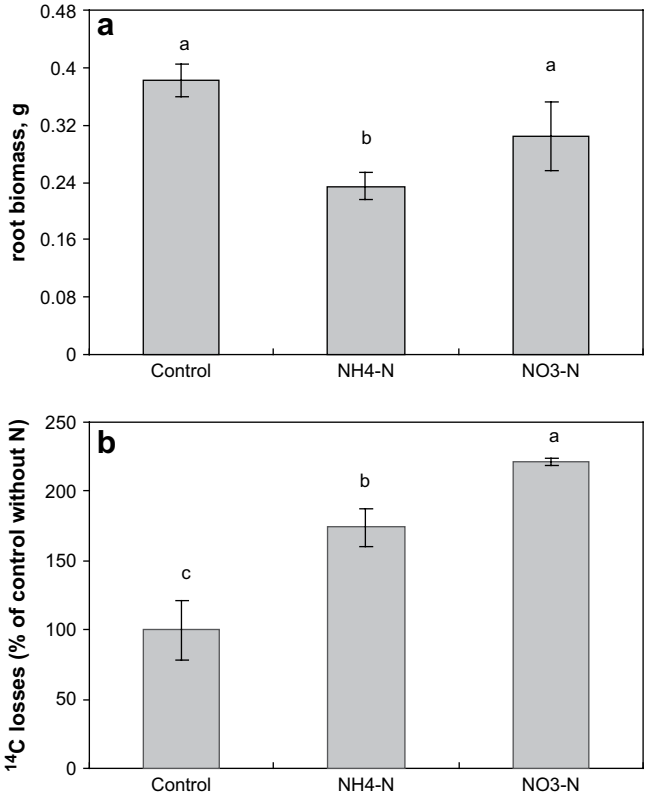

Fig. 4. (a) Belowground biomass of *Zea mays* (±SE) at the end of the experiment: 6 days after the labeling; (b) ^{14}C (peak values) respired from root-soil system with *Zea mays* in % of control without N, related for the unit of root biomass. Letters above indicate the significance of the differences at $p = 0.05$ between treatments.

3.3. ^{15}N uptake by plants

Significantly more ^{15}N was recovered from shoots and roots of lupine plants under NH_4^+-N ($p < 0.001$) (Fig. 7). The major part of the ^{15}N remained in roots (58% for NH_4^+-N and 64% for NO_3^--N).

Zea mays took up twice as much ^{15}N as the lupine, the distribution of ^{15}N also differed: around 70% of ^{15}N was allocated in aboveground biomass (Fig. 7). Significantly more ^{15}N was recovered from plants grown under NH_4^+-N ($p < 0.001$).

Maximum $^{14}CO_2$ efflux from plant-soil systems was related to the unit of N uptake (Fig. 7, in the corner). ^{14}C respiration under NO_3^--N was 2.6 times higher than the one under NH_4^+-N for lupine and 1.6 times higher for maize.

4. Discussion

4.1. Root-derived CO_2 – comparison of two methods

Two methods for estimating root-derived CO_2 efflux were used in this study: (1) pulse labeling in a ^{14}C atmosphere with subsequent tracing of recently assimilated $^{14}CO_2$ from soil; and (2) comparison between the CO_2 efflux from soil with plants and that from bare soil, the difference being accepted here as equal to the contribution of plant roots to the total CO_2 efflux.

In our experiment, both methods showed very similar results, with plants grown under NO_3^--N respiring more C than those grown under NH_4^+-N: for lupine the method based on ^{14}C gave an 47% increase in respiration for NO_3^--N relative to NH_4^+-N while the second method gave an 37% increase (Figs. 2b and 5c). For maize, both methods were also similar in magnitude: the ^{14}C method

