Direct acquisition of organic N by white clover even in the presence of inorganic N

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Abstract

Aim This study was conducted to answer the question of whether clover can absorb asparagine in the presence and absence of inorganic nitrogen, as well as what is the resulting concentration of post-uptake compounds closely involved in asparagine metabolism.

Methods Clover was grown at two asparagine concentrations (10 µ*M* and 1 m*M*) supplied in both the absence and presence of ammonium nitrate. Using dual-labeled $^{13}C^{15}N$-asparagine, the uptake rate was analyzed via bulk ^{15}N and ^{13}C excess and the detection of intact $^{13}C^{15}N$-asparagine in white clover.

Results The results from the two methods indicated greater utilization of $^{13}C^{15}N$-asparagine in the 10-µ*M* treatment than in the 1-m*M* treatment. The $^{13}C^{15}N$-asparagine uptake rate was higher when $^{13}C^{15}N$-asparagine was provided alone than when it was supplemented with inorganic nitrogen. Up to nine times lower uptake rates were obtained when intact $^{13}C^{15}N$-asparagine was measured than when bulk ^{15}N and ^{13}C excess were analyzed. The labeled amino acids that are closely related to $^{13}C^{15}N$-asparagine metabolism (aspartic acid, glutamic acid and glutamine) were detected in clover roots and shoots.

Conclusions Using two different methods, white clover's potential to absorb intact asparagine, even in the presence of inorganic nitrogen, was confirmed. The dual-methodology approach employed in this study demonstrates how the post-uptake metabolism can affect quantification of amino acid uptake.

Keywords

Amino acids, inorganic nitrogen, uptake, white clover, asparagine metabolism

Introduction

White clover (*Trifolium repens*) is a valuable species in agriculture. It can fix nitrogen (N) directly from the air via symbiosis with *Rhizobium* (Oldroyd and Downie 2004) , which is also reflected in the generally high N content of plants (Winters et al. 2004). All N-rich compounds may be released to surrounding plants either after clover decay or through exudation of nitrogenous compounds when the source plant is still growing and can act as a source of nutrients for subsequent crops (Paynel and Cliquet 2003; Rasmussen et al. 2013b). Therefore, N fixation by clover can also act as a complement or an alternative to inorganic N (IN) fertilizers (Jensen et al. 2012). Moreover, when sown with grasses, clover increases the forage quality because of its high N content (Louarn et al. 2014). Therefore, understanding the mechanisms that govern N fluxes under white clover cultivation is of major importance to enhance clover utilization in sustainable agriculture. It is known that organic N (ON) uptake by plants is an important component of the N cycle in some ecosystems (Nasholm et al. 2009). Consequently, acquisition of amino acids from soil in particular has recently attracted much attention. However, our knowledge about amino acid uptake in white clover is limited. The majority of the literature focuses on clover deposition, which includes both active and passive efflux of compounds from roots to soil (Gylfadottir et al. 2007; Hogh-Jensen and Schjoerring 2001; Rasmussen et al. 2013a). Thus the discussion of whether clover recaptures released compounds remains open. Answering this question could add a practical dimension to the role of white clover in N cycling and its contribution to the cycling of organic matter.

Previous studies have demonstrated the potential of white clover for amino acid uptake. Lesuffleur et al. (2007) measured the influx of glycine and serine under axenic conditions and reported influx rates for glycine and serine that ranged between 1 and 4 µmol g^{-1} DW h^{-1}. In comparison, Macduff et al. (2002) reported white clover nitrate absorption rate of 38 µmol g^{-1} DW h^{-1}. . However, the ability of clover to assimilate amino acids in the presence of IN forms is poorly characterized. Such an evaluation is important because soil solution contains different N compounds and their co-occurrence can influence the uptake of both IN and ON. Our knowledge about the interactions among different N forms during uptake in white clover is largely based on studies that have investigated the relationships between IN compounds and/or N_2 fixation (Griffith et al. 2000; Herrmann et al. 2002; Macduff et al. 2002). Leidi and RodrÍGuez-Navarro (2000) found a negative relationship between nitrate application and N_2 fixation in bean (*Phaseolus vulgaris* L. cv. Canellini). The focus on IN has also been driven by the classical paradigm of inorganic N being the source of plant N nutrition (Schimel and Bennett 2004), but the recent finding (Jones et al. 2005; Nasholm et al. 1998; Weigelt et al. 2003) that plants take up ON has reopened the discussion about the N

pools that are available to plants. Studies that investigated the importance of interactions between ON and IN sources have been performed on crop species such as perennial ryegrass (*Lolium perenne* L.) (Thornton and Robinson 2005) and wheat (*Triticum aestivum* L.) (Gioseffi et al. 2012). Thornton and Robinson (2005) reported that the glycine uptake in ryegrass, as a proportion of the total N uptake, increased when the ryegrass was supplied with NO_3^- and NH_4^+ compared with individually provided N sources. Evidence for interactions between uptake of ON and IN was also presented by Gioseffi et al. (2012). In both studies, the authors concluded that there was down-regulation of NO_3^- uptake in the presence of glycine. To our knowledge, no investigation of amino acid uptake in the presence of IN has been conducted on forage legumes, which are special in the sense of an expected net N outflow of the roots due to the N_2 fixation of these species.

Accurate measurement of the direct uptake of intact amino acids is the key to understanding the role of ON in plant N nutrition. One criticism of the literature regarding amino acid absorption is that some of the studies have measured recovery of ^{15}N in the target tissue after application of ^{15}N-labeled amino acids (Lesuffleur and Cliquet 2010a; Lesuffleur et al. 2007) or analyzed bulk ^{13}C and ^{15}N enrichments after exposure to ^{15}N,^{13}C-dual labeled amino acid (Nasholm et al. 1998; Nasholm et al. 2000). The main limitation of the bulk ^{15}N and/or ^{13}C measurements is that it cannot separate the acquisition of intact molecules from the acquisition of transformed molecules prior to uptake , which may lead to either uptake overestimation (Rasmussen et al. 2010; Sauheitl et al. 2009a) or underestimation due to respiration loss of ^{13}C (Warren 2012). Clear evidence for uptake is provided by the detection of intact labeled amino acids in plant material determined by, e.g., gas or liquid chromatography mass spectrometry (Nordin et al. 2004; Ohlund and Nasholm 2001; Persson et al. 2003; Persson and Nasholm 2001a; Warren 2012). However, such targeted analyses are also limited by the rapid catabolism of amino acids after absorption (rapidly after uptake); thus, rather short experiments must be performed. Such metabolic reactions will lead to either loss of ^{13}C (decarboxylation) or transfer of ^{15}N to other molecules (deamination and transamination). To account for label flux, an isotopologue analysis is a powerful tool to determine the rearrangements of the labeled elements among the compounds. Isotopologues are molecules that differ in isotopic composition, where one molecule has at least one atom with a different number of neutrons in comparison to parent molecule (Gold et al. 1987).

Consequently, the true uptake of intact amino acid is usually difficult to determine because of the pre- and post-uptake transformations of the labeled elements. In this sense, combination of both methods compound-specific analyses with bulk measurements of ^{13}C and ^{15}N can address the challenge of determining the uptake. Firstly, because they lead to different information, and secondly those information supplement each other.

In a number of studies (Lesuffleur et al. 2007; Paynel et al. 2001; Varin et al. 2010), asparagine (Asn) was reported to be the most abundant amino acid in clover root extracts. It is also the primary N transport molecule in other legumes such as lucerne, pea, and lupin (Lea et al. 2007). Therefore, universally labeled L-asparagine-$^{13}C_4$,$^{15}N_2$ ($^{13}C_4{}^{15}N_2$-Asn) was selected for the present study. Two experiments were conducted to determine the uptake of Asn by white clover with or without the presence of IN. In the first experiment, the objective was to assess white clover's potential for absorbing Asn and the resulting concentration of post-uptake compounds that are closely involved in Asn metabolism (aspartic acid, glutamine and glutamic acid) and their isotopologues when Asn was the only source of N supplied to the clover. The underlying hypothesis was that Asn would be an attractive nutrient to the clover because of its high abundance in the roots and therefore would be taken up by the clover. In the second experiment, the objective was to determine the uptake of Asn by clover and the concentration of its three metabolites and their isotopologues in the presence of ammonium nitrate (NH_4NO_3). The tested hypothesis was that Asn uptake would be reduced, although still discernible, when the amino acid was supplied along with NH_4NO_3 because IN is more easily assimilated by plants (Schimel and Bennett, 2004).

Materials and methods

Germination

White clover (*Trifolium repens*, cv. Rivendel) seeds were surface-sterilized by shaking them first for 10 min in 5% sodium hypochlorite and then for 10 min in 70% ethanol. Then, they were rinsed with sterilized water in a laminar flow cabinet (Pedersen et al. 2013). The seeds were sown on sterile petri dishes that contained 20 ml of sterilized growth medium (3 g/l phytagel, 5 g/l sucrose, and 1.9 g/l $MgSO_4$ dissolved in nutrient solution). The nutrient solution was modified according to Laine et al. (1994) and El-Naggar et al. (2009), and it contained the following macronutrients (the names of the macronutrients are preceded by their concentrations in units of μM): 150 K_2HPO_4, 1000 K_2SO_4, 400 KH_2PO_4, 500 $MgSO_4$, and 3000 $CaCl_2$. It also contained the following micronutrients: 14 H_3BO_3, 5 $MnSO_4$, 3 $ZnSO_4 \cdot 7H_2O$, 0.7 $CuSO_4$, 0.1 $CoCl_2$, $2x10^{-5}$ Fe-Na-EDTA, $4.95x10^{-7}$ $NiSO_4 \cdot 6H_2O$, $7.8x10^{-5}$NaCl, and $1.2x10^{-6}$ Na_2MoO_4. The petri dishes were sealed with parafilm and kept in the laboratory at room temperature for five days, exposed to light at a photon flux density of 70 µmol s^{-1} m^2 and a 16/8 h day/night cycle.

