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Organic nitrogen uptake by plants: reevaluation by position-specific labeling of amino acids

Reevaluation of organic N uptake by plants by position-specific labeling

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Abstract Current studies suggest that many plants are able to take up not only inorganic nitrogen (N) but also organic N. We used the novel tool of position-specific isotope labeling to improve the quantification of intact amino acid uptake and to deepen our understanding of the processes occurring at the root-soil-microorganism interface. Position-specific ^{14}C and ^{15}N labeled alanine enabled us to trace the uptake of C from individual molecule positions by *Zea mays*, *Lupinus albus* and *Cichorium intybus*. Uniformly ^{14}C labeled alanine and acetate and inorganic $^{15}NH_4^+$ and $^{15}NO_3^-$ were applied as controls. Equal uptake of uniformly ^{14}C labeled alanine and acetate showed that plant uptake of low molecular weight organic substances (LMWOS) is independent of N in the molecule. ^{14}C uptake from individual molecule positions of alanine strongly differed: this confirmed that soil microorganisms cleaved alanine within 6 h into transformation products, which were then taken up by the plants. Microbial utilization strongly outcompeted the plant uptake of LMWOS in agricultural soils. This study revealed that position-specific labeling is an innovative tool that enables separation of the intact uptake from the uptake of molecule fragments and

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improves the understanding of competing processes for LMWOS utilization in the rhizosphere.

Keywords Alanine · Chicory · Lupine · Maize · Organic N uptake · Position-specific labeling

Introduction

Over the past century, many studies have emphasized the role of dissolved inorganic nitrogen (DIN) in ecosystems (Matson et al. 1997; Vitousek et al. 1997, 1979). Ammonium (NH_4^+) and nitrate (NO_3^-) are the main representatives of mineral nitrogen. Ammonium is a reduced form of DIN and can be directly utilized by plants after uptake whereas nitrate needs to be reduced first. Nitrate reduction demands energy from plants (Doubnerova and Ryslava 2011; Liu et al. 2011; Tischner 2000) leading to additional CO_2 fluxes through the plant-soil system (Gavrichkova and Kuzyakov 2008, 2010). Both DIN species can be lost from ecosystems: nitrate by leaching into the ground water, denitrification to N_2O and N_2, or reduction to ammonium and ammonium can be lost by volatilization or irreversible fixation by soil minerals.

In ecosystems with low availability of DIN due to slow mineralization, including boreal or arctic ecosystems (Nasholm et al. 1998; Vitousek et al. 1979), plants also rely on other N forms such as dissolved organic nitrogen (DON). This is not only a short-circuit in the N nutrition pathways (the mineralization to NH_4^+ and NO_3^- is omitted), but also reduces potential N losses from ecosystems, e.g. by leaching.

In the past 20 years, there has been remarkable interest in DON as a plant N source (Chapin et al. 1993; Jones et al. 2005a; Nasholm et al. 1998; Paungfoo-Lonhienne et al. 2012; Schimel and Chapin 1996). Organic N can be found in many compounds in soil from macromolecules like proteins (Jones et al. 2005d) or humic substances (Szajdak et al. 2003) to low molecular weight organic substances (LMWOS) like amino acids (Doerr et al. 2012; Jones et al. 2005c; Lipson et al. 1999; Streeter et al. 2000), amino sugars (Roberts et al. 2007; Roberts and Jones 2012) and nucleic acids (Kuzyakov 1996).

Many amino acids have very fast cycling rates and the half-life of amino acid C in soils is in the range of few hours (Jones et al. 2009; Kuzyakov 1996). This fast cycling is connected with fast and almost complete uptake by microorganisms (Fischer et al. 2007). Furthermore, it was demonstrated that LMWOS at average soil concentrations in soil solution (below 10 μmol l^{-1}) were taken up by microorganisms at a rate of 82 % after 3 min (Fischer et al. 2010b), and the half-life of amino acids in soil solution ranges between 4 and 8 min (Jones et al. 2004). Due to this fast utilization, soil microorganisms are strong competitors for amino acids compared to plants (Biernath et al. 2008; Hodge et al. 2000; Jones et al. 2005a; Kuzyakov and Xu 2013). In the long-term this microbial biomass N is again released by the microorganisms and becomes available for plants. However, for some plants and ecosystems, like arctic sedge systems, the preferential use of organic N was shown (Chapin et al. 1993), which started the discussion about the relevance of intact amino acids as direct plant N source.

Further studies showed that boreal forest vegetation actively take up amino acids, probably due to a lack of other N sources (Delgado-Baquerizo et al. 2011; Nasholm et al. 1998). The ratio of uptake of DIN versus DON was shown to be strongly dependent on their availability (Kranabetter et al. 2007; Stahl et al. 2011). Therefore DON is described as less relevant for agricultural crops (Jones et al. 2005a). However, even within various agricultural management systems different nutrient availability and microbial communities may strongly affect the microbial cycle of DON compounds (Herrmann et al. 2014). In order to evaluate the ratio of DIN versus DON use a comparison between uptake of N-containing LMWOS and inorganic N was recommended (Glass et al. 2002; Jones et al. 2005a; Streeter et al. 2000).

Isotope labeling of LMWOS with ^{15}N coupled with ^{13}C or ^{14}C is a common tool to investigate uptake and allocation in plants as well as mineralization or microbial incorporation (Thede 2010; van Hees et al. 2005). The uptake of amino acids by plants was mainly investigated by dual-labeling with ^{15}N and ^{13}C (Nasholm et al. 1998; Streeter et al. 2000). It is tacitly assumed in this approach that the uptake of ^{13}C corresponds to the uptake of the intact amino acid. However, dual isotope labeling has a methodological shortcoming: it allows only to observe net uptake of C and N from soils into plants but cannot account for metabolization of the applied amino acids: neither in soil by microorganisms nor in the plants (Rasmussen et al. 2010). However, microbial metabolization

products formed in soil from individual parts of the alanine molecule can also contribute to the net uptake of C and N by plants (Sauheitl et al. 2009a). One opportunity to focus only on the uptake of the intact, unmodified amino acid is the compound-specific isotope analysis (CSIA) of the applied amino acid within the plant tissue (Sauheitl et al. 2009a). However, compound-specific ^{13}C and ^{15}N analysis has the disadvantage of being a time-consuming and expensive technique (Sauheitl et al. 2009a).

