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Advantages of compound-specific stable isotope measurements over bulk measurements in studies on plant uptake of intact amino acids

Leopold Sauheitl[1]*, Bruno Glaser[1] and Alexandra Weigelt[2]

[1]University of Bayreuth, Soil Physics Section, Germany
[2]Friedrich Schiller University Jena, Institute for Ecology, Germany

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Increasing interest in the ability of plants to take up amino acids has given rise to questions on the accuracy of the commonly used bulk method to measure and calculate amino acid uptake. This method uses bulk measurements of ^{13}C and ^{15}N enrichment in plant tissues after application of dual-labelled amino acids but some authors have recommended the use of compound-specific stable isotope (CSI) analysis of the plants' amino acids instead. However, there has never been a direct evaluation of both methods. We conducted a field study applying dual-labelled (^{13}C, ^{15}N) amino acids (glycine, valine, tyrosine and lysine) to soil of a Plantago lanceolata monoculture. Root and shoot samples were collected 24 h after label application and the isotope composition of the plant tissues was investigated using bulk and CSI measurements. Enrichment of ^{13}C in the case of CSI measurements was limited to the applied amino acids, showing that no additional ^{13}C had been incorporated into the plants' amino acid pool via the uptake of tracer-derived C-fragments. Compared with this rather conservative indicator of amino acid uptake, the ^{13}C enrichment of bulk measurements was 8, 5, 1.6 and 6 times higher for fine roots, storage roots, shoot and the whole plant, respectively. These findings show that the additional uptake of tracer-derived C-fragments will result in a considerable overestimation of amino acid uptake in the case of bulk measurements. We therefore highly recommend the use of CSI measurements for future amino acid uptake studies due to their higher accuracy. Copyright © 2009 John Wiley & Sons, Ltd.

Plants have the physiological capacity to take up amino acids in an intact form, not only in sterile hydroponics,[1] but also in natural soils. This was first studied *in situ* in soils of the sub-arctic region,[2] but it has since been demonstrated for soils of boreal,[3] temperate[4] and sub-tropic[5] climates, and even in agricultural systems.[6] A rising number of studies demonstrate the growing scientific interest in the use and possible importance of direct organic nitrogen uptake as an alternative organic nitrogen (N) source for plants. Amino acids might significantly contribute to the total plant N uptake, especially in poor soils with a small mineral N pool where this potential might have large effects on nutrient cycling.[2] Moreover, different plant species take up different amounts of amino acids, depending on their growth strategies[7] or habitat types,[8] which might facilitate nutrient partitioning and species coexistence. However, the correct measurement of direct amino acid uptake is the key to our understanding of such processes.

Dual stable isotope labelling (^{15}N, ^{13}C) is the most frequently used method to measure intact amino acid uptake. Labelled amino acid solution is injected into the soil and isotope enrichment of ^{15}N and ^{13}C is thereafter measured in bulk plant material. According to Näsholm *et al.*,[3] a significant linear correlation between ^{13}C and ^{15}N enrichment in plant samples is proof of the direct uptake of intact amino acids. The proportion of N taken up in an intact form is calculated by comparing the measured ratio of ^{13}C to ^{15}N excess in plant material with the theoretical ^{13}C:^{15}N ratio of the tracer molecule which is set to 100%. This technique allows the differentiation between N uptake from unlabelled soil N pools and simultaneous uptake of labelled amino acid derived N. Despite some flaws, the method published by Näsholm *et al.*[3] is most frequently applied to calculate intact amino acid uptake from this kind of data. The problems with this method are mainly due to the assumption that any ^{13}C enrichment found in plant material is caused by the uptake of ^{13}C as the intact amino acid tracer molecule. According to this assumption, changes in the plant's ^{13}C:^{15}N enrichment ratio could only be caused by changes in the intact uptake of ^{13}C or by changes in the uptake of mineral ^{15}N derived from microbial tracer decay in soil. As a result, the maximum ^{13}C:^{15}N enrichment ratio in plants will be the ratio of the used tracer itself (Fig. 1(A)). However, some reports have questioned this assumption. As an example, uptake calculations of tracer-derived N in the form of intact amino acid uptake sometimes result in maximum values of more than 100% uptake,[8,9] and thus the enrichment ratio in the plant was higher than that of the used

**Correspondence to*: L. Sauheitl, University of Bayreuth, Soil Physics Section, Universitätsstr. 30, 95448 Bayreuth, Germany.
E-mail: leopold.sauheitl@uni-bayreuth.de

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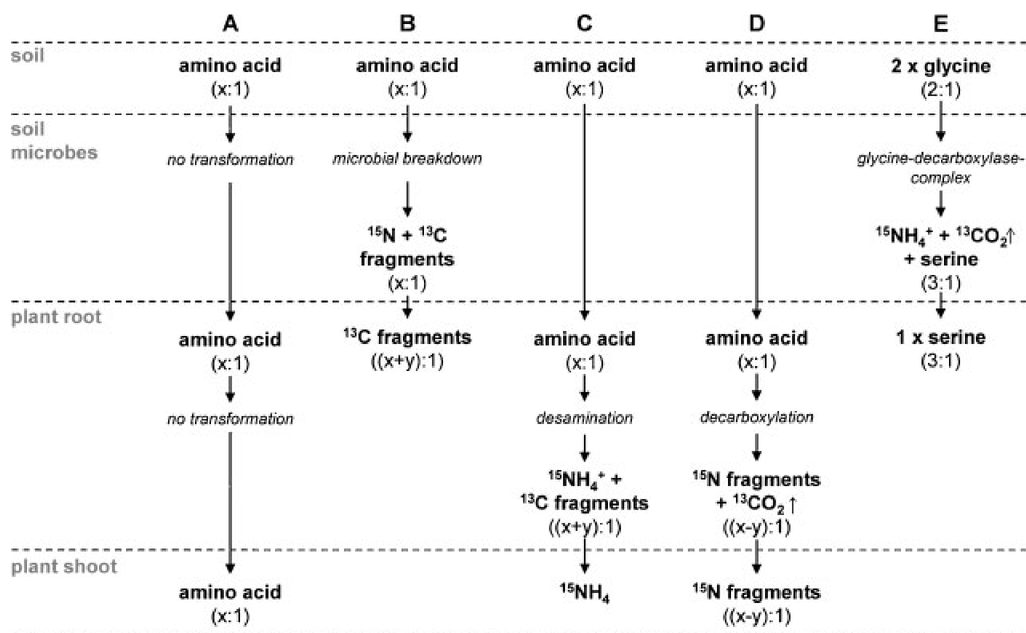