Asn uptake without IN – experimental setup

After five days of germination, seedlings were transferred under sterilized conditions to sterilized double-glass vials in a hydroponic setup based on El-Naggar et al. (2009). Two sets of Asn concentration treatments—10 µ*M* and 1 m*M*, each containing 16 vials—were prepared. Eighty milliliters of nutrient solution that contained Asn at a concentration of either 10 µ*M* or 1 m*M* was poured into the vials. Thus, immediately after the transfer, the plants were conditioned to grow on Asn as the only source of N (hereafter referred to as ON). The 10-µ*M* treatment was chosen because it reflects the amino acid concentration that is generally found in soil (Hill et al. 2011; Nasholm et al. 2009). The 1-m*M* treatment was included to sample a very high concentration and ensure that there was a theoretical possibility of detecting the uptake of intact Asn. After 5 seedlings per vial for each of the concentration treatments (10 µ*M* and 1 m*M* ON) were placed in the vials, the vials were sealed with parafilm, covered with black bags underneath , and kept under the same conditions as during germination for six weeks. Three times per week, the nutrient solution (pH maintained at 5-6) was changed under a laminar flow cabinet. During the plant growth, 4 of the 16 vials in the 10-µ*M* treatment were contaminated. Consequently, only 6 vials were used for determining the Asn natural abundance and 6 for the Asn uptake. In the 1-m*M* treatment, 6 vials were contaminated; thus, 5 vials were used for measuring the natural abundance of Asn and 5 for the Asn uptake. After 6 weeks of growth, the vials were transferred to the laminar flow cabinet. First, 6 (10 µ*M* ON) and 5 (1 m*M* ON) vials were selected, and plants from those vials were taken out and submerged in tubes that contained 15 ml of either 10 µ*M* or 1 m*M* $^{13}C_4{}^{15}N_2$-Asn (98 atom% ^{13}C, 98 atom% ^{15}N) sterilized nutrient solution, depending on the concentration of the solution in which they were grown, to determine the uptake of Asn (Persson and Nasholm 2001a). The remaining 6 and 5 vials from the 10-µ*M* and 1-m*M* ON treatments, respectively, were used to determine the natural abundance of Asn, its three metabolites and their isotopologues. Plants from those vials were then removed and immersed in the respective tubes that contained 15 ml of either 10 µ*M* or 1 m*M* sterilized unlabeled Asn solution (≥98%). After 60 min of exposure to either labeled or unlabeled amino acids, the shoots were cut off, and the roots thoroughly washed in 0.5 *M* $CaCl_2$ and dried with a paper towel. Shoot and root materials were immediately frozen in liquid N. The 10-µ*M* and 1-m*M* labeled and unlabeled solutions were also sampled and frozen for later use in the amino-acid-depletion calculations.

Asn uptake in the presence of IN – experimental setup

Clover seeds were germinated and pre-grown under the same conditions as above. Plants were then grown in the same nutrient solution that contained both Asn and NH_4NO_3; the latter served as an IN supplement. Two N supplementation treatments, (1) 10 µ*M* Asn + 10 µ*M* NH_4NO_3 and (2) 1 m*M* Asn + 1 m*M* NH_4NO_3, each containing 16 vials, were prepared (the combination is hereafter referred to as ON+IN). After 5 seedlings were placed in each of the treatments, the vials were kept under the same light and temperature conditions as mentioned above. During the plant growth in 7 and 4 vials with 10 µ*M* and 1 m*M* ON+IN, respectively, contamination was observed, and these vials were removed. Therefore, for the final uptake experiment, there were 9 vials for the 10-µ*M* ON+IN treatment and 12 for the 1-m*M* ON+IN treatment. Plant cultivation in the glass vials lasted 5 weeks. After that time, the clover's aboveground biomass reached the biomass of the plants from the ON experiment; therefore, we decided to terminate the growth and take the vials to the laminar flow cabinet. Five and 6 vials were selected from the 10-µ*M* and 1-m*M* ON+IN treatments, respectively. Plants from those vials were carefully taken out and submerged in the respective tubes that contained 15 ml of sterilized mixture of 10-µ*M* or 1-m*M* $^{13}C_4{}^{15}N_2$-Asn+NH_4NO_3 nutrient solution. The remaining 4 (10 µ*M* ON+IN) and 6 (1 m*M* ON+IN) vials were used to determine the natural abundance of Asn, its three metabolites and their isotopologues. Plants from those vials were then removed and immersed in the respective tubes that contained 15 ml of either 10-µ*M* or 1-m*M* sterilized unlabeled Asn + NH_4NO_3 solution. After 60 min of exposure to either labeled or unlabeled amino acids, the shoots were cut off and the roots thoroughly washed in 0.5 m*M* $CaCl_2$ (Persson and Nasholm 2001b) and dried with a paper towel. Shoot and root materials were immediately frozen in liquid N. The labeled and unlabeled solutions were also sampled and frozen for later use in the amino-acid-depletion calculations.

Quantification of bulk ^{15}N and ^{13}C in the roots and shoots

The roots and shoots were freeze-dried, weighed and ground to a fine powder. Two milligrams of samples were weighed in tin capsules and analyzed for bulk ^{15}N and ^{13}C with an Elementar Analyzer (Flash EA 2000, Thermo Fisher Scientific, Bremen, Germany) coupled with an Isotope Ratio Mass Spectrometer (EA-IRMS) (DeltaV, Thermo Fisher Scientific, Bremen, Germany).

Extraction and derivatization of amino acids in the root and shoot samples

One milligram of the ground plant material was extracted in a 1-ml extraction mixture of chloroform, methanol and water (1:3:1, v:v:v) that contained norvaline as an internal standard (0.25 pmol/µl) in Sarstedts Eppendorf tubes (Sarstedt AG & Co, Nümbrecht, Germany). One metal bead (a 3-mm tungsten carbide bead) was added to each tube. All tubes were shaken at a frequency of 30 Hz for 3 min in a MM 301 Vibration Mill (Retsch GmbH&Co. KG, Haan, Germany). After shaking, the metal beads were removed, and the tubes were centrifuged at 14,000 rpm at 4°C for 10 min. From each of the root and shoot extracts, 200 µl was transferred to liquid chromatography–mass spectrometry (LC/MS) vials, and the rest of the supernatant was stored at -80°C. In a vacuum centrifuge, 200 µl of extracts was evaporated to dryness. The dry extracts were then re-suspended in 20 µl of 20-m*M* HCl and derived with the AccQ•Tag Ultra DerivatizationKit (Waters Corp.) according to the manufacturer's protocol.

Derivatizationof amino acids in the hydroponic solutions

To facilitate transport of the solution samples, they were thawed, and 1 ml from each solution sample was placed in Sarstedts Eppendorf tubes and dried in a vacuum centrifuge (Thermo Scientific SPD121 P, SpeecVac Concentrator). Subsequently, the dried samples were re-dissolved in 1 ml of 20-m*M* HCl (samples of 1-m*M* solution were additionally diluted 100 times in Milli-Q water). From each re-suspended sample, 100 µl was placed in an LC/MS glass vial, and 10 µl of 5-µ*M* norvaline was added. Then, the solvent was again evaporated in a vacuum centrifuge. The dry samples were then re-suspended in 20 µl of 20-m*M* HCl and derived with the AccQ•Tag Ultra Derivatization Kit (Waters Corp.) according to the manufacturer's protocol.

Amino acid analysis in the derivatized samples

The derived root, shoot and solution samples were analyzed with an Agilent 6540 UHD Accurate Mass Q-TOF LC/MS using an electrospray ionization (Dual AJS ESI) probe in the positive mode. The acquisition method was based on Armenta et al. (2010) and Johansson et al. (manuscript in prep.). The derived amino acids were separated on a Phenomenex C18 column (2.1 mm x 100 mm, 1.7 µm). The following separation gradients were used: 0–0.54 min (99.9% A), 5.50 min (90.9% A), 7.70 min (78.8% A), 8.50–9.00 min (40.4% A), 9.50–10.00 min (20.0% A), and 10.50-15.00 (99.9% A). Eluent A consisted of Milli-Q water and 0.1% formic acid, eluent B consisted of acetonitrile and 0.1% formic acid, and the column flow rate was 0.5 ml/min. The column temperature was set to 55°C, and the sample injection volume was 2 µl. Full-scan analysis was performed to detect all of the

major components in the samples. The amino acids of interest (Asn, Asp, Gln, Glu) and their isotopologues were subsequently extracted using the Agilent Mass Hunter Workstation B.07 software package. In this context an "Asn isotopologue" means an Asn molecule which has at least one atom with a different number of neutrons in comparison to parent molecule of Asn. For example, Asn+1 represent a moleclue, for which the analyzed mass was the monoisotopic mass of Asn (132.053 u) with an addition of the mass of one neutron (1.008 u). In other words it represents an Asn molecule, where one element of either ^{12}C or ^{14}N was replaced with its isotope of ^{13}C or ^{15}N, respectively. A list of the compounds analyzed is presented in Table 1. After the analysis, subsamples of the derived root and shoot extracts were also analyzed after 40 and 250 times dilution due to the high amino acid (Asn, Asn+1, Gln, Glu) concentrations.

Amino acid quantification

The calibration curves of four unlabeled amino acids – Asn, Asp, Gln, and Glu (Sigma-Aldrich, Sweden) – were prepared from authentic standard compounds in the range of 0.1 – 10 pmol/2 µl injected into the column. The peak area of each isotopologue from the standard isotopic distribution was divided by the peak area of the internal standard (norvaline). The normalized peak areas of all amino acid isotopic variants were summed to yield a total amino acid peak area, and the total amino acid peak area of the standards was plotted against the standard concentration that was entered in the column. A linear function was applied to the calibration curves. To determine the concentrations of the amino acids in the samples, the peak area of each amino acid isotopic variant was normalized using the peak area of norvaline and calculated using the regression equations from the calibration of summed isotopic forms from the standards. For four of the amino acids (Asn, Asn+1, Gln, and Glu), the concentrations exceeded the range of the calibration curve; thus, subsamples were taken and diluted 40 or 250 times and used for the quantification.

The minimum level at which each of the amino acids could be reliably quantified was determined by the limit of detection (LOD). Based on the background noise level, the LOD was defined as the concentration of the analyte that generated a signal equal to three times the background noise. However, because of the limitations of the chemical analysis procedure, small concentrations of amino acids could not be precisely measured, which resulted in concentrations below the LOD. These concentrations were replaced with a constant value equal to the LOD divided by the square root of 2 (Croghan and Egeghy 2003).

Calculations and statistical analysis

The effects of the N source (ON vs. ON+IN) and the N concentration (10 µM vs. 1 mM) were analyzed by performing an analysis of variance (ANOVA) followed either by the t-test or Tukey's test. The Shapiro-Wilk test was performed to determine whether the data were normally distributed. The data were transformed, where necessary, to satisfy the assumptions of normality and analyzed using the R Studio software package (version 0.99.46 running R version 3.1.1).

The mean atom% values of ^{15}N and ^{13}C in the unlabeled root and shoot samples were calculated to determine the natural abundances of the isotopes. Subsequently, the respective means of the natural ^{15}N and ^{13}C abundances in the unlabeled plant material were subtracted from the ^{15}N and ^{13}C atom% values in the labeled root and shoot samples to determine the ^{15}N and ^{13}C excess. Uptake of Asn was verified by analyzing the relationship between the ^{13}C and ^{15}N excesses using linear regression analysis (McFarland et al. 2010; Nasholm et al. 1998; Weigelt et al. 2005).