To prove the uncertainties of the original $^{13}C/^{15}N$ uniformly-labeling approach, Rasmussen et al. (2010) proposed position-specific labeling as a potential tool to overcome the problem of molecule splitting. Thus, intact molecule uptake could be distinguished from uptake as partially degraded amino acid fragments i.e. decarboxylated fragments (Dippold and Kuzyakov 2013). Some recent studies clearly showed that position-specific ^{13}C and ^{14}C labeling enables tracing the fast microbial transformation of LMWOS in soils (Dijkstra et al. 2011a; Fischer and Kuzyakov 2010). These processes are also possible to modify the applied amino acids before uptake by plant roots. The application of position-specific ^{14}C labeling will enable us to identify transformations of amino acids in soil as well as in plants.

In order to consider the physiological differences of plant functional types (Weigelt et al. 2005), we performed our experiment with three plant species: maize (*Zea mays* L.), chicory (*Cichorium intybus* L.) and lupine (*Lupinus albus* L.). These species differ in their N uptake and transformation mechanisms as well as their physiology and morphology, especially in the root system: (1) maize, a grass species, has a fibrous root system and reduces NO_3^- in both, roots and shoots (He et al. 2011), (2); the herb chicory reduces NO_3^- in roots (Goupil et al. 1998) and has a taproot system, where it can store N-containing compounds for the next year (Ameziane et al. 1997) and (3) the legume lupine reduces NO_3^- in roots (Gavrichkova and Kuzyakov 2008) and has the ability to reduce atmospheric N_2 in root nodules through symbiosis with *Rhizobia*.

As organic N source, we used alanine—one of the most abundant amino acids (Fischer et al. 2010a) and ammonia and nitrate as inorganic N sources. To evaluate the preference of amino acid uptake compared to N-free LMWOS, we included additional treatments with acetate, which has a structural resemblance to alanine. If uptake of N-LMWOS (alanine) occurs mainly by unselective mechanisms, it should be in a similar range to N-free LMWOS (acetate).

The aims of this study were: (1) to determine the fate of amino acids at the root-soil interface with a special focus on the plant uptake of an initial substance versus the uptake of its transformation products, (2) to assess the ecological and physiological role of intact amino acid uptake by different plant species and (3) to evaluate the relevance of three N sources (alanine, ammonium and nitrate) for N nutrition of agricultural plants.

Materials and methods

Experiment preparation

Soil sampling

Soil samples were collected from an agricultural field site close to Hohenpölz (Bavaria, Germany at 49.907 N, 11.152 E, 501 m.a.s.l.) that had been long-term cultivated with cereals (barley, wheat, triticale). The soil is a loamy haplic Luvisol (FAO 2006). Soil was collected from 0 to 10 cm, sieved to 2 mm and roots were removed. The physicochemical characteristics of the soil are described in Table 1.

Plant and material preparation

After sieving, soil was immediately filled into transfer pipettes made of low density polyethylene 30 cm in length and 1 cm diameter, which were used as rhizotubes (Biernath et al. 2008; Kuzyakov and Jones 2006).

We used maize (*Zea mays* L.), lupine (*Lupinus albus* L.) and chicory (*Cichorium intybus* L.). Plant seeds were pre-germinated at constant temperature

Table 1 The physicochemical properties of the A-horizon of the haplic Luvisol

Soil parameters	Values
pH KCl	4.88 ± 0.12
pH H_2O	6.49 ± 0.11
Total organic carbon	1.77 ± 0.07 %
Total nitrogen	0.19 ± 0.01 %
Cation-exchange capacity	13.6 $cmol_c\ kg^{-1}$ soil
Microbial biomass C	42.5 ± 1.1 μmol C g^{-1} soil
Microbial C/N ratio	9.9 ± 0.3

(30 °C) and watered for 36 h (Gavrichkova and Kuzyakov 2008). Then, one sprout of each plant was inserted into the rhizotubes. The rhizotubes were submerged in a plastic container half-filled with cold water to maintain the soil temperature around 12 °C. Thus, microbial activity e.g. mineralization rates should resemble field conditions (Jones 1999). The pipette was connected with an air inlet (tube) at the bottom and directly under the soil surface (Biernath et al. 2008) to avoid water saturation of the soil and provide the soil and roots with air.

Chemicals and radiochemicals

The radiochemical stock solution had concentrations of 50 µM for alanine and acetate, both with 10^6 DPM ml^{-1} ^{14}C activity. Position-specific labeled alanine ([1-^{14}C], [2-^{14}C], [3-^{14}C]alanine, American Radiolabeled Chemical Inc., St Louis, USA), as well as uniformly labeled [U-^{14}C]acetate (Biotrend Köln, Germany) and [U-^{14}C]alanine (American Radiolabeled Chemical Inc., St Louis, USA) were used.

Nitrogen labeling was performed with a 99 atom% ^{15}N enriched tracer of either alanine $CH_3CH(^{15}NH_2)$-COOH as the organic N-source or ammonium sulfate $(^{15}NH_4)_2SO_4$ or potassium nitrate $K^{15}NO_3$ as inorganic N forms (Biotrend Köln, Germany). Amount of applied C and N was identical in each treatment and lower than average concentrations of alanine, acetate, NH_4^+ or NO_3^- in agricultural soils.

Experimental setup

Treatment and labeling

Each plant (chicory, lupine, maize) grew until their root system reached the rhizotube bottom at 25 cm (chicory 5 weeks, lupine 4 weeks and maize 3 weeks after germination). Each treatment (the three plants, the four ^{14}C-LMWOS-treatments [acetate, alanine C-1, alanine C-2, alanine C-3 and alanine uniformly labeled) and the three ^{15}N nitrogen sources (alanine, ammonium and nitrate)] were performed in four replicates. Each tube received the identical amount of nitrate, ammonium and alanine to exclude concentration effects on the quantified nitrogen uptake (Warren 2009). For each plant the respective backgrounds with addition of the same amount but unlabeled substances were performed in four replicates.

Each tube was sealed with silicon (NG 3170 Thauer & Co., Dresden) on the top to avoid C fixation of microbially respired $^{14}CO_2$ by the plants. The seals were applied 24 h before tracer application in order to avoid air leakage (Tian et al. 2013). Pipes were installed at the bottom of the top of the rhizotube, which were connected to a gas volume separated from the aboveground biomass, to enable a gas exchange between soil and atmosphere.

Finally, 100 µl of tracer solutions were injected at three locations along the tube (at 5, 10 and 15 cm depth) to spread the tracer over the entire rhizosphere.