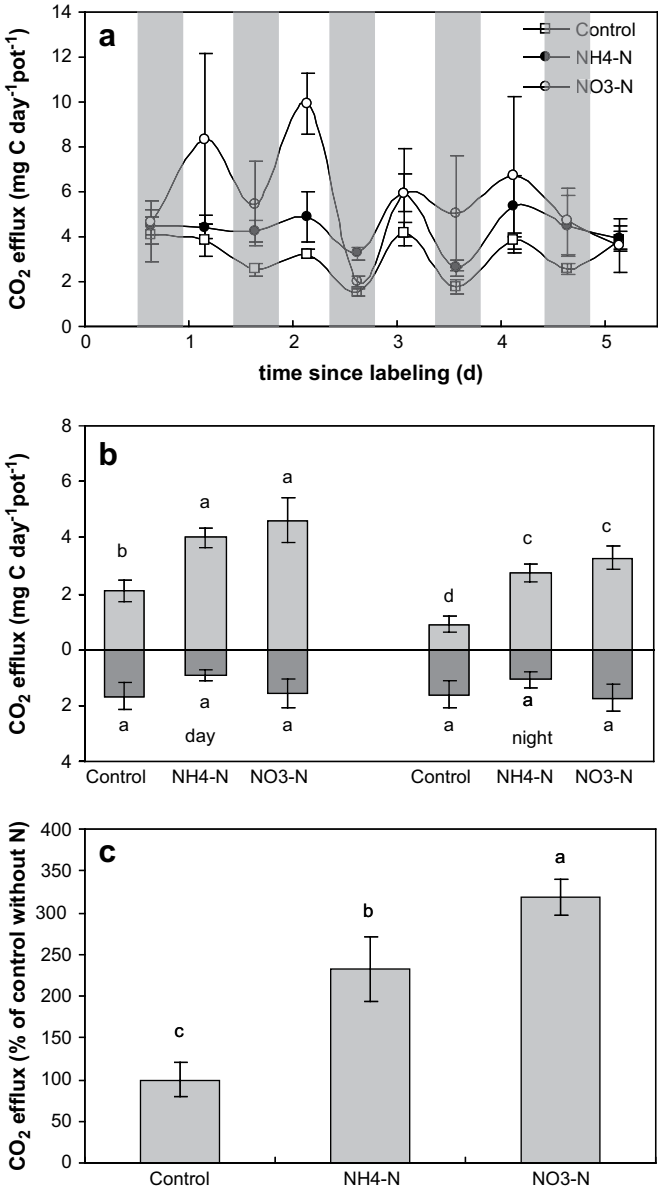

Fig. 5. (a) Total CO_2 efflux (±SE) from the soil with *Lupinus albus* under three N treatments: control, NH_4^+-N and NO_3^--N, day (white) and night (gray) periods are shown (b) positive values: root-derived CO_2 (as a difference between total and microbial respiration from bulk soil) (light gray), and negative values: microbial CO_2 (dark gray) efflux from the soil under *Lupinus albus* by three N treatments: averages for day and night periods. Letters above indicate the significance of the differences at $p = 0.05$ between the treatments, separately for root-derived and soil-derived CO_2; (c) root-derived CO_2 (as a difference between total and microbial respiration) from plant-soil system with *Lupinus albus* in % of control without N, recalculated for unit of root biomass. Letters above indicate the significance of the differences at $p = 0.05$ between the treatments.

giving a 27% difference between the two N fertilizer types and the second method giving 33% (Figs. 4b and 6c).

The suitability of ^{14}C or ^{13}C labeling and the following tracing of recently assimilated C in order to quantify root-originated CO_2 has been confirmed by many studies (Rygiewicz and Andersen, 1994; Kuzyakov and Cheng, 2001; Kuzyakov, 2006; Wang et al., 2005; Carbone and Trumbore, 2007). Labeling of plants is one of few approaches which potentially permit estimation of root-originated respiration minimizing the soil disturbance.

The second approach, comparing planted and unplanted soil, is a cheap and simple one, but it gives only a crude estimate of root-derived CO_2 and SOM-derived CO_2 from planted soil (Kuzyakov,