Figure 1. Depiction of possible pathways for amino acid uptake into plants. Numbers in brackets give the expected ^{13}C:^{15}N enrichment ratios in soil, soil microbes, plant roots and shoots where x stands for the number of C-atoms in an amino acid and y stands for the number of C-atoms present in the form of C-fragments.

amino acid tracer. This overestimation could be the result of two processes: First, labelled amino acids are subject to microbial cleavage, producing labelled C- and N-fragments in the soil. Plant uptake of these C-skeletons would lead to an overestimation of intact uptake.[10] (Fig. 1(B)). Second, plant internal transformation and degradation of the taken-up amino acids may occur. Desamination of the amino acids in the root followed by the transport of released $[^{15}NH_4]^+$ to the shoot would also lead to a relative enrichment of ^{13}C in the root and therefore overestimate intact amino acid uptake in the root material (Fig. 1(C)). For glycine, there is yet a third mechanism through microbial metabolism via the glycine decarboxylase pathway. Here, two molecules of glycine form one molecule of serine together with ammonia and carbon dioxide.[11] If this happens to two labelled glycine molecules and the produced serine is then taken up by plants, the resulting ^{13}C:^{15}N enrichment ratio of plant material will be 3:1 instead of 2:1 for glycine which will again result in an overestimation of the intact uptake (Fig. 1(E)). There is also the possibility of underestimating intact amino acid uptake because of decarboxylation of tracer amino acids in the plant and subsequent loss of $^{13}CO_2$ (Fig. 1(D)). However, this can be and is mostly controlled through proper experimental design. The problem with using bulk measurements is that we cannot differentiate between ^{13}C uptake in the form of the applied amino acids and uptake of other ^{13}C-enriched molecules derived from the microbial breakdown of tracer in soil. The different pathways illustrated in Fig. 1 thus cannot be separated via this method nor can we assess their importance.

A more general methodological constraint using the stable isotope technique is the detection of ^{13}C enrichment in plant material. This can be especially difficult when low levels of amino acid tracer are used in order to avoid alteration of the size of the natural amino acid pool in soil. Several studies found considerable amounts of ^{15}N in plant shoots while significant ^{13}C enrichment was not detected.[12–14] Apart from decarboxylation processes of amino acids in the plant root this mismatch is mainly related to a stronger dilution of the ^{13}C label than of ^{15}N. On the one hand, plant C content is much higher than plant N content (45–50% C compared with 3–5% N in the dry weight) and on the other hand the natural ^{13}C content of plants is higher (ca. 1.08% for C3 plants) than the ^{15}N content, leading to a dilution of ^{13}C that is 60–150 times higher than that of ^{15}N.[10]

All these problems are supposed to be overcome by using labelled amino acids in combination with compound-specific isotope (CSI) analysis to measure the uptake of amino acids into the plant material. This technique ensures that ^{13}C enrichment in plant material is only measured as part of the specific amino acids used as tracer. This largely avoids the overestimation of intact amino acid uptake through the inclusion of tracer C-skeletons into the calculations or the effect of transformation processes like the glycine decarboxylase pathway. Moreover, the dilution of incorporated tracer C is much reduced compared with bulk measurements as ^{13}C enrichment is only measured in the amino acid pool of the plant material. This strongly increases the resolution and allows the application of lower tracer amounts. However, the advantages of the CSI method come at the price of much higher costs due to material, man power and measurement time.

Despite the criticism of the Näsholm calculation method using bulk measurements,[15] and the suggestions that the more sensitive CSI method[9] should be used, there has been no consistent comparison of the two methods. Therefore, the goal of this study was to evaluate both methods in a field experiment using four types of dual-labelled amino acids.

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EXPERIMENTAL

Field site

In April 2006 we established a monoculture of *Plantago lanceolata* by sowing and planting seedlings on the Jena Experiment field site.[16] Seedlings were grown in pots for 8 weeks and transferred to the field simultaneously with the sowing of seeds (Rieger-Hofmann, Blaufelden-Raboldshausen, Germany). The field site is located on a flood-plain of the Saale River near Jena, Germany (11°34′60″ East; 50°55′60″ North; altitude 130 m a.s.l.). The soil of the experimental site was classified as Eutric Fluvisol[17] and it was highly fertilised during the last 40 years of agricultural utilisation until 2002 when the *Jena Experiment* started. In August 2006 undisturbed soil cores from the monoculture plot were collected by pressing a polyethylene (PE) tube (diameter 30 cm, height 30 cm) pneumatically into the soil. The base area of the soil cores was straightened by breaking off soil pieces using a spatula. This was done to avoid the sealing of macro pores with soil as a consequence of simply slicing the overlaying soil with a knife. Five soil cores were collected and brought to a 35 cm deep trench. The soil cores were placed upright in the trench leaving 5 cm of free space at the bottom to allow free drainage. After the installation the lateral free space between soil cores and the trench was filled with field soil to allow for natural soil temperature dynamics. As soil respiration might have been influenced by the cutting of roots when pressing the PE tube into the soil, the cores were allowed to equilibrate for 1 week before the labelling started.

Labelling

A mixture of four dual-labelled (^{13}C and ^{15}N, Spectra Stable Isotopes, Columbia, USA) amino acids (glycine, valine, tyrosine, lysine) was applied to four soil cores. Enrichment of the uniformly labelled amino acids was 98 at% both for ^{13}C and ^{15}N. The fifth soil core received a mixture of the four unlabelled amino acids and was used to measure natural isotope abundance values. We injected 34 mL aliquots of the tracer solution into each core using a 2.5 mL Luer-lock glass syringe (Microliter, Hamilton, Bonaduz, Switzerland) with a side-hole needle (length 5 cm, point style, 23 gauge), which was pierced into the soil to a depth of 5 cm and slowly withdrawn during injection. The aliquots were divided into 17 injections of 2 mL per core that were arranged circular around the centre to ensure a homogeneous dispersion of the amino acid solution. This led to a total application amount of approximately 0.06 mmol of each of the amino acids used.