Harvesting plant biomass

To identify the uptake of intact amino acids, we harvested 6 h after labeling: this shorter period compared to many studies using 24 h sampling time was chosen (1) because the half-life of amino acids C in soil ranges from 1 to 12 h (Jones et al. 2005b) and we focused on the uptake of intact amino acids and (2) to reduce the effect of post-uptake metabolism in plants (Warren 2012). Choosing 6 h sampling time is a compromise considering the fast kinetic of amino acid turnover but also the necessity to reach a detectable amount of ^{14}C incorporation. Attention has to be paid to the less sensitive detection of ^{13}C compared to ^{14}C which has to be considered in similar experiments based on stable isotopes. Aboveground biomass was cut at the top surface of the soil and immediately submerged in liquid nitrogen for 30 s. The entire rhizotube was also frozen in liquid N_2. Afterwards, all samples were stored at −20 °C and before further analysis, roots and soil were separated manually and roots were washed according to Sauheitl et al. (2009b) to remove sorbed LMWOS and ions.

Laboratory analysis

Chemical and radiochemical analysis

Soil and plant samples were freeze-dried and ball-milled for ^{14}C and ^{15}N analysis. To quantify ^{14}C incorporation, we combusted 500 mg of soil and 20 mg of roots/shoots at 600 °C for 10 min under a constant O_2 stream using an HT 1300 solid combustion module of a N/C analyzer 2100 (Analytik Jena AG, Jena Germany). The $^{14}CO_2$ was trapped in 10 ml of 1 M NaOH (two times 5 ml). For scintillation

analysis, 3 ml of this NaOH with soil-, root- and shoot-derived $^{14}CO_2$ and 6 ml of scintillation cocktail (Ecoplus, Roth Company, Germany) were mixed and measured on an LS 6500 scintillation counter (LS 6500, Beckman-Coulter, Krefeld, Germany). All samples were measured after 24 h of dark storage in order to remove chemiluminescence.

In parallel, ^{15}N was measured in 5–6 mg of soil and 0.25 mg of root/shoot samples using a Euro EA Elemental Analyser (Eurovector, Milan, Italy) which was coupled via a ConFlo III interface (Thermo-Fischer, Bremen, Germany) to a Delta V Advantage isotope ratio mass spectrometry (IRMS) (Thermo Fischer, Bremen, Germany) (Glaser 2005).

Calculations of ^{14}C and ^{15}N uptake

The percentage of incorporated ^{14}C from the applied ^{14}C in the pools (C_{inc_pools}) was calculated by the ratio of the ^{14}C activity in each pool (soil, root, shoots or total biomass) divided by the applied ^{14}C activity per rhizotube. Decomposition of alanine and acetate to CO_2 was calculated as the difference between ^{14}C added and ^{14}C recovered in soil and plant biomass. Please note that additional unconsidered losses in ^{14}C would lead to an overestimation of the calculated, mineralized CO_2.

All $\delta^{15}N$ values were converted into ^{15}N atom% (r_{pool}), considering the isotope composition of international reference standards (Fry 2006). The content of N (%) and the dry weight of the pools (DW_p) were used to calculate the total N ($[N]_{pool}$) content per sample. Thereafter, ^{15}N uptake (alanine, nitrate and ammonium) applied to the different pools was calculated following a mixing model in Eq. (1) (Amelung et al. 1999):

$$[N]_{inc-N} = [N]_{pool} \times \frac{r_{pool} - r_{pool-BG}}{r_{appl-N} - r_{pool-BG}} \quad (1)$$

N_{pool} represents the amount of N of an enriched pool and $[N]_{inc\text{-}N}$ is the amount of newly incorporated tracer-N. The variables r_{pool} and $r_{pool\text{-}BG}$ are the measured at % ^{15}N values of the labeled pool, its background (BG), and $r_{app\text{-}N}$ is the enrichment of the purchased tracer, respectively.

The calculation of the percentage of relative N incorporation per pool [$N_{incpool}$ (%)] is described in Eq. 2:

$$N_{incpool}\ (\%) = \frac{[N]_{inc-N}}{\sum\limits_{pools} [N]_{appl-N}} \times 100 \quad (2)$$

with $\sum\limits_{pools} [N]_{appl-N}$ being the sum of applied nitrogen measured in all of the pools.

Calculation of intact uptake of labeled substances

The calculation of intact uptake of alanine from the ^{14}C to ^{15}N ratio of the plant biomass ($R_{c/n}$) in Eq. 3 is based on the assumption of dual-isotope labeling that intact uptake is characterized by the parallel uptake of ^{15}N and ^{14}C. This parallel uptake can either be calculated by a linear regression between C excess and N excess (Nasholm et al. 1998) or by the ratio of C to N incorporation:

$$R_{C/N} = \frac{\overline{^{14}C}_{inc\_biomass}}{\overline{^{15}N}_{inc\_biomass}} \quad (3)$$

$R_{C/N}$ was multiplied by 100 to obtain percentage values $R_{C/N}$ (%). The standard error of the mean of this ratio was calculated by Gaussian error propagation. Based on position-specific ^{14}C labeling, this ratio can be calculated for each C position of alanine. The label signal of all three positions must be equal in the case of intact uptake. However, fragment uptake would cause position-specific differences in this ratio. Thus, only the lowest value of the three C positions represents the real intact uptake of added alanine; all higher values represent uptake of fragments.

There are still factors which may cause over- or underestimation of this approach: First plant metabolism can cause an underestimation because of preferential respiration of specific positions similarly to microorganisms. Second, overestimation of intact uptake can occur if all three C atoms are transferred to the plant in different fragments.

In order to have a better understanding of the quantitative relevance of organic N uptake compared to DIN, the percentage of intact uptake of alanine from the total incorporated N (ammonia, nitrate and alanine) was calculated. In addition, we calculated N uptake from mineralized alanine with Eq. 4:

$$DIN_{inc\_biomass} = N(ala)_{inc\_biomass}\ (\%) - R_{c/n}(\%) \quad (4)$$

where $DIN_{inc\_biomass}$ (%) is the percentage of alanine-N being mineralized and afterwards taken up by the plant as DIN; $N(ala)_{inc\_biomass}$ is the total ^{15}N

incorporation from ^{15}N-alanine into the plant biomass (in % of applied ^{15}N) and $R_{c/n}$ (%) is the alanine-^{15}N taken up as intact alanine into each plant.

In the same way, the C incorporated from alanine fragments was calculated:

$$C_{frag}(\%) = \left(^{14}C_{biomass}\right) - R_{c/n}\ (\%) \quad (5)$$

where C_{frag} is the percentage of an individual C position taken up as a fragment and $^{14}C_{biomass}$ is the total percentage of ^{14}C incorporation from each position into the plant biomass. Finally, comparison between the result for intact uptake derived from uniformly labeling (which resembles the mean of the three positions) and the calculation for intact uptake based on the C position with lowest uptake was calculated to assess the differences resulting from both labeling approaches.