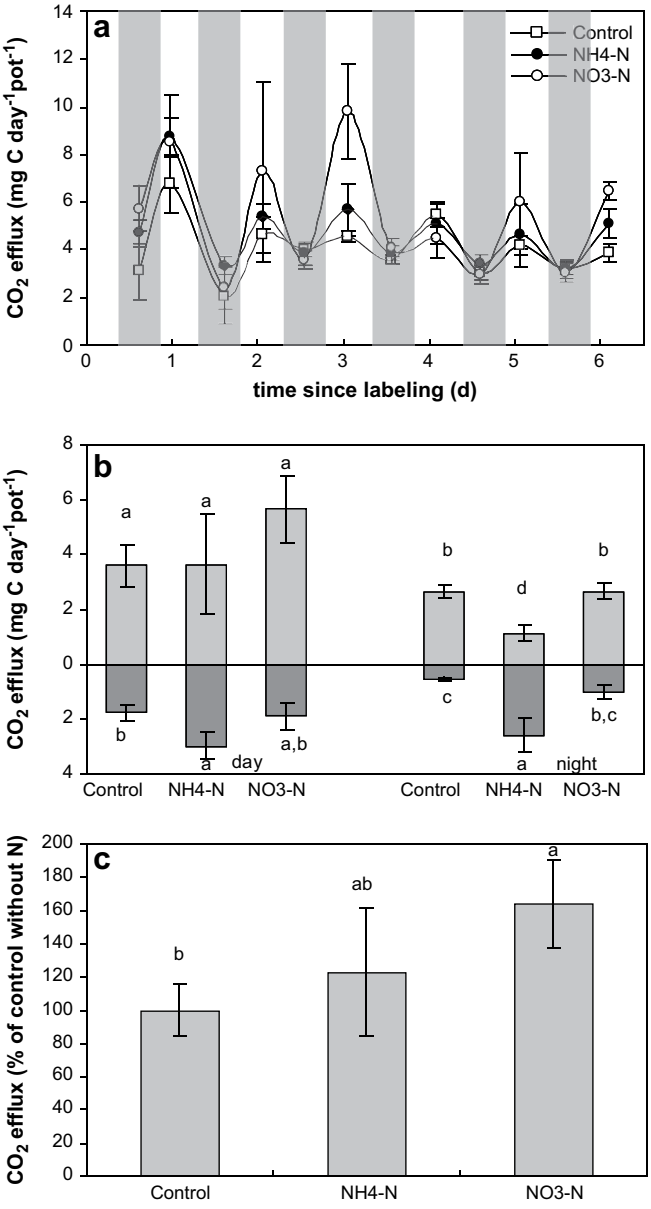

Fig. 6. (a) Total CO_2 efflux (±SE) from the soil with *Zea mays* under three N treatments: control, NH_4^+-N and NO_3^--N, day (white) and night (gray) periods are shown; (b) positive values: root-derived CO_2 (as difference between total and microbial respiration) (light gray), and negative values: microbial CO_2 (dark gray) efflux from the soil under *Zea mays* by three N treatments: averages for day and night periods. Letters above indicate the significance of the differences at $p = 0.05$ between the treatments, separately for root-derived and soil-derived respiration; (c) root-derived CO_2 (as a difference between total and microbial respiration) from plant-soil system with *Zea mays* in % of control without N, recalculated for the unit of root biomass. Letters above indicate the significance of the differences at $p = 0.05$ between treatments.

2006). Possible errors in the method come from the fact that it does not consider possible interactions between growing roots and SOM decomposition (Cheng et al., 2003), the so-called rhizosphere priming effects (Kuzyakov, 2002). The cycling of nutrients, the water regime, and temperature balance in the planted soil are also different from that in the unplanted soil (Fisher and Gosz, 1986; Ross et al., 2001; Paterson, 2003; Cheng and Kuzyakov, 2005). Additionally, it does not allow separating the rhizomicrobial respiration, associated with microbial decomposition of rhizodeposits and dead roots from the root respiration, which can be estimated using pulse labeling in the form of the first CO_2 evolved after the pulse, assuming the temporal difference between the CO_2 evolved from different sources.

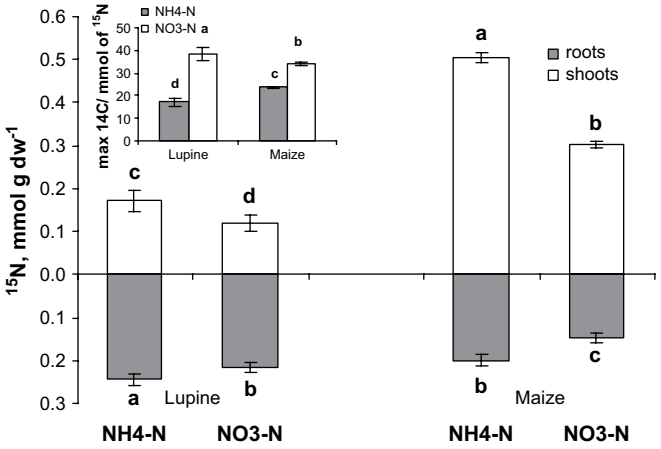

Fig. 7. ^{15}N content (±SE) in shoots (positive values, light gray bars) and roots (negative values, dark gray bars) of *Lupinus albus* and *Zea mays* under two types of N fertilizers applied: NH_4^+-N and NO_3^--N. Letters above indicate the significance of the differences at $p = 0.05$ between the treatments, separately for shoots and roots. In the upper corner: ^{14}C (peak values) respired from plant-soil system with *Zea may and Lupinus albus* per unit of ^{15}N recovered from roots. Letters above indicate the significance of the differences at $p = 0.05$ between treatments.