Sampling and sample preparation

Aboveground biomass of the four labelled soil cores and the unlabelled core was cut 24 h after application and immediately frozen in liquid N_2. The PE tubes containing the soil cores were cut open using a small circular saw and three 2 cm soil slices were cut from the centre to the edge of the core, serving as soil subsamples for all further soil analysis. The rest of the soil core was dried at 55°C for 7 days to measure the dry weight. An aliquot of the soil subsample was used to determine the gravimetric water content of the soil. The roots were extracted as outlined in Sauheitl *et al*.[18] After a second rinse with deionised water, the roots were separated into fine roots (<2 mm) and storage roots (>2 mm), frozen in liquid N_2 and stored at −40°C until further analysis. All plant material was freeze-dried and ground to fine powder with a ball mill shortly before amino acid extraction and bulk measurements were performed. The sampling resulted in a total of four replicates for the aboveground biomass and the two types of roots for the labelled soil cores, plus the samples from the unlabelled core.

Amino acid extraction, purification and derivatisation

Amino acid extraction of the plant material was carried out using hot acidic hydrolysis which ensured the extraction of free and protein-bound amino acid molecules. The hydrolysis, purification and derivatisation were carried out according to Amelung and Zhang[19] with some modifications: In brief 15 mg of shoot or 30 mg of root material were spiked with 35 μg Norvaline (Sigma Aldrich, Seelze, Germany) as the first internal standard (IS1) and then hydrolysed. After purification the liquid samples were freeze-dried and derivatised. The volumes of used derivatives were changed compared with Amelung and Zhang[19] to 500 μL water-free 4 M HCl and 150 μL pentafluoropropionic acid anhydride. Before derivatisation, 35 μg of *trans*-4-(aminomethyl)cyclohexane carboxylic acid (Sigma Aldrich) were spiked to each sample as the second internal standard (IS2). The resulting solution was then transferred to gas chromatography (GC) vials and measured on an isotope mass spectrometer (see below). In parallel with the samples a mixture of 12 amino acids at a concentration range of 13–106 μg mL^{-1} was derivatised, serving as reference standards for quantification and corrections of delta values as described in detail below.

Measurement

All compound-specific isotope (CSI) measurements of ^{13}C and ^{15}N were performed on an isotope ratio mass spectrometry (IRMS) instrument (Delta Plus^{TM}, ThermoFinnigan, Dreieich, Germany) which was coupled with a gas chromatograph (Trace GC 2000, ThermoFinnigan) via a combustion interface (GC Combustion III, ThermoFinnigan). Except for the gas chromatograph settings, detailed instrument setup and information on the referencing procedure during measurement can be found in Sauheitl *et al*.[20] Aliquots (2 μL) of the sample solution were injected into the gas chromatograph with an autosampler (AS 2000, ThermoFinnigan) working with a 10 μL syringe with a 70 mm needle length (Hamilton). Evaporation of the samples in the gas chromatograph was carried out at an injector temperature of 250°C in a glass liner that had been deactivated in 5% dimethylchlorosilane in toluene for at least 1 week. A BPX5 column (60 m long × 0.25 mm i.d. × 0.25 μm film thickness of a cross-linked polymer of 5% diphenyl- and 95% polysiloxane) was used for separation of the single amino acids. The helium (99.996% purity) flow rate through the column was kept constant at 1.1 mL min^{-1} and the starting temperature of the gas chromatograph oven was 80°C held for 1 min, then raised to 140°C at a rate of 2.0°C min^{-1}, raised to 200°C at a rate of 10°C min^{-1}, raised to 220°C at a rate of 15°C min^{-1}, held for 2 min, raised to 240°C at a rate of 20°C min^{-1}, held for 5 min, and finally raised to 300°C at a

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rate of 60°C min^{-1} and held for another 5 min. Each sample was measured in four-fold replication and ^{13}C and ^{15}N measurements were performed in successive runs in which the same gas chromatograph settings were used.

After peak separation the gas stream was fed into the isotope ratio mass spectrometer via an open split. For the measurement of ^{15}N enrichment an additional cooling trap operating with liquid N_2 was placed prior to the open split. This was done to withdraw any CO present in the helium stream which would interfere with all the measured isotope masses of N_2 (28, 29, 30) making accurate measurements of ^{15}N enrichment impossible.

Online calibration of delta values was carried out using CO_2 and N_2 reference gases as generally described in Glaser and Amelung.[21] To correct for the derivatisation C in the amino acid derivatives, the delta values of all the investigated amino acids were also measured in the underivatised amino acid standards using an elemental analyser (EA, Carlo Erba NC 2500, ThermoFinnigan) which was coupled with the isotope ratio mass spectrometer via a Conflo III interface (ThermoFinnigan) instead of the gas chromatograph. Details of the EA calibration used are given in Sauheitl *et al*.[18]

Calculations

Isotope measurements were performed using the δ notation as output. For all the following calculations this was transferred to at% heavy isotope according to Craig.[22]

Because for CSI measurements the amino acids have to be derivatised beforehand, the measured at% ^{13}C values include the added derivatisation C. This will change the original ^{13}C signal of the pure amino acid according to Eqn. (1):

$$Z_{derivative} * at\%_{derivative} = Z_{AA} * at\%_{AA} + Z_{PFPA} * at\%_{PFPA} + Z_{Isop} * at\%_{Isop} \quad (1)$$

with Z representing the number of C-atoms in the derivative ($_{derivative}$) and the pure amino acid ($_{AA}$) and showing the number of C atoms transferred during derivatisation in the form of pentafluoropropionic anhydride ($_{PFPA}$) and isopropanol ($_{Isop}$). At% gives the isotopic composition of each substance which, in the case of the amino acid and the derivatisation reagent, was measured using the EA-IRMS system. Glaser and Amelung[21] were able to remove this derivatisation effect using Eqn. (2):

$$at\%_{AA,\,s} = \frac{z_{tot} * (at\%_{AA,\,derivative,\,s} - f(x))}{z_{AA,\,s}} + at\%_{AA,\,Std} \quad (2)$$

where f(x) represents a linear or logarithmic function accounting for the influence of amount of substance on the at% values for a single amino acid. These functions were fitted by measuring derivatives of a standard mixture at different concentrations. The suffix s indicates that the respective amino acid is measured in a sample and z_{tot} gives the number of C-atoms in an amino acid derivative.

