Plant-microbe competition: does injection of isotopes of C and N into the rhizosphere effectively characterise plant use of soil N?

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Plant-microbe competition: does injection of isotopes of C and N into the rhizosphere effectively characterise plant use of soil N?

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Brief heading: Does rhizosphere injection of C and N isotopes effectively characterise plant use of soil N?

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Summary

- Despite considerable attention over the last 25 y, the importance of early protein breakdown products to plant N nutrition remains uncertain.
- We used rhizosphere injection of ^{15}N-, ^{13}C- and ^{14}C-labelled inorganic N and amino acid (L-alanine), with chase periods from 1 min to 24 h, to investigate the duration of competition for amino acid between roots (*Triticum aestivum* L.) and soil microorganisms. We further investigated how microbial modification of L-alanine influenced plant C and N recovery.
- From recovery of C isotopes, intact alanine uptake was 0.2-1.3% of added. Soil microbes appeared to remove alanine from soil solution within 1 min and release enough NH_4^+ to account for all plant ^{15}N recovery (over 24 h) within 5 min. Microbially-generated inorganic or keto acid C accounted for <25% of the lowest estimate of intact alanine uptake.
- Co-location of C and N labels appears a reasonable measure of intact uptake. Potential interference from microbially-modified C is probably modest, but may increase with chase period. Similarly, competition for L-alanine is complete within a few minutes in soil, whereas NO_3^- added at the same rate is available for >24 h, indicating that long chase periods bias outcomes and fail to accurately simulate soil processes.

Keywords: Organic nitrogen cycle; mineralisation; deamination; pulse-chase; wheat; respiration; N uptake

Introduction

A wide range of plants are now known to have the capacity to take up and utilise a variety of sources of N through their roots. These include L- and D-enantiomers of amino acids, short peptides, tertiary ammonium compounds and even intact proteins and soil microbes (Paungfoo-Lonhienne et al., 2008, 2010, 2012; Hill et al., 2011ac, 2013; Warren, 2013, 2014). Due to the dominance of protein as the form of N entering soil (in the absence of inorganic fertiliser additions), early breakdown products such as amino acids and short peptides probably represent the most quantitatively significant forms of organic N, which plants are able to utilise (Yu et al., 2002; Knicker, 2011; Warren, 2014). Consequently, mechanisms to successfully acquire protein components early in the breakdown process may provide a competitive advantage to plants in N-limited ecosystems (Chapin et al., 1993; Näsholm et al., 1998; Hill et al., 2011a; Weigelt et al., 2005). Evidence from plants growing in ecosystems where N mineralisation is slow tends to support this hypothesis with reports of equal or more rapid acquisition of amino acid or peptide N than inorganic N, especially NO_3^- (Chapin et al., 1993; Kielland et al., 2006; Näsholm et al., 2009b; Hill et al., 2011a). Similarly, microbes in soil from a wide range of ecosystems are able to acquire and utilise amino acids and short peptides with half-times in soil solution as short as 20 seconds, suggesting intense plant-microbe competition (Jones et al., 2009; Hill et al., 2011b, 2012; Farrell et al., 2011b, 2013; Warren, 2018; Wilkinson et al., 2014).

Although mixotrophy occurs in photosynthetic organisms and angiosperms appear able to utilise C acquired through roots as amino acids in respiration, soil amino acids are rarely likely to be a significant source of C to terrestrial plants (Raven et al., 2009; Hill et al., 2011c; Warren, 2012; Paungfoo-Lonhienne et al., 2012; Schmidt et al., 2013). In contrast, soil microbes are most frequently limited by available C and take up intact amino acids as a source of C, acquiring excess N which is generally excreted as NH_4^+ (Fig.1; Baraclough, 1997; Treseder, 2008; Geissler et al., 2009, 2010; Farrell et al., 2014). Although direct microbial amino acid uptake appears to predominate in the production of NH_4^+ from amino acids by soil microbes, extracellular deamination of amino acids may also generate NH_4^+ (Geisseler et al., 2009, 2010, 2012; Barraclough, 1997; Pingerra et al., 2015). In well-aerated soils, although microbial nitrifiers may compete with plants for NH_4^+, microbial reduction of NO_3^- is not favoured, and competition between plant roots and soil microbes for NO_3^- is low (Raven et al., 1992; Geisseler et al., 2010; Abaas et al., 2012). Direct use of amino acid N may be energetically favourable to plants in comparison to NO_3^- (Raven et al., 1992; Franklin

et al., 2017). Nevertheless, in well-aerated soils where soil microbes are C-limited with no significant N-limitation, large differences in microbial competition for different forms of N suggest that the selective pressure on plants to acquire N as organic protein breakdown products may be lower than that to acquire N as NO_3^-. Thus, although the capacity of plants to take up and metabolise amino acids and short peptides through their roots has been verified with sterile plants and root transporters have been characterised, the true importance of amino acid forms of N to the N nutrition of plants growing in soil remains elusive (Jones et al., 2005a; Biernath et al., 2008; Komarova et al., 2008; Rasmussen & Kuzyakov, 2009; Näsholm et al., 2009a; Tegeder & Rentsch, 2010; Svennerstam et al., 2011; Hill et al., 2011c; Kuzyakov & Xu, 2013; Franklin et al., 2017).

In most cases organic N uptake by plants is evaluated by exposing roots to N forms with an isotopic label (N, C or both) and measuring recovery in plant tissues by isotope ratio mass spectrometry (IRMS; Näsholm et al., 2009b). When roots are sterile, if investigated substrates are stable and not subject to extracellular modification by plant root enzymes, this methodology gives confidence that isotope recovery represents actual uptake of the moiety supplied. However, when plants are growing in soil, transformation of both inorganic and organic forms of soil N means that recovery of labelled N in plant tissues does not unequivocally indicate that the N was acquired by the plant in the same form in which it was added to soil. Commonly, direct, unmodified amino acid uptake by roots is estimated by use of dual-labelled organic forms of N with correlated co-location of C and N labels, in the same proportions as in the supplied amino acid, considered to be evidence of intact amino acid uptake (Weigelt et al., 2005; Näsholm et al., 2001, 2009b; Quinta et al., 2015; Wilkinson et al., 2015). In a much smaller number of investigations, compound specific recovery of isotopic labels in plant tissues has been used to estimate intact uptake (Persson & Näsholm, 2001; Persson et al., 2006; Sauheitl et al., 2009a; Warren, 2012; Czaban et al., 2016). Recovery of the supplied dual-labelled amino acid in plants by compound specific methods probably provides the most reliable proof of intact amino acid uptake (Persson & Näsholm, 2001; Warren, 2012; Czaban et al., 2016). However, in both bulk and compound-specific methods, interpretation is hindered by rapid loss of amino acid C in plant and microbial respiration, large plant and soil pools of C relative to tracers, and high background levels of the most frequently used C isotope, ^{13}C (Näsholm et al., 2009b; Sauheitl et al., 2009a; Warren, 2012; Wilkinson et al., 2014; Quinta et al., 2015; Moran-Zuloaga et al., 2015). Separation of C and N labels due to rapid post-uptake transformation of amino acids by plants is particularly problematic for quantification using compound-specific methods, with

comparisons showing lower estimates of uptake than by bulk tissue IRMS (Näsholm et al., 2009b; Sauheitl et al., 2009a; Warren, 2012; Czaban et al., 2016).