The concentrations (µmol g^{-1} DW) of the labeled naturally abundant $^{13}C_4{}^{15}N_2$-Asn in the unlabeled clover roots and shoots were subtracted from the concentrations of the $^{13}C_4{}^{15}N_2$-Asn in the labeled roots and shoots to determine the $^{13}C_4{}^{15}N_2$-Asn excess.

Results

Clover growth in the hydroponic solution

After 5 (ON+IN) and 6 (ON) weeks of growth, the clover's dry biomass was measured. The clover growth was greater and faster in the presence of IN ($p<0.001$) than when supplied with ON (Table 2). The dry root biomass was also as much as 16% greater in the 1-m*M* treatment than in the 10-µ*M* treatment, but this difference was not significant (Table 2). Moreover, the clover shoots that were grown in the 1-m*M* treatment accumulated approximately 33% more biomass than did those in the 10-µ*M* treatment (Table 2). The values of root:shoot ratio were also greater for the plants exposed to ON+IN than those solely with ON (Table 2). In contrast, the root:shoot ratio was found to be negatively related to the concentration: in the 1-m*M* treatment, the ratio was as much as 19% smaller than it was at 10 µ*M*.

279 Total profile of the four analyzed amino acid in clover roots and shoots

280

281 After the uptake experiment, total Asn, Asp, Gln, and Glu were quantified in the clover roots and shoots
282 that were grown with 10-µ*M* or 1-m*M* ON in both the presence and absence of IN (Figure 1). By "total", we mean
283 the sum of all isotopologues for each of the four analyzed amino acids (Asn, Asp, Gln, Glu). No significant
284 differences in the abundances of amino acids between labeled and unlabeled roots or shoots were detected.
285 Therefore, the data for the labeled and unlabeled roots and shoots were pooled and analyzed for the effects of
286 concentration (10 µ*M* vs. 1 m*M*) and N supplementation (ON vs. ON+IN) on the amino acid abundance. The most
287 abundant amino acid in the clover tissues was Asn, which constituted from 54% (in the roots) to 93% (in the shoots)
288 of all four amino acids (Figure 1). In the roots, only the concentration had an effect on the amino acid profile:
289 approximately 30% more Asn was accumulated in the 1-m*M* roots than in the 10-µ*M* treatment ($p<0.05$). In the
290 shoots, the effect of N supplementation on the amino acid distribution was more prominent than it was in the roots
291 (Figure 1). The clover plants that were supplied only with ON were characterized by significantly greater
292 percentages ($p<0.05$) of Asn in the shoots than were plants that were grown on ON+IN. Interestingly, the opposite
293 tendency was observed for Gln, whereas for Asp and Glu, no differences between the treatments were detected. For
294 absolute concentrations of four analyzed amino acids, please refer to Online Resource 1.

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296 $^{13}C^{15}N$-Asn uptake by clover analyzed using EA-IRMS

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298 Enhanced bulk ^{15}N and ^{13}C abundances were observed in clover roots in all $^{13}C_4{}^{15}N_2$-Asn treated plants,
299 both with and without IN (Table 3). Similar results were obtained for shoots except for one case, in which bulk ^{13}C
300 was not detectable in the shoots after 60 min of exposure to the 10-µ*M* $^{13}C_4{}^{15}N_2$-Asn. Significant effects of the
301 $^{13}C_4{}^{15}N_2$-Asn concentration (10 µ*M* vs. 1 m*M*) and N supplementation (ON vs. ON+IN) on the bulk ^{15}N and ^{13}C
302 excess in roots and shoots were observed. In the roots, significantly more ^{15}N was found when the clover was
303 supplied with 1-m*M* $^{13}C_4{}^{15}N_2$-Asn ($p<0.05$) compared with the 10-µ*M* treatments (Table 3). Moreover, the clover
304 roots contained significantly more ^{15}N when fed with only ON ($p<0.05$) compared with when they were additionally
305 supplied with IN. The same results were obtained for the ^{13}C excess in the roots (Table 3). In the shoots, similar
306 evidence for the effect of the treatment on the ^{15}N and ^{13}C excess was found (Table 3).

307 Regressions of the ^{13}C excess against the ^{15}N excess in roots and shoots were used to calculate the fraction
308 of $^{13}C_4{}^{15}N_2$-Asn that was assimilated as intact amino acid. Because one mole of added labeled Asn contains four

moles of ^{13}C and two moles of ^{15}N, the slope that corresponds to 100% uptake of intact amino acid equals two. The ratios between ^{15}N and ^{13}C that were recovered in the roots supplied with 1-m*M* $^{13}C_4{}^{15}N_2$-Asn were significant and equal to 2.01 (R^2=0.96, p<0.05) and 2.2 (R^2=0.95, p<0.05), respectively, for the treatments with the presence and absence of IN (Figure 2b). However, in the roots that received 10-µ*M* $^{13}C_4{}^{15}N_2$-Asn, the ^{13}C and ^{15}N ratios were lower, corresponding to 0.75 (R^2=0.44, p>0.05) and 1.2 (R^2=0.97, p<0.05), respectively, in the presence and absence of IN (Figure 2a). In the shoots, the ^{13}C and ^{15}N ratio was less pronounced than it was in the roots. In the 10-µ*M* treatment, the slopes were not significant and equal to 1.2 (R^2=2x10^{-6}, p>0.05) and -6.8 (R^2=0.32, p>0.05) for shoots in the absence and presence of IN, respectively (Figure 2c). In the shoots from the 1-m*M* treatment, the slopes were equal to 2.17 (R^2=0.92, p<0.05) and 2.93 (R^2=0.88, p>0.05) for the clover grown in the presence and absence of IN, respectively (Figure 2d).

Based on these data, the total $^{13}C_4{}^{15}N_2$-Asn uptake rate by clover was calculated (Table 4). In the 1-m*M* treatment, these uptake rates were equal to 32.6 (ON) and 12.3 (ON+IN) µmol g^{-1} DW h^{-1}, whereas in the 10-µ*M* treatments, they were 2.5 (ON) and 0.2 (ON+IN) µmol g^{-1} DW h^{-1}. These values account for the maximum uptake rates assuming that 100% of ^{15}N came from the acquisition of the intact $^{13}C_4{}^{15}N_2$-Asn. In both cases, the uptake rates were greater for the clover that was supplied only with ON than when the clover was supplemented with IN, being approximately twelve times greater for the 10-µM treatment and three times greater for the 1-mM treatment.

$^{13}C^{15}N$-Asn uptake and isotopologue profile in clover – quadrupole time-of-flight (Q-TOF) LC/MS analysis

No $^{13}C_4{}^{15}N_2$-Asn was found in the unlabeled clover plants (Online Resource 2), thus confirming that all $^{13}C_4{}^{15}N_2$-Asn detected in the labeled clover was derived from the labeled compound. Similarly, in the case of the EA-IRMS measurements, Q-TOF LC/MS revealed that there was a significant effect of concentration (10 µ*M* and 1 m*M*) and N supplementation (ON vs. ON+IN) on the amount of $^{13}C_4{}^{15}N_2$-Asn found in the clover roots (Table 3). In the 10-µ*M* treatment, nearly eight times more intact $^{13}C_4{}^{15}N_2$-Asn was found in the roots that were supplied only with ON compared with when IN was present (p<0.05); in the 1-m*M* treatment, the difference was less pronounced (Table 3). Moreover, approximately eight and fifty times more intact $^{13}C_4{}^{15}N_2$-Asn was found in the 1-m*M* roots than in the 10-µ*M* roots for the treatments in the absence and presence of IN, respectively (Table 3). Based on these measurements, the total $^{13}C_4{}^{15}N_2$-Asn uptake rate by clover was calculated (Table 4). Similar to the EA-IRMS data, the total $^{13}C_4{}^{15}N_2$-Asn uptake was greater in the absence than in the presence of IN. Comparing the results for total intact $^{13}C_4{}^{15}N_2$-Asn uptake rate from the LC-qTOF analysis with the total intact uptake based on

the bulk ^{15}N and ^{13}C excesses from the EA-IRMS measurements, it can be observed that up to nine (ON) and five (ON+IN) times lower uptake rates were estimated when using the values derived from the Q-TOF LC/MS data compared with the EA-IRMS values (Table 4).

Further Q-TOF LC/MS analyses focused on the amino acids from $^{13}C_4{}^{15}N_2$-Asn post-uptake metabolism. Comparison of amino acids in unlabeled and labeled plants indicated seven amino acid isotopologues (Asn+5, Asp+4, $^{13}C^{15}N$-Asp, Gln+4, Gln+5, Glu+5, Glu+6) that were found solely in the labeled clover tissues that originated from the $^{13}C_4{}^{15}N_2$-Asn (Online Resource 2). All of these compounds were present in the labeled clover roots (Figure 3), whereas in the shoots, only trace amounts of Asn+5, Asp+4, and $^{13}C^{15}N$-Asp were detected (data not shown). In general, all of the amino acid isotopologues were more abundant in the roots that were grown exclusively with ON than in the roots that were additionally supplied with IN. In particular, the Gln+4 and Gln+5 concentrations in the 10-µ*M* treatment and the $^{13}C^{15}N$-Asp and Asp+4 concentrations in the 1-m*M* treatment were significantly greater in the ON roots than in the ON+IN roots ($p<0.05$) (Figure 3). Moreover, when comparing the 10-µ*M* and 1-m*M* treatments, the amino acid isotopologues were approximately one order of magnitude more abundant in the roots that were exposed to 1-m*M* $^{13}C_4{}^{15}N_2$-Asn compared with those exposed to the 10-µ*M* treatment.

Amino acid isotopologue profile in the start labeled solutions

Three isotopologues were detected in the start labeled solution that was fed to the clover for the uptake experiment: Asn+5, Asp+4, and $^{13}C^{15}N$-Asp (Figure 4). These compounds were present in the labeled start solution but were not detected in the unlabeled solution (Online Resource 3). In the 10-µ*M* start solution, the occurrences of isotopologues were approximately one (Asn+5, $^{13}C^{15}N$-Asp) and two (Asn+4) orders of magnitude less than that of $^{13}C_4{}^{15}N_2$-Asn, whereas in the 1-m*M* treatment, they were one (Asn+5, $^{13}C^{15}N$-Asp) and up to three (Asp+4) orders of magnitude less than that of $^{13}C_4{}^{15}N_2$-Asn (Figure 4). Furthermore, the calculated ratios between Asn+5 to $^{13}C_4{}^{15}N_2$-Asn and Asp+4 to $^{13}C^{15}N$-Asp were similar in all of the start solutions and were approximately equal to 30 (Online Resource 4).