Statistical analysis

Data were checked for normal distribution with Kolmogorov–Smirnov Test and checked for outliers with Nalimov Test. Factorial ANOVA (with factors plant compartment and substance or position) with the HSD post hoc test for unequal N treatments were used for the data analysis. Calculations were performed by STATISTICA 8.0 (StatSoft Inc, Tulsa USA). Figures and tables were plotted using mean ± standard error of mean (SEM).

Results

^{15}N uptake in plants from organic and inorganic N sources

Total N uptake was higher for maize than for chicory and lupine. Nitrate was the preferred N source for all plant species. After 6 h, 34 % of the ^{15}N-NO_3^- injected into the rhizosphere was incorporated into shoots and up to 15 % in roots. On the other hand, less than 7 % of the ammonium was taken up at the same time and alanine N uptake reached a maximum of 7 % in roots and 4 % in shoots.

Significantly higher uptake of nitrate was found in maize than in lupine and chicory ($p < 0.001$), while alanine and ammonia were taken up in similar amounts by all three plant species (Fig. 1). Maize fed its high N demand mainly by uptake of nitrate in comparison with reduced N sources. The highest NO_3^- transport into shoots could be found in maize with a ^{15}N shoot/root ratio of 2.42 and lower ratios in chicory (1.35) and lupine (0.57) (Table 2).

Compared with inorganic N sources, alanine-derived N was preferentially incorporated into roots and to a lower amount allocated to the shoots. There was no species effect on the root/shoot ^{15}N ratio (Table 2) with the exception of preferential nitrate transport into the shoots of maize ($p < 0.001$).

Plant uptake of uniformly ^{14}C labeled alanine and acetate

After 6 h, 0.02–0.63 % of the added ^{14}C activity of alanine C were recovered in the shoots and 0.06–1.51 % in roots with significant differences among the three plant species ($p < 0.001$) (Fig. 2). Maize took up more acetate than alanine. There was no clear preference of acetate uptake for lupine or chicory. Higher uptake of uniformly labeled acetate was found in roots than shoots, reflecting its poor transport to the aboveground plant compartments. Plant uptake of uniformly labeled alanine was higher in lupine and lower in chicory with similar incorporation in roots and shoots by maize (Fig. 2). The LMWOS-C remaining in soil ranged from 42 to 65 %, and potential mineralization to CO_2 (calculated as difference between applied and recovered ^{14}C) occurred in 35–57 % of the applied tracer without significant differences between plants (Supplementary Figure).

^{14}C plant uptake of position-specific labeled alanine

We compared the mean uptake of position-specific labeled isotopomers (mean of positions in Fig. 3) with the results of uniform ^{14}C labeling (Fig. 2) to evaluate result quality and found no significant differences of the mean of the three positions to uniformly labeled alanine for any of the investigated pools.

Alanine C-3 was preferentially incorporated in maize and lupine shoots, whereas lupine roots preferred uptake of C-1 (Fig. 3). There was very low ^{14}C incorporation in chicory. In general, we observed higher incorporation of C-3 ($p < 0.001$) than of the

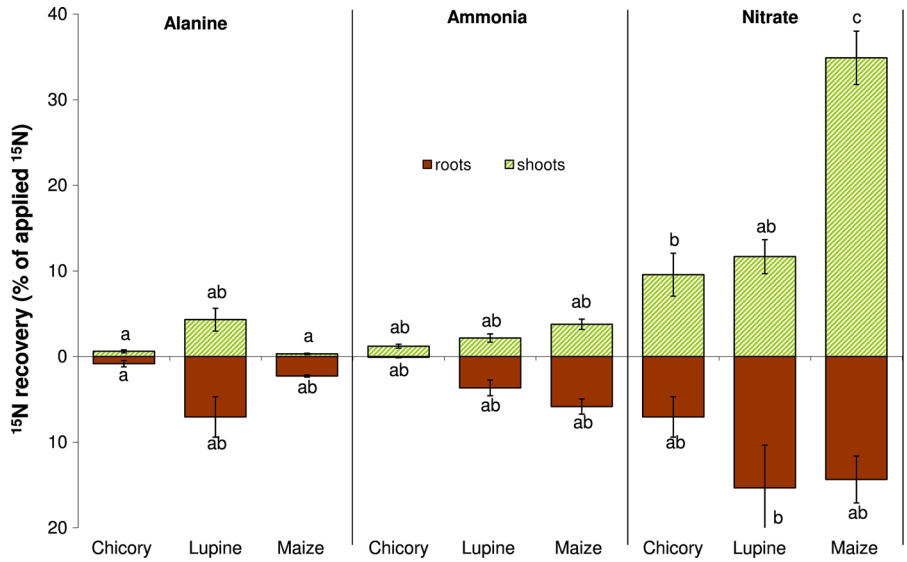

Fig. 1 Percentage of ^{15}N incorporation into roots and shoots as alanine-N, ammonia and nitrate in chicory, lupine and maize. *Letters* indicate significant differences ($p < 0.001$) between alanine, ammonia and nitrate within the plants

Table 2 Shoot/root ratio of ^{15}N from individual N sources

Shoot/root ^{15}N ratio	Chicory	Lupine	Maize
Alanine-15N	0.71 ± 0.24	0.61 ± 0.23	0.13 ± 0.06
Ammonium-15N	14.65 ± 11.81	0.59 ± 0.16	0.64 ± 0.12
Nitrate-15N	1.35 ± 0.37	0.76 ± 0.15	2.43 ± 0.35

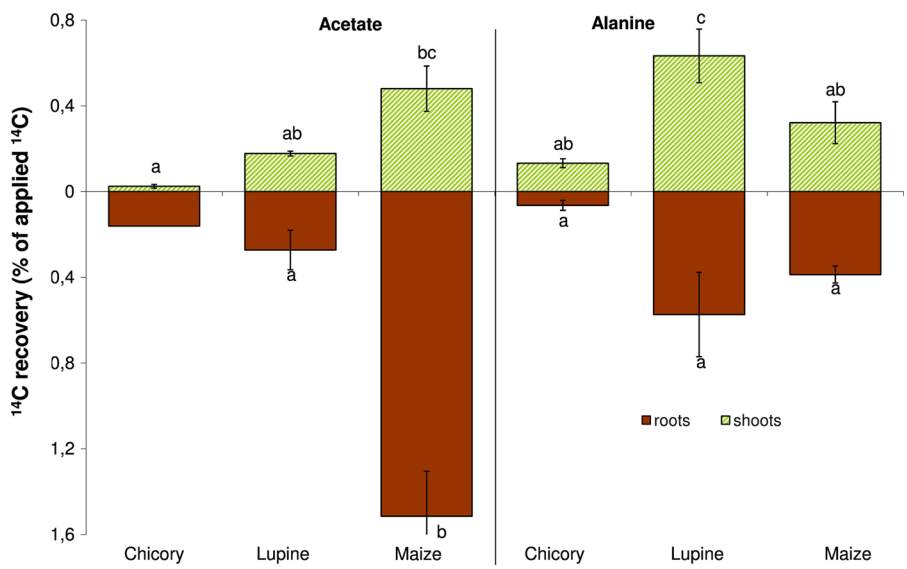

Fig. 2 Percentage of ^{14}C incorporation into roots and shoots after uniform ^{14}C labeling with acetate and alanine. *Letters* indicate the significant differences ($p < 0.001$) of acetate and alanine C between plants