It has been suggested that recovery of labelled C in plant tissues can result largely from dark fixation in roots of inorganic C produced during microbial respiration of added amino acids or by uptake of microbially-modified amino acid C following e.g. extracellular deamination of amino acids to keto acids (oxo-acids) by amino acid oxidases (Lee & Woolhouse, 1969; Rasmussen & Kuzyakov, 2009; Geissler et al., 2010; Rasmussen et al., 2010; Warren, 2012; Dippold & Kuzykov, 2013; Hossain et al., 2014; Moran-Zuloaga et al., 2015; Fig. 1). Dark fixation of inorganic C by roots of terrestrial plants has been reported, with phosphoenolpyruvate carboxylase (PEPc) identified as the likely primary carboxylating enzyme (Lee & Woodhouse, 1969). Keto acids generated from de-amination of amino acids are central to both plant C and N metabolism and transport within plant tissues and organelles is known to occur (Hanning et al., 1999; Fernie et al., 2004; Furumoto, 2016). However, their uptake by roots from soil has not been investigated to our knowledge. All uptake of microbially-modified C is obviously limited by both rates of microbial production (e.g. deamination and respiratory loss of CO_2) and plant uptake.

Bulk IRMS of tissue cannot account for separate uptake of N and C labels if recovered isotopes are in proportion with those in the added amino acid. Similarly, reliable quantification of labels entering plant C and N metabolism from intact amino acid uptake by compound-specific methods represents a formidable challenge when there is concurrent entry of products of soil microbe amino acid modification to closely connected pathways (Sauheitl et al., 2009a; Warren, 2012; Czaban et al., 2016).

A wide range of chase periods following experimental additions of isotopically-labelled amino acid to plant roots and soils have been employed, typically ranging from hours to days (Näsholm et al., 2001; Weigelt et al., 2005; Biernath et al., 2008; Harrison et al., 2007; Hill et al., 2011a; Moran-Zuloaga et al., 2015; Wilkinson et al., 2015). As the factors likely to confound accurate evaluation of the importance of direct use of amino acid forms of N result from plant or microbial modification of C and N, the temporal relationship between plant and microbe processes and the chase period is potentially of considerable importance.

Using a range of chase periods, this investigation aimed to critically evaluate the duration during which competition between wheat roots and soil microbes for amino acids takes place in a temperate agricultural soil. We further aimed to evaluate the degree to which potential uptake of microbially-modified amino acid C and N may influence results of pulse-chase experiments. We chose L-alanine as the specimen amino acid. L-alanine is abundant in a wide

range of proteins and in soil and has good precedent for use in plant and microbial amino acid uptake experiments (Persson et al., 2006; Fischer et al., 2007; Farrell et al., 2011a; Hill et al., 2011abc, 2012; Inselbacher & Näsholm, 2012a Dippold & Kuzyakov, 2013; Broughton et al., 2015; Chen et al., 2015; Moran-Zuloaga et al., 2015; Quinta et al., 2015; Warren et al., 2017). It also has an easily identifiable deamination product, pyruvate. Although it may not be the only organic compound released to soil following microbial modification of L-alanine C, it likely to be the overwhelmingly most abundant form in the short-term. Pyruvate is central to C metabolism and transporters in plants have been identified (Furumoto, 2016). Consequently, its acquisition by roots as a fragment following microbial modification of L-alanine in soil seems plausible.

We aimed to test the following hypotheses:

1. Due to rapid microbial uptake, competition between plants and soil microbes for intact amino acids is complete within minutes of their production.
2. Investigations using long chase periods fail to capture the importance of amino acid N to plant nutrition.
3. Long chase periods bias plant N uptake measurements in favour of N forms which are unattractive to soil microbes.
4. In well-aerated agricultural soil, plant acquisition of inorganic C is less than that acquired as intact amino acid.
5. Plant acquisition of amino acid C following extracellular deamination is less than that acquired as intact amino acid.

Materials and Methods

Soil.

Agricultural Brown Earth soil was sampled (0-10 cm; *n*=4) from Henfaes Agricultural Research Station, Abergwyngregyn, Bangor, UK (53° 14′N, 4° 01′W). The soil is classified as a Eutric Cambisol (FAO) or Dystric Eutrudepts (US Soil Taxonomy) and is derived from Ordovician post-glacial alluvial deposits. At the time of sampling, soil supported a sward of *Lolium perenne* L. The pH was 6.5, electrical conductivity was 24 µS cm^{-1}(1:2 soil to deionised water for pH and conductivity), and total C and N were 34 and 0.54 mg g^{-1} DW,

respectively. Soil was sieved to pass 2 mm, removing stones, earthworms, visible plant debris and vegetation.

Plant acquisition of added N from soil.

Seeds of wheat (*Triticum aestivum* L. var. Granary) were sown singly into rhizotubes (240 mm long; internal diameter 8 mm; Owen & Jones, 2001) containing *ca.*12 g FW soil (*ca.*8.5 g DW soil). The plants were grown at 20 °C, 70 % relative humidity and 16 h photoperiod (*ca.*500 µmol photons m^{-2} s^{-1} PAR). At the third leaf stage (65 ± 2 mg DW root and 67 ± 7 mg DW shoot with no difference between treatments) , 1 ml of either 1 mM $^{13}C^{15}N$ dual-labelled L-alanine, $^{15}NH_4Cl$ or $K^{15}NO_3$ (Cambridge Isotope Laboratories, Tewksbury, MA, USA) was injected into the rhizosphere in four equally-spaced (ca.40 mm apart) injections of 0.25 ml. An alanine concentration of 1 mM was chosen to provide sufficient label for analysis using short chase periods, whilst not exceeding an amino acid concentration which could reasonably be expected close to sites of cell lysis or protein degradation in soil (Jones et al., 2005b). Assuming soil concentrations in incubations without plants (see below) were similar to those in rhizotubes with plants, and alanine was 15% of total amino acids, we estimate that injected solutes (0.19 µmol N g^{-1} DW soil) increased, soil solution alanine, NH_4^+ and NO_3^- concentrations by 60-fold, 30-fold, and 10-fold, respectively (Jones et al., 2005b). No further water was added during the chase period. After 1 min, 5 min 10 min, 30 min, 1 h, 2 h, 4 h, 8 h and 24 h, roots of four plants receiving each form of N were removed from soil and washed thoroughly with water followed by 0.1 M $CaCl_2$ (we assume that this removed all labelled solutes adhering to the surface of roots). Roots and shoots were immediately placed on a hot (80 °C) steel surface to stop metabolic processes, oven dried (80 °C), weighed, ground in a ball mill and analysed for ^{13}C and ^{15}N content by PDZ Europa IRMS (Sercon Ltd., Cheshire, UK). Three further rhizotubes were each injected with four 0.25 ml injections of blue ink. The extent of penetration of the ink after *ca.*10 min was used to estimate the amount of soil and root accessed by injections.

To reduce uncertainties in alanine ^{13}C recovery over short chase periods, a further eight rhizotubes were injected with 1 ml of 1 mM 3 kBq ml^{-1} [U-^{14}C]L-alanine (American Radiolabeled Chemicals, St Louis, MO, USA). Plants were removed from soil as above after 1 and 5 min. Dry roots and shoots were combusted in a Harvey OX400 Biological Oxidiser (Harvey Instruments Corp., Hillsdale, NJ, USA). Liberated $^{14}CO_2$ was captured in Oxysolve C-400 Scintillant (Zinsser Analytic, Frankfurt, Germany) and ^{14}C activity measured by liquid scintillation counting in a Wallac 1404 scintillation counter (Perkin-Elmer Life Sciences, Boston, MA, USA).

Mineralisation of L-alanine N by soil microbes.