To correct for any shifts during measurements, the $at\%_{AA,\ derivative,\ s}$ values were corrected for the measured reference gas drift during one sample or standard run (Eqn. (3)) before Eqn. (2) was used.

$$at\%_{AA,\,derivative,\,s} = at\%_{AA,\,derivative,\,measured} - \left(t_{AA,\,derivative} * \frac{t_{refgas\,2} - t_{refgas\,1}}{at\%_{refgas\,2} - at\%_{refgas\,1}}\right) \quad (3)$$

In this equation the measured isotope composition of an amino acid derivative in a sample or standard ($at\%_{AA,\ derivative}$) is corrected for the drift of at% values between the first reference gas ($at\%_{refgas1}$) where t is the retention time of the amino acid derivative or the reference gas.

When plants take up ^{13}C- and ^{15}N-enriched material, this will mix with the already existing plant C and N pool. The resulting new isotope composition of the plant material can be calculated according to Gearing[23] via a two-component mixing system (Eqn. (4)):

$$R_{sample} = \frac{A_0 * heavy\ isotope_0 + A_T * heavy\ isotope_T}{A_0 * light\ isotope_0 + A_T * light\ isotope_T} \quad (4)$$

with A_0 representing the amount of plant C or N in mol and heavy/light $isotope_0$ giving its concentration of heavy and light isotope before tracer application in at%. A_T gives the amount of tracer C or N taken up into the plant and its isotope composition of heavy and light isotopes both as at%.

To calculate the amount of ^{13}C or ^{15}N taken up, Eqn. (4) has to be solved for A_T:

$$A_T = \frac{heavy\ isotope_0 * A_0 - R_{sample} * light\ isotope_0\ A_0}{R_{sample} * light\ isotope_T - heavy\ isotope_T} \quad (5)$$

We further refer to A_T as the *^{13}C or ^{15}N enrichment or excess*. In the case of CSI measurements, this was done separately for each of the four amino acids used. The total plant heavy isotope excess was calculated as the sum of the enrichments found in the single amino acids and plant compartments. For both methods, the assessed enrichments per plant tissue were expressed relative to the total uptake per plant and referred to as *proportion of total uptake*:

$$\text{Proportion of total uptake} = \frac{A_{T,\,compartment}}{\Sigma A_{T,\,whole\ plant}} \quad (6)$$

where the numerator gives the excess of heavy isotope in the individual plant compartment and the denominator represents the total heavy isotope enrichment in all plant tissues.

For single plant tissues the uptake of ^{13}C and ^{15}N in the form of individual amino acids was also expressed in relation to the total intact ^{13}C uptake of the whole plant. This was calculated according to Eqn. (7) and we further on will refer to this as the *proportion of total uptake of glycine, valine or tyrosine/lysine*.

$$\text{Proportion of total uptake of gly, val or tyr/lys} = \frac{A_{T,\,compartment,\,amino\ acid}}{\Sigma A_{T,\,whole\ plant,\,all\ amino\ acids}} \quad (7)$$

with $A_{T,\ compartment,\ amino\ acid}$ giving the heavy isotope enrichment of one individual amino acid in one plant compartment and the denominator giving the sum of enrichment of all used tracer amino acids in all plant tissues.

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Statistics

^{13}C enrichment quantifies the intact amino acid uptake in both methods and was thus used for statistical comparison of both methods. Statistical analyses were carried out using SPSS for Windows (version 10.0.1, SPSS GmbH, Munich, Germany) and differences between both methods were tested to be significant using a t-test for single independent samples. Differences in isotope enrichment between the used amino acids in each of the plant tissues were tested by performing an analysis of variance (ANOVA) with successive post hoc tests. To meet the assumptions of the t-test and the ANOVA, all data were tested for normal distribution (Kologorov-Smirnov test) and, in the case of the ANOVA, the Levene test was also used to test for homogeneity of variances. Depending on the result of the Levene test, the Scheffé test or the Games-Howell test was used to detect paired differences.

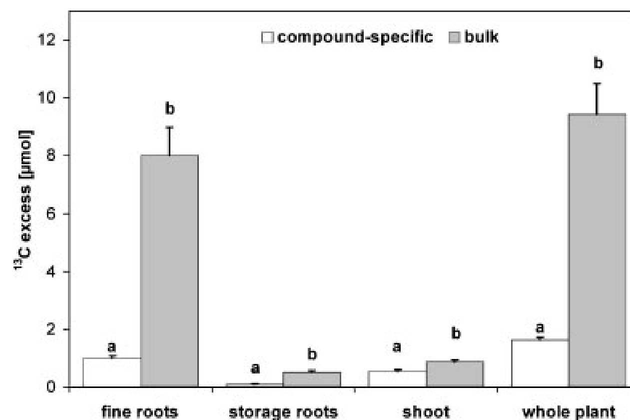

Figure 2. ^{13}C enrichment in three plant tissues and the whole plant measured via bulk (grey bars) or CSI measurement (open bars) of the target amino acids. Bars show mean values ± standard error; different lower case letters show significant differences between both measurements for one plant tissue ($p < 0.05$).

RESULTS

Comparison of bulk and CSI measurements

Correlations between ^{15}N and ^{13}C enrichment given as excess in µmol in root and shoot samples were highly significant ($p < 0.01$) with R^2 values of 0.943 and 0.999 for the fitted linear regressions for root and shoot samples, respectively. The enrichment of ^{13}C was significantly positive ($p < 0.05$) for all investigated plant tissues and for both methods. Both methods showed a declining trend of ^{13}C uptake from fine roots to shoots to storage roots (Fig. 2). We found significantly ($p < 0.05$) different ^{13}C uptake rates between the two methods in single plant compartments and the whole plant material, with bulk measurements resulting in 8, 5, 1.6 and 6 times higher ^{13}C enrichments than for CSI measurements for fine roots, storage roots, shoots and the whole plant, respectively. The relatively low allocation of ^{13}C to the shoot in the case of bulk measurements resulted in the lowest ^{13}C:^{15}N ratio of all plant tissues (Table 1).