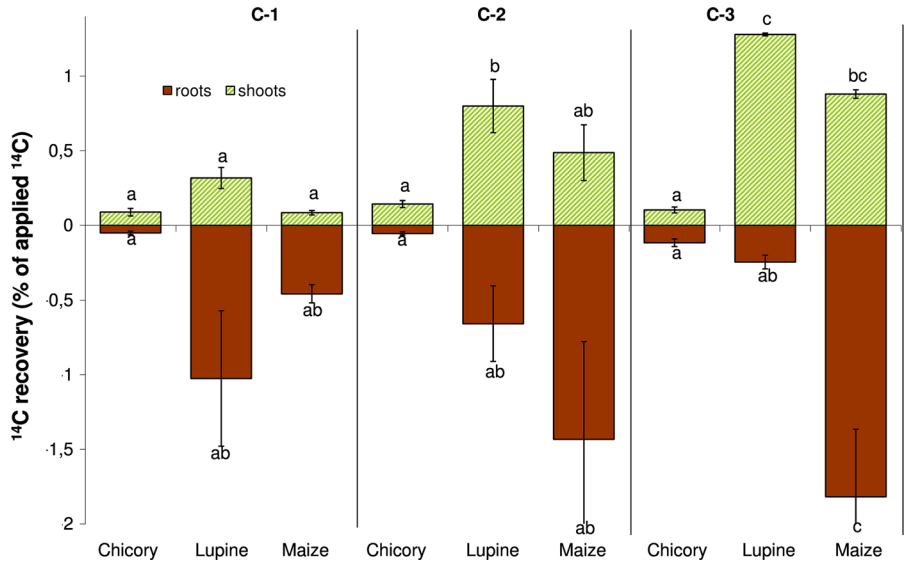

Fig. 3 Percentage of ^{14}C incorporation into roots and shoots after position-specific labeling with alanine. The alanine positions were C-1 (carboxyl group), C-2 (amino-bound group) and C-3 (methyl group). *Letters* indicate significant differences ($p < 0.001$) between alanine C positions

other positions, but with significant differences among the plant species ($p < 0.001$) (Fig. 3).

Plant species had no significant effect on the amount of mineralized ^{14}C (Supplementary Figure). Alanine showed significantly higher mineralization of C-1 (76 %) than C-2 (45 %) and C-3 (52 %).

In general, we observed that after 6 h, the individual molecule positions of alanine had strongly differing fates concerning plant uptake as well as the proportions remaining in the soil.

Intact uptake of alanine assessed by position-specific labeling

The ^{14}C/^{15}N ratio in the plant biomass (shoots and roots) reflects the proportion of ^{14}C of each individual position, which was taken up together with ^{15}N. Based on position-specific ^{14}C labeling, this calculation can be performed for each C position of alanine (Fig. 4). This ratio showed the pattern C-3 > C-2 > C-1 for each plant. We considered that a molecule of alanine could only be taken up intact if all three positions were incorporated into the plant. Thus, the minimum of the ^{14}C/^{15}N ratio reflects the maximum intact uptake of alanine in plants, which was the case for the ^{14}C/^{15}N ratio of C-1 position. These values were in a similar range for the three investigated plant species: 7–14 % of the alanine-N was taken up as intact alanine in the order maize < chicory < lupine (Table 3).

In order to compare the contribution of the three applied N sources, we estimated the tracer N nutrition budget. Comparing the role of alanine within the three investigated N sources, intact alanine uptake reached a maximum level of 0.25 % of N. Lupine showed the highest N uptake in the form of intact alanine followed by chicory and maize (Table 3). The range of plant-specific relevance of intact alanine uptake (0.04–0.25 %) reflected the plant-specific ability for N nutrition by organic sources.

The uptake of intact alanine reached a maximum of 13.7 % of the total ^{15}N uptake from alanine (Table 3). The majority of the alanine molecules were metabolized within 6 h, when the initially organic-bound N was taken up as mineralized ammonium or even already oxidized to nitrate. This degradation of alanine as a percentage of the applied alanine is illustrated in Fig. 5. Intact alanine as well as mineralized alanine-derived N uptake was highest for lupine. Once fragmented, the uptake of C-1 was only half of that of C-3. This corresponds to the highest decomposition of C-1. This different fate of individual molecule positions demonstrates splitting of LMWOS which may have occurred in plant or soil.

However, less than 1 % of the alanine C was recovered in plants at all, and the majority of the alanine (~99 %) remains in soil or microbial biomass. From the alanine fragments, 1.1–9.2 % of the mineralized N was taken up by plants, whereas C