Discussion

Knowledge of the below-ground plant-soil N pathways is important for understanding the availability of N for plant growth. White clover-based leys studies on this topic have mostly focused on N rhizodeposition, that is,

369 "What N forms are coming out from roots?" (Gylfadottir et al. 2007; Hogh-Jensen and Schjoerring 2001). Only
370 limited information is available on the reverse process, "What N forms are taken up by clover?" (Lesuffleur et al.
371 2007). Improving our understanding of both the efflux and influx of ON across the clover root is needed to better
372 manage the N input from legumes' N_2 fixation, especially in relation to soil organic matter cycling and soil N
373 fertility. The present study investigated the uptake of Asn by white clover in the absence and presence of IN in a
374 hydroponic system.
375
376 Enhanced growth of clover in the presence of IN
377
378 The aim of this study was to investigate uptake of Asn by clover, for this root size is of importance. After
379 5 weeks, the plants from the ON+IN treatment had reached similar size as the plants from the ON experiment grown
380 for 6 weeks (Table 2). Our results demonstrated that Asn in combination with IN caused greater root biomass
381 production than did Asn alone (Table 2). To deduce any preferences for IN uptake, one would have to include an
382 IN-only treatment; however, this was not the aim of the present study. Furthermore, when comparing the ON and
383 ON+IN treatments, the difference in the total N concentration must be considered; in the ON+IN treatment, the
384 available N was double that of the ON-only treatment. This higher N availability could have increased clover growth
385 when it was supplemented with IN (Ohlund and Nasholm 2001), and the nutrient accessibility controls biomass
386 production (Krapp et al. 2011).
387
388 Asn as the most abundant among four analyzed amino acids in clover roots and shoots
389
390 Previous studies (Lesuffleur et al. 2007; Paynel et al. 2001; Varin et al. 2010), showed that Asn, Asp, Gln
391 and Glu were the predominant among all free amino acids in clover with Asn being the most abundant and
392 representing up to 50% of the total amino acids. Simmilar results were also obtained by Lesuffleur and Cliquet
393 (2010b), who reported on Asn being the major amino acid compound in clover tissues (60% of the total free amino
394 acids). In addition, Glu, Gln, and Asp were the subsequent most prominent amino acids, representing together 33%
395 of total free amino acids in clover roots. Therefore, this study was focused on those four amino acids: Asn, Asp,
396 Gln, Glu, which according to previous investigations represent the biggest fraction of amino acids in clover. In
397 agreement with these findings is that Asn was the most abundant, representing from 54% (roots) to 93% (shoots)
398 of the total content of the four analyzed amino acids (Asn, Asp, Gln, Glu) (Figure 1). In addition to that, specific

differences in the amino acid profiles were observed (Figure 1). First, the lower abundance of Asn and greater abundance of Asp and Glu in the roots than in the shoots indicated differing utilization of Asn by these organs. It seems possible that clover uses Asn as the source of N that is intact and directed to the developing aboveground parts, whereas the Asn left in the roots is more extensively utilized because of the need for energy to assimilate other nutrients and root growth. This difference may be related to the fact that Asn is the major molecule that stores and transports N in the phloem in leguminous plants (Lea et al. 2007), which in turn can also trigger N uptake as a response to negative feedback control by the root Asn concentration (Tillard et al. 1998). The reverse pathway could explain the greater abundance of Asp and Glu in the roots because these amino acids can act as endogenous signals that regulate the Asn uptake and therefore are exported from shoots to roots (Tillard et al. 1998). Second, N supplementation (ON vs. ON+IN) affected the abundance of Asn, Asp, Gln and Glu in the shoots, whereas in the roots, only the concentration (10 µ*M* vs. 1 m*M*) influenced the Asn and amino acid profiles. It appears that root metabolism is more sensitive to nutrient quantity than quality; in the 10-µ*M* treatment, Asp, Gln, Glu accounted for 7–20% of all four amino acids, whereas in the 1-m*M* treatment, they ranged from only 2 to 7% (Figure 1). This difference arose possibly because the metabolism of Asn is more pronounced when Asn is provided in limited amounts (10 µ*M*) than when it is supplied in excessive concentrations (1 m*M*). Furthermore, in the shoots, the abundance of Asn varied among the different N supplementations, with more Asn found in the shoots of clover that was supplied only with ON (Figure 1). In a study with *Arabidopsis thaliana* seedlings, Cambui et al. (2011) reported that in plants grown on a split-root system that contained NO_3^- on one side and Gln on the other, a smaller fraction of the shoot N was N derived from Gln than from NO_3^-. A similar explanation could be applied to our findings, implying that when IN is present for clover uptake, it contributes more to clover nutrition and allocation in the shoots than in the roots. In summary, Asn, being the most abundant of the four measured amino acids, responded in an organ-specific manner that was affected by the concentration (10 µ*M* vs. 1 m*M*) and N form (ON vs. ON+IN) supplied to the clover.

Clover absorbs Asn even in the presence of IN

The presence of intact amino acid molecules within plant tissue is the strongest evidence for intact uptake (Rasmussen et al. 2010; Sauheitl et al. 2009a). For this reason, clover roots and shoots were analyzed for the intact $^{13}C_4{}^{15}N_2$-Asn using Q-TOF LC/MS. The results clearly indicated that clover took up intact $^{13}C_4{}^{15}N_2$-Asn from the solution (Table 3). However, the uptake rates of $^{13}C^{15}N$-Asn based solely on the $^{13}C^{15}N$-Asn were from 16 to 47%

smaller than when included other compounds closely related to $^{13}C^{15}N$-Asn post-uptake metabolism (Table 4). This shows that the post-uptake metabolism affects quantification of the amino acid uptake (Table 4). Seven amino acid isotopologues (Asn+5, Asp+4, $^{13}C^{15}N$-Asp, Gln+4, Gln+5, Glu+5, and Glu+6) were present solely in the labeled clover tissues and were not detected in unlabeled clover(Online Resource 2). Three of these isotopologues were also found in the start labeled solution that was fed to the plants (Asn+5, $^{13}C^{15}N$-Asp, and Asp+4) (Online Resource 3) although in very low in proportion to the amount of $^{13}C^{15}N$-Asn which was the dominating amino acid in the solutions (x-x % Online Resource 4). This result implies that these isotopologues could also have contributed to isotope uptake by clover. The isotopologue content in the roots was reflected in their composition in the start labeled solution (Figure 3 and 4) and the ratios between Asn+5 and $^{13}C^{15}N$ Asn in the solution corresponded to their ratios in the roots (Online Resource 4), which complicates the disentanglement of uptake of Asn+5 and post uptake metabolism of $^{13}C^{15}N$ Asn to Asn+5in the roots.. In contrast, Gln+4, Gln+5, Glu+5 and Glu+6 which were nort present in neither of the start solutions (labeled and unlabeled, Online Resource 3) were found only in clover roots exposed to the labeled solution (Figure 3); thus, their origine have to be from the internal $^{13}C^{15}N$-Asn metabolism. A possible route for this could be deamination of $^{13}C^{15}N$-Asn into $^{13}C^{15}N$-Asp, which is then converted to oxaloacetate (OA) that enters Krebs cycle, where subsequent reactions may lead to formation of 2-oxoglutarate, and then to Glu and Gln production (Hildebrandt et al. 2015). In addition, all of the isotopologues were found to be more abundant in the roots of clover supplied exclusively with ON (Figure 3). This result demonstrates that $^{13}C_4{}^{15}N_2$-Asn was utilized to a greater extent when it was provided alone than when it was supplied with IN because this was the only source of N available for the plants' biomass accumulation. However, when ON was accompanied by IN, it contributed to the plant's N budget to a lesser extent. This could have been because it was partially replaced by IN. This finding corroborates the data regarding $^{13}C_4{}^{15}N_2$-Asn uptake rates, which were less when clover was fed with ON and IN and thus less utilized in conversion into amino acids (Table 3). Although the total Asn were approximately the same in plants grown on both ON and ON supplied with IN (Online Resource 1). Not only post-uptake metabolism can affect the uptake rate, but pre-uptake processes as well. Asn can during some circumstances be easily degraded via abiotic factors but the composition of the uptake solution could also be changed due toplant-mediated changes secretion of exoenxymes or efflux of previously absorbed compounds. To quantify the impact of these factors on ON uptake was beyond the scope of this study but $^{13}C_4{}^{15}N_2$-Asn were the major labeled amino acid in all start as well as all end solutions x-x% (Online Resourse 4). Some of these processes could have taken place during uptake by clover as the ratios of Asn+5:$^{13}C^{15}N$-Asn and $^{13}C_4{}^{15}N$-Asp:Asp+4 changed in the solution after 60 min in comaprison to the start solution (Online Resource 4). Although, for this study it is not possible to determine

to what extent and which pre-uptake processes affetced the $^{13}C^{15}N$-Asn uptake, the conclusion of the potential of clover to abosrb $^{13}C^{15}N$-Asn does not change, only the reprted uptake rates might be understimated.

The presence of the isotopologues in the start labeled solution (Asn+5, $^{13}C^{15}N$-Asp, and Asp+4) and the pre- and post-uptake processes may have affected not only the uptake calculations based on the Q-TOF LC/MS analysis but also the bulk excess ^{13}C:^{15}N ratio in the plant. The bulk ^{15}N and ^{13}C excesses (EA-IRMS) were used by Nasholm et al. (1998) to calculate the fraction of the amino acids that was taken up intact. Intact uptake was implied if the slope of the correlation of the ^{13}C to ^{15}N excess in the plant corresponded to the ^{13}C to ^{15}N ratio in the intact amino acid. However, bulk measurements cannot differentiate between ^{13}C and ^{15}N taken up in the form of the amino acid applied or the uptake of it's degradation compounds but is rather a sum of it(Sauheitl et al. 2009b). Thus, during these circumstances it is not possible to calculate the uptake of intact amino acid based on bulk ^{15}N and ^{13}C excesses. However, combining IRMS measurements of ^{13}C and ^{15}N with the compound specific analysis can facilitate the interpretation of rapid metabolism and transport of added $^{13}C_4{}^{15}N_2$-Asn. As often is the case during short term uptake experiments, as this one, the correlation between the ^{13}C:^{15}N excess was more pronounced in the roots than in the shoots (Figure 2). A weak correlation in the shoots can be explained by (1) the rapid root $^{13}C^{15}N$-Asn deamination or transamination and transport of solely ^{15}N to the shoots, (2) decarboxylation of $^{13}C^{15}N$-Asn and loss of ^{13}C due to respiration, (3) dilution of the ^{13}C isotope in a large pool of unlabeled compounds that are therefore difficult to detect, and/or (4) the longer time needed for the isotope to move to the shoots than to the roots (Nasholm et al. 1998). These findings are consistent with those obtained by Nasholm et al. (2000) and Sauheitl et al. (2009a), who also found lower ^{13}C:^{15}N ratios in the shoots than in the roots. A significant correleation between ^{13}C and ^{15}N in the roots was observed for the clover supplied with 1-m*M* $^{13}C_4{}^{15}N_2$-Asn (Figure 2b). However, for the clover that received 10-µ*M* $^{13}C_4{}^{15}N_2$-Asn, the ^{13}C:^{15}N ratio was much lower, approximately 1 (Figure 2a), which may imply rapid amino acid metabolism with consequent loss of $^{13}CO_2$ (Persson and Nasholm 2001a; Warren 2012). The difference in the uptake between the 10-µ*M* and 1-m*M* treatments can be explained by the fact that the uptake rate increases with increasing concentration according to Michaelis-Menten kinetics, where more substrate for the membrane transporters is available for transport to the roots (Jamtgard et al. 2008). Furthermore, a significant correlation between ^{13}C and ^{15}N in the roots of clover, both in the presence and absence of IN (Figure 2b), further supports clover's potential to absorb $^{13}C_4{}^{15}N_2$-Asn even when IN is available for the plant.