Isotope enrichment in single amino acids

The CSI measurements showed that tracer C and N uptake into different plant compartments differed among individual amino acids (Table 1, Fig. 4(A)). Although peak separation

Table 1. ^{13}C and ^{15}N excess plus ^{13}C:^{15}N ratio for single and all (CSI total) target amino acids and in bulk samples as derived from the CSI and bulk measurements in three different plant tissues and the whole plant. Significant differences are indicated by different lower case letters as derived from post-hoc tests ($p < 0.05$)

Compartment	Source	^{13}C excess [µmol]	Sig. within[1]	Sig. between[2]	^{15}N excess [µmol]	Sig. within[1]	Sig. between[2]	^{13}C:^{15}N	Sig. within[1]	Sig. between[2]
Fine roots	gly	0.187 ± 0.013	a	a	0.30 ± 0.01	a	a	0.62 ± 0.04	a	a
	val	0.323 ± 0.037	ab	a	0.18 ± 0.03	b	a	1.79 ± 0.17	ab	a
	tyr/lys	0.497 ± 0.105	b	a	0.21 ± 0.01	b	a	2.28 ± 0.48	b	a
	CSI total	1.007 ± 0.082	a	a	0.69 ± 0.04	a	a	1.46 ± 0.16	a	ab
	bulk	8.019 ± 0.968	b	a	19.78 ± 2.00	b	a	0.41 ± 0.08	b	a
Storage roots	gly	0.018 ± 0.005	a	b	0.02 ± 0.00	a	b	0.90 ± 0.26	a	a
	val	0.032 ± 0.004	ab	b	0.002 ± 0.00	a	b	1.60 ± 0.39	ab	a
	tyr/lys	0.043 ± 0.006	b	b	0.02 ± 0.00	a	b	2.15 ± 0.52	b	a
	CSI total	0.093 ± 0.013	a	b	0.06 ± 0.01	a	b	1.55 ± 0.36	a	a
	bulk	0.503 ± 0.076	b	b	0.87 ± 0.15	b	b	0.58 ± 0.03	b	a
Shoot	gly	0.094 ± 0.014	a	c	0.58 ± 0.08	a	c	0.16 ± 0.00	a	b
	val	0.273 ± 0.001	b	a	0.41 ± 0.03	b	c	0.67 ± 0.05	b	b
	tyr/lys	0.184 ± 0.046	a	b	0.41 ± 0.06	b	c	0.44 ± 0.05	b	b
	CSI total	0.551 ± 0.059	a	c	1.4 ± 0.17	a	c	0.39 ± 0.01	a	b
	bulk	0.892 ± 0.038	b	b	8.24 ± 0.32	b	c	0.11 ± 0.00	b	b
Whole plant	gly	0.299 ± 0.018	a	n.c.[3]	0.91 ± 0.07	a	n.c.[3]	0.33 ± 0.03	a	n.c.[3]
	val	0.628 ± 0.031	b	n.c.	0.61 ± 0.05	b	n.c.	1.04 ± 0.06	b	n.c.
	tyr/lys	0.724 ± 0.104	b	n.c.	0.64 ± 0.05	b	n.c.	1.13 ± 0.20	b	n.c.
	CSI total	1.651 ± 0.095	a	n.c.	2.16 ± 0.21	a	n.c.	0.76 ± 0.09	a	n.c.
	bulk	9.414 ± 1.062	b	n.c.	28.88 ± 1.82	b	n.c.	0.33 ± 0.04	b	n.c.

[1] Significant differences (ANOVA) between amino acids or types of measurement within a compartment.
[2] Significant differences (ANOVA) for one specific amino acid or measurement between different compartments.
[3] ANOVA not conducted as 'whole plant' is not a specific plant compartment.

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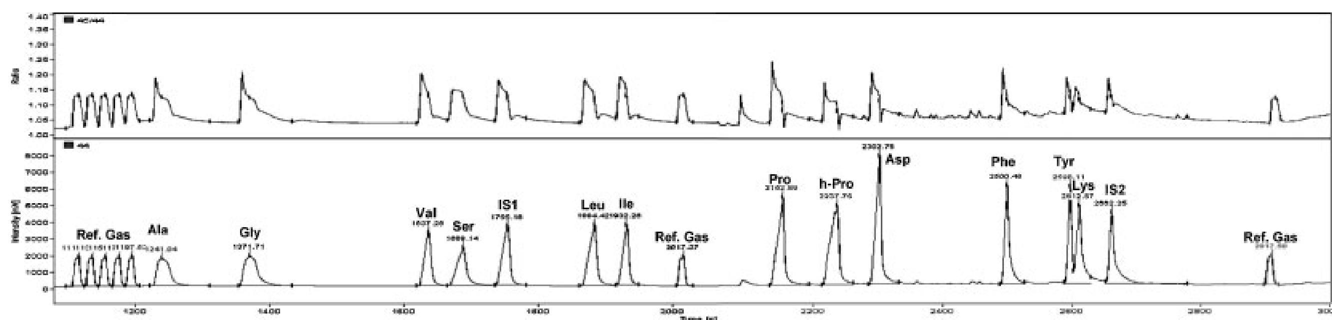

Figure 3. Chromatogram of an amino acid standard. Peaks give signal intensity [mV] of CO_2 derived from oxidation of alanine (Ala), glycine (Gly), valine (Val), serine (Ser), first internal standard (IS1), leucine (Leu), isoleucine (Ile), proline (Pro), hydroxyproline (h-Pro), aspartate (Asp), phenylalanine (Phe), lysine (Lys), tyrosine (Tyr), and second internal standard (IS2). CO_2 peaks of reference gas (Ref. Gas) are distributed throughout the chromatogram.

between tyrosine and lysine was not complete (Fig. 3), sensitivity analysis showed that the position of the parting line between the two peaks did not influence the overall enrichment of both peaks significantly. Although this poor peak separation did therefore not affect the outcome of our method comparison, we will only give the combined enrichment of both amino acids in all figures and tables, as this reflects the chromatographic potential of our method.