In our study, we did not use the absolute values of $^{14}CO_2$ and unlabeled CO_2, but instead related them to the changes in root-derived CO_2 induced by the change in the form of N fertilization. Therefore, despite their mentioned differences, both approaches for estimating the root-derived CO_2 showed similar results.

4.2. Diurnal changes of total and ^{14}C-CO_2 efflux from the soil

Many studies confirm that assimilation of CO_2 and the downward transport of C in plants, as well as the utilization of assimilated C by root respiration, are very rapid processes (Weixin et al., 1993; Gregory and Atwell, 1991; Kuzyakov et al., 1999, 2002; Kuzyakov and Cheng, 2001; Nguyen et al., 1999; Swinnen et al., 1994). The time lag between photosynthetic CO_2 uptake and the ensuing release of C through root respiration varies among studies from minutes to days. For example, Kuzyakov and Cheng (2001) found the first CO_2 evolution from soil with *Lolium perenne* within the first 4 h after labeling while Weixin et al. (1993) found the beginning of emission of CO_2 from winter wheat and rye to occur within the first 30 min. Field studies usually report lags higher than found in the laboratory; Tang et al. (2005) found evidence for time lags from 7 to 12 h up to 5–6 days, Horwath et al. (1994) reported a lag of 2–3 days for tree-soil systems. We found the maximum ^{14}C efflux from soil within 6 h after the labeling for lupine and within 26–30 h for maize. As the growing conditions, which could influence the soil CO_2 production rate and soil air vertical flow through the soil pot (soil water content, soil temperature) (Tang et al., 2003), were equal for both species, the difference in lags is connected to species-specific or growth-stage-specific differences in the transport rates of assimilates in lupine and maize. The difference in the lags cannot be ascribed to differences in path length or plant size (Farrar and Jones, 2000; Carbone and Trumbore, 2007) as both species were of a similar height at the labeling. The lupine plants were labeled on the eleventh leaf stage (v11) and maize on the fifth (v5). The growth stage could influence the metabolic orientation of plants, influencing source (photosynthetically active leaves, which supply a new C) – sink (developing organs of plants, which compete for the new C) interactions. The flow of C to sinks depends on the strength of the sink, the sink size, and the growth rate (Dickson, 1991; Farrar and Jones, 2000). In the case of maize in the present growth stage, intensively growing shoot cells could have preference

over roots in the competition for recently assimilated C. The root/shoot ratio of lupine was 0.3, indicating a possible intensive root-growing process, compared to 1.0 for maize with an already well-established root system. Its worth noting that the difference in the N acquisition strategy between lupine and maize makes the comparison between these species particularly complex. As an example other studies showed that symbiosis of lupine with rhizobacteria increases the C sink and so may accelerate downward transport of assimilates compared to non-legume plants like maize (Layzell et al., 1979), however the nodulation of lupine was not quantified here.

Diurnal changes in $^{14}CO_2$ and total efflux from planted soil were observed for both species and all N treatments (Figs. 1a, 3a, 5a and 6a). In our experiment, plants were grown at a single constant temperature during both day and night. As CO_2 efflux from unplanted soil was independent of a day/night changes (Figs. 5b and 6b), the daytime increase in ^{14}C evolution is attributed to the assimilation of C by photosynthesis and the ensuing rapid translocation to roots, with an associated signal in the root-derived CO_2. This observation was confirmed also by Kuzyakov and Cheng (2001).

4.3. Carbon costs of nitrate reduction – comparison between species and different N supplies

We chose two species with different sites of nitrate reduction. According to Pate (1973), *Lupinus albus* reduces the major part of incoming nitrate in roots. On the contrary, *Zea mays* reduce only half of the nitrate in roots and the other half translocates to the shoots for reduction. This difference in the reduction site could lead to differences in the quantity of CO_2 respired per unit of N absorbed, given that in non-green root cells and in darkness, the process of nitrate reduction is supplied by reducing equivalents from the degradation of carbohydrates with an additional CO_2 production (Aslam and Huffacker, 1982; Ninomiya and Sato, 1984), whereas the same process performed in leaves during the day is coupled directly with photosynthetic electron transport (Aslam and Huffacker, 1982; Atkins et al., 1979; Warner and Kleinhofs, 1992) without additional CO_2 evolution. Following these observations, we expected to observe differences in the quantity of CO_2 respired by a given species for different types of N supply and between two different species grown using either NO_3^--N or NH_4^+-N fertilizer.