Determining the uptake rate requires knowledge of what happens before and after the uptake as pre- and post-uptake processes can affect the $^{13}C_4{}^{15}N_2$-Asn absorption. The calculated uptake based solely on $^{13}C_4{}^{15}N_2$-Asn (Q-TOF LC/MS) ranged from 0.04 (ON+IN) to 0.4 (ON) and from 2.5 (ON+IN) to 3.8 (ON) µmol g^{-1} DW h^{-1} for

the 10-µ*M* and 1-m*M* treatments, respectively. However, these values are the minimum uptake rates of intact $^{13}C_4{}^{15}N_2$-Asn as they are resultant rates of the pre-and post-uptake processes. For example, metabolic conversion of the acquired $^{13}C^{15}N$-Asn, can yield lower-than-actual uptake rates. According to Zhang and Marsolais (2014), in higher plants Asn can be catabolized by two routes: (1) hydrolysis of Asn to Asp and NH_4, (2) transamination of Asn in the presence of oxo-acid producing 2-oxosuccinamic acid and other amino acid. Evidence for rapid N flow from Asn comes also from a study by Ta et al. (1984), in which pea plants (*Pisum sativum* CV Little marvel) were fed with either ^{15}N-amide Asn or ^{15}N-amine Asn for up to 60 min to trace the flow of ^{15}N into the metabolic products. The authors reported similar linear increases in ^{15}N accumulation in hydroxysuccinamic acid, Gln, Glu, Asp and ammonium in pea leaves fed with both ^{15}N-amide Asn and ^{15}N-amine Asn. Therefore, this result demonstrates that Asn metabolism occurs very rapidly, within minutes; hence, presented Q-TOF LC/MS analysis will likely yield underestimated results. If compound specific analysis supplement with bulk ^{13}C and ^{15}N measurements, then uptake interpretation in terms of post-uptake metabolism might be easier. However, the calculated uptake rates by EA-IRMS: 12.3 (ON+IN), 32.6 (ON) and 0.2 (ON+IN), 2.5 (ON) µmol g^{-1} DW h^{-1} for the 1-m*M* and 10-µ*M* treatments, respectively (Table 4), have to be interpreted as the maximum uptake rates obtained, assuming that 100% of ^{15}N in the clover came from the uptake of intact $^{13}C_4{}^{15}N_2$-Asn. Although, this senario is less likely, as part of the ^{13}C and ^{15}N might have entered the clover as other compounds than intact Asn (originating from the labeled Asn) , information from bulk measurements complements the results of fast movement of ^{13}C and ^{15}N from intact $^{13}C_4{}^{15}N_2$-Asn. It is concluded, that combination of compound specific analysis with the analysis of ^{13}C and ^{15}N excess confirmed clover potential to assimilate intact $^{13}C_4{}^{15}N_2$-Asn, even in the presence of IN, and that the pre- and post-upatke processes have to be considered when interpreting the uptake rate.

Biological implications of Asn uptake in the presence of IN

This study showed that even in the presence of IN, white clover acquires intact $^{13}C_4{}^{15}N_2$-Asn. Importantly, the 10-µ*M* treatment, which reflects the amino acid concentration that is normally found in soil solution (Jones et al. 2005), demonstrated that amino acids may be significant contributors to plant N nutrition.

Clover's ability to take up amino acids should also be tested in its natural environment to verify the ecological relevance of the present findings. This study was conducted under sterile hydroponic conditions; therefore, the results can be used as approximations in investigating clover amino acid uptake in soil and the factors involved in its regulation (the concentration and IN supplementation). We observed Asn uptake by clover occurring

even in the presence of IN, which emphasizes the potential for clover to take up amino acids under conditions in which the soil solution is a mixture of different N forms. Therefore, white clover N cycling appears to be much more dynamic than was previously thought, with, for example, fixed N exuded in the form of amino acids not being lost by the plant but rather most likely recaptured to some extent (Rasmussen et al. 2013a).

Conclusions

This study investigated the uptake of $^{13}C_4{}^{15}N_2$-Asn by white clover in the absence and presence of IN in a hydroponic system. Using different methods (EA-IRMS and QTOF LC/MS), we found that clover absorbed intact $^{13}C_4{}^{15}N_2$-Asn both when supplied alone and when supplemented with IN. Therefore, this study provides strong evidence for white clover's potential to acquire $^{13}C_4{}^{15}N_2$-Asn from the solution, which has important implications for our knowledge regarding N uptake. First, amino acids can contribute to white clover N nutrition even in the presence of IN, thereby adding to the plant's N supply. Second, amino acid utilization is organ-specific and differs depending on the concentration (10 µ*M* vs. 1 m*M*) and form (ON vs. ON+IN) given to the plant. Third, amino acids are more extensively utilized by clover when supplied at field-relevant concentrations (10 µ*M*) than they are when they are provided in excessive amounts (1 m*M*). Finally, the present study emphasizes that white clover possesses ON uptake systems that should be investigated in more detail to fully understand the amino acid transporters they contain and their expression and regulation.

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Conflict of Interest: The authors declare that they have no conflicts of interest.

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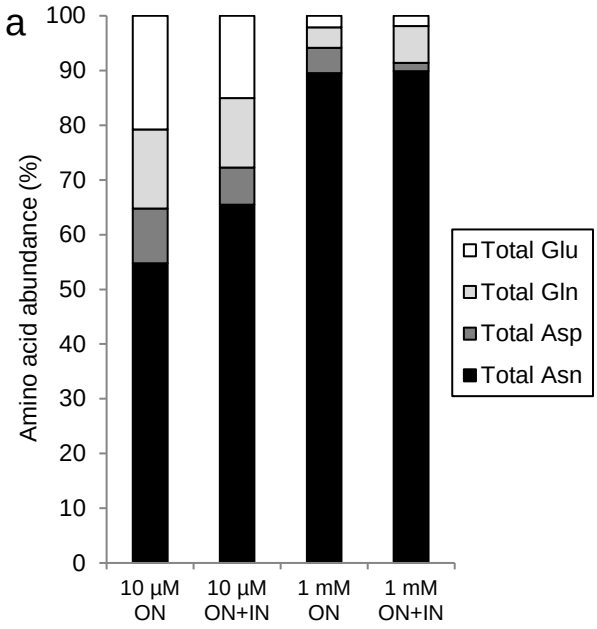
Figure captions

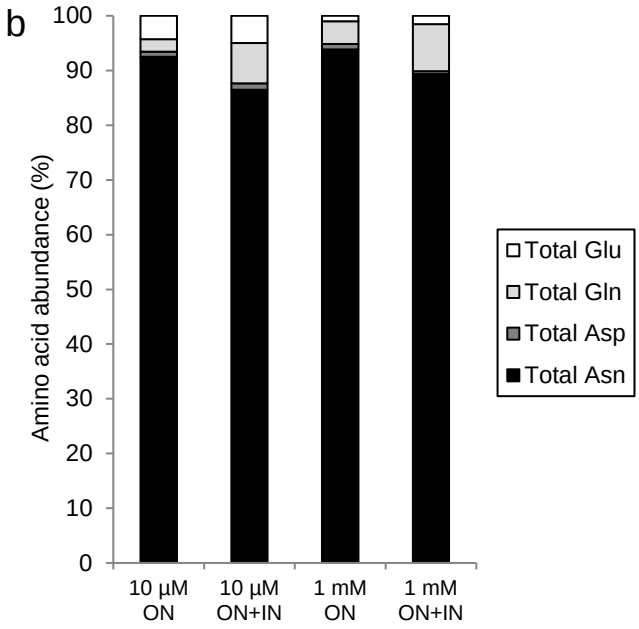
Fig. 1 Total amino acid profiles of Asn, Asp, Gln, and Glu in the clover roots (a) and shoots (b) fed with 10 µ*M* or 1 m*M* Asn in both the absence (ON) and presence (ON+IN) of NH_4NO_3. The data presented are the four total amino acid abundances as percentages. No significant differences in terms of amino acid percentage were detected between labeled and unlabeled clover roots or shoots; therefore, the data were pooled. For the 10-µ*M* treatments, the data are the means of 12 (ON) and 9 (ON+IN) replicates, whereas for the 1-m*M* treatments, the data are the means of 10 (ON) and 12 (ON+IN) replicates