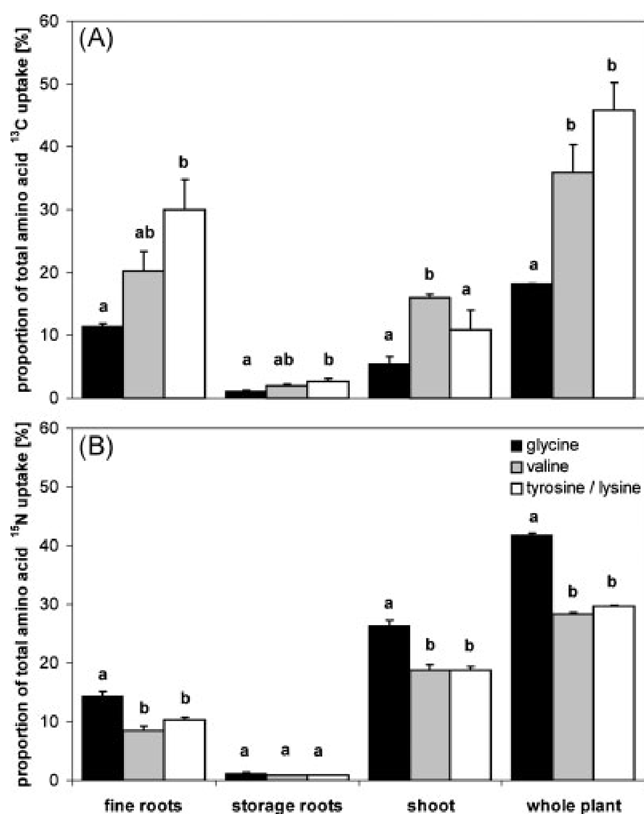

Figure 4. ^{13}C (A) and ^{15}N (B) enrichment of the target amino acids in each plant tissue based on the summed total ^{13}C and ^{15}N enrichment of all target amino acids for the whole plant, denoted as *proportion of intact amino acid* $^{13}C/^{15}N$ *uptake*. Bars show mean values ± standard error; different lower-case letters show significant differences between different amino acids for one plant tissue ($p < 0.05$).

The highest relative ^{13}C uptake was found in fine roots (61%), followed by shoots (33%) and storage roots (6%). In fine roots, tyrosine and lysine showed the highest relative uptake (30%) followed by valine (20%), with significantly less uptake of glycine (11%). This pattern was also found for storage roots with 2.6, 1.9 and 1.1% relative uptake for tyrosine/lysine, valine and glycine, respectively. In shoots the relative uptake of valine (17%) was highest followed by tyrosine/lysine (11%), with again significantly less uptake of glycine (6%) (Fig. 4(A)). The relative uptake of ^{15}N, however, shows a different picture (Table 1, Fig. 4(B)): Most tracer-derived ^{15}N that was coupled to the investigated amino acids was found in shoot material (64%) followed by fine roots (33%) and storage roots (3%). The highest portion of this overall uptake was bound to glycine (42%), while tyrosine/lysine and valine only accounted for 30 and 28%, respectively. The same distribution was found for fine roots and shoot material whereas the relative ^{15}N uptake of single amino acids was not different in storage roots. The ratio of ^{13}C:^{15}N enrichment in all plant tissues and for all applied amino acids was lower than that of the original tracer molecules. This ratio differed between amino acids and was significantly smaller in shoot than in root tissues (Table 1).

DISCUSSION

Validity of compared data

In the calculation method introduced by Näsholm *et al.*[3] the proportion of N taken up in the form of intact amino acids is calculated by comparing the ratio of ^{15}N:^{13}C enrichment in the plant with the ^{15}N:^{13}C ratio of the tracer molecule used. These authors take into account the fact that ^{15}N can either be taken up as intact amino acid or as mineral N originated from microbial tracer decay in the soil. However, in this method ^{13}C uptake is considered more conservative, i.e. if ^{13}C enrichment is detected in the plant it is attributed to the uptake of an intact amino acid C-skeleton. Therefore, we deliberately compared the ^{13}C enrichments calculated from data of both measurements as any difference in this enrichment directly will lead to a difference in calculated uptake values for intact amino acids, or rather amino acid N in case of the Näsholm calculation.

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According to Näsholm, a high correlation between ^{13}C and ^{15}N enrichment, as was found in our study, suggests the uptake of intact amino acids. Moreover, the slopes of linear regression never exceed 2.0 which is the lowest ^{13}C:^{15}N ratio possible if only glycine was taken up intact. Thus, the bulk measurement appeared fully valid according to the Näsholm method.

Compound-specific isotope measurements of amino acids in plants have been suggested as a more accurate measure of amino acid uptake.[9,10,15] However, even after corrections for the effects of derivatisation C, the amount dependency of isotope measurements and a calibration to international isotope standards, as in our investigations, the at% ^{13}C values of the CSI measurements could still lead to a false estimation of the amino acid uptake. Selective plant measurements of ^{13}C enrichment in the type of amino acids used in the tracer mixture (target amino acids) avoid the problem of defining non-amino acid bound ^{13}C uptake as uptake of intact amino acids as far as possible, but there still is a small chance of overestimating the amino acid uptake: If plants take up tracer-derived C-fragments into the roots, those might be oxidised and fed into the tricarbonic acid (TCA) cycle. By forming new amino acid skeletons this recycled ^{13}C theoretically can be incorporated into the target amino acids. However, this would also apply to all other newly synthesised amino acids and would lead to an enrichment of ^{13}C in non-target amino acids. The highest enrichments should then be expected for those amino acids that are being synthesised directly from molecules of the TCA cycle and for those that are intensively consumed for N assimilation and N transport from root to shoot. In both cases these are glutamic acid, aspartic acid and their respective amides.[24] However, we found no significant ^{13}C enrichment in aspartate or any other than the target amino acids (data not shown). Therefore, an overestimation of amino acid uptake is unlikely for CSI measurements.

It is also known that amino acids can be oxidised in the plant cells and fed back into the TCA cycle via an anaplerotic reaction.[25] If this is the case for a taken-up tracer amino acid, the ^{13}C enrichment measured via CSI analysis is likely to underestimate amino acid uptake in contrast to bulk measurements. Differences between both measurements could thus be due to an underestimation of amino acid uptake in CSI measurements. Usually, the re-feeding of amino acids into the TCA cycle happens after amino acids have been reallocated from mature plant tissues to sink organs like growing leaves or roots where they are transformed into molecules needed for the build-up of new biomass.[26] For root systems of mature plants, it is expected that unused amino acids are transported directly to the shoot.[26,27] Our study was performed in August and sampled mature *P. lanceolata* plants with clearly visible young growing leaves. If oxidation of target amino acids took place after uptake we would expect a higher difference between bulk and CSI measurements in shoot than in root tissues. Yet, our results show that differences between both methods are smaller for shoots than for fine roots, i.e. it is very unlikely that any significant oxidation of target amino acids appeared after uptake.