To estimate the rate at which N added as L-alanine was mineralised to inorganic N, 1 ml of 1 mM L-alanine was added to 8 g FW (*ca*. 5.5 g DW; a *ca.* 30% increase in soil moisture) portions of sieved (2 mm) soil in 50 ml polypropylene centrifuge tubes (approximately matching the ratio of solution to soil in rhizotube injections). Tubes were incubated at 20 °C. After periods of 0 (before adding alanine), 1, 5, 10, 30 min and 2 h, 4 h, 8 h and 24 h, 10 ml ice-cold (<4 °C) 0.5 M K_2SO_4 solution was added to each for four replicate tubes and shaken for 15 min. Resulting extracts were analysed colorimetrically for NH_4^+, NO_3^- according to Mulvaney (1996) and Miranda et al. (2001), respectively and fluorimetrically for total amino acids according to Jones et al. (2002).

Mineralisation of L-alanine C and keto acid (pyruvate) C by soil microbes.

Decomposition of L-alanine to CO_2 was measured according to Hill et al. (2008). 1 g FW (*ca.*0.7 g DW) soil was placed in each of three 10 ml glass tubes and 125 µl of 1 mM [U-^{14}C]L-alanine (1.3 kBq) were added to the surface of soil (approximately matching the ratio of solution to soil in rhizotube injections). Air was drawn over the soil at a rate of *ca.*100 ml min^{-1} and $^{14}CO_2$ was captured in two 3 ml vials of 0.1 M NaOH connected in series (described in detail in Hill et al., 2007). Vials of NaOH were changed 1, 5, 10, 20, 30, 40 and 60 minutes after addition of the alanine. Captured ^{14}C was measured by liquid scintillation counting after mixing with HiSafe 3 scintillation cocktail (Fisher Scientific, Loughborough, UK). The residence time in soil of respired $^{14}CO_2$ was investigated by injection of 125 µl (ca.0.3 kBq; 80 nmol) $NaH^{14}CO_3$ to three tubes of soil as above and comparing capture of liberated $^{14}CO_2$ after 1 min with that liberated from injection into 1 ml 1 M HCl (instant release of $^{14}CO_2$).

The rate of mineralisation of ^{14}C pyruvate (the keto acid generated following de-amination of alanine) by soil microbes was carried out by addition of 125 µl 1 mM (0.4 kBq) [1-^{14}C]sodium pyruvate (Perkin-Elmer) to 1 g FW soil using the same procedure as for alanine.

Plant uptake and respiration of L-alanine, pyruvate and inorganic C in the absence of competition from soil microbes.

In order to determine the capacity of plants to take up L-alanine and potential forms of C generated following modification by soil microbes, plants were exposed to substrates under sterile conditions. Wheat seeds were shaken in NaClO soln. (ca.8% free Cl) with 1 drop Tween 20 for 3 min followed by 80% ethanol for 1 min, and washed thoroughly with sterile tap water. Sterilised seeds were placed on sterile agar containing 2.1 g l^{-1} Murashige & Skoog basal medium (Sigma-Aldrich, Gillingham, UK), 1 mmol l^{-1} glucose and 47 µmol l^{-1} $NaSiO_3$

in Phytatrays (Sigma-Aldrich) and grown under the same conditions as rhizotubes. At the third leaf stage, plants (n=3) were carefully removed from agar, placed in sterile 6 ml vials containing 3 ml of either 1 mM [U-^{14}C]L-alanine (4 kBq ml^{-1}), [1-^{14}C]sodium pyruvate (3 kBq ml^{-1}) or K$H^{14}CO_3^-$ (10 kBq ml^{-1}) and sealed in 100 ml, clear polythene containers. Air was drawn through containers at 300 ml min^{-1} and bubbled through 15 ml Oxysolve C-400 to capture respired $^{14}CO_2$. CO_2 traps were changed after 1, 5, 10, 20 and 30 min and captured $^{14}CO_2$ measured by scintillation counting as above. After 30 min, plants were removed from solution and washed in water, followed by 0.1 M $CaCl_2$ and dried at 80 °C. Dry roots and shoots were analysed for ^{14}C activity as above. A plant-free control was included for $H^{14}CO_3^-$ to account for any potential abiotic generation of $^{14}CO_2$.

Plant uptake of microbially-modified L-alanine C from soil.

To assess the likelihood of alanine C being captured by plant roots following mineralisation to CO_2 by soil microbes, 1 ml of 1 mM L-alanine solution containing 1.2 kBq of $Na_2{}^{14}CO_3$ (which rapidly becomes $H^{14}CO_3^-$ and $^{14}CO_2$ in this soil) was injected into the rhizosphere of each of eight rhizotubes as above. After assimilation periods of 30 and 60 min (decided based on likely duration of a short pulse-chase experiment), roots were removed from soil, washed in water followed by 0.1 M $CaCl_2$ and dried (80 °C). Dry roots and shoots were analysed for ^{14}C activity as above.

Assessment of the possibility that recovery of alanine C in plants may take place following extracellular deamination by soil microbes was carried out by injection as above of 1 ml 1 mM L-alanine solution containing 0.3 kBq ml^{-1} [1-^{14}C]pyruvate into each of 12 rhizotubes. Plants were harvested and analysed as above after 1, 5 and 10 min. A further test of the extent to which pyruvate C could be acquired by plants in soil, assuming total deamination of alanine, was carried out by injection of 1 ml of 1 mM 0.3 kBq ml^{-1} [1-^{14}C]pyruvate (n=4) and harvesting as above after 30 min.

Statistical analysis.

Data were analysed by t-test or One-way ANOVA with Tukey HSD post-hoc test (SPSS v22; IBM, New York, USA) after testing for normality and homogeneity of variance with Shapiro-Wilk and Levene's test, respectively. Data not conforming were $\log_{10}$ transformed prior to analysis. Statistical differences were accepted at P<0.05.

Data accessibility

Data can be accessed by request from the corresponding author.

Results

Capture of ^{15}N, ^{13}C and ^{14}C by plants growing in soil.

Added ^{15}N was detected in plants in the first minute after addition (Fig. 2). Uptake of ^{15}N added as NH_4^+ was most rapid ($P \leq 0.001$) with 0.94 ± 0.09 % (mean ± SEM) recovered in plant tissue after a minute. Recovery of ^{15}N added as NO_3^- and alanine after a minute was the same, at 34% of recovery of ^{15}N added as NH_4^+ (0.33 ± 0.04 % of added). Recovery of ^{13}C added as alanine after a minute was 1.2 ± 0.4 %. Recovery of ^{13}C remained statistically the same as recovery of ^{15}N added as alanine for the first 10 minutes. After 30 min, however, recovery of ^{15}N added as alanine was approximately double (P=0.007) that of ^{13}C at 3% of that added. Recovery of ^{14}C added as alanine after 1 and 5 minutes was lower (P<0.05: 94 and 90% at 1 and 5 min, respectively) than that of ^{13}C at 0.075 ± 0.02 and 0.19 ± 0.02% of added (1 and 5 min, respectively). Recovery of ^{15}N was higher than that of ^{14}C after 1 min, but the same after 5 min. In contrast to ^{13}C, ^{14}C recovery increased (P=0.01) between 1 and 5 min.

Recovery of ^{15}N added as NH_4^+ after 30 min was double (P=0.01) that added as alanine at 6%, and that added as NO_3^- was double (P<0.001) again at 12%. However, after 8 h, only recovery of ^{15}N added as NO_3^- exceeded (P<0.04) that added as alanine and after 24 h recovery of ^{15}N was statistically the same from all substrates at 30 to 40 % of that added. Injection of ink suggested that 5.4 ± 0.2 g DW soil and 0.025 ± 0.002 g DW root came into contact with the injected solutions.

Mineralisation of L-alanine N by soil microbes.