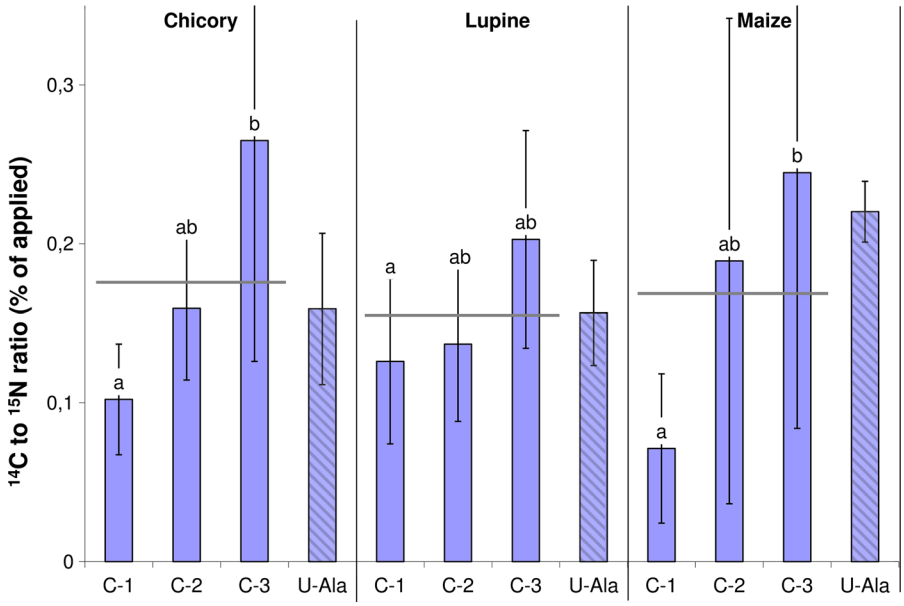

Fig. 4 Ratio of $^{14}C/^{15}N$ for individual alanine C positions and uniformly labeled alanine (U-Ala, marked by a *shaded bar*) incorporated into plant biomass. The alanine positions were C-1 (carboxyl group), C-2 (amino-bound group) and C-3 (methyl group). *Letters* indicate significant differences ($p < 0.001$) between alanine C positions. *Upper error bars* are cut but are symmetrical to those in negative direction

Table 3 Intact uptake of alanine by chicory, lupine and maize and estimated contribution of intact alanine uptake to total N nutrition of these plants with respect to the other N sources

	Chicory	Lupine	Maize
% ^{15}N uptake as intact alanine of total alanine-derived ^{15}N uptake	10.21 ± 3.48	13.70 ± 5.19	7.20 ± 4.70
% intact alanine of the three investigates N sources (alanine + ammonium + nitrate)	0.07 ± 0.04	0.25 ± 0.12	0.04 ± 0.02
Factor of overestimation of intact uptake based on uniform labeling	1.47 ± 0.25	1.14 ± 0.08	2.81 ± 1.89

incorporation in plants ranged only from 0.01 to 1.58 % (Fig. 5). Consequently, only a small portion of the applied ^{14}C but a relatively higher portion of the applied ^{15}N was taken up by plants and incorporated into their biomass after 6 h.

Discussion

Plant uptake of N-containing and N-free organic substances

Our results showed no preferential uptake of ^{14}C from N-LMWOS alanine compared to ^{14}C from acetate for any of the investigated plants. Biernath et al. (2008) found that maize had even higher uptake of acetate than alanine. This high uptake of acetate was mainly attributed to passive uptake mechanisms (Rasmussen et al. 2010). As shown by Jones et al. (2005c) and Ge et al. (2009), a higher concentration of LMWOS and well-developed root systems increases plant competitiveness for LMWOS compared to microorganisms (Kuzyakov and Xu 2013; Xu et al. 2011). In this case, passive uptake describes the phenomenon on the unspecific transport of LMWOS with the water flux towards the root surface, where they are taken up without any direct root-specific regulation of amino acid transport. There's evidence for passive co-transport of a range of amino acids together with water (Wegner 2014), which would increase due to an increasing amino acid or LMWOS concentration in the apoplast after unspecific transport with the water flux towards the root. If uptake is dominated by this passive flux of LMWOS towards the root surface, the concentration of free alanine and acetate in the apoplast, derived from soil solution would be the main driver for uptake. Alanine can strongly interact with the soil matrix by its amino group, whereas

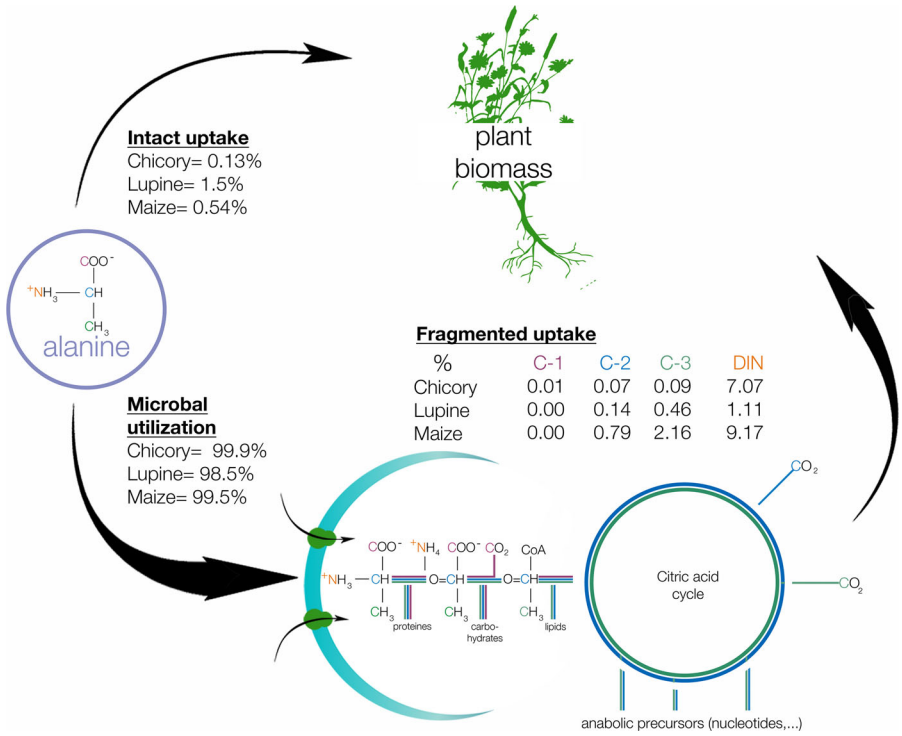

Fig. 5 Illustration of the fate of alanine tracer molecules, which are either taken up intact or degraded/mineralized to fragments and subsequently incorporated into plant biomass or microorganisms. Microbial metabolism of alanine by microorganisms is adapted from Dippold and Kuzyakov (2013)

acetate is less retained. In addition, microorganisms prefer alanine to acetate as a substrate (Fischer et al. 2010b; van Hees et al. 2002). Alanine may have been preferentially incorporated by microorganisms, as previously observed by Fischer et al. (2010b). Such processes would cause a lower concentration of free alanine than acetate in the soil solution and explain the slightly higher uptake of acetate. Thus, these results suggest that passive uptake of LMWOS plays a significant role in the rhizosphere and that this non-controlled uptake is independent of the existence of a N group in the LMWOS.

Fate of functional groups of alanine in soil

The loss of the carboxyl groups by mineralization is higher than that of the methyl groups in soil. Similar results were shown by Dippold and Kuzyakov (Dippold and Kuzyakov 2013) for alanine in lab experiments and Apostel et al. (2013) in a field study. Total decomposition in this experiment (6 h) was even higher than that observed for 3 days in a field experiment (Apostel et al. 2013) reflecting the higher microbial activity under rhizosphere conditions compared to root-free bulk soil (Blagodatskaya et al. 2009; Kuzyakov and Blagodatskaya 2015).