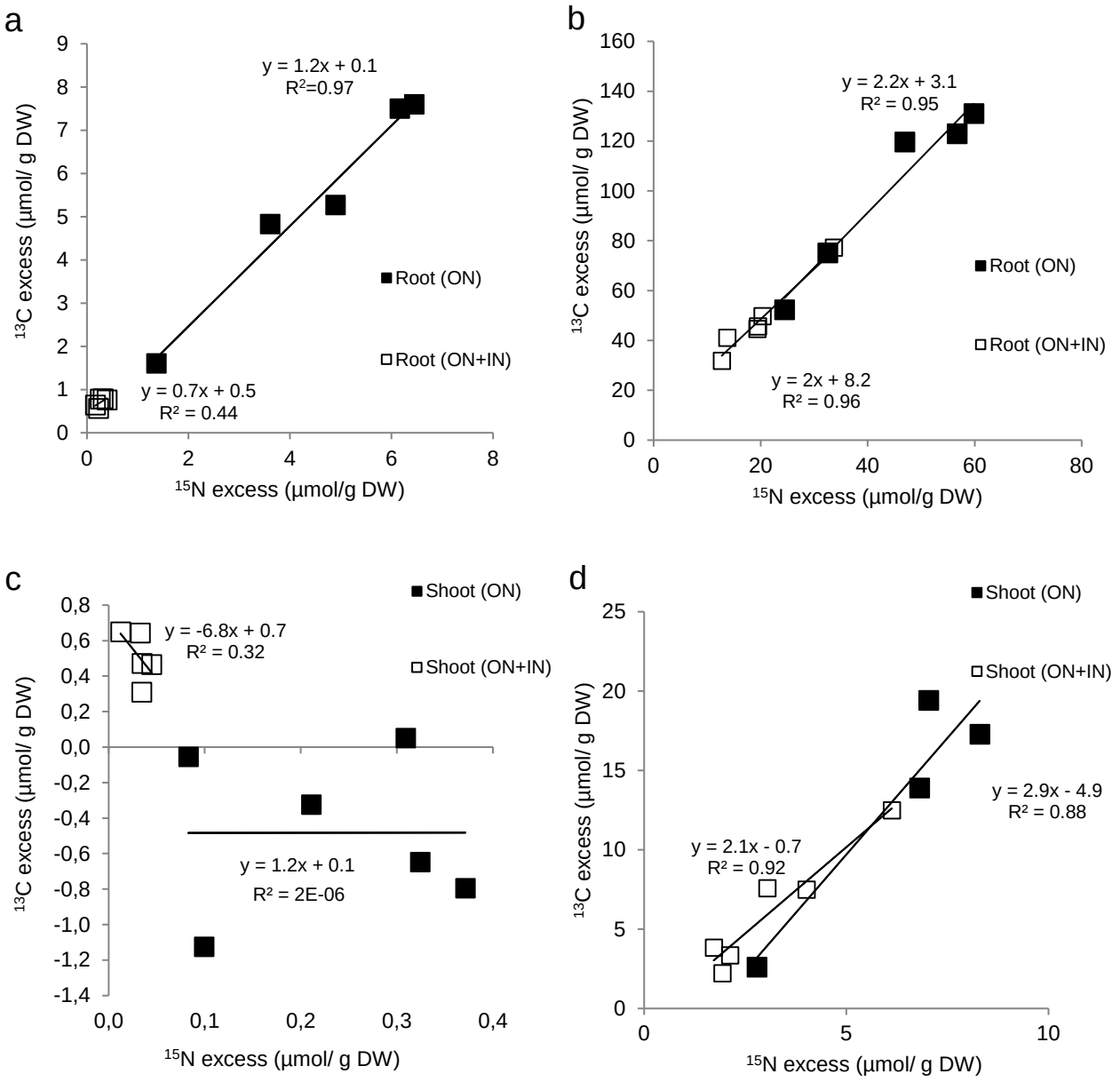
Fig. 2 The relationship between the ^{15}N and ^{13}C excesses (µmol/g DW) in clover roots (a, b) and shoots (c, d) after 60 min of exposure to the labeled solution containing 10 µ*M* (a, c) or 1 m*M* (b, d) $^{13}C_4{}^{15}N_2$-Asn in both the presence (open squares) and absence (filled squares) of NH_4NO_3. For the 10-µ*M* treatments, the data are the means of 6 (ON) and 5 (ON+IN) replicates, whereas for the 1-m*M* treatments, the data are means of 5 (ON) and 6 (ON+IN) replicates

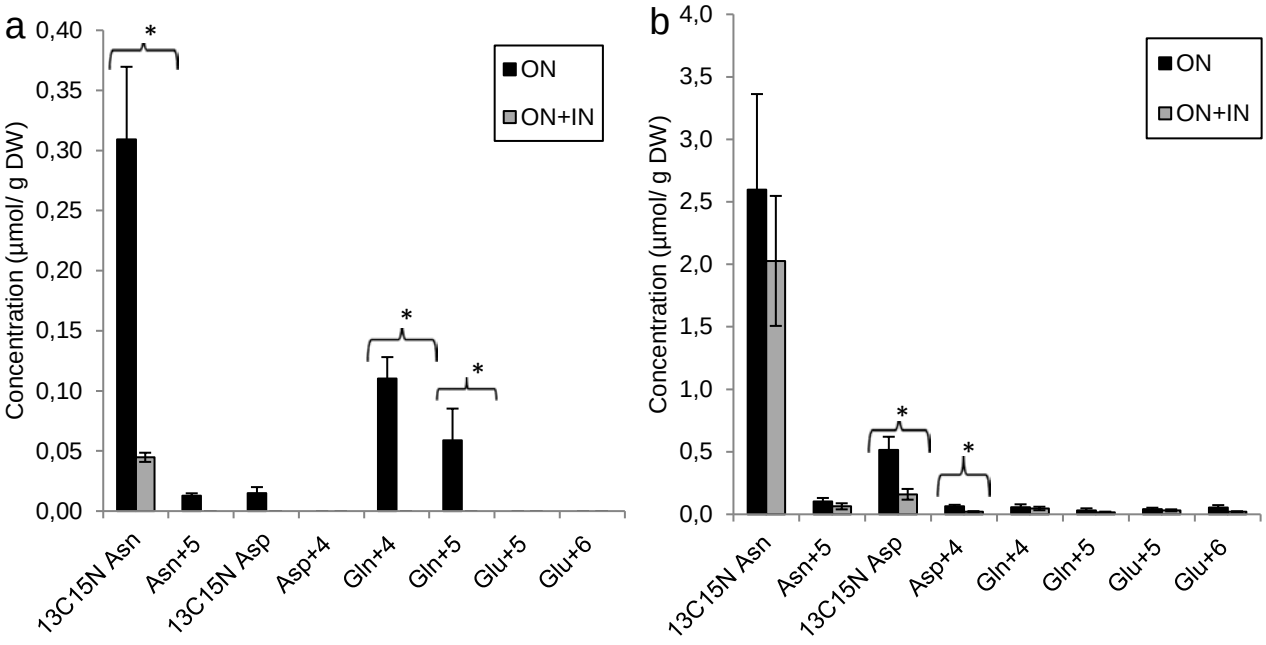
Fig. 3 $^{13}C^{15}N$-Asn and isotopologue concentration (µmol/g DW) in the clover roots after 60 min of clover exposition to the label in a concentration of 10 µ*M* (a) and 1 m*M* (b) in both the absence (ON) and presence (ON+IN) of NH_4NO_3. For the 10-µ*M* treatments, the data are the means of 6 (ON) and 5 (ON+IN) replicates, whereas for the 1-m*M* treatments, the data are the means of 5 (ON) and 6 (ON+IN) replicates. The bars indicate the standard error (SE), and the asterisks indicate significant differences in amino acid concentration between the ON and ON+IN treatments ($p<0.05$)

Fig. 4 Concentrations (µ*M*) of $^{13}C_4{}^{15}N_2$-Asn and isotopologues (Asn+5, $^{13}C^{15}N$-Asp, and Asp+4) in the start labeling solution containing either 10 µ*M* (a) or 1 m*M* (b) $^{13}C_4{}^{15}N_2$-Asn in both the absence (ON) and presence (ON+IN) of NH_4NO_3, in which clover roots were submerged for 60 min. Each value corresponds to one replicate


a
Amino acid abundance (%)
100
90
80
70
60
50
40
30
20
10
0
10 µM ON
10 µM ON+IN
1 mM ON
1 mM ON+IN
Total Glu
Total Gln
Total Asp
Total Asn


b
Amino acid abundance (%)
100
90
80
70
60
50
40
30
20
10
0
10 µM ON
10 µM ON+IN
1 mM ON
1 mM ON+IN
Total Glu
Total Gln
Total Asp
Total Asn


a
y = 1.2x + 0.1
R²=0.97
y = 0.7x + 0.5
R² = 0.44
Root (ON)
Root (ON+IN)
13C excess (µmol/ g DW)
15N excess (µmol/g DW)
b
y = 2.2x + 3.1
R² = 0.95
y = 2x + 8.2
R² = 0.96
Root (ON)
Root (ON+IN)
13C excess (µmol/ g DW)
15N excess (µmol/g DW)
c
y = -6.8x + 0.7
R² = 0.32
y = 1.2x + 0.1
R² = 2E-06
Shoot (ON)
Shoot (ON+IN)
13C excess (µmol/ g DW)
15N excess (µmol/ g DW)
d
y = 2.1x - 0.7
R² = 0.92
y = 2.9x - 4.9
R² = 0.88
Shoot (ON)
Shoot (ON+IN)
13C excess (µmol/ g DW)
15N excess (µmol/ g DW)


a
0,40
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
Concentration (µmol/ g DW)
ON
ON+IN
*
*
*
13C15N Asn
Asn+5
13C15N Asp
Asp+4
Gln+4
Gln+5
Glu+5
Glu+6
b
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
Concentration (µmol/ g DW)
ON
ON+IN
*
*
13C15N Asn
Asn+5
13C15N Asp
Asp+4
Gln+4
Gln+5
Glu+5
Glu+6

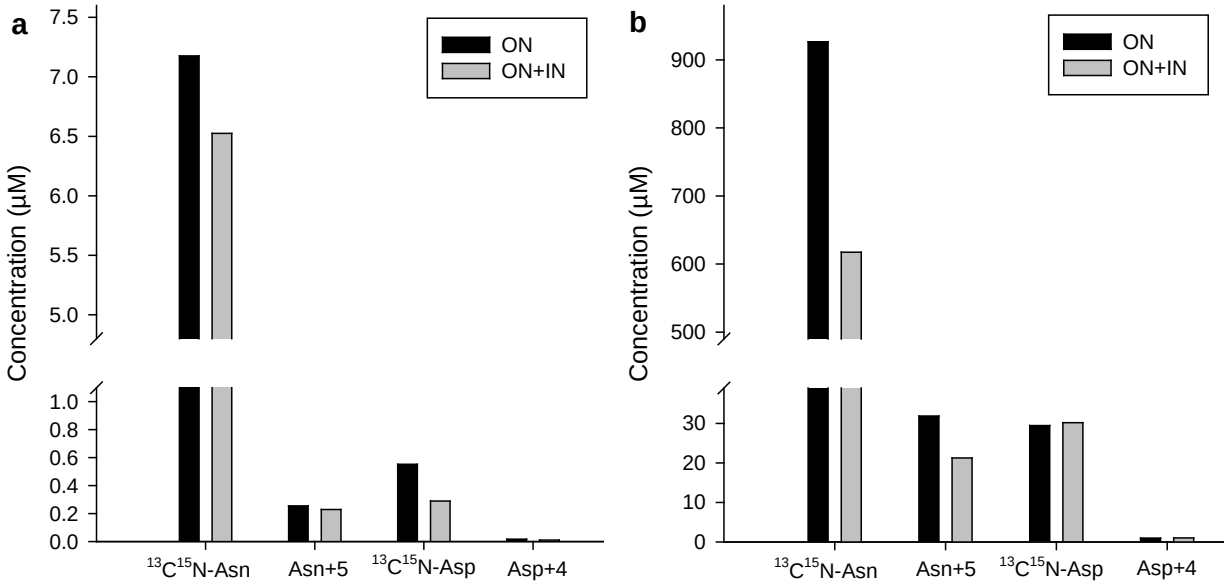

a
b
ON
ON+IN
Concentration (µM)
7.5
7.0
6.5
6.0
5.5
5.0
1.0
0.8
0.6
0.4
0.2
0.0
900
800
700
600
500
30
20
10
0
$^{13}C^{15}N$-Asn
Asn+5
$^{13}C^{15}N$-Asp
Asp+4