Mineralisation of L-alanine in soil appeared to be extremely rapid. Although, alanine concentration was not measured directly, total amino acids fell to background concentrations (0.020 ± 0.002 µmol N g^{-1} DW soil) a minute after alanine addition and NH_4^+ concentration had increased enough to account for ca.40% of N added as alanine after 5 min (Fig. 3). After 5 min, NH_4^+ concentration declined and remained statistically unchanged between 30 min and 24 h. In contrast, NO_3^- concentration increased over the 24 h incubation and had increased enough to account for >30% N added as alanine by the end.

Mineralisation of L-alanine and pyruvate C by soil microbes.

Mineralisation of ^{14}C added as both alanine and pyruvate by soil microbes was also rapid (Fig. 4). The production of $^{14}CO_2$ from alanine increased linearly over the first 40 min of incubation and reached a maximum of *ca.*12 % of added alanine ^{14}C after 1 h. The rate of mineralisation by soil microbes of ^{14}C added as pyruvate was initially very high, but reduced

over the incubation period to reach *ca.*43% after 1 h. However, as pyruvate was ^{14}C labelled only on the carboxyl group, which very likely mineralised faster than other pyruvate C atoms, the mineralisation rate for the whole pyruvate molecule was probably overestimated by a factor of around two (Dijkstra et al., 2011). Capture of $H^{14}CO_3^-$ - derived $^{14}CO_2$ from soil was the same as from addition to HCl solution. This indicates that the measured respiration rate was not influenced by the rate of travel of $^{14}CO_2$ from sites of production to the soil surface. However, to avoid any minor inaccuracies in short-term mineralisation due to difficulties in rapid CO_2 trap changes, generation of ^{14}C in respiration during the first minutes was estimated from functions fitted to data. A linear function ($y = 0.202x$; r^2=0.998) was fitted to the first 40 min of alanine mineralisation. A double exponential function ($y = 19.5441(1-e^{(-0.3304x)}) + 24.5306(1-e^{(-0.0496x)})$; r^2=0.999) was fitted to all pyruvate mineralisation data. Loss of substrate ^{14}C as $^{14}CO_2$ over the first minute was estimated to be 0.202 and 6.69% of added ^{14}C for alanine and pyruvate, respectively.

Uptake and loss as CO_2 of L-alanine, pyruvate and inorganic C by plants with sterile roots.

Uptake of alanine by plants with sterile roots over 30 min was 29.1 ± 0.38 nmol g^{-1} DW root min^{-1}. Surprisingly, the uptake rate of inorganic C was the same as alanine at 32.9 ± 3.8 nmol g^{-1} DW root min^{-1} (about a third of the C taken up as alanine), and uptake of pyruvate was five-fold higher at 161 ± 41 nmol g^{-1} DW root min^{-1}. Loss of alanine ^{14}C as $^{14}CO_2$ over 30 min was much lower (6.23 ± 1.3% of uptake; P<0.001; Fig. 5) than that added as pyruvate or inorganic C (56.2 ± 3.0 and 65.7 ± 5.9 % of uptake, respectively), which were not different (although, as mentioned above, loss of pyruvate ^{14}C is very likley overestimated by a factor of around two due to preferential mineralisation of the carboxyl group; Dijkstra et al., 2011). Proportional loss of alanine and pyruvate $^{14}CO_2$ (the slope of lines shown in Fig. 5) increased over the 30 min chase period, but was constant for inorganic C. The proportion of alanine and pyruvate C taken up during the first minute lost as $^{14}CO_2$ was calculated after fitting 2nd order polynomials to data for the whole 30 min period (r^2> 0.998). Assuming a constant rate of uptake over the 30 min, we estimate that 4.9 and 20.9% of uptake (alanine and pyruvate, respectively) over the first minute was lost in respiration. This would fall to around 10% for pyruvate if corrected for preferential mineralisation of the carboxyl group (Dijkstra et al., 2011)

Capture of inorganic C and pyruvate C by plants growing in soil.

Recovery of inorganic ^{14}C injected into the rhizosphere in plant tissues was the same after 30 min and 60 min at 0.33 ± 0.04 and 0.34 ± 0.10% of added, respectively. ^{14}C delivered as

pyruvate was detectable in plants after 1 min (0.017 ± 0.002% of added), but recovery after 1 minute was 77 and 98% less (P<0.05) than ^{14}C and ^{13}C added as alanine, respectively. All other additions (5, 10 and 30 min) of pyruvate ^{14}C were recovered in the same quantity (0.19 ± 0.01% of added) whether added with unlabelled alanine or pyruvate. Post- one minute recovery of pyruvate ^{14}C was the same as recovery of alanine ^{14}C after 5 min, but, less than half (P=0.01) the recovery of ^{14}C added as inorganic C and about 17% of recovery of alanine ^{13}C.

Discussion

Timeframe of plant-microbe competition and rate of L-alanine uptake by roots.

After 24 h, plants were able to acquire similar amounts of ^{15}N from all added substrates, including about 36% of that added as L-alanine. However, the much lower recovery of alanine ^{13}C suggests that less than about 1.3% of alanine ^{15}N was taken up by plant roots as the unmodified amino acid. The lack of a change in recovery of ^{13}C in plant tissues after the first minute, and the rapid removal of added alanine from soil solution by soil microbes, probably indicates that all competition for the amino acid was over within about a minute. However, it is clear that measurements with different isotopes give somewhat different results. The continued increase in alanine ^{14}C recovered in plants between 1 and 5 minutes may suggest competition continued beyond one minute, and that alanine removal from solution was not complete during the first minute.

If the recovery of ^{15}N during the first minute was all taken up as intact amino acid, the rate of uptake was 84 nmol g^{-1} DW root min^{-1} without accounting for any isotopic pool dilution from existing pools of soil amino acid (based on control starting soil concentrations, correction for dilution would increase calculated rates of alanine, NO_3^- and NH_4^+ uptake by about 1.5, 10 and 3%, respectively). However, soil N transformations were so rapid that some uptake of alanine N as NH_4^+ cannot be entirely excluded even over chase periods as short as a minute (we make the assumption that measured increases in inorganic N following alanine addition to soil were derived from the added amino acid). The lower rate of uptake of alanine measured in sterile plants also suggests that some of the alanine ^{15}N entered plants growing in soil as inorganic N.

Effects of differential isotopic discrimination are generally considered to be trivial in labelling experiments (Kruger et al., 2007; Feng & Tang, 2011). However, due to a very large pool of plant and soil C (relative to isotope additions) and the relatively high and variable

natural abundance of ^{13}C, ^{13}C is unlikely to be the most reliable measure of amino acid uptake. If ^{14}C recovery is used as the most conservative measure of intact alanine uptake, the rate of plant acquisition during the first minute after addition was 30 nmol g^{-1} DW root min^{-1}. If we assume post-uptake losses of alanine-^{14}C as plant respiration in sterile conditions are a good estimate of those for plants growing in soil, this figure rises to *ca*. 31.5 nmol g^{-1} DW root min^{-1}, a rate very close to that measured in sterile culture (29 nmol g^{-1} DW root min^{-1}). Uptake of inorganic C and pyruvate by plants with sterile roots was rapid enough to support suggestions that recovery of the C label in plants results from uptake of microbially-processed C (Näsholm et al., 2001; Rasmussen & Kuzyakov, 2009; Rasmussen et al., 2010; Moran-Zuloaga et al., 2015). Recovery of ^{14}C in plant tissues following injection of inorganic ^{14}C into the rhizosphere of plants growing in soil, suggests that almost 1% of mineralised C may have been captured by plant roots during the minute or so during which competition for alanine appears to have taken place (assuming the same proportional loss in respiration as in sterile plants and recovery over the 30 min chase period was indicative of that after 1 min). However, only 0.2% of alanine ^{14}C was mineralised by soil microbes over the minute following addition to soil. Consequently, dark fixation of mineralised amino acid C by roots cannot explain more than about 0.002% of C added as alanine; 2.5% of the C which can be attributed to intact alanine uptake (that C recovered in plants after a minute using the ^{14}C label; 0.16% if calculated using the ^{13}C label).