The decarboxylation of the C-1 is an extremely fast process in soil (Dippold and Kuzyakov 2013) and microbial uptake is assumed to be faster than plant uptake (Jones et al. 2005a). In contrast, methyl groups represent reduced C and thus a preferential C source for microbial anabolism (Apostel et al. 2013; Dijkstra et al. 2011b). Therefore, C-3 was preferentially incorporated into microbial metabolites—also into those metabolites released by microorganisms into soil solution. In addition, partial extracellular oxidation of alanine may have contributed to the higher amount of alanine-derived C-3 in soil (Dippold and Kuzyakov 2013). These metabolization fragments account for the preferential incorporation of the C-3 position (Figs. 3, 4). This preferential C-3 uptake seems to predominate the dark-fixation i.e. by PEP Carboxylase in roots of microbially respired CO_2 (which would consequently have a C-1 enrichment) (Werner and Gessler 2011).

In summary, microbial uptake and utilization were the main processes affecting the fate of individual C positions of LMWOS in soil. Preferential oxidation of

C-1 and preferential incorporation of C-3 by microorganisms are likely the cause of the preferential loss of C-1 and accumulation of C-3 in the entire plant-soil system.

Allocation and transformation of C and N within plants

The preference of crop plants for NO_3^- uptake has been reported in many previous studies (Ge et al. 2008; Hermans et al. 2006; Jones et al. 2005a). A high portion of NO_3^- reduction by maize in shoots was corroborated by our findings on preferential nitrate allocation into shoots for maize. Confirming Ameziane et al. (1997), we found that chicory kept the majority of ^{15}N in its roots (shoot/root ratio 0.3), even 8 days after labeling. In contrast, reduced N sources like NH_4^+ or alanine showed no clear preference for allocation from root to shoot.

Svennerstam et al. (2007) stated without experimental evidence that incorporation of amino acids after intact uptake occurred mainly as intact molecules. Intact incorporation without further transformation was first shown by Persson and Nasholm (2001) by CSIA via GC–MS. Sauheitl et al. (2009a), who performed similar experiments by GC–C–IRMS, also found no indication for oxidation of incorporated amino acids within the plant metabolism. Both studies could prove that no C from the applied amino acid was found in the backbone of other amino acids. However, there is a remaining uncertainty of the effect of plant metabolism, which may contribute to an underestimation of the calculated intact uptake if preferential C-1 oxidation occurred in plants (Warren 2012).

Position-specific labeling in this experiment provided the first information about plant transformation of alanine by comparing the fate of individual molecule positions within the plant compartments. In lupine (Fig. 2), position C-1 was preferentially kept in the root and among position C-1 to C-3, an increasing allocation from root to shoot could be observed. Hence, either individual fragments of alanine, taken up by the root, were allocated differently within the plant or intact alanine was partially cleaved during 6 h by the plant metabolism and the plant metabolization products were allocated differently within the plant. Ge et al. (2008) and Warren (2012) found that amino acids can be transformed to other compounds to be transported to shoots. However, Warren (2012) and Sauheitl et al. (2009a) also indicate that transaminations are the most likely metabolic transformation within plants and that oxidation of the C skeleton is much lower.

Maize showed increasing amounts of ^{14}C from alanine C-1 to C-3 in both, shoots and roots. This could either result from a fast, preferential oxidation of C-1 and C-2 after intact uptake or from a preferred uptake of C-3 fragments and their untransformed allocation into the shoots.

In summary, our results indicate that even if intact uptake occurs, plants tend to transform LMWOS rather quickly in their metabolism (Wegener et al. 2010), but mainly by transamination (Sauheitl et al. 2009a). The molecular nature of the newly formed metabolites can only be clarified by further studies performing CSIA of the transformation products.

Intact uptake of alanine in plants

The physiological ability of plants to uptake intact alanine is well known. Recent studies mainly focused on the relevance of N nutrition from intact amino acids under natural conditions using dual-isotope but uniformly labeled ^{13}C- and ^{15}N-tracers (Bardgett et al. 2003; Nasholm and Persson 2001; Weigelt et al. 2003). Calculating the $^{14}C/^{15}N$ ratio of plant uptake (Fig. 4) is based on this approach (Nasholm et al. 1998). In the case of position-specific labeling, the averaging of all alanine C positions would correspond to the uniform labeling. This averaging results in an intact uptake of around 15–18 % of alanine-derived N without any species-specific differences in this study (Fig. 4). If one assumed microbial metabolization in soil is much faster than plant metabolization, the calculation of the intact alanine uptake has to be based on the C-1 position, i.e. the position with the lowest uptake. This calculation results in an intact uptake of 7–14 % of alanine-derived N, which is a factor of 1.2–3 lower, than the respective uniformly labeling result.

Rasmussen et al. (2010) expected the highest plant uptake of the C-1 position. They postulated C-1 mineralization causing an increase in HCO_3^- from C-1 in the soil solution, which can be passively taken up by plants (Demidchik and Maathuis 2007). Our results contradict this expectation as we observed the highest incorporation rate of C-3. Thus, either a fast exchange of mineralized $H^{14}CO_3^-$ with atmospheric CO_2 leads to fast ^{14}C losses from mineralized molecules or dark fixation of CO_2 in roots is a quantitatively irrelevant

process (Werner and Gessler 2011). The highest uptake of C-3 supports the idea of plant uptake of molecule fragments, i.e. microbial transformation products, by passive uptake mechanisms. Not having alanine but a high diversity of microbial metabolization products would furthermore support the concept of passive uptake, as such products are not (or in lower amounts) present in the plant xylem sap and thus a favorable concentration gradient, supporting uptake, from apo- to symplast would exist.

Position-specific C-2-labeling of glycine revealed that ~20 % of the glycine-derived N was taken up as the intact amino acid by *Triticum aestivum* (Nasholm et al. 2001). Notably, C-2 of glycine—as a methyl group—resembles C-3 of alanine, which had the highest uptake. Thus, labeling of reduced C positions (Nasholm et al. 2001) is likely to imply highest intact amino acid uptake. In addition, intact reduced competitiveness of soil microorganisms for glycine compared to alanine (Hocking and Jeffery 2004) and the increased passive uptake due to its smaller molecular weight may favor intact glycine uptake. The applied amino acid concentration may explain the higher range of intact uptake observed in many previous studies as it increases plant competitiveness for amino acids (Kuzyakov and Xu 2013; Warren 2009). Thus, amino acid uptake quantified at high concentrations may not resemble natural conditions as free amino acid concentrations rarely exceed 100 µM in soils (Jones and Willett 2006) and bioavailable amino acid are even less concentrated (Hobbie and Hobbie 2012).