Table 1 List of compounds of interest (available/unavailable standards and calculated masses) used in the analyses of clover roots and shoots and solution

	Available/ not available standard	Compound name*	Short name	Molecular mass (Da)	Molecular mass with mass of Tag (Da)	Retention time (min)
Unlabeled compounds	available	asparagine	Asn	132.053	303.109	3.02
	available	aspartic acid	Asp	133.037	304.093	3.92
	available	glutamine	Gln	146.069	317.124	3.50
	available	glutamic acid	Glu	147.053	318.108	4.24
	available	norvaline	Nor	117.078	288.134	7.50
Labeled compounds	available	$^{13}C_4\,^{15}N_2$ -asparagine	$^{13}C_4{}^{15}N_2$-Asn	138.153	309.209	3.02
	available	$^{13}C_4\,^{15}N$-aspartic acid	$^{13}C_4\,^{15}N$-Asp	138.077	309.133	3.92
	available	$^{13}C_5\,^{15}N_2$-glutamine	$^{13}C_5\,^{15}N_2$-Gln	153.125	324.180	3.50
	available	^{15}N-glutamic acid	^{15}N-Glu	148.061	319.116	4.24
	not available	asparagine + 1	Asn+1	133.061	304.117	3.02
	not available	asparagine + 2	Asn+2	134.069	305.125	3.02
	not available	asparagine + 3	Asn+3	135.077	306.133	3.02
	not available	asparagine + 4	Asn+4	136.085	307.141	3.02
	not available	asparagine + 5	Asn+5	137.093	308.149	3.02
	not available	aspartic acid + 1	Asp+1	134.045	305.101	3.92
	not available	aspartic acid + 2	Asp+2	135.053	306.109	3.92
	not available	aspartic acid + 3	Asp+3	136.061	307.117	3.92
	not available	aspartic acid + 4	Asp+4	137.069	308.125	3.92
	not available	glutamine + 1	Gln+1	147.077	318.132	3.50
	not available	glutamine + 2	Gln+2	148.085	319.140	3.50
	not available	glutamine + 3	Gln+3	149.093	320.148	3.50
	not available	glutamine + 4	Gln+4	150.101	321.156	3.50
	not available	glutamine + 5	Gln+5	151.109	322.164	3.50
	not available	glutamine + 6	Gln+6	152.117	323.172	3.50
	not available	glutamic acid + 2	Glu+2	149.069	320.124	4.24
	not available	glutamic acid + 3	Glu+3	150.077	321.132	4.24
	not available	glutamic acid + 4	Glu+4	151.085	322.140	4.24

	not available	glutamic acid + 5	Glu+5	152.093	323.148	4.24
	not available	glutamic acid + 6	Glu+6	153.101	324.156	4.24

* Compounds named …+1, +2, +3, +4, +5, +6 represent analyzed compounds, for which the analyzed mass was the monoisotopic mass with an addition of the mass of the same number of neutrons (1.008 u)

Table 2 The root and shoot dry biomass (mg) and root:shoot ratio of clover grown in hydroponics for 6 weeks in 10-µ*M* or 1-m*M* asparagine solution in both the absence (ON) and presence (ON+IN) of NH_4NO_3. For the 10-µ*M* treatment, the mean biomass was calculated based on plant material taken from 12 (ON) and 9 (ON+IN) vials, whereas for the 1-m*M* treatment, the mean biomass was measured based on material taken from 10 (ON) and 12 (ON+IN) vials. The data are presented as the mean ± SE

	N concentration	**N source**	
		ON	ON+IN
Roots	10 µM	2.5 (±0.2) [b]	5.6 (±0.6) [a]
	1 mM	2.9 (±0.9) [b]	6.1 (±0.7) [a]
Shoot	10 µM	5.4 (±0.3) [a]	7.2 (±0.9) [a]
	1 mM	6.9 (± 0.7) [a]	9.2 (±1.1) [b]
Root:shoot ratio	10 µM	0.48 (±0.04) [b]	0.85 (±0.1) [a]
	1 mM	0.4 (±0.05) [b]	0.69 (±0.06) [a]

Table 3 Concentrations of $^{13}C^{15}N$-Asn, ^{15}N and ^{13}C excess (µmol/g DW) in clover roots and shoots after immersion in 15 ml solution containing either 10-µ*M* or 1-m*M* $^{13}C^{15}N$–asparagine in both the absence (ON) and presence (ON+IN) of NH_4NO_3 for 60 min. For the 10-µ*M* treatment, the data are the means ± SE of 6 (ON) and 5 (ON+IN) replicates, whereas for the 1-m*M* treatments, the data are the means ± standard error of 5 (ON) and 6 (ON+IN) replicates

	$^{13}C^{15}N$ Asn*		^{15}N		^{13}C	
Roots	ON	ON+IN	ON	ON+IN	ON	ON+IN
10 µM	0.62 (±0.12)[b]	0.08 (±0.008)[c]	4.5 (±0.9) [c]	0.27 (±0.04)[d]	5.4 (±1.1)[c]	0.71 (±0.04)[d]
1 mM	5.2 (±1.6)[a]	4.1 (±1)[a]	44 (±15)[a]	20 (±7.3) [b]	100 (±15.4)[a]	48 (±6.3) [b]
Shoots						
10 µM	0.01 (±0.006)[b]	n.d.	0.23 (±0.05) [c]	0.03 (±0.005) [d]	-0.48 (±0.2) [d]	0.51 (±0.06)[c]
1 mM	0.6 (±0.2)[a]	0.6 (±0.2)[a]	6.2 (±1.2) [a]	3.2 (±0.7) [b]	13 (±3.7) [a]	6.2 (±1.6) [b]

*The values are presented as µmol N/g DW; therefore, to calculate the concentration of the $^{13}C^{15}N$-Asn molecule, the values must be divided by 2

Table 4 Total $^{13}C^{15}N$ Asn and amino acid isotopologue uptake rates by clover (µmol g^{-1} DW h^{-1}) based on the bulk ^{13}C and ^{15}N excess measurements (EA-IRMS) and detection of intact molecules (Q-TOF LC/MS) after immersing clover roots in 15 ml solution containing either 10-µ*M* or 1-m*M* $^{13}C^{15}N$ –asparagine in both the absence (ON) and presence (ON+IN) of NH_4NO_3. For the 10-µ*M* treatment, the data are the means ± SE of 5 (ON) and 5 (ON+IN) replicates, whereas for the 1-m*M* treatments, the data are the means ± SE of 4 (ON) and 6 (ON+IN) replicates

	EA-IRMS	Q-TOF LC/MS								
	$^{13}C^{15}N$ Asn	$^{13}C^{15}N$ Asn	Asn+5	$^{13}C^{15}N$ Asp	Asp+4	Gln+4	Gln+5	Glu+5	Glu+6	Total
10 µM										
ON	2.5±0.5	0.4 ±0.1	0.01±0.002	0.02±0.005	n.d.	0.11±0.02	0.07±0.03	n.d.	n.d.	0.59±0.1
ON+IN	0.2±0.02	0.04±0.004	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.04±0.004
1 mM										
ON	32.6±3.7	3.8±1.5	0.11±0.04	0.62±0.13	0.12±0.02	0.07±0.03	0.04±0.02	0.05±0.01	0.07±0.02	4.8±1.8
ON+IN	12.3±2	2.5±0.7	0.08±0.03	0.18±0.04	0.02±0.005	0.05±0.01	0.02±0.005	0.03±0.007	0.02±0.003	2.9±0.8

Supplementary Files

Online Resource 1 Total amino acid concentrations (µmol g^{-1} DW) of Asn, Asp, Gln, and Glu in the clover roots and shoots fed with 10 µ*M* or 1 m*M* Asn in both the absence (ON) and presence (ON+IN) of NH_4NO_3. The "total" means sum of concentrations of all isotopologues for each of the four measured amino acid (Asn, Asp, Gln, Glu). No significant differences in terms of amino acid concentrations were detected between labeled and unlabeled clover roots or shoots; therefore, the data were pooled. For the 10-µ*M* treatments, the data are the means of 12 (ON) and 9 (ON+IN) replicates ± SE, whereas for the 1-m*M* treatments, the data are the means of 10 (ON) and 12 (ON+IN) ± SE

	Roots			
	10 µ*M*		1 m*M*	
	ON	ON+IN	ON	ON+IN
Total Asn	8.5 ± 2.4 [a]	14.5 ± 3 [a]	289.2 ± 60.5 [a]	204.5 ± 23.9 [a]
Total Asp	1.2 ± 0.1 [b]	1.5 ± 0.3 [b]	12.4 ± 1.9 [b]	3.5 ± 0.5 [b]
Total Gln	2 ± 0.2 [b]	3.2 ± 0.3 [b]	12 ± 2.5 [b]	15.6 ± 2.4 [b]
Total Glu	2.6 ± 0.3 [b]	3.6 ± 0.6 [b]	5.3 ± 0.6 [b]	4.3 ± 2.4 [b]
	Shoots			
	10 µ*M*		1 m*M*	
	ON	ON+IN	ON	ON+IN
Total Asn	99.8 ± 28.8 [a]	75.6 ± 23.9 [a]	398.4 ± 132.8 [a]	400.2 ± 115 [a]
Total Asp	1 ± 0.3 [b]	1 ± 0.3 [b]	3.4 ± 1 [b]	1.9 ± 0.6 [b]
Total Gln	2.4 ± 0.7 [b]	8 ± 2.5 [b]	23.8 ± 7.9 [b]	36 ± 10.4 [b]
Total Glu	4.3 ± 1.2 [b]	3.7 ± 1.2 [b]	2.7 ± 0.9 [b]	5.5 ± 1.6 [b]

Supplementary Files
Online Resource 2
Amino acid profile in the clover. Presented is the concentration (µmol g^{-1} DW)of four analyzed amino acids in the roots and shoots after immersing white clover for 60 min in solutions containing either $^{13}C^{15}N$–asparagine (L) or $^{12}C^{14}N$–asparagine (U) at two concentrations (10 µ*M* and 1 m*M*) in both the absence (ON) and presence (ON+IN) of NH_4NO_3. For the 10-µ*M* treatment, the data are the means ± SE of 6 (ON) and 5 (ON+IN) replicates, whereas for the 1-m*M* treatments, the data are the means ± SE of 5 (ON) and 6 (ON+IN) replicates.