Overestimation of direct amino acid uptake by bulk measurements

We found a higher ^{13}C enrichment for all plant compartments with bulk than with CSI measurements suggesting that this might be the result of one common process affecting all plant compartments. This leads to an overestimation of amino acid uptake as well as an overestimation of the proportion of intact N uptake using bulk measurements and the Näsholm equation, as already reported by some authors.[8,9] These authors partly found relative direct uptake values for ^{15}N of more than 100% and Nordin *et al*.[9] presented some thoughts on the possible causes of this overestimation. The overestimation of amino acid ^{13}C uptake as found in our study did not lead to relative direct uptake rates of ^{15}N >100%. However, the reasons for an overestimation of direct amino acid uptake as given by Nordin *et al*.[9] could potentially also apply to our investigation. These authors investigated only shoot biomass and they ascribed their partly unrealistically high proportions of intact N uptake (>100%) to different compartmentation characteristics of amino acid C and N after uptake into the root. They suggested that most of the ^{15}N taken up as intact amino acid was transferred to the shoot after de- and transamination of the taken-up tracer molecule while ^{13}C was incorporated into the root biomass (see Fig. 1(C)). Although our calculated intact N uptake using the Näsholm equation was within realistic ranges, we also found lower ^{13}C:^{15}N enrichment ratios in shoot than in root material for bulk measurements which would support the ideas of Nordin *et al*.[9] However, because of Nordin's results we deliberately investigated not only shoot material but all plant compartments. As the ratio of ^{13}C to ^{15}N enrichment was not unrealistically high in either of these compartments we have to conclude that the supposed compartmentation might explain the high uptake rates by Nordin *et al*.,[9] but we believe that this is not appropriate in our investigation.

Nordin *et al*.[9] also suggested that the enrichment ratio between ^{13}C and ^{15}N in plant tissue might be influenced by microbial conversion processes of glycine before plant uptake (Fig. 1(E)). Microorganisms are able to transform two molecules of glycine into one molecule of serine plus ammonium and carbon dioxide via the glycine decarboxylase pathway.[11] If this process takes place in the plants' mycorrhiza, plants would take up serine with a C:N ratio of 3:1 instead of the originally applied glycine with a 2:1 ratio. Following Näsholm *et al*.,[3] the plant's enrichment ratio would then be compared with the ratio of the used tracer (2:1) which would mean that 150% of the taken-up ^{15}N was taken up in the form of amino acids. In addition, the uptake of one serine instead of one glycine molecule would also lead to a higher absolute amount of ^{13}C uptake which could explain the higher ^{13}C enrichment of bulk measurements than of CSI analyses in our investigation. However, we found no ^{13}C enrichment in serine for any of the plant compartments. This might indicate either that mycorrhizal amino acid uptake is of minor importance, as suggested by Persson and Näsholm,[28] or that the glycine decarboxylation in our investigations might not have been intense enough to influence the ^{13}C:^{15}N enrichment significantly. However, our data are not adequate to allow a decision on this

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question, let alone the fact that no glycine decarboxylation as suggested by Nordin *et al.*[9] appeared in our investigations.

We believe that our investigations show that bulk measurements overestimate intact amino acid uptake as they imply uptake of ^{13}C as intact amino acid whereas in fact ^{13}C has been taken up only as amino acid fragment: As the major part of the amino acids undergoes an incomplete breakdown via decarboxylation[29] followed by deamination and oxidation in the soil, the remaining products will be organic acids belonging to the low molecular weight (LMW) pool of soil. It has been suggested that plants not only lose a vast amount of these LMW compounds to the rhizosphere, but might also compensate this loss via active uptake of these compounds.[30] Several studies have shown that plants are able to take up not only amino acids, but also carbohydrates and organic acids,[31,32] and that this uptake is not by chance or unspecific.[33] Any incomplete decay of ^{13}C-labelled amino acids will therefore probably lead to an uptake of tracer-derived ^{13}C-enriched organic acids. However, bulk measurements can not differentiate between this ^{13}C uptake and the uptake of intact amino acid ^{13}C, thus leading to an overestimation of ^{13}C enrichment in bulk compared with CSI measurements.

The microbial metabolism of amino acids in soil also produces CO_2 which accounts for a loss of up to 25% of the applied tracer C within 24 h.[34] On its way to the soil surface this CO_2 can be dissolved in soil water by forming bicarbonate.[35] Vuorinen *et al.*[36] have shown that this bicarbonate can be taken up by plant roots and thereafter be incorporated into root biomass via the phosphoenolpyruvate carboxylase (PEP) pathway (dark fixation). In addition to other abiotic and biotic factors, the activity of PEP has been shown to be mainly controlled by the amount of mineral N uptake.[37–41] In this context a number of authors showed that NO_3^- nutrition leads to higher contents in malate and other organic acid ions.[42–45] In detail, Cramer *et al.*[46] found that NH_4^+-dominated nutrition enhances the uptake of bicarbonate followed by an equal distribution of the incorporated C to organic acid and amino acid synthesis with the highest enrichment in asparagine. On the other hand, predominant NO_3^- uptake led to a lower PEP activity and an increased incorporation of the taken-up C into organic acids. Our data provide no evidence for any amino acid enrichment other than of the target ones. Thus, plants might have taken up mainly NO_3^-, bicarbonate fixation was low and ^{13}C enrichment was limited to organic acids. As Cramer *et al.*[46] did not use labelled amino acids together with bicarbonate application, it remains unclear to what extent dark fixation can explain the methodological differences in ^{13}C enrichment found in our investigations.