The rate of microbial mineralisation of alanine in this soil appears to have been sufficiently rapid to convert 40% to free NH_4^+ within 5 min. Although we expect direct uptake of alanine by soil microbes, it seems plausible that extracellular deamination could take place as rapidly as intracellular processing followed by excretion of NH_4^+. Thus, under the scenario that all alanine was extracellularly deaminated, it is quantitatively possible that a portion of alanine C recovered in plants could have entered as pyruvate. However, although uptake of pyruvate by plants with sterile roots was surprisingly rapid, recovery of pyruvate ^{14}C during the first minute following injection into the rhizosphere was only about a fifth of recovery of alanine ^{14}C. Consequently, even assuming instant availability of pyruvate after injection of alanine into the rhizosphere (we assume the same loss of pyruvate ^{14}C in plant respiration as in sterile plants), acquisition of alanine C as pyruvate cannot account for more than about a quarter of recovery of alanine ^{14}C. Thus, it is reasonable to suggest that the rate of intact alanine uptake over the first minute of the chase period was a minimum of 23 nmol g^{-1} DW root min^{-1}. Mineralisation of pyruvate to CO_2 by soil microbes was rapid so that, under the assumption of instant pyruvate availability, it is also quantitatively possible some pyruvate ^{14}C recovered

in plants entered as inorganic C. Although significant plant uptake of alanine C following de-amination to pyruvate and mineralisation by soil microbes within a minute is perhaps implausible, over the longer chase periods used in most experiments this cannot be ruled out. Figure 6 shows estimated maximum fluxes of alanine C and N into wheat roots over a 30 min chase period, which is shorter than used in the majority of previous investigations (Näsholm et al., 2001; Weigelt et al., 2005; Biernath et al., 2008; Harrison et al., 2007; Hill et al., 2011a; Moran-Zuloaga et al., 2015; Wilkinson et al., 2015).

Comparisons of amino acid and inorganic forms of N.

The release in the soil incubation of enough NH_4^+ to account for 40% of the N added as alanine within 5 minutes (again we assume increased soil NH_4^+ originated from added alanine) and cessation of ^{13}C recovery injected into the rhizosphere, suggests that all subsequent plant ^{15}N acquisition was as inorganic N. Slower nitrification of NH_4^+ to NO_3^- further suggests that the wheat acquired the alanine ^{15}N as both $^{15}NH_4^+$ and $^{15}NO_3^-$ over the course of the 24 h experiment. Similarly, continued increase in recovery in plants of ^{15}N added in inorganic forms indicates that $^{15}NH_4^+$ and/or $^{15}NO_3^-$ remained available in the soil for considerably longer than alanine. This clearly demonstrates that isotopic pulse chase experiments aiming to evaluate use of organic N by plants are unable to reliably assess direct use of amino acid N by plants with only an N label, even when very short chase periods are used (very short e.g. at least sub one minute chase periods with compound-specific methods might be an exception to this). Perhaps more importantly, it also suggests that comparisons between short-lived N forms such as amino acids and N forms with much longer residence in soil, such as NO_3^-, may fail to accurately evaluate the relative importance of these forms of N to plant N nutrition.

Significance of amino acid N to wheat growing in mineral soil.

High costs of labelled compounds mean that investigations of plant use of amino acid N tend to be restricted to one or, a few amino acids. L-alanine is only one of many amino acids and their enantiomers present in soil solution; probably about 15% of total amino acids in this soil (Jones et al., 2005b; Fischer et al., 2007; Warren, 2014; Broughton et al., 2015). It is common in proteins, but smaller than some amino acids and uncharged at the pH of this soil, perhaps indicating more availability to plant roots and microbes than some amino acids. However, although it is clear that not all amino acids have the same transport system, there is evidence that suggests most amino acids are taken up by roots at rates of at least the same order of magnitude (Lee et al., 2007; Näsholm et al., 2009b; Sauheitl et al., 2009ab; Svennerstam et al., 2011). Thus, although we do not know exactly how representative of the broader range of

soil amino acids L-alanine is, it is clear that wheat roots have the capacity to acquire and use some amino acid N at rates comparable with inorganic forms of N (Näsholm et al., 2001; Hill et al., 2011c). Indeed, even with strong competition from soil microbes, ^{15}N recovery suggests that in the first minute of this experiment root uptake of L-alanine took place at a similar rate to NO_3^-.

We injected alanine at a concentration at least an order of magnitude greater than the concentration of free amino acids in our soil (probably closer to two orders of magnitude greater than alanine concentrations) or likely to be present in bulk soil solution (Jones et al., 2005b; Jones et al., 2009). The effect of concentration on plant amino acid capture is not clear. Increased concentration has been reported to favour capture by plants, favour capture by soil microbes, and to have no effect on competitive success (Jones et al., 2005b; Näsholm et al., 2009b; Sauheitl et al., 2009b; Hill et al., 2011a). Whether high rates of alanine addition favoured plants or soil microbes, and despite 1 min uptake rates comparing favourably with NO_3^-, only a small proportion of alanine was captured intact by plants. Further, from rates of microbial N mineralisation and plant uptake, it appears that within 10 min of alanine addition to our soil, as much alanine N could have been acquired as NH_4^+ as was acquired as intact alanine.

Assuming that L-alanine adequately represents other amino acids, this calls into question the benefit to wheat N nutrition of maintaining root amino acid transporters (Jones et al., 2005a; Tegeder & Rentsch, 2010; Perchlick et al., 2014). Obvious possibilities are (1) that amino acids do not represent a significant source of N nutrition in soils with a highly active microbial biomass and root transporters are a relic of plants growing in ecosystems where N mineralisation is slower; (2) that root amino acid transporters are only important for recovery of N and C lost in passive exudation or root damage (Jones et al., 2005a); (3) that the commonly used methodology of flooding the rhizosphere with a pulse of isotopically-labelled substrates does not adequately simulate the processes taking place in these soils.

Although, these possibilities are not mutually exclusive and all have the potential to be true, microbial competition was so fierce in our soil that it may indicate that root amino acid transporters neither acquire soil amino acids nor recover exudates if there is a sufficiently active microbial community at the root surface. Nevertheless, we suggest that it is also likely that methodology is limiting. Our results highlight the importance of investigation at a finer temporal scale, but although we can broadly estimate the proportion of root and soil contacted by solutions, it is questionable how well these methods address the spatial controls on root amino acid acquisition.

Soil solution amino acid and peptide concentrations are generally established from extracts which integrate over at least several cm^3 of soil (Jones et al., 2009; Farrell et al., 2013). However, we know that low concentrations belie the high flux through the soil solution and sites of protein cleavage are almost certainly not evenly located within the rhizosphere, with heterogeneity increasing as finer scales are considered (Jones et al., 2005a; Hill et al., 2012; Näsholm et al., 2009b; Inselbacher & Näsholm, 2012b; Wilkinson et al., 2014). Similarly, estimates of microbial colonisation of roots show that coverage of the root surface can be far from complete with much spatial heterogeneity (Liljeroth, 1990). Consequently, although many root amino acid transporters may experience low amino acid concentrations and acquire little amino acid N due to interception by microbes, it is likely that some portions of root growing very close to lysing cells or a site of protein cleavage experience much higher concentrations with less microbial competition, and thus probably acquire much more amino acid N.