	Roots																Shoots													
	10 µM								1 mM								10 µM								1 mM					
	ON				ON+IN				ON				ON+IN				ON				ON+IN				ON				ON+IN			
	U		L		U		L		U		L		U		L		U		L		U		L		U		L		U		L	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
Asn	4,83	1,03	8,73	3,85	7,33	4,46	14,27	3,50	327,28	81,03	163,59	34,33	210,72	28,15	118,97	13,64	80,72	11,30	89,34	15,95	83,79	24,52	48,65	17,65	365,40	94,87	323,37	105,26	386,74	41,46	293,25	82,02
Asn+1	0,78	0,18	1,45	0,63	1,24	0,75	2,19	0,52	53,71	12,65	26,92	5,47	33,48	4,35	20,13	2,23	12,35	1,90	14,22	2,63	13,97	4,17	7,87	3,08	58,68	15,73	50,61	16,78	60,15	6,43	46,88	12,94
Asn+2	0,15	0,03	0,22	0,07	0,15	0,08	0,29	0,06	2,00	0,27	1,78	0,55	1,30	0,06	1,02	0,13	0,79	0,08	0,79	0,10	0,51	0,23	0,78	0,10	1,83	0,47	1,87	0,54	1,75	0,14	1,33	0,22
Asn+3	0,01	0,002	0,02	0,01	0,01	0,01	0,03	0,01	0,19	0,02	0,18	0,06	0,13	0,01	0,10	0,01	0,07	0,01	0,07	0,01	0,05	0,02	0,07	0,01	0,13	0,02	0,14	0,04	0,17	0,01	0,13	0,02
Asn+4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Asn+5	-	-	0,01	0,00	-	-	-	-	-	-	0,10	0,03	-	-	0,09	0,03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0,01	0,01
$^{13}C^{15}N$ Asn	-	-	0,31	0,06	-	-	0,04	0,01	-	-	2,60	0,77	-	-	2,57	0,50	-	-	0,01	0,002	-	-	-	-	-	-	0,30	0,10	-	-	0,35	0,14
Asp	1,16	0,05	0,79	0,10	2,26	0,36	0,45	0,07	12,21	1,73	7,38	1,99	3,99	0,49	1,27	0,13	0,76	0,15	0,98	0,25	1,58	0,70	0,37	0,03	2,22	0,57	2,57	0,68	1,71	0,32	1,52	0,28
Asp+1	0,19	0,01	0,16	0,02	0,33	0,05	0,06	0,02	2,48	0,53	1,51	0,42	0,59	0,09	0,30	0,02	0,12	0,02	0,15	0,03	0,11	0,05	0,06	0,01	0,32	0,08	0,41	0,11	0,25	0,04	0,23	0,04
Asp+2	0,03	0,00	0,02	0,01	0,05	0,01	0,01	0,002	0,32	0,06	0,26	0,07	0,08	0,01	0,07	0,01	0,02	0,004	0,02	0,005	0,01	0,01	0,01	0,001	0,05	0,01	0,06	0,01	0,03	0,01	0,04	0,01
Asp+3	-	-	-	-	-	-	-	-	0,03	0,01	0,03	0,01	-	-	0,04	0,01	-	-	-	-	-	-	-	-	-	-	0,01	0,002	-	-	-	-
Asp+4	-	-	-	-	-	-	-	-	-	-	0,06	0,01	-	-	0,03	0,01	-	-	-	-	-	-	-	-	-	-	0,05	0,03	-	-	-	-
$^{13}C^{15}N$ Asp	-	-	0,02	0,01	-	-	-	-	-	-	0,51	0,11	-	-	0,21	0,05	-	-	-	-	-	-	-	-	-	-	0,04	0,01	-	-	0,01	0,002
Gln	1,53	0,43	1,22	0,30	3,38	0,83	1,92	0,23	11,12	3,49	7,50	2,08	16,92	3,00	6,39	1,01	1,93	0,35	1,94	0,33	7,58	3,86	6,29	2,34	26,96	11,60	15,44	5,61	46,54	9,67	14,76	6,70
Gln+1	0,32	0,06	0,60	0,09	0,61	0,14	0,47	0,05	1,78	0,51	2,34	0,54	2,52	0,32	2,42	0,36	0,30	0,05	0,34	0,06	1,05	0,46	1,06	0,33	2,92	1,07	2,84	1,08	4,45	0,78	2,16	0,86
Gln+2	0,04	0,01	0,15	0,04	0,08	0,02	0,06	0,01	0,22	0,06	0,78	0,26	0,31	0,04	0,41	0,06	0,04	0,01	0,04	0,01	0,13	0,05	0,13	0,04	0,35	0,13	0,37	0,12	0,53	0,09	0,27	0,10
Gln+3	-	-	0,05	0,02	-	-	-	-	-	-	0,17	0,06	0,03	0,003	0,11	0,02	-	-	-	-	-	-	-	-	0,03	0,01	0,04	0,01	0,05	0,01	0,03	0,01
Gln+4	-	-	0,11	0,02	-	-	-	-	-	-	0,06	0,02	-	0,00	0,06	0,01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gln+5	-	-	0,06	0,03	-	-	-	-	-	-	0,03	0,02	-	0,00	0,02	0,01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gln+6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glu	2,10	0,31	1,88	0,38	3,03	0,57	3,17	0,85	4,56	0,86	3,43	0,36	4,29	0,40	1,58	0,34	3,65	0,56	2,93	0,67	3,00	0,48	2,88	0,38	2,31	0,20	1,97	0,27	4,72	0,68	4,18	0,23
^{15}N Glu	0,50	0,07	0,57	0,09	0,68	0,10	0,74	0,17	0,90	0,14	1,15	0,12	0,89	0,07	0,75	0,20	0,76	0,11	0,68	0,16	0,61	0,10	0,66	0,03	0,48	0,08	0,50	0,04	0,83	0,12	0,97	0,04
Glu+2	0,07	0,01	0,09	0,02	0,09	0,01	0,10	0,02	0,12	0,02	0,24	0,04	0,11	0,01	0,19	0,04	0,10	0,01	0,09	0,02	0,08	0,01	0,09	0,005	0,06	0,01	0,07	0,01	0,10	0,01	0,13	0,01
Glu+3	-	-	0,03	0,01	0,01	0,00	0,01	0,00	-	-	0,11	0,03	0,01	0,001	0,10	0,02	-	-	0,01	0,002	0,01	0,0002	0,01	0,001	-	-	0,01	0,002	0,01	0,001	0,02	0,001
Glu+4	-	-	0,03	0,01	-	-	-	-	0,01	0,01	0,13	0,05	-	-	0,11	0,02	-	-	-	-	-	-	-	-	0,02	0,01	-	-	-	-	-	-
Glu+5	-	-	-	-	-	-	-	-	-	-	0,04	0,01	-	-	0,04	0,01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glu+6	-	-	-	-	-	-	-	-	-	-	0,05	0,02	-	-	0,02	0,004	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Supplementary Files

Online Resource 3

Amino acid profile in the start solution. Presented is the concentration (µ*M*) of the four analyzed amino acids and their isotopologues in the start solutions containing $^{13}C^{15}N$–Asn (Labeled solution) or $^{12}C^{14}N$–Asn (Unlabeled solution) at two concentrations (10 µ*M* and 1 m*M*) in both the absence (ON) and presence (ON+IN) of NH_4NO_3. Each concentration corresponds to one replicate.

	Labeled solution				Unlabeled solution			
	10 µ*M*		1 m*M*		10 µ*M*		1 m*M*	
	ON	ON+IN	ON	ON+IN	ON	ON+IN	ON	ON+IN
Asn	-	-	-	-	7,60	8,69	964,81	896,29
Asn+1	-	-	-	-	1,18	1,30	151,83	140,58
Asn+2	-	-	-	-	0,16	0,17	19,57	18,61
Asn+3	-	-	-	-	0,02	0,02	1,74	1,70
Asn+4	-	-	-	-	-	-	-	-
Asn+5	0,26	0,23	31,88	21,27	-	-	-	-
$^{13}C^{15}N$ Asn	7,18	6,52	926,38	617,45	-	-	-	-
Asp	0,01	0,03	-	-	0,37	0,94	25,86	64,34
Asp+1	-	-	-	-	0,06	0,16	4,29	10,87
Asp+2	-	-	-	-	-	-	-	-
Asp+3	-	-	-	-	-	-	-	-
Asp+4	0,02	0,01	0,97	1,03	-	-	-	-
$^{13}C^{15}N$ Asp	0,55	0,29	29,47	30,22	-	-	-	-
Gln	-	-	-	-	-	-	-	-
Gln+1	-	-	-	-	-	-	-	-
Gln+2	-	-	-	-	-	-	-	-
Gln+3	-	-	-	-	-	-	-	-
Gln+4	-	-	-	-	-	-	-	-
Gln+5	-	-	-	-	-	-	-	-
Gln+6	-	-	-	-	-	-	-	-
Glu	0,004	0,004	-	-	-	0,01	0,46	-
^{15}N Glu	-	-	-	-	-	-	-	-
Glu+2	-	-	-	-	-	-	-	-
Glu+3	-	-	-	-	-	-	-	-
Glu+4	-	-	-	-	-	-	-	-
Glu+5	-	-	-	-	-	-	-	-
Glu+6	-	-	-	-	-	-	-	-

Supplementary Files

Online Resource 4 Ratios of $^{13}C_4{}^{15}N_2$-Asn:Asn+5, $^{13}C_4{}^{15}N$-Asp:Asp+4 and $^{13}C_4{}^{15}N_2$-Asn: $^{13}C_4{}^{15}N$-Asp in the start and end labeled solution containing either 10 µ*M* or 1 m*M* $^{13}C_4{}^{15}N_2$-Asn in both the absence (ON) and presence (ON+IN) of NH_4NO_3 fed to the clover for uptake experiments and clover roots that were immersed in the start solution for one hour.

	10 µ*M*		1 m*M*	
	ON	ON+IN	ON	ON+IN
Start solution				
$^{13}C_4{}^{15}N_2$-Asn:Asn+5	28.10	28.55	29.06	29.03
$^{13}C_4{}^{15}N$-Asp:Asp+4	33.29	29.51	30.54	29.41
$^{13}C_4{}^{15}N_2$-Asn: $^{13}C_4{}^{15}N$-Asp	12.98	22.56	31.43	20.43
End solution				
$^{13}C_4{}^{15}N_2$-Asn:Asn+5	28.55	28.47	28.13	28.47
$^{13}C_4{}^{15}N$-Asp:Asp+4	26.21	0	30.13	0
$^{13}C_4{}^{15}N_2$-Asn: $^{13}C_4{}^{15}N$-Asp	15.98	22.29	11.60	20.14
Root				
$^{13}C_4{}^{15}N_2$-Asn:Asn+5	23.50	n.d.	24.72	25.87
$^{13}C_4{}^{15}N$-Asp:Asp+4	n.d.	n.d.	8.35	8.26
$^{13}C_4{}^{15}N_2$-Asn: $^{13}C_4{}^{15}N$-Asp	28.31	n.d.	5.81	13.53