Additional information from CSI measurements

As bulk measurements cannot differentiate between the uptake of single amino acids, they demand the application of amino acid mixtures in which only one amino acid is labelled. For each additional amino acid to be investigated, a new treatment is needed with a different mixture of labelled amino acids. Therefore, it is not possible to directly compare the uptake characteristics of different amino acids in true replicates. CSI measurements can trace the uptake of single amino acids even if applied in a mixture. Our results show that less glycine than valine was taken up. This supports the findings of Sauheitl *et al.*,[18] but contradicts Lipson *et al.*,[47] who hypothesised that uptake rates of amino acids with lower C:N ratios are higher than for those revealing high C:N ratios.

CSI measurements also give information on the amount of tracer-derived ^{15}N fixed to the target amino acids. Näsholm *et al.*[3] used bulk ^{15}N enrichment to determine the relative proportion of ^{15}N taken up in the form of amino acids to total ^{15}N uptake. Based on the fact that we measured ^{15}N enrichment in the target amino acids and showed that the ^{13}C enrichment in these amino acids reflects the amount of direct uptake, one should expect that the enrichment ratio of ^{13}C:^{15}N in the target amino acids reflects the C:N ratio of the original tracer molecule. In contrast, our measurements show higher ^{15}N than ^{13}C enrichment leading to ^{13}C:^{15}N ratios <1 for glycine and <3 for valine, tyrosine and lysine in all plant tissues. In the bulk method this would imply that less than 100% of ^{15}N fixed to the target amino acids had been taken up in an intact form. Basically, there are two possible ways to explain this finding: Either the C-skeletons of the tracer amino acids have been oxidised after uptake by the plant, or tracer-derived mineral ^{15}N has been added to newly synthesised target amino acids. In the former the low ratios of ^{13}C:^{15}N enrichment in the target amino acids could only be explained if the carboxylic group was split off in an oxidative reaction catalysed by a peroxidase[48] leaving the amino group fixed to the remaining C-skeleton. However, the amines formed in this reaction would no longer be identified as the original target amino acid which makes it necessary that the carboxylic group is added back to the same molecule in an anabolic reaction using a ^{12}C-atom. In the case of glycine this would result in a ^{13}C:^{15}N ratio of 1:1 instead of the original ratio of 2:1. Although this could explain the measured low ^{13}C:^{15}N ratio of just below 1 in storage roots, the whole taken-up amount of tracer glycine would need to be run through the depicted reactions which is unlikely. Moreover, this process cannot explain the enrichment ratios <1 of glycine in all other plant tissues or of the other target amino acids as these molecules have even higher C:N ratios than glycine.

While no ^{13}C enrichment was detected in any other than the target amino acids, which is a clear sign of the relatively low turnover of amino acid C in the plant, all 12 analysed amino acids showed significant ^{15}N enrichments in all plant tissues (data not shown). This can be explained by the fact that if any tracer-derived mineral ^{15}N is taken up by the plant it will partially be used for the formation of amino acids either in the shoot or in the root. In the case of NH_4^+ uptake, nitrogen will quickly be assimilated in the form of amino acids due to its high plant toxicity.[25] As shoots only have a limited capacity for the disposal of protons[49] evolving from the assimilation reaction this is mainly done in the roots. As nitrate is not plant-toxic it can either be stored in plant tissues after uptake or be transported to the shoot or be reduced to ammonium by the nitrate- and nitrite-reductase.[50] In any case of ammonium emerging from plant uptake or transformation reactions, this ammonium will first be assimilated in the form of glutamate and glutamine via

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glutamate dehydrogenase or glutamate synthase and glutamine synthetase.[51] Both molecules are central amino acids for the transport of nitrogen from root to shoot[24] and serve as N source for the synthesis of new amino acids. As the bulk of this synthesis takes place in the chloroplasts of photosynthetic active plant tissues,[52] it is very likely that a large part of the tracer-derived ^{15}N taken up is fixed to newly formed C-skeletons in the shoot. This C is mainly derived from the glycolysis and therefore is largely composed of photosynthetically bound ambient CO_2 revealing natural abundance ^{13}C contents. Therefore, it can be expected that these newly formed amino acids dilute the ^{13}C enrichment found in the target amino acids, leading to smaller ^{13}C:^{15}N enrichment ratios in the target amino acids found in shoot tissues. We believe this to be the main mechanism explaining the generally low ^{13}C:^{15}N ratio in target amino acids in all plant compartments as well as the significantly lower ^{13}C:^{15}N ratio in shoot than in root tissue. However, even the ratios found in the fine roots are too high to be caused by a pure uptake of intact amino acids which clearly shows that 24 h after label application newly formed amino acids have already been transported from the shoot to the root via the phloem.[26]

CONCLUSIONS

Our findings comparing the compound-specific isotope measurement with the ^{13}C bulk measurement lead us to four central conclusions concerning the accuracy and applicability of the two methods:

1. Compound-specific isotope measurements of ^{13}C in the applied amino acids in plant tissues are an accurate indicator of direct plant amino acid uptake that is not affected by the uptake of tracer C-fragments.
2. In contrast, ^{13}C enrichment in bulk measurements was up to 8-fold higher than that of the CSI measurements leading to overestimations of direct amino acid uptake.
3. This overestimation is caused by the uptake of tracer-derived ^{13}C-fragments. Possible species for this uptake are organic acids or bicarbonate ions, both originating directly or indirectly from tracer decay in soil. However, as already requested by Rasmussen and Kuzyakov[53] and Näsholm,[54] further research is needed to show in detail which of these components account for most of the overestimation of amino acid uptake.
4. We were able to show that ^{15}N enrichment in the plant amino acids as derived from compound-specific measurements cannot be used to calculate the proportion of ^{15}N taken up in an intact form as done in the Näsholm equation. This is due to the high turnover of amino acid-bound ^{15}N and mineral tracer-derived ^{15}N in the plant.

Our investigations therefore demonstrate the general accuracy of CSI measurements not only in calculating direct amino acid uptake by plants, but also for studying plant internal allocation of amino acid N. These advantages of CSI measurements justify the higher costs of this measurement.

Although our results show that the combination of bulk measurements with the Näsholm equation[3] tends to overestimate amino acid uptake in a soil of the temperate zone, this does not indicate the inaccuracy of the method per se. It rather shows that Näsholm *et al.*[3] specifically developed the method in soils with low annual mean soil temperature and low pH and therefore a low microbial activity. Future studies therefore should carefully check if this quick and simple method is really suitable for their kind of environment.

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