Additional spatial complexity may be added due to the potential location of soil microbial proteases in cell walls, which may increase capture of protein cleavage products (amino acids and peptides) by the organism investing in extracellular protease production (Francoeur et al., 2001). There are also questions about availability in soil of other C and N forms, which may affect amino acid use by both plants and soil microbes (Thornton & Robinson, 2005; Gioseffi et al., 2012; Hill et al., 2012; Farrell et al., 2014; Czaban et al., 2016). Further, both soil solution pools and fluxes of N from other plant-available protein cleavage products (short peptides) can exceed those of free amino acids by an order of magnitude, but we have almost no spatial or compositional detail about this pool (Hill et al. 2011a, 2012; Farrell et al., 2013; Warren, 2014; Wilkinson et al., 2014; Carswell et al., 2016; Jämtgård et al., 2018). Thus, although conventional approaches have some validity, we suggest that progress in truly understanding the contribution of early protein breakdown products to plant nutrition cannot be achieved without consideration of the rhizosphere at a finer temporal and spatial scale.

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Author contributions

PWH and DLJ identified the knowledge gap and conceived the investigation. PWH carried out the majority of experimentation and wrote the manuscript first draft. Both authors contributed to the final manuscript.

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744 Figure 1

745 Potential routes for root uptake of C and N added to soil solution as L-alanine.

746

747 Figure 2

748 Recovery of stable isotope labels in wheat plants following injection of solutions into the
749 rhizosphere. Values are mean ± SEM; *n*=4. Insert shows the first 30 min of data only.

750

751 Figure 3

752 Concentrations of N forms in soil extracts at different incubation times following addition of
753 alanine to soil. Values are mean ± SEM; *n*=4. Insert shows the first 30 min of data only.

754

755 Figure 4

756 Mineralisation of alanine and pyruvate ^{14}C to $^{14}CO_2$ in soil. Values are mean ± SEM; *n*=3.
757 Lines are linear and exponential (alanine and pyruvate, respectively) fits to data as described
758 in the text.

759

760 Figure 5

761 Loss of alanine, pyruvate and inorganic ^{14}C, following uptake by wheat plants with sterile
762 roots. Values are mean ± SEM; *n*=3. Lines are linear (inorganic C) and 2nd order polynomial
763 (alanine and pyruvate) fits to data as described in the text.

764

765 Figure 6

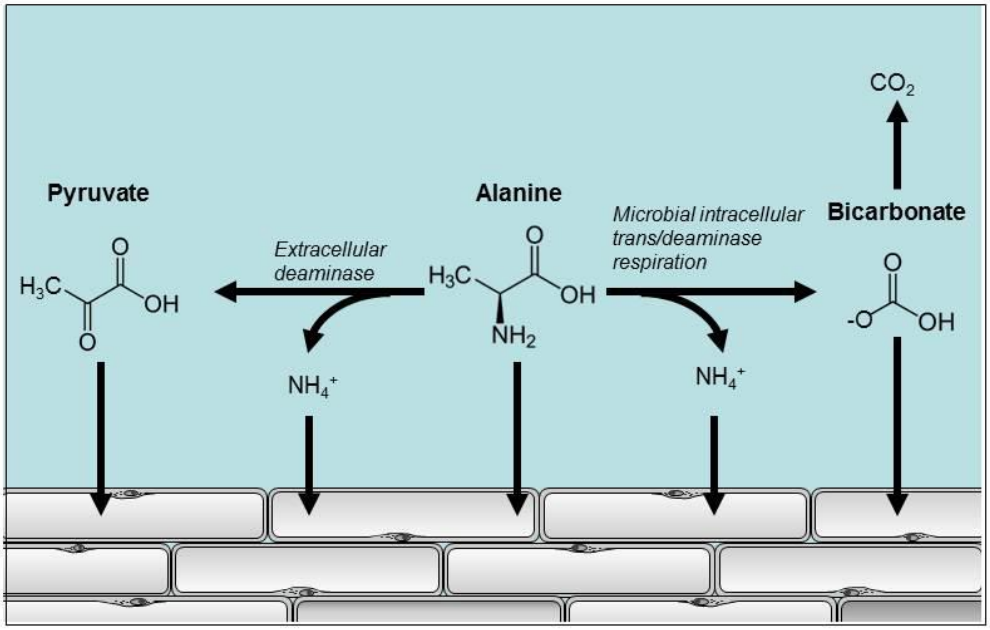
766 Maximum potential fluxes of L-alanine C and N into wheat roots by different routes over a 30
767 min chase period. Because of uncertainties about the route of uptake, fluxes are not
768 necessarily additive. Details of the rationale for flux estimates are presented in Supporting
769 Information Notes S1.

770

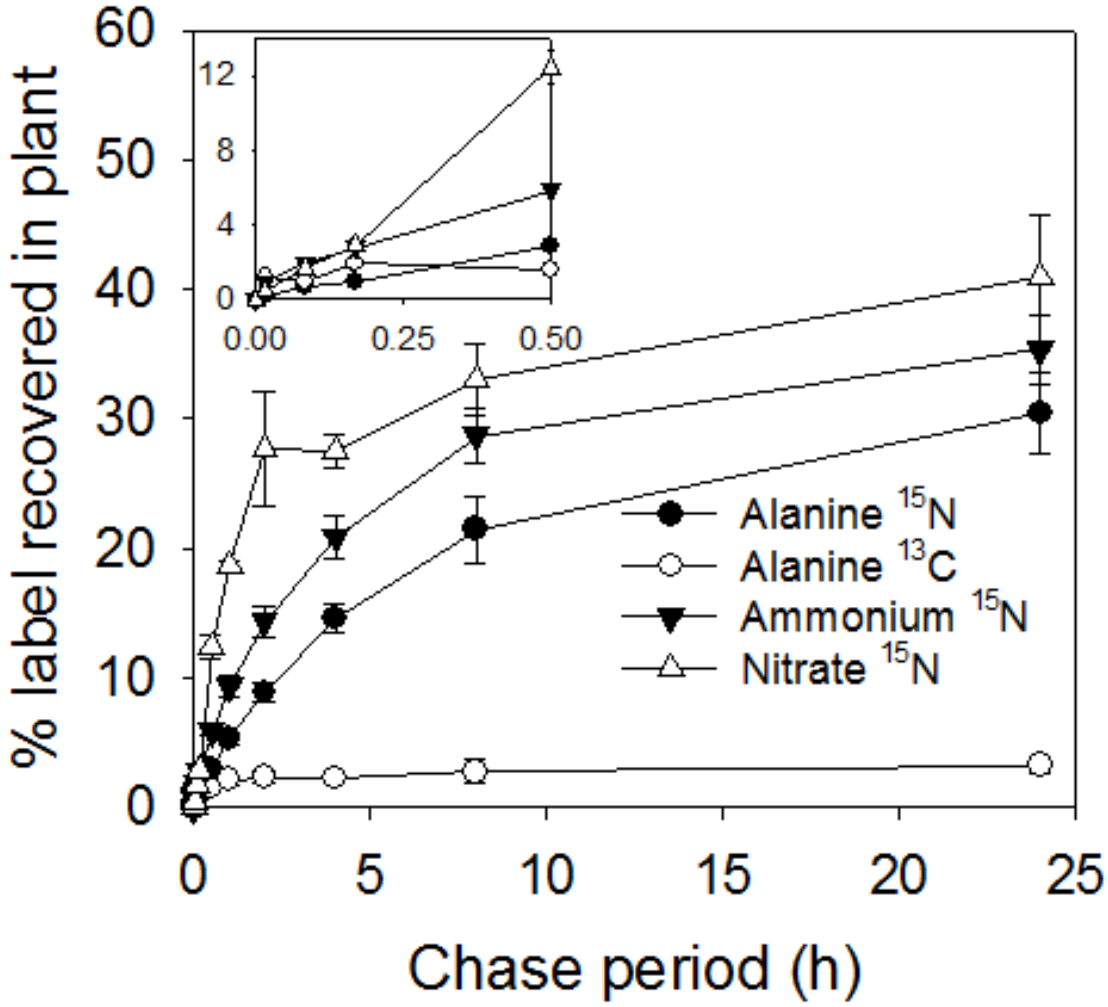
771 Supporting Information Notes S1:

772 Rationale for estimation of maximum values for potential fluxes of C and N into wheat roots
773 over a 30 minute chase period following injection of L-alanine into the rhizosphere

774


Pyruvate
Alanine
Bicarbonate
CO_2
Extracellular deaminase
Microbial intracellular trans/deaminase respiration
H_3C
O
OH
NH_2
NH_4^+
NH_4^+
-O

775


% label recovered in plant
Chase period (h)
Alanine ^{15}N
Alanine ^{13}C
Ammonium ^{15}N
Nitrate ^{15}N
0
10
20
30
40
50
60
0
5
10
15
20
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0
4
8
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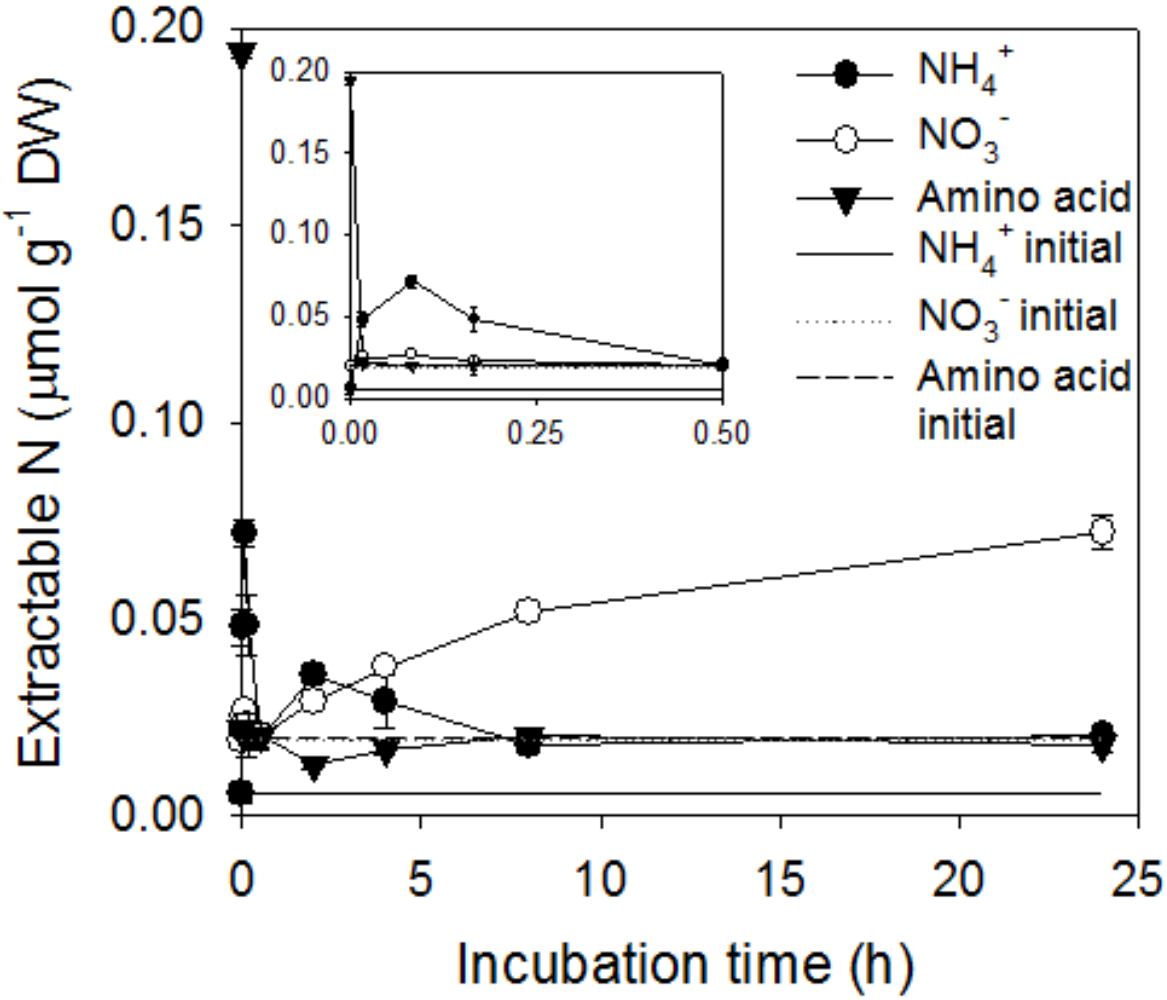
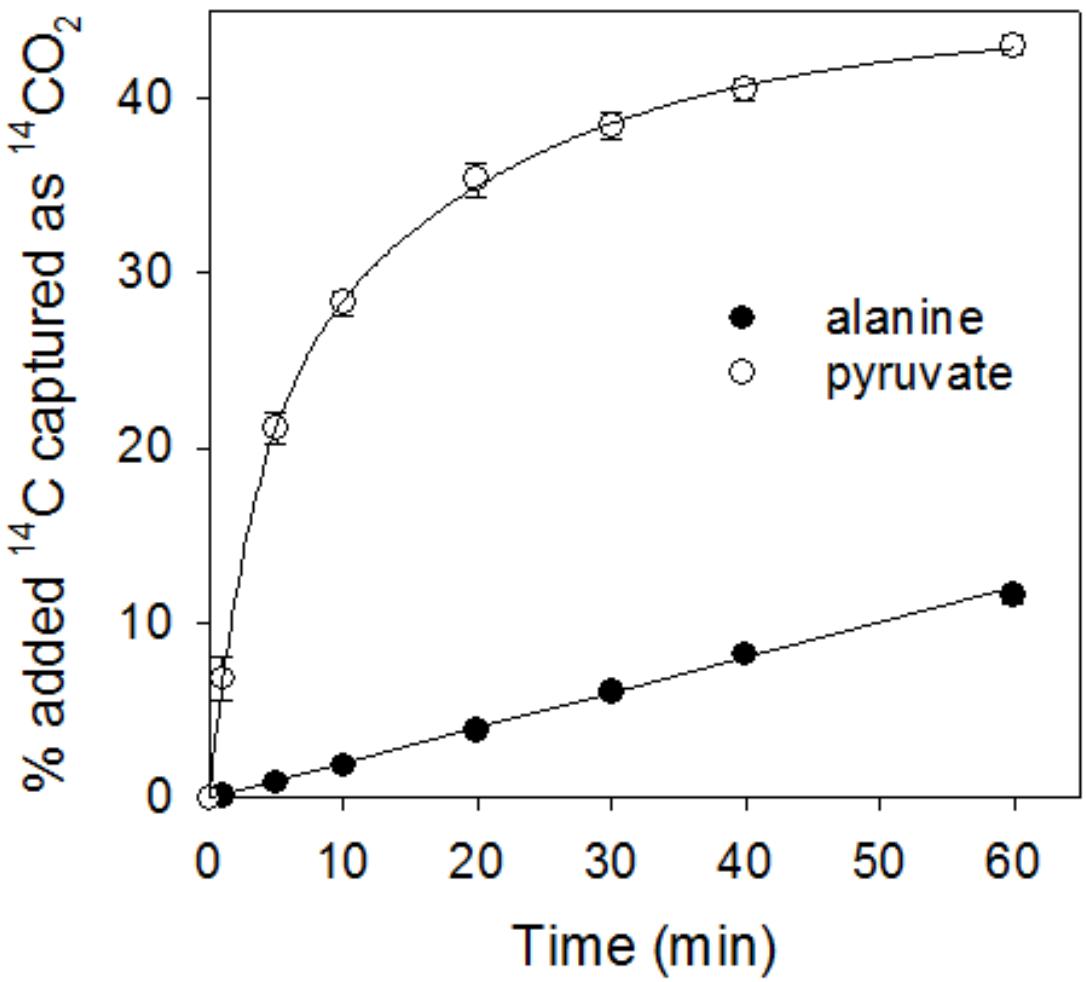
0.00
0.25
0.50