As no effect of N form was observed on the respiration from unplanted soil we can conclude that the form of N (NO_3^- or NH_4^+) affected mainly root-derived respiration and not SOM-derived one. But, a major question in this work is whether the differences in respiration reflect actual root respiration rather than exudation and microbial respiration in the rhizosphere. Its clear that N form affects respired C, but both plants and microbes have to reduce NO_3^- to NH_4^+ before assimilating it, and the C costs might be similar, making it difficult to simply conclude that any extra C respired has a root-origin. Thus, while we cannot establish the answer to the question definitively, we believe that the time course of respiration, as viewed in the light of previous studies, strongly suggests the observed differences are due to changes in root respiration rather than microbial one. After Kuzyakov et al. (1999) and Kuzyakov and Domanski (2002) the $^{14}CO_2$ efflux after pulse labeling originating from different sources appears at different time after the labeling: $^{14}CO_2$ from root respiration occurs earlier than $^{14}CO_2$ from microbial respiration by decomposition of root exudates because the latter consists of a chain of successive processes: exudation from the root, intake by microorganisms, and only then respiration by microorganisms. It was shown on *Lolium perenne* that the actual root respiration affects the $^{14}CO_2$ efflux curve only during the first 24 h after pulse labeling and the maximum effect of exudation on rhizomicrobial respiration predominates in the $^{14}CO_2$ efflux only after about 1–2 days after the pulse labeling (Kuzyakov et al., 2001; Kuzyakov and Domanski, 2002). In our study, for both plant species, N treatment affected $^{14}CO_2$ efflux most during periods when the dominant source of $^{14}CO_2$ was likely root respiration. Therefore, the results are consistent with N form having a significant effect on respiratory costs of plants which is associated with NO_3^--N reduction and assimilation.

Additionally, the results of the ^{15}N analyses in shoots and roots demonstrated that all plants took up much more ^{15}N under NH_4^+-N than under NO_3^--N, making the difference between two types of N applied in the quantity of the respired C even more dramatic. This could be used as an extra prove of the costliness of nitrate reduction. Adding this, its worth to note that we are operating with a total amount of recovered ^{15}N, the plants were harvested on the 6th day after the fertilizing and the distribution of ^{15}N between shoots and roots during this period could change significantly.

Between species comparisons demonstrated a significant difference in the amount of CO_2 evolved under NO_3^--N and NH_4^+-N supply: respiration under NO_3^--N relative to that under NH_4^+-N was 2.6 times higher for lupine and 1.6 times higher for maize. Although microbes also pay the cost for assimilation of NO_3^--N and might cause an increase in ^{14}C respired, the effect would be the same across plant species and so we consider the observed variation in respired ^{14}C to be determined by plant physiology. A higher difference between the two N fertilizers in the case of lupine could be explained by the fact that lupine is referred to the plants reducing the major quantity of nitrates in roots, resulting in an enhanced demand for reducing equivalents of the carbohydrates degradation-origin with a subsequent evolution of CO_2 (Pate, 1973; Ninomiya and Sato, 1984). The result achieved by ^{14}C pulse labeling was supported also by an independent method based on the measurements of unlabeled total CO_2 respired from planted and unplanted soil, although between species variation was not so pronounced (Figs. 5c and 6c).

It should be mentioned also that the location of the nitrate reduction site is not species- but rather cultivars-dependent (Schilling et al., 2006; Silveira et al., 2001). Moreover, environmental conditions and the quantity of N could also affect and change the proportion of nitrate reduced in root and shoots. Atkins et al. (1979, 1980) and Oscarson and Larsson (1986) observed an increased portion of nitrate reduction in shoots when more NO_3^- became available. So, attention must be paid when choosing the species and cultivation conditions.

We conclude that the form of N supply has a strong effect on the amount of root-derived CO_2 respired from two plants characterized by different nitrate reduction sites. This was determined from two plant species using two independent methods based on recently assimilated (^{14}C labeled) and total (unlabeled) CO_2.

4.4. Conclusions

Fertilization of lupine and maize with labeled nitrate ($K^{15}NO_3$) and ammonium ($(^{15}NH_4)_2SO_4$), combined with pulse labeling of plants in $^{14}CO_2$ atmosphere allowed evaluating the effect of N form on recently respired CO_2 efflux from the rhizosphere. In respect to ammonium, nitrate addition significantly augments root-derived respiration from both plants, influencing also the contribution of autotrophic respiration to the total CO_2 efflux. This makes essential to account for the form in which the soil N was available for plant uptake and for the location of nitrate reduction site in plants in modeling and while separating estimation of individual CO_2 sources which contribute to total soil CO_2 efflux.

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