After glycine application to *Plantago lanceolata*, Sauheitl et al. (2009a) quantified intact uptake around 16.5 % of glycine-derived N using ^{13}C- and ^{15}N-CSIA of amino acids. This percentage is up to sixfold lower than values gained by bulk isotope analysis and thus confirms the overestimation of intact uptake by the use of uniformly-labeled amino acids combined with bulk isotope analysis.

We also found significant species-specific differences in the proportion of intact alanine-^{15}N to mineralized alanine-^{15}N uptake (Table 3). Lupine had the highest uptake of intact alanine followed by chicory and maize (Table 3). Maize is known to take up either amino acids or their degradation fragments (Adamczyk et al. 2012; Godlewski and Adamczyk 2007) and the highest growth and water uptake of maize also supports the idea of a high portion of microbially transformed C-3 fragments taken up.

In contrast, lupine had a high incorporation of alanine-derived N as well as high uptake of intact alanine, resulting from its cluster roots (Hawkins et al. 2005) and the very efficient amino acid transport systems, characteristic for legumes (Day et al. 2001). Thus, plant ecophysiological characteristics can increase their ability to use organic N as a relevant N source and substantial interspecific variation in the relevance of amino acids N is likely.

In summary, the use of position-specific ^{13}C or ^{14}C labeling improved the quantification of intact uptake of amino acids by plants by revealing the contribution of fragment uptake, which was significant for two of the three investigated plant species.

Relevance of amino acids as a N source for agricultural plants

Within the three applied N sources, nitrate was preferred by each of the three plants irrespective of their ecophysiology. This preference of crops for nitrate has been shown in many previous studies (Gavrichkova and Kuzyakov 2008; Ge et al. 2009; Glass et al. 2002; Jamtgard et al. 2008). The Luvisol, developed from loess, contains clay minerals (mainly illites) which can fix NH_4^+ and reduce its plant availability. In addition, also species specific preferences for N sources are in accordance with previous studies in grasslands (Weigelt et al. 2005): fast growing species—in our study maize—showed the highest uptake of nitrate.

The uptake of alanine-^{15}N was in the same range as ammonium-^{15}N. This indicates that presumably the majority of alanine-^{15}N was very quickly mineralized to and taken up as ammonium. This was also shown by a previous study for tundra species (Schimel and Chapin 1996). Thus, ^{15}N uptake confirms the position-specific ^{14}C results (Fig. 3) that mainly partially metabolized or mineralized fragments are taken up. This is consistent with studies from Jones et al. (2004), who determined amino acid half-lives of 4–8 min in soil. Thus, within 1 h, applied amino acids are completely removed from soil solution and either incorporated into microorganisms, mineralized to NH_4^+ or fixed by the soil matrix.

Table 1 shows a small C:N-ratio of the microbial biomass (~9.9) and thus a low N demand of the microbial community. Hence, the main fate of microorganisms using N-LMWOS is the C skeleton, which was similarly shown for P-containing LMWOS

(Spohn and Kuzyakov 2013). Thus, the majority of alanine-^{15}N will be mineralized and released as $^{15}NH_4^+$, being available for plants. Therefore, the N supply of the soil microbial community is a crucial factor for the incorporation of amino acids N occurs intact or mineralized.

In summary, the majority of alanine-derived N was taken up by plants after mineralization and less than 1.5 % of the applied alanine as intact alanine. Thus, intact uptake of amino acids was the least relevant N source, contributing to less than 0.25 % to the total plant N nutrition. The maximal relevance of amino acid-based N nutrition can be calculated assuming that all 20 proteinogenous amino acids have an uptake similar to alanine (although some of them have much lower concentrations in the soil than alanine). Thus, multiplying the alanine uptake by 20 gives an estimate of the total amino acid uptake. Comparing this with the ammonium and nitrate uptake measured in this study revealed that a maximum of 5 % of plant N nutrition can be expected from all amino acids.

Conclusions

This study emphasizes that position-specific labeling is a novel and unique technique to gain detailed insight into the importance of organic N sources and the uptake of LMWOS by roots from soil. The precision of previous estimates of intact uptake can be strongly enhanced using this new labeling approach without performing time- and cost-consuming measurements like compound-specific isotope $^{13}C/^{15}N$ analyses.

The comparison of N-LMWOS versus N-free LMWOS uptake revealed no significant differences in the ^{14}C incorporation from these sources. This supported the concept of passive uptake as a relevant uptake mechanism for LMWOS by plants.

Position-specific ^{14}C labeling revealed that a minor portion of amino acids was taken up intact, whereas the majority of alanine was used by soil microorganisms and—in parts—released as microbial metabolization fragments back to soil solution, from where they can be taken up by plants. For two of the three investigated plant species uniformly dual-isotope labeling causes an up to threefold overestimation of the intact amino acid uptake. Some uncertainties remain as plant metabolization like root dark fixation (leading to an overestimation of intact uptake) and plant respiration (leading to an underestimation of intact uptake) cannot be quantified by this approach.

Mineralized N as well as metabolization fragments of the C skeleton were available in the soil solution for root uptake. Lupine, as the representative of the legumes in this study, confirmed the general trend for a greater preference of legumes for organic N sources compared to non-legumes. This might be attributed to their ecophysiological capability for amino acid transfer between nodules and roots. Maize, a plant species with fast growth, high N demand and water uptake showed a higher contribution of passive uptake and thus uptake of microbial transformation products (^{14}C-fragment and DIN). Thus, substantial interspecific variations in the preferred N source and the role of DON as N source could be exhibited.

In summary, comparing the relevance of DIN and amino acids for each of the investigated plants, irrespective of their ecophysiological specifics, the role of intact amino acid uptake within N nutrition was rather low. Our study suggests N uptake from organic sources is of minor importance for N nutrition of agricultural plants. These results cannot be transferred from agricultural soils to natural boreal or arctic ecosystems where the availability and delivery of the investigated N sources is frequently contrasting. In each of these ecosystems the ecophysiological role of amino acid uptake cannot be fully understood as long as the uptake and allocation mechanisms (passive/active transport, metabolization within the plant) as well as their regulating factors are not identified. Therefore, investigations with a broad spectrum of position-specific labeled LMWOS coupled with CSIA of plant and microbial transformation products are needed.

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