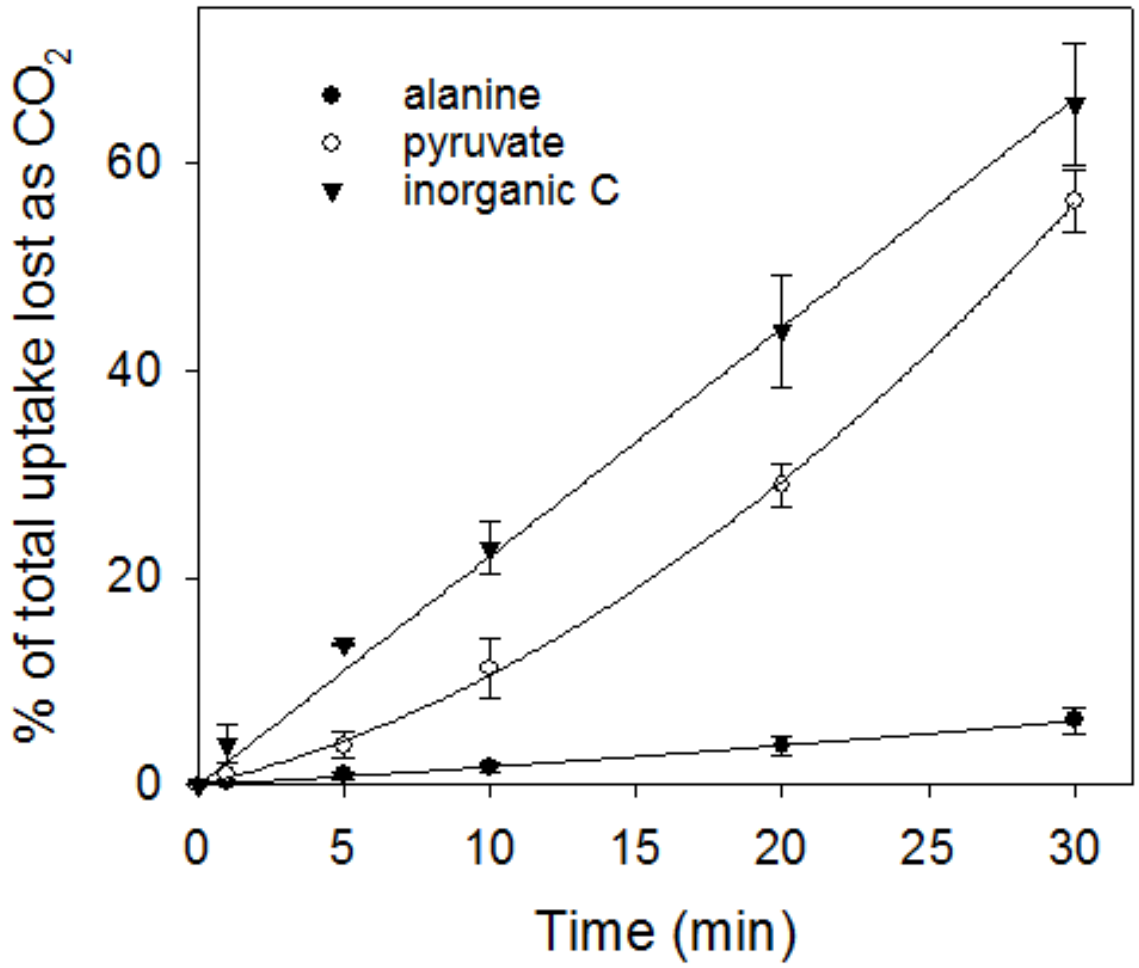
776


Extractable N (µmol g-1 DW)
Incubation time (h)
0.20
0.15
0.10
0.05
0.00
0
5
10
15
20
25
0.00
0.25
0.50
NH4+
NO3-
Amino acid
NH4+ initial
NO3- initial
Amino acid initial

777


% added ^{14}C captured as $^{14}CO_2$
0
10
20
30
40
0
10
20
30
40
50
60
Time (min)
alanine
pyruvate

778



779

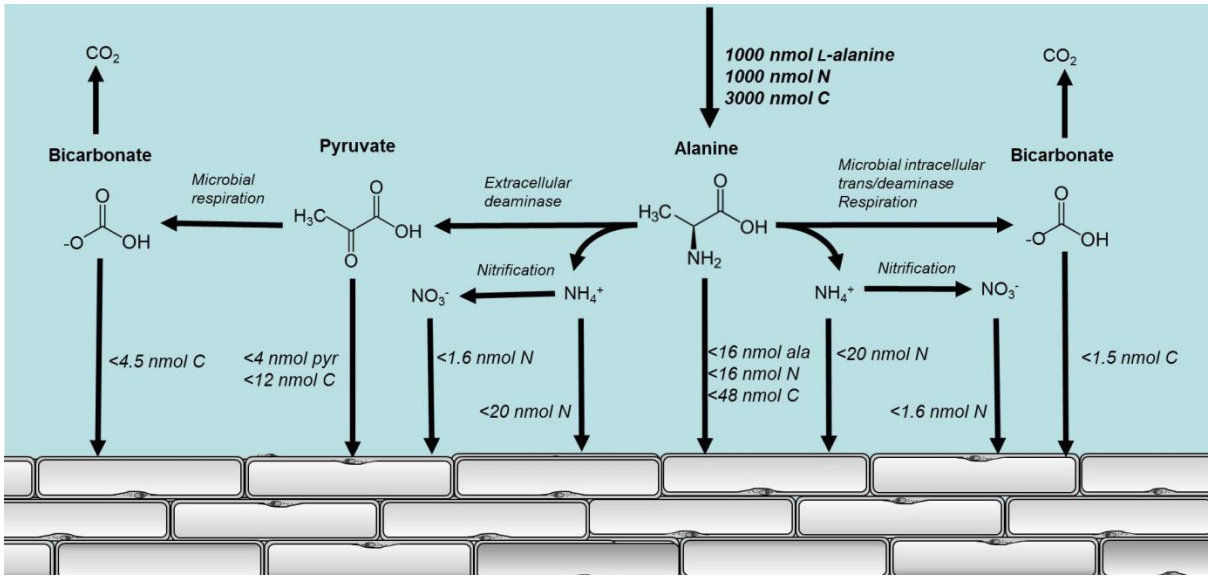
780

781

782

783

784


1000 nmol L-alanine
1000 nmol N
3000 nmol C
CO2
Bicarbonate
Pyruvate
Alanine
CO2
Bicarbonate
Microbial respiration
Extracellular deaminase
Microbial intracellular trans/deaminase Respiration
Nitrification
Nitrification
NO3-
NH4+
NH4+
NO3-
<4.5 nmol C
<4 nmol pyr
<12 nmol C
<1.6 nmol N
<20 nmol N
<16 nmol ala
<16 nmol N
<48 nmol C
<20 nmol N
<1.6 nmol N
<1.5 nmol C